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ORIGINAL PAPER

Pasteuria, a bacterial parasite of plant-parasitic nematodes: its occurrence in Australian sugarcane soils and its role as a biological control agent in naturally-infested soil

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Abstract In a survey of all sugar production areas in Australia, *Pasteuria* was detected in 56% of the fields sampled. Endospores were seen on root-knot nematode (*Meloidogyne* spp.), root-lesion nematode (*Pratylenchus zeae*), stunt nematode (*Tylenchorhynchus annulatus*) and spiral nematode (*Helicotylenchus dihystera*). In most cases infestation levels were relatively low, as less than 5% of the nematodes usually had spores attached. However, the results of a bioassay with soil from a site heavily-infested with *P. penetrans* suggested that root-knot nematode was being suppressed to some extent at this site. When second-stage juveniles of *M. javanica* had to move 4 and 8 cm through the soil to reach sugarcane roots, egg production was reduced by 55 and 85%, respectively. Sugarcane had been grown at most of the surveyed sites for more than 100 years but the highest levels of spore encumbrance on root-lesion nematode were observed in two fields that were previously grass pasture and had only grown sugarcane for 18 and 22 years. Pasture and sugarcane soil from one of these sites was bioassayed by inoculating *Pratylenchus zeae* into soil that had been heated at 60 °C to kill any nematodes present. When the added nematodes were extracted 40 days later, approximately 50% were either parasitised by *Pasteuria thornei* or had endospores attached, indicating that the biocontrol agent was present at relatively high levels in both soils. Since grazed pastures are never tilled and the two sugarcane fields had been subjected to much less tillage than the other surveyed sites, research should be undertaken to determine whether the tillage practices commonly used in sugarcane are limiting the capacity of *Pasteuria thornei* to increase to high levels and provide some nematode control.

Keywords *Meloiodgyne* · *Pratylenchus* · *Pasteuria penetrans* · *Pasteuria thornei* · Suppressive soil · Pasture · Tillage

Introduction

The bacterial genus *Pasteuria* contains a group of obligate parasites of invertebrates, many of which parasitise nematodes (Dickson et al. 2009). Hundreds of nematode-attacking strains have been recognised, with each strain relatively specific to particular hosts (Chen and Dickson 1998). *Pasteuria* is known to infect all important nematode pests of agricultural crops and because it prevents its parasitised host from reproducing, and produces endospores that are resistant to environmental stresses such as heat and dryness, it is a potentially useful biocontrol agent (Stirling 2014).

Pasteuria is found worldwide and several studies have shown that it can multiply to levels capable of suppressing plant-parasitic nematodes. *P. penetrans* is widespread in South Australian vineyards (Stirling and White 1982) and was found to be largely responsible for the decline in root-knot nematode (*Meloidogyne* spp.) populations that occurs as vineyards age (Stirling 1984; Bird and Brisbane 1988). In Florida, *P. penetrans* was able to suppress root-knot nematode in soils used for tobacco and peanut production (Chen et al. 1994; Weibelzahl-Fulton et al. 1996; Dickson 1998), while a 7-year study in Illinois showed that *P. nishizawae* reduced populations of soybean cyst nematode (*Heterodera glycines*) by an average of 43% (Noel et al. 2010).

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Although sugarcane is widely grown in topical and subtropical regions of the world and plant-parasitic nematodes are important pests (Cadet and Spaull 2005), *Pasteuria* has rarely been studied in sugarcane soils. The first observations were made by Williams (1960), who noticed that the bodies of about one-third of the root-knot nematode females in a sugarcane field in Mauritius were tightly packed with innumerable numbers of spores. Infected nematodes were milky white in colour and easily distinguished from healthy hosts, which were dull in colour and more or less translucent. Williams believed the parasite was a protozoan and referred to it as *Duboscqia penetrans*, but it was later found to be prokaryotic (Mankau 1975a, b) and eventually renamed *Pasteuria penetrans* (Sayre and Starr 1985; Starr and Sayre 1988).

