INTERACTIONS BETWEEN *POCHONIA CHLAMYDOSPORIA* AND *MELOIDOGYNE CHITWOODI* IN A CROP ROTATION SCHEME

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ABSTRACT

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Studies on the use of *Pochonia chlamydosporia* isolates as biological control agents of root-knot nematodes (RKN, *Meloidogyne* spp.), showed that their efficacy relies on selected key factors such as fungal proliferation in soil, establishment in the rhizosphere, and ability to parasitize RKN eggs. A pot experiment was conducted to evaluate the performance of a Portuguese *P. chlamydosporia* isolate (Pc2) and of a non-native isolate (Pc10) against *M. chitwoodi* in a potato-maize-potato-potato crop rotation. The growth of *P. chlamydosporia* in soil and roots was monitored throughout the experiment by assessing the numbers of colony forming units (cfu). The prevalence in *M. chitwoodi* eggs and nematode reproduction was measured at the end of each crop. The cfu/g of soil varied over time and was particularly low for both isolates during the spring and summer 2009, when temperatures above 30ºC were registered. Native isolate Pc2 was more effective in establishing in soil than Pc10. Growth and survival of the fungus did not seem to depend on the presence of nematode or host plant species. Establishment of *P. chlamydosporia* in soil was slow and was only achieved after the full cropping sequence. The two *P. chlamydosporia* isolates revealed subtle differences in host preference, plant compatibility, or tolerance to abiotic conditions that may affect their efficacy as biocontrol agents in a long-lasting management programme.

Key words: biological control, integrated pest management, potato, rhizosphere colonization, root-knot nematodes.

RESUMEN

Vieira dos Santos, M. C., I. Esteves, B. Kerry, and I. Abrantes. 2014. Interacciones entre *Pochonia chlamydosporia* y *Meloidogyne chitwoodi* en un esquema de rotación de cultivos. Nematropica 44:37-46.

Estudios sobre el uso de aislados de *Pochonia chlamydosporia* como agentes de control biológico de nematodos formadores de agallas en las raíces (*Meloidogyne* spp.), mostraron que su eficacia depende de determinados factores clave, como la proliferación del hongo en suelo, su establecimiento en la rizosfera y su capacidad para parasitar los huevos del nematodo. Se llevó a cabo un experimento en macetas para evaluar el comportamiento de un aislado portugués (Pc2) y un aislado no-nativo (Pc10) de *P. chlamydosporia* frente a *M. chitwoodi* en una rotación de cultivos patata-maíz-patata-patata. El crecimiento de *P. chlamydosporia* en suelo y en raíces se monitorizó durante el experimento mediante la determinación del número de unidades formadoras de colonias (ufc). La prevalencia en huevos de *M. chitwoodi*, y la reproducción del nematodo se midieron al final de cada cultivo. La ufc/g de suelo varió a lo largo del tiempo y fue particularmente baja para ambos aislados durante la primavera y verano de 2009, cuando se registraron temperaturas sobre 30ºC. El aislado nativo Pc2 fue más efectivo al establecerse en el suelo que el aislado Pc10. El crecimiento y supervivencia del hongo no pareció depender de la presencia del nematodo o de plantas hospedadoras. El establecimiento de *P. chlamydosporia* en suelo fue lento y se alcanzó sólo tras la secuencia de cultivos completa. Los dos aislados de *P. chlamydosporia* mostraron pequeñas diferencias respecto a preferencia de hospedador, compatibilidad con plantas, o tolerancia a condiciones abióticas que pudieran afectar su eficacia como agentes de biocontrol en un programa de manejo prolongado.

Palabras clave: control biológico, manejo integrado de plagas, patata, colonización rizosférica, nematodos formadores de agallas en raíces.

INTRODUCTION

Root-knot nematodes (RKN), *Meloidogyne* spp., are ubiquitous plant pests that affect a wide range of crops. In addition to direct root damage, these gall-forming sedentary endoparasites elicit infection by secondary pathogens such as fungi and bacteria and, therefore, act as a major cause of preventable crop disease and yield loss (Nicol *et al*., 2011). Among RKN, *M. chitwoodi* is a quarantine organism that parasitizes potato. This nematode has been reported from Argentina, Belgium, Germany, Netherlands, USA, Mexico, Portugal, and South Africa (OEPP/EPPO, 2009). Management practices involve the use of highly toxic nematicides, but their negative impact on the environment led to a total ban or restricted use of most products (Chitwood, 2003). As a consequence, there is an urgent need for safer and more effective alternatives for nematode control, like the use of fungal antagonists that hold promising ability to act as nematode biological control agents (Stirling, 2011).

