# The motility, development and infection of *Meloidogyne incognita* encumbered with spores of the obligate hyperparasite *Pasteuria penetrans*

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SUMMARY

The effects of *Pasteuria penetrans* spore burden on the motility, the number of developing females and infection of *Meloidogyne incognita* is reported. The motility of second-stage juveniles, in tubes containing sand at a suction pressure adjusted for maximum nematode movement, was significantly reduced when juveniles were encumbered with an average of seven spores per juvenile. The length of time taken for second-stage juveniles to become encumbered with spores in a water suspension of spores was dependent on juvenile age. *Pasteuria* spore burden led to a significant reduction on the number of females developing in the roots compared to unencumbered controls. As the length of time, between inoculating soil with second-stage juveniles and the planting of tomato seedlings, increased, there was a significant reduction in the number of females developing on the roots and also a reduction in infection by the hyperparasite. The watering regime, to which experimental plants were treated, affected the number of females developing in roots, and the rate of development of *Pasteuria* in infected females was slower when soils were maintained at field capacity.

RÉSUMÉ

*Mobilité, développement et pouvoir infestant* de Meloidogyne incognita
*portant des spores de l'hyperparasite obligé* Pasteuria penetrans

Cet article traite des conséquences de la charge en spores de *Pasteuria penetrans* sur la mobilité, le nombre des femelles formées et le pouvoir infestant de *Meloidogyne incognita*. Dans des tubes contenant du sable où une succion est ajustée à la valeur optimale pour la mobilité des nématodes, cette mobilité des juvéniles de deuxième stade est diminuée de manière significative quand ces juvéniles portent une moyenne de sept spores par individu. Le temps mis par des juvéniles de deuxième stade pour se charger en spores dans une suspension aqueuse de celles-ci dépend de l'âge de ces juvéniles. La charge en spores de *Pasteuria* conduit à une diminution significative du nombre de femelles formées dans les racines en comparaison des témoins sans spores. L'accroissement du temps écoulé entre l'inoculation du sol avec les juvéniles de deuxième stade et la mise en place des plantules de tomate a pour conséquence une diminution significative du nombre des femelles se développant dans les racines et aussi de l'infestation par l'hyperparasite. Le régime hydrique appliqué aux plantes en expérience retentit sur le nombre de femelles formées dans les racines, et le mode de développement des femelles infestées par *Pasteuria* est plus lent dans le cas de sols maintenus à la capacité au champ.

The bacterial parasites, *Pasteuria* spp., produce spores which adhere to the cuticle and infect many species of nematode, a number of which are important crop pests (Sayre & Starr, 1988). The bacterium has been observed in both tropical and temperate regions of the world and has considerable potential as a biological control agent against both, cyst (Bhattacharya & Swarup, 1988; Davies *et al.*, 1990), and root-knot nematodes (Mankau, 1975; Sayre, 1980; Stirling, 1984). The natural suppression of root-knot nematodes in W. Africa (Mankau, 1980) and on vines in S. Australia (Stirling & White, 1982) have been associated with a large proportion of spore-encumbered second-stage juveniles in soil. Biological control using *Pasteuria* will be dependent on the number and distribution of spores in the soil and the time the target nematode is active. *Pasteuria* has been found to reduce nematode populations not only by prohibiting infected root-knot females from producing eggs (Mankau, 1980; Sayre, 1980) but also by reducing the numbers that invade roots (Mankau & Prasad, 1977; Brown & Smart, 1985; Davies, Kerry & Flynn, 1988). Stirling (1984) found that invasion of roots decreased as the distance nematodes had to move through spore infested soil increased. As few as fifteen spores per second-stage were found to reduce invasion of root-knot by more than 70 % when juveniles encumbered with spores were added to soil around tomato plants (Davies, Kerry & Flynn, 1988). This reduction in invasion was

density dependent and only tended to occur at high nematode densities; there was no obvious explanation for this effect, but it could result from competition for invasion sites. An understanding of the host-parasite relationship is fundamental to increase the efficiency of the bacterium as a control agent. Competition and other factors which may weaken the juveniles and predispose them to infection may be important in exploiting the relationship (Davies, Kerry & Flynn, 1988).

This paper therefore reports the interactions of *Pasteuria* with age of second-stage juvenile and its effect on motility, root invasion and infection. The results are discussed in relation to the prospects of increasing the efficiency of *Pasteuria* as a control agent.