At about the same time as these taxonomic studies were being undertaken, *P. penetrans* was found in sugarcane fields in several countries, including South Africa, the United States and Papua New Guinea (Spaull 1981, 1984; Birchfield 1984; Bridge 1986). These results, together with those of Williams (1960), showed that the bacterium was relatively common in coarse-textured sugarcane soils where root-knot nematode is usually the dominant nematode pest.

In Australia, root-knot nematode and several other plant-parasitic nematodes are widely distributed on sugarcane (Blair et al. 1999a, b; Blair and Stirling 2007), but little is known about the distribution of *Pasteuria* in sugarcane fields. Thirty fields in the Bundaberg region were surveyed in 2008 and 43% of the root-knot nematodes and 33% of the root-lesion nematodes (*Pratylenchus zeae*) were found to be encumbered with endospores (Stirling 2009; Stirling unpublished data). However, in most of these fields, less than 5% of the nematodes had spores attached and infested nematodes were usually encumbered with only 1 or 2 spores. Given the localised nature of the previous survey and the limited number of sites sampled, a more comprehensive survey was undertaken and the results are presented in this paper.

Although surveys of this nature are useful, they are based on attachment of endospores to the nematode cuticle rather than parasitism. Thus, once the survey was completed, soil from the sites most heavily-infested with *Pasteuria* was bioassayed to determine whether the bacterium was able to parasitise the nematode and produce endospores within the body of its host. A second objective was to determine whether *Pasteuria* was likely to be suppressing populations of the plant-parasitic nematodes present at the selected sites. The nematodes used in the bioassays were *M. javanica* and *P. zeae*, the most important nematode pests of sugarcane in Australia (Blair and Stirling 2007).

Host-specific suppressive agents such as *Pasteuria* tend to be found more commonly in perennial crops than annual crops, presumably because their host nematode is always present and the soil environment is not disturbed by tillage (Stirling 2014; Stirling et al. 2016). Since nematodes capable of hosting *Pasteuria* have been present in many Australian sugarcane fields for more than 100 years, a further objective of this study was to consider why the bacterium has generally not increased in cane-growing soils to levels capable of providing some nematode control.

Methods

Survey for *Pasteuria*

Soil samples (a composite of at least 15 cores to a depth of 15 cm from an area of about 1 ha) were collected from 126 fields in all cane-growing districts of Queensland and New South Wales. To avoid soils that had recently been disturbed by tillage, the crops sampled were generally in their third to seventh ratoon. Nematodes were extracted by spreading a 200 mL sample of each soil on a tray (Whitehead and Hemming 1965) and after incubation for two days at 24–28 °C, nematodes were retrieved by sieving twice on a 38 μm-aperture sieve. All remaining soil was air-dried and kept for future analyses. The nematode suspension obtained from each sample was concentrated to a volume of less than 0.5 mL and a drop was placed on a slide and covered with a cover slip. A representative sample of the four most widely distributed nematodes was then checked under a microscope for the presence of *Pasteuria* endospores. Root-knot and root-lesion nematodes were examined together with stunt nematode (*Tylenchorhynchus annulatus*) and spiral nematode (*Helicotylenchus dihystera*). Between 50 and 80 nematodes were usually examined at each site, with the number checked for each species dependent on their population density.

A bioassay with soil naturally-infested with *P. penetrans*

A sandy loam soil from a field about 3 km south-east of Port Bundaberg was chosen for further study because the survey results indicated that it was heavily infested with *P. penetrans*. Further soil was collected from this site and the tube technique of Stirling (1984) and Stirling et al. (1990) was used to determine whether the endospore concentration was high enough to affect multiplication of root-knot nematode. Sugarcane plantlets (var. Q208) grown from single-eye setts were planted in pots filled with pasteurised sand and a single 25 mm diameter tube of various lengths was placed vertically in each pot. The tubes were filled with soil from the Port Bundaberg field, with autoclaved soil from the same field used as a non-infested control. Thus, the experiment consisted of two *Pasteuria* treatments (naturally-infested soil and autoclaved soil) × 4 tube lengths (1, 2, 4 and 8 cm) × 5 replicates.

