Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9275
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v37i4.25042

Pochonia chlamydosporia promotes the growth of tomato and lettuce plants

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ABSTRACT. The fungus *Pochonia chlamydosporia* is one of the most studied biological agents used to control plant-parasitic nematodes. This study found that the isolates Pc-3, Pc-10 and Pc-19 of this fungus promote the growth of tomato and lettuce seedlings. The isolate Pc-19 colonized the rhizoplane of tomato seedlings in only 15 days and produced a large quantity of chlamydospores. This isolate was able to use cellulose as a carbon source, in addition to glucose and sucrose. Scanning electron microscopy (SEM) revealed that hyphae of the *P. chlamydosporia* isolate Pc-10 penetrated the epidermal cells of the tomato roots. These three *P. chlamydosporia* isolates promote the growth of tomato and lettuce.

Keywords: *Lactuca sativa*, nematophagous fungus, rhizosphere colonization, *Solanum lycopersicum*, *Verticillium chlamydosporium*.

Pochonia chlamydosporia promove crescimento de tomateiro e alface

RESUMO. O fungo *Pochonia chlamydosporia* é um dos mais estudados agentes de controle biológico de fitonematoides. Neste estudo foi demostrado que os isolados Pc-3, Pc-10 e Pc-19 do fungo promovem o crescimento de plântulas de tomate e alface. O isolado Pc-19 colonizou o rizoplano de mudas de tomate em apenas 15 dias e produziu grande quantidade de clamidósporos. O isolado foi capaz de utilizar celulose como fonte de carbono, tanto quanto glicose e sacarose. Em estudos realizados por microscopia eletrônica de varredura (MEV), foi possível observar que hifas do isolado Pc-10 penetraram as células epidérmicas das raízes de tomateiro. Os três isolados de *P. chlamydosporia* promovem o crescimento de tomateiro e alface.

Palavras-chave: *Lactuca sativa*, fungo nematófago, colonização da rizosfera, *Solanum lycopersicum*, *Verticillium chlamydosporium*.

Introduction

Pochonia chlamydosporia Zare and Gams is one of the most studied biological agents used to control plant-parasitic nematodes. It can produce chlamydospores, which enable it to survive in the soil without additional energy sources (MANZANILLA-LÓPEZ et al., 2013). However, the ability of *P. chlamydosporia* to control endoparasitic nematodes, such as *Meloidogyne* spp. Goeldi, depends on rhizosphere colonization (MANZANILLA-LÓPEZ et al., 2013).

The *P. chlamydosporia* fungus can colonize the rhizoplane and the internal tissues of the roots of some plants, in the manner of an endophyte (BORDALLO et al., 2002; ESCUDERO; LOPEZ-LLORCA, 2012; MACIÁ-VICENTE et al., 2009b; MONFORT et al., 2005). Once inside the root tissues, this antagonistic fungus shares the same niche as root-knot nematodes and is less subject to competition from soil microorganisms (ESCUDERO; LOPEZ-LLORCA, 2012). From the perspective of the biocontrol of nematodes, the endophytic colonization of roots by the fungus is a competitive advantage. The majority of the putatively highly expressed *P. chlamydosporia* genes are related to its endophytic behaviour, including the production of hydrolytic enzymes, transporters, proteases, chitinases and a large number of secondary metabolites (LARRIBA et al., 2014). As a result of this interaction with its host, the fungus can enhance the tolerance of the crop to biotic and abiotic stresses and promote plant growth (ESCUDERO; LOPEZ-LLORCA, 2012; MACIÁ-VICENTE et al., 2009b; MANZANILLA-LÓPEZ et al., 2013; MONFORT et al., 2005).

Pochonia chlamydosporia acts as a growth-promoting agent of monocot and dicot crops, such as barley (MACIÁ-VICENTE et al., 2009b), wheat

(MONFORT et al., 2005), lettuce (DIAS-ARIEIRA et al., 2011), pistachio (EBADI et al., 2009) and tomato (ESCUDERO; LOPEZ-LLORCA, 2012). However, the extent to which plant growth is promoted depends on the combination of the crop and the particular *P. chlamydosporia* isolate (MANZANILLA-LÓPEZ et al., 2011).