Pochonia chlamydosporia (Goddard) Gams and Zare is a facultative nematophagous fungus with a complex behaviour in soil including endophytism, saprophytism, or a parasitic activity against eggs and sedentary females of nematodes (Rosso *et al*., 2011; Ward *et al*., 2012). It has been associated with soils that suppress the multiplication of economically important plant-parasitic nematodes, including cyst nematode populations (Kerry and Crump, 1977; Kerry *et al*., 1982). The biocontrol potential of *P. chlamydosporia* has been widely studied in pots and field experiments. Although the efficacy of selected isolates against nematodes has been demonstrated, consistency is a problem since the fungus may fail to establish in the soil (Bourne and Kerry, 1999; Atkins *et al*., 2003b; Tzortzakakis and Petsas, 2003; Verdejo-Lucas *et al*., 2003; Tobin *et al*., 2008; Tzortzakakis, 2000, 2007, 2009; Puertas and Hidalgo-Díaz, 2009). *Pochonia chlamydosporia* isolates differ in their ability to grow in soil and tend to be more abundant in organic than in mineral soils. (De Leij *et al*., 1993; Kerry *et al.*, 1993; Bourne and Kerry, 2000).

In the rhizosphere, *P. chlamydosporia* seems to be able to proliferate by using nutrients released from root exudates (De Leij *et al*., 1993; Kerry *et al*., 1993; Bourne and Kerry, 2000). In addition to the high variability shown among isolates in rhizosphere colonization, their pathogenicity can also differ significantly, and the outcome of these variations in the regulation of nematode populations is not fully understood. Nonetheless, a number of key factors affecting the plant-nematode-fungus interactions and *P. chlamydosporia* activity as a biocontrol agent have been identified (Abrantes *et al*., 2002; Kerry and Hirsch, 2011). The fungal ability to colonize the rhizosphere rather than to proliferate in soil significantly affects its impact on nematode reproduction. Rhizosphere colonization is essential for nematode control and is mediated by the plant species (De Leij and Kerry, 1991; Bourne *et al.*, 1994, 1996; Kerry and Bourne, 1996; Bourne and Kerry, 1999). When *P. chlamydosporia* contacts a nematode egg mass in the rhizosphere, the fungus switches its saprophytic behaviour to a parasitic phase. The factors that trigger this switch are not well understood, but nutrition and nematode host preference at the fungal infra-specific level may be involved (Kerry, 2000; Mauchline *et al*., 2004, Ward *et al*., 2012). Fungal abundance is not always related to the extent of its prevalence in eggs although the fungus was more abundant on nematode-infected than healthy roots (Bourne *et al*., 1996; Bourne and Kerry, 1999; Atkins *et al.*, 2003b, Kerry and Hirsch, 2011).

Even though *P. chlamydosporia* does not prevent the initial infection of roots by nematode second-stage juveniles (J2), it affects nematode reproduction, causing a decrease of the nematode population in soil. Thus, the biocontrol efficacy of the fungus should be enhanced by combining the fungus with other control measures that prevent initial infestations, such as crop rotation with poor hosts for the nematode (Kerry and Bourne, 1996). Crop rotation is a traditional control method that prevents, to some extent, the build-up of large nematode population density. A successful combination of crop rotation with the application of *P. chlamydosporia* could lead to environmentally friendly, sustainable RKN management. Therefore, understanding how a crop rotation sequence can affect the establishment and control efficacy of isolates of the fungus is of key importance. The objective of this study was to evaluate the survival of two isolates of *P. chlamydosporia* from different nematode hosts in soil and their effect on the multiplication of *M. chitwoodi*, in a crop rotation scheme (potato-maize-potato-potato) in a pot experiment that simulated field conditions.

MATERIALS AND METHODS

Meloidogyne chitwoodi *Population*

A population of *M. chitwoodi*, obtained from infected potato tubers collected in Porto, Portugal (Conceição *et al*., 2009), was propagated on a susceptible tomato cultivar (*Solanum lycopersicum* cv. Easypeel) in pots filled with sterilised sandy loam soil and sand (1:1). Pots, inoculated with 10 egg masses each, were placed in a glasshouse at 25ºC, 12 h:12 h (day:night) photoperiod, and watered regularly. After 60 d, eggs were extracted from infected roots using 0.52% sodium hypochlorite (Hussey and Barker, 1973).

Pochonia chlamydosporia *Isolates*

Two isolates of *P. chlamydosporia* (Pc2 and Pc10) were used in this study. Pc2 was isolated from *Globodera rostochiensis* eggs from a Portuguese potato field and Pc10 (IMI 331547), originally

isolated from *M. incognita* eggs (Brazil), was kindly supplied by Rothamsted Research, UK. Both isolates were maintained in 1.7% corn meal agar (CMA, Oxoid, UK) at 25ºC. Their identities were checked using specific diagnostic primers derived from the ß-tubulin gene (Hirsch *et al.*, 2000) and confirmed to be *P. chlamydosporia* using PCR (Vieira dos Santos *et al*., 2013). Chlamydospores were produced using a barley:sand substrate (1:1) and extracted following the method described by De Leij and Kerry (1991). Chlamydospore viability and germination were assessed on sorbose agar with antibiotics (Abrantes *et al.*, 2002).

