Mycol. Res. **107** (2): 206–212 (February 2003). © The British Mycological Society
DOI: 10.1017/S095375620300724X *Printed in the United Kingdom.*

Approaches for monitoring the release of *Pochonia chlamydosporia* var. *catenulata*, a biocontrol agent of root-knot nematodes

Simon D. ATKINS[1]*, Leopoldo HIDALGO-DIAZ[2], Ian M. CLARK[3], C. Oliver MORTON[1], Nivian MONTES de OCA[2], Paul A. GRAY[1] and Brian R. KERRY[1]

[1] *Nematode Interaction Unit, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK.*
[2] *CENSA, San Jose de Las Lajas, Apto 10, La Habana, Cuba.*
[3] *AEN Department, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK.*
E-mail: *simon.atkins@bbsrc.ac.uk*

Received 20 July 2002; accepted 13 December 2002.

Pochonia chlamydosporia var. *catenulata* is a potential biocontrol agent against root-knot nematodes. Diagnosis of isolates has relied on morphological identification, and is both time-consuming and difficult. β-tubulin primers have been developed for the identification of this fungus that were specific enough to distinguish between varieties of the fungus within the same species. Separate primers have been developed for the specific detection of *P. chlamydosporia* var. *catenulata* based on ITS sequences, which were able to detect the fungus in soil from various sites in Cuba where the biocontrol agent had been added. When the PCR diagnosis was combined with serial dilution of soil samples on selective medium, colonies were rapidly identified. The fungus was still present, albeit at low densities, in soils inoculated five years previously. The development of a baiting method allowed quick *in situ* screening of the isolates' ability to infect nematode eggs, and when combined with PCR diagnosis both varieties of the fungus could be detected in infected eggs. RFLP analysis of ITS sequences from *P. chlamydosporia* provided an extra level of discrimination between isolates.

INTRODUCTION

Root-knot nematodes are widespread and important pests of both annual and perennial crops in Cuba where they cause extensive damage to coffee and vegetable crops (Fernandez *et al.* 1998). 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. There is, therefore, a need to develop alternative methods to control populations of root-knot nematodes.

In Cuba, a network of approximately 200 small production plants produce several microbial control agents including *Bacillus thuringiensis*, *Metarhizium anisopliae*, *Lecanocillium lecanii* and *Trichoderma harzianum* for pest and disease control by local growers (Rosset 1997). The production of *Pochonia chlamydosporia* as a biocontrol agent of root-knot nematodes is also being investigated (Hidalgo-Diaz 2000, Atkins *et al.* 2002a). Residents in Havana have direct access to fresh vegetables via small intensively cropped 'organoponic' holdings within the city limits which are seen as a means of improving the diet of the urban poor (Murphy 1999). These holdings are small, often only around 2 ha, and the control of root-knot nematodes in an integrated pest management system including the use of *P. chlamydosporia* var. *catenulata* is being investigated (Hidalgo-Diaz 2000, Atkins *et al.* 2002a).

The nematophagous fungus *Pochonia chlamydosporia* var. *chlamydosporia* (syn. *Verticillium chlamydosporium*) is a widespread and naturally occurring facultative parasite of root-knot nematode eggs and has shown potential as a biocontrol agent against these nematodes (De Leij & Kerry 1991, De Leij *et al.* 1993). A number of molecular approaches have been developed to monitor it in the environment (Atkins *et al.* 2000, Hirsch *et al.* 2000, 2001, Mauchline, Kerry & Hirsch 2002). In a recent survey, a number of *Pochonia* species were isolated from Cuban soils and screened against *Meloidogyne* spp. (Hidalgo-Diaz *et al.* 2000); these included isolates of *P. chlamydosporia* var. *chlamydosporia*, var. *catenulata*, var. *catenulata* biotype A (which produces unusually large chlamydospores), *P. suchlasporia* var. *catenulata*, and *Verticillium psalliotae*. *P. chlamydosporia* var. *catenulata* was shown to be the most numerous species isolated from Cuban soils. One isolate (no. 392) had most potential as a biocontrol agent against

* Corresponding author.

Table 1. Fungal isolates and their sources.

