Crop Protection 52 (2013) 97–102

Contents lists available at SciVerse ScienceDirect

Crop Protection

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

Biological control of the false root-knot nematode *Nacobbus aberrans* by *Pseudomonas protegens* under controlled conditions

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ARTICLE INFO

Article history:
Received 25 September 2012
Received in revised form
21 February 2013
Accepted 24 February 2013

Keywords:
Biological control
Fluorescence microscopy
PCR–RFLP
Plant–parasitic nematode
Rhizobacterium
Tomato

ABSTRACT

The use of antagonistic biological agents, such as fungi and bacteria, offers an economical and safe strategy to manage plant–parasitic nematodes in infested fields. The false root-knot nematode, *Nacobbus aberrans*, is a damaging parasite of many agronomic and horticultural crops in South and North America. The management of this nematode is challenging and often not profitable for farmers. In greenhouse tests, conducted in Argentina, applications of the strain *Pseudomonas protegens* CHA0 and its isogenic derivative ARQ1 (used as control) at a rate of 10^8 cfu ml^{-1} suppressed infection and reproduction of *N. aberrans* on tomato roots. However, neither of the strains promoted plant root or shoot growth of the treated plants. Root colonization by the bacteria was assessed by specific PCR–RFLP protocols and fluorescence microscopy. The results obtained in this preliminary study were encouraging and showed the potential of *P. protegens* CHA0 to be used for the management of the false root-knot nematode on tomato.

1. Introduction

Some plant–parasitic nematodes are important pests, causing severe crop losses to food and fiber crops (Graham, 2011), annually estimated to be US $78 billion worldwide (Barker et al., 1998). *Nacobbus aberrans* is a sedentary endoparasite that induces galls in the roots of its hosts. Because the external symptoms it causes on roots are similar to those produced by species of the genus *Meloidogyne* (the root-knot nematode), *N. aberrans* is commonly referred as the false root-knot nematode. The species is native to the Americas (Sher, 1970) and is present in Argentina, Bolivia, Chile, Ecuador, Mexico, Peru, and USA (EPPO, 2011). It causes significant losses in crops such as potato (*Solanum tuberosum*) (up to 80%), tomato (*Solanum esculentum*) (50–90%) and bean (*Phaseolus vulgaris*) (35%) (Manzanilla-López et al., 2008). It is considered an A1 quarantine organism (EPPO, 2011) and is under international phytosanitary regulations to avoid its introduction to other countries (CABI and EPPO, 1997). The species has a wide host range and can establish under different environmental conditions (Manzanilla-López et al., 2002). These features, combined with the existence of physiological races/pathotypes/groups (terms given by different authors) (Inserra et al., 1985; Manzanilla-López et al., 2002; Lax et al., 2011a), make its management difficult.

Management of plant–parasitic nematodes in crop production systems relies primarily on host-plant resistance, crop rotation and nematicides (Timper, 2011). Although chemical nematicides are effective, many are being withdrawn from the market in some developed countries primarily due to concerns about human health and environmental safety (Nico et al., 2004). The search for novel, environmentally friendly alternatives to manage plant–parasitic nematode populations has, therefore, become increasingly important (Tian et al., 2007). Biological control using microbial antagonists might be a potential alternative to nematicides (Burkett-Cadena et al., 2008).

Plant growth-promoting rhizobacteria are free-living bacteria that colonize roots and stimulate plant growth (Lugtenberg and Kamilova, 2009). Certain fluorescent pseudomonads produce a variety of extracellular metabolites; many of them are inhibitory to other microorganisms and some of them are involved in the biological control of plant pathogens (Haas and Défago, 2005).

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0261-2194/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cropro.2013.02.020

Pseudomonas spp. isolates have been found to act as biological agents against plant–parasitic nematodes (Aalten et al., 1998; Samaliev et al., 2000; Ali et al., 2002; Vagelas et al., 2007; Siddiqui et al., 2009). Under greenhouse conditions, cell suspensions of different *Pseudomonas fluorescens* strains have been found to be effective in suppressing populations of *Meloidogyne incognita* (Siddiqui and Shaukat, 2003a, b; 2004; Ashoub and Amara, 2010) and *Meloidogyne javanica* (Siddiqui and Shaukat, 2002). The antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) produced by *Pseudomonas protegens* (ex-*fluorescens*) strain CHA0 (Ramette et al., 2011) would have an important role in inducing systemic resistance in roots and, therefore, reducing nematode infection (Siddiqui and Shaukat, 2003b).

