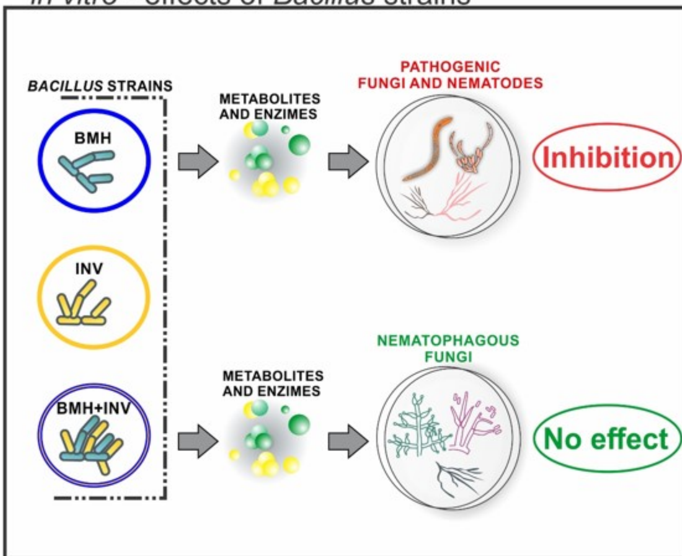
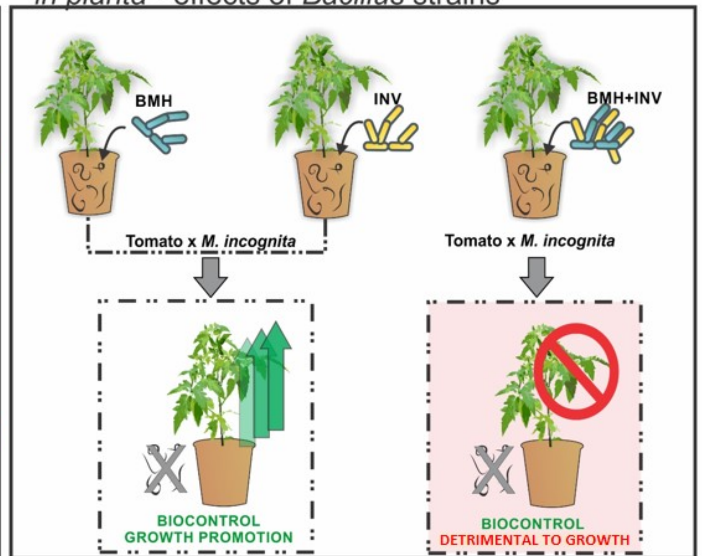
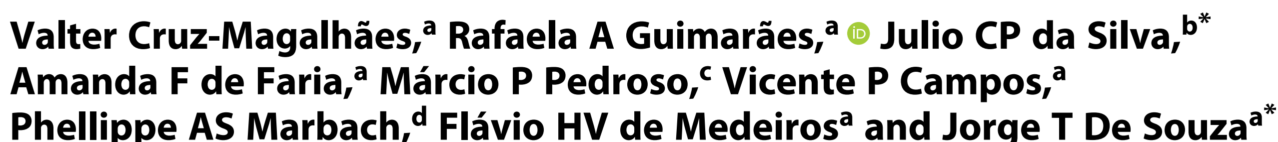

in vitro - effects of Bacillus strains
BACILLUS STRAINS
BMH
INV
BMH+INV
METABOLITES AND ENZIMES
PATHOGENIC FUNGI AND NEMATODES
Inhibition
NEMATOPHAGOUS FUNGI
No effect


in planta - effects of Bacillus strains
BMH
INV
BMH+INV
Tomato x M. incognita
Tomato x M. incognita
BIOCONTROL
GROWTH PROMOTION
BIOCONTROL
DETRIMENTAL TO GROWTH

Received: 6 July 2021 Revised: 18 September 2021 Accepted article published: 24 October 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.6685

The combination of two *Bacillus* strains suppresses *Meloidogyne incognita* and fungal pathogens, but does not enhance plant growth



Valter Cruz-Magalhães,[a] Rafaela A Guimarães,[a] Julio CP da Silva,[b*] Amanda F de Faria,[a] Márcio P Pedroso,[c] Vicente P Campos,[a] Phellippe AS Marbach,[d] Flávio HV de Medeiros[a] and Jorge T De Souza[a*]

Abstract

BACKGROUND: The combination of biocontrol agents is a desirable strategy to improve control efficacy against the root-knot nematode (RKN) *Meloidogyne incognita* under field conditions. However, strains compatibility is generally tested *in vitro* and incompatible combinations are normally not further examined in experiments *in planta*. Therefore, there is virtually no information on the performance of incompatible strains. In this study, we evaluated two *Bacillus* strains previously described as incompatible *in vitro* for effects on plant growth and suppression of *M. incognita*, pathogenic fungi and nematophagous fungi.