The pots, together with the tubes they contained were set up in a glasshouse using a randomised block design and the plants were left for 5 weeks to establish a root system. A

0.5 mL suspension containing about 5000 eggs and second-stage juveniles of *M. javanica* (cultured on tomatoes in the greenhouse) was then added to the top of the tube in each pot, which meant that the nematodes had to move down the full length of the tube to reach the sugarcane roots. Plants were grown for a further 51 days and then roots were removed from the pots and checked for the presence of parasitised females of root-knot nematode. Roots were then submerged in 1% NaOCl for 5 min and nematode eggs were retrieved on a 38 μm-aperture sieve.

Further assessment of soils where *Pasteuria* was detected on *Pratylenchus*

Results from the survey showed that the sites with the highest levels of spore encumbrance on *P. zeae* were located at Mareeba and Mutarnee in north Queensland and so air-dried soil that had been retained was used to check whether parasitised nematodes were present at these sites. The soils were moistened with water and 2 days later, nematodes were extracted from 25 g sub-samples using a centrifugal-flotation technique in which the sucrose concentration had been increased to a specific gravity of 1.26 (1362 g sucrose in 1 L of water) so that spore-filled nematodes would be recovered (Oostendorp et al. 1991).

Background information on the Mareeba and Mutarnee sites indicated that they had only grown sugarcane for about 20 years, which was much less than the other surveyed sites. Since cattle had previously grazed on grass pastures at both sites and pastures in adjacent fields were still being used for the same purpose, they were checked for the presence of *Pasteuria* and *Pratylenchus*. Paired samples from both the sugarcane fields and adjacent fields under grass pasture were collected and the samples were processed in the same way as in the survey. The pasture at the Mareeba site was dominated by Guinea grass (*Megathyrsus maximus*) while the grasses in the Mutarnee pasture were Pangola *Brachiaria* (*Digitaria eriantha*) and Creeping signal grass (*Brachyaria humidicola*).

A bioassay was also set up to compare levels of *Pasteuria* infestation in the sugarcane and pasture soils from Mareeba. A small quantity (10 g) of air-dried soil was added to 50 mL centrifuge tubes and the soils were then moistened with water. Since previous observations had shown that some plant-parasitic nematodes survived the air-drying process, the tubes containing the moistened soil were kept for a day to allow any surviving nematodes to revive and then heated at 60 °C to kill them. Each tube was inoculated with 500 *P. zeae* obtained from carrot cultures (Moody et al. 1973) and the open tubes were then placed in a humid chamber to prevent the soil from drying and incubated at a temperature of 26 °C for 40 days. A centrifugation and sugar flotation technique was then used to extract the nematodes, except that the method of Oostendorp et al. (1991) was modified to reduce the number of fine organic particles recovered with the nematodes. Thus, the soil from a tube was first washed into a beaker containing about 50 mL of water and decanted over a 710 μm-aperture sieve to remove most of the large particles of organic matter. After a second decanting process, the liquid passing through the sieve was washed into 100 mL tubes and centrifuged at 3000 rpm for 4 min. The supernatant was then poured off, a concentrated sugar solution was added (specific gravity 1.26) and the sample was re-centrifuged at 1750 rpm for 30 s. The nematodes in the supernatant were retrieved on a 38 μm-aperture sieve, washed free of sucrose and checked for *Pasteuria* under a microscope.

Statistical analyses

For the bioassay with root-knot nematode and different tube lengths, the data were analysed by ANOVA using GenStat 8.1 (Genstat 2005). Relationships between nematode egg production and the distance moved in soil were determined using the regression analysis feature in Microsoft Excel.