There are no published reports on the antagonistic action of rhizobacteria against *N. aberrans*. In order to determine the effect of the biocontrol agent *P. protegens* CHA0 in suppressing root penetration, development and reproduction of *N. aberrans* on tomato roots and promoting plant growth in the presence of the nematode, two experiments were conducted under greenhouse conditions. The reference biocontrol strain CHA0 was used in experiment 1, which was repeated, and its isogenic derivative ARQ1 (used as control) in experiment 2, which was not repeated.

2. Materials and methods

2.1. Nematode inocula for both experiments

An *N. aberrans* isolate from the locality of Lisandro Olmos (province of Buenos Aires, Argentina), that is known to have the ability to reproduce on tomato (Lax et al., 2011a), was used for the study. Nematodes were maintained on tomato cv Platense under greenhouse conditions. Plants were uprooted and washed to remove adhering soil particles. Egg masses were removed from galls and placed in Petri dishes containing distilled water. They were maintained at room temperature (25 ± 2 °C) to allow hatching. Active second-stage juveniles (J2) of the nematode were extracted and used for inoculation the same day.

2.2. Bacterial strains and culture conditions for both experiments

P. protegens strain CHA0 is a wild-type isolate from the rhizosphere of tobacco plants (Stutz et al., 1986; Ramette et al., 2011). Strain ARQ1, used as a control, is an isogenic derivative of CHA0 that was chromosomally tagged with a miniTn7 cassette expressing *gfp* and a kanamycin resistance gene (Jousset et al., 2006), which enables the detection of the inoculated strain on the root surface in the presence of other pseudomonads. Strains CHA0 and ARQ1 were maintained on nutrient agar at 28 °C (Agaras et al., 2012). For plant inoculation, bacterial suspensions were prepared from overnight cultures in nutrient yeast broth grown at 28 °C with agitation (200 rpm) (Jousset et al., 2006). Cultures were washed once with saline solution (NaCl 0.85% w/v) and suspensions were normalized to $OD_{600} = 0.1$.

2.3. Soil preparation for both experiments

Sandy loam soil was used for all experiments. Soil physicochemical characteristics were as follows: pH (H_2O) 7.4, clay (%) 15, silt (%) 35, sand (%) 50, organic matter (%) 14, nitrogen (%) 0.66 and available P (mg kg^{-1}) 15. The soil was air-dried, powdered and sieved through a 2-mm pore sieve, and steam-sterilized three times (120 °C, 1-h with 24-h between the three treatments). The steamed soil was mixed with autoclaved sand (60 min at a pressure of 2 atm) in a 3:1 ratio.

2.4. Source of materials, nematode and rhizobacterium initial densities, treatments set up and recorded parameters in experiment 1

Seeds of tomato cv Platense were sterilized in 10% NaOCl for 5 min, washed several times with sterilized distilled water and sown in trays containing sterile soil to promote germination. Four-leaf seedlings were transplanted individually in plastic pots (20 cm long × 4 cm wide, with a capacity of 125 g of soil) containing the sterilized soil mix.

At the time of transplanting, the roots of each seedling were infested with the nematode by spraying the root surface with 1.5 ml of water containing 100 J2. This standard initial nematode density was sufficient to produce root galls and for nematode multiplication in previous studies (Lax et al., 2011a, b). Roots were covered with the soil mix. Then, tomato seedlings were inoculated with the rhizobacterium by drenching the surface of each pot with 10 ml of bacterial suspension with a total of *ca.* 1×10^9 cells of *P. protegens* strain CHA0. The roots of the seedlings in the non-inoculated controls and in plants inoculated only with the nematode received an equivalent amount of water on the surface of each pot. Four treatments were evaluated: 1) plants free of both *N. aberrans* and *P. protegens* CHA0 (Non-inoculated control), 2) plants inoculated with *N. aberrans* (NEM), 3) plants inoculated with *P. protegens* CHA0 (CHA0), 4) plants inoculated with both *N. aberrans* and *P. protegens* CHA0 (NEM + CHA0). Treatments were set up in a completely randomized design in a greenhouse with five replicates.