RESULTS: Strains BMH and INV were shown to be closely related to *Bacillus velezensis*. These strains, when applied individually, reduced the number of galls and eggs of *M. incognita* by more than 90% in tomato roots. When BMH and INV were combined (BMH + INV), RKN suppression and tomato shoot weight were lower compared to single-strain applications. Additionally, metabolites in cell-free supernatants and volatile organic compounds (VOCs) from strains BMH and INV had strong effects against the plant pathogens *M. incognita*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsiii*, but not against three species of nematophagous fungi. Although strain INV and the combination BMH + INV emitted fewer VOCs than strain BMH, they were still capable of killing second-stage juveniles of *M. incognita*.

CONCLUSIONS: *Bacillus* strains BMH and INV inhibited *M. incognita* and fungal pathogens, and promoted tomato growth. However, strain INV emitted fewer VOCs and the combination BMH + INV did not enhance the activity of the biocontrol strains against the RKN or their capacity to promote plant growth.
© 2021 Society of Chemical Industry.
Supporting information may be found in the online version of this article.

Keywords: biocontrol; root-knot nematodes; *Bacillomycin*; metabolites; volatile organic compounds

1 INTRODUCTION

Plant-parasitic nematodes are estimated to cause billions of dollars in agricultural losses.[1] The root-knot nematodes (RKN; *Meloidogyne* spp.) are responsible for causing great losses to horticulture and grain crops worldwide.[2,3] *Meloidogyne incognita* is by far the most widely distributed and destructive species due to its extensive host range.[4] Nematode control strategies are mainly based on the use of resistant varieties, crop rotation and soil sterilization by chemicals or solarization.[5] The use of resistance genes is limited or impractical in annual crops due to few RKN-resistance genes and the difficulty of introducing them into susceptible crops.[6] Also, the effectiveness of crop rotation is not always successful due to the wide host range of RKN, while chemical nematicides can have negative impacts on the local microbiota.[7–9] For these reasons, the demand for chemical-free agricultural products has increased. Therefore, the use of microbial-based products in agricultural systems to control RKNs is of great interest.[3,9–12] In addition to the possibility of promoting plant growth, biocontrol agents can also stimulate microbial interactions.[13]

* Correspondence to: JCP da Silva, Department of Phytosanitary Defense, CCR, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil, E-mail: julio.c.silva@ufsm.br; or JT de Souza, Department of Phytopathology, Universidade Federal de Lavras, Lavras, MG, Brazil. E-mail: jorge.souza@ufla.br

a Department of Phytopathology, Universidade Federal de Lavras, Lavras, Brazil

b Department of Phytosanitary Defense, CCR, Universidade Federal de Santa Maria, Santa Maria, Brazil

c Department of Chemistry, Universidade Federal de Lavras, Lavras, Brazil

d Center for Agricultural, Biological and Environmental Sciences, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, Brazil

 © 2021 Society of Chemical Industry.

Species of the genus *Bacillus* occur in diverse types of agricultural soils, associated with different plant species, in different environmental conditions and play an important role in increasing crop productivity.[14–16] *Bacillus* spp. have a great ability to colonize roots and versatility in protecting plants against pathogens by multiple mechanisms.[17–21] Currently, there are more than 300 validly described species divided into two species complexes: the *Bacillus subtilis* group and the *Bacillus cereus* group.[22] The beneficial effects associated with plants make this genus one of the most interesting for the development of biological control agents.[23,24] Several strains of *Bacillus* spp. are used as the basis for different commercial formulations aimed at promoting plant growth and plant disease control,[23,25–28] including nematode-induced diseases.[10,29–33]

Bacillus-based products represent approximately half of the commercially available bacterial biological control agents.[12] However, the application of *Bacillus* can cause dramatic changes in rhizosphere microbial populations.[34] Studies on the effects of *Bacillus* are generally focused on the reduction of pathogen populations and ignore the potential effects on the structure and function of the microbial community.[35] *Bacillus* may also suppress beneficial fungi, such as RKN parasites, requiring further investigation. This interaction should be better explored to improve the biocontrol of RKN. Furthermore, as different species of *Bacillus* possess different mechanisms, the combination of strains is an alternative to improve the biocontrol effects.[29] Usually, the compatibility of combined strains is associated with an increase in disease control by the synergism between the produced metabolites and other possible mechanisms.[36] However, bacterial strains labeled as incompatible are normally evaluated only by *in vitro* assays and under limited conditions to effectively prove their incompatibility.[37,38] There is little information on the behavior of incompatible strains combined beyond *in vitro* tests. *In planta* assays are more appropriate to verify compatibility among strains by evaluating the mechanisms affected by the combination.

