Crop Protection 42 (2012) 102–107

Contents lists available at SciVerse ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Screening of *Pochonia chlamydosporia* Brazilian isolates as biocontrol agents of *Meloidogyne javanica*

Rosangela Dallemole-Giaretta [a], Leandro G. Freitas [b], Everaldo A. Lopes [c,*], Olinto L. Pereira [b], Ronaldo J.F. Zooca [b], Silamar Ferraz [b]

[a] Universidade Estadual do Centro-Oeste, Campus CEDETEC, Guarapuava, PR 85040-080, Brazil
[b] Universidade Federal de Viçosa, Viçosa, MG 36570-000, Brazil
[c] Universidade Federal de Viçosa, Campus Rio Paranaíba, Instituto de Ciências Agrárias, Rodovia MG 230, km 07, Caixa Postal 22, Rio Paranaíba, MG 38810-000, Brazil

ARTICLE INFO

Article history:
Received 9 February 2012
Received in revised form
31 May 2012
Accepted 2 June 2012

Keywords:
Biological control
Nematophagous fungi
Root-knot nematodes

ABSTRACT

Pochonia species can occur in many *Meloidogyne*-infested soils throughout the world. The objective of this work was to isolate *Pochonia* species from different *Meloidogyne*-infested soil locations in Brazil and to screen for potential isolates effective in controlling *Meloidogyne javanica* under glasshouse conditions. A total of 29 isolates were identified as *Pochonia chlamydosporia*. Among them, 65.52% were identified as *P. chlamydosporia* var. *chlamydosporia* and 34.48% as *P. chlamydosporia* var. *catenulata*. For the glasshouse experiments, fungal chlamydospores were incorporated into the soil (5000 g soil^{-1}) and one tomato seedling was transplanted in 300 mL pot. After one week, each plant was inoculated with 1000 eggs of *M. javanica*. The isolates 1, 2, 3, 4, 9, 10, 21, 24, 28 (from Brazil) and 64 (from Spain) were the most efficient in reducing the number of eggs of the nematode. These isolates were re-evaluated in another glasshouse experiment, this time the number of eggs was increased (3000), inoculating both eggs and chlamydospores at the same time. After one week of inoculation, a tomato seedling was transplanted in each pot. In this experiment, isolates 64 and 10 were the most efficient in reducing the number of eggs by 72% and 60%, respectively.

1. Introduction

The nematophagous fungus *Pochonia chlamydosporia* Zare & Gams (syn. *Verticillium chlamydosporium* Goddard) is a facultative parasite of nematode eggs. Since it was first found to be associated with the infection of plant-parasitic nematodes (Willcox and Tribe, 1974; Kerry, 1975), this fungus has been extensively studied as a potential biological control agent for cyst and root-knot nematodes (Godoy et al., 1983; De Leij and Kerry, 1991; Crump and Irving, 1992; Hidalgo-Diaz et al., 2000; Sosnowska and Sikora, 2001; Sun et al., 2006; Lopes et al., 2007).

This antagonistic fungus has been isolated from a wide range of soil types throughout the world, and can survive as a saprophyte in the absence of the nematode host (Verdejo-Lucas et al., 2003). Some isolates of the fungus are rhizosphere competent (Bourne et al., 1996) as well as easily cultivated in vitro (Bourne et al., 1999) and produce chlamydospores, which are the resistant resting spores that prolong their survival in the field under stress situations and storage. Nevertheless, there is a great variation among *P. chlamydosporia* isolates in their ability to colonize nematode eggs (Kerry, 1995; Hallmann et al., 2009). Thus, there is a need for screening effective isolates for nematode control that are adapted to a range of field conditions (Kerry and Bourne, 2002).

Generally, the screening process is initiated by 'in vitro' tests containing a large number of isolates. However, the results obtained under laboratory conditions are not frequently similar to those obtained under either glasshouse or field conditions (Hidalgo-Diaz et al., 2000; Pérez-Rodríguez et al., 2007). Although it is more space and time consuming, the screening of *P. chlamydosporia* isolates under a glasshouse can provide more reliable results for further studies under field conditions.

Thus, the main goal of this work was to isolate *P. chlamydosporia* species from *Meloidogyne*-infested soils and to select, under glasshouse conditions, a fungal isolate potentially effective to control of *Meloidogyne javanica* (Treub) Chitwood.

* Corresponding author. Tel.: +55 34 3855 9322; fax: +55 34 3855 9317.
E-mail address: everaldolopes@ufv.br (E.A. Lopes).

0261-2194/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cropro.2012.06.002

2. Materials and methods

2.1. Isolation, identification and morphological characterization of *P. chlamydosporia*

Soil samples were collected in *Meloidogyne*-infested areas for *P. chlamydosporia* isolation from three Brazilian vegetable production regions located in Viçosa – Minas Gerais (20°45′ S, 42°51′W, 650 m), Mariópolis – Paraná (26°21′ S, 52°33′W, 850 m) and Venda Nova do Imigrante – Espírito Santo (20°19′ S, 41°07′W, 730 m) (Table 1). In each site, soil samples (500 g each) were randomly collected from the rhizosphere of *Meloidogyne*-infected plants.