Name	Isolate	Substrate	Source[a]
Pochonia chlamydosporia var. *chlamydosporia*	10	*Meloidogyne incognita* eggs	1
	200	*Heterodera avenae* infested soil	1
	338	*Meloidogyne incognita* eggs	2
	378	*Meloidogyne incognita* eggs	2
P. chlamydosporia var. *catenulata*	392	*Meloidogyne incognita* eggs	2
Verticillium psalliotae biotype A	334	*Meloidogyne incognita* eggs	2
V. psalliotae biotype B	388	*Meloidogyne incognita* eggs	2
Pochonia suchlasporia var. *suchlasporia*	381	*Meloidogyne* eggs	2
P. chlamydosporia var. *catenulata*	336	*Meloidogyne* sp. eggs	2
	353	*Meloidogyne* sp. eggs	2
P. chlamydosporia var. *catenulata* biotype A	364	*Meloidogyne incognita* eggs	2
	370	*Meloidogyne* sp. eggs	2
	374	*Meloidogyne* sp. eggs	2
Verticillium dahliae	327	Strawberry	3
Lecanocillium lecanii	17.3	Barley	4
Penicillium chrysogenum		Soil	5
Metarhizium anisopliae	ME1	Pecan weevil	6
Paecilomyces lilacinus		*Globodera pallida* eggs	7
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
Clonostachys rose[a]	10.1	Wheat	4

[a] 1, Rothamsted *Pochonia chlamydosporia* culture collection with the isolate number indicated: Rothamsted Research; 2, L. Hidalgo, CENSA, Cuba; 3, D. J. Barbara, HRI Wellesbourne; 4, W. Dawson, Rothamsted Research; 5, D. K. Arora, Rothamsted Research; 6, J. Pell, Rothamsted Research; 7, S. D. Atkins, Rothamsted Research. Isolates have also been deposited with CABI Bioscience, Egham (IMI).

Table 2. Description of soils from Cuba.

Designation	Site	Treatment
A	Censa[a]	Inoculated with 10 g *Pochonia chlamydosporia* var. *catenulata* colonized rice m^{-2} (1.4×10^7 chlamydospores g^{-1} rice)
B	Censa	Control soil
C	Censa	Inoculated with 20 g *P. chlamydosporia* var. *catenulata* colonized rice m^{-2} (1.4×10^7 chlamydospores g^{-1} rice)
S	Organoponico-Vivero-Alamar[b]	Inoculated with *P. chlamydosporia* var. *catenulata* 2 yr previously (6.18×10^6 chlamydospores g^{-1} rice)
B6	Organoponico-Vivero-Alamar	Inoculated with *P. chlamydosporia* var. *catenulata* 5 yr previously (6.18×10^6 chlamydospores g^{-1} rice)

[a] CENSA, San Jose de Las Lajas, Apto 10, La Habana, Cuba.
[b] Urban Farm, Organoponico-Vivero-Alamar, Havana Cuba.

root-knot nematodes and was shown to significantly reduce the number of nematodes (Hidalgo-Diaz *et al.* 2000) and was developed and tested in an integrated pest management system for the control of root-knot nematodes in small plot tests (Hidalgo-Diaz 2000, Atkins *et al.* 2002a).

To determine the efficacy of a biocontrol agent it is important to develop methods to monitor and identify the isolate released into the environment and to re-isolate it from soil and nematode samples (Avis, Hamelin & Belanger 2001). This has been done with a range of other fungi using selective media (Mitchell, Kannwischer-Mitchell & Dickson 1987, Kerry *et al.* 1993, Steadman, Marcinkowska & Rutledge 1994) and PCR detection including *P. chlamydosporia* var. *chlamydosporia* (Hirsch *et al.* 2000). Baiting techniques (Rodriquez-Kabana *et al.* 1994, Sikora, Schuster & Kiewnick 1994, Atkins *et al.* 2002b) have also enabled the pathogenic capability of isolates to be assessed *in situ*.

Here, we report on a range of tests to monitor the efficacy of *P. chlamydosporia* var. *catenulata*, including the development of specific PCR primers combined with serial dilution on selective media and baiting of soil.

