RESEARCH

Real-time PCR to monitor and assess the efficacy of two types of inoculum of the nematophagous fungus Pochonia chlamydosporia *var.* catenulata *against root-knot nematode populations in the field*

Belkis Peteira[1], Ana Puertas[1,2], ✉ Leopoldo Hidalgo-Díaz[1], Penny Hirsch[3], Brian Kerry[3], Simon Atkins[3,4]

[1]National Research Centre of Plant and Animal Health, CENSA
AP 10, San José de las Lajas, Havana, Cuba
[2]Facultad de Ciencias Agrícolas,
Granma University, AP 21, Bayamo, Granma, Cuba
[3]Nematode Interactions Unit, Rothamsted Research,
Harpenden, Hertfordshire, AL5 2JQ
[4]Environmental Biotechnology Research Unit, Rhodes University,
PO Box 94, Grahamstown, 6140, South Africa
E-mail: lhidalgo@censa.edu.cu

ABSTRACT

The fungus *Pochonia chlamydosporia* var. *catenulata* has a potential as a biological control agent against root-knot nematodes. If the fungus was applied on rice established in the soil, it would provide a cheaper form of application of the fungus for local farmers. Two types of fungal inocula were tested: a) the fungus added on colonised rice grain and, b) the purified chlamydospore powder. Each was added to plots infested with root-knot nematodes and the plots planted with two consecutive tomato crops. The population of *P. chlamydosporia* var. *catenulata* was monitored using real-time PCR and plating soil onto a selective medium for the fungus. The fungal inoculum added to rice established and proliferated more successfully than it did when it was added as a chlamydospore powder. However, there was no significant difference between the proportion of eggs infected after both fungal treatments at the first harvest, or in fungal densities measured as CFU or DNA levels by the time of the planting of the second tomato crop. Baiting the soil with nematode egg masses at different times after application demonstrated that the fungus applied in both forms infected significantly more eggs than soil from the untreated control plots. *Pochonia chlamydosporia* var. *catenulata* significantly reduced pest nematode populations and could be successfully used as a soil amendment.

Key words: Biological control agent, real-time PCR, root-knot nematodes, *Pochonia*

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RESUMEN

PCR en Tiempo Real para el monitoreo y la evaluación de la eficacia de dos tipos de inoculo del hongo nematófago *Pochonia chlamydosporia* var. *catenulata* contra poblaciones de nematodos formadores de agallas en campo. El hongo *Pochonia chlamydosporia* var. *catenulata* tiene potencial como agente de control biológico contra nematodos formadores de agallas. Aplicar el hongo al arroz, establecido en suelo, pudiera proveer a los agricultores locales de una forma económica de aplicación del mismo. Se evaluaron dos formas de inóculo del hongo: a) el hongo adicionado sobre grano de arroz colonizado y b) polvo de clamidosporas puras. Cada una fue adicionada a los surcos de suelo infectados con nematodos formadores de agallas, los cuales fueron plantados con dos cultivos sucesivos de tomate. La población de *P. chlamydosporia* var. *catenulata* se monitoreó usando la PCR en Tiempo Real y la siembra de muestras de suelo sobre medio semi-selectivo para el hongo. El hongo adicionado como arroz colonizado se estableció y proliferó de forma más exitosa que cuando se adicionó como polvo de clamidosporas. Sin embargo, no se observaron diferencias significativas entre la proporción de huevos infectados para ambos tratamientos del hongo en la primera cosecha, ni en la densidad del hongo medida a través de las UFC o los niveles de ADN a partir de la siembra del segundo cultivo de tomate. La técnica del cebo aplicada al suelo usando masas de huevos de nematodos a diferentes tiempos después de la aplicación demostró que los tratamientos con ambas formas de inóculo infectaron significativamente más huevos que el suelo a partir del tratamiento control. *Pochonia chlamydosporia* var. *catenulata* redujo significativamente las poblaciones del nematodo plaga y pudiera ser usada de forma exitosa como enmiendas al suelo.

Palabras clave: Agente de control biológico, PCR en Tiempo Real, nematodos formadores de agallas, *Pochonia*

*I*ntroduction

Not only root-knot nematodes are widespread, but also important pests in both annual and perennial crops worldwide. In Cuba, they cause extensive damage to coffee and vegetable crops [1]. The control of these pests in perennial crops or in rotations with nematode susceptible crops is difficult, as nematicides must be applied several times during a growing season, which is often not practicable or economic. Therefore, there is a need to develop alternative methods to control populations of root-knot nematodes.