Keeping in mind all these aspects of *Bacillus* spp. as biocontrol agents, it is important not only to verify the biocontrol performance of new strains against RKN, but also to understand the mechanisms of action and their interactions with other microorganisms and among themselves. In this study, *Bacillus* strains BMH and INV were used to assess their biocontrol activity against *M. incognita* either alone or in combination and to verify their possible biocontrol mechanisms. Strains BMH and INV were shown to be incompatible in previous *in vitro* tests[38] and therefore not further studied in combination in *in planta* experiments. Also, the biocontrol potential of these strains has not been verified against RKN. The focus of the present study was on the combination of these two *Bacillus* strains in experiments against *M. incognita* utilizing *in vitro* and *in planta* experiments. Additionally, we evaluated the effects of the *Bacillus* strains against selected pathogenic and beneficial fungi.

2 MATERIAL AND METHODS

2.1 Bacterial strains, mass production and cell-free supernatants

Bacillus strains BMH and INV were isolated from soil of the semiarid region in the northeast of Brazil.[38] The strains were routinely grown on Luria-Bertani (LB) agar.[39,40] Mass production of the strains was done in Erlenmeyer flasks containing 75 mL of nutrient (N) broth medium[41] incubated at 25 °C with shaking (150 rpm) for 48 h. The correspondence between optical density in a spectrophotometer at 600 nm (OD_{600}) and dilution plating on nutrient agar (NA)[41] was determined for both bacterial strains. An $OD_{600} = 0.7$ for both bacterial strains corresponded to approximately 10^8 CFU mL^{-1}. This equivalence was used to facilitate the preparation of the bacterial suspensions. To produce the cell-free supernatants, each strain was grown in N broth as described above and 48 h later the cultures were adjusted to the same cell concentration (10^8 CFU mL^{-1}) and centrifuged twice at 4500 rpm for 5 min. The pelleted cells were discarded and the cell-free supernatants were used in the experiments. The mixture of strains BMH + INV was prepared by combining equal volumes of each strain. Only supernatants without any bacterial growth were used in the experiments, which was confirmed by plating aliquots of each bacterial suspension on NA and incubating at 25 °C for 24 h.

2.2 Molecular identification of *Bacillus*

Strains BMH and INV were grown on NA medium for 24 h at 25 °C and used for genomic DNA extraction as previously described.[41] Briefly, a loopful of each strain was transferred to 1.5-mL microcentrifuge tubes containing 100 μL of extraction buffer (0.05 mol L^{-1} NaOH + 0.25% SDS). The tubes were incubated at 97 °C under agitation (800 rpm) for 15 min, cooled to room temperature for 2 min and centrifuged at 10 000 rpm for 1 min. The DNA in the supernatant was diluted 20× in TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) and stored at −20 °C until use. The universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) were used to amplify and sequence the 16S rRNA gene as previously described.[42,43] Different primers were used to detect biosynthetic antibiotic genes by PCR-based screening and amplify the Zwittermicin-A resistance gene (Supplementary Text). Sequences were obtained using an ABI370 sequencer following the manufacturer's instructions (Applied Biosystems, Waltham, MA, USA). Comparisons with other sequences deposited in the nonredundant database were done with the BLASTN algorithm.[44] The sequences were deposited in GenBank under the following accession numbers: KU207996 (*Bacillus* sp. BMH) and KU207997 (*Bacillus* sp. INV). The program MEGA v.7[45] was used in the alignment and to perform the phylogenetic analyses with the maximum likelihood method. All the other 16S rRNA sequences used in the phylogenetic analysis were from type strains recovered from the List of Prokaryotic names with Standing in Nomenclature (LPSN).[46]

2.3 Effect of *Bacillus* on tomato growth

Two-week-old tomato seedlings (*Solanum lycopersicum* cv. Santa Clara) were transplanted to 500 mL cups containing sterile substrate (60% pine bark, 15% vermiculite and 25% humus; Terra do Paraíso; Holambra, SP, Brazil). For inoculation of *Bacillus* spp., an aliquot of 200 μL of suspension of each strain containing 10^8 CFU mL^{-1} was inoculated in each pot around the tomato roots after 2 days of seedling tranplantation. The treatments included strains BMH, INV, BMH + INV and the control, where water without any bacterial strain was applied. Pots were arranged in a completely randomized design and kept in a greenhouse for 45 days at 25 ± 2 °C, where they received irrigation and fertilization according to the technical recommendations.[46] After this period, plants were removed from the pots and shoots were separated from the roots. The roots were carefully washed and dried on paper towels. The plant parts were dried at 60 °C for 72 h and the dry weight of shoots and roots was recorded after reaching constant weight. The experiments were conducted with

© 2021 Society of Chemical Industry.

five replicates per treatment and the experiment was performed twice at different times.

