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## ORIGINAL ARTICLE

# Potential of rhizobacteria native to Argentina for the control of *Meloidogyne javanica*

María P. Borrajo<sup>a,b</sup>, Eduardo A. Mondino<sup>c</sup>, Guillermo A. Maroniche<sup>a,b</sup>,  
Macarena Fernández<sup>a,b</sup>, Cecilia M. Creus<sup>a,\*</sup>

<sup>a</sup> Laboratorio de Bioquímica Vegetal y Microbiana, Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Argentina

<sup>b</sup> Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

<sup>c</sup> Laboratorio de Nematología, Estación Experimental Agropecuaria INTA Balcarce, Argentina

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### KEYWORDS

*Bacillus*;  
*Pseudomonas*;  
Biocontrol;  
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Lettuce

**Abstract** Biocontrol of the nematode *Meloidogyne javanica* was studied using the Argentinean strains *Pseudomonas fluorescens* MME3, TAE4, TAR5 and ZME4 and *Bacillus* sp. B7S, B9T and B19S. *Pseudomonas protegens* CHA0 was used as a positive control. Egg hatching and juvenile mortality were evaluated *in vitro* by exposure of nematodes to bacterial suspensions or their cell-free supernatants (CFS). The effect of bacteria on nematode infestation of lettuce was also studied. Results showed that most of the tested strains and CFS reduced egg hatching and juvenile survival *in vitro*. The bacterial suspension of *Bacillus* sp. B9T produced the lowest hatching of eggs. Juvenile mortality was higher when *M. javanica* was exposed to *Bacillus* sp. than to *Pseudomonas* spp. suspensions. Except for CFS of B9T, all filtrates inhibited hatching at levels similar to or higher than the biocontrol strain *P. protegens* CHA0. The CFS of CHA0 showed the highest level of juvenile mortality followed by *Bacillus* sp. strains and *P. fluorescens* TAE4. None of the inoculated rhizobacteria reverted the negative effect of infestation on the aerial dry weight of lettuce plants. However, inoculation impacted on reproduction of *M. javanica* by reducing the development of galls and egg masses on roots and diminishing the number of individuals both on roots and in the substrate, as well as the reproduction factor. These results show that most of the analyzed native strains can control the nematode *M. javanica*. Among them, *P. fluorescens* TAE4 and *Bacillus* sp. B9T showed the most promising performances for the biocontrol of this pathogen and have a potential use in the formulation of commercial products. © 2021 Asociación Argentina de Microbiología. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

\* Corresponding author.

E-mail address: [ccreus@mdp.edu.ar](mailto:ccreus@mdp.edu.ar) (C.M. Creus).

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## PALABRAS CLAVE

*Bacillus*;  
*Pseudomonas*;  
Biocontrol;  
Nematodo de las  
agallas;  
Lechuga

## Potencial de rizobacterias nativas de Argentina para el control de *Meloidogyne javanica*

**Resumen** Se estudiaron las cepas argentinas *Pseudomonas fluorescens* MME3, TAE4, TAR5 y ZME4 y *Bacillus* sp. B7S, B9T y B19S para el control del nematodo *Meloidogyne javanica*. *Pseudomonas protegens* CHA0 se utilizó como control positivo. La eclosión de huevos y la mortalidad de juveniles se evaluaron *in vitro* al exponerlos a suspensiones bacterianas y a sus sobrenadantes libres de células (SLC). Asimismo, se estudió la inoculación bacteriana sobre la infestación del nematodo en lechuga. Los resultados *in vitro* indicaron que la mayoría de las cepas, así como sus SLC redujeron la eclosión y la supervivencia de *M. javanica*. La suspensión de *Bacillus* sp. B9T produjo los menores niveles de eclosión. La mortalidad de juveniles fue mayor al exponerlos a suspensiones de *Bacillus* sp. respecto de *Pseudomonas* spp. Los SLC inhibieron la eclosión de huevos en niveles similares o superiores a *P. protegens* CHA0, excepto por el de B9T. La exposición a SLC de CHA0 registró la mayor mortalidad, seguido por las cepas de *Bacillus* sp. y *P. fluorescens* TAE4. La inoculación bacteriana no revertió el efecto de la infestación sobre el peso seco aéreo de las plantas, sin embargo, afectó la multiplicación de *M. javanica* lo que redujo el desarrollo de agallas y las masas de huevos, y disminuyó el número de individuos presentes tanto en la raíz como en el sustrato, así como el factor de reproducción. Los resultados indican que la mayoría de las cepas nativas evaluadas son capaces de controlar a *M. javanica*. Entre ellas, *P. fluorescens* TAE4 y *Bacillus* sp. B9T, se presentan como las más promisorias para el control de este patógeno, con potencialidad para ser utilizadas en la formulación de productos biológicos.

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## Introduction

Root knot nematodes *Meloidogyne* spp. are cosmopolitan phytopathogens that cause significant crop losses worldwide. In Argentina, they are widely distributed through several regions, affecting both extensive and intensive crops<sup>6-8,27</sup>. The most widespread species in the country are *Meloidogyne arenaria*, *Meloidogyne hapla*, *Meloidogyne incognita* and *Meloidogyne javanica*, the latter two showing the greatest incidence on horticultural crops such as lettuce<sup>8</sup>.