## Materials and methods

### Culturing and extraction of nematodes and *Pasteuria*

*Meloidogyne incognita* (Race 2 No 1135, North Carolina State University, Raleigh USA) was cultured on the roots of tomato plants, cv. Pixie. Second-stage juveniles were obtained by gently washing away the soil around infected roots; picking off egg masses, and suspending them in a small volume of tap water on a nylon 100 µm aperture sieve (Hooper, 1986). *Pasteuria penetrans*, population PP1 (obtained from Dr S. R. Gowen, University of Reading, UK) was increased following an adapted method of Stirling and Wachtel (1980). *M. incognita* second-stage juveniles were agitated in a suspension of PP1 spores until they had between five and ten spores attached to their cuticles. They were added to compost (3:1 peat sand mixture) around tomato roots cv. Pixie; the bacteria multiplied in the developing female nematodes within the roots. Infected females were obtained by collecting the galled roots after 8 weeks and digesting them in 25 % Pectinex (Novo Enzyme Products Ltd., Farnham, UK) for 24 h at room temperature. The resultant slurry, and root fragments were gently homogenised, washed, and the females collected following the method of Davies, Kerry and Flynn (1988). PP1 spores were concentrated by collecting parasitised females and homogenising them in tap water. Spore samples in which the sporangia were retained were sonicated before use with a Kerry's ultrasound generator fitted with a logarithmic probe (Davies, Kerry & Flynn, 1988).

### Influence of *Pasteuria* on motility

The movement of second-stage juveniles encumbered with different numbers of *Pasteuria* spores was measured in polythene tubes (6 mm diameter) containing sand (particle size 150-400 µm), divided into 1 cm sections (Evans, 1969). The suction pressure was adjusted to 1.7 pF, which is the point of inflection on the moisture characteristic curve for sand of this particle size (Robinson, Atkinson & Perry, 1987), and is the point at which nematode movement is maximal (Wallace, 1963). Four replicates of 100 and 300 freshly hatched second-stage juveniles encumbered with 0, 5-10 and 11-20 spores per individual juvenile were introduced into the tubes. Spores of *Pasteuria* were attached to the second-stage juveniles by aerating a $10^6$ spore $ml^{-1}$ suspension, to which juveniles had been added, in a 100 ml conical flask (Stirling & Wachtel, 1980). When the required number of spores had attached to a sample of 20 juveniles the nematodes were washed with water and collected on a 10 µm aperture sieve. Juveniles were resuspended in tap water and the volumes adjusted to give, 4 000 and 12 000 juveniles per ml, before 25 µl of the appropriate suspension was pipetted on to the end of the tube. After 72 h at 20 °C the sand cores were divided into 1 cm sections and the juveniles in each section counted. A motility index (MI) was determined for each replicate using the formula of Townshend and Webber (1971) :

$$MI = \frac{(no\ in\ 1\ cm \times 0) + (No\ in\ 2\ cm \times 1) \ldots etc + (No\ in\ 6\ cm \times 5)}{Total\ number\ recovered}$$

### Effect of age of second-stage juvenile on attachment of *Pasteuria* spores

To investigate the effects of age on the adhesion of *Pasteuria* spores, galled roots with egg masses were collected and stored at 4 °C. Four age cohorts (0-7; 8-14; 15-21; 22-28 days old) of second-stage juveniles were obtained by placing egg masses, from standard cultures stored at 4 °C, on a hatching tray at room temperature as previously described, and allowing them to hatch over a seven day period. The juveniles which had hatched were aged in tap water at 25 °C in a controlled temperature room without any aeration. The attachment of spores was carried out by bubbling air into a $10^6$ spores $ml^{-1}$ suspension containing each group of second-stage juveniles as previously described. Every 30 min, ten second-stage juveniles were removed from each age cohort sample, and the spores adhering to ten individuals counted using a high power microscope ( × 400).

### Influence of age and *Pasteuria* on the fate of *M. incognita*

#### *Aging in water at 25 °C*

Four age cohorts (0-7, 8-14, 15-21 and 22-28 days old) of second-stage juveniles were obtained as described above. *Pasteuria* spores were attached to second-stage juveniles from each age cohort by agitating the juveniles in a spore suspension and when they had between 5-10 and 11-20 spores adhering to their cuticles (the latter took up to 5 h) the juveniles were inoculated around the

roots of six week old tomato plants at 1000 and 2000 juveniles per tomato plant. Control plants were inoculated with spore-free juveniles of each age cohort; each treatment consisted of four replicates.