2.4 Effect of *Bacillus* against *Meloidogyne incognita*

The second-stage juveniles (J_2) of *M. incognita* used in the experiments were obtained from a pure population multiplied in tomato 'Santa Clara' maintained in a greenhouse for 2 months. To obtain eggs, the roots were gently washed, cut into 1- to 2-cm pieces and ground in a blender with a 0.5% NaClO solution for 30 s. The eggs were then separated from the root debris by centrifugation[47] and incubated in hatching chambers at 28 °C. Only *M. incognita* J_2 hatched within a 24-h period were used in the experiments. Tomato seedlings and the same bacterial treatments were prepared as described above for the experiments on the effect of *Bacillus* on plant growth. Two days after the application of the bacteria, plants were inoculated with 2 mL of a suspension containing 100 *M. incognita* J_2 around the roots. The negative control was only the *M. incognita* J_2 suspension in water without any bacterial strain. Pots were arranged in a completely randomized design and kept in a greenhouse for 45 days at 25 ± 2 °C as described above. After this period, the number of galls on roots was determined by direct counting. Eggs were extracted as described by Boneti and Ferraz,[47] and enumerated in a Peters chamber under a light microscope. The experiment was conducted with five replicates per treatment and was performed twice.

2.5 Effect of bacterial supernatants and VOCs against *Meloidogyne incognita*

The effect of *Bacillus* cell-free supernatants and emission of volatile organic compounds (VOCs) were evaluated by two different techniques. To evaluate the cell-free supernatants on the motility and mortality of *M. incognita*, aliquots of 100 μL of the supernatant of each bacterial treatment (BMH, INV and BMH + INV) prepared as described above were mixed with 900 μL of an aqueous suspension containing 100 *M. incognita* J_2 in 1.5 mL microcentrifuge tubes. Controls contained only 100 μL of N broth medium mixed with the suspension. The microcentrifuge tubes were incubated at 25 °C for 48 h. The effect of VOCs produced by *Bacillus* strains on the motility and mortality of *M. incognita* was studied in bi-compartmented Petri plates.[46] Aliquots of 100 μL of suspensions of BMH, INV or BMH + INV were spread on NA medium in one compartment of the Petri plate. A 2 mL suspension containing 100 *M. incognita* J_2 was placed in the other compartment of the same plate. In control plates, 2 mL of water were placed on the NA medium in one of the compartments and the *M. incognita* J_2 suspension in the other compartment. The plates were sealed with parafilm and incubated at 25 °C for 48 h.

In both experiments, after 48 h of incubation, the J_2 were washed with tap water through a 500 mesh (25 μm opening) sieve to remove the metabolites from the suspension and collected in tap water. Then, the motility and mortality of *M. incognita* J_2 were evaluated by counting the number of immotile *M. incognita* J_2 in a Peters chamber. The *M. incognita* J_2 that remained immotile after 24 h of the first count were considered dead. The experiments were arranged in a completely randomized design with five replicates and the experiments were conducted twice.

2.6 Effect of supernatants and VOCs against fungi

The effect of cell-free supernatants and VOCs from BMH, INV and BMH + INV was evaluated on mycelial growth and conidia formation by plant pathogens and biocontrol agents. Three tomato pathogens, *Fusarium oxysporum* f.sp. *lycopersici* CML1875, *Rhizoctonia solani* CML551 and *Sclerotium rolfsii* VC01, and three fungi antagonistic to RKN, *Trichoderma atroviride* IMI206040, *Purpureocillium lilacinum* BC01 and *Arthrobotrys conoides* CML1659, were used in these experiments. All fungi were grown on potato dextrose agar (PDA) for 5 days at 25 °C.[48] Fungal mycelial discs, 5 mm in diameter, obtained from the edges of actively growing colonies, were transferred to the center of PDA plates. After 2 h, 10 μL aliquots of cell-free supernatants from the bacterial strains and their mixture were deposited on the mycelial discs. To evaluate the effect of VOCs on mycelial growth, aliquots of 100 μL of suspension from each bacterial strain and their mixture adjusted to 10^8 CFU mL^{-1} were spread on NA medium in one of the compartments of split Petri plates. The other compartment, containing PDA medium, received a mycelial disc from the fungi listed above. Plates with each fungus in one compartment and a noninoculated NA medium in the other compartment were used as controls.

The plates were incubated at 25 °C and the final evaluation was performed when the fungus in the control treatment reached at least one of the edges of the plate or after 7 days. Conidia formation was analyzed 2 days after the evaluation of mycelial growth by extracting discs of 5 mm from the edge of each sporulating fungal culture and counting in a Neubauer chamber. The experiments were arranged in a completely randomized design with five replicates and were conducted twice.

