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ORIGINAL PAPER

Nematicidal effect of rhizobacteria on plant-parasitic nematodes associated with vineyards

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Abstract The action of metabolites and exoenzymes from rhizobacteria on different plant-parasitic nematodes has an influence on the nematicidal efficacy of the microbe. Seven rhizobacteria, divided into two bacterial groups, were evaluated in vitro for nematicidal activity on *Meloidogyne ethiopica* and *Xiphinema index*. The direct effect of their filtrates on egg hatching and juveniles of *M. ethiopica* as well as mobile stages of *X. index* was evaluated during a 72-h period. The production of four exoenzymes and two metabolites associated with nematode mortality was investigated. Molecular characterization of three isolates was performed, and the physiological profiles and lipase activity of all isolates were obtained using the BIOLOG EcoPlate system. While chitinase and collagenase were measured using the BIOLOG MT2 plate system, protease, hydrogen cyanide and hydrogen sulphide were directly determined in Petri dishes. Nematode mobile stages exposure to the bacterial filtrate revealed a nematicidal effect up to 93.7% on *X. Index* and up to 83.3% on *M. ethiopica*. The control of egg hatching varied between 35 and 85%. A positive correlation was found between the mortality of both nematode mobile stages and the concerted activities of the bacterial enzymes as well as the level of the volatile metabolites. The nematicidal effect of rhizobacteria strains varies by nematode genera and among the developmental stages evaluated.

Keywords Biological control · Exoenzymes · *Meloidogyne ethiopica* · Metabolites · Plant-parasitic nematodes · *Xiphinema index*

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Introduction

Plant growth-promoting rhizobacteria (PGPR) produce antibiotics, antimicrobial metabolites and hydrolytic enzymes, some of which have been reported to control plant-parasitic nematodes (PPN) (Siddiqui and Shaukat 2003). The increasing demand for environmentally friendly alternatives to control plant pathogens has led to an increase in research and development of new biopesticides from which bacteria currently represent 74% of the world market (Thakore 2006). Investigation into rhizobacteria control of PPN has mainly focused on endoparasitic nematodes of the genus *Meloidogyne* while the effectiveness of bacterial strains in the control of other nematode species is poorly understood.

Xiphinema index Thorne and Allen and *Meloidogyne ethiopica* Whitehead are two of the most important nematodes in Chilean crops (Aballay et al. 2011; Aballay and Merino 2015). These nematodes have different feeding habits, found outside and inside of roots, respectively, and causing big damage when they are present in the same host, as it occurs in grapevines. This would imply considering various strategies by the organism used in biological control. The ectoparasitic nematode, *X. index*, causes damage and atrophy of root meristematic zones and is also the vector of grape fanleaf virus (GFLV), a Nepovirus that can cause more than 90% loss in vineyard yield and decrease their production period by 50% (Fiore et al. 2011). On the other hand, the endoparasitic *M. ethiopica*, is listed as the most aggressive species of the genus found in the country due to the size of galls and the number of eggs produced per gram of root (Aballay et al. 2013). Previous works that have proven the efficacy of PGPR in controlling several *Meloidogyne* species, showed over 70% mortality of larval *M. incognita* (Radwan et al. 2012), *M. javanica* (Siddiqui et al.

2003) and *M. ethiopica* (Aballay et al. 2013) using bacterial suspensions and filtrates of *Bacillus* and *Pseudomonas* species mainly, and a similar control level was found on egg hatching. Meanwhile, assessments of the rhizobacteria effect on ectoparasites, particularly *X. index* in greenhouse conditions, found an 80% control level (Aballay et al. 2011). The nematicidal effect of *Bacillus* and *Pseudomonas* genera has been associated with gaseous metabolites and exoenzymes (Mnif and Ghribi 2015). The presence of collagenases (Galper et al. 1990) and proteases (Lian et al. 2007) has been reported in the control of PPN. The production of hydrogen cyanide in *Pseudomonas aeruginosa* is associated with *M. javanica* mortality under in vitro condition (Siddiqui et al. 2003). Studies performed with *B. megaterium* have concluded that protease production is responsible for controlling *M. graminicola* (Padgham and Sikora 2007); in addition, *B. subtilis* chitinase production is associated with the control of *M. incognita* (Wei et al. 2014). The metabolite 2,4-diacetylphloroglucinol has also been implicated in the control of *M. javanica* juveniles under in vitro conditions. (Siddiqui and Shaukat 2003). Further identification of the bacterial compounds thought to play roles in nematicidal activity may orient the biological control investigation towards isolates or consortia that produce the compounds, thus generating an antagonistic effect over PPN.

The effect of determined rhizobacteria on distinct nematode genera was found to be variable; the same bacteria may have antagonistic effects on one nematode genus and may present no or fewer effects on another. From one point of view, the specificity of the bacterial effect is considered of useful character, selectively lowering certain pathogen nematode populations, while the beneficial nematodes in the soil are not affected (McSorley et al. 2006, 2008). Nevertheless, highly restrictive bacteria-nematode specificity is unfavourable in soil populations where many pathogen nematode genera coexist, limiting the PPN control to a single nematode species. Research conducted under field conditions using *Burkholderia tropica* bacterium in sugar cane cultivation found positive control rates on *Helicotylenchus dihystera* and *Pratylenchus zeae* but negative results for *Xiphinema elongatum* (Omarjee et al. 2008). Studies on PPN control using combinations of bacteria (Siddiqui and Akhtar 2009; Sarma et al. 2015) or bacterial consortia in commercial products (Burkett-Cadena et al. 2008) have found better results, likely due to the wide variety of compounds and strategies implicated in minimizing populations of different PPN species. Rhizobacteria are valuable tools for PPN control; however, further research must be directed to select strains or develop combinations with a broad spectrum of PPN control.

Despite advances in the use of rhizobacteria in PPN control, the results obtained under controlled conditions have not been consistent with field trials. These limitations are attributed to the natural specificity of the microorganisms for their niche in the plant rhizosphere, their energy supplying habitat and other factors (Siddiqui 2000).

The objectives of the present work were to evaluate the in vitro nematicidal activity of several native strains of rhizobacteria on *X. index* and different developmental stages of *M. ethiopica*, to investigate the bacterial enzymes and metabolic by-products reported to play antagonistic roles on nematodes and to verify if any correlations exist between bacterial enzymatic activity or by-product liberation and nematicidal effect in vitro. Additionally, the physiological and molecular characterization of three of the considered bacterial strains is presented.

Materials and methods

Rhizobacteria strains

Seven bacterial strains were divided into two groups and evaluated as follows: firstly, the nematicidal effect of three rhizobacteria strains, identified in this study as *Bacillus weihenstephanensis* FB25M, *Pseudomonas fluorescens* FP805PU and *Brevibacterium frigoritolerans* FB37BR on eggs and second stage juveniles of *M. ethiopica* and mobile stages of *X. index* was evaluated. Secondly, the nematicidal effect of four rhizobacteria was evaluated on eggs and J2 stages of *M. ethiopica* in vitro. All the strains belong to our own bacteria collection and the second group was previously tested in vitro on *X. index* and molecularly identified as *Bacillus amyloliquefaciens* consortium FR203A, *Bacillus megaterium* FB133M and *Bacillus thuringiensis* isolates FB833T and FS213P (Castaneda-Alvarez et al. 2016). To distinguish between the two series of bacteria evaluated in the present work, the first bacterial group was denoted FBG and the second SBG.

Molecular identification

DNA extraction and sequencing

DNA was extracted from bacteria growing on 50% tryptic soy broth (TSB) agar (Becton Dickinson & Co., USA) medium at 30 °C for 48 h. A single colony of each strain was removed and suspended in lysis buffer [10 $mmol^{-1}$ Tris HCl (pH 8.0), 1 $mmol^{-1}$ EDTA (pH 8.0), 1% Triton X-100], heated for 13 min at 95 °C and centrifuged at 16,000×*g* for 10 min (Helgason et al. 2004). The supernatant was stored at −20 °C.