Results

Survey for *Pasteuria*

Pasteuria was found in all regions where sugarcane is grown and was detected in 56% of the 126 fields sampled. Endospores were seen attached to root-knot, root-lesion, stunt and spiral nematodes (Table 1). In most cases less than 5% of the nematodes were infested and most nematodes only had one endospore attached. However, there were three fields where more than a third of the root-knot nematodes were encumbered with endospores of *P. penetrans* and at those sites some nematodes had more than 10 spores attached.

P. zeae was by far the most common nematode observed in the survey but in most cases relatively few nematodes were encumbered with endospores. The highest levels of encumbrance were observed in fields at Mareeba and Mutarnee where 33 and 21% of the nematodes, respectively, had endospores attached. Interestingly, these fields had only grown sugarcane for 18 and 22 years, respectively, whereas the other surveyed sites had grown sugarcane for at least 50 years and in most cases for more than 100 years.

Most of the fields surveyed were farmed conventionally (i.e. the crop was ploughed out after about 5 years, the soil was tilled and sugarcane was replanted 2–6 months later) but a new farming system that incorporated wide row spacings, controlled traffic, minimum tillage and legume rotation crops (Stirling 2008) was being used in 14 of the fields. *Pasteuria* was detected in 71% of these fields, a much higher percentage infestation than the conventionally-managed fields. Those

Table 1 Occurrence of nematodes encumbered with *Pasteuria* endospores in a survey of 126 Australian sugarcane fields

	Root-knot *Meloidogyne* spp.	Root-lesion *Pratylenchus zeae*	Spiral *Helicotylenchus dihystera*	Stunt *Tylenchorhynchus annulatus*
No. of fields with the nematode	33	119	108	96
% of the nematode-infested fields with *Pasteuria*	30	47	15	19
Total no. of nematodes examined	484	3647	1273	1011
No. of nematodes with *Pasteuria*	53	146	41	54
% of nematodes examined encumbered with endospores	11.0	4.0	3.2	5.3
Highest level of encumbrance[a]	56	33	43	64

[a] Highest percentage of nematodes encumbered with endospores in a sample of at least 10 nematodes

fields also tended to have the highest levels of spore encumbrance and often two or three nematode species had spores attached. For example, in one field where the new farming system had been in place for 15 years, *Pasteuria* was observed on *P. zeae*, *H. dihystera* and *T. annulatus*, with more than 5 endospores attached to some specimens of the latter species.

A bioassay with soil naturally-infested with *P. penetrans*

Root-knot nematode females parasitised by *P. penetrans* were observed in the roots of bioassay plants but were only found in galls where no egg mass was apparent, and were only observed in pots where the nematode had been inoculated into 4 or 8 cm tubes filled with non-autoclaved soil. Analyses of the nematode egg data obtained from these roots showed that both tube length and the presence of *P. penetrans* had significant effects on the number of eggs recovered ($P = 0.041$ and 0.029, respectively). Fewer eggs were recovered as tube length increased and the presence of *P. penetrans* reduced the number of eggs recovered by 49%. These effects are apparent in Fig. 1. In *P. penetrans*-free soil, the distance travelled by second-stage juveniles did not affect the number of eggs produced ($P = 0.307$) whereas in soil naturally infested with *P. penetrans* there was a significant reduction in egg production ($P = 0.012$) as distance increased (Fig. 1a, b).

Further assessment of soils where *Pasteuria* was detected on *Pratylenchus*

When two soils with the highest levels of spore encumbrance in the survey were checked to see whether they contained parasitised nematodes, spore-filled cadavers were recovered from both sites. *P. zeae* was the only parasitised nematode seen at Mareeba whereas at the Mutarnee site, endospores of *Pasteuria* were observed in *P. zeae*, *T. annulatus* and *H. dihystera*. An example of a spore-filled cadaver of *P. zeae* that was squashed under a cover slip to partly release the endospores is shown in Fig. 2.

