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Development of a new management strategy for the control of root-knot nematodes (*Meloidogyne* spp) in organic vegetable production†

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Abstract: The nematophagous fungus, *Pochonia chlamydosporia* (Goddard) Zare & Gams, has been investigated as a potential biological control agent for use in integrated pest management strategies for *Meloidogyne incognita* (Kof & White) Chitwood in vegetable crops. The release of the fungus as a biological control agent requires a diagnostic method to monitor its spread in the environment and to gain knowledge of its ecology. Only molecular methods are sufficiently discriminating to enable the detection of specific isolates of fungi in soil. A method to extract DNA from soil was developed to increase the efficacy of PCR-based diagnostic tests that use specific primers. A selected isolate of *P chlamydosporia* var *catenulata* was applied at densities similar to those that occur naturally in nematode-suppressive soils. The fungus significantly reduced nematode infestations in soil following a tomato crop, in a strategy that combined the use of the fungus with crop rotation. The survival of the fungus in soil was also examined in controlled conditions in which it remained in soil in densities significantly greater than its original application rate for at least 5 months. Hence, it seems that populations of this fungus may be built up in soil and have significant effects on the regulation of root-knot nematode populations.
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Keywords: root-knot nematodes; biological control; *Pochonia chlamydosporia*; molecular diagnostics; integrated pest management; vegetables

1 INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp) are major pests of vegetable crops world-wide. In southern Europe and other developed horticultural industries, the general soil fumigant, methyl bromide, is used extensively for the control of these pests but alternative methods are urgently needed as this chemical will be banned in Europe and the USA in 2005 because of its depleting effect on the ozone layer.[1] The control of root-knot nematodes in vegetable production is being examined in small, intensively cropped 'organoponic' holdings within the city limits of Havana, Cuba. These units use no synthetic pesticides for crop protection, are usually <2 ha in area and are situated in each of the residential areas of the city. They provide direct access to fresh vegetables and are seen as a method of improving the diet of the urban poor.[2] Several methods, including soil amendments and trap cropping, are used to manage root-knot infestations (L Hidalgo-Diaz, unpublished results). In Cuba, a network of approximately 200 small production plants produce several microbial control agents, including *Bacillus thuringiensis* Berliner, *Metarhizium anisopliae* (Metsch) Sorokin, *Verticillium lecanii* (Zimm) Viegas and *Trichoderma harzianum* Rifai for pest and disease control by local growers.[3]

The nematophagous fungus, *Pochonia chlamydosporia* (Goddard) Zare & Gams[4] (synonym: *Verticillium chlamydosporium* Goddard) has been widely reported as a parasite of root-knot nematodes, and its potential as a biological control agent has been examined.[5,6] The fungus is a facultative parasite of nematodes that

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colonises the rhizosphere of a wide range of crop species and infects nematode egg masses on the surface of the roots. Egg masses that remain within galled roots are not colonised and this fungus provides poor control in situations where large galls are formed, such as in heavily infested soil and on highly nematode-susceptible crops. Therefore, *P chlamydosporia* must be integrated with other control methods to maximise its impact on nematode populations. A strategy that combines the fungus with poor hosts for the nematode, to reduce infestations in soil before the next fully susceptible crop is grown, has provided significant control in glasshouse situations.[7] *Pochonia chlamydosporia* is widespread in Cuba, and a range of isolates was obtained as pure cultures that differed significantly in important characteristics that affected their biological control potential.[8] Indigenous isolates were screened and an isolate (Res 392) of *P chlamydosporia* var *catenulata* selected for further evaluation. Two varieties, *P chlamydosporia* var *chlamydosporia* and *V chlamydosporia* var *catenulata* were identified from distinct morphological differences[4] but the biological activities of both varieties were similar. To monitor the activity of the fungus in the field requires the development of specific diagnostic tests to detect the presence of the biological control agent amongst a background of the residual soil microflora. Molecular techniques are essential for the identification of specific isolates of the fungus. PCR-based methods using specific primers in conjunction with standard dilution plating techniques have enabled *P chlamydosporia* var *chlamydosporia*[9,10] and *P chlamydosporia* var *catenulata* (SD Atkins unpublished results) to be detected after application to soil. These methods do not allow specific isolates of the fungal taxa to be identified directly from DNA extracted from soil, but PCR fingerprinting using primers from arbitrary enterobacterial repetitive intergenic consensus sequences (ERIC) has been used to discriminate isolates from colonies on a selective medium.[11] ERIC have highly conserved nucleotide sequences, but their chromososmal location differs between isolates, which allows the construction of specific diagnostic fingerprints. The use of ERIC PCR fingerprinting of colonies developing on the selective medium for the fungus provides a two-stage isolation and diagnostic procedure that has enabled different isolates to be detected on the same root and within a single nematode egg mass. Hence, there are molecular methods available to monitor specific isolates of *P chlamydosporia sensu lato* after their application to soil. However, there is a need to optimise the extraction of fungal DNA from soil to increase the sensitivity of PCR-based tests. The resistance of fungal spores and conidia to lysis and the potential presence of inhibitory compounds in these cells may significantly impair PCR methods designed for their detection. Hyphae, conidia and multi-cellular chlamydospores of *P chlamydosporia* occur in soil.[7] The latter are the preferred form of inoculum of the fungus in field trials, as they are the resting stage of this fungus and are able to survive in soil in the absence of nematode hosts. They are composed of 10–20 haploid cells that have thick cell walls and, as such, pose a problem for DNA extraction. It is crucial that DNA is extracted from every form of the fungus in order to measure changes in its abundance and to study its molecular ecology by PCR. Haughland *et al*[12] found that DNA extraction using bead beating to lyse cells out-performs other methods such as sonication, freeze-thaw lysis or liquid nitrogen grinding. Kerry and Crump[13] considered that the presence of nematodes increased the numbers of chlamydospores in soil and improved the long-term survival of the fungus.