The extracted DNA was used to PCR amplify the *16 S rRNA* gene using universal primers published by Lane (1991) (Table 1). The *16 S rRNA* gene sequences were

Table 1 Primers used for PCR amplification

Fragment	Sequence (5–3′) and primer code	References
16 S rRNA (1528 bp)	AGAGTTTGATCMTGGCTCAG (27 f)	Lane (1991)
	AAGGAGGTGWTCCARCC (1525r)	
gyrB (1200 bp)	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAART-TYGA (UP1-F)	Yamamoto and Harayama (1995)
	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTC-NGTCAT (UP2-R)	
gyrB (PB) (400 bp)	GTTTCTGGTGGTTTACATGG (gyr-B-F)	Manzano et al. (2003)
	CAACGTATGATTTAATTCCACC (gyr-B-R)	
rpoB (1200 bp)	ATCGAAACGCCTGAAGGTCCAAACAT (rpoB1-f)	Ki et al. (2009)
	ACACCCTTGTTACCGTGACGACC (rpoB1-r)	
adk (570 bp)	CAGCTATGAAGGCTGAAACTG	Helgason et al. (2004)
	CTAAGCCTCCGATGAGAACA	
plcR (450 bp)	AAAAAGGAAGAATATCATC (BA-plcRF)	Ko et al. (2004)
	ATGCATCTTCAATCTCTG (BA-plcRR)	
16 S–23 S ITS (450 bp)	TCGCTAGTAATCGCGGATCAGC (L516SF)	Xu and Coté (2003)
	GCATATCGGTGTTAGTCCCGTCC (L523SR)	
16 S–23 S ITS (1590 bp)	GGGCCCGCACAAGCGGTGG (16F945)	Lane et al. (1985)
	CTTTCCCTCACGGTAC (23R458)	

used to identify the bacteria genus, and additional genes were sequenced to identify the species. Up to five genes were amplified and sequenced for each isolate as follows: *gyrB* coding for the DNA gyrase B subunit and the internal transcribed spacer between the 16 S and 23 S *(ITS*) were amplified for the FP805PU strain using UP1-F and UP1-R primers and 16F945 and 23R458 primers, respectively. For *Bacillus* FB37BR, the *ITS* region was amplified with L516SF and L523SR primers. Four housekeeping genes were used to identify the FB25M strain as follows: *gyrB* with primers for the *Bacillus* genus, *rpoB* that codes for the β subunit of bacterial RNA polymerase, *adk* coding for adenylate kinase enzyme and *plcR*, a specific pleiotropic gene responsible for virulence in species of the *B. cereus* group. All primers used are shown in Table 1.

Amplified DNA fragments were purified using an EZNA Gel Extraction Kit (Omega Bio-tek, Inc., GA, USA), cloned into the pGEM-T (Promega, USA) vector and transformed into *E. coli* One Shot® TOP10 chemically competent cells (Invitrogen). Recombinant plasmids were purified with EZNA® (Plasmid DNA Mini Kit, Omega Bio-tek, Inc., GA, USA) according to the manufacturer's instructions.

Strain identification

The plasmids were sequenced by Macrogen USA Corp. (Rockville, Maryland, USA) services using universal primers T7 and SP6 (Schenborn and Mierendorf 1985). The sequences were aligned using the Geneious version 7 (Biomatters Ltda.©) program and analysed in the NCBI database using the Blastn algorithm (Morgulis et al. 2008). For the FB25M strain that belongs to the *B. cereus* group, additional phylogenetic analyses were performed using the Neighbour-Joining method of MEGA version 6 (Tamura et al. 2013). Six GenBank sequences of representative *B. cereus* species were included. Additional tests were performed to complement molecular identification: bacterial growth at extreme temperatures (5 and 45 °C) and identification by BBL crystal plate kits (Dickinson, Franklin Lakes, MD, USA).

The nucleotide sequences were deposited into GenBank with the following accession numbers: KT881299 to KT881301 for *16 S rRNA*; KT881297, KT881298 and KU985060 for *ITS*; KT881305 and KT881306 for *gyrB*; KT881304 for *adk*; KT881303 *rpoB* and KT881302 for *plcR*.

The effect of rhizobacteria filtrates on mobile stages of *X. index* and *M. ethiopica*.

Preparation of cell free filtrates of rhizobacterial inoculum for in vitro nematode test

Bacterial strains were cultivated in the dark for 48 h at 22 °C on 50% TSB agar to verify their purity. Each strain was inoculated in 15% TSB liquid media, and suspensions were grown overnight with orbital shaking at 180 rpm and an average temperature of 20 °C. The bacterial concentration was adjusted to 1×10^6 CFU ml^{-1}, and the suspension was centrifuged twice for 15 min at 4000×*g* in the cold.

The cell free filtrate was collected and maintained under refrigeration. The filtrate was used to verify the direct effect of bacterial metabolites and enzymes on PPN.

Direct effect of rhizobacterial cell free filtrates on PPN mobile stages

To determine the nematicidal effect on juveniles and adults of *X. index*, mobile stages were extracted from fresh soil samples from the root zone of *Ficus carica* using modified Cobb's sieving and decanting method for extraction of Longidorid nematodes (Brown and Boag 1988). To obtain the juveniles (J2) stage of *M. ethiopica*, eggs were extracted from infested grapevine roots according to the method described by Hussey and Barker (1973). The eggs were placed on top of a 90-micron sieve on a Baermann funnel and incubated at room temperature for 48 h. Thereafter, the emerged J2 juveniles were collected. The mobile stages of both nematodes species were surface sterilized with 0.001% chlorhexidine digluconate, washed three times and kept in sterile tap water for 24 h at 4 °C (Huettel and Rebois 1985). The *X. index* population was a mix of approximately 70% adults and 30% of different larval stages, while the *M. ethiopica* population was homogeneous, composed only of J2 stages. Both species were tested separately for their susceptibility to the bacterial filtrate as follows: a 500-µl aliquot containing approximately 50 individuals was placed in a 35 mm Petri dish, and a volume of 1.5 ml of each bacterial filtrate was added to each plate in the presence of 200 µg ml^{-1} streptomycin (final concentration, to ensure that no bacterium cell could develop into the filtrate and control medium), strains sensitivity previously determined. The plates were incubated at 23 °C, then the nematode mortality was recorded every 24 h, for 3 days. A nematode was considered dead when it did not move when touched repeatedly with a needle. Control treatment consisted on a 15% TSB suspension of the nematodes.

Effect of rhizobacteria filtrates on egg hatching of M. ethiopica

One thousand eggs were deposited onto a filter paper disc placed on top of a 40 mm diameter sieve (90-µ opening), which was introduced in a Baermann funnel. The entire capacity of the funnel was filled with bacterial filtrate diluted in sterile distilled water (three volumes filtrate: one of water), and a streptomycin solution (100 µg ml^{-1} final concentration). The eggs were then incubated in bacterial filtrate for 72 h at 26 °C. After this period, the liquid in the funnel was removed and replaced by 30 ml of sterile tap water. Every 48 h during 12 days, the emerging juveniles were collected and counted with a stereoscopic microscope (Carl Zeiss, Stemi 2000-C, Gottingen, Germany) using 90X magnification. Total egg hatching was recorded for each treatment at the end of the 72-h period. Control treatment consisted on a 15% TSB suspension of the nematode eggs.

Experimental design and statistical analysis

A completely randomized design was used with the four treatments (three bacteria and one control treatment) of the FBG and the five treatments (four bacteria and one control treatment) of the SBG; for each bacteria group, a series of six replicates was performed three times. The data were subjected to analysis of variance (ANOVA), and means were compared with the control treatment according to Dunnett's test ($p \leq 0.05$). The data were transformed to arcsine of the percentage data. MINITAB statistical software version 17 (Minitab, Inc., PA, USA) was used for the analysis.

Rhizobacteria metabolites and enzymes

Three bacterial strains (FBG) were subjected to a series of biochemical tests to verify the liberation of two secondary metabolites (hydrogen sulphide and cyanide) and the presence of four exoenzymes (chitinases, collagenases, proteases and lipase). All tests were repeated four times, and the results were organized into four categories according to the intensity of the biochemical response of the measured compound.