In a previous study, we screened different isolates of *P. chlamydosporia* for the ability to manage the root-knot nematode *Meloidogyne javanica* (DALLEMOLE-GIARETTA et al., 2012). However, little is known about the effects of these isolates on plant growth. Thus, the aims of this study were to evaluate the colonization of tomato and lettuce roots by three isolates of *P. chlamydosporia* var. *chlamydosporia* and to characterize the effects of these organisms on plant development.

Material and methods

Fungal isolates

Pochonia chlamydosporia var. *chlamydosporia* Pc-3, Pc-10 and Pc-19 were isolated from soil naturally infested with *Meloidogyne* spp. and cultivated with vegetables in Viçosa, Minas Gerais State, Brazil (DALLEMOLE-GIARETTA et al., 2012). The fungal isolates were recovered by incubating pieces of colonized filter paper (SMITH; ONIONS, 1994) on corn meal agar (CMA, Difco, Detroit, MI, USA) at 25°C in darkness for 21 day.

Promotion of the growth of tomato seedlings

Two assays were performed to assess the potential of Pc-3, Pc-10 and Pc-19 to promote the growth of tomato cv. Santa Clara under laboratory conditions. Glass tubes (23 mm in diameter x 150 mm in length) were filled with 1.5 g of coconut fiber (Table 1), which was moistened with 7.5 mL (assay 1) or 6.5 mL (assay 2) of distilled water. After 12h, the tubes were capped and autoclaved at 121°C for 30 min. This process of sterilization was performed twice with an interval of 24h.

Table 1. Chemical characterization of the coconut fiber used as a substrate for the growth of tomato and lettuce plants.

Macronutrients (g kg^{-1})						
Nitrogen	Phosphorus	Potassium	Calcium	Magnesium	Sulphur	Sodium
7.3	3.3	11.7	11.6	4.8	5.1	0.4
Micronutrients (mg kg^{-1})						
Zinc	Iron	Manganese	Copper	Boron		
502	3668	250	228	126		
C/N				pH		
26.49				5.01		

Fungal inoculum was applied to the substrate either as mycelial discs or as a conidia suspension. In assay 1, four discs (5 mm in diameter) were cut from the edge of a 21-day-old culture of *P. chlamydosporia* on CMA, and each disc was placed at a depth of 2 cm in a tube containing the substrate. In assay 2, *P. chlamydosporia* conidia were collected and suspended in sterile water, and the suspension was adjusted to provide 1.52 x 10^6, 1.83 x 10^5 and 1.92 x 10^6 conidia per tube of the isolates Pc-3, Pc-10 and Pc-19, respectively. Sterile PDA discs and distilled water were used as controls. One pre-germinated tomato seed was added in each tube at a depth of 0.5 cm. The tomato seeds were previously disinfected with 70% alcohol (v v^{-1}) for 1 min., then immersed for 10 min. in sodium hypochlorite diluted to 1% active chlorine (v v^{-1}) and finally rinsed with sterile water. The seeds were then pre-germinated for 2 d in the culture medium described by Bordallo et al. (2002). For controls, tomato seeds were placed in the substrate without fungus. The tubes were transferred into a growth chamber at 25°C with a 16-h photoperiod.

The height and the fresh mass of the aboveground parts of the seedlings were recorded after 15 day. The experiment was carried out in a completely randomized design with 10 (assay 1) or eight (assay 2) replicates.

Root colonization and the use of carbon sources by *P. chlamydosporia* isolate Pc-19

Ten root segments, 0.5 cm in length, subjected to colonization by Pc-19 in assay 1 were incubated in Petri dishes on semi-selective medium (GASPARD et al., 1990) at 25°C, and the number of colonized segments was recorded after three days. The remaining root segments were stored in a solution containing formaldehyde, lactic acid and alcohol (FAA 50; 5:5:90, v v^{-1} v^{-1}) to study rhizospheric colonization. The root segments were prepared according to a protocol used for the observation of mycorrhizal associations (BRUNDRETT et al., 1996).