Traditionally, the control of nematodes in horticulture has been done using chemical compounds that have known deleterious effects on the environment and human health. Therefore, it is desirable to find environmentally friendly options that promote integrated pest and disease management<sup>40</sup>. Biological control is an interesting alternative to chemical nematicides. Beneficial bacteria known as plant growth promoting rhizobacteria (PGPR) have been successfully used worldwide for the biocontrol of various plant pathogens. Among the most prominent biocontrollers are members of the *Pseudomonas* and *Bacillus* genera<sup>16,23,36</sup>.

Different mechanisms were proposed as responsible for the biocontrol of nematodes by PGPR. Direct control of bacteria on nematodes through the production of secondary metabolites (*i.e.*, antibiotics and toxins) and hydrolytic enzymes, has been reported<sup>16,40,42</sup>. Furthermore, indirect mechanisms such as niche and nutrient competition, induction of plant systemic resistance and palliative effects by stimulating root growth, are also recognized<sup>36,37,40</sup>.

The study of native organisms as biological resources is important to contribute with biocontrol alternatives that are adjusted to the environmental conditions of the region. In the past years, different biological alternatives for the control of *Meloidogyne* spp., such as native arbuscular mycorrhizal fungi<sup>27</sup>, entomopathogenic nematodes<sup>7</sup> and essential oils of *Tagetes minuta*<sup>26</sup>, were studied in Argentina. However, the antagonistic action of rhizobacteria native to Argentina against the root knot nematode *M. javanica* has not been explored yet.

In the present work, the antagonistic potential of several native strains of fluorescent *Pseudomonas* spp. and *Bacillus* sp. was evaluated against *M. javanica* under *in vitro* and *in planta* conditions. The objectives were: (i) to evaluate the effect of bacteria and their cell-free supernatants on egg hatching and juvenile mortality *in vitro*, and (ii) to determine if bacterial inoculation protects lettuce plants against the disease caused by nematode infestation.

## Materials and methods

### Bacterial strains, isolates and molecular identification

The strains of *Pseudomonas fluorescens* used in this study have been previously characterized as PGPR<sup>25</sup> and are deposited in the collection of IMyZA, INTA-Castelar, Argentina. Strains TAE4 and TAR5 were both isolated from wheat, strain MME3 was recovered from tomato and strain

ZME4 from maize<sup>25</sup>. The reference biocontrol strain *Pseudomonas protegens* CHA0 was used as a positive control<sup>39</sup>.

*Bacillus* sp. strains were isolated from wild potato (*Solanum tuberosum*) or surrounding typic paleargid soil in Abra pampa (Jujuy province, Argentina) and stored at  $-80^{\circ}\text{C}$  in 20% glycerol slabs. Briefly, tuber and soil homogenates were pasteurized in a water bath at  $80^{\circ}\text{C}$  for 20 min and serial dilutions were cultivated on nutrient agar (NA) with 50 mM glucose. Isolates B7S, B19S and B9T were subjected to molecular identification by 16S rRNA gene sequencing. Genomic DNA extracts were obtained by suspending bacterial colonies in 50  $\mu\text{l}$  of ultrapure water and boiling for 10 min. Universal primers 27F and 1492R were then used for 16S rRNA gene amplification from the extracted DNA by PCR<sup>10</sup>. The PCR products were visualized by TAE-agarose electrophoresis, excised and purified with QIAEX II gel extraction kit (Qiagen) for subsequent capillary sequencing at the Genomics Unit of the Biotechnology Institute of CNIA-INTA (<http://www.inta.gov.ar/biotec>). The retrieved 16S rRNA gene sequences of strains B7S, B19S and B9T were deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) under accession numbers MT160747, MT160746 and MT160745, respectively.

The derived sequences were compared to 16S rRNA gene sequences of type strains in the GenBank database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were also analyzed with the EzTaxon tool available in the EzBioCloud portal (<https://www.ezbiocloud.net/>).

### Selection of rifampicin-resistant mutants

Natural rifampicin-resistant mutants were isolated for all the studied strains. Bacterial cultures were plated on a rifampicin gradient (0–200  $\mu\text{g}/\text{ml}$ ) and single colonies were isolated from the area with higher concentration of antibiotic. The selected clones were subcultured on antibiotic-free media for 20 generations and then tested for resistance stability on medium with and without rifampicin (50  $\mu\text{g}/\text{ml}$ )<sup>9</sup>. The Rif<sup>r</sup> mutants were stored in glycerol stocks at  $-80^{\circ}\text{C}$  on LB supplemented with 50  $\mu\text{g}/\text{ml}$  rifampicin.

### Culture conditions and preparation of cell-free supernatants

*Pseudomonas* spp. were cultured on LB agar at  $30^{\circ}\text{C}$  while *Bacillus* spp. isolates B7S, B19S and B9T were grown at  $37^{\circ}\text{C}$  on potato-dextrose-agar (PDA). Pure colonies were transferred to LB broth (adding 50 mM sucrose for *Bacillus* spp. isolates) and incubated with orbital agitation at 120 rpm for 24 h. Cells were harvested by centrifugation at 6000 rpm for 15 min and resuspended in sterile water. Bacterial concentration was estimated by measuring absorbance at 600 nm and adjusted to 1 to obtain suspensions with  $10^9$  CFU/ml for *Pseudomonas* spp. and  $10^8$  CFU/ml for *Bacillus* spp. isolates.