When nematodes were extracted from paired sites under grass pasture or sugarcane, *P. zeae* and *P. brachyurus* were recovered from the pasture sites at both Mareeba and Mutarnee whereas only *P. zeae* was found in the adjacent sugarcane fields. *Pasteuria* was detected in all four soils and levels of spore encumbrance in the pasture and sugarcane soils were similar, as 14 and 15% of the *Pratylenchus* from Mareeba and 34 and 29% of the *Pratylenchus* from Mutarnee had endospores attached, respectively. In the pasture soils, *Pasteuria* was observed on both *Pratylenchus* species.

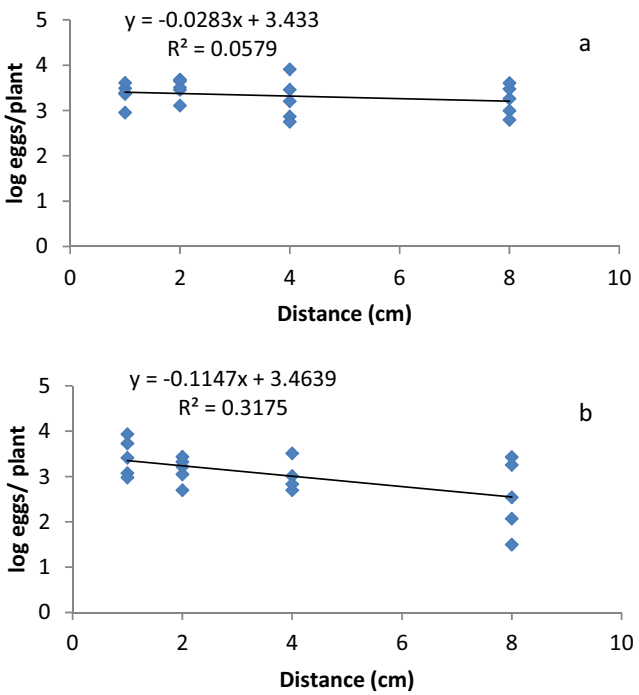

Fig. 1 Relationships between egg production by *Meloidogyne javanica* on sugarcane and the distance travelled by juvenile nematodes through soil where *Pasteuria* had been removed by autoclaving (**a**) and through *Pasteuria*-infested soil (**b**)

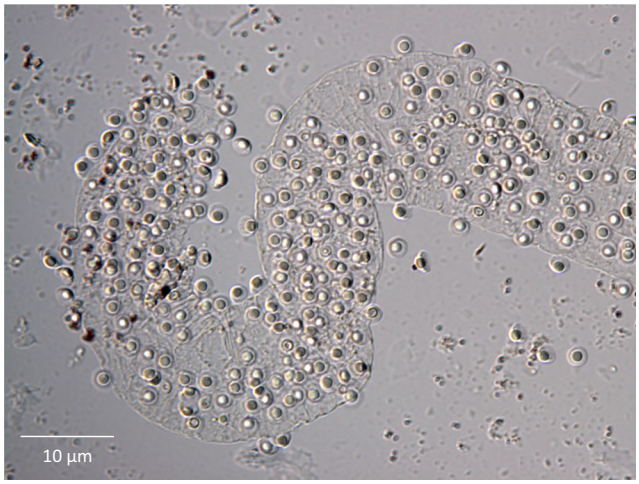

Fig. 2 Endospores of *Pasteuria* associated with a cadaver of root-lesion nematode (*Pratylenchus zeae*) recovered from a sugarcane field at Mutarnee, Queensland

In the bioassay in which *P. zeae* was inoculated into defaunated pasture and sugarcane soils from Mareeba and extracted 40 days later, *Pasteuria* endospores were observed within the bodies of some nematodes and on the cuticles of others (Table 2). *Pasteuria* levels were relatively high in both soils, as around 50% of the added nematodes had either been parasitised or had endospores attached.