*Aging in soil at 25 °C*

Freshly hatched (i.e. less than 48 h old) second-stage juveniles were encumbered with 0, 5-10 or 11-20 spores per individual juvenile, as described above, and 500 and 1000 second-stage juveniles were added to compost in plant pots in the greenhouse. The pots were then placed in polythene bags to reduce evaporation. After the juveniles had aged for zero, 6, 12 and 18 days the polythene bags were opened and a six week old tomato seedling planted into the compost. Before the bags were resealed each tomato plant was watered with 100 ml of water. After nine days the polythene bags were opened and the tomato plants watered as necessary. The experiment consisted of three replicates. Both experiments were conducted in the glasshouse at approximately 25 °C with a 16 hour day photoperiod. Roots were harvested four weeks after the addition of juveniles, and washed, blotted dry, weighed and the females collected as previously described. The residue was resuspended in 100 ml water from which a 10 ml sample from each treatment was placed in a counting tray (Doncaster, 1962) and the number of females determined. Twelve females were then removed from each sample, individually dissected on a microscope slide in a small droplet of water, and examined (× 400) for *Pasteuria* infection.

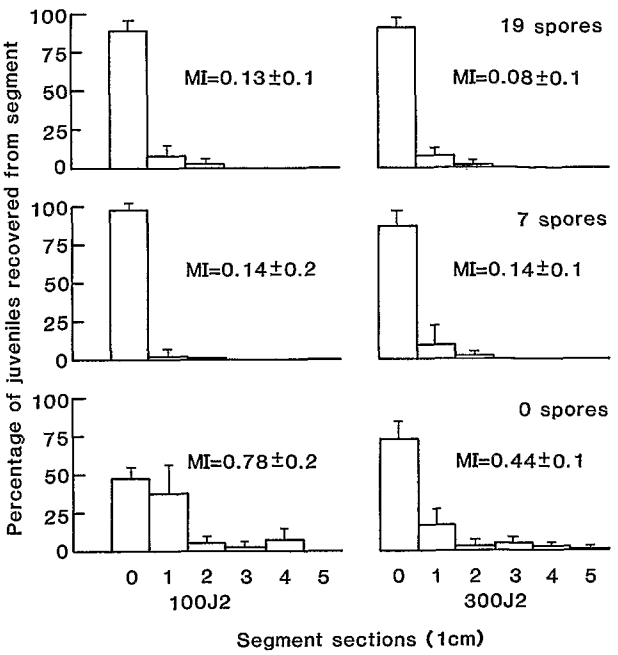


Fig. 1. Percentage of *Meloidogyne incognita* second-stage juveniles recovered from six, 1 cm sections of sand filled tubes with a suction pressure of 1.7 pF, 72 hours after inoculation, at two population densities (approx. 100 & 300), and encumbered with a mean number of *Pasteuria* spores, 0, 7 and 19; and the mean motility index (MI, +/— standard error).

INFLUENCE OF WATERING REGIME ON THE FATE OF JUVENILES ENCUMBERED WITH *PASTEURIA* SPORES

Seven week old tomato plants were grown under three watering regimes (high, 200 ml; medium, 100 ml; low, 50 ml of tap water every 24 h in a 3:1 peat/sand compost) the highest of which had previously been found to maintain the compost at, or above, water holding capacity. Second-stage juveniles (2000), encumbered with between 5-15 spores as previously described, were added around the roots of tomato plants. Control treatments consisted of juveniles not exposed to *Pasteuria* spores. Six weeks after inoculation the root systems were harvested, washed free of soil and digested in Pectinex; the number of females and percentage of infection was