MATERIALS AND METHODS

Isolates and media

Fungal isolates were taken either from the Rothamsted culture collection or from other sources stated in Table 1. Isolates were stored at 4 °C on corn meal agar (Oxoid,

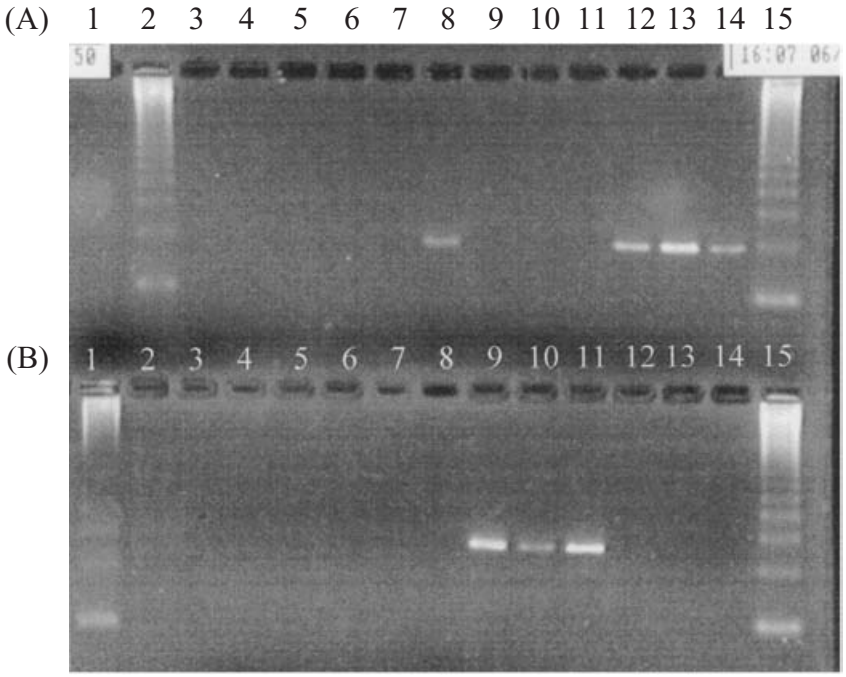

Fig. 1. Example of screening of fungi listed in Table 1 using primers for the specific detection of: (A) *Pochonia chlamydosporia* (Hirsch *et al*. 2000); lane 1 negative control, lanes 2 and 15 123 bp marker, lanes 3–14 isolates 381, 388, 334, 374, 370, 378, 392, 353, 336, 200, 10, 338; lanes 8, 12–14 var. *chlamydosporia*; (B) *P. chlamydosporia* var. *catenulata*; lanes 1 and 15 123 bp marker, lane 2 negative control, lanes 3–14 isolates 381, 388, 334, 374, 370, 378, 392, 353, 336, 200, 10, 338; lanes 9–11 var. *catenulata*.

Basingstoke) plates until needed, then subcultured onto fresh corn meal agar plates and incubated at 28 °.

Bi-phasic growth of cultures for chlamydospore production

For inoculation of soil, the fungus was cultured on sterilized cracked rice for 3 wk at 25 ° in 100 g aliquots in 500 ml conical flasks according to Hidalgo-Diaz (2000). The rice was then washed through a series of sieves and the chlamydospores collected on a 10 μm sieve. The sieves were blotted dry to remove excess water from the spores, the spores removed, weighed then mixed in 10 times their weight with fine sterilized sand for storage at 4 °. An aliquot (1 g) was mixed with 9 ml water and the number of chlamydospores counted in suspension in a haemocytometer. The level of germination was determined by plating an aliquot (200 μl) of the chlamydospore suspension onto sorbose agar (12 g technical agar, 2 g sorbose l^{-1}, autoclaved then 50 mg streptomycin, 50 mg chloramphenicol, 50 mg chlortetracycline added), incubated for 48 h at 28 ° then counting the proportion (%) of chlamydospores that had developed germ tubes, of 100 spores selected at random.

RFLP analysis of ITS sequences

DNA was extracted from a pure culture of each fungus using the method described by Arora, Hirsch & Kerry (1996). The conditions for RFLP analysis of the ITS sequence were as described by Zare, Gams & Evans (2001). The ITS sequences were digested with the enzyme *Hin*f1 (Roche, Lewes) at 37 ° for 1 h and the PCR products separated on 2% agarose gels.

