Biological Control 69 (2014) 72–77

Contents lists available at ScienceDirect

Biological Control

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

Use of *Pochonia chlamydosporia* to control *Meloidogyne javanica* in cucumber

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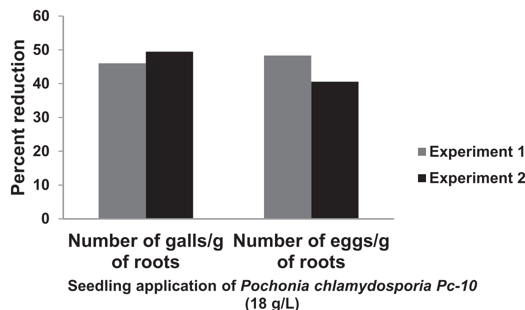
HIGHLIGHTS

- Seedling treatment with Pc-10 (18 g/L) controlled *Meloidogyne javanica* in both the experiments.
- Numbers of galls/g of roots and eggs/g of roots were reduced from 40.58% to 49.44%.
- Soil application of Pc-10 was not required for a consistent control of the nematode.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 July 2012
Accepted 17 November 2013
Available online 27 November 2013

Keywords:
Biological control
Cucumis sativus
Nematophagous fungi
Root-knot nematode

ABSTRACT

This study was done to evaluate the effect of the bionematicide Pc-10, based on *Pochonia chlamydosporia* var. *chlamydosporia*, on the control of *Meloidogyne javanica*. The fungus was applied by two methods: (1) drenching the substrate of seedlings with an aqueous suspension of Pc-10 (0, 4.5, 9.0, 13.5 and 18.0 g/L), or (2) incorporating Pc-10 into the soil of potted plants at a rate of 5000 chlamydospores/g of soil. The soil of each pot was infested with 3000 eggs of *M. javanica*, and one cucumber seedling was transplanted to each pot after 1 week. Increasing the doses of Pc-10 in the seedlings reduced the numbers of galls/g and eggs/g in the roots of cucumber plants in both experiments, regardless of the application method of the fungus in the soil. The application of 18 g/L of Pc-10 in the seedlings reduced the number of galls/g of roots by 46.04% and 49.44%, and the number of eggs/g of roots by 48.32% and 40.58% in the first and second experiments, respectively. Soil application of Pc-10 reduced the number of eggs/g of roots by 19.42% in the first experiment only. Drenching the substrate of seedlings with Pc-10 at 18 g/L controls *M. javanica* in cucumber and does not require additional fungus application in the soil.

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1. Introduction

The cucumber (*Cucumis sativus* L.) is a tropical cucurbit originally from India. It grows best at high temperatures but can also be cultivated in regions with lower temperatures, although it cannot tolerate freezing conditions or frost. Its fruit is usually consumed raw in salads or preserved in the form of pickles (Filgueira, 2003).

Root-knot nematodes (RKNs), especially *Meloidogyne javanica* (Treub) Chitwood and *Meloidogyne incognita* (Kofoid & White) Chitwood, are widely distributed in soils cultivated with vegetables in Brazil, causing losses of up to 100% of crops (Sikora and Fernández, 2005). The life cycle of these species starts when second-stage juveniles (J_2) hatch from eggs and invade the root. After inducing the formation of feeding sites, the J_2 become sedentary and develop into J_3, J_4 and adult females that produce eggs which are embedded in a gelatinous matrix (Teillet et al., 2013).

Pochonia chlamydosporia Zare and Gams (syn. *Verticillium chlamydosporium* Goddard) is a promising biological control agent

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1049-9644/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.biocontrol.2013.11.004

for RKNs. This fungus parasitizes eggs and exposed nematode females (Kerry, 2001). It produces chlamydospores, resistant survival structures that are the most effective type of propagule for establishing fungus in the soil. The most efficient concentration for field use is approximately 5000 chlamydospores per gram of soil (Kerry, 2001).