2.7 Detection of enzymes, siderophores and phosphate solubilization activity

Assays to detect the production of cellulases, chitinases, lipases, siderophores and phosphate solubilization activity were done on media supplemented with the substrate of each enzyme under study and 20 g L^{-1} of agar. All experiments were carried out by transferring aliquots of 8 μL of bacterial suspensions (10^8 CFU mL^{-1}) to four equidistant points on a Petri plate and incubating at 25 °C as previously described.[49] The cellulolytic activity was evaluated on the medium described by Mandels and Reese,[50] supplemented with carboxymethylcellulose (CMC; Sigma-Aldrich, Burlington, MA, USA) according to Teather and Wood.[51] Proteases were detected on a medium containing skimmed milk, as described by Dune *et al*.,[52] and visualized after 72 h of incubation. Chitinases were detected after 7 days of incubation on media containing colloidal chitin.[53] Lipases were detected by incubating the bacteria for 7 days on a medium containing Tween 80.[54] Siderophores were detected by incubating the bacteria for 24 h on a medium containing Chrome Azurol S (CAS).[55] Phosphate solubilization was evaluated by incubating for 10 days on medium GL (glucose yeast medium).[56,57] The formation of a halo around the colonies was indicative of positive reactions. Four replicates consisting of one plate per replicate were used for each assay.

2.8 Identification of VOCs produced by *bacillus*

For chromatographic analyses of the VOCs, strains BMH, INV and BMH + INV were grown for 2 days at 25 °C on NA medium placed inside Supelco tubes (Supelco Inc., Bellefonte, PA, USA). The

extraction was done by solid-phase microextraction (SPME) and analyzed by gas chromatography coupled to a mass spectrometer (GC/MS). NA medium without the bacterial strains was the negative control. A 2 cm SPME fiber (Supelco Inc.,) coated with divinylbenzene/polydimethylsiloxane/carboxen (DVB/PDMS/CAR) was used for the extraction of the VOCs. The SPME fiber was exposed to the headspace of the Supelco tube for 35 min at 55 °C and then inserted into the GC/MS injector for analyte desorption (2 min), separation and detection. The GC–MS system consisted of a Shimadzu GCMS QP2010 Ultra (Shimadzu, Columbia, MD, USA) equipped with a split-splitless injector, an AOC-5000 autoinjector and an HP-5MS (5% phenyl-95% dimethylsiloxane) fused-silica capillary column (30 m × 0.25 mm × 0.25 μm). Helium 5.0 grade was used as carrier gas at 1.0 mL min^{-1}. The injector was operated in splitless mode. The injector, the transfer line and the ion source were kept at 250, 240, and 200 °C, respectively. The oven temperature was programmed from 40 to 160 °C at 3 °C min^{-1} and then to 240 °C at 10 °C min^{-1}. Mass spectrometry scan range was set between 40 and 400 *m/z*. To identify the VOCs in the samples, the mass spectrum of each chromatogram peak was extracted through the Automated Mass Spectral Deconvolution and Identification System (AMDIS) v. 2.63. The VOC identification was performed by comparing the mass spectra of the sample peaks with National Institute of Standards and Technology (NIST) library spectra by the Mass Spectral Search Program (NIST, Washington, DC, USA) and by comparing experimentally obtained retention indices (RI Exp.) with the retention indices in the literature (RI Lit.).[58,59]

2.9 Statistical analysis

All the data sets were tested for normality (Shapiro–Wilk's test) and homogeneity (Bartlett's test). Once the assumptions were met, the F test was applied through analyses of variance (ANOVA). The experiment repetitions (experiments 1 and 2) were submitted to ANOVA and if there was no difference between them, a combined analysis was performed ($N = 10$). When the significance level in the F test ($P < 0.05$) was reached, means of each treatment were compared with the Tukey's test at 5% probability. The multivariate ordination was done by a principal components analysis (PCA) in the software PAST 4.0.[60]

3 RESULTS

3.1 *Bacillus* BMH and INV are closely related to *B. velezensis*

The 16S rRNA phylogenetic tree showed that strains BMH and INV belong in the clade containing sequences of type strains of *Bacillus amyloliquefaciens*, *B. siamensis* and *B. velezensis* deposited in the LPSN database (Fig. 1). The identity between strains BMH and INV was 99.1%, whereas the identity between each of these strains and the two closest matches in the LPSN database, which were the type strains *B. amyloliquefaciens* NBRC15535 and *B. velezensis* NRRL B-41580, was 99.4% for strain BMH and 99.5% for strain INV. These identities show that strains BMH and INV are closely related to *B. velezensis* and the other species in this clade.