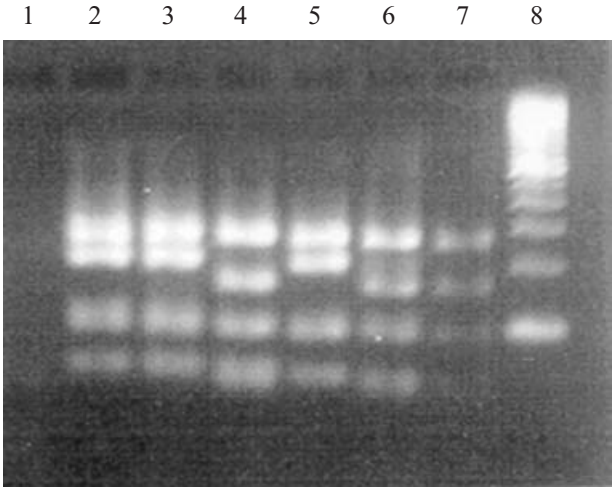

Fig. 2. Examples of RFLP analysis of ITS sequences digested with *Hin*f1 from *Pochonia chlamydosporia* isolates: lane 1 negative control, lane 2 var. *chlamydosporia* isolate 338, lane 3 var. *catenulata* biotype A isolate 370, lane 4 var. *catenulata* isolate 391, lane 5 var. *catenulata* biotype A isolate 374, lane 6 var. *catenulata* isolate 353, lane 7 var. *catenulata* isolate 392, lane 8 100 bp marker (Roche).

DNA extraction for PCR

Positive control DNA was prepared from pure culture as described previously (Arora *et al*. 1996). DNA was

Table 3. Summary of PCR analysis of isolates using primers specific for the detection of *Pochonia chlamydosporia* var. *chlamydosporia* and var. *catenulata*, and RFLP analysis of ITS PCR products using *Hin*fI.

Isolate no. (IACR-Rothamsted)	Country	*Pochonia chlamydosporia* var.	Tested with var. *chlamydosporia*-specific primers	Tested with var. *catenulata*-specific primers	ITS *Hin*fI digest grouping
327	Cuba	*chlamydosporia*	+	–	B
338	Cuba	*chlamydosporia*	+	–	B
363	Cuba	*chlamydosporia*	+	–	B
368	Cuba	*chlamydosporia*	+	–	B
372	Cuba	*chlamydosporia*	+	–	B
373	Cuba	*chlamydosporia*	+	–	B
378	Cuba	*chlamydosporia*	+	–	B
387	Cuba	*chlamydosporia*	+	–	B
207	Italy	*chlamydosporia*	+	–	B
204	Italy	*chlamydosporia*	+	–	B
10	Brazil	*chlamydosporia*	+	–	B
336	Cuba	*catenulata*	–	+	A
341	Cuba	*catenulata*	–	+	A
353	Cuba	*catenulata*	–	+	A
391	Cuba	*catenulata*	–	+	A
392	Cuba	*catenulata*	–	+	A
364	Cuba	*catenulata* Biotype A	–	–	B
366	Cuba	*catenulata* Biotype A	–	–	B
370	Cuba	*catenulata* Biotype A	–	–	B
374	Cuba	*catenulata* Biotype A	–	–	B
382	Cuba	*catenulata* Biotype A	–	–	B

Table 4. *Pochonia chlamydosporia* densities in soils supplied from Cuba on selective media plates and confirmed by PCR analysis.

Cuban soil sample	CFU g^{-1} soil	Number of colonies tested	Number of colonies shown to be *P. chlamydosporia* var. *catenulata*
A	3.75×10^3	4	3
B	0	0	0
C	9×10^3	4	3
S	2.5×10^2	1	1
B6	1.25×10^3	6	4

extracted from a range of soil fungi (Table 1) taken from the Rothamsted culture collection. Cultures were incubated at 28 ° in 100 ml Czapek Dox liquid medium (Oxoid) and shaken at 130 rpm for 1 wk.