In order to obtain these isolates, 10 g of soil samples were placed in 200 mL Erlenmeyer flasks containing 90 mL of sterilized water and mixed in an orbital shaker (Tecnal, Piracicaba, Brazil) for 30 min. Subsequently, a serial dilution was prepared to produce a 10^{-4} solution and 1 mL of each diluted soil sample was plated on four Petri dishes (9 cm diameter) containing semi-selective medium (Gaspard et al., 1990).

After two weeks of incubation at 25 °C in a BOD incubator (Fanem, São Paulo, Brazil), growing colonies of *P. chlamydosporia* were transferred to Petri dishes containing Potato-Dextrose-Agar (PDA, Vetec, Duque de Caxias, Brazil). The fungal isolates were allowed to grow for one week at 25 °C, and were stored long-term on sterile filter paper discs (0.5 cm diameter) at 8 °C for subsequent studies (Smith and Onions, 1994; Dhingra and Sinclair, 1995).

Initially, *Pochonia* isolates were characterized by the measurement of the radial growth and color of each colony. Then, we recorded the presence of chlamydospores and fungal reproductive structures (Zare and Gams, 2004). To perform this characterization, fungal isolates were recovered by incubating pieces of colonized filter paper on Corn-Meal-Agar (CMA, Difco, Detroit, USA) at 25 °C in the darkness for five days. Then, a 5 mm-diameter mycelium disc, obtained from the edge of the freshly activated colonies, was placed at the center of Petri dishes-containing PDA and incubated in the darkness at 25 °C during 10 days. The diameter of each colony was measured twice in opposing radial orientations with a ruler. The presence of chlamydospores was also evaluated. This experiment was conducted in a completely randomized block design with three repetitions and it was performed after the glasshouse screening.

CMA microculture slides were used to measure the reproductive structures of the isolated *P. chlamydosporia* species. This method consisted of covering the mycelium with a glass cover slip and incubating in the dark at 25 °C. After 21 days, glass cover slips containing vegetative and reproductive mycelia were stained with lactophenol cotton blue and examined using a light microscope (400×) for taxonomic identification of the isolates (Zare and Gams, 2004).

2.2. Glasshouse screening of *P. chlamydosporia* isolates

In order to assess *P. chlamydosporia* isolates as potential biocontrol agents of *M. javanica*, three experiments were conducted in a glasshouse at the Department of Plant Pathology at Universidade Federal de Viçosa, Viçosa – MG, Brazil. In addition to the isolates obtained in this work, other three isolates were used in the experiments (52, 64 and 75, provided by L. V. López-Llorca – Universidad de Alicante, Spain) for comparison against the performance of the Brazilian isolates.

For a primary selection of the *P. chlamydosporia* isolates, two glasshouse experiments were carried out in a completely randomized block design with eight repetitions. In experiment 1, the treatments consisted of 15 isolates (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 and 16) and experiment 2 included 14 additional isolates (18, 19, 20, 21, 22, 23, 24, 13, 26, 28, 29, 52, 64 and 75). In experiments 1 and 2, the control treatment consisted of a "soil mixture" (detailed below) without the addition of the fungus.

Table 1
Pochonia chlamydosporia isolates from *Meloidogyne* spp.-infested fields from three vegetable production regions in Brazil.

Isolate	Locality (Municipality – State – Region)	Geographical coordinates	Dominant crops in the filed[a]
1	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
2	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
3	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
4	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
5	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
6	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
7	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
8	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
9	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
10	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
11	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
12	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
13	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
14	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
15	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
16	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
17	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
18	Venda Nova do Imigrante – Espírito Santo – Southeast	20°19′ S, 41°07′W	Tomato
19	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
20	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
21	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
22	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
23	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
24	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
25	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
26	Viçosa – Minas Gerais – Southeast	20°45′ S, 42°51′W	Lettuce
27	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
28	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
29	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
30	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha
31	Mariópolis – Paraná – South	26°21′ S, 52°33′W	Arracacha

[a] Lettuce (*Lactuca sativa*), Arracacha (*Arracacia xanthorrhiza*) and Tomato (*Solanum lycopersicum*).

In both experiments, a mixture of soil (Yellow red oxisol, ph 6.5) and sand at the ratio 1:1 (w:w), previously treated with methyl bromide, was placed in 5 L plastic bags containing the antagonist's chlamydospore suspension, previously calibrated to 5000 chlamydospores (g soil^{-1}) (Kerry and Bourne, 2002). The inoculum of *P. chlamydosporia* was produced according to Kerry (1991). The final mixture was then homogenized manually for approximately 3 min and placed in plastic pots containing 300 g of soil. From each treatment, a 100 g soil sample was taken to evaluate the fungal density (Colony-Forming Units – CFU) in a semi-selective medium (Gaspard et al., 1990; Kerry, 1991). After soil sampling, a tomato seedling (*Solanum lycopersicum* L., nematode susceptible cultivar Santa Clara) was transplanted into each pot (Lopes et al., 2007). After one week, a suspension containing 1000 eggs of *M. javanica* was applied into opened holes in the soil around each plant. Populations of the nematode were maintained on tomato (cv. Santa Clara) in the glasshouse. Nematode eggs were extracted from tomato roots with a sodium hypochlorite solution (Hussey and Barker, 1973).

These experiments were conducted for 45 days. The average air temperature was 35 ± 2 °C and average minimum temperature was 21 ± 2 °C and natural photoperiod (12 h light alternating with 12 h darkness).