To investigate which carbon sources can be used by the isolate Pc-19, the culture medium was prepared using glucose (control), sucrose, cellulose or pectin and contained 8 g L^{-1} of carbon, 2 g L^{-1} of $NaNO_3$, 1 g L^{-1} of K_2HPO_4, 0.5 g L^{-1} of $MgSO_4$, 0.5 g L^{-1} of KCl, 0.01 g L^{-1} of $FeSO_4$ and 17 g L^{-1} of agar in distilled water (SUN; LIU, 2006). A mycelial disc 5 mm in diameter was cut from the edge of a 15-day-old culture of the fungus and was placed at the centre of each 9-cm-diameter Petri dish. The fungal biomass was recorded after 15 d of culturing at 25°C in the dark. To measure the fungal biomass, the culture medium was dissolved in 200 mL of boiling water, and the mycelium was transferred to a

crucible and held at 105°C until a constant mass was reached. The assay was arranged in a randomised design with seven replicates per treatment.

Root colonization of tomato plants by the Pc-10 isolate as determined by scanning electron microscopy (SEM)

Root segments from assay 1 and colonized by Pc-10 were fixed in phosphate buffer solution (0.05 mol. L^{-1}, pH 7.1) with 2.5% of glutaraldehyde for one hour at room temperature. They were washed six times in phosphate buffer solution (0.05 mol. L^{-1}, pH 7.1) for ten min. each time, dehydrated in an ethanol series of 30 and 50% for 10 min. at each concentration and placed in 70% ethanol in the refrigerator until the following day. Dehydration was then continued with an additional ethanol series of 80 and 95% for 10 min. each, followed by 100% ethanol for 15 min., three times. The samples were dried in a critical point dryer (Bal-Tec, model CPD 030, Germany) and mounted on aluminium stubs, sputter-coated in gold and viewed under a scanning electron microscope (Zeiss, model LEO 1430 VP, England).

Promotion of the growth of lettuce seedlings

The potential of the isolate Pc-10 to promote the growth of lettuce seedlings was assessed using the cultivars Verônica, Americana, Regina and Manteiga (Isla Sementes, Porto Alegre, Rio Grande do Sul State, Brazil). Each cultivar was evaluated separately, using the methodology described for assay 1. Root segments subjected to colonization by Pc-10 were incubated in Petri dishes on semi-selective medium (GASPARD et al., 1990) at 25°C, and the number of colonized segments was recorded after three days.

Statistical analysis

The data for each variable were tested using the Kolmogorov-Smirnov test to determine whether they were normally distributed. The Bartlett test was used to determine whether variances were homogeneous. The data were subjected to a one-way analysis of variance (ANOVA, F-test, $p < 0.05$). Treatment means were compared using tukey's HSD test ($p < 0.05$). Statistical analyses were performed using R software, version 2.12.2 (R DEVELOPMENT CORE TEAM, 2011).

Results and discussion

Promotion of tomato seedling growth

All isolates of *P. chlamydosporia* increased the height (from 1,179 to 1,404%) and the aboveground mass (from 1,350 to 1,650%) of tomato seedlings in assay 1, when mycelial discs were used as the source of inoculum, and from 72 to 103, and 100 to 148%, respectively, in assay 2, when the fungus was added to the substrate via conidia suspension (Table 2). No significant difference among the isolates was observed in either assay. Despite the differences between the assays, we concluded that the growth of tomato seedlings was promoted by the *P. chlamydosporia* var. *chlamydosporia* isolates, excluding the sole effect of the nutrients from the culture medium (CMA).

Microorganisms can stimulate plant growth both directly (i.e., via the production of plant hormones, biological nitrogen fixation, phosphorus solubilization, acceleration of the process of mineralization and siderophore synthesis) and indirectly (i.e., via the induction of systemic resistance, the production of antibiotics and antagonism in relation to pathogens) (HAYAT et al., 2010). Pochonia chlamydosporia can promote the growth of wheat seedlings via direct mechanisms, such as the production of growth regulators associated with the activity of peroxidase (MONFORT et al., 2005). In this study, it is likely that the increase in plant growth was related to an increase in the area of absorption of the roots, allowing them to overcome possible water stresses in the tubes.