Studies are still needed to find practical, efficient and integrated methods of introducing *P. chlamydosporia* into soil in commercial production systems. According to Kerry and Bourne (2002), several techniques have been tested, including applying *P. chlamydosporia* to the soil surface in an aqueous suspension or in the form of granules, immersing roots in alginate slurry containing the chlamydospores and transplanting seedlings in blocks of colonized peat. However, these techniques have not provided entirely satisfactory results. Therefore, further investigation is required to optimize methods of application. Campos and Campos (1997) used inoculated pre-cooked wheat grains and a suspension of spores from *P. chlamydosporia* (2.0×10^6 spores/mL) in three applications to introduce the fungus to soil infested by *Meloidogyne exigua* and cultivated with coffee. Lopes et al. (2007) and Dallemole-Giaretta et al. (2008) applied the fungi by mixing the substrate (colonized by the fungi) with soil. Coutinho et al. (2009) applied the fungi to soil in the form of a suspension mixture of chlamydospores and flour from papaya seeds, which are a natural nematicide. Dallemole-Giaretta et al. (2010b) applied the fungi to the soil using a mixture of rice colonized by the fungus and pumpkin seed flour. Another method by Dallemole-Giaretta et al. (2010a) consisted of incorporating colonized coffee husks into the soil.

There are products being developed and tested for commercial production systems. The introduction of the isolate Pc-10 of *P. chlamydosporia* var. *chlamydosporia* to the soil has been tested by applying the fungus via irrigation water of the seedlings in a nursery with the plants under a central pivot. This bionematicide successfully controlled *M. javanica* on tomatoes, carrots and beets in the field (Podestá et al. 2009; Coutinho et al. 2009; Dallemole-Giaretta et al. 2010a,b).

Cucumbers and other vegetables are implanted in the field mainly through seedlings grown in polystyrene trays with different numbers of cells and sizes, with various types of substrates, either commercial or formulated by the farmer themself (Seabra Junior et al., 2004). This technique is already widely used by Brazilian farmers and may be a technically and economically feasible alternative for introducing *P. chlamydosporia* to the soil. The objective of this study was to evaluate the effect of *P. chlamydosporia* var. *chlamydosporia* for controlling *M. javanica* when applied to the substrate of seedlings or incorporated into the soil for growing cucumber plants.

2. Materials and methods

2.1. Location of the experiments

The experiments were conducted in a climate-controlled greenhouse located in the experimental field of the Department of Plant Pathology, Universidade Federal de Viçosa, Minas Gerais State, Brazil. Minimum and maximum air and soil temperatures inside the greenhouse were recorded daily.

2.2. Acquisition of the Pc-10 product

A commercial product (Rizotec®, Rizoflora Biotecnologia S.A., Viçosa, Brazil) consisting of chlamydospores from the isolate Pc-10 of *P. chlamydosporia* var. *chlamydosporia* (hereafter referred to as Pc-10) was used in the experiments. The mean concentration was 3.1×10^8 viable chlamydospores per gram of product, from the August 2010 batch.

2.3. Cucumber seedling production

A commercial substrate was used in the production of cucumber seedlings that were moistened with 5% water (v:v). Samples were collected from the substrate used in the experiments, with and physical and chemical analyses were performed at the Laboratory of Soil Analysis Viçosa Ltda (Table 1).

Seeds from the cucumber cv. Caipira were planted manually in five trays of cut styrofoam (128 cells) with 56 cells and capacity for approximately 1.8 L of substrate. One seed was planted per cell at a depth of 1 cm, standardized with the use of a wooden ruler. After planting, the trays were placed in a screened greenhouse, covered with black nylon and irrigated. Emergence occurred 2 days after sowing, at which point the nylon was removed. Plants were irrigated daily using a hose fitted with a sprinkler nozzle.

2.4. Applying Pc-10 to the seedlings

Pc-10 was applied to the cucumber seedlings in each tray by irrigation using a 500 mL PET (Polyethylene terephthalate) bottle with a perforated cover, containing a 250 mL suspension with different doses of Pc-10: 0, 4.5, 9.0, 13.5 and 18.0 g of PC-10/L of water. Each Pc-10 dose was divided into three equal parts and applied at 3, 7 and 12 days after planting, in order to supply the propagules of the fungus at different stages of seedling development.

2.5. Plant development

For cucumber plant growth, 2 L plastic pots containing the substrate composed of soil and sand at the proportion of 1:1 (v:v), previously fertilized with a 120 g simple superphosphate for each 20 L (w:v) of the mixture, were used. Seven days prior to transplanting, the soil of each pot was infested with a suspension volume containing 3000 eggs of *M. javanica*. The *M. javanica* inoculum was composed of eggs obtained from pure populations and collected from the roots of tomato plants kept in pots in a greenhouse, extracted by the technique of Hussey and Barker (1973), modified by Bonetti and Ferraz (1981). Soil from 35 of the pots was inoculated with a 20 mL suspension formulated with Pc-10 and calculated to provide 5000 chlamydospores per gram of soil per pot, while the soil of the other 35 pots was not infested with the fungi. The soil in the pots was maintained moist at near 60% field capacity.