This paper describes a bead-beating method to extract, directly from soil, DNA of a quality and in quantities sufficient for detection of specific varieties of *P chlamydosporia* by PCR. Data are presented on the effects of (a) the presence of *Meloidogyne incognita* (Kof & White) Chitwood on different crops grown in two soils, and (b) chlamydospore size, on the survival of the fungus in controlled conditions. Also, a selected isolate of *P chlamydosporia* var *catenulata* is evaluated as a potential biological control agent for use against root-knot nematodes in organic vegetable production.

2 EXPERIMENTAL METHODS

2.1 Detection limits of *Pochonia chlamydosporia* in soil and the effect of bead-beating on the integrity of extracted DNA

In order to determine the detection limits of *P chlamydosporia* in soil using molecular diagnostic methods,[9] known amounts of chlamydospores of *P chlamydosporia* were seeded into soil free of the fungus. A flinty silty clay loam of the Batcombe-Carstens Series, a Typic Paleudalf[14] from Sawyers field at IACR-Rothamsted, Hertfordshire, UK was partially sterilised. Soil was sieved, batches (5 kg) sealed in plastic bags and γ-irradiated at a dose of 1 Mrad (Irradiated Products Ltd, Swindon, UK). The soil had been stored at ambient temperature for several years, during which time surviving residual bacteria and fungi stabilised at 10^2 Cfu g^{-1} soil (I Clark, pers comm).

The fungus was grown on a sand + bran (1 + 1 by volume) medium for 3 weeks at 25 °C, and the chlamydospores extracted as described by de Leij & Kerry.[15] The spores were added to the γ-irradiated soil at rates ranging between 10^1 and 10^5 chlamydospores g^{-1} soil, and mixed thoroughly by shaking in plastic bags. In preliminary experiments to examine chlamydospore lysis, glass beads of different sizes (from 38 μm to 3 mm diameter) were added to a suspension of chlamydospores and subjected to bead-beating for 5 min at 5 mm amplitude in a Mikro-dismembrator II (B Braun, Melsungen, Germany). Aliquots of the suspension were viewed at ×400 magnification using a light microscope to assess the proportion of spores disrupted. Subsequently, DNA was extracted from soil

samples (0.25 g) using a commercial kit (Ultra Clean™ Mo Bio Laboratories Inc, Solana Beach, California, USA) and the Mikro-dismembrator II. The kit uses a bead-beating based protocol with beads of various sizes greater than 0.5 mm. It was largely used according to the manufacturer's instructions. However, the entire sample was processed each time and the DNA extracted was suspended in 200 µl instead of the recommended 100 µl of Tris buffer (100 mM; pH 8). This was necessary to dilute out PCR inhibitors.