Table 2. Height and mass of the aboveground portion of tomato seedlings cv. Santa Clara grown on a coconut fiber substrate infested using mycelial discs (assay 1) or a conidia suspension of *Pochonia chlamydosporia* var. *chlamydosporia* isolates Pc-3, Pc-10 and Pc-19.

Treatments	Plant height (cm)	Increase (%)	Aboveground mass (g)	Increase (%)
Assay 1				
Control	0.69 (± 1.21) b	-	0.004 (± 0.006) b	-
Isolate Pc-3	9.45 (± 3.15) a	1,269	0.063 (± 0.028) a	1,475
Isolate Pc-10	10.38 (± 1.36) a	1,404	0.070 (± 0.021) a	1,650
Isolate Pc-19	8.83 (± 2.68) a	1,179	0.058 (± 0.022) a	1,350
Assay 2				
Control	2.87 (± 1.69) b	-	0.023 (± 0.022) b	-
Isolate Pc-3	5.83 (± 1.07) a	103	0.057 (± 0.023) a	148
Isolate Pc-10	5.00 (± 1.52) a	74	0.046 (± 0.020) a	100
Isolate Pc-19	4.95 (± 1.81) a	72	0.048 (± 0.018) a	109

Means (± standard deviations) within a column in each assay followed by the same letter do not differ by tukey's HSD test ($p < 0.05$).

Root colonization and the use of carbon sources by *P. chlamydosporia* isolate Pc-19

The fungus colonized all root segments of the tomato plants and also the coconut fiber used as a substrate for the plants (Figure 1A). A large quantity of chlamydospores was produced in the rhizoplane (Figure 1B), and the hyphae of the fungus penetrated into cells of the root cortex (Figure 1C). The fungus was able to use cellulose as a carbon source, as much as glucose and sucrose, with an average mycelial production (dry mass) of 0.135, 0.111 and 0.138 g using each of these carbon sources, respectively. However, poor mycelial growth was recorded when the fungus was grown in culture medium amended with pectin as the carbon source (0.009 g of dry mass).

This study provides evidence that Pc-19 can colonize tomato roots and use cellulose as a carbon source. Different species of plants differ in their abilities to allow the growth of *P. chlamydosporia* in the rhizosphere. Tomato, cabbage, crotalaria, beans, corn, kale, potato and pea are plants that favour the development of the fungus, whereas soybean, wheat, sorghum, aubergine and okra are plants that do not permit satisfactory development in the rhizosphere (MANZANILLA-LÓPEZ et al., 2013). In addition to corroborating earlier findings of the ability of tomato to support *P. chlamydosporia* colonization (MANZANILLA-LOPEZ et al., 2013), we found that the fungus can form a tangle of hyphae similar to those formed by arbuscular mycorrhizae. It has been suggested that this nematode antagonist may act as an endophyte (MACIÁ-VICENTE et al., 2009a) and may increase the ability of the tomato to germinate, absorb water from the substrate and resist stress due to the deleterious effects of salts present in the coconut fiber substrate (DOMEÑO et al., 2009; HERNÁNDEZ-APAOLAZA et al., 2005). In addition, enhanced development of the plants may be associated with the chemical degradation of cellulose from the coconut fiber by enzymes produced by the fungus (ESTEVES et al., 2009; LARRIBA et al., 2014) and the subsequent translocation of carbohydrates, as well as nutrients like nitrogen and phosphorus, to the roots of the tomato plants, as has been observed with arbuscular mycorrhizal fungi (SCHREINER, 2007).

Root colonization of tomato plants by Pc-10 as determined by scanning electron microscopy (SEM)

After 15 days, *P. chlamydosporia* var. *chlamydosporia* isolate Pc-10 colonized the roots of tomato seedlings, forming a mantle of hyphae and producing conidia in some parts of the rhizosphere (Figure 1D and E). Thus, like Pc-19, this isolate was able to colonize tomato roots; this information can be used to guide the choice of crop rotation system to support good rhizosphere development. Greater development and reproduction of the fungus in the rhizosphere can improve the control of plant-parasitic nematodes (DALLEMOLE-GIARETTA et al., 2011; VIGGIANO et al., 2014).