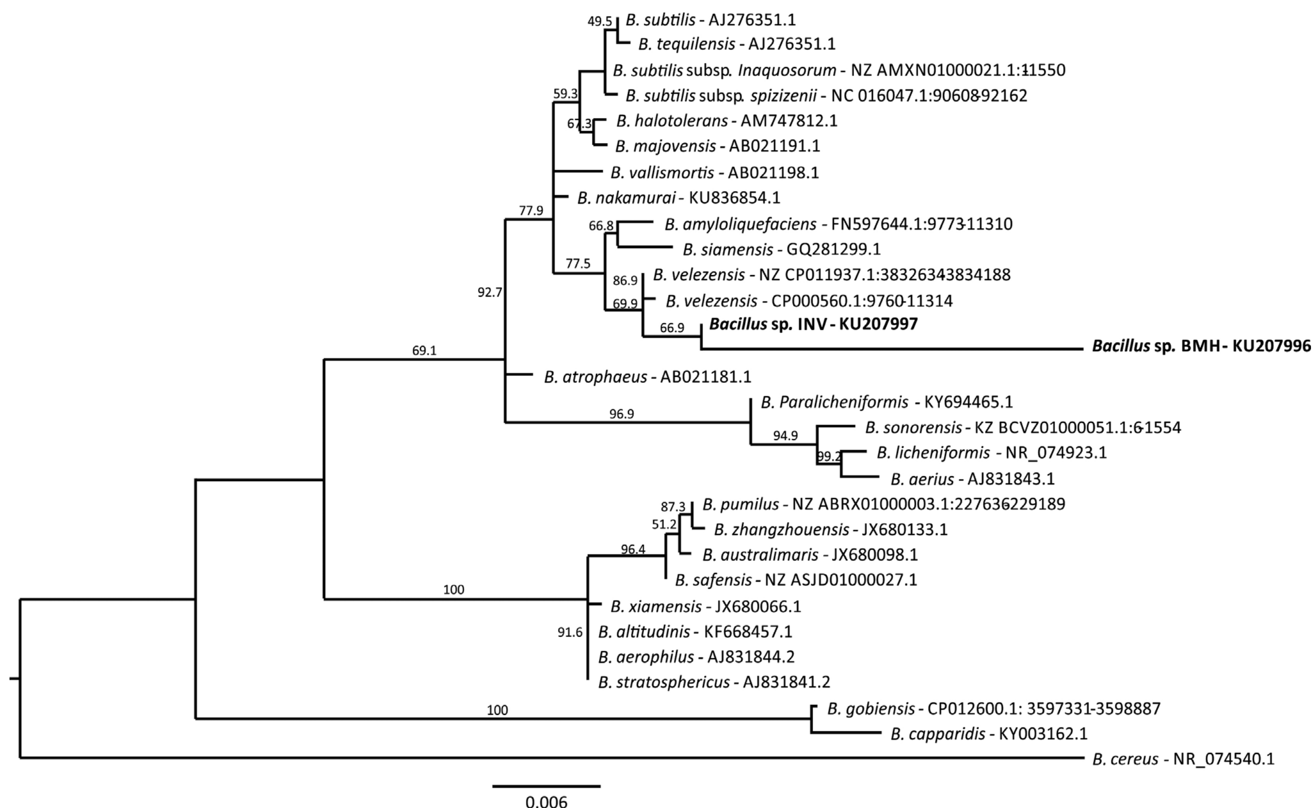


Figure 1. Phylogenetic tree showing the relationship of strains BMH and INV with species of the genus *Bacillus*. The tree was generated with 16S rDNA sequences of type strains available on the LPSN site (List of Prokaryotic Names with Standing in Nomenclature). The molecular phylogenetic analysis was constructed by using the maximum likelihood method. The tree was inferred with 1343 aligned nucleotides. The nucleotide substitution model used was GTR + G + I. The bootstrap analyses were performed with 1000 replicates. The tree was rooted with a 16S rDNA sequence of *Bacillus cereus*. The scale bar represents the number of expected substitutions per site. The analysis was conducted in MEGA v.7[45] and the tree was edited using the FigTree v1.4.4 program.[61]

 © 2021 Society of Chemical Industry.