After preliminary experiments on the size and extent of the soil-beating process to release DNA from fungal tissue, the following bead-beater parameters were tested: 5, 10 and 15 mm amplitudes each for 10, 15 and 20 min, using the Mo Bio kit. The resulting DNA samples were run on 1% agarose gels and the integrity of DNA examined. In addition to this, samples were tested in β-tubulin PCR assays to confirm the origin and quality of the DNA using the primers tub1f and tub1r, which are specific for *P chlamydosporia* var *chlamydosporia*.[9]

2.2 Survival of *Phonia chlamydosporia* in two soils following growth on cabbage, bean and tomato plant roots in the presence and absence of *Meloidogyne incognita*

Chlamydospores of the fungus were produced on the sand-bran medium as described in Section 2.1 and added as an aqueous suspension at a rate of 5000 spores g^{-1} soil to pots (12.5 cm diameter) containing either a peat or a sand-based compost. A 4-week-old tomato seedling cv Tiny Tim or a 2-week-old seedling of cabbage cv Durham Early or a local line of *Phaseolus* bean from Malawi was transplanted into each pot. There were 48 pots for each plant cultivar in each soil (96 pots) and a similar number of pots were left unplanted. One week later, 3000 second-stage juveniles of *M incognita* were added to half the pots. The nematodes had been extracted from cultures maintained on Aubergine cv Purple Ruby in the glasshouse and the eggs hatched using standard techniques.[16] Each treatment combination was replicated four times and the experiment was conducted in randomised blocks on a glasshouse bench. The pots were watered as required and treated with a slow-release fertiliser.

After 9 weeks, the plant shoots were removed and the fungal densities in soil estimated in four replicates of each treatment selected at random using the dilution plate method on a selective medium.[17] Thereafter, to determine the survival of the fungus in soil, similar samples were processed by the same method at monthly intervals on five occasions. Throughout the experiment the pots were kept moist and soil temperatures were maintained above 20 °C.

2.3 The effect of chlamydospore size on the establishment of *Pochonia chlamydosporia* in soil

As the extraction of small chlamydospores from the sand–bran medium is difficult and labour intensive, a comparison was made of the ability of spores of different size-ranges to establish the fungus in the soil and rhizosphere. If small spores were ineffective as a soil inoculum, the extraction of chlamydospores from the medium would be greatly eased by the use of sieves coarser than the 10-µm aperture ones currently used, which retain all chlamydospores. Spores washed from the sand–bran medium were collected on nested sieves that divided them into three size classes, >30 µm, 20–30 µm and 10–20 µm. The viability of each group was checked by plating the suspension of chlamydospores (0.2 ml) onto sorbose dextrose agar amended with antibiotics,[8] and the proportion of spores germinated after 2 days of incubation at 25 °C was determined. Spores of a given class were added to a sand-based compost (5×10^3 spores g^{-1}) as before, and a tomato seedling cv Tiny Tim planted in each pot (12.5 diameter) of treated soil. Six replicates of each size class and a further six pots untreated with the fungus were set up in a randomised block in the glasshouse and watered and supplied with slow-release fertiliser, as necessary. After 1 week, 2500 second-stage juveniles of *M incognita* were added to all pots, as before.

The experiment was harvested after 8 weeks, the fresh weight of the tomato shoots and roots measured, and the density of the fungus on roots and in soil estimated using standard techniques.[15] The roots were cut into 1-cm sections, thoroughly mixed and a sample (1 g) taken at random to estimate the number of nematode egg masses, which were counted at ×50 magnification using a dissecting microscope. The egg masses were broken up mechanically and the eggs released in a suspension in water, and counted.[15] At least 200 eggs were plated onto water agar (Oxoid; 2 g $litre^{-1}$) and incubated at 25 °C for 3 days to estimate those colonised by the fungus.

2.4 An evaluation of *Pochonia chlamydosporia* var *catenulata* for control of root-knot nematodes in organic vegetable production

A preliminary evaluation of the impact of *P chlamydosporia* var *catenulata* on the control of root-knot nematodes was conducted at the 'Organico-Vivero-Alamar' in Havana, Cuba. A red beet crop on the test site immediately before the experiment commenced had been severely damaged due to infestation of the soil by second-stage juveniles of *M incognita* (300 juveniles g^{-1} soil). A selected isolate (Res 392) of *P chlamydosporia* var *catenulata* was cultured on sterilised, cracked rice grain (100 g) for 3 weeks at 25 °C in conical flasks and produced *ca* 6×10^6 chlamydospores g^{-1} medium. The nematode-infested area was divided into eight plots ($2\,m^2$), which were separated by plastic sheets, 50 cm high, buried to a depth of 30 cm to prevent spread of the fungus between plots. *Pochonia chlamydosporia* on rice grains, prepared as above, was applied ($10\,g\,m^{-2}$) in a mixture with cattle manure to four plots, selected at random, and the mixture was incorporated to a depth of 15 cm. Control plots received the cattle manure alone. After the single