Temperature in the greenhouse was 24 ± 5 °C (10-h photoperiod). Plants were watered daily. After 60 days, tomato plants were uprooted and roots were gently washed with water to remove adhering soil particles. The plant growth parameters measured were length and dry weight of root and shoot. Nematode extraction from soil of each replicate was performed using the centrifugal-flotation technique (Jenkins, 1964). Numbers of galls and egg masses present on the entire root system were counted under a stereoscopic microscope. Egg masses were removed from the roots with a needle and submerged in 1% NaOCl solution for 4 min (Hussey and Barker, 1973) to release and count the eggs. Final nematode population was assessed by adding the number of eggs recovered from each root system to the number of nematodes extracted from the soil in order to determine the nematode reproduction factor (RF = final population/initial population). The effect of CHA0 on *N. aberrans* (Nematode response) was calculated following the formula of Hol and Cook (2005): the difference between nematode numbers on non-treated plants and nematode numbers on treated plants divided by the nematode numbers on non-treated plants and multiplied by 100; this measure represents the percentage of reduction in nematode number on plants treated with CHA0. The experiment was repeated to validate the results.

2.5. Source of materials, nematode and rhizobacterium initial densities, treatments set up and recorded parameters in experiment 2

The same materials, procedures, cultivar of tomato seedlings, nematode isolate, initial densities, treatments and experimental design as described above were used to determine the effect of strain ARQ1. Each treatment was replicated 10 times. At 15 days after inoculation, five randomly selected plants of each treatment were sampled to assess root colonization. The remaining plants were harvested at 60 days after inoculation and processed as described above for strain CHA0. Nematode response was calculated for this strain. This experiment was not repeated.

2.6. Assessment of rhizosphere colonization by P. protegens CHA0 and ARQ1 in both experiments

For experiments in which tomato plants were inoculated with strain CHA0, a total of 2.5 g of roots were randomly sampled from different parts of the root system of each replicate plant to evaluate root colonization using a PCR–RFLP protocol targeting the pseudomonads-specific gene *oprF* (Agaras et al., 2012). Roots were further processed to obtain colonies on S1 agar plates to serve as a source of DNA for the PCR–RFLP analysis. A pure culture of strain CHA0 served as the positive control. PCR products (10 μl) were treated with *HaeIII* or *TaqI* endonucleases following the instructions of the manufacturer, and the RFLP pattern was analyzed by electrophoresis in 2% (w/v) agarose gels.

Rhizosphere colonization by strain ARQ1 was assessed qualitatively by fluorescence microscopy, and quantitatively by selective plate count of rhizosphere suspensions. Roots from all treatments were randomly sampled and analyzed at 15 and 60 days after inoculation. The bacterial density of rhizospheric ARQ1 cells was estimated based on Agaras et al. (2012), using pseudomonads-selective S1 agar plates (Gould et al., 1985) supplemented with kanamycin (50 μg/ml) and chloramphenicol (20 μg/ml) to specifically allow colony development from ARQ1 cells. Colony counts were recorded after 48 h of incubation at 28 °C, and were expressed as CFU on a root fresh weight basis. For fluorescence microscopy, randomly selected root samples were deposited onto S1 plates supplemented with kanamycin and chloramphenicol. After overnight incubation at 28 °C, root pieces were mounted on glass slides with deionized water, covered with cover slips, and examined with a Leica DMI6000B fluorescence microscope. Images of GFP-labeled ARQ1 bacterial cells were obtained with an excitation wavelength of 470 nm and an emission filter of 525 nm. The images were captured with a coupled CCD camera DFC345-FX controlled by the Leica Applications Suite 3.7.0 software.

2.7. Data analysis for both experiments

Normal distribution of data and homogeneity of variance were checked before statistical analyses. Data on shoot and root dry weight did not show a normal distribution and were transformed to $log_{10}(x + 1)$ before analysis. Data were analyzed statistically by analysis of variance ($P \leq 0.05$). Significant differences occurred between the two replicated experiments with CHA0 strain; therefore, results of each trial were analyzed separately. Tukey's test ($P \leq 0.05$) was used to determine differences between treatments. All these analyses were performed using InfoStat program (InfoStat, 2002).

