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FULL RESEARCH PAPER

The potential of combining *Pasteuria penetrans* and neem (*Azadirachta indica*) formulations as a management system for root-knot nematodes on tomato

Nazir Javed · Said El-Hassan · Simon Gowen · Barbara Pemproke · Muhammed Inam-ul-Haq

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Abstract Initial applications of 10^4 spores g^{-1} of *Pasteuria penetrans*, and dried neem cake and leaves at 3 and 2% w:w, respectively, were applied to soil in pots. Juveniles of *Meloidogyne javanica* were added immediately to the pots (500, 5,000 or 10,000) before planting 6-week-old tomato seedlings. The tomatoes were sampled after 64 days; subsequently a second crop was grown for 59 days and a third crop for 67 days without further applications of *P. penetrans* and neem. There was significantly less root-galling in the *P. penetrans* combined with neem cake treatment at the end of the third crop and this treatment also had the greatest effect on the growth of the tomato plants. At the end of the third crop, 30% of the females were infected with *P. penetrans* in those treatments where spores had been applied at the start of the experiment. The effects of neem leaves and neem cake on the nematode population did not persist through the crop sequences but the potential for combining the amendments with a biological control agent such as *P. penetrans* is worthy of further evaluation.

Keywords Biological control · IPM · *Meloidogyne javanica* · Natural products · *Pasteuria penetrans*

N. Javed · S. El-Hassan (✉) · S. Gowen · B. Pemproke · M. Inam-ul-Haq
School of Agriculture, Policy and Development,
The University of Reading,
Earley Gate, P.O. Box 236, Reading,
Berkshire RG6 6AT, UK
e-mail: s.el-hassan@reading.ac.uk

Introduction

Root-knot nematodes are notoriously difficult to manage because of their high reproductive potential and wide host ranges (Whitehead 1998). Economic damage on tomato can occur with root-nematode densities of 0.1–1.0 nematodes cm^{-3} soil at planting (Sikora and Fernandez 2005). Whitehead (1998) suggested that 99.9% control is required in order to prevent the subsequent buildup of damaging populations because of the reproductive potential of *Meloidogyne* spp. Gowen et al. (1998) observed that application of *Pasteuria penetrans* (Sayre and Starr 1985) may not achieve good control when conditions are favourable for nematode reproduction but it may have a useful role in nematode management if deployed with other control measures (Tzortzakakis and Gowen 1994).

Pasteuria penetrans, an endospore-forming obligate hyperparasite, has been found effective in reducing the root-knot nematode populations in pots (Eddaoudi and Bourijate 1998; Gowen et al. 1998; Giannakou et al. 1999) and field micro-plots (Brown et al. 1985; Nishizawa 1987; Melki et al. 1998; Chen et al. 1996; Weibelzahl-Fulton et al. 1996). Spores of *P. penetrans* attach to second stage root-knot nematode juveniles as they move in the soil. The spores are not motile so the attachment can only occur if spore and nematode come into contact. Infection success or biocontrol efficacy will therefore depend on the density and distribution of the spores in the soil. Juveniles encumbered with spores may find and invade

a host root. After root invasion, the spores germinate and penetrate the cuticles of the developing root-knot nematodes. Depending on temperature, the parasite multiplies in the living female nematode and its life cycle is completed in approximately 35 days at 30°C (Stirling 1981). The female may continue to feed for up to 88 days and >2 million spores may be contained in its body (Darban et al. 2004). Eventually these spores will enter the soil after the female has died and the root in which the nematode was feeding has senesced.

Melki et al. (1998) demonstrated the increase of *P. penetrans* in soil in a pot experiment over three crop cycles. After 47 days the plants in the first crop cycle were sampled and percent infection of females was determined. After drying, the root systems (containing *P. penetrans* infected females) were mixed in the soil, and tomato seedlings were planted in the pots which were then inoculated with *Meloidogyne javanica* juveniles. These plants were then grown for a similar period and the process was repeated again for a third crop cycle. The increase in the spore density in the pots was indicated by a progressive increase in the numbers of spores attaching to nematodes over the three crops.