Table 1. The cropping sequence in the field trial following a red beet crop heavily damaged by the root-knot nematode, *Meloidogyne incognita*

Crop [a]	*Planting date (duration/days)*	*Plant density (plants m^{-2})*
1 Beans, *Vigna ungiculata sesquipedalis* cv Lilia	03/07 (52)	7
2 Chinese cabbage, *Beta vulgaris cicla* cv PK-7	31/08 (40)	16
3 Tomato, *Lycopersicon esculentum* cv Campbell-28	13/10 (96)	5

[a] *Pochonia chlamydosporia* var *catenulata* applied to soil before the bean crop.

application of the fungus, two poor hosts for the nematode (beans, cabbage) were grown before the fully susceptible tomato crop (Table 1); 16- to 25-day-old transplants were used for each crop, which was provided with drip irrigation. The tomato crop was planted 96 days after the application of the fungus.

At each harvest, the shoots from five plants selected at random from within each plot were removed and weighed and the roots were lifted along with the adhering soil. Soil samples (3×10 g) were taken from around the plant roots from each plot, the second-stage juveniles extracted for 24 h using a Baermann funnel[17] and the nematodes counted at $\times 50$ magnification. The roots were washed and the root-gall index determined[18] before the number of external egg masses and the number of healthy and infected eggs were estimated, as in the experiment above. Ten infected eggs were selected at random from each plate and grown on PDA at 25 °C for 10 days and then examined microscopically ($\times 400$ magnification) to confirm the identity of the fungus.

3 RESULTS

In the preliminary tests, small beads of 38 µm and 50 µm were ineffective for the disruption of chlamydospores whereas larger beads (0.5 mm and 3 mm) were effective, although lysis was not complete with bead-beating for 5 min. The Mo Bio kit contained beads of the appropriate sizes for spore disruption, and was used in subsequent experiments. Increased bead-beating time and intensity resulted in decreased genomic DNA integrity, as indicated by increased smearing of DNA samples on agarose gels (Fig 1). After 10 and 15 min, DNA integrity was greater than after 20 min where increased smearing was noted. Amplitudes of 15 mm for 15 or 20 min and 12 mm for 20 min caused too much DNA degradation as no PCR amplification was detected and increased smearing of genomic DNA was observed on 1% agarose gels. Bead-beating at an amplitude of 10 mm for up to 15 min and 12 mm for 10 min permitted detection of only 10^4 chlamydospores g soil^{-1}, indicating that chlamydospores had not been sufficiently disrupted to release all their genomic DNA. The best bead-beating protocols were beating at 12 mm for 15 min and 15 mm for 10 min, as these released sufficient DNA from chlamydospores to detect as few as 10^2 chlamydospores g^{-1} soil. However, 12 mm for 15 min was the chosen protocol as this caused less stress on the Mikro-dismembrator II. *Pochonia chlamydosporia* was undetectable in γ-irradiated control samples when using this optimal bead-beating protocol. Any indigenous population of the fungus was probably killed in the