3. Results

3.1. Effect of P. protegens CHA0 and ARQ1 on tomato seedling growth and N. aberrans infection and reproduction

Under greenhouse conditions, the application of strain CHA0 had no significant effect on root and shoot growth of tomato plants in both replicate experiments (Table 1). Roots of all the tomato plants inoculated with *N. aberrans* had galls and egg masses produced by the nematode (Fig. 1). Strain CHA0 significantly reduced nematode root invasion compared to the control (NEM) (Table 1). This effect was evident in the number of galls and egg masses, as well as in nematode reproduction factor (RF) values, all of which were significantly smaller in plants inoculated with the strain compared to the nematode infested plants without bacteria. There was a 64% reduction in nematode number on plants treated with CHA0 (Nematode response). A similar reduction in nematode population densities (60%) was observed in the replicate experiment.

As observed in the two repeated trials with strain CHA0, strain ARQ1 did not significantly increase plant growth (Table 1), although at 60 days after inoculation, this strain significantly reduced the number of galls, egg masses and RF of *N. aberrans* (Table 1). Nematode final population densities were reduced by 52%. Thus, strain ARQ1 basically showed a similar effect to that of strain CHA0 in plants infested with *N. aberrans*. No growth suppression of tomato seedlings was observed in pots infested with *N. aberrans* in all the experiments, regardless of whether the nematode was inoculated alone or concomitantly with the rhizobacteria, indicating that the initial nematode density used in the experiments did not reach damaging levels and was well tolerated by the tomato seedlings during 60 days.

Table 1
Tomato root colonization by *Pseudomonas protegens* CHA0 and ARQ1 and effect on plant growth, number of galls (Galls), egg masses (EM), final population, and reproduction factor (RF) of *Nacobbus aberrans* (NEM).[a]

Inoculation treatment	Root colonization ARQ1 (cfu/g)		Root		Shoot		Nematode			
	15 DAI	60 DAI	Total length (cm)	Dry weight (g)	Total length (cm)	Dry weight (g)	Galls	EM	Final population	RF
CHA0: Trial 1										
Non-inoculated control	–	–	20.4 ± 2.9a	1.2 ± 0.3a	19.9 ± 2.6a	0.7 ± 0.2a	–	–	–	–
NEM	–	–	20.8 ± 1.2a	1.1 ± 0.1a	20.6 ± 4.2a	0.8 ± 0.1a	15 ± 4.7a	9 ± 2.5a	3121a	31.2 ± 12.6a
CHA0	–	–	21.8 ± 2.2a	1.3 ± 0.2a	21.2 ± 2.5a	0.9 ± 0.2a	–	–	–	–
NEM + CHA0	–	–	19.4 ± 1.4a	1.1 ± 0.1a	21.7 ± 2.9a	0.8 ± 0.2a	6 ± 2.8b	3 ± 1.7b	1109b	11.1 ± 5.7b
CHA0: Trial 2										
Non-inoculated control	–	–	22 ± 0.7a	0.9 ± 0.2a	23.5 ± 0.9a	0.7 ± 0.2a	–	–	–	–
NEM	–	–	20.9 ± 1.3a	0.9 ± 0.1a	25.4 ± 1.4a	0.6 ± 0.2a	40 ± 7.2a	26 ± 5.5a	8772a	87.7 ± 19.9a
CHA0	–	–	21.7 ± 1.9a	1.2 ± 0.4a	26.6 ± 1.9a	0.7 ± 0.2a	–	–	–	–
NEM + CHA0	–	–	22.3 ± 2.1a	0.9 ± 0.1a	24.8 ± 2.8a	0.7 ± 0.2a	15 ± 5.1b	10 ± 4.8b	3545b	35.5 ± 17.8b
ARQ1: Trial 1										
Non-inoculated control	n.d.	n.d.	30.6 ± 11.8a	0.7 ± 0.4a	20.3 ± 3.7a	0.8 ± 0.3a	–	–	–	–
NEM	n.d.	n.d.	24.6 ± 3.6a	0.5 ± 0.2a	20.1 ± 2.5a	0.7 ± 0.1a	34 ± 4.6a	19 ± 3.8a	6516a	65.2 ± 14.3a
ARQ1	$1.7 \pm 0.1 \times 10^6$	$2.1 \pm 0.6 \times 10^4$	25.4 ± 3.3a	0.8 ± 0.3a	22.9 ± 2.5a	0.8 ± 0.2a	–	–	–	–
NEM + ARQ1	$1.0 \pm 0.2 \times 10^6$	$2.9 \pm 0.7 \times 10^4$	26 ± 2.9a	0.5 ± 0.1a	23.2 ± 1.8a	0.7 ± 0.1a	20 ± 5.2b	11 ± 3.5b	3096b	31.0 ± 13.6b