One cucumber seedling (15-days-old) was transplanted in each recipient, making a 2×5 factorial design: soil without Pc-10 (0, 4.5, 9.0, 13.5 and 18.0 g/L of Pc-10 applied to the seedlings) and soil with Pc-10 (0, 4.5, 9.0, 13.5 and 18.0 g/L of Pc-10 applied to the seedlings). After transplanting, the recipients were placed in a screened greenhouse and irrigated daily, using a hose fitted with a sprinkler nozzle.

Two experiments were done, one on October 1st, 2010 and the other on October 18th, 2010. Topdressing was performed at 33 days after transplanting in both experiments, using 3 g of 10-54-10 foliar fertilizer per liter of water and applying 20 mL of this solution to each pot. The foliar fertilizer had the following chemical characteristics: 10% N, 54% P_2O_5, 10% K_2O, 0.1% Fe, 0.02% B and 0.05% Cu. During the experiments the presence of a powdery mildew (*Sphaerotheca fuliginea*) (Schlectend: Fr.) Pollacci was observed on the leaf surface of the cucumber plants. To control the fungus, plants were sprayed twice with raw cow's milk (Bettiol, 2004) at concentrations of 10% and 5% (v/v), respectively,

Table 1
Physic-chemical analysis of the commercial substrate used in the production of cucumber seedlings of the experiments, performed at the Laboratory of Soil Analysis Viçosa Ltda.

pH H_2O	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	S (%)	T.O.C. (%)	C/N –	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Density (g/mL)
5.54	0.55	0.26	0.22	1.40	0.77	0.44	13.10	23.81	51	8516	105	29	13.6	0.64

Total concentrations, determined in the acid extract (nitric acid with perchloric acid); N, by the Kjeldahl method; T.O.C. (Total organic carbon) by the Walkley–Black method.

in Experiment 1 at 33 and 40 days after transplanting, and in Experiment 2 at 15 and 22 days after transplanting.

In Experiment 1, the average minimum and maximum temperatures during the cucumber plant development were 23.1 °C and 36.0 °C for the air, and 23.9 °C and 32.3 °C in the soil. In Experiment 2, the average minimum and maximum temperatures were 23.5 °C and 36.7 °C for the air, and 24.5 °C and 33.0 °C in the soil.

2.6. Experiment evaluation

To determine the CFU of *P. chlamydosporia* var. *chlamydosporia* per gram of substrate in the seedlings treated with different doses of Pc-10, ten seedlings were randomly selected from each of the trays. The substrate bonded to the respective root systems of the collected plants, creating a sample composed of the substrate per dose of Pc-10 used in the seedlings. Composite samples of the substrates were collected on the first and seventh days after seedling transplant in experiments 1 and 2, respectively. The number of CFU/g of substrate was determined as described by Kerry and Bourne (2002) using semi-selective medium according to Gaspard et al. (1990).

The cucumber plants were collected 45 days after transplanting. Their height, fresh weight of shoots (FWS) and fresh weight of roots (FWR) were determined. Nematode damage was measured by counting the number of galls and eggs per whole root system and expressed as number of galls or eggs per gram of root. Nematode eggs were extracted from cucumber roots according to Hussey and Barker (1973) and were counted under a dissecting microscope at 4× magnification. Root galls were counted with the naked eye. During root extraction, samples from the soils of the respective treatments were collected to determine the number of colony forming units (CFU) of the fungus per gram of soil. The number of CFU/g of soil was determined according to the previously described methodology.

2.7. Experimental design

The experimental design consisted of randomized blocks with seven replicates per treatment. Each experimental unit consisted of one plant/pot. Each experiment was performed twice.

2.8. Statistical analysis

Values for the plant growth parameters, nematological variables and CFU/g of substrate were tested for normality of the error (Kolmogorov–Smirnov test), homogeneity of variances (Bartlett test) and subjected to analysis of variance (ANOVA, *F* test at 5% probability). Square-root or $\log_{10}$ transformations were performed before ANOVA when the assumptions were not satisfied. Data were analyzed by means of a two-way ANOVA (doses of Pc-10 in the seedlings x soil with or without Pc-10 and their interactions). Significant relationships between doses of Pc-10 in the seedlings and the parameters were described by linear regression models.