Leaves and seeds of neem (*Azadirachta indica*) contain a number of terpenoids some of which are recognised as having nematicidal effects although the precise compound(s) remain uncertain (Akhtar 2000; Chitwood 2002). Neem products, particularly the seed and the residues after oil extraction (neem cake) are widely used as a soil amendment. Crops in such amended soil benefit from the release of anti-microbial and insecticidal compounds and plant nutrients (Schmutterer 1990). In greenhouse trials, addition of 1% neem cake to soil caused a 67–90% reduction in the number of lesions (*Pratylenchus penetrans*) and root-knot nematodes (*Meloidogyne hapla*) in tomato roots grown in three different soils (Abbasi et al. 2005).

This glasshouse study was intended to evaluate the combination of spores of *P. penetrans* with neem products on the efficacy of root-knot nematode management in soils containing different initial nematode densities over three crop cycles.

Materials and methods

The neem products were dry leaf and cake (a by-product left after the extraction of oil from neem seed). Fresh neem leaves were collected from trees in Pakistan and neem cake was imported from India and had been stored in a metal container in a refrigerator for 6 months. In brief, leaves were dried in the sun for 10 days and then converted into powder using a Glen Creston grinder (Dalton Garden, Stanmore, UK) fitted with a 2 mm pore size sieve. Neem cake was also passed through the same process. The moisture contents of dry leaves and cake formulations were 7.37 and 6.34%, respectively. The resulting products were stored in metal containers at 4°C. From preliminary experiments, it was decided to apply neem leaves and cake powder at 2 and 3% w:w, respectively, in 1.3 kg loam-based proprietary compost, John Innes No. 2 (Roffey Ltd., Dorset, Bournemouth, UK); a standard combination of loam, peat, sand, lime, fertilizers and traces of elements. Thorough mixing was achieved by shaking in a plastic drum for 2–3 min prior to placing in the pots.

Pasteuria penetrans (a population originally from South Africa and found on *M. javanica*) was prepared as a spore-laden powder of tomato roots following the method of Stirling and Wachtel (1980). A subsample of 0.5 g of this powder was ground in a pestle and mortar in 10 ml water; the slurry was then suspended in 100 ml water. The root debris from the slurry was removed by sieving the suspension through 38 μm aperture mesh and the volume of spore suspension was made up to 500 ml. For the *Pasteuria* treatments 10 ml of this suspension was mixed in 10 g of fine sand and then this sand was mixed in 1.3 kg soil by shaking in a plastic drum as previously described. The concentration of spores applied was equivalent to approximately 10,000 spores g^{-1} of soil. The treatments used for nematode control were neem cake, neem leaf, *P. penetrans* and neem cake + *P. penetrans*. After filling pots with the amended soil, each was placed in a saucer and irrigated by adding water to the saucer; this system of watering was maintained throughout the experiment.

After 6 days the pots were inoculated with approximately 500, 5000 or 10,000 freshly hatched *M. javanica* juveniles. Control plants were uninoculated or inoculated only with nematodes. Two days after infestation, 6-week-old tomato seedlings (with five to six true leaves) were transplanted into these pots. Each treatment was replicated five times. The pots were completely randomised in a heated glasshouse in which the temperatures were generally 20–32°C but extremes of 17°C and 43°C were recorded during a few nights/

days of the experimental period. The plants were harvested after 64 days (May–July); a second crop was grown for 59 days (July–September) and then a third crop for 67 days (September–December). Natural light was supplemented with 400 W mercury halide lamps during the third crop (September–December). When necessary the plants were protected against thrips and red spider mites using the biocontrol agents *Amblyseius cucumeris* and *Phytoseiulus persimilis*, respectively (Novartis BCM, England).

Assessment of plant growth and nematode damage

Plants were not watered one day before harvesting when they were removed from pots and the root ball shaken until most of the soil had been dislodged from the roots. This soil was put back into the same pots. The roots were washed and fresh root and shoot weights were taken. The roots were assessed for intensity of root-galling on a 0–10 scale (Bridge and Page 1980) and placed in a solution of phloxin B to stain egg-masses (Hartman and Sasser 1985). Then these roots were cut into 1 cm pieces, mixed thoroughly and numbers of egg masses were counted on a 1 g subsample. After taking subsamples half of the root system was reincorporated into half of the soil from the pot and then this soil was diluted with an equal volume of fresh soil and placed back into the pots. The pots were kept covered for 3 days in the glasshouse before replanting with 6-week-old tomato seedlings (three to five true leaves) for the second and third crops.