Chitinase and collagenase activity

Chitinase and collagenase activities were determined using the MT2 MicroPlate™ plate system (Biolog, Inc., CA, USA), whose principle is that aerobic respiration reduces a redox tetrazolium dye from colourless to violet if bacteria are able to use the specific carbon source added in the microplate wells. The plates have been successfully used in tests of chemical toxicity on microflora (dos Santos et al. 2002). The MT2 test was performed using a freshly prepared bacterial suspension with a final concentration of 1×10^6 CFU ml^{-1} for each bacterial strain or consortia. The carbon sources were as follows: colloidal and triturated solid chitin to verify the chitinase activity and collagen to verify collagenase activity. Two bacterial chitinase substrates were used: (1) a sterile 6 mg ml^{-1} suspension of colloidal chitin and (2) a sterile 0.5% (w/v) suspension of Chilean king crab (*Lithodes santolla* Molina) exoskeleton triturate, obtained by exhaustive grinding in liquid nitrogen. The substrate for bacterial collagenase was a sterile 0.6% suspension of a collagen-based commercial product from bovine tracheal cartilage (Sigma-Aldrich, MO, USA). One entire MT2 plate was used for each enzyme, all the 96-wells being distributed among the bacterial treatments

and the controls, to obtain a high number of repetitions for a reliable result. In practice, a volume of 40 µl of substrate solution was added to each MT2 well, followed by 110 µl of bacterial suspension at 10^6 CFU ml^{-1}. Bacterial control and substrate control wells were considered, and each was repeated twice within the same plate. The plates were incubated at 24 °C, then examined every 12 h for violet colour development for 2 days. The optical density was measured with a microplate spectrophotometer (EpochTM, Biotek®) at 590 nm. Three arbitrary ranges of colour intensity level were established and expressed as percentages of the maximal intensity achieved on the plate and considered as reference. Therefore, the ranges were as follows: (−) negative, without colour; (+) 0–25% positive low activity; (++) positive medium activity 25–60%; and (+++) high activity >60%.

Hydrogen cyanide (HCN) liberation

HCN gaseous metabolite production was determined in sealed Petri plates by the method of Millar and Higgins (1970), modified by Kloepper et al. (1991). Strains were streaked onto 50% TSBA supplemented with 4.4 g l^{-1} glycine. A disk of No. 1 Whatman filter paper was placed on the inside of the Petri dish lid and humidified with a solution of 0.5% v/v picric acid and 2% w/v Na_2CO_3. The plates were incubated for 96 h at 24 °C, and a change of the filter paper colour from yellow to brown was observed. The results were classified as (−) negative if the filter paper colour remained yellow (original colour) or (+) positive if the colour turned brown.

Hydrogen sulphide (H_2S)

The production of hydrogen sulphide was determined using lead acetate paper tape (McBride and Edwards 1914). The bacterial strains were streaked on 50% TSBA, and a 3-cm long strip of lead acetate paper (Macherey-Nagel GmbH & Co. Düren, Germany) was stuck onto the inner side of the lid. Plates were sealed and incubated at 22 °C for 4 days. Formation of a dark precipitate of lead-sulphide determines a change of the indicator paper colour in the presence of H_2S metabolites. *Escherichia coli* enterobacteria strain Top10 (Invitrogen®) was used as a positive control for H_2S production. Three positives ranges of colour intensity were established: low (+), medium (++) and strong (+++).

Proteases

Protease production was determined on 15 g l^{-1} skim milk (Merck, Germany) agar plates (Smibert and Krieg 1994). Bacterial isolates were inoculated in the centre of the plates and incubated for 5 days at 22 °C.The presence of a clear halo around the colony indicated proteolytic activity; using the radius of the clear halo area as an indicator of enzymatic activity, a scale was established as follows: radius <1 cm, low production (+); 1–4 cm, medium production (++); >4 cm, high production (+++); without halo, negative activity.

Physiological characterization and lipase evaluation

A physiological profile of the evaluated rhizobacteria was performed using the EcoPlate™ plates system (Biolog, Inc., CA, USA). The Ecoplate consists of a 96-well field divided into three identical sectors, each of them being a pattern of 31 different carbon sources commonly used by soil microorganisms. The control wells do not contain a carbon source. As with the MT2 plates, the principle of the EcoPlates is that aerobic respiration reduces a redox tetrazolium dye if the bacteria are able to use the specific carbon sources present in each well. Their ability to use the provided carbon sources is evaluated, thus generating a characteristic physiological profile.

Additional data on the bacteria exoenzyme activity may be generated using the Ecoplate system: Tween 40 and Tween 80, which are esters of palmitic and oleic acids, respectively, are included among the carbon sources; therefore, lipase activity may be deduced.

One entire Ecoplate was used to assay a single bacterial strain, ensuring three repetitions in a single procedure, as follows: a volume of 150 µl of 1×10^6 CFU ml^{-1} bacterial suspension was inoculated in each well according to the manufacturer's instructions. The plates were incubated for 5 days at 22 °C and visually evaluated from the second day for colour development. At the end of the incubation period, an evaluation of each well's colour was performed using a microplate spectrophotometer (EpochTM, Biotek®) at 590 nm, according to the manufacturer's instructions. The use of each carbon source was evaluated as follows: (−) negative, without colour; (+) 0–25% positive low activity; (++) positive medium activity 25–60%; and (+++) high activity >60%, where the colour intensities are expressed as the percentages of the maximal intensity achieved on the plate.

To determine metabolic preferences for certain carbon sources among the investigated strains, the Ecoplates results were analysed through principal component. Moreover, to verify relationships between the metabolite and/or enzyme production and the nematicidal properties, the results were analysed through principal component, as well. All statistical analysis was performed with Minitab version 17 (Minitab, Inc., State College, PA).

Results

Molecular identification of rhizobacteria strains

Identification based on *16 S rRNA* sequencing determined that the FP805PU strain belongs to the genus *Pseudomonas*, whereas the FB37BR and FB25M strains belong to the genera *Brevibacterium* and *Bacillus*, respectively.

The FP805PU *Pseudomonas* strain was identified as *Pseudomonas fluorescens* due to 99% identity in the *16 S rRNA* and *ITS* sequences and 93% identity in the *gyrB* sequence. However, the sequences were also related to *P. brassicacearum*. The results obtained using BBL Crystal plates identified the strain as *P. fluorescences* with 60% reliability. Growth behaviour at extreme temperatures revealed that FP805PU grows well at 5 °C but does not grow at temperatures above 41 °C.

The FB25M strain was identified as *B. weihenstephanensis*. The characterization based on *16 S, gyrB, rpoB* and ITS yielded 99% identity with species of the *B. cereus* group. The *plcR* and *adk* gene analysis separated the FB25M strain from other species of the *B. cereus* group, with a margin of 2% in each gene, and identified the isolate as *B. weihenstephanensis* (Fig. 1). Growth at extreme temperatures defined the psicrotolerant character of the FB25M isolate.

The FB37BR strain was identified as *Brevibacterium frigoritolerans*. The *16 S* gene shared 99% identity with both *Brevibacterium frigoritolerans* and *B. simplex*. ITS sequence analysis showed 98.9% identity with the *Bacillus simplex* strain SH-B26 (Genbank Accession Number CP011008), but no information is available for the *Brevibacterium frigoritolerans* ITS sequence. BBL Crystal plates indicated that the strain belongs to *Corynebacterium jeikeum* (97.2% confidence).

Effect of rhizobacteria filtrates on X. index and M. ethiopica

The effect of rhizobacterial filtrates was evaluated on mobile stages of *X. index* and J2 of *M. ethiopica* every 24 h over a 72-h period. The effect on *M. ethiopica* egg hatching was verified directly at 72 h of filtrate exposure. Different filtrates revealed distinct levels of nematicidal effect on the mobile stages of both species of nematodes at the end of the evaluation period, as manifested by damage of the cuticle and loss of mobility. After 72 h, damage of the egg vitelline membrane and decreased mobility of the emerged larvae under the effect of some of the filtrates were observed (data not shown).

The FBG filtrates that were effective against mobile stages were not necessarily effective on *M. ethiopica* egg hatching (Table 2). The nematicidal effect of *P. fluorescens* PF805PU was observed from 24 h on *X. index* and from 72 h on *M. ethiopica* larvae, reaching 75.8 and 93.7%, respectively, at the end of the filtrate exposure. *P. fluorescens* PF805PU had no effect on *M. ethiopica* egg hatching. Filtrates of *B. weihenstephanensis* FB25M showed nematicidal effect on the mobile stages of both nematodes species at 72 h of exposure. FB25M filtrate affected egg hatching in 66.4% compared to untreated controls. *Brevibacterium frigoritolerans* FB37BR manifested an approximately 50% control rate on the mobile population of both nematode species, which is the lowest recorded mortality value. However, this strain showed the highest control values over *M. ethiopica* egg hatching, diminishing the larvae emergence by 83% at the end of the evaluation period.