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✉ Corresponding author

In Cuba, a network of approximately 270 small production plants produce several microbial control agents including *Bacillus thuringiensis*, *Metarhizium anisopliae, Lecanicillium lecanii* and *Trichoderma harzianum* for pest and disease control by local growers [1]. The production of *Pochonia chlamydosporia* var. *catenulata* as a biological control agent (BCA) of root-knot nematodes is being investigated [2-4].

One isolate (IMI SD 187) has shown potential as a BCA against root-knot nematodes and significatively reduced populations. This isolate was developed and tested in an integrated pest management system for the control of root-knot nematodes in small plot tests [2-4].

The development of a commercial product is underway. Currently, the fungus is grown in a biphasic process, which includes solid fermentation on rice from which chlamydospores are extracted. The chlamydospores are then extracted from the rice using a MycoHarvester (www.dropdata.net/mycoharvester) to provide a powder of chlamydospores to be applied to the soil. This process can be scaled up using larger commercially available MycoHarvesters (www.mycoharverter.info). It provides a very pure product of chlamydospores that can be furtherly formulated for application to the field, or for its storage and export. Chlamydospores were used as the preferred form of inoculum for field application of the fungus, as they have sufficient food reserves to enable the fungus to become established without the addition of other energy sources [5]. They can be easily extracted from the colonised rice using the MycoHarvester, and collected in quantities suitable for field application. Both, conidia and hyphal fragments, have low survival rates in soil, and are subject to fungistasis when they are added to the soil without a supplementary energy source [6].

Once a BCA has been released it is important, especially for registration purposes, to have tools available to monitor its spread and activity. Reliance on just one method is not recommended and previous research [7, 8] has clearly demonstrated the need for a multi-method approach in assessing the activity of a BCA in the soil. The ranges of molecular methods now available provide rapid diagnostic techniques for fungi and are reviewed by Anderson *et al.* 2004 [9]. Real-time PCR provides a rapid, accurate and quantitative method for monitoring fungi in the soil, providing results on the amount of specific fungal isolates within hours of sampling, whereas more conventional methods such as serial plate dilutions of soil onto selective media may take up to three weeks to provide data, and still may need further analysis of colonies to identify them accurately. A comparison of conventional methods and molecular methods was carried out by Zhang *et al.* [10]; since then, the cost of many of the molecular methods compared has significantly decreased making the methods more common and cost effective. Real-time PCR has been used to successfully determine the amount of a number of fungal species [7, 8, 11, 12, 14-16] and to investigate their ecology in the soil [7, 13, 14].

Here, the development of a TaqMan® based real time PCR probe and primer set for the fungus *P. chlamydosporia* var. *catenulata* is reported, and its use to monitor the fungus after application over the course of two successive tomato crops is described and compared with results derived from plating soil onto a selective medium. The effect of the fungus upon the nematode population was also assessed.

The aim of this paper is to determine if there was a difference in the control of the pest nematode population based on the inoculum type, and also to compare the detection of the fungus by real-time PCR and plating soil onto the selective media.

Materials and methods

Isolates and media

Fungal cultures were taken from the Rothamsted culture collection or from other sources stated in table 1. Isolates were stored at 4 °C on corn meal agar (CMA-

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Table 1. Fungal isolates and their sources: (1) *Pochonia chlamydosporia* culture collection with the isolate number indicated: Rothamsted Research; (2) Dr. L Hidalgo, CENSA, Cuba; (3) Dr. DJ Barbara, HRI Wellesbourne, UK; (4) Dr. W Dawson, Rothamsted Research, UK; (5) Prof. DK Arora, Rothamsted Research, UK; (6) Dr. J Pell, Rothamsted Research, UK; (7) Dr. SD Atkins, Rothamsted Research, UK; (8) CABI Bioscience, Genetic Resource Collection, UK.