[a] Means of five replicates (each trial). Means in the same column followed by the same letter did not differ according to a Tukey test ($P \leq 0.05$) for each trial. DAI: days after inoculation; n.d.: not detected (the detection limit of the plating method was 10^2 cfu/g).

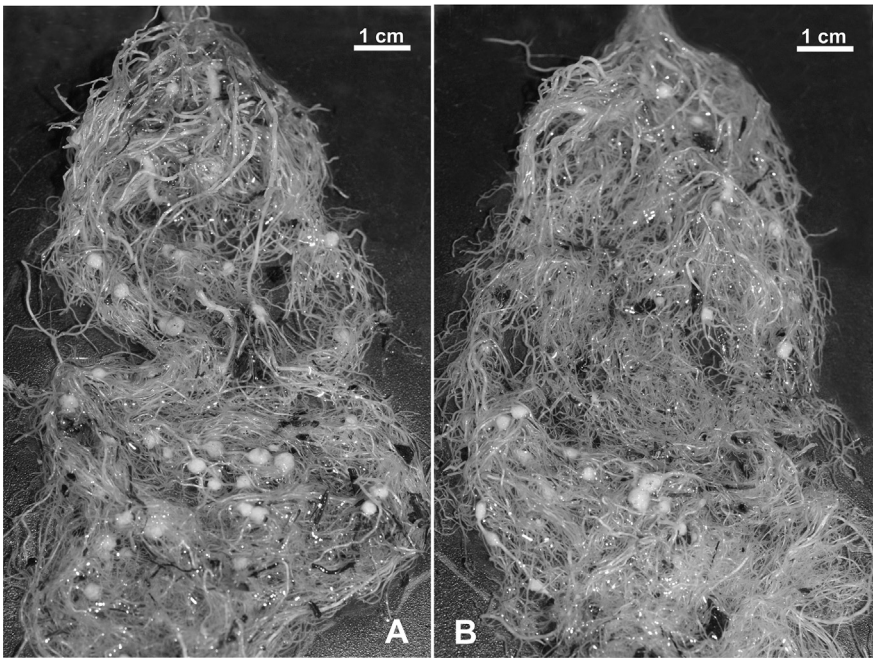

Fig. 1. Tomato roots with galls induced by *Nacobbus aberrans*. A: Control plant (NEM). B: Treated plant (NEM + CHA0).

3.2. Analysis of rhizosphere colonization by P. protegens strains CHA0 and ARQ1

According to results of the PCR–RFLP applied to tomato roots at 60 days after inoculation, the rhizosphere of all experimental groups, even of those that received no inoculation, were colonized by other pseudomonads (Fig. 2). However, RFLP signals consistent with the presence of DNA from CHA0 cells were only detected in plant roots inoculated with this strain. This finding indicates that these contaminating pseudomonads escaped the action of the sterilizing effects of the agents and procedures used when the experiments were set up.

A similar level of tomato rhizosphere colonization was achieved in both treatments inoculated with ARQ1 (ARQ1 and NEM + ARQ1), whereas no colonies were recovered from roots of non-inoculated control plants or from plants inoculated only with nematodes. ARQ1 counts at 15 days after inoculation ($1.0–1.7 \times 10^6$ cfu/g) were *ca.* two orders of magnitude higher than at 60 days after inoculation ($2.1–2.9 \times 10^4$ cfu/g) (Table 1). Images of GFP-labeled ARQ1 bacterial cells (Fig. 3) revealed a widespread colonization of different parts of the tomato root system 15 days after inoculation. Fluorescent colonies were not detected in non-inoculated roots. Samples collected 60 days after inoculation also showed fluorescent microcolonies in those treatments inoculated with ARQ1 cells, but the intensity was lower than that observed in 15-day sampling date.