The group of four bacteria (SBG) was investigated to verify the control levels on *M. ethiopica* mobile stages and egg hatching, and production of the previously mentioned metabolites and enzymes was evaluated. At the end of the 72-h evaluation period, FR203A, FS213P and

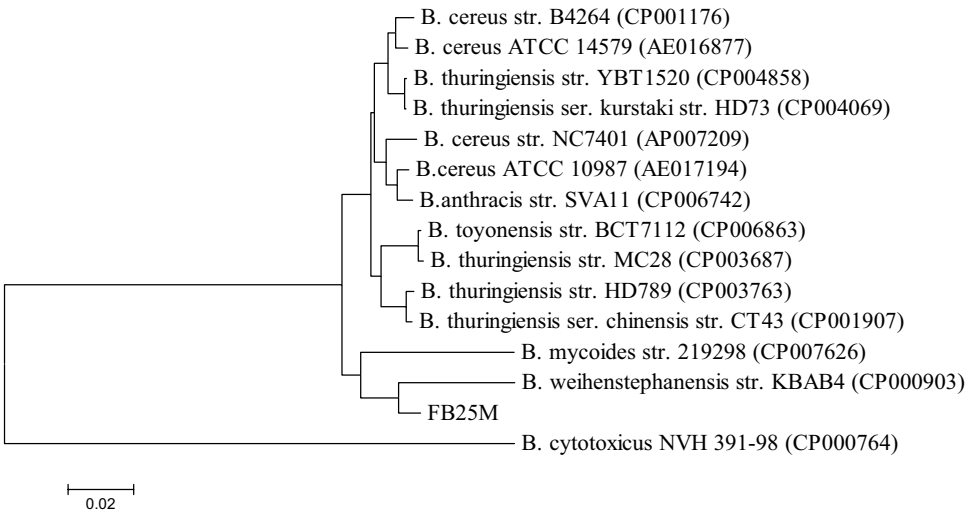

Fig. 1 Phylogenetic analysis of *B. weihenstephanensis* FB25M belonging to the *B. cereus* group. Tree contains six concatenated sequences of the *16 S rRNA, gyrB, rpoB, plcR, adk* genes and 16 S–23 S intergenic region. The evolutionary history was inferred by the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary distances were calculated using the maximum likelihood composite method (Tamura et al. 2004)

Table 2 Effect of rhizobacterial filtrates on mobile stages of *X. index* and *M. ethiopica* and egg hatching of *M. ethiopica*

Treatments	*M. ethiopica*				*X. index* mortality (%)		
	J2 Mortality (%)			Hatching (%)			
	24 h	48 h	72 h	72 h	24 h	48 h	72 h
Control	0.0a	0.4a	0.4a	89.9a	1.0a	3.3a	9.7a
P. fluorescens FP805PU	0.8a	5.8a	75.8	53.9a	29.8	83.8	93.7
B. weihenstephanensis FB25M	0.8a	21.5a	54.2	30.2	9.3a	48.7	80.5
B. frigoritolerans FB37BR	0.3a	3.2a	53.0	15.1	6.3a	30.0	53.7

The data correspond to the means of six replicates. Means without letters are significantly different from the mean level of the control according to Dunnett's test ($P \leq 0.05$)

Table 3 Effect of rhizobacterial filtrates of SBG on the mortality of juveniles (J2) and eggs hatching of *M. ethiopica*

Treatments	Mortality (%)			Hatching (%)
	24 h	48 h	72 h	
Control	0.0a	0.2a	0.2a	91.1a
B. megaterium FB133M	1.0a	39.7a	40.7a	34.1
B. thuringiensis FB833T	0.7a	38.0a	55.0	65.0a
B. amyloliquefaciens FR203A	1.0a	52.7	83.3	25.5
B. thuringiensis FS213P	1.7a	36.8a	54.0	28.3

The data correspond to the means of six replicates. Means without letters are significantly different from the mean level of the control according to Dunnett's test ($P \leq 0.05$)

FB833T strains registered nematicidal effects on the target J2 population, while the *B. megaterium* FB133M strain did not. (Table 3), while a lethal effect of *B. amyloliquefaciens* FR203A consortium on mobile nematodes was found after 48 h exposure period. Concerning egg hatching, the *B. amyloliquefaciens* FR203A consortium, *B. thuringiensis* FS213P and *B. megaterium* FB133M strains decreased larvae emergence between 62.6 and 72% compared to the untreated control, while the *B. thuringiensis* FB833T strain did not showed a nematicidal effect.

Rhizobacterial metabolites and enzymes

All rhizobacteria strains from the first bacteria group showed activity in at least four of the six tested metabolites and enzymes (Table 4). *P. fluorescens* FP805PU was positive for almost all considered metabolites and enzymes except protease activity. Collagenase, chitinase and lipase activities as well as hydrogen cyanide production were strong, recording the highest general activity among the tested strains. The *B. weihenstephanensis* FB25M strain presented activity of all examined enzymes, with proteases being the strongest, while gaseous metabolite production was negative. The *B. frigoritolerans* FB37BR strain was active for almost all of the compounds, except hydrogen cyanide production; nevertheless, the recorded activity level was generally low.

According to the principal component analysis (Fig. 2), which registered enzyme and metabolite production of the three rhizobacteria (FBG) and the effect of their filtrates on nematodes, a characteristic pattern was registered for each strain. FP805PU was localized

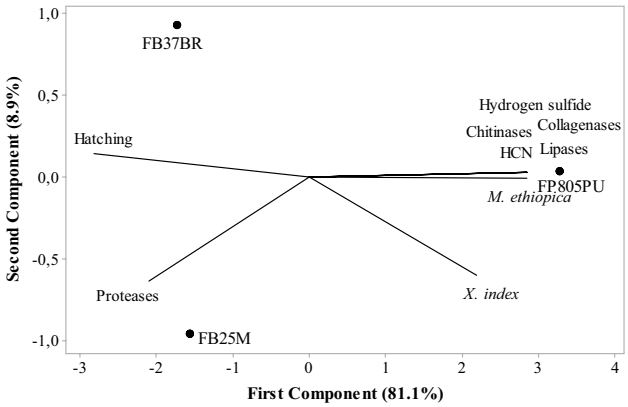

Fig. 2 Principal component analysis of the relationship between enzyme and metabolite production and the nematicidal effect in both nematode species by rhizobacteria tested

Table 4 Production of metabolites and extracellular enzymes of rhizobacterial strains tested on *X. index* and *M. ethiopica*

Strain	Metabolites/enzymes					
	HCN	H_2S	Collagenases	Chitinases	Proteases	Lipases
P. fluorescens FP805PU	+++*	+	+++	+++	−	+++
B. weihenstephanensis FB25M	−	−	+	+	+++	+
B. frigoritolerans FB37BR	−	+	+	+	+	+

*(−) Negative, (+) Low production, (++) Medial production and (+++) High production

in a sector of high metabolite and enzyme production, FB25M in a zone of especially high protease activity and FB37BR in a site of low production of all evaluated compounds.

The highest mortality of *M. ethiopica* mobile stages was achieved by the *P. fluorescens* FP805PU strain, which also presented the highest production of collagenases, lipases, chitinases and HCN. This correlation may promote the hypothesis—to be verified in future work—that J2 *Meloidogyne* mortality and metabolite production levels are closely related. On the other hand, the *P. fluorescens* FP805PU filtrate was not effective in egg hatching compared to control. Additionally, a positive correlation was observed (Fig. 2) between metabolite and enzyme presence and the mortality of *X. index;* one exception was that protease production was not related to larvae mortality but to egg hatching control of *M. ethiopica*.