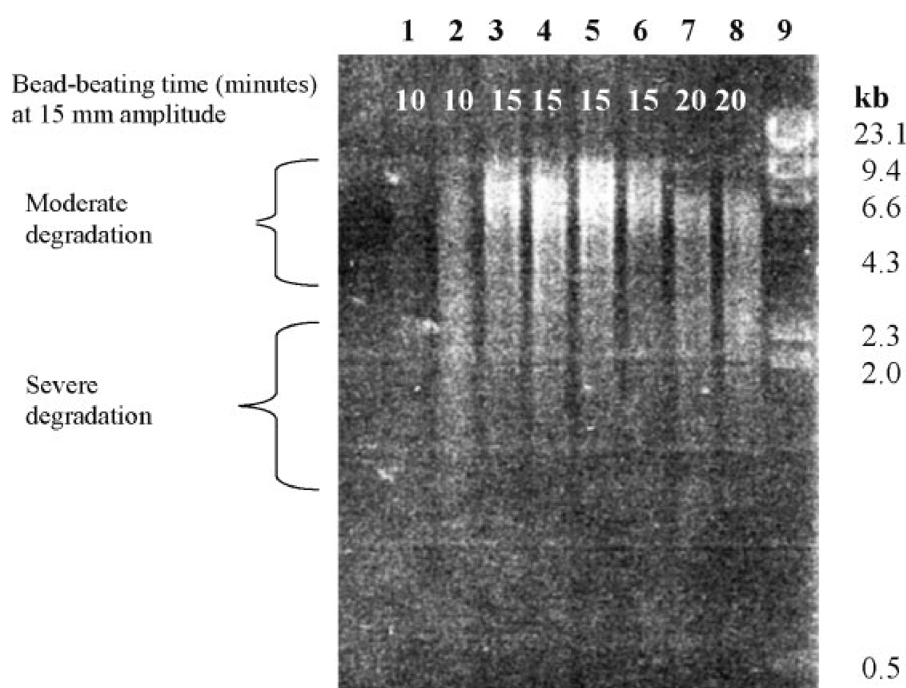

Figure 1. Shearing of DNA extracted by bead beating soil inoculated with chlamydospores of *Pochonia chlamydosporia* for different times.

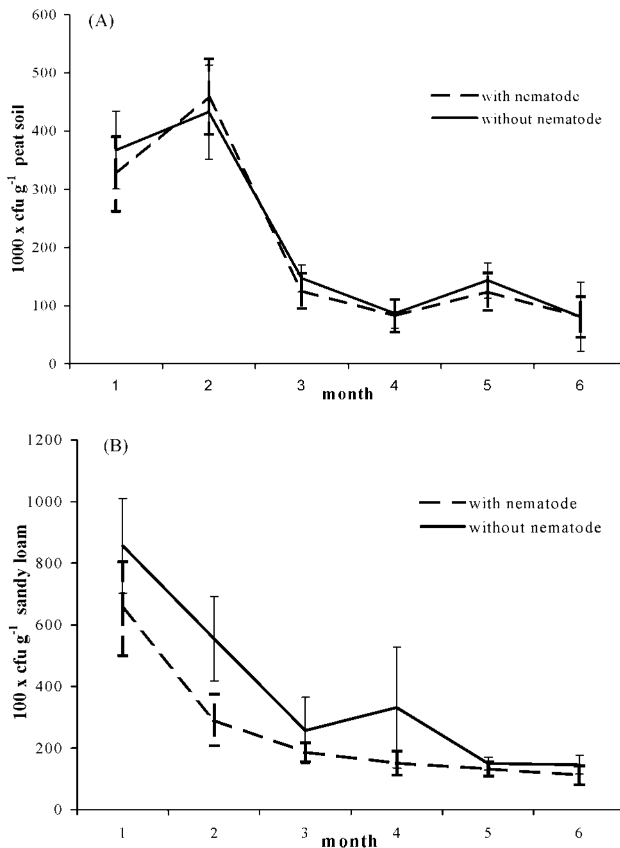

Figure 2. Changes in the density of *Pochonia chlamydosporia* in (A) peat and (B) sandy loam following healthy and *Meloidogyne incognita* infested crops (means of 12 replicates).

irradiation process and genetic material from these cells degraded by any surviving residual microflora.

The survival of the fungus was similar on all three plant cultivars tested and the data presented are overall means. *Pochonia chlamydosporia* was significantly more abundant in the peat-based compost than in that based on sand, in which maximum populations reached only 80×10^3 cfu g^{-1} compared to 400×10^3 g^{-1} in the peat (Figs 2(A, B)). The presence of the nematode host had no significant effect on the abundance or survival of the fungus. In general, populations of the fungus in soil declined for the first 2 months after the plants were harvested and then stabilised or declined more slowly for the remaining 3 months of observations. At the end of the experiment, the fungus was still more prevalent in both soils than at the time immediately after its application. On several of the sampling occasions the standard errors associated with the estimates of fungal densities were large, which suggests that the fungal distribution in soil was aggregated even in a pot experiment in which the initial inoculum was thoroughly mixed with the contents of the pot. Although there was a trend that smaller chlamydospores were more effective than large ones for establishing the fungus in the rhizosphere (Fig 3), the differences were not statistically significant and there were no significant differences in the numbers of nematode eggs produced on the tomato roots or on the proportion infected.