Table 1. Fresh and dry shoot weight of tomato seedlings inoculated with different *Bacillus* strains

	Shoot weight (g)			
	Experiment 1[†]		Experiment 2	
Strains	Fresh ± SEM[‡]	Dry ± SEM	Fresh ± SEM	Dry ± SEM
BMH	12.1 ± 0.31 a	01.6 ± 0.06 a	09.4 ± 0.21 a	02.7 ± 0.07 a
INV	12.0 ± 0.33 a	01.6 ± 0.05 a	09.8 ± 0.53 a	02.8 ± 0.03 a
BMH + INV	10.3 ± 0.40 b	01.2 ± 0.10 b	06.5 ± 0.62 b	02.3 ± 0.34 ab
Water	10.4 ± 0.24 b	01.2 ± 0.05 b	08.4 ± 0.31 ab	02.2 ± 0.23 ab
F (*P* value)	6.11 (<0.01)	9.38 (<0.01)	3.43 (0.04)	3.70 (0.02)

[†] Experiments were done twice at different times.
[‡] Mean values followed by the same letter are not significantly different according to Tukey's test at 5%. SEM represents the standard error of the means.

3.2 *Bacillus* BMH and INV promote shoot growth when applied individually, but not in combination

Inoculation of the bacterial strains on the roots of tomato increased shoot weight compared with the water control in both experiments (Table 1). Strains BMH and INV increased fresh shoot weight by 27% and dry shoot weight by 25% compared to the water control. In general, shoot weight did not differ from the control when the BMH + INV was applied (Table 1). Fresh and dry weights of tomato roots did not differ from the water control in any of the bacterial treatments ($P > 0.05$).

3.3 The *Bacillus* strains interfere in *Meloidogyne incognita* suppression

All bacterial treatments significantly reduced the number of *M. incognita* galls and eggs compared with the water control in both experiments ($P < 0.01$) (Fig. 2). Individual applications of both strains BMH and INV reduced the numbers of galls and eggs by more than 93% in relation to the control. In the same experiment the combination BMH + INV reduced the number of galls to levels similar to that achieved by the application of the strains alone, but the number of eggs was reduced only by 84%. In the second experiment, the reduction in the number of galls and eggs was on average 79% for BMH and INV, and approximately 60% for the BMH + INV. In general, these experiments show that the application of the strains alone or in combination resulted in a reduction in the number of galls and eggs of *M. incognita* in relation to the control, but the combination BMH + INV was worse than single applications.

3.4 Bacterial cell-free supernatants and VOCs had pronounced effects against *M. incognita* and fungal pathogens

The cell-free supernatants of all bacterial treatments significantly ($P < 0.01$) increased immotility and the mortality of *M. incognita* J_2 compared with the controls (Fig. 3(A)). The VOCs produced by BMH and BMH + INV immobilized almost 100% and killed 80% of *M. incognita* J_2, while volatiles from INV immobilized only 30% of *M. incognita* J_2 (Fig. 3(B)).

Cell-free supernatants, when tested against fungal biocontrol agents, only had a significant negative effect against the mycelial growth of *A. conoides* ($P = 0.05$), with a growth reduction of 20% compared with the control (Fig. 4(A)). However, the number of conidia was not significantly different for *A. conoides* and *P. lilacinum*, but the number of conidia of *T. atroviride* was significantly increased ($P < 0.01$) by approximately 60% (Fig. 4(B),(C)). Pathogenic fungi were generally negatively affected by the bacterial cell-free supernatants. There was a 20% reduction in mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* ($P < 0.02$) and a 32% reduction in the number of conidia ($P < 0.01$) when treated with the bacterial cell-free supernatants (Fig. 4(D)). Nonsporulating