Physiological characterization

The tested bacterial strains from FBG and SBG generally manifested low to median affinity for the carbon sources provided by the Ecoplate (Table 5). An exception was that *P. fluorescens* FP805PU and *B. amyloliquefaciens* consortium FR203A presented a higher affinity, using more than 50% of the carbon sources. Precisely, FP805PU used 81% of the carbon sources, while FR203A consortium presented affinity for 17 of the 31 carbon sources. The metabolic activity of strain FR203A was low in the positive wells, while *B. megaterium* FB133M and *B. frigoritolerans*

Table 5 Physiological characterization of bacterial strains (first and second group) based on the carbon sources used in the Ecoplate plates

Carbone source	Strain						
	FP805PU	FB25M	FB37BR	FR203A	FB833T	FB133M	FS213P
Pyruvic Acid Methyl Ester	+++*	+	+	+++	−	+	+++
D-Galacturonic Acid	+++	−	+	+	+	−	++
γ-Hydroxybutyric Acid	+	−	−	+	++	−	+
D-Glucosaminic Acid	+++	+	−	−	−	−	−
Itaconic Acid	−	−	−	+	−	+	−
α-Ketobutyric Acid	−	−	−	++	++	+	−
D-Malic Acid	+++	−	−	−	−	−	−
Phenylethyl-amine	++	−	−	+	+	+	−
Putrescine	+++	+	+	−	−	+	−
L-Arginine	+++	−	−	−	−	+	−
L-Asparagine	+++	−	−	−	−	−	−
L-Phenylalanine	++	+	−	−	−	−	−
L-Serine	+++	+	−	+	−	−	+
L-Threonine	+++	+	−	−	+	−	−
Glycyl-L-Glutamic Acid	+++	−	−	−	−	−	−
β-Methyl-D-Glucoside	−	+	+	−	−	−	−
D-Galactonic Acid γ-Lactone	+++	−	−	−	−	−	−
D-Xylose	+++	+++	+++	+++	+++	++	+++
i-Erythritol	+	+	−	+	−	−	−
D-Mannitol	+++	−	−	+	−	−	−
N-Acetyl-D-Glucosamine	+++	−	−	+	+	−	−
D-Cellobiose	+	−	−	+	−	−	+
Glucose-1-Phosphate	−	−	−	−	−	−	−
α-D-Lactose	+	−	−	−	−	+	+
D,L-α-Glycerol Phosphate	++	+	−	−	−	+	+
2-Hydroxy Benzoic Acid	−	+	−	−	−	−	−
4-Hydroxy Benzoic Acid	+++	−	−	+	−	−	−
Tween 40	+++	++	−	+++	+++	−	+++
Tween 80	++	−	+	+	+	−	+
α-Cyclodextrin	−	−	+	+	++	−	+
Glycogen	+	+	−	++	−	−	−

*(−) Negative, (+) Low, (++) Medium and (+++) High affinity

FB37BR strains recorded even lower metabolic activity in the positive wells.

The Ecoplates carbon sources consist mainly of carbohydrates, carboxylic acids and amino acids, and the most common compound used by all strains was D-xylose. Conversely, glucose 1-phosphate was not metabolized by any bacterial strain. Although there were no real trends in the use of the carbon sources, the physiological profiles identified a predilection for certain sources manifested by phylogenetically nearby bacteria. Principal component analysis of the physiological profile of all six rhizobacteria strains (Fig. 3) showed that *P. fluorescens* FP805PU localized in one extreme of the principal component PC 1, separated from the group of *Bacillus* species in the other extreme. Principal component PC2 divided the *Bacillus* species into three groups: *B. amyloliquefaciens* FR203A consortium; FB133M, FB833T, FB25M and FB37BR strains at the opposite end; and FS213P was a half distance between the two groups.

Discussion

Microorganisms, such as fungi and bacteria, have been investigated in the biological control of PPN (Costa et al. 2012; Zhang et al. 2014). The effect of different rhizobacteria on distinct PPN has been previously investigated in our group under variety of conditions, and a certain level of PPN control was certified by these previous investigations. Nevertheless, the principles of the antagonistic effect of rhizobacteria on nematodes remained to be assessed. The present work aimed to determine the antagonistic activity of the rhizobacterial filtrate against different developmental stages of ecto- and endoparasitic nematodes—represented by *X. index* and *M. ethiopica*, respectively—and to evaluate the presence of four rhizobacterial exoenzymes and two metabolic by-products in the cell-free bacterial filtrate.

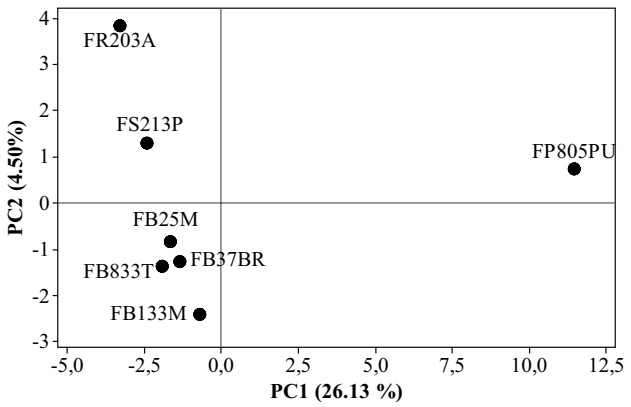

Fig. 3 Principal component analysis of the physiological profile of rhizobacterial strains (FBG and SBG)

Results of the present investigations indicated that the rhizobacterial filtrates had the ability to induce in vitro mortality on *X. index* adults and larvae and on *M. ethiopica* J2 stages. Egg hatching of *Meloidogyne* was also affected compared to an untreated control. However, variability existed in the control rates between different nematode genera and between developmental stages within the same genus. Previous studies using rhizobacteria against *X. index* (Aballay et al. 2012), *M. ethiopica* (Aballay et al. 2013) and other species of the genus *Meloidogyne* (Siddiqui and Shaukat 2003; Radwan et al. 2012; Almaghrabi et al. 2013) also reported this variability.

Results of the in vitro assay of *X. index* exposure to the rhizobacteria filtrates showed that, after 24 h, only *P. fluorescens* FP805PU presented a nematicidal effect. After 48 h, all of the strains showed a certain level of nematode control, a trend that continued and increased at 72 h. The in vitro assay on *M. ethiopica* showed no significant difference between filtrate and control treatments after 24 and 48 h; the filtrates began to affect the nematodes after 72 h of exposure. This clearly indicates cuticle structural differences between these two species of nematodes, the cuticle being a structure that widely varies between genera, even between individuals of the same species (Decraemer and Hunt 2006).

The nematode cuticle is composed primarily of collagen and is considered a complex structure due to the amount of genes encoding this polypeptide chain. In free living nematodes, such as *Caenorhabditis elegans*, more than 150 genes are involved in the creation of this structure (Ray and Hussey 1995), while 122 collagen coding genes were identified in *M. incognita* (Abad et al. 2008). In the present study, all the tested bacteria manifested collagenase activity. A direct correlation was found between high collagenase activity and high mortality in the mobile stages of the two nematodes. *P. fluorescens* FP805PU manifested high collagenase activity and also recorded the highest mortality on mobile stages. However, *M. ethiopica* egg hatching was poorly affected. The collagenase activity may be related to nematode mobile stage control, and few works have focused on this enzyme. A positive effect of collagenases on *M. javanica* control has been observed under field and laboratory conditions (Galper et al. 1990). Although collagenases may play a role in PPN control, the effect may be symbiotic or supplemented by other enzymes.

Investigations using rhizobacteria suspensions and cell-free filtrates have involved the presence of proteases in the control of nematode egg hatching (Galper et al. 1990; Siddiqui et al. 2005) and mobile stages (Paiva et al. 2013). Similarly, proteolytic degradation of the egg cuticle has been implicated in the fungal colonization of nematodes

(Lopez-Llorca et al. 2008). The egg is broadly considered the stage of increased resistance in the nematode life cycle (Bird and Bird 1991). The effect of bacterial filtrates on egg hatching was strong, with *B. frigoritolerans* FB37BR manifesting the highest egg damage effect among the two groups of tested bacteria (FBG and SBG). Within the second group of evaluated bacteria (SBG), the highest control percentages were achieved by the *B. amyloliquefaciens* FR203A consortium and *B. thuringiensis* FS213P strains, which manifested high protease activity in previous work (Castaneda-Alvarez et al. 2016). *Bacillus weihenstephanensis* FB25M also manifested high protease activity and a moderate-to-high damage effect on eggs. On the other hand, *P. fluorescens* FP805PU lacked protease activity and had no significant effect on the eggs. These correlations may suggest a participation of bacterial proteases in egg-hatching control. Tikhonov et al. (2002) found that proteolytic activity had an effect on the outer layer of nematode eggs; moreover, they found wider damage when proteases were combined with chitinases. Chitinase activity by itself may be restricted because the chitin layer is not directly exposed to the exterior, but is embedded in a protein matrix. Nematode egg shells are mainly comprised of chitin (Stirling 2014), however, the chitin/protein ratio varies in different species of nematodes (Curtis et al. 2011).