Fungal species	Isolate	Original host	Source
Pochonia chlamydosporia var. *chlamydosporia*	10	*Meloidogyne incognita eggs*	1
P. chlamydosporia var. *chlamydosporia*	327	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	338	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	363	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	368	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	372	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	373	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	378	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	387	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *chlamydosporia*	207	*Meloidogyne incognita eggs*	1
P. chlamydosporia var. *chlamydosporia*	204	*Meloidogyne incognita eggs*	1
P. chlamydosporia var. *catenulata*	336	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata*	341	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata*	353	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata*	391	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata*	IMI SD 187	*Meloidogyne incognita eggs*	8
P. chlamydosporia var. *catenulata* Biotype A	398	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata* Biotype A	364	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata* Biotype A	366	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata* Biotype A	370	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata* Biotype A	374	*Meloidogyne incognita eggs*	2
P. chlamydosporia var. *catenulata* Biotype A	382	*Meloidogyne incognita eggs*	2
Verticilium dahliae	327	Strawberry	3
Penicillium chrysogenum		Soil	5
Plectosphaerella cucumerina		*Globodera pallida eggs*	7
Pythium sp.	31.5	Wheat	4
Aspergillus niger		Soil	5
A. versicolor		Soil	5
Botrytis sp.	C1650	Soil	6
Fusarium poae	24.1	Wheat	4
Gliocladium roseum	10.1	Wheat	4

Oxoid, Basingstoke, UK) plates until needed, then sub-cultured onto fresh corn meal agar and incubated at 28 °C.

Experimental design of field trials

The field site was located at CENSA (National Research Centre of Plant and Animal Health). The area had been previously planted with tomatoes and sweet peppers which had both been infested by root-knot nematodes. Prior to the establishment of the experiment the field had been planted with cucumbers to increase the level of root-knot nematodes in the soil. The total area of the site was 720 m^2, divided into plots of 55.50 m^2 consisting of three beds of 12 m^2. The field trial was based on a Latin square design. The experiment consisted of three treatments: 1) untreated control; 2) addition of *P. chlamydosporia* var. *catenulata* IMI SD 187 on colonised rice; and 3) addition of the same isolate as pure chlamydospores. Each treatment consisted of three replicates. The fungus was grown on rice according to the protocol outlined [2]. Chlamydospores were extracted from colonised rice after 16 days of incubation at 25 °C using a MycoHarvester. Chlamydospores were counted using a haemocytometer. For the colonised rice, 1 g was added to 10 mL water and vortexed vigoursly to release the spores prior to counting. The application dosage was of 5000 chlamydospores g^{-1} soil to a depth of 15 cm. The fungus was mixed with organic matter (cattle manure) prior to application at a rate of 1 Kg organic matter m^2. The control received only organic matter at the stated application rate. The plots were planted with two consecutive tomato crops cv. Amalia. Soil cores were taken at random from each plot at the time of fungal application two months later, and at the harvest of the first crop. Similarly, cores were taken from the second crop at planting time two months later, and at the harvest.

The soil was pooled and mixed. The soil cores from each plot were plated onto selective media as described by Kerry *et al*. 1993 [5] with 2 replicates. Colonies that arose on the medium were tested using the *P. chlamydosporia* var. *catenulata* specific PCR primers as described by Atkins *et al*. 2003 [2], and *P. chlamydosporia* var. *chlamydosporia* specific primers as described Hirsch *et al*. 2000 [17]. A DNA profile was derived from all *Pochonia chlamydosporia* var. *catenulata* positive isolates using the arbitrary ERIC (Enterobacterial repetitive Intergenic Consensus) primers [18] and compared to the profile derived from a pure culture of IMI SD 187.

The egg infection percentage and number of juveniles (J2s) in 100 mL soil were determined at the end of each plant harvest using standard techniques [19]. Soil samples collected before the application of the BCA, and from each consecutive harvest and planting, were baited with *Meloidogyne incognita* egg masses in accordance to the outlined protocol [19]. All data were compared and analysed by one way analysis of variance using the Genstat® program [20].

Design and testing specificity of the TaqMan® primer and probe set

TaqMan® PCR primers and probes were designed by comparing *P. chlamydosporia* var. *catenulata* ITS sequences (Internal Transcribed Spacer) from the GenBank/EMBL database (Accession Nos AJ292398 and AJ292399), using the Primer Express software (Perkin-Elmer Biosystems, Norwalk, CT, USA), in accordance with the criteria required for real-time PCR primer design. Primers and probe were compared with other sequences in the database through BLAST and FASTA searches to confirm specificity, and their design was optimised using the NetPrimer software (Biosoft International, www.premierbiosoft.com/netprimer.html). The fluorogenic probe (CATrtP) was labelled at the 5' end with the fluorogenic reporter dye FAM (6-carboxy-fluorescein), and the 3' end was modified with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine). The primer and probe sequences were as follows: CATrtF 5' CAG TGG TTT GGT GTT GGG 3'; CATrtR 5' ACG AGA CCG CCA ATT CAT TTC 3'; CATrtP 5' FAM AGA GGC GGA TGG GGA CTT GTG TAMRA 3'. The probe was designed over the same region as the reverse primer described by Atkins *et al*.[2] for the variety-specific PCR primer set for *P. chlamydosporia* var. *catenulata*.