Plant Material

Potato plants (*S. tuberosum* cv. Désirée) were grown from a single sprout per pot. Maize plants (*Zea mays* cv. Belgrano) were grown from seeds, in plastic containers for 3 wk, in a greenhouse at 25ºC and a 16 h:8 h (day:night) photoperiod before being transplanted to the pots. The host suitability of these crop cultivars to *M. chitwoodi* was assessed on the basis of their reproduction factor (Rf = Pf/Pi where Pi = initial inoculum of 5,000 J2 and Pf = the population at the end of the experiment). The study was conducted under greenhouse conditions for 60 d. Both cultivars were considered as susceptible hosts with Rf values of 10.8 (maize) and 39.1 (potato).

Experimental Conditions

The experiment was conducted from April 2009 to May 2010 and the crop rotation scheme selected was similar to regional crop schemes: potato (April-June 2009)-maize (June-November 2009)-potato (December 2009-March 2010)-potato (March-May 2010). Pots (5-L vol) were filled with non-sterilized sandy loam soil (83.1% sand, 8.4% clay and 8.5% silt with 1.7% organic matter) collected from a field in Carapinheira, Coimbra, where potato had been the primary crop for a number of years. The absence of RKN was verified using a modified Whitehead and Hemming tray method (Abrantes *et al*., 1976). The use of a soil dilution plating technique on a semi-selective medium (De Leij and Kerry, 1991) did not detect any *P. chlamydosporia*. Each pot was partially buried in a larger pot (40 L) containing sand to mitigate rapid temperature fluctuations. Pots were placed under a shading net in a field located in "Escola Superior Agrária" of Coimbra, Portugal, during spring and summer 2009.

In the winter, due to low temperatures and high rainfall that were unsuitable for the potato crop, pots were transferred to a shelter without controlled temperature. Temperatures registered in the region ranged from -2.0ºC (December 2009) to 33.1ºC (May 2010) (http://www.ipma.pt). The following treatments were applied: i) *P. chlamydosporia* (isolate Pc2 or Pc10) + *M. chitwoodi* + crop; ii) *P. chlamydosporia* (isolate Pc2 or Pc10) + no nematode + crop; iii) *P. chlamydosporia* (isolate Pc2 or Pc10) + no nematode + no crop. Controls were prepared for each treatment without application of the fungus. Fungal inocula were applied three times at different rates: a rate of 5,000 chlamydospores/g of soil at the time of first crop (potato) planting (March 2009); 2,500 chlamydospores/g of soil at the time of second crop (maize) planting (June 2009); and 5,000 chlamydospores/g of soil at the time of third crop (potato) planting (November 2009). One week after the first fungal application, the pots were inoculated with 4,000 *M. chitwoodi* eggs. Five replicates of each treatment were set up in a total of 45 pots arranged in a completely randomised design.

Pochonia chlamydosporia *Survival, Proliferation, and Parasitism*

To monitor the survival and proliferation of the fungus in the soil, samples were collected from each pot at the beginning of each crop, then every 4 wk, and at harvest. Root samples were collected at harvest and processed to estimate the fungal population. The numbers of cfu/g of soil and cfu/g of root were evaluated by dilution plating (De Leij and Kerry, 1991). Egg parasitism was assessed by plating *M. chitwoodi* eggs (ca. 200 eggs/plate), extracted from infected roots, on 0.8 % technical agar and antibiotics (Abrantes *et al.*, 2002) for three days, at 25°C. Three plates per root system were prepared. The percentage of parasitized eggs was determined by examining 100 eggs per plate for signs of fungal colonization using an inverted microscope (Olympus, Japan) at 200× according to standard methods (Kerry and Crump, 1977).

Meloidogyne chitwoodi *Reproduction*

At the end of each crop, roots were collected to evaluate nematode reproduction. The number of galls and egg masses per root system were counted. Eggs were extracted from each root system using 0.52% sodium hypochlorite as described previously, and counted to estimate the final population density (Pf). The number of eggs/egg mass was calculated for each plant. Also, population densities in soil were assessed by counting the number of J2 extracted from soil samples (100 cm^3) collected at harvest using a modified Whitehead and Hemming tray method (Abrantes *et al*., 1976).

Data Analysis

Data on cfu/g of soil, cfu/cm of root, egg parasitism, and nematode reproduction were compared by ANOVA using the General Linear Model command in SPSS (IBM® SPSS® Statistics 19, SPSS Inc., USA).

A square root transformation was used when needed to ensure a normal distribution and constant variance of the data that, otherwise, would not meet the ANOVA assumptions. Statistically significant differences among treatments were computed using the LSD test ($P<0.05$).