Assessment of juveniles in soil

The remaining half of the soil from each pot was mixed with the other half of the root system and kept in plastic bags. Four days later a 50 ml subsample of soil was taken and placed on extraction trays (Southey 1986) to assess nematode density prior to planting the next crop. Juveniles were collected after 24 h. After counting the total number of nematodes, half of the nematode suspension was poured in an open counting dish and spore attachment was recorded on the first 20 juveniles encountered.

Assessment of infected females

The 1 g subsamples of root were placed in a freezer overnight to soften the root tissue. Upon defrosting they were cut into small pieces and were dissected in water to release the females. This suspension was placed in a counting dish and the first 20 females were picked singly onto glass slides and crushed under a cover slip. Females were observed at ×400 for the presence of endospores.

A similar procedure was repeated before and after planting the second and third crops. During the third crop some plants senesced prematurely.

Statistical analysis

During the third crop cycle some of the plants in the neem cake, neem leaves and control treatments were dead and therefore data analysis was based on missing values. Data on number of egg masses g^{-1} of root, egg masses per root system and number of juveniles per 50 ml soil were log transformed [$\log_{10}(x+1)$]. The data were subjected to analysis of variance (ANOVA) by using GenStat 8th Edition (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK). If P values indicated a significant difference ($P \leq 0.05$), means were separated by Fisher's protected least significant difference (LSD) test.

Results

The number of *Pasteuria*-infected females was lower in the second and third cropping cycles but even in the third cycle, 30% of the females in the root system were infected with *P. penetrans* (Fig. 1a). In all treatments except neem leaf and cake, the number of egg masses g^{-1} of root increased in the second and third cropping cycles (Fig. 1b).

The numbers of spore-encumbered juveniles (1–10 spores per juvenile) recovered throughout the course of experiment declined from approximately 60% in the first crop to 24% in the *P. penetrans* only and 8% in the *P. penetrans* + neem cake in the third crop (data not presented).

The root and shoot weights were not affected by the different inoculum densities (Fig. 2a,b; Tables 1 and 2). In all treatments including the uninoculated control the root and shoot fresh-weights in the second and third crops were significantly lower than those in the first (Fig. 2a,b; Table 3). The treatments had no effect on root growth in the first crop cycle but in the third crop, the *P. penetrans* + neem cake treatment

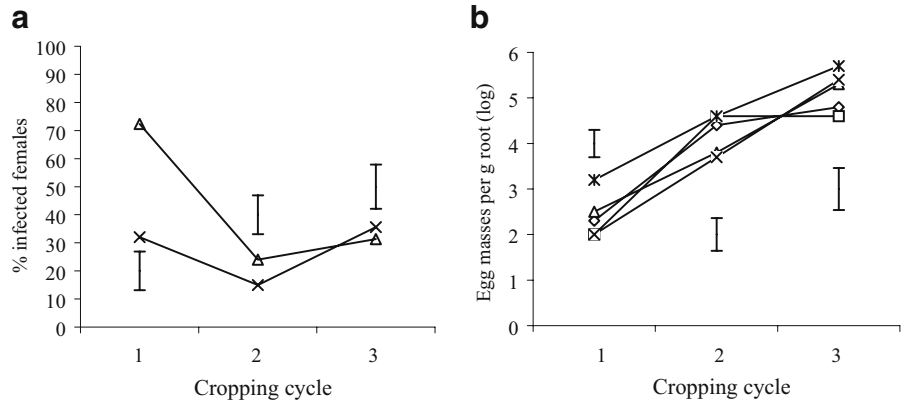

Fig. 1 Effect of *Pasteuria penetrans* and neem formulations on: (**a**) Percent of *Pasteuria*-infected root-knot nematode females in tomato root systems over three crops, (**b**) Root-knot nematode egg masses g^{-1} of root over three crops. (*asterisk*) Nematodes only, (*open triangle*) *Pasteuria penetrans* + nematodes, (*open square*) Neem leaves + nematodes, (*open diamond*) Neem cake + nematodes, (*multiple*) *P. penetrans* + neem cake + nematodes. Data of each crop cycle are the means of five replications of each initial nematode inoculum density (each with five subsamples). Error bars represent standard error of differences of means (SED)