Cell-free supernatants (CFS) were obtained by growing strains on 100 ml of LB in 250 ml flasks (adding 50 mM sucrose for the *Bacillus* spp. isolates), with 120 rpm agitation at  $30^{\circ}\text{C}$  during 48 h. The bacterial cultures were centrifuged at 12 000 rpm at  $4^{\circ}\text{C}$  for 15 min. The supernatants were collected and filtered with Minisart Syringe Filter 0.2  $\mu\text{m}$ .

### Nematode inoculum

The *M. javanica* population was isolated from yacon *Smallanthus sonchifolius* (Tucumán) and maintained on susceptible tomato *Lycopersicon esculentum* cv. 'Platense' in the Nematology Lab, EEA-INTA Balcarce, Argentina. For inoculum preparation, egg masses were collected from the galls formed on the infected roots, pooled, and agitated in 0.5% NaOCl for 4 min<sup>18</sup>. Egg suspensions were sifted through a 100-mesh sieve over 500-mesh coupled sieves, washed, and collected on water. Second stage juveniles (J2) were obtained from hatched eggs on sterile water at  $28^{\circ}\text{C}$ , J2 were collected and refrigerated until use.

### In vitro antagonism assays

Assays were conducted in 24-well tissue culture plates (Costar®). To facilitate the counting of individuals, grid lines 2 mm apart were scratched onto the underside of the plates. All assays were conducted using sterile materials in a laminar flow chamber.

To determine the effect on egg hatching, wells were supplied with 600  $\mu\text{l}$  of bacterial suspensions or CFS and 300  $\mu\text{l}$  of sterile water containing 100 *M. javanica* eggs. The percentage of hatched eggs was evaluated after 3, 6 and 9 days of incubation in a wet chamber at  $28^{\circ}\text{C}$ , under a stereomicroscope (Zeiss Stemi SV6, Carl Zeiss, Jena, Germany).

To study the effect on juvenile mortality, assays were set up similarly but replacing eggs with an equal number of second stage juveniles (J2). Nematodes were considered dead when they remained immobile after being touched with a fine needle. Number of dead juveniles was counted after 2 days of incubation and percentages of mortality were calculated.

The cell suspensions and CFS of *Bacillus* sp. B7S, B19S and B9T strains, *P. fluorescens* MME3, TAE4, TAR5 and ZME4 strains, and *P. protegens* CHA0 were tested against eggs and juveniles of *M. javanica*. Sterile water with nematode individuals without bacteria or CFS was used as control treatment (WC). Fresh LB was used as control (MC) for CFS assays. The treatments were tested in quadruplicate and each trial was repeated two times. All *in vitro* assays were carried out as completely randomized designs with rhizobacteria as factor. Data were checked for normality and homogeneity of variances and analyzed with one-way ANOVA and means compared by the LSD Tukey test ( $\alpha=0.05$ ). All statistical analyses were performed using the statistical software R version 2.15.2<sup>33</sup>.

### In planta biocontrol assay

Seeds of *Lactuca sativa* L. cv. Elisa were disinfected in 1% NaOCl for 2 min and washed four times with sterile distilled water. The seeds were then inoculated by immersion for 90 min in a bacterial suspension containing  $1 \times 10^9$  CFU/seed for strains of *Pseudomonas* spp. (Rif<sup>r</sup>) and  $1 \times 10^8$  CFU/seed for strains of *Bacillus* spp. (Rif<sup>r</sup>). Uninoculated control seeds were imbibed in sterile water for 90 min.

The assay consisted of seven treatments: strains B9T, B19S, TAE4, MME3, CHA0 (as positive control), control of infestation (MJC, with nematode but without rhizobacteria)

and absolute control (PC, plant control without nematode or rhizobacteria).

After inoculation, lettuce seeds were individually sown in plug trays (cell size 288) with perlite: sterile sand (1:1) and cultivated in a growth chamber under 12:12 h light–dark photoperiod and  $25 \pm 2^\circ\text{C}$ . Two weeks after sown, plants were transplanted to 2 l plastic pots containing the perlite:sand mixture, and were re-inoculated at the stem base with 1 ml of  $1 \times 10^9$  CFU/ml for *Pseudomonas* spp. or  $1 \times 10^8$  CFU/ml for *Bacillus* spp. strains. Control pots received the same amount of water. Plants were irrigated with distilled water by capillarity and fertilized with Hoagland solution (dilution 1:3) when necessary.

Bacterial colonization of roots was analyzed in a set of plants by CFU count using the drop plate method<sup>14</sup> five days post-transplantation and one day after nematode inoculation. Plant roots were extracted from pots, agitated to release adhered substrate, gently washed, and weighed. Roots homogenates were obtained by grinding with sterile water (1:10) (w/v) using a mortar. Serial dilutions with sterile distilled water were seeded in triplicate as a drop (10  $\mu\text{l}$ ) on LB or PDA adding rifampicin (50  $\mu\text{g}/\text{ml}$ ) for *Pseudomonas* spp. and *Bacillus* spp., respectively. After 24 h of incubation at  $30^\circ\text{C}$ , colonies were counted, and CFU/g root calculated for each treatment.