Development of selective primers

Primers (forward primer *PcatF*: GTG AAC TTA TAC CAT TTT TTG; reverse primer *PcatR*: CAC AAG TCC CCA TCC GC) were designed by the comparison of *P. chlamydosporia* var. *catenulata* ITS sequences from the GenEMBL database (accession nos AJ292398 and AJ292399) with other *Verticillium* ITS sequences (accession nos AJ291800, AJ291806, AJ292397, AF108468 and AJ303054) and other fungal ITS sequences contained in the same database. The primers were compared to the database using FASTA and BLASTA to confirm specificity. PCR reactions of 20 µl contained 0.1 µM each primer, 2 µl ×10 PCR reaction buffer (Roche, 1.5 mM Mg^{2+}), 0.1 µM each dNTP, 1 U *Taq* polymerase (Roche) and 20 ng template DNA. PCR conditions were optimized as follows: 95 ° followed by 35 cycles of 94 ° for 1 min, 60 ° for 1 min, 72 ° for 1 min, with a final incubation at 72 ° for 5 min.

Detection of varieties using specific primer sets

DNA was extracted from a range of fungi (Table 1) and used in PCR reactions with either *Pochonia chlamydosporia* var. *catenulata* or var. *chlamydosporia* (Hirsch *et al*. 2000) specific primer sets.

Detection of var. catenulata *in soil*

Soils were collected from two sites in Havana (Table 2) where isolate *Pochonia chlamydosporia* var. *catenulata* 392 had been used to control nematodes in previous experiments (Atkins *et al*. 2002a). Soil was inoculated with colonized rice media at a rate of 10 and 20 g m^{-2}; the density of chlamydospores g^{-1} rice is shown in Table 2. A control soil where the fungus had not been previously applied was also sampled. Soil was serially diluted and plated onto *P. chlamydosporia* selective media (Kerry *et al*. 1993) and incubated for 11 d at 28 °.

After 11 d, colonies were counted and then lifted from the agar plate using a sterile tooth pick and used to inoculate 10 ml sterile Czapek Dox broth (Oxoid). Cultures were incubated for 4 d at 28 °, and DNA extracted from a small amount of mycelium using the method of Klimyuk *et al*. (1993). DNA was then used in PCR reactions using *P. chlamydosporia* var. *catenulata* primers.

DNA was extracted from triplicate 0.25 g soil samples from all the soil sites using the soil DNA extraction kit from Mo-Bio (Mo-Bio Laboratories, CA). An

Table 5. Egg infections (%) recorded from bait experiment in soils treated with *Pochonia chlamydosporia* isolates, and recovery of var. *catenulata* from Cuban soils C and S.

Treatment	% egg infection (±SE)	F. pr from control (SED)	Comments
Soil inoculated with *P. chlamydosporia* var. *chlamydosporia* isolate 132	71 ± 2.3	<0.001 SED (5.18)	4 of the 20 infected eggs identified as var. *chlamydosporia* by PCR analysis
Soil inoculated with *P. chlamydosporia* var. *catenulata* isolate 392	79 ± 1.6	<0.001 SED (4.89)	7 of the 20 infected eggs were identified as var. *catenulata* by PCR analysis
Control	26 ± 4.6		No var. *chlamydosporia*, or var. *catenulata* detected by PCR analysis
Cuban C	49	Na	None of the 20 eggs screened was identified as var. *catenulata* by PCR analysis
Cuban S	68	Na	None of the 20 eggs screened was identified as var. *catenulata* by PCR analysis

aliquot from each sample (20 ng) was used in PCR reactions using primers specific for the detection of each variety.