Based on the results from the previous experiments, the isolates 1, 2, 3, 4, 9, 10, 21, 24, 28 and 64 were re-evaluated in experiment 3 following the methodology described before with some

modifications. We used pots containing 500 g of soil added with 3000 eggs of the nematode and 5000 chlamydospores (g soil^{-1}) of the fungus. The soil was watered and maintained at 60% of the field capacity. After one week, one tomato seedling was transplanted to each pot. This experiment was conducted for 60 days. The average air temperature was 33 ± 2 °C and the average minimum temperature was 15 ± 2 °C.

At the end of all experiments, the tomato plant response was evaluated by weighting the whole fresh roots of all plants. Nematode parameters were measured by counting the number of galls and eggs per root system. Eggs were extracted from each root system according to Hussey and Barker (1973). Soil samples were taken again to determine the fungus population in the soil (CFU g^{-1}) following the same procedure as described above.

In all experiments, the plants were watered as needed (usually twice a day) and fertilized weekly using a commercial water-soluble fertilizer (15-15-20 + Ca, S, Mg, Zn, B, Fe and Mn; 3 g l^{-1}; 30 mL pot^{-1}, Ouro Verde, Assis, Brazil).

All data were tested for normality (Shapiro–Wilks' test) to check the need for a transformation. Then, they were subjected to analysis of variance and treatment means were compared by Scott-Knott's test at 5% level of probability (Scott and Knott, 1974).

3. Results

3.1. Isolation, identification and morphological characterization of *P. chlamydosporia*

Thirty-one isolates of *Pochonia* were obtained from the soil samples. However, isolates 8 and 25 could not be recovered from storage, and so they were not morphologically identified. All 29 isolates produced chlamydospores in aerial mycelium after cultivation on BOD at 25 °C in the dark for ten days. Fungal colonies appeared white, cream or yellowish in color while their diameter varied from 3.0 to 4.5 cm depending on the isolate (Table 1). All isolates belonged to *P. chlamydosporia* (Tables 1 and 2). Nineteen isolates (65.52%) had globose to ellipsoid hyaline conidia that measured 1.0–5.0 × 1.0–4.0 μm. Chlamydospores varied in size (8.0–26.0 × 8.0–31.0 μm) and the phialides had 3.0–60.0 μm in length, producing conidial aggregation in heads, belonged to *P. chlamydosporia* var. *chlamydosporia* (Zare and Gams, 2004) (Table 2). Ten isolates (34.48%) that had globose to sub-globose hyaline conidia sized 1.0–4.0 × 1.0–4.0 μm, chlamydospores were 5.0–31.0 × 6.0–32.0 μm and phialides that varied from 3.0 to 92.0 μm in length, producing conidia arranged in chains, belonged to *P. chlamydosporia* var. *catenulata* (Table 2).

3.2. Glasshouse screening of *P. chlamydosporia* isolates

Twelve out of the 15 *P. chlamydosporia* isolates studied in experiment 1 reduced the number of eggs of *M. javanica* when compared to the control (Table 3). The number of eggs of the nematode was reduced from 39 to 67.3% in plots treated with the isolates 1, 2, 3, 4, 9 and 10, in comparison to the control (Table 3). Number of galls for root system was not reduced only by the isolates 6, 8, 14 and 15 (Table 3). Reduction of the number of galls ranged from 18.4% (isolate 2) to 34.3% (isolate 1).

In experiment 2, isolates 28, 64, 24 and 21 reduced the number of eggs from 57.3% (isolate 28) to 75.2% (isolate 21) (Table 4). Eight isolates (18, 19, 21, 22, 23, 24, 28 and 29) reduced the number of galls induced by *M. javanica* on tomato roots from 11.6% (isolate 29) to 25.0% (isolate 21), when compared to the control (Table 4).

Based on the results of these two experiments, especially regarding to the ability of isolates in reducing the number of eggs of the pathogen, the isolates 1, 2, 3, 4, 9, 10, 21, 24, 28 and 64 were re-evaluated in experiment 3. None of these isolates reduced the number of galls of *M. javanica* in this experiment (data not shown). On the other hand, all fungal isolates reduced the number of eggs of the nematode in tomato roots (Fig. 1), especially isolates 2, 3, 10 and 64, with reductions from 54 to 72%. Isolate 10, for example, reduced the number of eggs by 39% and 60% in experiment 1 and experiment 3, respectively.

None of the treatments affected the weight of the tomato roots in all experiments (data not shown).

Soil establishment of *P. chlamydosporia* throughout the experiments is shown in Table 5. The colony-forming unit values varied

Table 2
Characterization of the morphology and variety of the *Pochonia chlamydosporia* isolates from root-knot nematode-infested soils from Brazil.