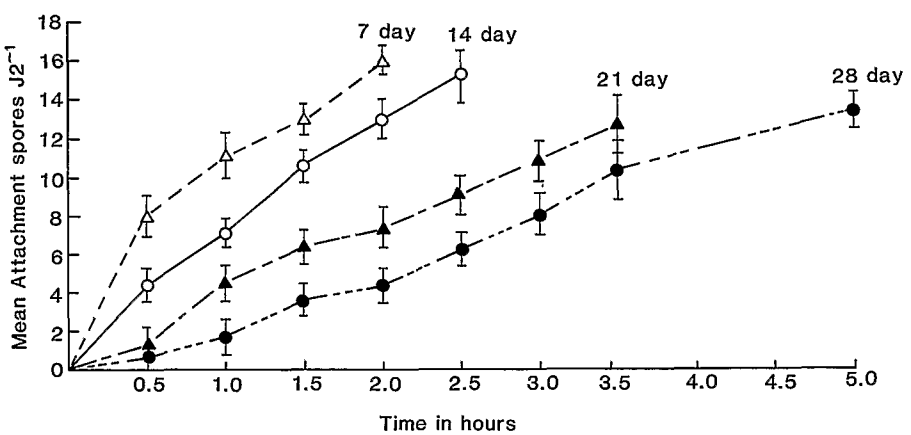


Fig. 2. The mean of attachment of *Pasteuria* spores to 0,7, 8, 14, 15, 21, 22, 28 day old

Table 1

The mean number of females recovered from tomato roots after being inoculated with second-stage juveniles (J2) of *Meloidogyne incognita* encumbered with two levels of spores and a control, at two densities, in four age cohorts (0-7, 8-14, 15-21 and 22-28 days).

| Nematode density (per pot) | spores per J2 | Age cohorts | | | |
|---|---|---|---|---|---|
| | | 0-7 | 8-14 | 15-21 | 22-28 |
| 1000 | 0 | 148 (2.2)* | 182 (2.2) | 168 (2.2) | 235 (2.3) |
| | 5-10 | 35 (1.5) | 68 (1.8) | 55 (1.6) | 68 (1.4) |
| | 11-20 | 4 (0.6) | 10 (0.6) | 15 (0.9) | 1 (1.5) |
| 2000 | 0 | 320 (2.5) | 363 (2.6) | 328 (2.5) | 375 (2.6) |
| | 5-10 | 70 (1.8) | 195 (2.3) | 110 (2.0) | 70 (1.8) |
| | 11-20 | 7 (0.3) | 15 (1.1) | 35 (1.5) | 38 (1.5) |

* data skewed : figures in parenthesis = log (females + 1); SED = 0.3.

Table 2

The mean number of females infected with *Pasteuria*, and percentage infection, recovered from tomato roots after being inoculated with second-stage juveniles (J2) of *Meloidogyne incognita* encumbered with two levels of spores and a control, at two densities, in four age cohorts (0-7, 8-14, 15-21 and 22-28 days).

| Nematode density (per pot) | spores per J2 | Age cohorts | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0-7 | | 8-14 | | 15-21 | | 22-28 | |
| | | infected ♀♀ | % infected | infected ♀♀ | % infected | infected ♀♀ | % infected | infected ♀♀ | % infected |
| 1000 | 0 | 0.0 (0)* | 0 | 0.0 (0) | 0 | 0.0 (0) | 0 | 0.0 (0) | 0 |
| | 5-10 | 27.9 (1.4) | 79 | 54.1 (1.7) | 80 | 43.1 (1.5) | 80 | 47.3 (1.3) | 79 |
| | 11-20 | 0.1 (0) | 84 | 7.5 (0.5) | 75 | 11.5 (0.9) | 81 | 12.2 (1.0) | 65 |
| 2000 | 0 | 14.0 (0.7) | 5 | 0.0 (0) | 0 | 6.5 (0.4) | 3 | 0.0 (0) | 0 |
| | 5-10 | 55.3 (1.7) | 75 | 136.5 (2.1) | 70 | 79.3 (1.8) | 78 | 61.0 (1.7) | 87 |
| | 11-20 | 4.5 (0.4) | 69 | 10.9 (1.0) | 76 | 27.8 (1.1) | 77 | 24.8 (1.4) | 72 |

* data : skewed : figures in parenthesis = log (infected females + 1); SED = 0.3

Table 3

The mean number of females recovered from tomato roots, that were planted into soil containing second-stage juveniles (J2)

Table 4

The mean number of females infected with *Pasteuria*, and percentage infection, recovered from tomato roots that were planted into soil containing second-stage juveniles (J2) of *M. incognita* encumbered with two levels of spores and a control, at two nematode densities, at 0, 6 and 12 days between inoculation of the soil with juveniles and the planting of tomato seedlings.