had significantly heavier roots and shoots than the nematode-only control (Fig. 2a,b). With the neem leaves and neem cake treatments, root and shoot weights were generally similar to those plants inoculated only with *M. javanica* (Fig. 2a,b). Also, 10 plants (out of 30) died prematurely in these treatments and two (out of 15) in the nematode-only control; there were no plant losses in the treatments with *P. penetrans*. The death of these plants can be attributed to the greater intensity of root damage caused by the root-knot nematodes in these treatments (Fig. 3). However, in the *M. javanica* infested plants the percent decreases in root weights in the *P. penetrans* + cake and *P. penetrans* treatments over the uninoculated control were 22 and 48%, respectively; less than those without *P. penetrans* (Table 1). Root-galling was less severe in the *P. penetrans* + cake and *P. penetrans* treatments (Fig. 3); a difference that was significant at the end of the third crop. By the end of the third crop, the numbers of egg masses g^{-1}

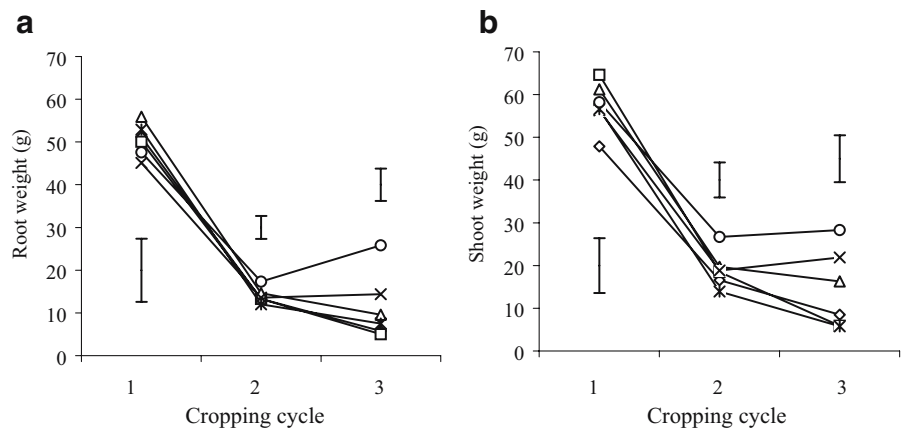

Fig. 2 Effect of *Pasteuria penetrans* and neem formulations on root and shoot weights of tomato plants over three crops. (**a**) Root weights, (**b**) Shoot weights. (*open circle*) Uninoculated plants, (*asterisk*) Nematodes only, (*open triangle*) *Pasteuria penetrans* + nematodes, (*open square*) Neem leaves + nematodes, (*open diamond*) Neem cake + nematodes, (*multiple*) *P. penetrans* + neem cake + nematodes. Data of each crop cycle are the means of five replications of each initial nematode inoculum density (each with five subsamples). Error bars represent standard error of differences of means (SED)

Table 1 Effect of *Pasteuria penetrans* and neem formulations on root weight of tomato in artificially infested loam-based compost soil with root-knot nematodes over three crop cycles

Treatment	% Decrease in root weight over uninoculated control									Over all means
	Initial inoculum levels of root-knot nematodes									
	500			5,000			10,000			
	Cycle1	Cycle2	Cycle3	Cycle1	Cycle2	Cycle3	Cycle1	Cycle2	Cycle3	
Cake	−5.6	−10	81.4	−11.1	39.9	72.6	−23.6	16.0	73.2	25.9
Leaves	−24.6	−0.2	90.6	5.5	14.5	68.9	−10.2	28.6	70.0	27.0
Pp	−28.4	−16.0	69.1	30.0	30.3	63.1	−15.6	10.2	47.7	14.5
Pp + cake	3.6	12.2	57.2	−7.2	12.9	46.4	−3.3	19.8	22.2	18.2
Nematodes only	−4.5	19.5	65.2	−25.7	4.0	78.8	−32.9	54.4	63.3	23.7

Each value is an average of results from five replications.