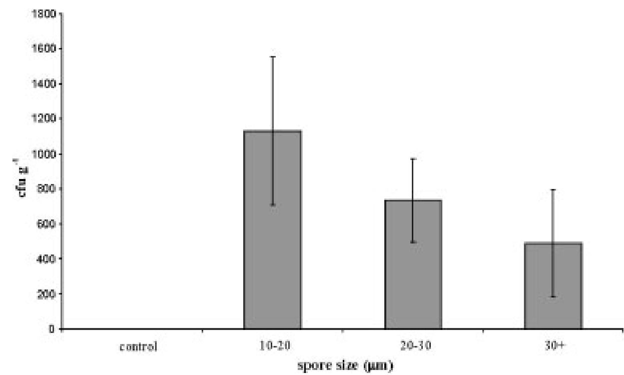

Figure 3. Effect of chlamydospore size on the establishment of *Pochonia chlamydosporia* on tomato roots after 9 weeks.

The bean and cabbage crop in the cropping cycle significantly reduced the nematode infestation in soil following the red beet crop (Fig 4). The application of the fungus before the bean crop had a small additional effect in reducing the population of the nematode after the two poor nematode hosts in the cropping cycle, and prevented the population increasing on the subsequent tomato crop. In untreated soil, the nematode population increased significantly to the level that had occurred after the damaged red beet crop. The proportions of nematode egg masses and eggs colonised by the fungus were $> 70\%$ and significantly greater ($P < 0.001$) than the numbers of nematodes colonised in untreated soil (Fig 5). *Pochonia chlamydosporia* var *catenulata* was found in most eggs from treated soil, but was absent from untreated soil, in which $<7\%$ of the eggs were colonised by fungi and contained *Fusarium* spp and *Paecilomyces lilacinus* (Thom) Samson. Although the root gall index of the tomato roots was significantly reduced from 4 in untreated plots to 2.5 in plots treated with the fungus, there was no significant effect on yields.

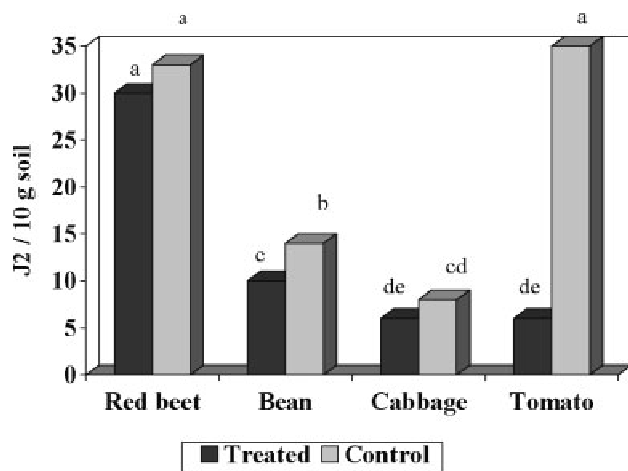

Figure 4. Numbers of second-stage juveniles of *Meloidogyne incognita* in untreated soil and soil treated with *Pochonia chlamydosporia* var *catenulata* (Res 392) applied before the bean crop in the rotation.

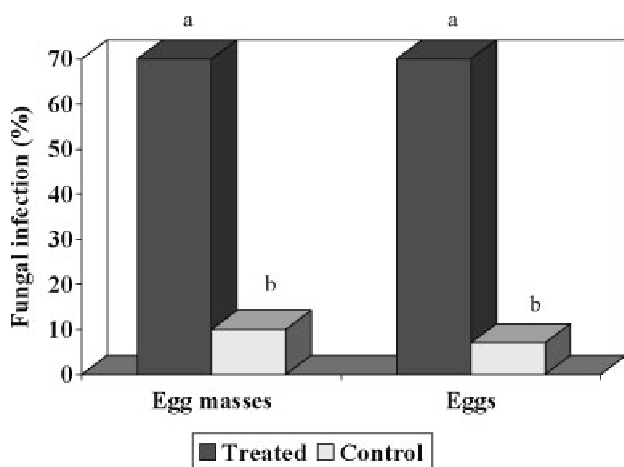

Figure 5. Proportions of *Meloidogyne incognita* egg masses colonised and eggs killed by fungi on roots of tomato plants grown in untreated soil and soil treated with *Pochonia chlamydosporia* var *catenulata* (isolate Res-392).