Real-time PCR was performed in Bioplastics (EU) 96 x 0.2 mL PCR plates capped with Bioplastics EU Optical thin wall, 8-cap strip, low background (Bioplastics Landgraaf, Netherlands) with the automated ABI Prism 7700 sequence detector (PE Applied Biosystems). JumpStart™ *Taq* ReadyMix™ (Sigma, UK) for quantitative PCR was used for real time PCR reactions. Primer and probe concentrations were optimised according to the manufacturer's guide. Primers CATrtF and CATrtR were included at a final concentration of 900 and 300 nM, respectively, and the TaqMan® probe (CATrtP) was used at 225 nM. PCRs were performed in 25 µl volumes. Reference dye was added in accordance to the manufacturer's recommendations for its use with the ABI 7700. The reaction was made up to 25 µl using ultrapure dH2O. The manufacturer's recommended universal thermal cycle protocol was used for PCR amplification: stage 1; 94 °C for 2 min (activation of *Taq* DNA polymerase); and stage 2; 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 1 min. Reactions were performed with 3 replicates. The specificity of the primers and probes were tested by using DNA extracted from all the cultures listed in table 1. DNA was extracted from the pure cultures as described by Klimyuk *et al*. 1993 [21].

Construction of a standard curve for the quantification of *Pochonia chlamydosporia* var. *catenulata*

DNA was extracted from a pure culture of *P. chlamydosporia* var. *catenulata* using the CTAB extraction method described by Lee and Taylor (1990) [22]. The DNA was quantified using a spectrophotometer and serial diluted in 10 mM Tris HCl (pH 7.5). DNA was extracted from 0.25 g soil taken from the control plot at the CENSA field trial that had been shown not to contain *P. chlamydosporia* var. *catenulata* by PCR using the variety-specific primers [2] and plating onto the selective medium. *P. chlamydosporia* var. *catenulata* DNA was added to 5 ng soil DNA at a range of 1 ng to 100 fg. Additional $MgCl_2$ was added to increase the concentration to 4 mM. Reactions were performed using the previously outlined conditions.

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The standard curve was derived from the CT value (Threshold cycle) (set at 0.2) for each of the DNA concentrations. Reactions were performed with 3 replicates. A negative control of 5 ng soil DNA without additional *P. chlamydosporia* var. *catenulata* DNA was included to determine if any erroneous signal was derived and a template control (water added to the PCR instead of DNA) was performed to act as a control for the PCR.

Detection and quantification of *Pochonia chlamydosporia* var. *catenulata* in the field trial

DNA was extracted from 0.25 g soil taken from the pooled sample from each treatment and at each time point. Aliquots (5 ng) were added to each real-time PCR reaction using the conditions described above. The CT value for each point was compared to the standard curve to derive the level of *P. chlamydosporia* var. *catenulata* DNA in the soil. All PCR reactions were performed with 3 replicates.

Results

The primers CATrtF and CATrtR generated a PCR product of 97 bp from all the *P. chlamydosporia* var. *catenulata* isolates (Table 1). No fluorescence was recorded from the DNA samples extracted from the other fungal isolates, neither from the *P. chlamydosporia* var. *chlamydosporia* isolates, or from the *Pochonia chlamydosporia* var. *catenulata* Biotype A isolates. The CT was set at 0.2, measuring amplification during the logarithmic phase of the PCR. A standard curve for *P. chlamydosporia* var. *catenulata* was generated by using data derived from the serial dilution of *P. chlamydosporia* var. *catenulata* DNA in soil. The equation of the line was y = -3.933 log(x) + 49.558. The linear correlation coefficient of the standard curve was r^2 = 0.998, demonstrating the accuracy of the PCR based quantification. Based on three sample replications, the ABI Prism fluorescence detection system automatically calculated the starting concentrations of *P. chlamydosporia* var. *catenulata* DNA by comparing the CT values for the unknown samples with those values on the standard curve. By using this method, it was possible to detect concentrations of 100 fg of *P. chlamydosporia* var. *catenulata* DNA.