When *P. zeae* was parasitised by *Pasteuria*, endospores usually completely filled the body of the nematode but there were cases where up to a third of the body had few or no endospores. Some infected individuals were dead but more commonly, nematodes packed with endospores were still moving slowly. Most of the infected nematodes were females that had not reproduced, as eggs were never observed in their ovaries. Spores within the body were so closely-packed that it was impossible to count the number of endospores in spore-filled nematodes, but estimates from a limited number of samples suggested there were 180–200 endospores in the body cavity of juveniles and 400–650 endospores in parasitised females.

Table 2 Percentage of *Pratylenchus zeae* infected by *Pasteuria* or encumbered with *Pasteuria* endospores 40 days after the nematodes were added to heat-treated soil from adjacent sugarcane or Guinea grass pasture fields near Mareeba, Queensland

	Infected (%)	Spore encumbered (%)	Total with *Pasteuria* (%)
Sugarcane	36.9 ± 6.6	13.4 ± 3.9	50.3 ± 9.1
Pasture	24.5 ± 4.6	18.2 ± 6.9	42.7 ± 7.3

Discussion

Our survey results indicate that *P. penetrans* is reasonably common in Australian sugarcane soils, as it was found in 30% of the fields where root-knot nematode was detected. However few of the second-stage juveniles extracted from soil were encumbered with endospores, indicating that infestation levels were generally relatively low. Nevertheless, relatively high levels of spore attachment were observed in a few fields and this finding prompted us to check whether *P. penetrans* was present at levels that were high enough to provide some control of the nematode. Since the probability of an endospore adhering to a second-stage juvenile increases with spore concentration and the distance the nematode moves before entering a root, we added nematodes to tubes of different length and assessed their capacity to move to roots and reproduce (Stirling 1984; Stirling et al. 1990). The results in Fig. 1 showed that the presence of *P. penetrans* had little impact on the nematode when second-stage juveniles only had to move 1 cm to a root but egg production was reduced by about 55 and 85% when tube lengths were increased to 4 and 8 cm, respectively.

Although the results from our bioassay suggest that *P. penetrans* can increase in sugarcane soil to levels capable of reducing populations of root-knot nematode, marked reductions in egg production were only obtained when second-stage juveniles moved more than 4 cm to roots. Since sugarcane has a fibrous root system and most juvenile nematodes are likely to establish feeding sites after moving relatively short distances to roots, it is not clear whether the spore concentration in the field where this soil was collected was high enough to provide useful levels of nematode control. Results of experiments aimed at addressing that issue can be found in an accompanying paper (Bhuiyan et al. 2017).

P. penetrans is the most widely studied species of *Pasteuria* because its root-knot nematode host is a serious pest of most of the world's food and fibre crops. However, many other nematodes also host the bacterium, with a review by Chen and Dickson (1998) indicating that *Pasteuria*-like organisms were associated with 323 nematode species in 116 genera. Since Spaull (1981) observed *Pasteuria* endospores on eight nematode genera other than *Meloidogyne* in South African sugarcane fields, it was not surprising that we found endospores attached to *P. zeae*, *T. annulatus* and *H. dihystera* in Australia. However, as *P. zeae* is probably the most important nematode pest of sugarcane worldwide (Stirling 2017), perhaps the most significant finding was that *Pasteuria* was found at almost half the sites where this nematode was detected.

On the basis of morphological and developmental differences between *P. penetrans* and the bacterium parasitising *Pratylenchus*, the species which attacks root-lesion nematodes

was named *Pasteuria thornei* (Starr and Sayre 1988; Sayre et al. 1988). However, despite the fact that *Pratylenchus* spp. are important pests of many crops and numerous species in the genus are known to host *Pasteuria* (Sayre and Starr 1988; Chen and Dickson 1998), high levels of infestation have rarely been reported in the literature. One exception was a study by Ornat et al. 1999, who found more than 75% of the *Pratylenchus neglectus* in a Spanish greenhouse were encumbered with *Pasteuria* spores. Spores were also observed filling the body cavity of fourth-stage juveniles and adult females.