3. Results

3.1. Number of CFU/g of substrate of the cucumber seedlings

There was no contamination of the experimental control samples with Pc-10. The fungus was recovered only from seedlings treated with the bionematicide.

Increasing doses of Pc-10 in the seedlings increased the number of CFU/g of substrate applied to cucumber seedlings in both experiments (Fig. 1). The separate applications of Pc-10 to the plants, consisting of approximately 1.21×10^6 (dose 4.5 g/L of Pc-10) to 4.84×10^6 chlamydospores/g of substrate (dose 18.0 g/L of Pc-10), resulted in the production of 6.5×10^4–16.8×10^4 CFU/g of substrate in Experiment 1, and 3.0×10^4–6.16×10^4 CFU/g of substrate in Experiment 2. In both experiments, the application of high concentrations of Pc-10 to the seedlings did not impair cucumber seedling development.

3.2. Cucumber plant development

No significant interaction ($p < 0.05$) was observed between the application of Pc-10 in the seedlings and the additional treatment with the fungus in the soil.

The application of Pc-10 to the soil or seedlings did not significantly affect ($p < 0.05$) the height and fresh weight of the shoots (FWS) of cucumber plants in both experiments (data not shown).

Applying Pc-10 to the soil and seedlings did not significantly affect the fresh weight of the roots (FWR) of cucumber plants in Experiment 1 (data not shown). In Experiment 2, however, the independent effect of the two factors on the FWR was observed. Applying Pc-10 to the soil increased cucumber root mass by 12.03% when compared to non-treated plants (16.39 × 14.63). The highest FWR was observed in seedlings treated with 18.0 g/L of Pc-10, according to a linear model (Fig. 2).

3.3. Controlling M. javanica in cucumber plants

Increasing the doses of Pc-10 in the seedlings reduced the number of galls/g of cucumber plant roots in both experiments according to linear models (Fig. 3), regardless of the application of the fungus in the soil. The application of 18 g/L of Pc-10 reduced the number of galls/g of roots by 46.04% and 49.44% in the first and second experiments, respectively, compared to the control.

Nematode reproduction was also reduced by the application of Pc-10, with no significant interaction between the two factors (seedling × soil application of the fungus). The number of eggs/g of roots was reduced by increasing doses of the bionematicide in the seedlings in both experiments (Fig. 3). Reductions of 48.32% and 40.58% were observed in plants treated with the highest dose of Pc-10 (18 g/L) in experiments 1 and 2, respectively. The soil application of Pc-10 reduced the number of eggs/g of roots in the first experiment only (19.42%) compared to the control (14,178 × 17,595).

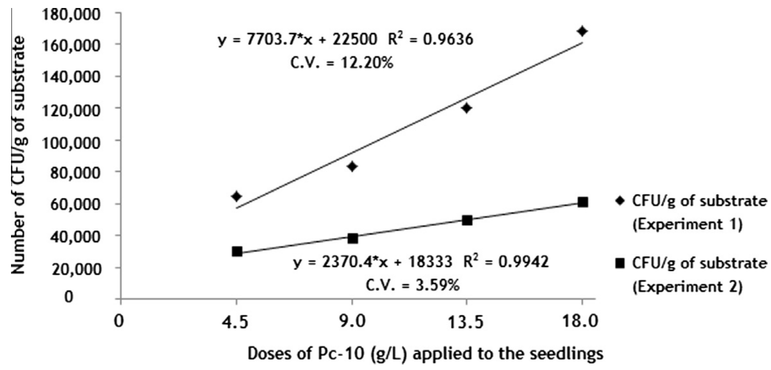

Fig. 1. Number of colony forming units (CFU) of Pochonia chlamydosporia var. chlamydosporia (isolate Pc-10) per gram of substrate in the cucumber seedlings subjected to difference concentrations of Pc-10 (g/L). Each point represents the mean of three repetitions. C.V.: Coefficient of variation (%).