To infest lettuces, the inoculum of *M. javanica* was obtained as previously described. Suspensions consisting of a mix of eggs and juveniles were adjusted to infest at a dose of 1500 individual/plant. Four days after transplantation, the nematode inoculum was placed on two holes around the lettuce stem that were covered by substrate following the application. Sixty-five days after nematode infestation the number of galls and egg masses per root, egg and J2 per grams of root or substrate, fresh and dry weight of root and shoot per plant were determined.

Number of galls and egg masses per radical system were counted using a standing magnifier lamp  $2\times$ . Eggs were extracted from a portion of the middle root using Hussey and Barker's method<sup>18</sup>. Individuals of *M. javanica* were recovered from substrate using the sugar flotation-centrifugation technique<sup>20</sup>. Eggs and juveniles of *M. javanica* were counted on a 1 ml counting slide under a stereomicroscope (Zeiss Stemi SV6, Carl Zeiss, Jena, Germany), and expressed as eggs or juveniles per gram of root or substrate. The reproduction factor was calculated as  $\text{RF} = \text{final population}/\text{initial population}$ , where the final population was the sum of the initial population (used for infestation) and the individuals present in root and substrate at the end of the assays.

The trial was designed as a randomized complete block with seven treatments. The experiment was repeated twice, each consisting of 11 replicates (3 of which were used for bacterial recount) with a total of 77 pots. Data were compared by one-way analysis of variance (ANOVA) followed by the Tukey-LSD test ( $\alpha = 0.05$ ).

## Results

### Molecular identification of *Bacillus* sp. isolates

Isolates B7S, B19S and B9T were selected from a collection of potato-associated bacilli due to their strong *in vitro*

antifungal activity against *Botrytis cinerea* and *Fusarium* sp. (data not shown). Analysis of 16S rRNA gene sequences showed that the three isolates exhibit high identity to members of the genus *Bacillus*. Although the sequences obtained from B9T and B19S were identical, they exhibited differences in colony morphology (data not shown). Based on the results of the EzBioCloud database, B9T and B19S isolates belong to the *Bacillus siamensis* species, sharing 99.93% identity with *B. siamensis* KCTC 13613<sup>T</sup>. Moreover, the BLAST alignments of these sequences in the NCBI GenBank database showed 99.72% identity with both *Bacillus subtilis* subsp. *subtilis* and *Bacillus amyloliquefaciens* 168<sup>T</sup>. Analysis of the 16S rRNA gene sequence of strain B7S indicated that it belongs to the *atrophaeus* species, showing 99.93% identity to *Bacillus atrophaeus* JCM 9070<sup>T</sup> in both the EzBioCloud and NCBI GenBank databases.

### *In vitro* antagonism assays

All rhizobacterial suspensions showed a significant inhibition of egg hatching when compared to the control (WC) (Table 1). The lowest rates of *M. javanica* egg hatching were produced by B9T treatment during the whole period of incubation. Levels of hatching of B9T were statistically similar to B19S and TAR5 on day 3, to MME3 and TAE4 on day 6, and to B19S, MME3 and TAR5 on day 9.

The effectiveness of bacterial suspensions in controlling egg hatching decreased with incubation time. Three days after incubation with PGPR suspensions, egg hatching was 47–73% less than control, while hatching inhibition dropped to 26–53% and 24–42% on days 6 and 9, respectively.

The mortality of *M. javanica* juveniles was also significantly affected by all tested PGPR suspensions (Table 1). Mortality was higher when juveniles were exposed to *Bacillus* sp. than to *Pseudomonas* sp. suspensions.

All CFS from bacteria tested exhibited inhibitory activity of egg hatching considering both controls (MC and WC), except for CFS from B9T on days 6 and 9 (Table 2). Overall, treatment with *Pseudomonas* filtrates showed the lowest hatching values, matching or outperforming the positive control CHA0 (Table 2).

Bacterial metabolites increased juvenile mortality by more than 31.3% regarding water control (WC) and by 18.2% when compared to medium control (MC) (Table 2). Metabolites of the positive control CHA0 showed the highest level of mortality. Metabolites from *Bacillus* spp. isolates caused greater deaths of juveniles than *Pseudomonas* spp. filtrates (Table 2), except for TAE4, which matched the mortality levels caused by B7S and B9T.

### *In planta* biocontrol assay

Considering the best *in vitro* antagonist behavior (hatching and mortality) against *M. javanica*, one strain per bacterial genus and per bioassay was selected to continue with *in planta* analyses. Strains B9T and MME3 were selected from the suspension bioassay and strains B19S and TAE4 from the CFS bioassay. Strain CHA0 was again included as a positive control.

One day after nematode infestation, the levels of bacterial count were in the order of  $10^5$  CFU/g of roots for *Bacillus* spp. and  $10^7$ – $10^8$  CFU/g for *Pseudomonas* spp. (Table 3).