RESULTS

Pochonia chlamydosporia *Survival, Proliferation, and Parasitism*

The cfu/g soil values varied throughout the experiment, and were particularly low between the second and third inoculations for both isolates, though higher for Pc2 (Fig. 1). However, *P. chlamydosporia* spores/hyphal fragments remained viable in soil between inoculations and survived the temperatures above 30ºC registered during the spring and summer 2009 (Fig. 1). The number of cfu/g of soil of Pc2 was significantly higher in pots inoculated with *M. chitwoodi* after the first and third inoculations than Pc10 (Fig. 1a). During the second crop, although differences were observed between Pc2 and Pc10 (Fig. 1a–c), the abundance and survival of the fungus did not seem to be influenced either by the presence of the nematodes or by the plant host. After the third inoculation, the number of cfu/g of soil was above the initial fungal inoculation rate for both isolates. After planting of the fourth crop (March 2010), there was a decrease in the cfu/g soil, which was significantly more pronounced for isolate Pc10 than for Pc2 in the presence of *M. chitwoodi* (Fig. 1a). Fungal abundance in soil was similar in all treatments. High variability was observed among replicates of the same treatment, and the isolates could not be detected in some of the pots.

Similar results to those of low soil cfu densities were obtained from roots. A high variability among pots of the same treatment was found, and the fungus was not found in some of the roots. In the third and fourth crops, Pc2 was present in higher rates than Pc10 (Fig. 2). The number of cfu/cm^2 was numerically, but not statistically, higher in the presence of the nematode for isolate Pc10 in all crops, whereas for Pc2, the reverse was observed except for the fourth crop (Fig. 2).

After the first crop, infection prevalence of eggs was less than 4% in all pots and treatments (Fig. 3). After the third and fourth crops, parasitism remained low (< 3% and 12%, respectively). Significant changes in egg parasitism levels were only detected after the last potato crop, when a 10-fold difference was found between parasitism by Pc2 and Pc10 isolates (Fig. 3).

Meloidogyne chitwoodi *Reproduction*

Meloidogyne chitwoodi soil population densities fluctuated during the experimental period (Fig. 4). After the first crop, the number of J2/100 cm^3 of soil was lower in nematode-infested pots without the fungus compared to pots treated with Pc2 or Pc10. A decrease was observed after the second crop followed by an increase after the third crop except for pots treated with Pc2. At the end of the fourth crop, soil population densities were low in all treatments (Fig. 4).

The numbers of galls and eggs/egg mass were also lower in pots treated with Pc2 when compared to pots treated with Pc10 (Table 1). However, significant differences were only found after the fourth crop.

Table 1. Number of galls, egg masses, and eggs/egg mass of *Meloidogyne chitwoodi* in roots of potato cv. Désirée and maize cv. Belgrano alone or inoculated with *Pochonia chlamydosporia* isolates (Pc2 and Pc10) in a 14-mon pot experiment of a crop rotation scheme: potato cv. Désirée (April/June 2009), maize cv. Belgrano (June/November 2009), potato cv. Désirée (December 2009-March 2010), potato cv. Désirée (March/May 2010).

Treatment	Galls/g of root	Egg masses/g of root	Eggs/egg mass
Pc2 + Potato (1st) [y, z]	51.6 ± 4.0 a	51.7 ± 4.4 a	369.5 ± 25.9 c
Pc2 + Maize (2nd) [y]	undetected	undetected	undetected
Pc2 + Potato (3rd) [y]	46.8 ± 11.6 a	47.5 ± 11.9 a	36.0 ± 9.3 a
Pc2 + Potato (4th) [y]	55.4 ± 26.9 a	47.2 ± 22.2 a	41.2 ± 14.7 a
Pc10 + Potato (1st) [y, z]	40.0 ± 11.7 a	54.3 ± 19.5 a	270.6 ± 61.5 b
Pc10 + Maize (2nd) [y]	undetected	undetected	undetected
Pc10 + Potato (3rd) [y]	58.7 ± 45.7 a	60.7 ± 47.7 ab	97.4 ± 33.7 a
Pc10 + Potato (4th) [y]	150.4 ± 28.9 b	143.3 ± 29.5 c	49.9 ± 8.9 a
Potato (1st)[z]	38.9 ± 10.7 a	43.0 ± 11.8 a	250.4 ± 45.1 b
Maize (2nd)	undetected	undetected	undetected
Potato (3rd)	61.8 ± 23.3 a	61.3 ± 23.1 ab	131.0 ± 36.7 a
Potato (4th)	149.9 ± 32.3 b	119.3 ± 25.8 bc	57.8 ± 17.1 a

Values are replicates of 5 pots/treatment ± standard error; values in the same column followed by the same letter within a treatment (with or without the fungus) are not significantly different according to LSD test ($P<0.05$).
[y]Inoculated at planting in 1st, 2nd, and 3rd crops (5,000, 2,500, and 5,000 chlamydospores/g of soil, respectively).
[z]Soil was inoculated with 4,000 *M. chitwoodi* eggs/pot.