Isolates	Phialide length (μm)	Conidia (μm)		Chlamydospores (μm)		Variety
		Length	Width	Length	Width	
1	6–38	1.0–2.5	1.0–4.0	18.0–23.0	20.5–27.0	*catenulata*
2	6–28	1.0–4.0	1.0–4.0	20.5	23.0	*chlamydosporia*
3	3–28	1.0–3.0	1.0–2.0	11.5–19.0	14.0–24.0	*chlamydosporia*
4	9.5–22	2.5–4.0	1.0–2.0	14.0–18.0	17.0–24.0	*chlamydosporia*
5	9.5–28	2.0–2.5	1.0–2.5	19.0–28.0	23.0–32.0	*catenulata*
6	9.5–20.5	2.5–2.5	2.5–2.5	5.0–19.0	6.0–20.5	*catenulata*
7	3–16	2.0–4.0	1.0–2.0	17.0–19.0	15.0–24.0	*chlamydosporia*
9	5–28	2.0–4.0	1.0–2.0	17.0–20.5	19.0–26.0	*chlamydosporia*
10	6–22	1.0–4.0	1.0–2.0	17.0–22.0	23.0–31.0	*chlamydosporia*
11	5–22	1.0–3.0	1.0–3.0	18.0–2.40	17.0–29.0	*chlamydosporia*
12	9.5–25	1.0–4.0	1.0–4.0	18.0–24.0	22.0–27.0	*catenulata*
13	6–32	1.0–5.0	1.0–2.5	–	–	*chlamydosporia*
14	6–38	2.0–3.0	1.0–4.0	18 0–20.5	19.0–27.0	*catenulata*
15	3–28	1.0–4.0	1.0–2.5	14.0–26.0	15.0–28.0	*chlamydosporia*
16	9.5–32	2.0–2.5	2.0–3.0	19 0–31.0	24.0–28.0	*catenulata*
17	6–54	2.5–5.0	1.0–4.0	–	–	*chlamydosporia*
18	5–32	1.0–4.0	1.0–4.0	18.0–24.0	19.0–27.0	*catenulata*
19	3–38	1.0–4.5	1.0–2.0	18.0–23.0	19.0–28.0	*chlamydosporia*
20	6–92	2.0–4.0	2.0–4.0	18.0–28.0	23.0–29.0	*catenulata*
21	3–28	1.0–5.0	1.0–2.5	11.5–20.5	14.0–26.0	*chlamydosporia*
22	6–25	2.0–4.0	1.0–2.5	17.0–260	18.0–31.0	*chlamydosporia*
23	6–25	1.0–4.5	1.0–2.0	10.0–22.0	11.5–29.0	*chlamydosporia*
24	5–28	1.0–3.0	1.0–2.5	10.0–23.0	11.5–24.0	*catenulata*
26	6–60	1.0–4.0	1.0–4.0	8.0–22.0	8.0–26.0	*chlamydosporia*
27	3–47	2.0–4.0	2.0–4.0	–	–	*catenulata*
28	6–54	2.0–4.5	1.0–4.0	9.0–23.0	8.0–28.0	*chlamydosporia*
29	6–35	1.0–4.5	1.0–2.5	14.0–24.0	14.0–31.0	*chlamydosporia*
30	5–38	1.0–4.5	1.0–2.5	14.0–21.0	19.0–27.0	*chlamydosporia*
31	5–32	2.0–5.0	1.0–2.5	–	–	*chlamydosporia*

Table 3
Number of root galls and *Meloidogyne javanica* eggs per tomato root system after application of 5000 chlamydospores (g soil^{-1}) of the *Pochonia chlamydosporia* isolates and cultivation of tomato in soil infested with 1000 eggs of the nematode under glasshouse conditions in Viçosa, Brazil (Experiment 1).

Treatment	Number of nematode eggs	Number of root galls
Control	351,167 a	499 a
Isolate 12	352,999 a	393 b
Isolate 7	350,636 a	387 b
Isolate 8	321,055 a	483 a
Isolate 16	288,624 b	371 b
Isolate 5	263,359 b	354 b
Isolate 11	250,627 b	399 b
Isolate 15	243,862 b	437 a
Isolate 6	241,130 b	430 a
Isolate 14	238,028 b	443 a
Isolate 10	214,190 c	376 b
Isolate 9	170,412 c	347 b
Isolate 4	150,099 c	372 b
Isolate 2	143,053 c	407 b
Isolate 3	142,255 c	361 b
Isolate 1	114,814 c	328 b
Coefficient of variation (%)	22.3	20.1

Values are means of eight repetitions. Means followed by different letters in the column indicate significant differences among treatments according to Scott-Knott's test ($P < 0.05$).

Table 4
Number of root galls and *Meloidogyne javanica* eggs per tomato root system after application of 5000 chlamydospores (g soil^{-1}) of the *Pochonia chlamydosporia* isolates and cultivation of tomato in soil infested with 1000 eggs of the nematode under glasshouse conditions in Viçosa, Brazil (Experiment 2).

Treatment	Number of nematode eggs	Number of root galls
Control	634,869 a	587 a
52	666,187 a	618 a
75	587,467 a	564 a
26	579,658 a	546 a
20	467,977 b	538 a
13	442,696 b	589 a
18	433,229 b	512 b
29	409,740 c	519 b
23	372,770 c	486 b
19	371,605 c	507 b
22	329,604 c	442 b
28	270,810 d	511 b
64	248,380 d	616 a
24	201,185 d	454 b
21	157,255 d	440 b
Coefficient of variation (%)	28.8	19.5

Values are means of eight repetitions. Means followed by different letters in the column indicate significant differences among treatments according to Scott-Knott's test ($P < 0.05$).

from 4.80 × 10^4 to 2.58 × 10^5 and 3.07 × 10^4 and 4.01 × 10^5 (g soil^{-1}) at 45 days after soil infestation with the fungus in experiments 1 and 2, respectively. In experiment 3, the establishment of the fungus in soil was also confirmed at 60 days, with colony-forming unit values that ranged from 4.2 × 10^4 to 2.4 × 10^5 (g soil^{-1}). *P. chlamydosporia* was not isolated from the soil of non-infested plots at the end of all experiments (Table 5).