**Table 1** *In vitro* effect of PGPR suspensions on egg hatching and juvenile J2 mortality of *M. javanica*.

| Treatments | Egg hatching (%)         |                          |                           | Mortality of J2 (%)      |
|------------|--------------------------|--------------------------|---------------------------|--------------------------|
|            | 3 <sup>rd</sup> day      | 6 <sup>th</sup> day      | 9 <sup>th</sup> day       |                          |
| WC         | 55.5 ± 3.5 <sup>a</sup>  | 70.8 ± 5.7 <sup>a</sup>  | 74.5 ± 3.2 <sup>a</sup>   | 33.8 ± 2.1 <sup>g</sup>  |
| B19S       | 21.3 ± 2.4 <sup>cd</sup> | 42.4 ± 2.1 <sup>bc</sup> | 44.9 ± 2.9 <sup>de</sup>  | 85.4 ± 2.5 <sup>b</sup>  |
| B7S        | 27.0 ± 3.7 <sup>bc</sup> | 51.8 ± 3.7 <sup>b</sup>  | 56.4 ± 2.3 <sup>b</sup>   | 98.6 ± 0.8 <sup>a</sup>  |
| B9T        | 14.7 ± 1.0 <sup>d</sup>  | 32.7 ± 4.3 <sup>d</sup>  | 42.8 ± 1.3 <sup>e</sup>   | 72.1 ± 4.7 <sup>c</sup>  |
| CHA0       | 27.7 ± 1.0 <sup>bc</sup> | 49.7 ± 3.1 <sup>b</sup>  | 52.2 ± 2.0 <sup>bc</sup>  | 58.5 ± 2.5 <sup>de</sup> |
| MME3       | 23.8 ± 2.2 <sup>bc</sup> | 38.7 ± 3.5 <sup>cd</sup> | 45.2 ± 2.1 <sup>de</sup>  | 64.4 ± 3.8 <sup>cd</sup> |
| TAE4       | 27.9 ± 1.2 <sup>bc</sup> | 36.8 ± 1.5 <sup>cd</sup> | 51.6 ± 2.1 <sup>bcd</sup> | 61.6 ± 3.7 <sup>de</sup> |
| TAR5       | 21.8 ± 2.3 <sup>cd</sup> | 43.0 ± 4.2 <sup>bc</sup> | 49.4 ± 2.3 <sup>cde</sup> | 55.8 ± 2.7 <sup>e</sup>  |
| ZME4       | 29.2 ± 2.9 <sup>b</sup>  | 44.9 ± 1.8 <sup>bc</sup> | 51.3 ± 1.7 <sup>bcd</sup> | 47.2 ± 1.4 <sup>f</sup>  |

WC: water control (without bacteria, with nematode). Results are presented as mean ± standard error. Within each column, different letters indicate significant differences in means according to the LSD-Tukey's test ( $p < 0.05$ ).

**Table 2** *In vitro* effect of cell-free supernatants of bacterial on egg hatching and juvenile J2 mortality of *M. javanica*.

| Treatments | Egg hatching (%)         |                          |                           | Mortality of J2 (%)      |
|------------|--------------------------|--------------------------|---------------------------|--------------------------|
|            | 3 <sup>rd</sup> day      | 6 <sup>th</sup> day      | 9 <sup>th</sup> day       |                          |
| MC         | 28.5 ± 2.9 <sup>a</sup>  | 41.7 ± 3.0 <sup>b</sup>  | 43.7 ± 4.1 <sup>b</sup>   | 50.0 ± 1.9 <sup>f</sup>  |
| WC         | 32.5 ± 3.6 <sup>a</sup>  | 51.9 ± 3.3 <sup>a</sup>  | 52.8 ± 3.2 <sup>a</sup>   | 45.0 ± 1.0 <sup>f</sup>  |
| B19S       | 8.2 ± 1.8 <sup>cd</sup>  | 17.6 ± 4.0 <sup>c</sup>  | 19.8 ± 2.7 <sup>cde</sup> | 84.7 ± 1.6 <sup>b</sup>  |
| B7S        | 11.3 ± 1.6 <sup>c</sup>  | 12.3 ± 2.5 <sup>cd</sup> | 21.4 ± 2.6 <sup>cd</sup>  | 79.3 ± 3.7 <sup>bc</sup> |
| B9T        | 19.5 ± 3.0 <sup>b</sup>  | 36.7 ± 2.6 <sup>b</sup>  | 43.8 ± 1.7 <sup>b</sup>   | 74.6 ± 2.3 <sup>c</sup>  |
| CHA0       | 13.7 ± 1.8 <sup>bc</sup> | 17.9 ± 0.6 <sup>c</sup>  | 23.2 ± 2.2 <sup>c</sup>   | 95.1 ± 0.4 <sup>a</sup>  |
| MME3       | 7.9 ± 0.4 <sup>cd</sup>  | 10.1 ± 1.7 <sup>d</sup>  | 17.2 ± 2.1 <sup>cde</sup> | 59.1 ± 3.5 <sup>e</sup>  |
| TAE4       | 4.3 ± 2.2 <sup>d</sup>   | 5.9 ± 0.7 <sup>d</sup>   | 18.9 ± 3.8 <sup>cde</sup> | 76.1 ± 2.8 <sup>c</sup>  |
| TAR5       | 4.8 ± 1.4 <sup>d</sup>   | 5.5 ± 1.4 <sup>d</sup>   | 12.0 ± 2.4 <sup>e</sup>   | 66.9 ± 3.0 <sup>d</sup>  |
| ZME4       | 8.2 ± 1.5 <sup>cd</sup>  | 8.2 ± 1.5 <sup>d</sup>   | 14.5 ± 2.1 <sup>de</sup>  | 61.1 ± 3.2 <sup>de</sup> |