Fig.1

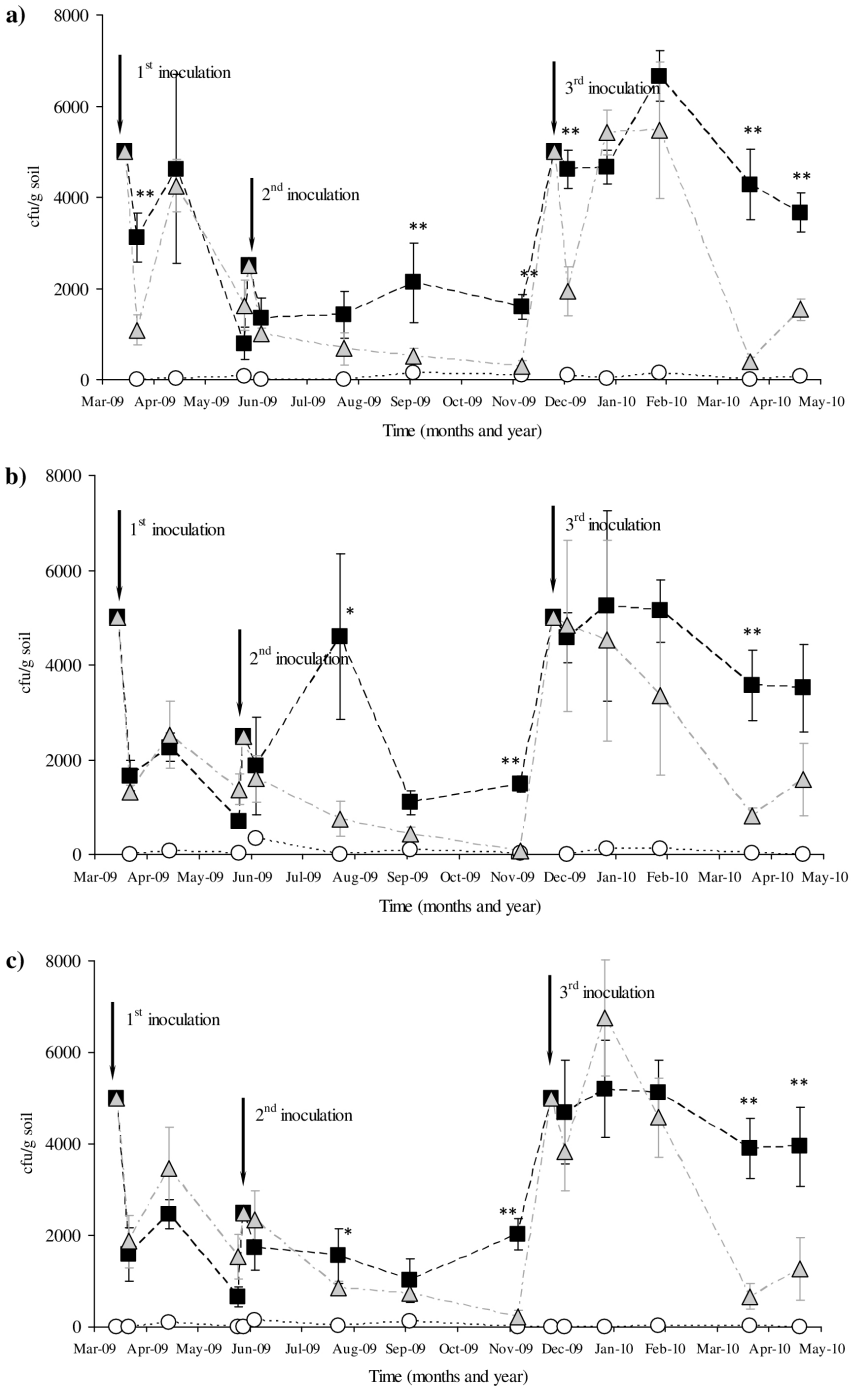

Fig. 1. Numbers of colony-forming units (cfu) of *Pochonia chlamydosporia* isolates Pc2 (■) and Pc10 (▲) and of control (no fungus) (○) in soil in a 14-mon pot experiment of a crop rotation: potato cv. Désirée (April/June 2009), maize cv. Belgrano (June/November 2009), potato cv. Désirée (December 2009/March 2010), potato cv. Désirée (March/May 2010). Treatments: a) *P. chlamydosporia* + *Meloidogyne chitwoodi* + crop; b) *P. chlamydosporia* + no nematode + crop; and c) *P. chlamydosporia* + no nematode + no crop. Values are means of five replicates/treatment. Bars represent standard error of means. Where the bars are not shown, they were smaller than the symbol size. Arrows show dates of fungal inoculum application. One and two asterisks indicate significant differences according to Fisher LSD test ($P<0.05$ and $P<0.01$, respectively).