The *B. weihenstephanensis* FB25M strain registered activity for four of the five exoenzymes and metabolites evaluated, and it manifested a nematicidal effect on both nematode species. The proteolytic activity of FB25M was the highest recorded in this work; nevertheless, it was not the most effective against *M. ethiopica* egg hatching. The chitinolytic activity of this isolate was low; nevertheless, the reflected damage on egg hatching was moderate-to-high. These findings strengthen the theory of the concerted action of different enzymes, such as chitinases and proteases, to generate the control phenomenon. The use of chitinases in biological control is likely to be complemented by other bioactive peptides and lytic enzymes produced by the same bacteria or found in the natural systems where the nematode-bacteria interaction occurs (Herrera-Estrella and Chet 1999).

Lipase activity was present in all tested strains, and the highest level of activity coincided with the highest mortality of the nematode mobile stages. On the other hand, the *B. megaterium* FB133M (SBG) strain had low lipase activity, and no significant mortality of *M. ethiopica* J2 was recorded. These results are consistent with previous reports in our group that registered low mortality of FB133M on *X. index* (Castaneda-Alvarez et al. 2016). Lipases may be a component of the enzymatic pool involved in nematode in vitro control, as these enzymes can damage the nematode morphological structures. In this sense, lipase activity was found in rhizobacteria with nematicidal effects on the pinewood nematode *B. xylophilus* (Paiva et al. 2013). There are few studies on lipases, but they continue to be reported in PPN control in bibliographic works (Beneduzi et al. 2012).

Pseudomonas fluorescens is one of the most studied rhizobacteria in the biological control of nematodes. In the present study, *P. fluorescens* FP805PU had nematicidal effects on both nematode species assayed, with a higher control level on *X. index*. This was the only strain that produced HCN, a metabolite already reported to have a nematicidal effect on PPN (Siddiqui et al. 2003) and on the free-living nematode *C. elegans* (Romanowski et al. 2011). Hydrogen cyanide a secondary metabolite, is insufficient to control nematode egg hatching (Collange et al. 2011), likely due to its volatility, and the results of our assay on *M. ethiopica* egg hatching corroborated this conclusion. Nevertheless, because HCN biosynthesis requires a glycine precursor that is normally found in the rhizosphere exudates, the investigation of cyanogenic bacteria may open new fields into biological control (Blumer and Haas 2000). The production of hydrogen sulphide has also been reported in PPN control (Rodriguez-Kabana et al. 1965). This gaseous metabolite is related with plant defence signalling against pathogen attack (Bloem et al. 2012; Calderwood and Kopriva 2014). In the present work, hydrogen sulphide production was positively correlated with the mortality of juvenile *M. ethiopica* and mobile stages of *X. index*.

Concerning the FB37BR isolate, ambiguity was recognized in the phylogenetic characterization because the *16 S rRNA* sequence revealed a high nucleotide identity with two distantly related families (*Bacillaceae* and *Brevibacteriaceae*). Gelsomino et al. (2004) reported that the *Brevibacteriaceae* family is misclassified; in fact, the *Brevibacterium* species belong to the *Bacilliaceae* family. Phylogenetic analysis of the *ITS* region sequence of FB37BR was performed, but information for this genomic region of *Brevibacterium frigoritolerans* was unavailable in the National Center for Biotechnology Information (NCBI). However, *BLAST* analysis restricted to *Brevibacteriaceae* proved 100% identity of the first 160 bp of the *ITS* sequence (data not shown). BBL Crystal plates indicated that the FB37BR isolate belongs to the species *Corynebacterium jeikeum* (97.2% confidence). The *Corynebacterium* sensu lato group includes *Arthrobacter, Brevibacterium, Cellulomonas, Curtobacterium, Microbacterium* and *Corynebacterium* genera. Bacteria of this group are not easily identifiable by classical phenotypic methods, and traditional genotyping methods using the *16 S* gene do not work for all species of the *Brevibacterium* genus (Gelsomino et al. 2004). However, the results of the physiological profile (Table 5) and metabolite production assays (Table 4) determined that FB37BR does not belong to *B. simplex* due to its opposite behaviour in

verified aspects: *B. simplex* does not produce H_2S, casein digestion is variable and it does not metabolize D-xylose (Heyrman 2005). Additionally, *B. simplex* hardly grows at temperatures below 20 °C, having an optimal growth temperature of 30 °C (Sikorski and Nevo 2007), while the FB37BR isolate has the ability to grow at 5 °C but not at temperatures higher than 40 °C. These results consolidate the pertinence of our isolate to *Brevibacterium frigoritolerans*.

For FP805PU isolate identification, a protease assay and growth at extreme temperatures helped to discriminate between *Pseudomonas fluorescens* and *P. brassicacearum*, which are closely related to FP805PU according to the *16 S* and *gyrB* sequences. Background information regarding the *P. fluorescens* strain Pf29Arp (Marchi et al. 2013) indicates that this strain is missing the genes that encode extracellular proteases, while they are present in *P. brassicacearum* strain J12 (Zhou et al. 2012). From this point of view, FP805PU is closer to the Pf29Arp strain since both isolates are missing protease activity. In addition, growth tests at extreme temperatures showed that FP805PU grows well at 5 °C but not at temperatures above 41 °C, a characteristic that also separates our isolate from the *P. brassicacearum* J12 strain, which has the ability to grow from 4 °C to 45 °C (Zhou et al. 2012). Phylogenetic analysis of the *gyrB* sequence identified P805PU as a variant of *P. fluorescens*.

Phylogenetic analysis of concatenated genes (Fig. 1) separated FB25M from other *B. cereus* species and defined the isolate as *B. weihenstephanensis*, which is closely related to *B. mycoides*. The morphologic feature of FB25M on solid media is rhizoid, and authors have stated that the rhizoid appearance is characteristic only of *B. mycoides* (Lechner et al. 1998). On the other hand, the same authors have stated that *B. weihenstephanensis* is the only psychrotolerant species within the *B. cereus* group. The ambiguity that resulted from the psychrotolerance and rhizoid morphology of FB25M was solved by studies carried out by Soufiane and Côté (2013), which proved that more than one genetically linked psychrotolerant species exists within the *B. cereus* group, some of which belong to *B. weihenstephanensis*, questioning that the rhizoid aspect is only typical for *B. mycoides*.

Bacillus megaterium FB133M reduced *M. ethiopica* egg hatching by 60% compared to the untreated control. This value coincides with the control level on *M. graminicola* eggs under in vitro conditions informed by Padgham and Sikora (2007). Although the FB133M filtrate controlled egg hatching, the control effect on *M. ethiopica* J2 larvae was not observed. However, strain FB133M showed a nematicidal effect on *X. index* larvae and adults, as reported in previous studies (Castaneda-Alvarez et al. 2016). In practice, *B. megaterium* is already an active ingredient in commercial products that reduce *M. incognita* J2 populations in the soil (Radwan et al. 2012) and nematode damage in tomato plants.

Brevibacterium frigoritolerans FB37BR had a modest in vitro effect on the nematode compared to strains that reached mortality values of approximately 90% on larvae and adults of *X index* and *M. ethiopica*. However, its control effect on *M. ethiopica* egg hatching was the highest among all analysed strains. FB37BR manifested the ability to grow at temperatures below 10 °C, which can be an interesting aspect worth evaluating at the field level. *B. frigoritolerans* has also been reported for its nematicidal properties on *M. incognita* (Ma et al. 2012), along with entomopathogenic features on first instar larvae of *Anomala dimidiata* and *Holotrichia longipennis* (Selvakumar et al. 2011).

Among the investigated bacteria, the *P. fluorescens* FP805PU strain stood out concerning its nematicidal effect. Furthermore, prominent metabolites and exoenzyme activity were verified, and an extensive field of metabolic options was reflected by its physiological profile. These features may represent an advantage in the process of bacteria establishment in the rhizosphere in the field.