4. Discussion

All fungal isolates obtained in our semi-selective medium survey were identified as *P. chlamydosporia*, the most common species of this genus. Conidial and chlamydospore dimensions of the isolates were similar with those described by Zare and Gams (2004). Both varieties of *P. chlamydosporia* (var. *chlamydosporia* and var. *catenulata*) were isolated from Brazilian soil samples. Conidia of *P. chlamydosporia* var. *catenulata* are arranged in chains and are generally globose to subglobose, while conidia of *P. chlamydosporia* var. *chlamydosporia* are arranged in heads and more ellipsoidal shaped (Zare and Gams, 2004). In Mexico, Franco-Navarro et al. (2009) also isolated both varieties of the fungus from soil samples where *P. chlamydosporia* var. *chlamydosporia* was also the commonest variety, similarly to our findings.

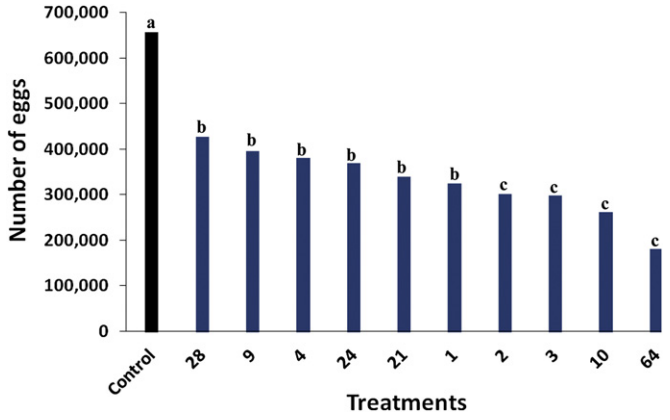

Fig. 1. Number of *Meloidogyne javanica* eggs per tomato root system after application of 5000 chlamydospores (g soil^{-1}) of the *Pochonia chlamydosporia* isolates (Treatments) and cultivation of tomato in soil infested with 3000 eggs of the nematode under glasshouse conditions in Viçosa, Brazil (Experiment 3). Values are means of eight repetitions. Means followed by different letters in the column indicate significant differences among treatments according to Scott-Knott's test ($P < 0.05$). Coefficient of variation = 12.4%.

In this study, we found variability among the *P. chlamydosporia* isolates in controlling the root-knot nematode under glasshouse conditions and it has been previously reported by several authors (De Leij and Kerry, 1991; Hidalgo-Diaz et al., 2000; Lopes et al., 2007). Hidalgo-Diaz et al. (2000) reported that all isolates of *P. chlamydosporia* obtained from Cuban soils were effective in colonizing eggs of *Meloidogyne incognita*. In addition, these authors found that the most efficient isolates belonged to *P. chlamydosporia* var. *catenulata* and not to *P. chlamydosporia* var. *chlamydosporia*. Here, among the most efficient isolates in controlling *M. javanica*, only two belonged to *P. chlamydosporia* var. *catenulata* (isolates 1 and 24). However, the ability of the fungus in parasitizing nematode eggs is more related to the isolates and nematode biotype rather than only to the variety of the fungus (Hidalgo-Diaz et al., 2000; Lopes et al., 2007).

Although the results of the number of CFUs of *P. chlamydosporia* (g soil^{-1}) cannot measure the fungal activity on the exact population density in the soil (Viaene and Abawi, 2000; Kerry, 2001), it can be stated that all isolates establish themselves in the soil, except isolate 13 (Bourne et al., 1996; Dallemole-Giaretta et al., 2011). The combination of semi-selective media plating method and qPCR in further studies can provide relevant information on *P. chlamydosporia* abundance in the environment (Morton et al., 2003; Manzanilla-López et al., 2009).

Isolates of *P. chlamydosporia* can vary in their ability of colonizing the rhizosphere of nematode-infected plants and parasiting nematode eggs (Hidalgo-Diaz et al., 2000; Kerry, 2001). The main distinction among the isolates in controlling *M. javanica* could be related to the parasitism of eggs. The production of extracellular enzymes (chitinases and proteases) involved in degradation of the outer layers of nematode eggs is strongly related to the ability of a specific isolate in colonizing nematode eggs and differences in proteolytic activity (Segers et al., 1996; Olivares-Bernabeu and López-Llorca, 2002; Tikhonov et al., 2002; Esteves et al., 2009).

The ability to colonize the rhizosphere of certain host plant, to produce chlamydospores, and to infect root-knot nematode eggs are important parameters during the screening of *P. chlamydosporia* isolates (Kerry, 2001). PCR-based methods can also be useful for screening of *P. chlamydosporia* in soil and root samples and for fingerprinting the selected isolates (Arora et al., 1996; Hirsch et al., 2000; Atkins et al., 2003; Manzanilla-López et al., 2009). These assays can be performed in the laboratory prior to further testing in glasshouse. However, experiments 1 and 2 of this study were designed to select potential isolates of *P. chlamydosporia* under glasshouse conditions using lower amount of nematode inoculum (1000 eggs per plant) instead of screening isolates based on the laboratory tests. Although the approach used in this work is more time and space consuming, we assumed that it would be more useful for our purposes to initially evaluate the antagonist under glasshouse because some isolates that perform well 'in vitro' may not be effective in the soil (Kerry, 2001; Wang et al., 2005).