The presence of *P. chlamydosporia* var. *catenulata* was not detected in the soil from untreated plots in the field trial by plating onto the selective medium (Figure 1), or by real-time PCR (Figure 2). The colonies lifted from the selective medium were shown to be *P. chlamydosporia* var. *catenulata* after PCR with the variety specific PCR primers. When tested using the ERIC PCR primers all of then gave the same profile as an IMI SD 187 isolate demonstrating that all the recovered fungi belonged to the added isolate. Both, plating soil onto the selective medium (Figure 1), and real time PCR results (Figure 2) demonstrated an increase in the amount of the fungus after its application on colonised rice. This increase was significant on sampling at two months and at the first harvest (F.pr 0.03 ± 5160 and 0.013 ± 2152, respectively) for the CFU counts (Colonies Former Units) on the selective medium from soil taken from the plots treated with colonised rice compared to plots treated with chlamydospore

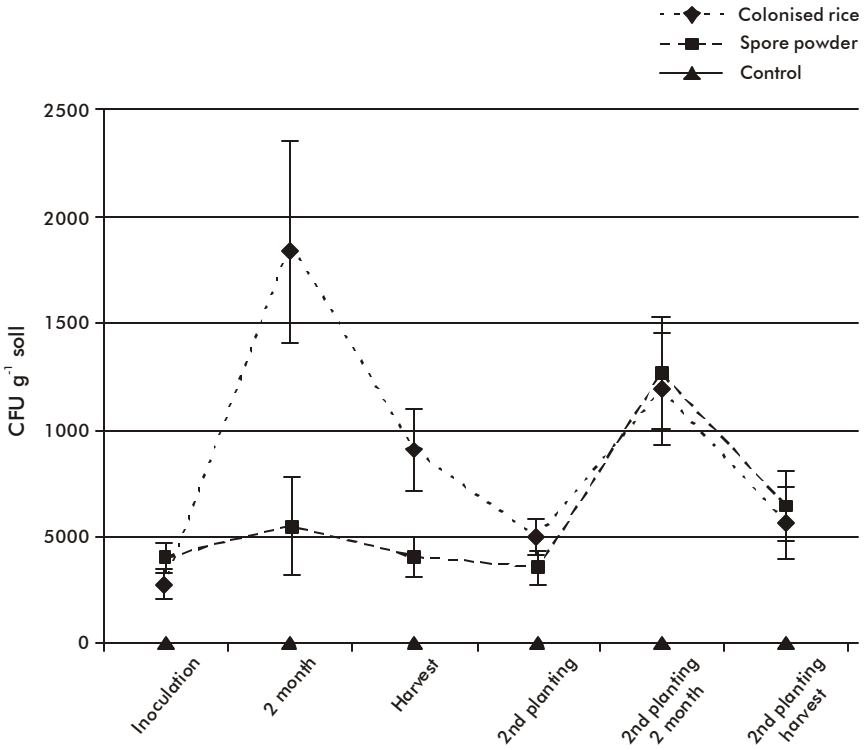

Figure 1. Plot of *P. chlamydosporia* var. *catenulata* CFU. g-1 soil levels obtained for colonized rice, chlamydospores powder and control treatments over six months in a time course experiment.

powder. Significantly more fungal DNA was detected by real-time PCR in the soil taken from the plots treated with chlamydospore powder, as compared to plots treated with colonised rice at the time of inoculation (F.pr 0.012 ± 1.761), but at two months more fungal

19. Abrantes IM De O, Alho L, Bourne JM, Brito I, Ciancio A, Hirsch PR, *et al*. A Manual for Research on *Verticillium chlamydosporium*, a Potential Biological Control Agent for Root-Knot Nematodes. Kerry BR and Bourne JM (Eds.). IOBC/WPRS, Gent 2002;84pp.