Principal component statistical analysis of the distribution of used carbon sources in the EcoPlates clearly separated the *Bacillus* and *Pseudomonas* genera into two physiologically distinct fields. Considering the six *Bacillus* isolates, three assemblages were delineated according to the used carbon source (Fig. 3). The trends in physiological profiles may generate valuable data about the specific substrates required for bacterial establishment in the rhizosphere. Knowledge about the optimal conditions for bacterial establishment is important for the development of biological products targeted to the rhizosphere. By analysing the characteristic patterns of the EcoPlates, information is generated concerning the most common substrates used by the investigated microorganisms. Ultimately, all the data will be used to promote those specific strains that are physiologically appropriate for different crops and conditions.

The rhizobacteria evaluated during the present work produce metabolites and enzymes with in vitro nematicidal effects on *M. ethiopica* and *X. index*, the most important PPN in Chilean vineyards. The important task of improving and transferring PPN control from the laboratory to the field is the next goal, and steps are already underway. in vitro assays complemented with assays in the greenhouse in the open field are promoting rhizobacteria as a competitive alternative to the use of synthetic nematicidal products. The results indicate that several bacterial enzymes or metabolites may be involved in nematode control, individually or synergistically, and further studies on bacterial metabolism to detect other metabolites, not considered in this research, may generate information and amplify criteria to find new controller strains with broader spectrum over PPN. Physiology studies may contribute to

the identification of rhizobacteria colonization and multiplication patterns, allowing for future selection of strains specialized to certain soil or crop conditions. Nevertheless, long and reliable field assays still need to be considered to introduce their use in the field. Rhizobacteria themselves, as biological controllers or as producers of nematicidal metabolites—if the technological transfer strategies require it—represent a sustainable option against the indiscriminate use of pesticides.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

References

Abad P, Gouzy J, Aury J-M et al (2008) Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nat Biotechnol 26:909–915. doi:10.1038/nbt.1482

Aballay E, Merino C (2015) Efficacy and crop tolerance of 1,3-dichloropropene applied at low rates in established vineyards for the control of plant-parasitic nematodes. Crop Prot 78:78–83. doi:10.1016/j.cropro.2015.08.009

Aballay E, Martensson A, Persson P (2011) Screening of rhizosphere bacteria from grapevine for their suppressive effect on *Xiphinema index* Thorne & Allen on in vitro grape plants. Plant Soil 347:313–325. doi:10.1007/s11104-011-0851-6

Aballay E, Prodan S, Martensson A, Persson P (2012) Assessment of rhizobacteria from grapevine for their suppressive effect on the parasitic nematode *Xiphinema index*. Crop Prot 42:36–41. doi:10.1016/j.cropro.2012.08.013

Aballay E, Ordenes P, Mårtensson A, Persson P (2013) Effects of rhizobacteria on parasitism by *Meloidogyne ethiopica* on grapevines. Eur J Plant Pathol 135:137–145. doi:10.1007/s10658-012-0073-7

Almaghrabi OA, Massoud SI, Abdelmoneim TS (2013) Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions. Saudi J Biol Sci 20:57–61. doi:10.1016/j.sjbs.2012.10.004

Beneduzi A, Ambrosini A, Passaglia LMP (2012) Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. Genet. Mol Biol 35:1044–1051

Bird AF, Bird J (1991) The egg. The structure of nematodes, 2nd edn. Elsevier, London, pp 7–43

Bloem E, Haneklaus S, Kesselmeier J, Schnug E (2012) Sulfur fertilization and fungal infections affect the exchange of H_2S and COS from agricultural crops. J Agric Food Chem 60:7588–7596. doi:10.1021/jf301912h

Blumer C, Haas D (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch Microbiol 173:170–177. doi:10.1007/s002039900127

Brown DJ, Boag B (1988) An examination of methods used to extract virus-vector nematodes (nematoda: longidoridae and trichodoridae) from soil samples. Nematol Mediterr 16:93–99.

Burkett-Cadena M, Kokalis-Burelle N, Lawrence KS et al (2008) Suppressiveness of root-knot nematodes mediated by rhizobacteria. Biol Control 47:55–59. doi:10.1016/j.biocontrol.2008.07.008

Calderwood A, Kopriva S (2014) Hydrogen sulfide in plants: from dissipation of excess sulfur to signaling molecule. Nitric Oxide Biol Chem 41:72–78. doi:10.1016/j.niox.2014.02.005

Castaneda-Alvarez C, Prodan S, Rosales IM, Aballay E (2016) Exoenzymes and metabolites related to the nematicidal effect of rhizobacteria on *Xiphinema index* Thorne & Allen. J Appl Microbiol 120:413–424. doi:10.1111/jam.12987

Collange B, Navarrete M, Peyre G et al (2011) Root-knot nematode (*Meloidogyne*) management in vegetable crop production: the challenge of an agronomic system analysis. Crop Prot 30:1251–1262. doi:10.1016/j.cropro.2011.04.016

Costa SR, Kerry BR, Bardgett RD, Davies KG (2012) Interactions between nematodes and their microbial enemies in coastal sand dunes. Oecologia 170:1053–1066. doi:10.1007/s00442-012-2359-z

Curtis RHC, Jones JT, Davies KG et al (2011) Plant nematode surfaces. In: Davies K, Spiegel Y (eds) Biological control of plant-parasitic nematodes: building coherence between microbial ecology and molecular mechanisms, progress in biological control. Springer, Dordrecht, p 311

Decraemer W, Hunt DJ (2006) Structure and Classification. In: Perry RN, Moens M (eds) Plant Nematology, 2nd edn. CABI, Wallingford, p 447

dos Santos LF, Defrenne L, Krebs-Brown A (2002) Comparison of three microbial assay procedures for measuring toxicity of chemical compounds: ToxAlert®10, CellSense and Biolog MT2 microplates. Anal Chim Acta 456:41–54. doi:10.1016/S0003-2670(01)00907-2

Fiore N, Zamorano A, Rivera L et al (2011) Grapevine viruses in the Atacama region of Chile. J Phytopathol 159:743–750. doi:10.1111/j.1439-0434.2011.01834.x

Galper S, Cohn E, Chet I (1990) Nematicidal effect of collagen-amended soil and the influence of protease and collagenase. Rev Nematol 13:67–71.

Gelsomino R, Vancanneyt M, Vandekerckhove TM, Swings J (2004) Development of a 16 S rRNA primer for the detection of *Brevibacterium* spp. Lett Appl Microbiol 38:532–535. doi:10.1111/j.1472-765X.2004.01533.x

Helgason E, Tourasse NJ, Meisal R et al (2004) Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. Appl Environ Microbiol 70:191–201. doi:10.1128/AEM.70.1.191-201.2004

Herrera-Estrella A, Chet I (1999) Chitinases in biological control. In: Jollès P, Muzzarelli RAA (eds) Chitin and Chitinases. Birkhauser, Basel, pp 171–184

Heyrman J (2005) Study of mural painting isolates, leading to the transfer of "*Bacillus maroccanus*" and "*Bacillus carotarum*" to *Bacillus simplex*, emended description of *Bacillus simplex*, re-examination of the strains previously attributed to "*Bacillus macroides*" and descr. Int J Syst Evol Microbiol 55:119–131. doi:10.1099/ijs.0.63221-0

Huettel R, Rebois R (1985) Culturing plant parasitic nematodes using root explants. In: Zuckerman BM, Mai MBH WF (eds) Plant nematology: laboratory manual. University Of Massachusetts Agricultural Experiment Station, Amherst, pp 155–158

Hussey RS, Barker KR (1973) A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Dis Report 57:1025–1028.