Although the extent to which isolate Pc-3 colonizes rhizosphere has not been evaluated, based on the results of the growth promotion assays it is possible to speculate that, like Pc-10 and Pc-19, this isolate may efficiently colonize the rhizosphere of tomato plants (Table 2).

Promotion of the growth of lettuce seedlings

Pc-10 increased the aboveground mass of all the lettuce cultivars from 100 to 330% and the height of the cultivar Regina by 80% (Table 3). Colonization of the roots by the isolate was observed in 100% of the root system segments of the lettuce seedlings, for all the cultivars tested, when plated on semi-selective medium.

In addition to the growth promotion of the plants, the ability of *P. chlamydosporia* isolates to colonize tomato and lettuce roots may protect the hosts against nematodes and other soil pathogens. MONFORT et al. (2005) reported that the *P. chlamydosporia* isolate 4624 improved barley growth and reduced root colonization by the phytopathogenic fungus *G. graminis* var. *tritici*. The protection of the root system by *P. chlamydosporia* against fungal pathogens probably occurs via supplementation with nutrients and competition for infection sites, as has been observed with arbuscular mycorrhizal fungi (BORGES et al., 2007). Moreover, *P. chlamydosporia* induces a defense reaction in the plant, with the formation of papillae in the cell wall of the root system (BORDALLO et al., 2002). Further studies are needed to understand the mechanisms of action of the *P. chlamydosporia* isolates Pc-3, Pc-10 and Pc-19.