The final step of the screening aimed to evaluate the efficiency of the isolates selected in our previous experiments using a higher amount of nematode inoculum in glasshouse (3000 eggs per plant). Thus, nine Brazilian isolates (1, 2, 3, 4, 9, 10, 21, 24 and 28) and Spanish isolate 64 were re-evaluated in experiment 3. All Brazilian isolates reduced the number of galls and eggs of the nematode induced by *M. javanica* in tomato plants when the fungus was introduced into the soil before the nematode and under a lesser density of inoculum of the pathogen (1000 eggs, experiments 1 and

Table 5
Pochonia chlamydosporia population in *Meloidogyne javanica*-infested soil (CFU g^{-1}) after application of 5000 chlamydospores (g $soil^{-1}$) and cultivation of tomato at 0, 45 or 60 days after application of the fungus.

Isolates	Days after fungus application	
Experiment 1	0	45
1	9.32×10^3	1.04×10^5
2	1.60×10^4	1.73×10^5
3	2.00×10^3	2.12×10^5
4	4.00×10^3	2.06×10^5
5	3.32×10^3	1.06×10^5
6	3.32×10^3	1.87×10^5
7	3.32×10^3	1.17×10^5
8	2.66×10^3	7.27×10^4
9	6.60×10^2	9.40×10^4
10	3.32×10^3	1.34×10^5
11	1.00×10^4	8.27×10^4
12	1.32×10^3	1.38×10^5
14	3.32×10^3	4.80×10^4
15	4.00×10^3	2.58×10^5
16	1.32×10^3	6.80×10^4
Control	0	0
Experiment 2	0	45
18	1.32×10^3	3.60×10^4
19	2.00×10^3	3.07×10^4
20	5.32×10^3	1.59×10^5
21	1.32×10^3	2.67×10^5
22	4.00×10^3	4.27×10^4
23	1.32×10^3	1.56×10^5
24	3.32×10^3	1.50×10^5
13	1.21×10^5	7.60×10^4
26	1.47×10^4	4.01×10^5
28	3.80×10^4	1.93×10^5
29	2.07×10^4	5.53×10^4
52	4.00×10^3	3.87×10^4
64	4.93×10^4	2.63×10^5
75	3.20×10^4	2.25×10^5
Control	0	0
Experiment 3	0	60
1	1.47×10^4	2.40×10^5
2	3.67×10^4	9.40×10^4
3	4.00×10^3	7.53×10^4
4	1.20×10^4	1.11×10^5
9	2.00×10^4	4.20×10^4
10	8.00×10^3	7.80×10^4
21	3.27×10^4	5.20×10^4
24	1.13×10^4	7.67×10^4
28	2.73×10^4	9.13×10^4
64	2.13×10^4	6.93×10^4
Control	0	0

CFU = colony-forming units. Coefficient of variation = 32.0%.

2). However, the same efficiency was not observed when chlamydospores and *M. javanica* eggs were simultaneously added to the soil and the inoculum density of the nematode was higher (3000 eggs per plant, experiment 3). Viaene and Abawi (2000) also reported that the ability of *P. chlamydosporia* in reducing final population densities of *Meloidogyne hapla* on lettuce was dependent of the initial nematode density, even when 10,000 chlamydospores (g $soil^{-1}$) of the fungus was introduced into the soil 20 days before the nematode. The fungus is likely to be less effective at higher nematode densities on susceptible tomato crops compared to low densities (Kerry and Bourne, 2002; Dallemole-Giaretta et al., 2011). *P. chlamydosporia* must colonize the pathogen eggs in the soil before hatching in order to prevent the nematode penetration, and then to reduce the number of galls due to a reduction of J_2 invading the roots. Consequently, the efficiency of the biological control agent can be inversely proportional to the density of the nematode in the soil, such as our findings observed in the present study.

In experiments 1 and 2, the reduction of the number of *M. javanica* eggs in cultivated plants in soils treated with the same nine Brazilian isolates, mentioned above, was due to the colonization of the nematode eggs (Kerry and Bourne, 2002). However, only isolates 2, 3, 10 and 64 (from Spain) reduced the reproduction of the nematode in a higher density of inoculum. Lopes et al. (2007) and Dallemole-Giaretta et al. (2011) also reported the reduction of the number of eggs of *M. javanica* in tomato plants induced by *P. chlamydosporia* under glasshouse conditions in Brazil.

Based on the results of our experiments, isolates 10, 2 and 3 were identified as the most promising isolates for future development of bionematicides. With regard to isolate 10 obtained in our study, a chlamydospore-based formulation has been evaluated under field conditions and has already been prepared to be commercialized in Brazil for the management of the root-knot nematode (Freitas et al., 2009). By coincidence, a reference isolate of *P. chlamydosporia* belonged to Rothamsted Research (Harpenden, United Kingdom) collection, also named Pc-10 and from Brazil, has proven to be a very efficient biological control agent of root-knot nematode (De Leij et al., 1993; Kerry and Bourne, 1996; Viaene and Abawi, 2000; Morton et al., 2003).