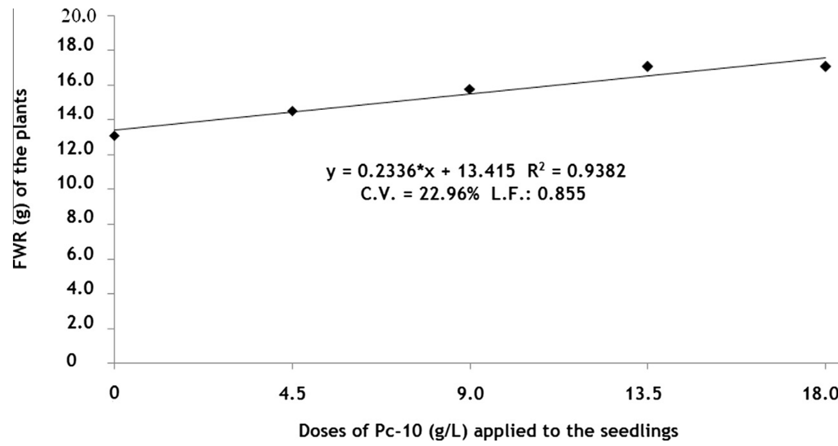

Fig. 2. Fresh weight of the root system (FWR) in the cucumber plants of experiment 2, subjected to different doses of Pc-10 (g/L) applied to the seedlings at 45 days after transplanting. Each point represents the mean of seven repetitions. C.V.: Coefficient of variation (%), L.F.: Lack of fit; *Significant according to the t-test at 5% probability.

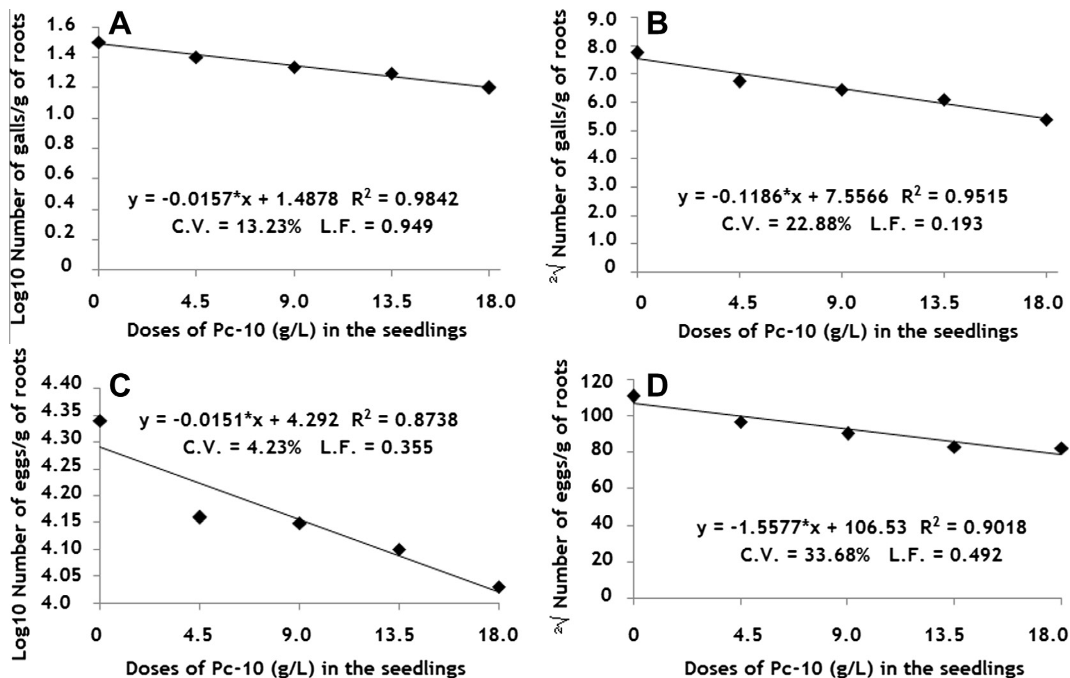

Fig. 3. Number of galls/g of roots and eggs/g of roots (log10 x) of Meloidogyne javanica in cucumber plants treated with different doses of Pc-10 (g/L) applied to seedlings in experiments 1 (A and C) and 2 (B and D), 45 days after transplanting. Each point represents the mean of seven repetitions. CV: Coefficient of variation (%); LF: Lack of fit. *Significant according to the t-test at 5% probability.