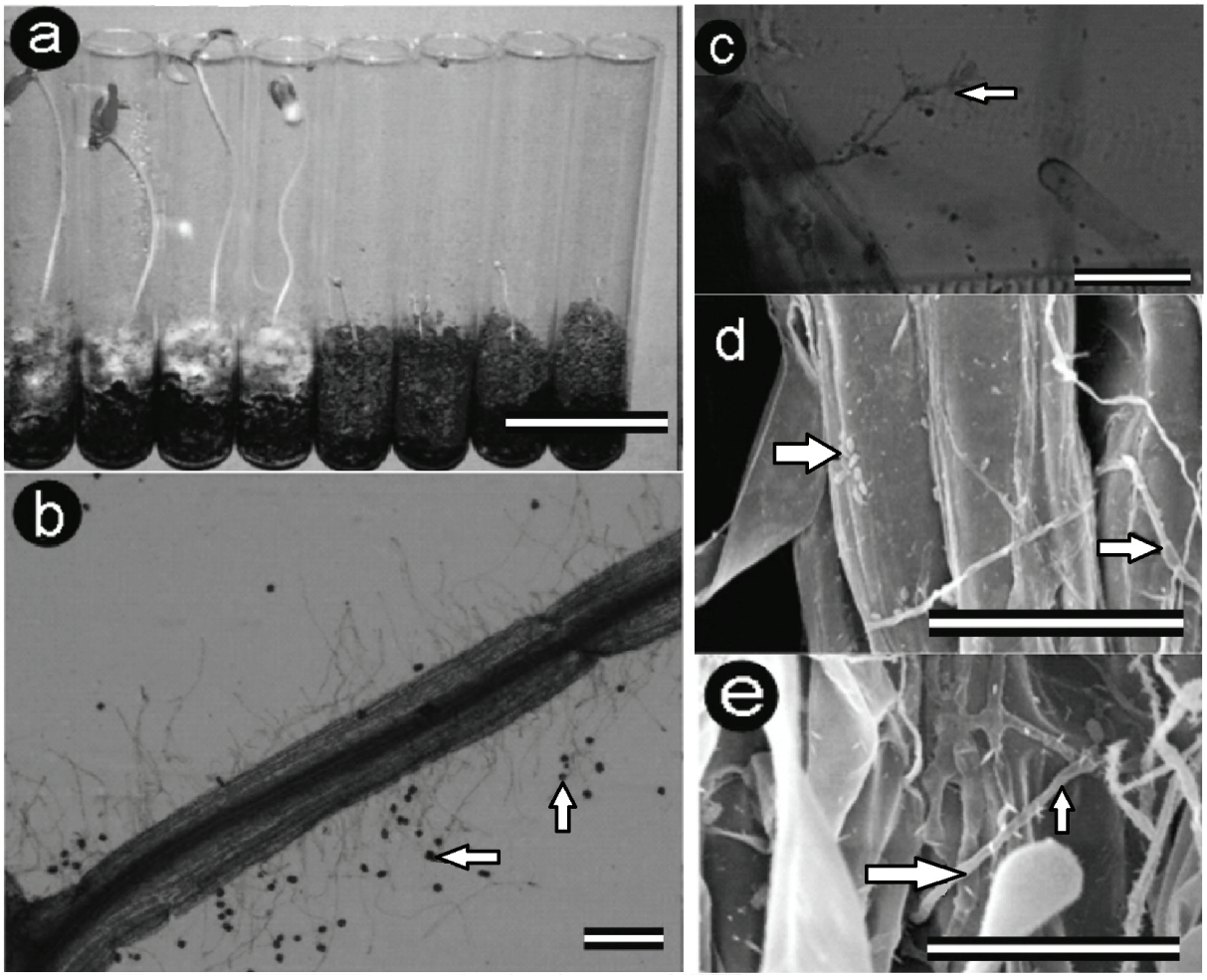

Figure 1. Tomato seedlings grown on a coconut fiber substrate infested with *P. chlamydosporia* var. *chlamydosporia* isolate Pc-19. (A) The four culture tubes on the left contain the isolate Pc-19, while the four on the right do not contain the antagonist (scale bars represent 1 cm). (B) Rhizoplane colonization of tomato seedlings by Pc-19, with the formation of chlamydospores (scale bar represents 250 μm). (C) Phialides of Pc-19 formed on the surface of the tomato root (scale bar represents 100 μm). (D) and (E) Scanning electron microscopy images of tomato roots colonized by *P. chlamydosporia* var. *chlamydosporia* isolate Pc-10. Hyphae and conidia of the fungus in the rhizoplane of the roots (D) and hyphae penetrating into cells of the root cortex of tomatoes (E; scale bars represent 50 μm).

Table 3. Height and fresh mass of the aboveground portion of the lettuce cultivars* Verônica, Americana, Regina and Manteiga grown on a coconut fiber substrate infested with mycelial discs of *Pochonia chlamydosporia* var. *chlamydosporia* isolate Pc-10 (Pc).

Treatments	Plant height (cm)	Increase (%)	Aboveground mass (g)	Increase (%)
Verônica + Pc	1.23 (± 0.678) a	-	0.02 (± 0.013) a	100
Verônica - Pc	0.83 (± 0.453) a	-	0.01 (± 0.007) b	-
Americana + Pc	1.45 (± 0.626) a	-	0.03 (± 0.017) a	200
Americana - Pc	1.34 (± 0.637) a	-	0.01 (± 0.005) b	-
Regina + Pc	1.78 (± 0.646) a	80	0.03 (± 0.016) a	200
Regina - Pc	0.99 (± 0.321) b	-	0.01 (± 0.006) b	-
Manteiga + Pc	1.08 (± 0.436) a	-	0.043 (± 0.016) a	330
Manteiga - Pc	1.21 (± 1.115) a			-

*Each cultivar was evaluated in a separate assay. Means (± standard deviation) within a column in each cultivar followed by the same letter do not differ by F-test ($p < 0.05$).

Conclusion

The isolates Pc-3, Pc-10 and Pc-19 of *P. chlamydosporia* promote the growth of tomato and lettuce seedlings. This fungus colonizes the roots of both plant species and produces hyphae and chlamydospores in the rhizoplane of tomato plants.

Acknowledgements

The authors would like to thank the National Council for Scientific and Technological Development (CNPq), the Foundation for Research Support of the State of Minas Gerais (Fapemig) and the Coordination for the Improvement of Higher

Education Personnel (Capes) for their financial support.

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Received on September 24, 2014.
Accepted on November 30, 2014.