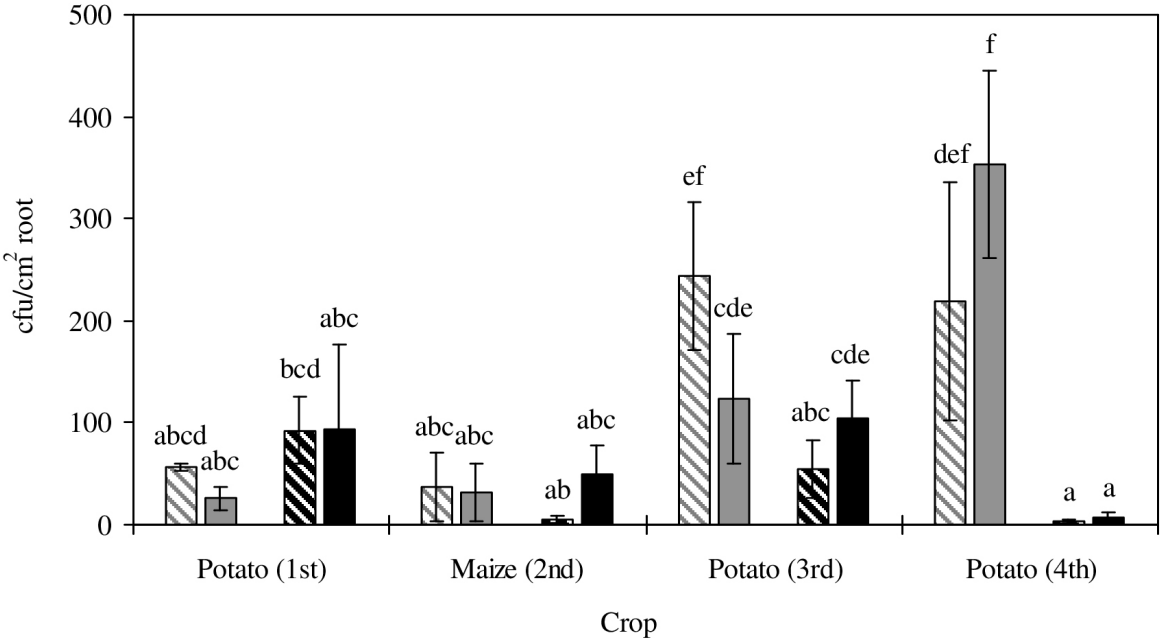

Fig. 2. Numbers of colony forming units (cfu) of *Pochonia chlamydosporia* Pc2 and Pc10 isolates in roots at the end of each crop in a 14-mon pot experiment of a crop rotation: potato cv. Désirée (April/June 2009), maize cv. Belgrano (June/November 2009), potato cv. Désirée (December 2009-March 2010), potato cv. Désirée (March/May 2010) inoculated with 4,000 *Meloidogyne chitwoodi* eggs/pot 1 wk after planting the first crop. (▨) Pc2 + no nematode; (▩) Pc2 + *M. chitwoodi*; (▨) Pc10 + no nematode; (■) Pc10 + *M. chitwoodi*. Values are means of five replicates/treatment. Bars represent standard error of means. Bars with the same letter are not significantly different according to LSD test ($P<0.05$).