4. Discussion

In *P. protegens* (ex-*fluorescens*) strain CHA0, as well as in other biocontrol pseudomonads, secondary metabolites such as 2,4-DAPG, hydrogen cyanide, and pyoluteorin, are important for biocontrol activity (Haas and Défago, 2005). In addition, as *P. protegens* is a plant-growth promoting rhizobacterium, its application may also

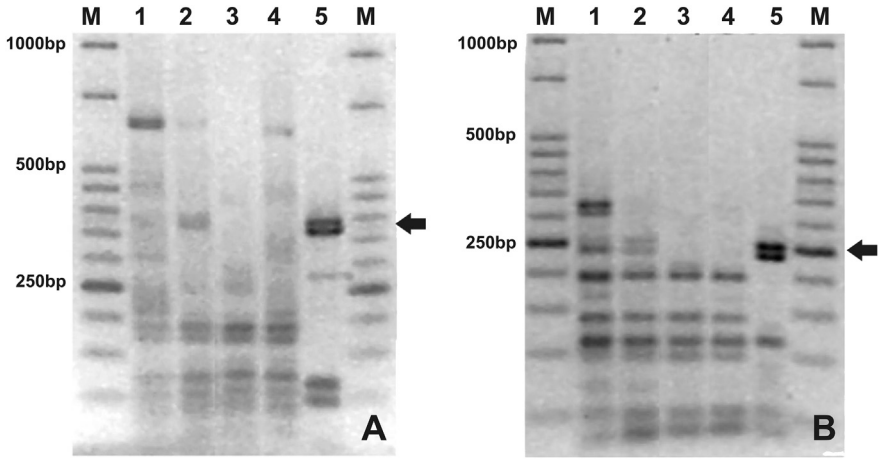

Fig. 2. RFLP patterns of *oprF* PCR product of root-colonizing pseudomonads recovered at 60 days after inoculation with CHA0 on tomato plants. A: *Hae*III endonuclease. B: *Taq*I endonuclease. Lines: 1, CHA0; 2, NEM + CHA0; 3, NEM; 4, Non-inoculated control; 5, positive control (pure culture of strain CHA0). Arrows indicate CHA0 signal in treatments CHA0 and NEM + CHA0. M: Molecular marker.

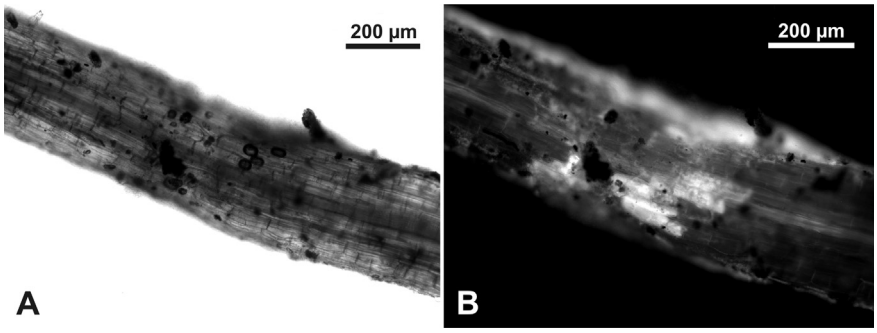

Fig. 3. Colonization by *gfp*-tagged *Pseudomonas protegens* ARQ1 cells on tomato roots infected with *Nacobbus aberrans* at 15 days after inoculation. A: White light. B: Blue light showing fluorescent microcolonies attached to the root surface.

enhance growth of plants infected with plant–parasitic nematodes (Jonathan et al., 2000; Abo-Elyousr et al., 2010; Muthulakshmi et al., 2010; Khan and Haque, 2011). However, increased plant growth does not always occur, as observed in the present work. Infestation of the soil with a cell suspension of *P. protegens* did not significantly enhance biomass or length (root and aerial part) of tomato plants. Similar results were obtained in roots parasitized by *M. incognita* growing in a non-sterilized sandy loam soil treated with CHA0 (Siddiqui and Shaukat, 2004). Lack of plant growth response has also been reported with other *P. fluorescens* strains, which did not favor shoot and root fresh weight growth of egg plant (*Solanum melongena*) infected with *M. incognita* (Ashoub and Amara, 2010).