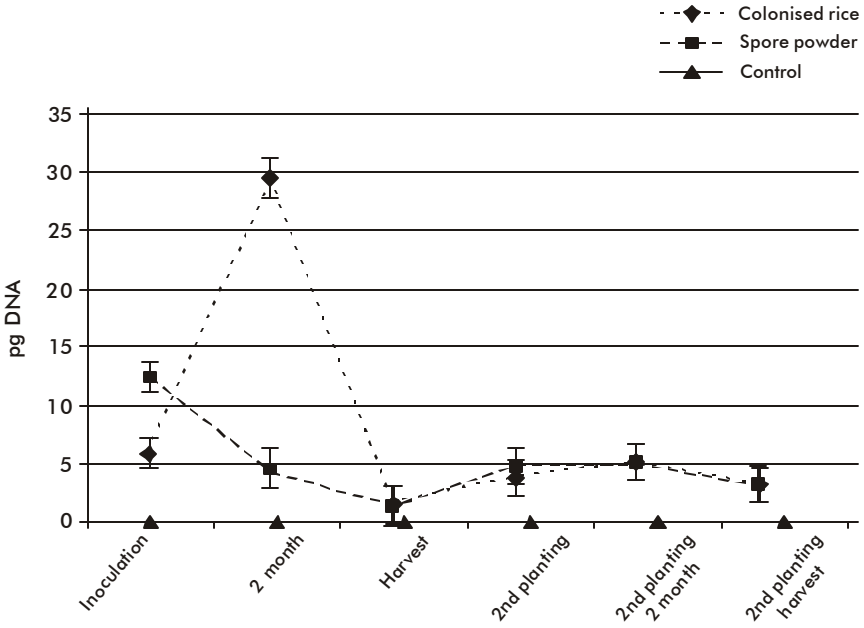

Figure 2. Plot of *P. chlamydosporia* var. *catenulata* DNA levels (in pg) obtained for colonized rice, chlamydospores powder and control treatments over six months in a time course experiment.

DNA was detected in the soil taken from the plots treated with colonised rice (F.pr <0.001 ± 1.91).

In both fungal treatments there was a significant increase in nematode egg infection compared to the control at the first and second harvest, (Table 2). This level of egg infection was associated with significant differences in the number of nematode juveniles (J2s) in the soil as compared to the control for the colonised rice treatment (F.pr 0.004 ± 43.9), although not for the chlamydospore powder treatment (F.pr 0.061 ± 45.1) at the first harvest. Egg infection increased in both fungal treatments between the two harvests (Colonised rice F.pr < 0.001 ± 6.74; chlamydospore powder F.pr 0.009 ± 8.03) and the level of J2s within the soil, significantly decreased, compared to the control trait (Colonised rice F.pr < 0.001 ± 27.4; chlamydospore powder F.pr < 0.001 ± 27.3). The number of juveniles within the soil at each harvest did not differ for the control (F.pr 0.342 ± 49.7), whereas for both fungal treatments there was a significant decrease in their numbers (Colonised rice F.pr 0.011 ± 14.4; chlamydospore powder F.pr < 0.001 ± 17.8) between harvests. At both harvest times, there were no statistical differences recorded following the level of egg infection between the two fungal treatments (First harvest: F.pr 0.241 ± 6.5; Second harvest: F.pr 0.963 ± 8.18).

There were also significant increases recorded in the proportion of eggs colonised in the baits in both fungal treatments as compared to the control (Figure. 3). More eggs were infected in both, the fungal treatments at the first harvest compared to the initial control (Colonised rice F.pr < 0.001 ± 5.31; chlamydospore powder F.pr < 0.001 ± 3.65), and the control at the first harvest (Colonised rice F.pr 0.005 ± 5.60; chlamydospore powder F.pr <0.001 ± 4.06). There was no significant difference in the proportion of eggs infected in the soil treated with either of fungal treatments at the first harvest, but more eggs were infected in both fungal treatments at the second harvest as compared to those in untreated soil (Colonised rice F.pr 0.005 ± 4.34; chlamydospore powder F.pr <0.001 ± 2.82), and the control at the second harvest as compared to the two fungal treatments at the second harvest (Colonised rice F.pr <0.001 ± 6.34; chlamydospore powder F.pr <0.001 ± 2.40). There was no difference in the level of egg infection between the two fungal treatments at the planting of the second harvest. There was a significant increase in the level of egg infection between the colonised rice treatment at the second planting compared to the colonised rice treatment at the second harvest (F.pr 0.043 ± 7.41). There was no difference in the level of eggs infected in the control plots when compared at each time point and to the initial soil count.