Pp= P. penetrans

of root were generally higher in the nematodes-only and *Pasteuria* treatments although this difference was not always significant ($P \leq 0.05$; Fig. 1b).

Discussion

This glasshouse pot experiment was done to assess the potential of combining *P. penetrans* and neem (leaves and cake) formulations as a management system for root-knot nematodes and growth of tomato plants over three crop cycles. *Pasteuria penetrans* can suppress root-knot nematodes; this has been reported under natural conditions and in micro-plot trials (Stirling 1991; Trudgill et al. 2000). The nutritional and pesticidal properties of neem are well documented (Schmutterer 1990) and there are numerous articles that claim to demonstrate the nematicidal effects of neem (Akhtar 2000). Many farmers in India and Pakistan consider that the application of neem cake is a pre-requisite to ensure a profitable crop. *Pasteuria penetrans* is not capable of producing the rapid 'knock-down' effect that is associated with a nematicide; however in the longer term it is capable of limiting nematode egg production. Gowen et al. (1998) showed decreased numbers of egg masses in a two crop-cycle pot experiment where initially, soil had been amended with 1.1 and 5.5×10^4 *P. penetrans* spores cm^{-3} soil. Neem products have been shown to have an effect on juvenile behaviour resulting in a

Table 2 Effect of *Pasteuria penetrans* and neem formulations on shoot weight of tomato in artificially infested loam-based compost soil with root-knot nematodes over three crop cycles

Treatment	% Decrease in shoot weight over uninoculated control									Over all means
	Initial inoculum levels of root-knot nematodes									
	500			5,000			10,000			
	Cycle1	Cycle2	Cycle3	Cycle1	Cycle2	Cycle3	Cycle1	Cycle2	Cycle3	
Cake	15.3	19.8	82.9	17.4	45.0	50.3	11.6	34.5	76.0	39.1
Leaves	−14.6	−3.8	91.9	−6.3	52.4	71.0	−19.4	26.8	62.4	28.9
Pp	−10.2	−21.00	62.4	−7.6	51.9	58.0	−4.7	24.7	−1.3	16.9
Pp + cake	−16.0	10.3	51.4	2.3	30.0	14.3	11.9	37.1	−7.7	14.8
Nematodes only	16.0	30.4	80.4	−20.0	23.3	86.9	3.9	67.3	70.8	39.9
LSD										13.99
Probability										$P \leq 0.05$

Each value is an average of results from five replications.

Pp= P. penetrans

Table 3 % Decrease in root and shoot weight in all *Pasteuria penetrans* and neem formulation treatments over the uninoculated control over three crop cycles

	Root weight	Shoot weight
Cycle 1	−14.2	−1.3
Cycle 2	15.1	28.5
Cycle 3	64.6	56.6
LSD	12.41	10.83
Probability	$P \leq 0.05$	$P \leq 0.05$

Each value is an average of results from five replications of all treatments.

reduction in nematode invasion (Akhtar 2000), for this reason it may well complement treatments with *P. penetrans*.

Unexpectedly there was no effect of the different initial nematode densities; it was thought that the neem treatments would have had a greater effect on the nematode infection in the first crop cycle. The differences in initial nematode density were also not reflected in significant differences in root-galling or plant growth. In hindsight, an assessment of the infection success at the different inoculum densities a week after inoculation would have been useful. Using root weight as an assessment of plant health is of dubious value when working with root-knot nematodes due to the increased weight resulting from the galling response, often resulting in infested plants being heavier than the controls. Nevertheless, the nematode burden that developed over the three crops at all densities was sufficient to cause plant damage, particularly by the end of the third crop. *Pasteuria* is seen as a long-term strategy, requiring more than one crop to establish. In the first crop cycle the initial spore-density of 10^4 spores gave encouraging results with >70% of females in the *Pasteuria*-only treatment infected. Significantly fewer females (32%) were infected in the combined *Pasteuria* and neem treatment; it is conceivable that the spore-encumbered juveniles were more susceptible to the effects of the neem. The data presented have shown that by the third crop cycle *P. penetrans* had a significant effect on plant growth and root-galling.