The use of biological control agents in the agriculture potentially reduces the use of different chemical inputs and can provide economic and ecological benefits. However, the performance of a particular biocontrol agent must be confirmed under different conditions, especially under field conditions (Ferraz et al., 2010).

In conclusion, the isolates of *P. chlamydosporia* found in our survey, especially 10, 2 and 3, can potentially be used to control *M. javanica*. The combination of the fungus with other control strategies can enhance the management of the pathogen (Dallemole-Giaretta et al., 2011). Further studies are needed to assess the potential of these isolates or based-by bionematicides under field conditions, as well as preparing different types of formulations in order to improve the control performance of the fungus.

Acknowledgments

The authors would like to thank the CNPq, FAPEMIG, CAPES and FUNARBE for their financial support, L. V. López-Llorca for providing three *P. chlamydosporia* isolates, and Cristiano Caixeta Nunes (North Caroline State University – USA) for language help.

References

Arora, D.K., Hirsch, P.R., Kerry, B.R., 1996. PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. Mycol. Res. 100, 801–809.
Atkins, S.D., Hidalgo-Diaz, L., Clark, I.M., Morton, O., Montes de Oca, N., Gray, P., Kerry, B.R., 2003. Approaches for monitoring the release of *Pochonia chlamydosporia* var. *catenulata*, a biological control agent of root-knot nematodes. Mycol. Res. 107, 206–212.
Bourne, J.M., Kerry, B.R., De Leij, F.A.A.M., 1996. The importance of the host plant on the interaction between root-knot nematodes (*Meloidogyne* spp.) and the nematophagous fungus, *Verticillium chlamydosporium* Goddard. Biocontrol Sci. Tech. 6, 539–548.
Bourne, J.M., Kerry, B.R., Galloway, J., Smith, C., Marchese, G., 1999. Evaluation of application techniques and materials for the production of *Verticillium chlamydosporium* in experiments to control root-knot nematodes in glasshouse and field trials. Int. J. Nematol. 9, 153–162.
Crump, D.H., Irving, F., 1992. Selection of isolates and methods of culturing *Verticillium chlamydosporium* and its efficacy as a biological control agent of beet and potato cyst nematodes. Nematologica 38, 367–374.
Dallemole-Giaretta, R., Freitas, L.G., Lopes, E.A., Ferraz, S., Podestá, G.S., Agnes, E.L., 2011. Cover crops and *Pochonia chlamydosporia* for the control of *Meloidogyne javanica*. Nematology 13, 919–926.
De Leij, F.A.A.M., Dennehy, J.H., Kerry, B.R., 1993. Effect of watering on the distribution of *Verticillium chlamydosporium* in soil and the colonization of egg masses of *Meloidogyne incognita* by the fungus. Nematologica 39, 250–265.
De Leij, F.A.A.M., Kerry, B.R., 1991. The nematophagous fungus *Verticilllium chlamydosporium* as a potential biological control agent for *Meloidogyne arenaria*. Revue de Nematol 14, 157–164.
Dhingra, O.D., Sinclair, J.B., 1995. Basic Plant Pathology Methods, second ed. CRC Press, Boca Raton.

Esteves, I., Peteira, B., Atkins, S.D., Magan, N., Kerry, B., 2009. Production of extracellular enzymes by different isolates of *Pochonia chlamydosporia*. Mycol. Res. 113, 867–876.

Ferraz, S., Freitas, L.G., Lopes, E.A., Dias-Arieira, C.R., 2010. Manejo sustentável de fitonematoides. Editora UFV, Viçosa.

Franco-Navarro, F., Vilchis-Martínez, K., Miranda-Damián, J., 2009. New records of *Pochonia chlamydosporia* from Mexico: isolation, root colonization and parasitism of *Nacobbus aberrans* eggs. Nematropica 39, 133–142.

Freitas, L.G., Dallemole-Giaretta, R., Ferraz, S., Zooca, R.J.F., Podestá, G.S., 2009. Controle biológico de nematóides: estudo de casos. In: Zambolim, L., Picanço, M. (Eds.), Controle biológico de pragas e doenças - Exemplos práticos. Suprema Gráfica e Editora Ltda, Visconde do Rio Branco, pp. 41–82.

Gaspard, J.T., Jaffee, B.A., Ferris, H., 1990. Association of *Verticillium chlamydosporium* and *Paecilomyces lilacinus* with root-knot nematode infested soil. J. Nematol. 22, 207–213.

Godoy, G., Rodríguez-Kábana, R., Morgan-Jones, G., 1983. Fungal parasites of *Meloidogyne arenaria* eggs in an Alabama soil: a mycological survey and greenhouse studies. Nematropica 13, 201–213.

Hallmann, J., Davies, K.G., Sikora, R., 2009. Biological control using microbial pathogens, endophytes and antagonists. In: Perry, R.N., Moens, M., Starr, J.L. (Eds.), Root-knot Nematodes. CAB International, Wallingford, pp. 380–411.

Hidalgo-Diaz, L., Bourne, J.M., Kerry, B.R., Rodríguez, M.G., 2000. Nematophagous *Verticillium* spp. in soils infested with *Meloidogyne* spp. in Cuba: isolation and screening. Int. J. Pest Manage. 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. Mycol. Res. 104, 435–439.