Table 2. Egg infection (%) and number of *Meloidogyne* J2s in 100 ml soil from the control and fungal treatments at both tomato harvest times. Results were analysed by ANOVA. (F.pr; frequency of probability SED: standard error of deviation; ±SE: ± standard error).

Treatment	Crop	% egg parasitism (±SE)	F.pr	SED	J2 100 ml soil (±SE)	F.pr	SED
Colonised Rice	1	31.2 (1.74)	<0.001	1.68	64.5 (13.5)	0.004	43.9
	2	62.3 (5.8)	<0.001	5.83	25.4 (4.7)	<0.001	27.4
Chlamydospore powder	1	39.1 (6.57)	<0.001	5.98	114.9 (16.9)	0.061	45.1
	2	62.6 (3.82)	<0.001	2.91	34.1 (4.2)	<0.001	27.3
Control	1	0			202.9 (41.4)		
	2	0			154.9 (26.8)		

Discussion

With a new legislation governing the use of many chemical nematicides coming into play this year and in the future [23], the use of alternative methods to control pest nematode populations is under investigation. The results from this paper demonstrate the effectiveness of applying *P. chlamydosporia* var. *catenulata* to soil to significantly reduce populations of root-knot nematodes. A single application of the fungus was shown to infect < 60% of the eggs by the harvest of the second crop and have a significant effect upon the number of infective juveniles within the soil. This work correlates well with other published studies using *P. chlamydosporia* var. *catenulata* [2-4] and *P. chlamydosporia* var. *chlamydosporia* [5, 24-26] as a BCA against pest nematode populations.

Previous papers [5, 25] have shown that the control of root-knot nematode colonisation of the rhizosphere is imperative, as this is where the contact between the fungus and nematode egg masses is established. The results in this paper demonstrate that once the fungus was added to the soil it was able to colonise the rhizosphere of the tomato plants and to infect the nematode eggs. The results also demonstrate that the fungus remained viable in the soil at levels that could colonise a fresh crop and infect subsequent nematode populations.

The ability of the fungus to become established within the soil was affected by the inoculum type. When the fungus was added on rice, both the CFU data and the real-time PCR data indicated that it became readily established and proliferated. When the fungus was added as chlamydospores there was little proliferation. By the time the second tomato crop had

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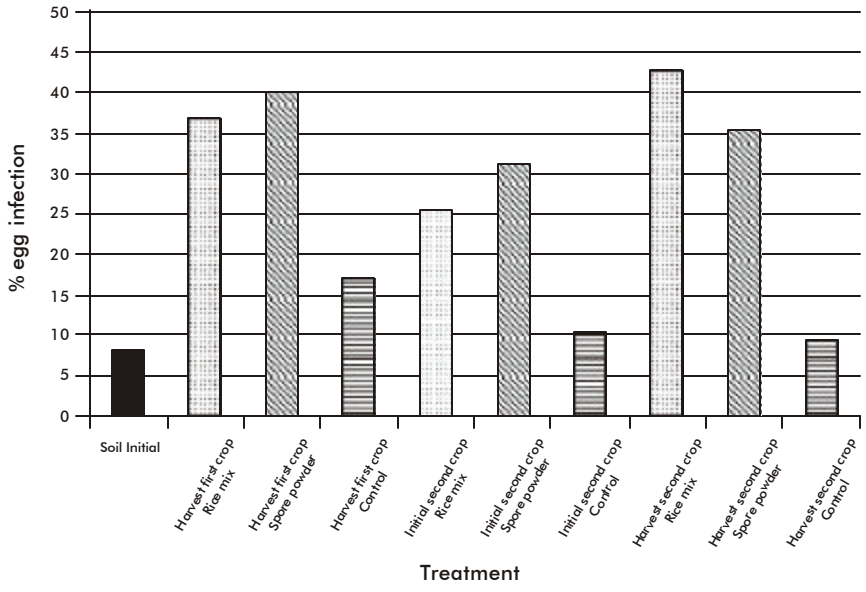

Figure 3. Percentage egg infection in baits added in the lab to soil taken from control and fungal treatment plots originating in the time course experiment.

been planted, the amount of fungus in the colonised rice treatment had declined to the same density as that in the other inoculum treatment. In the second crop, the fungus in both treatments fluctuated in the same manner and the detection of the fungus was the same in both diagnostic methods. An increase in the fungal population was demonstrated by both methods of monitoring during the second month of the second crop due to the proliferation of the fungus in the rhizosphere, after which, the levels returned to post first harvest densities. This may demonstrate a threshold level for the fungus that the soil can support as previously reported by Mauchline *et al.* [6].