Ki JS, Zhang W, Qian PY (2009) Discovery of marine *Bacillus* species by 16S rRNA and rpoB comparisons and their usefulness

for species identification. J Microbiol Methods 77:48–57. doi:10.1016/j.mimet.2009.01.003

Kloepper JW, Rodríguez-Kábana R, McInroy JA, Collins DJ (1991) Analysis of populations and physiological characterization of microorganisms in rhizospheres of plants with antagonistic properties to phytopathogenic nematodes. Plant Soil 136:95–102. doi:10.1007/BF02465224

Ko KS, Kim JW, Kim JM et al (2004) Population structure of the *Bacillus cereus* group as determined by sequence analysis of six housekeeping genes and the plcR Gene. Infect Immun 72:5253–5261. doi:10.1128/IAI.72.9.5253

Lane DJ (1991) 16 S/23 S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics, 1st edn. Wiley, New York, pp 115–175

Lane J, Pace B, Olsen GJ et al (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955–6959

Lechner S, Mayr R, Francis KP et al (1998) *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int J Syst Bacteriol 48(Pt 4):1373–1382

Lian LH, Tian BY, Xiong R et al (2007) Proteases from Bacillus: a new insight into the mechanism of action for rhizobacterial suppression of nematode populations. Lett Appl Microbiol 45:262–269. doi:10.1111/j.1472-765X.2007.02184.x

Lopez-Llorca LV, Maciá-Vicente JG, Jansson H-B (2008) Mode of action and interactions of nematophagous fungi. In: Ciancio A, Mukerji KG (eds) Intregated management and biocontrol or vegetable and grain crops nematodes. Springer, Berlin, p 356

Ma L, Fu W, Huang Y et al (2012) A strategy to discover potential nematicidal fumigants based on toxic volatiles from nematicidal bacteria. African J Microbiol Res 6:6106–6113. doi:10.5897/AJMR12.1328

Manzano M, Cocolin L, Cantoni C, Comi G (2003) *Bacillus cereus, Bacillus thuringiensis and Bacillus mycoides* differentiation using a PCR-RE technique. Int J Food Microbiol 81:249–254. doi:10.1016/S0168-1605(02)00222-2

Marchi M, Boutin M, Gazengel K, et al (2013) Genomic analysis of the biocontrol strain *Pseudomonas fluorescens* Pf29Arp with evidence of T3SS and T6SS gene expression on plant roots. Environ Microbiol Rep 5:393–403. doi:10.1111/1758-2229.12048

McBride RS, Edwards JD (1914) The lead acetate test for hydrogen sulphide in gas. J Franklin Inst 178:639–642. doi:10.1016/S0016-0032(14)90280-0

McSorley R, Wang K, Kokalis-Burelle N, Church G (2006) Effects of soil type and steam on nematode biological control potential of the rhizosphere community. Nematropica 36:197–214.

McSorley R, Wang K-H, Church G (2008) Suppression of root-knot nematodes in natural and agricultural soils. Appl Soil Ecol 39:291–298. doi:10.1016/j.apsoil.2008.01.002

Millar RL, Higgins VJ (1970) Association of Cyanide with Infection of birdsfoot trefoil by *Stemphylium loti*. Phytopathology 60:104. doi:10.1094/Phyto-60-104

Mnif I, Ghribi D (2015) Potential of bacterial derived biopesticides in pest management. Crop Prot 77:52–64. doi:10.1016/j.cropro.2015.07.017

Morgulis A, Coulouris G, Raytselis Y et al (2008) Database indexing for production MegaBLAST searches. Bioinformatics 24:1757–1764. doi:10.1093/bioinformatics/btn322

Omarjee J, Balandreau J, Spaull VW, Cadet P (2008) Relationships between *Burkholderia* populations and plant parasitic nematodes in sugarcane. Appl Soil Ecol 39:1–14. doi:10.1016/j.apsoil.2007.11.001

Padgham JL, Sikora RA (2007) Biological control potential and modes of action of *Bacillus megaterium* against *Meloidogyne graminicola* on rice. Crop Prot 26:971–977. doi:10.1016/j.cropro.2006.09.004

Paiva G, Proença DN, Francisco R et al (2013) Nematicidal bacteria associated to pinewood nematode produce extracellular proteases. PLoS ONE 8:e79705. doi:10.1371/journal.pone.0079705

Radwan MA, Farrag SAA, Abu-Elamayem MM, Ahmed NS (2012) Biological control of the root-knot nematode, *Meloidogyne incognita* on tomato using bioproducts of microbial origin. Appl Soil Ecol 56:58–62. doi:10.1016/j.apsoil.2012.02.008

Ray C, Hussey RS (1995) Evidence for proteolytic processing of a cuticle collagen in a plant-parasitic nematode. Mol Biochem Parasitol 72:243–246

Rodriguez-Kabana R, Jordan JW, Hollis JP (1965) Nematodes: biological control in rice fields: role of hydrogen sulfide. Science (80-) 148:524–526. doi:10.1126/science.148.3669.524

Romanowski A, Migliori ML, Valverde C, Golombek D a (2011) Circadian variation in *Pseudomonas fluorescens* (CHA0)-mediated paralysis of *Caenorhabditis elegans*. Microb Pathog 50:23–30. doi:10.1016/j.micpath.2010.09.001

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. doi:10.1093/oxfordjournals.molbev.a040454

Sarma BK, Yadav SK, Singh S, Singh HB (2015) Microbial consortium-mediated plant defense against phytopathogens: readdressing for enhancing efficacy. Soil Biol Biochem 87:25–33. doi:10.1016/j.soilbio.2015.04.001

Schenborn ET, Mierendorf RC (1985) A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure. Nucleic Acids Res 13:6223–6236

Selvakumar G, Sushil SN, Stanley J, et al (2011) *Brevibacterium frigoritolerans* a novel entomopathogen of *Anomala dimidiata* and *Holotrichia longipennis* (Scarabaeidae: Coleoptera). Biocontrol Sci Technol 21:821–827. doi:10.1080/09583157.2011.586021

Siddiqui IA (2000) Effects of cell suspension and cell-free culture filtrate of *Pseudomonas aeruginosa* in the control of root rot-root knot disease complex of tomato (*Lycopersicon esculentum* Mill.). Acta Agrobot 53:47–55.

Siddiqui Z a., Akhtar MS (2009) Effect of plant growth promoting rhizobacteria, nematode parasitic fungi and root-nodule bacterium on root-knot nematodes *Meloidogyne javanica* and growth of chickpea. Biocontrol Sci Technol 19:511–521. doi:10.1080/09583150902887792

Siddiqui IA, Shaukat S (2003) Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: importance of bacterial secondary metabolite, 2,4-diacetylpholoroglucinol. Soil Biol Biochem 35:1615–1623. doi:10.1016/j.soilbio.2003.08.006

Siddiqui IA, Shaukat SS, Khan GH, Ali NI (2003) Suppression of *Meloidogyne javanica* by *Pseudomonas aeruginosa* IE-6S+ in tomato: the influence of NaCl, oxygen and iron levels. Soil Biol Biochem 35:1625–1634. doi:10.1016/j.soilbio.2003.08.007

Siddiqui IA, Haas D, Heeb S (2005) Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*. Appl Environ Microbiol 71:5646–5649. doi:10.1128/AEM.71.9.5646-5649.2005

Sikorski J, Nevo E (2007) Patterns of thermal adaptation of *Bacillus simplex* to the microclimatically contrasting slopes of "Evolution Canyons" I and II, Israel. Environ Microbiol 9:716–726. doi:10.1111/j.1462-2920.2006.01193.x

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Gerhardt P (ed) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC, p 791

Soufiane B, Côté J-C (2013) *Bacillus weihenstephanensis* characteristics are present in *Bacillus cereus* and Bacillus mycoides strains. FEMS Microbiol Lett 341:127–137. doi:10.1111/1574-6968.12106

Stirling GR (2014) Biological control of plant-parasitic nematodes: soil ecosystem management in sustainable agriculture. CABI, Wallingford

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035. doi:10.1073/pnas.0404206101

Tamura K, Stecher G, Peterson D et al (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. doi:10.1093/molbev/mst197

Thakore Y (2006) The biopesticide market for global agricultural use. Ind Biotechnol 2:194–208. doi:10.1089/ind.2006.2.194

Tikhonov VE, Lopez-Llorca LV, Salinas J, Jansson H-B (2002) Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. Fungal Genet Biol 35:67–78. doi:10.1006/fgbi.2001.1312

Wei L, Shao Y, Wan J et al (2014) Isolation and characterization of a rhizobacterial antagonist of root-knot nematodes. PLoS ONE 9:e85988. doi:10.1371/journal.pone.0085988

Xu D, Coté J-C (2003) Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences. Int J Syst Evol Microbiol 53:695–704. doi:10.1099/ijs.0.02346-0

Yamamoto S, Harayama S (1995) PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of Pseudomonas putida strains. Appl Environ Microbiol 61:1104–1109

Zhang S, Gan Y, Xu B, Xue Y (2014) The parasitic and lethal effects of *Trichoderma longibrachiatum* against *Heterodera avenae*. Biol Control 72:1–8. doi:10.1016/j.biocontrol.2014.01.009

Zhou T, Chen D, Li C et al (2012) Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. Microbiol Res 167:388–394. doi:10.1016/j.micres.2012.01.003