Application rates of the biocontrol bacteria and plant–parasitic nematode population density play an important role in the degree of suppression of the nematode (Siddiqui and Shaukat, 2003a). These authors did not observe a marked difference in the efficacy of strain CHA0 at doses of 10^8 or 10^9 cfu ml^{-1}, suggesting that an application rate of 10^8 cfu ml^{-1} is optimal for the suppression of root-knot nematodes. In the present study, with the latter dose, *P. protegens* CHA0 caused significant suppression of the *N. aberrans* population; these results were supported by molecular detection of the bacterium in the rhizosphere of the treated plants, even in the presence of competing bacterial flora. A similar antagonistic effect on the parasite also was observed when the experiment was repeated. The number of galls was reduced by 60–62% with strain CHA0; these values were higher than those reported for *M. javanica* by Siddiqui and Shaukat (2003a), who detected a reduction of about 38% in galling in tomato using the same dose of CHA0. Doses of 10^{12} cfu ml^{-1} of *P. fluorescens* reduced Gall Index of *M. incognita* in tobacco by approximately 44–56%, depending on the cultivar (Khan and Haque, 2011). Production of egg masses of *N. aberrans* was reduced by 62–67% with CHA0 relative to its respective control (NEM). Similar effects were observed with other plant–parasitic nematodes. The application of *Pseudomonas oryzihabitans* (10^6 cells ml^{-1}) on tomato at the time the plants were inoculated with *Meloidogyne* spp. J2 resulted in significantly fewer (56%) egg masses in roots (Vagelas et al., 2007). In addition, soil infestation with *P. fluorescens* significantly reduced egg mass production of *M. incognita* in tobacco cultivars (Khan and Haque, 2011) and egg plant (*S. melongena*) (Ashoub and Amara, 2010). According to the present results, the lower number of galls would indicate that the bacteria affected root penetration of *N. aberrans* infective stages (juveniles and immature females). Strain CHA0 was shown to be significantly effective in inhibiting hatching and inducing mortality of *Meloidogyne* spp. J2 in *in vitro* bioassays (Siddiqui et al., 2005; Siddiqui and Shaukat, 2003a, 2004). Cells of *P. protegens* CHA0, and/or its extracellular metabolites may have caused mortality of inoculated *N. aberrans* juveniles before they could penetrate the host tissues, which might have led to a reduced production of number of eggs and, therefore, a reduction in final population and RF (60–64%).

P. fluorescens strains often form microcolonies or discontinued biofilms in the grooves between epidermal cells at the root surface; certain strains are also capable of endophytic colonization (Couillerot et al., 2009). *P. protegens* ARQ1 cells were observed colonizing the tomato root surface. At 15 days after inoculation, good levels of colonization in the rhizosphere were detected ($\sim 10^6$ cfu/g of root), and then declined to $\sim 10^4$ cfu/g of root at 60 days after inoculation. Similar colonization levels to those measured at 60 days after inoculation were reported in tomato seedlings at 30 days after inoculation with different *P. aeruginosa* isolates (Singh and Siddiqui, 2010). Colonization by ARQ1 on tomato was also associated with a significant suppressive effect on *N. aberrans* (a reduction of about 41–42% in the number of galls and egg masses, respectively, and of 52% in RF). A successful and fast establishment of *P. protegens* CHA0 (or ARQ1) cells in the rhizosphere of seedlings may be crucial to achieving a significant reduction in root penetration by nematodes.

No similar work with rhizobacteria has been published for this plant–parasitic nematode. The present results show the effect of *P. protegens* CHA0 in suppressing *N. aberrans* infestation and reproduction under controlled conditions, suggesting its potential for the management of the nematode on tomato. However, further studies are necessary to confirm the biocontrol potential of the strain in soils naturally infested with this plant–parasitic nematode (both in the field and under greenhouse conditions where its population levels can be very high), where an interaction and/or competition with other microorganisms may occur.

Acknowledgments

This work was financially supported by the Ministerio de Ciencia y Tecnología (Province of Córdoba), the Secretaría de Ciencia y Técnica (Universidad Nacional de Córdoba), Universidad Nacional de Quilmes, and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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