MC: medium control (LB medium without bacteria, with nematode). WC: water control (without bacteria, with nematode). Results are presented as mean ± standard error. Within each column, different letters indicate significant differences in means according to the LSD-Tukey's test ( $p < 0.05$ ).

**Table 3** Colonization and effects of rhizobacteria on the growth of *M. javanica* infested-lettuce.

| Treatments | Root colonization (CFU/g)   | Aerial dry weight (g/plant) | Root dry weight (g/plant)  |
|------------|-----------------------------|-----------------------------|----------------------------|
| PC         | –                           | 2.19 ± 0.15 <sup>a</sup>    | 0.83 ± 0.07 <sup>c</sup>   |
| MJC        | –                           | 1.74 ± 0.12 <sup>b</sup>    | 1.12 ± 0.09 <sup>a</sup>   |
| B19S       | 1.4 ± 0.4 × 10 <sup>5</sup> | 1.73 ± 0.06 <sup>b</sup>    | 0.99 ± 0.07 <sup>abc</sup> |
| B9T        | 6.6 ± 1.0 × 10 <sup>5</sup> | 1.70 ± 0.06 <sup>b</sup>    | 1.04 ± 0.08 <sup>ab</sup>  |
| CHA0       | 7.0 ± 3.9 × 10 <sup>7</sup> | 1.64 ± 0.08 <sup>b</sup>    | 0.97 ± 0.04 <sup>abc</sup> |
| MME3       | 2.2 ± 0.8 × 10 <sup>8</sup> | 1.73 ± 0.08 <sup>b</sup>    | 0.93 ± 0.06 <sup>bc</sup>  |
| TAE4       | 1.2 ± 0.4 × 10 <sup>7</sup> | 1.71 ± 0.08 <sup>b</sup>    | 1.02 ± 0.11 <sup>ab</sup>  |

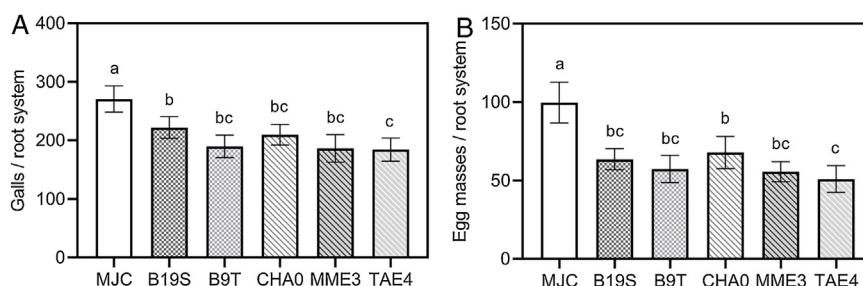
PC: plant control (without bacteria, without nematode). MJC: nematode infestation control (without bacteria, with nematode). Results are presented as mean ± standard error. Within each column, different letters indicate significant differences in means according to the LSD-Tukey's test ( $p < 0.05$ ).

Lettuce aerial dry weight was significantly affected by infestation with *M. javanica* ( $p < 0.01$ ). The inoculation of lettuce with rhizobacteria did not compensate for losses in aerial dry weight caused by nematode infestation (Table 3).

A significant increase in root dry weight was produced by nematode infestation as can be observed by comparison to the control (PC vs MJC) (Table 3,  $p < 0.05$ ). Inoculation with

strains B19S, CHA0 and MME3 restored root dry weight to the PC control value; however, only MME3 ameliorated the effect of nematode infestation on this parameter (Table 3).

Inoculation with PGPR strains statistically reduced the number of galls and egg masses caused by *M. javanica* compared to the infestation control (Fig. 1). The level of galling on lettuces inoculated with strains of *Bacillus* spp. and *Pseudomonas* spp. was similar to that recorded for positive



**Figure 1** Effect of rhizobacteria inoculation on the number of galls (A) and egg masses (B) on the root system of lettuce infested with *M. javanica*. MJC: nematode infestation control (with nematode, without bacteria). B19S: *Bacillus* sp. strain B19S. B9T: *Bacillus* sp. strain B9T. CHA0: *P. protegens* strain CHA0. MME3: *P. fluorescens* strain MME3. TAE4: *P. fluorescens* strain TAE4. Error bars correspond to standard error means. Different letters indicate significant differences in means according to the LSD-Tukey's test ( $p < 0.05$ ).