Additionally, in the third crop cycle 30% of the females in *Pasteuria* treatments were infected. The length of the crop cycles was probably too short to allow the maximum reproduction of the bacterium under the glasshouse conditions in the UK at that time of year. Darban et al. (2004) showed that numbers of spores in infected females were >2 million and increasing at 88 days after inoculation of plants in similar growing conditions. It is possible that in this experiment a longer growing period might have led to greater spore numbers and greater chances of the release of the spores from spore-filled cadavers. If this was the case, the levels of infection might have been higher. At temperatures below 20°C, *P. penetrans* may take >100 days to complete its life cycle (Stirling 1981). Slow release of spores from the cadavers of the root-knot nematode females during the second and third crops may have accounted for less spores accumulating in the system (Pembroke et al. 1998; Giannakou et al. 1997). The spore density would have been less in the second and third crops because of the replacement of half the soil with fresh soil before replanting. Spore attachment does not necessarily result in nematode infection; Pembroke (personal communication) found that even if all juveniles were encumbered with (a mean of) six spores there was

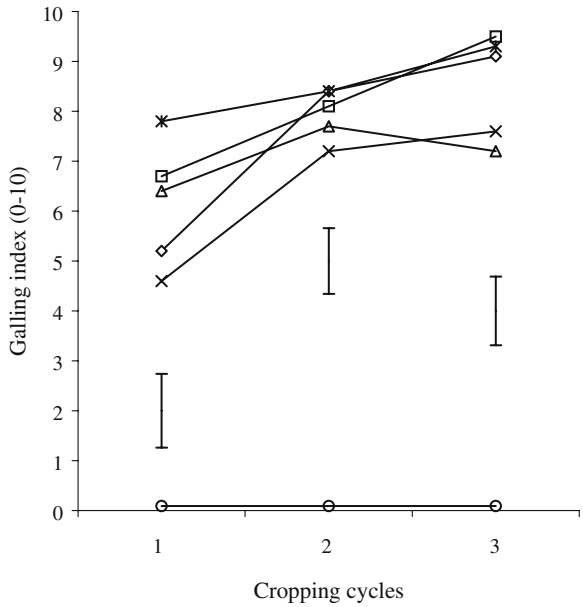

Fig. 3 Effect of *Pasteuria penetrans* and neem formulations on root-galling index over three crops. (*open circle*) Uninoculated plants, (*asterisk*) Nematodes only, (*open triangle*) *Pasteuria penetrans* + nematodes, (*open square*) Neem leaves + nematodes, (*open diamond*) Neem cake + nematodes, (*multiple*) *P. penetrans* + neem cake + nematodes. Data of each crop cycle are the means of five replications of each initial nematode inoculum density (each with five subsamples). Error bars represent standard error of differences of means (SED)

only 60% infection of the females that subsequently developed in the roots. This might account for the decline in numbers of spore-encumbered juveniles in the second and third crops and possibly the relatively high numbers of egg masses in the *Pasteuria* treatments. This suggests that large numbers of unencumbered juveniles were invading the roots and developing to maturity in the healthier plants.

In the third cycle, plants treated only with neem products showed relatively greater levels of root galling than those with the *Pasteuria* which suggests that the nematicidal compounds in these amendments had disappeared. It may be significant that no plants died in *Pasteuria* treatments and the plants from these treatments were the most vigorous and had significantly lower galling damage. Because of this, the concept of combining neem with *P. penetrans* in a long-term root-knot nematode management strategy is worthy of further development and should be evaluated over longer crop cycles so that the potential for increasing spore densities of *P. penetrans* can be realised. In Pakistan and India, where use of neem is an established practice, frequently repeated treatments would supplement the management achieved with *P. penetrans*.

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