Baiting of soils and detection of isolates from infected eggs

Chlamydospores prepared from *Pochonia chlamydosporia* var. *chlamydosporia* isolate 132 and var. *catenulata* isolate 392 were collected from the rice medium as above and mixed 1:10 (w/w) with sand. The mixture was used to inoculate 50 g sandy loam soil at a rate of 5×10^3 chlamydospores g^{-1} soil, mixed into the soil by shaking in a plastic bag, then 10 g aliquots were added to five separate boiling tubes (25 mm diam, length 150 mm). A bait containing five *Meloidogyne incognita* egg masses freshly removed from an infected aubergine root and enclosed in a nylon mesh of pore diameter 30 μm was added to the soil, and the soil packed around it. The boiling tubes were plugged with cotton wool and incubated for 7 d at 28 °. After 7 d the baits were removed, the egg masses broken open using forceps and the eggs suspended in 1 ml water. The eggs were spread onto water agar containing antibiotics (8 g technical agar, 50 mg streptomycin, 50 mg chloramphenicol, 50 mg chlortetracycline l^{-1}) and incubated for 48 h at 28 °. Infected eggs were then counted. A control of uninoculated soil was also baited at the same time. Infection data collected were analysed using one way analysis of variance (ANOVA) using the Genstat programme (Genstat 5 Committee 1993). An aliquot of soil from each sample C and S previously inoculated with the fungus from Cuba (Table 2) was also baited. To confirm identification of the fungi in colonized eggs, infected eggs (20 in total) were taken from each treatment and individual eggs used to inoculate 10 ml Czapex Dox broth as above. The cultures were incubated and DNA extracted as described above. The DNA was used in PCR reactions using primers for the specific detection of each of the two varieties. The DNA extraction process was checked by using 1 μl from each extraction in a PCR reaction using universal ITS primers as described by Arora *et al*. (1996).

RESULTS

The specific ITS primers for the detection of *Pochonia chlamydosporia* var. *catenulata* generated a single PCR product of 352 bp in size. The product was only generated from DNA extracted from this variety and not from that of any other fungi tested. In particular, the primers did not detect the presence of var. *catenulata* biotype A (Hidalgo-Diaz *et al*. 2000). The β-tubulin primers designed for the detection of var. *chlamydosporia* only generated a PCR product for isolates of that species (Fig. 1A). Both primer sets, therefore, demonstrated their specificity and diagnostic capability (Fig. 1A, B).

RFLP analysis of the ITS PCR product separated *P. chlamydosporia* var. *chlamydosporia* from var. *catenulata* isolates by distinctive band fragments as demonstrated by Zare *et al*. (2001). Band sizes generated from all var. *chlamydosporia* isolates tested were: 270, 220, 90 and 50 bp. Var. *catenulata* band sizes were: 270, 180, 90 and 50 bp. Var. *catenulata* biotype A band fragments were the same size as those produced by var. *chlamydosporia* isolates (Fig. 2, Table 3), but this biotype did not generate a PCR product when tested with primers specific for the detection of var. *chlamydosporia* (Fig. 1A) which generated a PCR product of 260 bp from isolates of that variety.

P. chlamydosporia isolates were detected in all soils supplied from Cuba apart from the uninoculated control soil (B) when plated onto selective medium for the detection of this fungus. The majority of isolates were identified as var. *catenulata* (Table 4) when screened with primers specific for this taxon.

The species specific PCR primers did not detect *P. chlamydosporia* var. *chlamydosporia* in any of the soil samples. However, var. *catenulata* was detected in two of the three samples from treatment C and one from treatment A by direct PCR using DNA extracted from soils using *P.* var. *catenulata* specific primers; var. *catenulata* was not detected in any other soil samples by direct PCR.

The proportion of eggs infected in the baits from treatments inoculated with *P. chlamydosporia* var. *chlamydosporia* isolate 132 and var. *catenulata* isolate

392 were significantly greater than in the control (Table 5). The level of infection in Cuban soils C and S was also greater than in the control. Of the eggs taken from each treatment for those inoculated with isolate 132, 20% were identified by PCR analysis as var. *chlamydosporia*. Of the eggs taken from the treatment inoculated with isolate 392, 35% were identified by PCR analysis as being infected by var. *catenulata*. Neither variety was detected in any of the infected eggs from the baits from Cuban soils C and S, or the control. PCR products were produced from all DNA extractions when DNA was used in a PCR reaction using universal ITS primers demonstrating the efficiency of the extraction method.

DISCUSSION

Verticillium chlamydosporium and *V. catenulatum* were considered varieties of one species, *V. chlamydosporium*, by Gams (1988). He considered that the distinction between conidial chains and heads was of limited taxonomic value as there was no sharp delineation between isolates. In introducing the generic name *Pochonia* for these fungi, (Zare *et al.* 2001) recognized the similarities between the two varieties of *P. chlamydosporia*. The primers developed permit a quick discrimination between the varieties which had previously proved difficult to differentiate morphologically. In addition, this provides support for the separate naming of the two organisms, an important development if isolates are to be registered as biocontrol agents.