3.4. Number of CFU/g of soil in the cucumber plants

The fungus was not found in the soil of the control plants. Data from the other treatments did not fit any of the regression models (data not shown). Overall, the numbers of CFU/g of soil in treatments with Pc-10 in the substrate and soil averaged 40,000 ± 5323 and 38,500 ± 8466 CFU/g of soil in experiments 1 and 2, respectively.

4. Discussion

This study provided evidence that applying 18 g/L of Pc-10 to the seedlings resulted in greater control of *M. javanica* in cucumber, without additional applications of the fungus in the soil.

The efficiency of *P. chlamydosporia* as a biological control agent of RKNs depends on the interaction among the host plant, the nematode and the fungus (Kerry, 2000).

Some plant species, including cabbage, kale, sunn hemp, corn and tomato permit extensive colonization of *P. chlamydosporia* in their rhizosphere and are considered good hosts for the fungus. In these situations, the inoculum of the fungus is increased in the soil without additional applications (Dallemole-Giaretta et al., 2011). Other plants, such as okra, sorghum, beans and eggplant do not permit satisfactory rhizosphere growth (Kerry and Bourne, 1996; Bourne and Kerry, 1999). The number of CFU/g in the cucumber roots was not evaluated in either experiment and there exists no information about the host status of this plant to the fungus (Bourne and Kerry, 1999). Based on the results of the present study regarding the number of CFU/g of the soil or substrate, it is likely that cucumber cv. Caipira allows poor to moderate rhizospheric Pc-10 development. The combination of the semi-selective media plating method and qPCR in further studies can provide relevant information on Pc-10 abundance in the cucumber rhizosphere (Morton et al., 2003; Manzanilla-López et al., 2009; Dallemole-Giaretta et al., 2012).

Nevertheless, even on a good host for *M. javanica* and a putative moderate to poor host for the fungus, the nematode population was reduced after the application of Pc-10 (Fig. 3). Many large galls were formed in the roots of cucumber cv. Caipira (data not shown), evidencing this genotype is very susceptible to *M. javanica*, as in several cucumber cultivars used in Brazil (Charchar and Aragão, 2005).

These considerations suggest that controlling *M. javanica* in the cucumber cv. Caipira can be achieved with applications resulting in high concentrations of the fungus in the soil, especially in the seedlings. Bourne and Kerry (1999) also reported that increasing doses of *P. chlamydosporia* (5000; 10,000 and 50,000 chlamydospores/g soil) enhanced nematode control and the colonization of soil and the rhizosphere (CFU/g of soil or root) of different host plants at the end of the experiments.

Seedling treatment gave a competitive advantage to Pc-10 in relation to *M. javanica*, allowing contact between fungus and plant before nematode infection. This strategy enabled the establishment of the fungus in the substrate at levels that made it possible to reduce the number of galls and eggs in the roots (Fig. 3) without requiring additional applications in the soil. This is significant for Pc-10, because in previous experiments with this fungus, the number of galls of the root-knot nematode was reduced only when the antagonist was applied in soils infested with eggs of the pathogen and the seedlings were transplanted after 1 (Podestá et al., 2009) or 2 weeks (Dallemole-Giaretta et al. 2008; Coutinho et al., 2009; Dallemole-Giaretta et al. 2010b). In addition to nematode control, seedling treatments with increased doses of Pc-10 improved the development of cucumber roots in Experiment 2 (Fig. 2). Dallemole-Giaretta et al. (2008) also reported that the isolates Pc-3, Pc-10 (the same as the present study) and Pc-19 of *P. chlamydosporia* colonized the rhizosphere and effectively promoted the growth of tomato seedlings.

The application of Pc-10 in the seedlings (18 g/L) may be a technically and economically feasible alternative for introducing this fungus into the soil of areas cultivated with cucumber. Nevertheless, the biological control should be combined with other control methods (crop rotation, wet fallow, soil solarization) to maximize the effect of the fungus, especially under high pressure of nematode inoculum and in crops highly susceptible to the pathogen (Dutra and Campos, 1998; Kerry and Bourne, 2002; Dutra and Campos, 2003).

5. Conclusion

Seedling treatment with Pc-10, at 18 g/L, controls *M. javanica* in cucumber and does not require additional fungus application in the soil.

Acknowledgments

The authors thank Paulo Afonso Ferreira for his collaboration on the statistical procedures and review of this paper; Rizoflora Biotecnologia S.A. for providing the Pc-10 product for the experiments and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for providing a scholarship to the first author.

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