4 DISCUSSION AND CONCLUSIONS

The speed and duration of bead-beating were critical for optimal extraction of DNA from a soil sample and subsequent use in PCR-based diagnostics. Small beads (<0.5 mm) were ineffective at disrupting chlamydospores, presumably because they were held in suspension and unable to collide with chlamydospores at a high enough velocity to break them open. The optimisation of spore lysis is important in the development of quantitative molecular diagnostic methods for monitoring the fungus.

Quantitative molecular methods were more discriminating than a selective medium[17] for estimating fungal growth in soil but may detect dead or moribund propagules. More discriminating methods are required to detect individual isolates in soil and to quantify them without first isolating colonies on the selective medium. Diagnostic tests that use specific primers which distinguish the two varieties of *P chlamydosporia* will provide powerful tools for detecting and quantifying these fungi in soil.[9,10] Such methods, currently being developed, are essential for understanding the role of different isolates in the regulation of host nematode populations and for providing information on the fate of applications of the fungus in the environment. This information is essential to enable regulatory authorities to make decisions on the widespread release of biological control agents. The concentration of 5×10^3 chlamydospores g^{-1} soil applied in the field trial is similar to the densities of the fungus ($2–8 \times 10^3$ cfu g^{-1} soil) that eventually build up in naturally suppressive soils.[17] Development of more discriminating methods based on PCR and DNA extracted directly from soil will allow verification that the biological control agent is predominant, rather than an increase in numbers of indigenous *P chlamydosporia*. Such discrimination is important for the purposes of registration of any product based on a specific, selected isolate of the fungus and for its commercial evaluation.

Kerry and Crump[13] suggested that chlamydospore production by *P chlamydosporia* was associated with nematode infection, and that few chlamydospores were produced during saprophytic growth of the fungus in the rhizosphere. Hence, the presence of nematodes on roots might be expected to affect the long-term survival of the fungus in soil significantly. However, in the pot experiment, applications of the fungus around different infected host plants with different nematode susceptibilities and, indeed, the presence or absence of nematodes had no significant effects on the survival of the fungus. Fungal proliferation and chlamydospore production on the roots may not be apparent from sampling bulk soil and the presence of nematodes may have no effect on fungal growth in soil compared to the rhizosphere. The two types of compost used in the experiment may have had relatively low residual microbial populations to compete with the introduced agent, which could therefore proliferate. However, as has been observed in naturally suppressive soils,[13] it would appear that *P chlamydosporia* is able to survive between crops, and populations can be increased in soil when nematode-susceptible crops are grown in infested soil. Hence, it may be possible to induce nematode-suppressive soils as a result of a limited number of applications of the fungus. This would be an important consideration if the mass culture of the fungus remains relatively inefficient and time-consuming. Spore size had no significant effect in establishing the fungus in soil, and the use of only large chlamydospores that were easier to extract from the medium than the smaller spores would result in much loss of inoculum. Although application of chlamydospores alone has proved useful experimentally to demonstrate the efficacy of the fungus, it may prove more effective to apply all forms of the fungus on the colonised medium.

In the field test, the inclusion in the rotation of beans and cabbage, which supported fungal growth in their rhizospheres but reduced nematode populations, enabled *P chlamydosporia* var *catenulata* to increase in soil sufficiently to reduce the numbers of nematodes developing in the following tomato crop. This latter crop was planted 96 days after the original application of the fungus, which indicates that *P chlamydosporia* var *catenulata* maintains sufficient densities in soil to infect large numbers of nematode eggs for a prolonged period of time. Although the application of cattle manure to the plots is likely to have increased the microbial activity in soil, there is no evidence that it affected *M incognita* populations. There were no significant yield benefits obtained from the fungal treatment in any of the crops. However, it is assumed that the significantly larger nematode infestation left in the untreated soil following the tomato crop would result in lower yields in a subsequent crop compared to those in treated soil. If so, the combined use of poor nematode hosts and the fungus, as recommended by Kerry,[19] may be a useful strategy for the management of root-knot nematodes, especially in organic produc-

tion where control options for nematodes are limited. The strategy needs much more extensive evaluation and, as an equivalent of $100\,\text{kg ha}^{-1}$ of colonised medium was used, methods to improve inoculum production efficacy and reduce rates of application are required. The quantitative, molecular tools being developed to monitor the fungus after its application and to understand the key factors affecting its ecology in soil are essential for the successful development of *P chlamydosporia* as a biological control agent.

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