Although the fungal content based on estimates of CFU of the two inoculum types was similar (Figure 1), a significant difference was demonstrated in the *P. chlamydosporia* var. *catenulata* DNA content recovered in the soil immediately after inoculation between the two treatments (Figure 2). The soil inoculated with colonised rice would contain a mixture of conidiospores, mycelium and chlamydospores and proportionally the level of chlamydospores recovered in the samples would be lower than the level recovered from the soil inoculated with just pure chlamydospores. Chlamydospores are large multinucleate structures consisting of 10-20 cells [27], and would proportionally contain significantly more copies of the ITS than mycelium or uninucleate conidiospores. As the sample taken from the chlamydospore powder treated soil would be expected to contain only chlamydospores, the ITS copy number should exceed that in the sample taken from the colonised rice treated soil, which would result in a significant difference in the level of *P. chlamydosporia* var. *catenulata* DNA between the two treatments seen at the time of inoculation.

The effect on the nematode population was the same for both fungal treatments regardless of the fungal population density. Both treatments significantly infected a greater number of eggs than seen in the control and significantly reduced the number of infective juveniles in the soil.

Although, the amount of fungal DNA reached an important level, after the first harvest it decreased to 2-4 pg, which may represent the fungal density threshold that the soil can support. Both, the real-time PCR and CFU data, showed similar trends for monitoring the fungus. The correlation between the two data sets was low ($r^2 = 0.502$) if the first time point is taken into consideration. The reasons for this difference in data sets have previously been discussed. After this time point though, the two data sets were more strongly correlated ($r^2 = 0.704$).

The advantage of using real-time PCR is that it provided a rapid quantitative detection of the fungus in three hours, whereas, colonies took over two weeks to grow on the selective medium and further PCR diagnostic methods were needed to determine if those colonies arising corresponded to *P. chlamydosporia* var. *catenulata*. It was assumed that the detection of *P. chlamydosporia* var. *catenulata* in the soil samples was due only to the presence of the added isolate. All colonies arising on the selective medium gave the same DNA fingerprint as those of the isolate IMI SD 187 thus proving this assumption to be correct. Further work is necessary to develop a primer and probes set that is specific to this isolate. At present, sequence data are limited with only ITS and serine protease genes [28] having been sequenced and would not provide regions suitable for selective primers to be developed.

Formulation of a BCA is fundamental to the commercialisation of the product. This experiment demonstrates the ability of the fungus to become established in the soil and influence pest nematode populations after the application of different inocula. The addition of the fungus on a growth media such as rice enables a cheap local product to be supplied directly to farmers. The separation of the chlamydospores from the rice medium requires more processing, time, and cost, but offers several advantages. The shelf life of the product can be increased by formulation, there is less potential for contamination and the product is easier to transport.

The inclusion of the fungus on a nutrient substrate such as rice helps the fungus to become established in the soil. Other reports have demonstrated that the inclusion of the fungus on such a source can have a detrimental effect upon its establishment, as the food source influences indigenous species, which may outcompete the fungus, or encourage fungal predators [29]. The results demonstrate that incorporating the fungus in either of the two inocula provides similar rates of control of the pest nematode population.

This work further demonstrates *P. chlamydosporia* var. *catenulata* has potential for developing as a BCA against root-knot nematodes, and that it can be added to the soil either as a pure spore inoculum or as a mixture of mycelium and spores with no effect on its ability to become established and to control pest nematode populations. The real-time PCR diagnostic methods have been shown to provide rapid quantitative data on the fungus in a fraction of the time needed for more conventional methods. Therefore, this diagnostic method can be used to monitor the fungus after application, an important criterion in the registration of any potential BCA.

Conclusions

The results obtained from the Real Time PCR and CFU were correlated. It is possible to use this technique to monitor the BCA released in the soil.

By the second harvest, the inoculum type had no effect on fungal abundance or on the percentage of eggs parasitism (more than 60% in both treatments).

One application of Pochonia chlamydosporia var. catenulata IMI SD isolate, at a rate of 5000 chlamydospores/g of soil, was effective for at least six months.

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