**Table 4** Effect of bacterial inoculation on the reproduction factor of *M. javanica* in lettuce plants.

| Treatments | Reproduction factor        |
|------------|----------------------------|
| MJC        | 17.00 ± 2.07 <sup>a</sup>  |
| B19S       | 11.57 ± 0.57 <sup>bc</sup> |
| B9T        | 11.13 ± 2.14 <sup>bc</sup> |
| CHA0       | 10.80 ± 1.66 <sup>bc</sup> |
| MME3       | 14.25 ± 2.11 <sup>ab</sup> |
| TAE4       | 7.50 ± 1.02 <sup>c</sup>   |

MJC: nematode infestation control (without bacteria, with nematode). Nematode reproduction factor (RF) was calculated as final population/initial population. Results are presented as mean ± standard error. Different letters indicate significant differences in means according to the LSD-Tukey's test ( $p < 0.05$ ).

control treatment CHA0 (Fig. 1A). A similar behavior was found when analyzing the number of egg masses (Fig. 1B), except for TAE4.

Bacterial treatments reduced the number of eggs and juveniles present at the root in the range of 31–41% regarding the infestation control (MJC) (Fig. 2A). The presence of individuals in the substrate was also significantly lowered by B9T, CHA0 and TAE4 (Fig. 2B). Consequently, a reduction in RF values can be observed (Table 4).

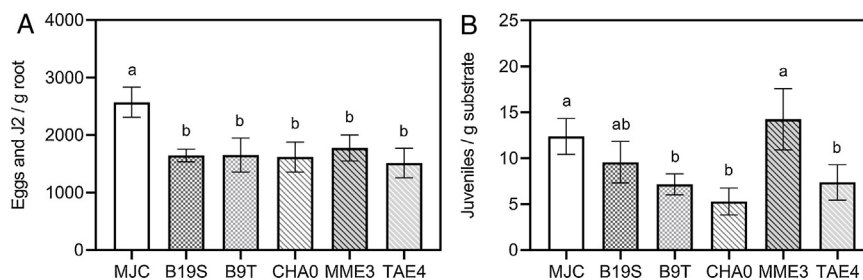
## Discussion

In the quest for indigenous microbes as a natural resource of root-knot nematode biocontrol agents adapted to the edaphic conditions of Argentina, we challenged *Meloidogyne javanica* with locally isolated strains of *Pseudomonas* spp. and *Bacillus* spp. This study demonstrated that several of these strains are capable of successfully limiting the growth and survival of the nematodes *in vitro* and also of reducing parasitism in lettuce plants.

Both bacterial suspensions and cell-free supernatants inhibited egg hatching and increased juvenile mortality. In general, hatching inhibition was greater when cell-free supernatants were used, particularly those produced by *Pseudomonas* spp. strains. It has been reported that antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide (HCN) and protease AprA produced by *P.*

*protegens* CHA0 contribute to the biocontrol activity against eggs and juveniles of *Meloidogyne* spp., which would explain their performance in this study<sup>34–36</sup>. However, the other *P. fluorescens* strains used in this work are not expected to generate 2,4-DAPG<sup>2</sup> while only strain MME3 produces HCN<sup>25</sup>. Our results show that some *P. fluorescens* strains inhibited the hatching of *M. javanica* eggs at a higher level than *P. protegens* CHA0, suggesting the action of other metabolites. Several compounds, including diketopiperazines<sup>13,19</sup>, antibiotics<sup>21</sup>, cyclic lipopeptides<sup>16,22</sup> and hydrolytic enzymes such as proteases, lipases, and chitinases<sup>16,34,40</sup> could determine the ability and efficacy of CFS to control *M. javanica* by both *P. fluorescens* and *Bacillus* spp. strains. An interesting case is the behavior of *Bacillus* sp. B9T on egg hatching, because CFS had no effect on this parameter while the bacterial suspension exhibited the highest inhibition. The fact that *Bacillus* sp. B9T may require physical contact with *M. javanica* to exert its biocontrol mechanism cannot be ruled out. Although, diverse mechanisms that require cell to cell contact have been described in gram-positive bacteria<sup>12</sup>, to the best of our knowledge, no data is available about the role of this type of mechanisms on nematode antagonism by *Bacillus* sp.

Except for *P. protegens* CHA0, all cell suspensions caused higher juvenile mortality rates than their respective CFS, especially with *Bacillus* spp. strains. This behavior could be explained by *in situ* generation of compounds with volatile characteristic that may have been lost during the filtration of CFS. Volatile organic compounds (VOC) such as hydrogen cyanide, hydrogen sulfide, dimethylsulfide, decanal, 2-undecanone and 2-nonanone have been reported to have nematicidal activity against *Meloidogyne* spp.<sup>1,5,17,35,43</sup> Any of these metabolites could be produced by the *Bacillus* spp. and *Pseudomonas* spp. strains studied here. Alternatively, the production of hydrolytic enzymes may be induced in bacterial cell suspensions under a starving state. *Pseudomonas* spp. and *Bacillus* spp. enzymes such as chitinases<sup>1,24,29</sup>, proteases<sup>34</sup>, collagenases and lipases<sup>1</sup> were already described for the control of *Meloidogyne* spp. eggs and juveniles. In addition to the diffusible secondary metabolites described above, intermediates of purine biosynthesis<sup>41</sup>, sphingosine<sup>11</sup>, uracil, dihydrouracil and 9H-purine<sup>30</sup> produced by *Bacillus* spp. have nematicidal properties on juveniles of *Meloidogyne* spp. and may contribute to the control exerted by our strains. The