| Nematode density (per plot) | Spores per J2 | 0 days | | 6 days | | 12 days | |
|---|---|---|---|---|---|---|---|
| | | infected ♀♀ | % infected | infected ♀♀ | % infected | infected ♀♀ | % infected |
| 500 | 0 | 0.0 (0)* | 0 | 0.0 (0) | 0 | 0.0 (0) | 0 |
| | 5-10 | 20.5 (1.3) | 19 | 5.5 (0.6) | 14 | 0.0 (0) | 0 |
| | 11-20 | 21.1 (1.3) | 39 | 1.7 (0.4) | 11 | 0.0 (0) | 0 |
| 1 000 | 0 | 0.0 (0) | 0 | 0.0 (0) | 0 | 0.0 (0) | 0 |
| | 5-10 | 26.6 (1.4) | 19 | 6.0 (0.8) | 17 | 0.0 (0) | 0 |
| | 11-20 | 73.8 (1.8) | 47 | 11.4 (0.8) | 14 | 0.0 (0) | 0 |

* Skewed data : parenthesis = log (infected females + 1); SED = 0.2.

Table 5

The mean number of *M. incognita* females per root system and percentage infection by *Pasteuria*, when second-stage juveniles (J2) encumbered with between 5-15 spores were inoculated around the roots of tomatoes grown under three watering regimes (high = 200 ml; medium = 100 ml and low = 50 ml), and its developmental stage at harvest.

## Results

### Influence of *Pasteuria* on motility

In the experiment monitoring the movement of second-stage juveniles down tubes containing sand approximately 32 % of the juveniles were recovered. *Pasteuria* spore burden greatly affected the motility of second-stage juveniles (Fig. 1); there was no significant differ-

INFLUENCE OF WATERING REGIME ON THE FATE OF *M. INCOGNITA*

When second-stage juveniles, aged in water at 25 °C for different lengths of time with different burdens of *Pasteuria* spores, were inoculated around the roots of tomato plants an increase in age did not significantly reduce the number of females that developed. However, significantly fewer females developed on plants attacked by juveniles encumbered with spores than those without; the effect was significantly greater when juveniles had 11-20 spores on their cuticles than 5-10 spores, and these differences were consistant for all ages of juveniles. This occurred at both nematode densities (Table 1). There was no significant difference in the percentage infection of developing females, originating from spore encumbered juveniles, due either to the age of juveniles or their burden of spores (Table 2). Aging the second-stage juveniles in soil prior to planting had a large effect on the number of developing females and the percentage of infection (Tables 3 & 4). Significantly fewer females developed as the time from inoculating the soil with juveniles to the planting of seedlings increased; the percentage of infected females also decreased and this was the case irrespective of the nematode density. The nematode, and the watering regime all had an effect on the fate of *M. incognita* and its infection by *Pasteuria*. *Pasteuria* burden greatly reduced the motility of second-stage juveniles through tubes of sand. The overall distances moved in the tubes were not very great; *M. javanica* and other root-knot species are capable of migrating relatively large distances (up to 40 cm) in conditions approximating to those in the field in which tomato plants were present (Prot & Netscher, 1979); the lack of motility in the tubes was probably due to the lack of a stimulus. Using motility tubes it was found that *Globodera rostochiensis* males rarely migrated more than 1 cm without a stimulus, but in the presence of white females the distance moved increased; within 72 hours males had migrated a distance of 6 cm (Evans, 1969). The reduction in the movement of juveniles encumbered with *Pasteuria* spores is consistant with the results of Stirling, Sharma and Perry (1990) where the migration of second-stage juveniles through spore infested sand reduced invasion. The results suggest that seven spores adhering to the second-stage juvenile cuticle is sufficient to obtain a measurable reduction in movement, without a stimulus, and that increasing the spore burden further only had a small effect on reducing the number of females developing on the roots. It would therefore

likely that the reduction in survival was caused by antagonists, as it has been shown that survival is reduced in non-sterile systems (de Guiran, 1975).

Soil moisture, as has been reviewed elsewhere (Wallace, 1963), has a considerable effect on the movement of nematodes through the soil and it is therefore not surprising that watering regime had a large effect on the number of nematodes developing in the roots. It is interesting, however, that watering regime also had an effect on the rate of development of *Pasteuria* within the developing females; the rate of development of *Pasteuria* was faster in the treatments with a low watering regime. In the high watering regime, where the soil was at field capacity for much longer periods of time, there may have been a depletion of oxygen which led to reduced respiration inhibiting both the developing nematode and its parasite. Increasing *Pasteuria* spore burden led to a significant increase in percentage infection of females in the roots only where the juveniles were stored in water prior to inoculation. When juveniles were aged in soil, spore burden had very little effect on female infection; the number of females developing in these roots was very small, even in the treatments where juveniles were unencumbered with spores. The second-stage juveniles which survive in the soil, and manage to invade the roots, were less likely to be infected by *Pasteuria*. This, therefore, represents a group of second-stage juveniles that, either, have not become encumbered with spores, or, have some mechanism which avoids infection.