Primers specific for the detection of var. *catenulata* isolates have demonstrated excellent discrimination from other soil fungi, and other isolates of *Pochonia*, in much the same way as the β-tubulin primers described by Hirsch *et al.* (2000) have for var. *chlamydosporia*. The primers tested in our study could be used to detect the fungus within the soil directly from DNA extracted from the soil, but this was unreliable due to the low densities of the fungus, heterogeneity in its spatial distribution and the small sample size. Therefore, such PCR-based methods alone should not be relied on to detect the fungus in soil samples. As shown in this study, it is important to combine PCR diagnostics with methods such as baiting and plating soil onto selective media to provide information on the population size and viability of the fungus in environmental samples.

The use of the specific primers to identify colonies taken from plates inoculated with Cuban soils allowed a rapid screening that would otherwise have been both time-consuming and unreliable as identification based on morphology is not always accurate. The var. *catenulata* was recovered and identified from all soils, and although the enumeration using serial dilution of the fungus showed that the densities were low, particularly in the experimental plots S and B6, the screen demonstrated that the fungus was still present up to 5 yr after its application, an important factor in the establishment of a soil suppressive to root-knot nematodes.

The baiting method described allowed a rapid screen of the infection potential of isolates. Detection of var. *chlamydosporia* in the screened eggs taken from the treatment inoculated with a var. *chlamydosporia* isolate demonstrates that detection and identification by PCR analysis is possible. The significant difference in the amount of egg infection recorded in the treatments inoculated with var. *chlamydosporia* and var. *catenulata* when compared to the control treatment demonstrated that the presence of the fungus significantly reduced the healthy nematode population.

Hidalgo-Diaz *et al.* (2000a) hypothesized that the isolate of var. *catenulata* that was morphologically distinct from other var. *catenulata* isolates represented a new biotype (biotype A). The analysis in this paper demonstrates that the fungus is genetically distinct from either named variety in that it did not generate a PCR signal when tested with the two sets of specific primers.

The potential of *P. chlamydosporia* var. *catenulata* as a biocontrol agent is still under investigation, and as with all such potential agents requires careful monitoring within its target environment. In this paper several approaches including baiting, selective media and biovar specific PCR have been developed for evaluating the population, host pathogenicity and viability of the variety within soil. The use of these has demonstrated the importance of using a combination of monitoring techniques to acquire a fuller understanding of the fate and survival of the released organism.

ACKNOWLEDGEMENTS

Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the UK. L.H.-D. was part funded by the British Council.

REFERENCES

Arora, D. K., Hirsch, P. R. & Kerry, B. R. (1996) PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. *Mycological Research* **100**: 801–809.

Atkins, S. D., Clark, I. M., Sosnowska, D. & Kerry, B. R. (2002b) Initial testing of potential fungal biological control agents for potato cyst nematodes. *Brighton Crop Protection Conference 2002: Pests and Diseases*: 79–84.

Atkins, S. D., Peberdy, J., Kerry, B. R. & Hirsch, P. R. (2000) Development of a transformation system for the nematode biological control fungus *Verticillium chlamydosporium*. In *Biology of Plant–Microbe Interactions* (P. J. G. de Wit, T. Bisseling & W. J. Stiekema, eds) **2**: 336–341. International Society for Molecular Plant–Microbe Interactions, St Paul, MN.

Atkins, S. D., Hidalgo-Diaz, L., Kalisz, H., Mauchline, T. H., Hirsch, P. R. & Kerry, B. R. (2002a) Development of a new management strategy for the control of root-knot nematodes (*Meloidogyne* spp.) in organic vegetable production. *Pest Management Science*: in press.

Avis, T. J., Hamelin, R. C. & Belanger, R. R. (2001) Approaches to molecular characterization of fungal biocontrol agents: some case studies. *Canadian Journal of Plant Pathology* **23**: 8–12.

De Leij, F. A. M. M. & Kerry, B. R. (1991) The nematophagus fungus *Verticillium chlamydosporium* as a potential biological

control agent for *Meloidogyne arenaria*. *Revue de Nematologia* **14**: 157–164.