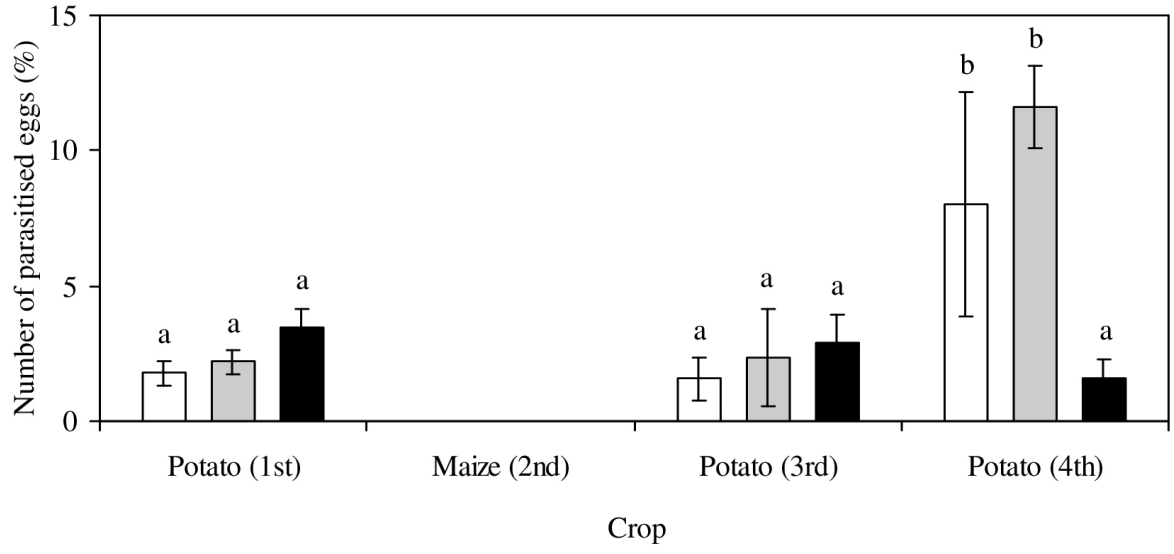

Fig. 3. Prevalence of *Pochonia chlamydosporia* isolates Pc2 and Pc10 in *Meloidogyne chitwoodi* eggs in roots, at the end of each crop as shown by pot experiments in a crop rotation (potato cv. Désirée, maize cv. Belgrano, potato, potato) inoculated with 4,000 *M. chitwoodi* eggs/pot 1 wk after planting the first crop. *Pochonia chlamydosporia* isolates were applied at a rate of 5,000 chlamydospores/g of soil at planting of first and third crops and 2,500 chlamydospores/g at the second crop. (□) no fungus; (▩) Pc2; (■) Pc10. Values are means of five replicates/treatment. Bars represent standard error of means. Bars with the same letter are not significantly different according to LSD test ($P<0.05$).

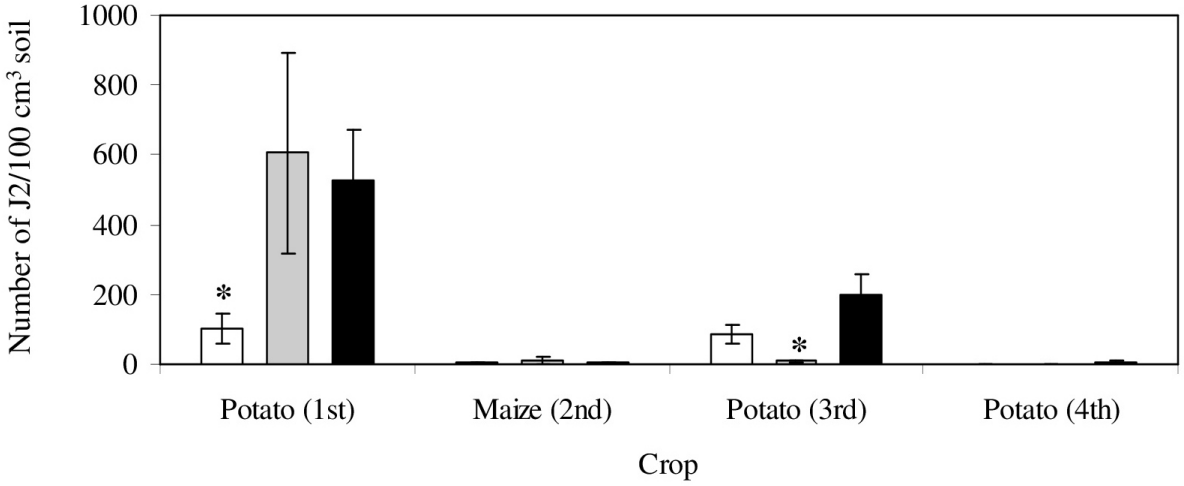

Fig. 4. Final population densities of *Meloidogyne chitwoodi* second stage juveniles (J2) in soil at the end of each crop as shown by pot experiments in a crop rotation (potato cv. Désirée, maize cv. Belgrano, potato, potato) inoculated with 4,000 *M. chitwoodi* eggs/pot 1 wk after planting the first crop. *Pochonia chlamydosporia* isolates were applied at a rate of 5,000 chlamydospores/g of soil at planting of first and third crops and 2,500 chlamydospores/g at the second crop. (□) no fungus; (▩) Pc2; (■) Pc10. Values are means of five replicates/treatment. Bars represent standard error of means. One asterisk indicates significant differences according to Fisher LSD test (P<0.05).

Meloidogyne chitwoodi was not detected in roots of maize (second crop) despite the host susceptibility observed in greenhouse conditions. After the first crop, the number of eggs/egg mass was higher in treatments with Pc2 compared to the other treatments. A decrease was observed in all treatments after the third and fourth crops. Although no significant differences were found among treatments, this decrease was higher in pots treated with Pc2 (Table 1).

DISCUSSION

Establishment in soil, rhizosphere, and the ability to parasitize nematode eggs are considered key factors that greatly influence the potential of *P. chlamydosporia* as a biological control agent against RKN (Abrantes *et al*., 2002). The interactions between two isolates of *P. chlamydosporia* and the RKN, *M. chitwoodi*, were assessed in a crop rotation scheme for the first time. Fungal population densities in soil varied over time, and the two isolates tested were able to increase the initial fungal inoculum rate only after the third inoculation. Following their incorporation into soil, a decrease in the detectable number of cfu/g of soil was generally observed for both isolates. These fluctuations have already been reported and could be related to the use of the dilution-plate technique (Kerry *et al*., 1993; Viaene and Abawi, 2000; Sorribas *et al*., 2003; Mauchline *et al*., 2004). On the other hand, these trends can be related to soil conditions. The nutrient-poor sandy loam soil (<2% organic matter) used in the pot experiments may have limited the fungus proliferation, which is related, to a certain extent, to nutrients available in the different soil types (De Leij *et al*., 1992). The presence of the plant alone or inoculation of the plant with the nematode did not seem to affect fungal proliferation although fungal abundance increased in coarse sand in the presence of a plant as the only source of nutrients (Manzanilla-López *et al*., 2011). Our data also suggest that successful fungal establishment in soil may require several applications and may take a longer time interval than the cropping rotation scheme.