The bioassay results obtained in this study clearly show that a significant proportion of the root-lesion nematodes in some sugarcane and pasture fields are affected by the parasite, as approximately 50% of the *P. zeae* added to soils from one site were either encumbered with endospores of *Pasteuria thornei* or parasitised by the bacterium. However, it is likely that we underestimated levels of parasitism because the extraction method used probably did not recover all infected nematodes. Also, spore-filled nematodes were difficult to find amongst the many fine particles of organic matter that were recovered with the nematodes.

Our results also add to our knowledge of the relationship between *Pasteuria thornei* and its nematode host. When *P. zeae* was added to soil infested with the bacterium, we found that it took about 40 days at 26 °C for the parasite to infect the nematode, grow within the body and sporulate. Some spore-filled nematodes were still moving when they were recovered, suggesting that the parasitised nematodes continued to feed as infection progressed. Few estimates of spore production by *Pasteuria* in vermiform nematodes have been published but our observations suggested that the bodies of spore-filled females usually contained about 600 endospores. This is much lower than the several thousand spores observed in cadavers of *Belonolaimus longicaudatus*, a much larger nematode (Giblin-Davis et al. 2001).

Although *Pasteuria thornei* was found to be reasonably widespread in sugarcane soils, infestation levels were generally relatively low, raising the question as to why this host-specific parasite was not flourishing in fields that were first planted to sugarcane in the late nineteenth century, and where host nematodes have been present, often at high population densities, for more than 100 years. Since most sugarcane soils in Australia are aggressively cultivated during the replanting process to alleviate compaction caused by harvest machinery, we hypothesised that tillage was disrupting the interaction between nematode and parasite. Results from our survey supported that hypothesis as *Pasteuria* was more common on farms where growers had moved to a minimum till farming system 10–15 years ago. However, minimum tillage was being used in only a small number of fields and so further work is required to confirm that *Pasteuria* becomes more prevalent when tillage is minimised.

More compelling evidence that tillage is detrimental to *Pasteuria* was obtained from studies at the two survey sites with the highest infestations on *Pratylenchus*. These sites had previously been grass pastures and had only grown sugarcane for about 20 years. Spore-encumbered and infected specimens of *P. zeae* were readily recovered from pasture soils similar to those into which sugarcane had been planted, indicating that *Pasteuria thornei* thrived when host nematodes were present but the soil was not tilled. This result also suggested that the high infestation levels at the two sugarcane sites were due to carry over from the pasture.

Our suggestion that tillage is detrimental to *Pasteuria thornei* is supported by observations made in a tillage trial that was established in a Spanish greenhouse following a crop of French beans. More than 75% of the *Pratylenchus neglectus* were encumbered with *Pasteuria* endospores but the percentage of nematodes with spores attached was significantly higher in untilled than tilled plots (Ornat et al. 1999).

Since factors other than tillage may have favoured the build-up of *Pasteuria* in soils under grass pasture, more work is required to assess the effect of tillage in agricultural soils. However, we would argue that it is likely to be having a negative impact on *Pasteuria*. Spore-filled nematodes are most likely to be found within or near the roots of the nematode host and because plant roots form channels in the soil during growth and those roots later decay to form open biopores (Cresswell and Kirkegaard 1995), concentrations of *Pasteuria* endospores are likely to be highest at microsites within root channels utilised by previous crops. When a root from the next crop grows into such a channel, there is a reasonable chance that an endospore will adhere to a nematode as it attempts to invade the root. However, when endospores are dispersed by tillage, the endospore concentration at a microsite level will decline markedly and nematodes are much less likely to come into contact with an endospore.

From a practical perspective, the results reported here provide yet another reason why sugar growers should adopt the farming system developed by the Sugar Yield Decline Joint Venture (Garside et al. 2005). Controlling traffic and reducing tillage provides many soil health benefits (Stirling 2008; Stirling et al. 2016) but the results of this study suggest that root health should also improve due to an increase in the number of root-knot and root-lesion nematodes parasitised by *Pasteuria*.

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