De Leij, F. A. A. M., Kerry, B. R. & Dennehy, J. A. (1993) *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita* and *M. hapla* in pot and microplot tests. *Nematologica* **39**: 115–126.

Fernandez, E., Perez, M. & Gandarilla, H. (1998) Guia para disminuir infestaciones de *Meloidogyne* spp. Mediante el empleo de cultivos no susceptibles. *Instituto Nacional de Investigaciones de la Sanidad Vegetal, Boletin Tecnico* **4**: 1–48.

Gams, W. (1998) A contribution to the knowledge of nematophagous species of *Verticillium*. *Netherlands Journal of Plant Pathology* **4**: 123–148.

Genstat 5 Committee (1993) *Genstat 5 Release 3 Reference Manual*. Clarendon Press, Oxford.

Hidalgo-Diaz, L. (2000) *Potencialidades de cepas autotonas de Verticillium chlamydosporium* (*Goddard*) *como agents de control biologico de Meloidogyne spp*. PhD thesis, University Agraria, La Habana.

Hidalgo-Diaz, L., Bourne, J. M., Kerry, B. R. & Rodriguez, M. G. (2000) Nematophagous *Verticillium* spp. in soils infested with *Meloidogyne* spp. in Cuba: isolation and screening. *International Journal of Pest Management* **46**: 277–284.

Hirsch, P. R., Mauchline, T. H., Mendum, T. A. & Kerry, B. R. (2000) Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode-infested plant roots using PCR. *Mycological Research* **104**: 435–439.

Hirsch, P. R., Atkins, S. D., Mauchline, T. H., Morton, C. O., Davies, K. G. & Kerry, B. R. (2001) Methods for studying the nematophagous fungus *Verticillium chlamydosporium* in the root environment. *Plant and Soil* **232**: 21–30.

Kerry, B. R., Kirkwood, I. A., De Leij, F. A. A. M., Barba, J., Leidjens, M. B. & Brookes, P. C. (1993) Growth and survival of *Verticillium chlamydosporium* Goddard, a parasite of nematodes in soil. *Biocontrol Science and Technology* **3**: 355–365.

Klimyuk, V. I., Carroll, B. J., Thomas, C. M. & Jones, J. D. G. (1993) Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *Plant Journal* **3**: 493–494.

Mauchline, T. H., Kerry, B. R. & Hirsch, P. R. (2002) Quantification in soil and the rhizosphere of the nematophagous fungus, *Verticillium chlamydosporium* by competitive PCR and comparison with selective plating. *Applied and Environmental Microbiology* **68**: 1846–1853.

Mitchell, D. J., Kannwischer-Mitchell, M. E. & Dickson, D. W. (1987) A semi-selective medium for isolation of *Paecilomyces lilacinus* from soil. *Journal of Nematology* **19**: 255–256.

Murphy, C. (1999) *Cultivating Havana: urban agriculture and food security in the years of crisis*. [Institute for Food and Development Policy, Development Report No. 12.] Food First Books, Oakland, CA.

Rodriguez-Kabana, R., Kokalis-Burelle, N., Kiewnick, S., Schuster, R. P. & Sikora, R. A. (1994) Alginate films for delivery of root-knot nematode inoculum and evaluation of microbial interactions. *Plant and Soil* **164**: 147–154.

Rosset, P. (1997) Cuba: ethics, biological control and crisis. *Agriculture and Human Values* **14**: 291–302.

Sikora, R. A., Schuster, R. P. & Kiewnick, S. (1994) Indexing biodiversity and antagonistic potential in agricultural soil of Madagascar. *Mededelingen Faculteit Landbouwwet-tenschappen Universiteit Gent* **59** (2b): 781–790.

Steadman, J. R., Marcinkowska, J. & Rutledge, S. (1994) A semi-selective medium for isolation of *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology* **16**: 68–70.

Zare, R., Gams, W. & Evans, H. C. (2001) A revision of *Verticillium* section *Prostrata*. V. The genus *Pochonia*, with notes on *Rotiferophthora*. *Nova Hedwigia* **73**: 51–86.

Corresponding Editor: N. Magan