The survival of both fungal isolates, during the spring and summer 2009 when temperatures above 30ºC were registered, reflects the importance of temperature on the performance of *P. chlamydosporia*. *In vitro* studies revealed that spores and hyphal fragments of both isolates remained viable after being exposed for nearly 1 mon to temperatures known to limit fungal growth (10 and 33ºC) and isolates resumed growth after incubation at 25ºC (Vieira dos Santos *et al*., 2013). Tolerance to extreme temperatures can have a great impact on the establishment of an organism in the field and can compromise its efficacy as a biocontrol agent (Magan, 2001). Data presented here also show that the local isolate Pc2 was more effective in establishing in soil than the non-native Pc10. Therefore, our results are in agreement with previous findings that suggest that, when applied to the soil, growth of non-native isolates is lower compared to native isolates. This may occur because a fungus may be better suited to

the local habitat in natural environmental conditions (Sorribas *et al*., 2003). The high variability observed in soil among replicates of the same treatment could be attributed to an aggregated distribution and lack of spreading and colonization, stressing the need for more detailed methods to monitor fungal development and establishment in soil (Atkins *et al*., 2003a; Tzortzakakis, 2009).

Low cfu numbers were recovered from roots, which may explain the low levels of egg parasitism observed. Effective control against RKN can only be achieved after successful root colonization (De Leij and Kerry, 1991). Egg prevalence with Pc10 was higher in the first and third crops, although not significantly different from egg parasitism by Pc2. Previous *in vitro* studies revealed no significant differences in the ability to parasitize *M. chitwoodi* eggs between isolates Pc2 and Pc10 (Vieira dos Santos *et al*., 2013). The increase in egg parasitism detected in the last crop in pots treated with Pc2 and not with Pc10 could be attributed to rhizosphere colonization (Figs. 2 and 3). In the last crop, egg parasitism was also high in two of the control pots. The fungus was not found in plant roots but was recovered from soil. Initially, *P. chlamydosporia* was not detected in the control pots, and it is unclear whether this represents a contamination from the surrounding environment or the multiplication of a native fungus that eluded the initial detection efforts.

As noted above, maize cv. Belgrano was considered a susceptible host for *M. chitwoodi* but, in this experiment, the nematode was not found on the roots of any of the treatments. The adverse atmospheric conditions during the maize crop were probably responsible for the lack of nematode reproduction.

Although isolate Pc10 did not control *M. chitwoodi*, it has been proven to be effective against *M. incognita* in pot and microplot trials (De Leij and Kerry, 1991; De Leij *et al.*, 1993). In contrast, there are reports of its failure in controlling *M. javanica*; thus, the ability of a given isolate could vary in experiments carried out under different conditions or when different nematode populations are involved (Tzortzakakis, 2000, 2009; Tzortzakakis and Petsas, 2003; Verdejo-Lucas *et al.*, 2003). Subtle differences in host preference, plant compatibility, or tolerance to abiotic conditions were detected between the isolates. Colonisation of the rhizosphere of crop plants was low, which may explain the generally low levels of egg parasitism. Growth and proliferation in soil were slow and were only achieved, for both isolates, after multiple inoculations. Nutrient availability and temperature had a strong effect on the efficacy of both isolates, as expected from the above bioassays and previous studies (De Leij *et al*., 1992; Magan, 2001). These results suggest that this fungus is overall a poor competitor in soil, and its application can reach higher biocontrol efficiency when applied after the summer, even if native isolates, putatively better adapted to local conditions, are used, which is in accordance with results obtained in studies in Mediterranean conditions (Verdejo-Lucas *et al*., 2003; Tzortzakakis, 2009).

Pochonia chlamydosporia has been successfully developed as a biological control agent against RKN in integrated management strategies that include poor host crops for the nematode, which support extensive rhizosphere growth of the fungus (Hérnandez and Hidalgo-Diaz, 2008). As plant species differ in their ability to support *P. chlamydosporia* growth in their rhizosphere (Bourne *et al*., 1996; Kerry and Bourne, 1996; Bourne and Kerry, 1999), crop rotation requires a careful selection of cultivars that should not only be less susceptible to the nematode but also that support extensive growth of the fungus in their rhizosphere (Manzanilla-López *et al*., 2011).

Factors affecting establishment, performance, and biocontrol efficacy under field conditions need to be further investigated at the single isolate level. A greater understanding of the interactions between the various biotic and abiotic components of the system by combining population dynamics observations with molecular tools may help to determine the conditions for optimal fungal performance and help in designing efficient and sustainable management strategies.

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