**Figure 2** Effect of bacterial inoculation on the number of *M. javanica* individuals present in the root of lettuce (A) or in the substrate (B). MJC: nematode infestation control (with nematode, without bacteria). B19S: *Bacillus* sp. strain B19S. B9T: *Bacillus* sp. strain B9T. CHA0: *P. protegens* strain CHA0. MME3: *P. fluorescens* strain MME3. TAE4: *P. fluorescens* strain TAE4. Error bars correspond to standard error means. Different letters indicate significant differences in means according to the LSD-Tukey's test ( $p < 0.05$ ).

potential presence of these secondary metabolites together with VOC and hydrolytic enzymes in cell suspensions could be the reason why *Bacillus* spp. strains affect juvenile survival to a greater extent than *Pseudomonas* spp. strains. The specific case of *P. fluorescens* MME3, whose aggressiveness against juveniles was notably higher in the cell suspension than CFS with respect to other native *Pseudomonas* spp. strains, may be explained by *in situ* production of HCN<sup>25</sup>. Although *P. protegens* CHA0 also produces HCN, its CFS showed higher juvenile mortality than the bacterial suspension, an exception to general behavior observed for other strains. This may be due to the additional production of 2,4-DAPG, pyrrolnitrin and pyoluteorin in CFS, metabolites known not only to affect hatching but also to induce mortality in nematode juveniles<sup>28,36,37</sup>.

The successful colonization of the plant rhizosphere is a prerequisite for pathogen suppression by rhizobacteria. Lettuce roots were effectively colonized by the bacterial strains studied in this work. *Pseudomonas* sp. showed better survival than *Bacillus* sp. one day after infestation with *M. javanica*. Once located in the roots, bacteria can interfere with nematode vital cycle through the production of secondary metabolites and hydrolytic enzymes, by competing for niche and nutrients, or by inducing the plant systemic resistance<sup>36,37,40,42</sup>. Additionally, rhizobacteria can also have a palliative effect by promoting plant growth. In the present study, the losses on lettuce aerial biomass caused by *M. javanica* could not be recovered through inoculation with any of the bacterial strains. Similar results were reported with field-cultivated lettuce infested by *Meloidogyne* spp. and treated with *B. subtilis* and *Pseudomonas aeruginosa*<sup>32</sup>. Nevertheless, other authors reported the effect of growth promotion by *Bacillus* spp. and *Pseudomonas* spp. as attenuating the infestation by *Meloidogyne* spp. in lettuce and tomato<sup>3,4,15,31,38</sup>. An unexpected behavior was observed for root dry weight as infestation with *M. javanica* incremented this parameter compared to healthy plants, while bacterial inoculation did not show clear trends. It is possible that the formation of galls on the radical system with a consequent decrease in the ability to absorb water could have incremented root dry weight. In addition, a growth-promoting effect of bacteria on root might have been slanted by the increase in dry weight due to *M. javanica* infestation. Similarly, Hoffmann-Hergarten et al.<sup>15</sup>, described an increase in fresh root weight of cherry tomato produced by *M. incognita* infection.

Species of *Bacillus* spp. and *Pseudomonas* spp. were reported as suppressors of root knot infestation in various crops, with magnitudes that depend on the mode and dose of bacterial application<sup>3,31,36</sup>. Our results show that all bacterial strains studied here controlled the infestation and reproduction of *M. javanica* on lettuce. Bacterial inoculation decreased nematode infestation by reducing the number of galls developed on roots by 18–32%. This effect may be due to direct mechanisms on free nematodes in soil, as suggested by our *in vitro* results. Nevertheless, we cannot rule out the possibility of an indirect action on plants, such as the induction of systemic resistance<sup>36,37,40</sup>. The reproduction of *M. javanica* in infested lettuce was also affected by bacterial inoculation, as revealed by the lower number of egg masses, individuals in the roots, juveniles in the substrate and reproduction factor. In particular, the presence of *Bacillus* sp. B9T, *P. protegens* CHA0 and *P. fluorescens* TAE4 would seem to affect to a greater extent the multiplication of *M. javanica*, as shown by the number of individuals in the substrate and the reproduction factor.

More research is necessary to validate the potential of these rhizobacteria for the biocontrol of *M. javanica* under natural conditions. In addition, combinations with other PGPR in order to find environmentally friendly alternatives that point to a double purpose, promotion of plant growth and biocontrol of nematodes, could be an outstanding goal in next studies.

## Conclusion

Argentinean strains of *Pseudomonas* spp. and *Bacillus* spp. could control the root knot nematode *Meloidogyne javanica*, either under *in vitro* or *in planta* conditions. Bacterial suspensions and their cell-free supernatants exerted a direct antagonism on eggs and juveniles of *M. javanica*. These PGPR strains were also effective for reducing *Meloidogyne javanica* reproduction on inoculated lettuce plants; however, they did not show positive effects on plant growth. *Bacillus* sp. B9T and *P. fluorescens* TAE4 are the most promising native strains for the control of this pathogen.

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## Conflict of interest

The authors declare that they have no conflicts of interest.

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