Hussey, R.S., Barker, K.R., 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Dis. Rep. 57, 1025–1028.

Kerry, B.R., 1975. Fungi and the decrease of cereal cyst-nematode populations in cereal monoculture. EPPO Bull. 5, 353–361.

Kerry, B.R., 1991. Methods for studying the growth and survival of the nematophagous fungus. *Verticillium chlamydosporium* Goddard in soil. Bull. SROP. 14, 34–38.

Kerry, B.R., 1995. Ecological considerations for the use of nematophagous fungus, *Verticillium chlamydosporium*, to control plant parasitic nematodes. Can. J. Bot. 73, S65–S70.

Kerry, B.R., 2001. Exploitation of the nematophagous fungus *Verticillium chlamydosporium* Goddard for the biological control of root-knot nematodes (*Meloidogyne* spp.). In: Butt, T.M., Jackson, C., Magan, N. (Eds.), Fungi as Biocontrol Agents. CAB International, Wallingford, pp. 155–167.

Kerry, B.R., Bourne, J.M., 1996. The importance of rhizosphere interactions in the biological control of plant parasitic nematodes – a case study using *Verticillium chlamydosporium*. Pestic. Sci. 47, 69–75.

Kerry, B.R., Bourne, J.M., 2002. A Manual for Research on *Verticillium chlamydosporium*, a Potential Biological Control Agent for Root-knot Nematodes. IOBC/WPRS, Gent.

Lopes, E.A., Ferraz, S., Ferreira, P.A., Freitas, L.G., Dhingra, O.D., Gardiano, C.G., Carvalho, S.L., 2007. Potencial de isolados de fungos nematófagos no controle de *Meloidogyne javanica*. Nematol. Bras 31, 78–84.

Manzanilla-López, R.H., Atkins, S.D., Clark, I.M., Kerry, B.R., Hirsch, P.R., 2009. Measuring abundance, diversity and parasitic ability in two populations of the nematophagous fungus *Pochonia chlamydosporia* var. *chlamydosporia*. Biocontrol Sci. Tech. 19, 391–406.

Morton, C.O., Mauchline, T.H., Kerry, B.R., Hirsch, P.R., 2003. PCR-based DNA fingerprinting indicates host-related genetic variation in the nematophagous fungus *Pochonia chlamydosporia*. Mycol. Res. 107, 198–205.

Olivares-Bernabeu, C.M., López-Llorca, L.V., 2002. Fungal egg-parasites of plant-parasitic nematodes from Spanish soils. Rev. Iberoam. Micol. 19, 104–110.

Pérez-Rodríguez, I., Doroteo-Mendoza, A., Franco-Navarro, F., Santiago-Santiago, V., Montero-Pineda, A., 2007. Isolates of *Pochonia chlamydosporia* var. *chlamydosporia* from Mexico as potential biological control agents of *Nacobbus aberrans*. Nematropica 37, 127–134.

Scott, A.J., Knott, M.A., 1974. A cluster analysis method for grouping means in the analysis of variance. Biometrics 30, 507–512.

Segers, R., Butt, T.M., Kerry, B.R., Bechett, A., Peberdy, J.F., 1996. The role of the proteinase VCP1 produced by the nematophagous *Verticillium chlamydosporium* in the infection process of nematode eggs. Mycol. Res. 100, 421–428.

Smith, D., Onions, A.H.S., 1994. The Preservation and Maintenance of Living Fungi. CAB International, Wallingford.

Sosnowska, D., Sikora, R., 2001. The role of fungi in reduction of sugar beet nematode (*Heterodera schachtii* Schmidt) population. Bull. OILB-SROP 24, 151–156.

Sun, M.H., Gao, L., Shi, Y.X., Li, B.J., Liu, X.Z., 2006. Fungi and actinomycetes associated with *Meloidogyne* spp. eggs and females in China and their biocontrol potential. J. Invert. Pathol. 93, 22–28.

Tikhonov, V.E., López-Llorca, L.V., Salinas, J., Jansson, H.B., 2002. Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fung. Genet. Biol. 35, 67–78.

Verdejo-Lucas, S., Sorribas, F.S., Ornat, C., Galeano, M., 2003. Evaluating *Pochonia chlamydosporia* in a double-cropping system of lettuce and tomato in plastic houses infested with *Meloidogyne javanica*. Plant Pathol. 52, 521–528.

Viaene, N.M., Abawi, G.S., 2000. *Hirsutella rhossiliensis* and *Verticillium chlamydosporium* as biocontrol agents of the root-knot nematode *Meloidogyne hapla* on lettuce. J. Nematol. 32, 85–100.

Wang, K., Riggs, R.D., Crippen, D., 2005. Isolation, selection, and efficacy of *Pochonia chlamydosporia* for control of *Rotylenchulus reniformis* on cotton. Phytopathology 95, 890–893.

Willcox, J., Tribe, H.T., 1974. Fungal parasitism in cysts of *Heterodera*. I – preliminary investigations. Trans. Brit. Mycol. Soc. 62, 585–594.

Zare, R., Gams, W., 2004. A Monograph of *Verticillium* Section Prostrata. Plant Pests and Diseases Research Institute, Tehran.