*Pasteuria* spores reduce motility and the longer second-stage juveniles encumbered with spores are active in the soil the greater is the chance that they will prohibit invasion. Previous studies had suggested that a minimum of 15 spores were required to affect invasion (Davies, Kerry & Flynn, 1988); the results here, suggest that fewer spores will have a similar effect if the distance that the juveniles have to migrate to locate a root is

biology and control. *Indian Journal of Nematology,* 18 : 61-70.

Brown, S. M. & Smart, G. C. (1985). Root penetration by *Meloidogyne incognita* juveniles infected with *Bacillus penetrans. Journal of Nematology,* 17 : 123-126.

Davies, R. G., Flynn, C. A., Laird, V. & Kerry, B. R. (1990). The life-cycle, population dynamics and host specificity of a parasite of *Heterodera avenae,* similar to *Pasteuria penetrans. Revue de Nématologie,* 13 : 303-309.

Davies, K. G., Kerry, B. R. & Flynn, C. A. (1988). Observations on the pathology of *Pasteuria penetrans* a parasite of root-knot nematodes. *Annals of applied Biology,* 112 : 491-501.

Doncaster, C. C. (1962). A counting dish for nematodes. *Nematologica,* 7 : 334-336.

Evans, K. (1969). Apparatus for measuring nematode movement. *Nematologica,* 15 : 433-435.

Himmelhoch, S., Kisiel, M. J., & Zuckerman, B. M. (1977). *Caenorhabditis briggsae;* electron microscope analysis of changes in negative surface charge density of the outer cuticle membrane. *Experimental Parasitology,* 41 : 118-123.

Hooper, D. J. (1986). Extraction of free-living stages from soil. In : Southey, J. F. (Ed.). *Laboratory Methods for Work with Plant and Soil Nematodes.* London, HMSO : 5-30.

de Guiran, G. (1979). Survie dès nématodes dans les sols secs et saturés d'eau : œufs et larves de *Meloidogyne incognita. Revue de Nématologie,* 2 : 65-77.

Mankau, R. (1975). *Bacillus penetrans* n. comb. causing a virulent disease of plant-parasitic nematodes. *Journal of Invertebrate Pathology,* 26 : 333-339.

Mankau, R. (1980). Biological control of *Meloidogyne* populations by *Bacillus penetrans* in West Africa. *Journal of Nematology,* 12 : 230.

Mankau, R. & Prasad, N. (1977). Infectivity of *Bacillus penetrans* in plant-parasitic nematodes. *Journal of Nemato-*

STIRLING, G. R. (1981). Effect of temperature on infection of *Meloidogyne javanica* by *Bacillus penetrans*. *Nematologica*, 27 : 458-462.

STIRLING, G. R. (1984). Biological control of *Meloidogyne javanica* with *Bacillus penetrans*. *Phytopathology*, 74 : 55-60.

STIRLING, G. R. (1985). Host specificity of *Pasteuria penetrans* within the genus *Meloidogyne*. *Nematologica*, 31 : 203-209.

STIRLING, G. R., SHARMA, R. D. & PERRY, J. (1990). Attachment of *Pasteuria penetrans* to the root-knot nematode *Meloidogyne javanica* in soil and its effects on infectivity. *Nematologica*, 36 : 246-252.

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STIRLING, G. R. & WACHTEL, M. F. (1980). Mass production of *Bacillus penetrans* for the biological control of root-knot nematodes. *Nematologica*, 26 : 308-312.

STIRLING, G. R. & WHITE, A. M. (1982). Distribution of a parasite of root-knot nematodes in South Australian vineyards. *Plant Disease*, 66 : 52-53.

TOWNSHEND, J. L. & WEBBER, L. R. (1971). Movement of *Pratylenchus penetrans* and the moisture characteristics of three Ontario soils. *Nematologica*, 17 : 47-57.

WALLACE, H. R. (1963), *The Biology of Plant Parasitic Nematodes*, London, Edward Arnold Publ., VIII + 280 p.