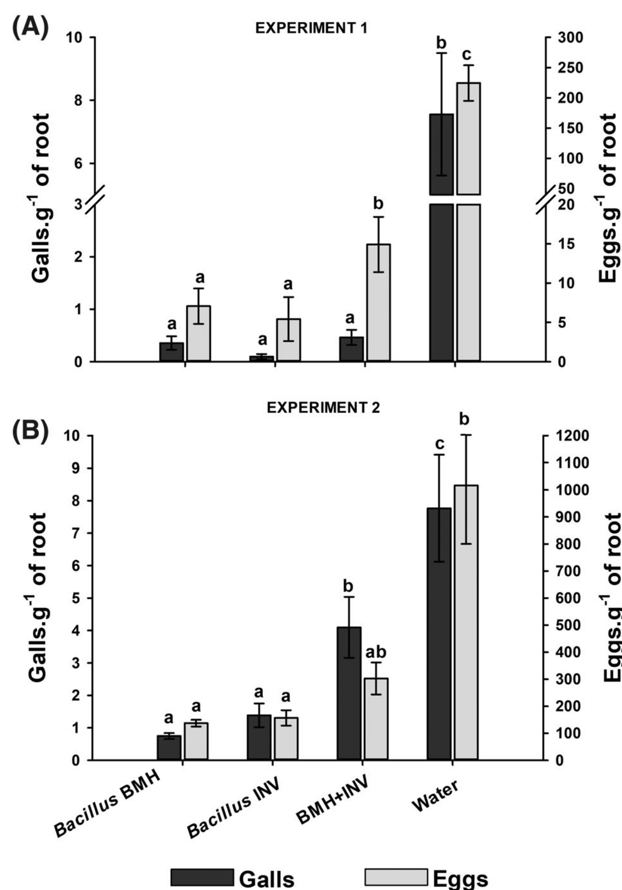


Figure 2. Influence of *Bacillus* strains on the development of *Meloidogyne incognita* in tomato roots. Cell suspensions of the *Bacillus* strains BMH, INV and BMH + INV were applied around the roots of tomato and the number of galls and eggs was evaluated after 45 days. In the controls, water was applied without the *Bacillus* strains. (A) and (B) represent two independent experiments. Mean values followed by the same letter are not significantly different according to Tukey's test at 5%. Error bars represent the standard error of the means.

© 2021 Society of Chemical Industry.

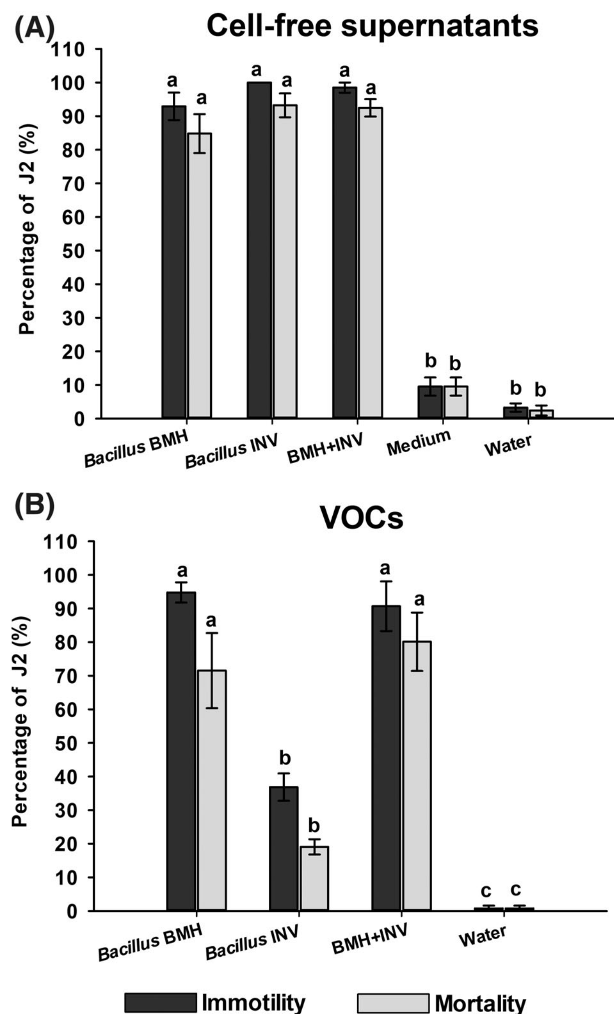


Figure 3. Activity of cell-free supernatants and volatile organic compounds (VOCs) produced by *Bacillus* strains against *Meloidogyne incognita* second-stage juveniles (J_2). (A) Immotility and mortality of *M. incognita* J_2 exposed to cell-free supernatants. Cell-free supernatants were mixed with a suspension of J_2 and the number of immotile and dead J_2 was determined under a microscope. (B) VOCs produced by the *Bacillus* strains were tested against *M. incognita* J_2 in plates split into two compartments and after the exposure the number of immotile and dead J_2 was determined. Mean values followed by the same letter are not significantly different according to Tukey's test at 5%. Error bars represent the standard error of the means. Results represent a joint analysis of two experiments.

pathogenic fungi had reductions in mycelial growth on average of 65% for *S. rolfsii* ($P < 0.01$) and 30% for *R. solani* ($P < 0.03$) for all bacterial cell-free supernatants. The exception was strain INV, for which suppression was not different from the control ($P > 0.05$) for *S. rolfsii* (Fig. 4(E),(F)).

VOCs did not significantly ($P > 0.05$) affect mycelial growth and conidiation of fungal biocontrol agents (Fig. 4(G)–(I)). Pathogenic fungi, however, with the exception of *S. rolfsii* (Fig. 4(K)), were negatively affected by the bacterial VOCs. Mycelial growth of *F. oxysporum* f. sp. *lycopersici* was reduced on average by 27% ($P < 0.04$), whereas conidiation decreased on average by 64% ($P < 0.01$) after exposure to the bacterial VOCs (Fig. 4(J)). Mycelial growth of *R. solani* was decreased on average by 30% ($P < 0.01$) when exposed to the bacterial VOCs (Fig. 4(L)).

A PCA analysis was performed to group the treatments according to their response in experiments selected for their significant differences among the bacterial strains. The components 1 and 2 of the PCA accounted for more than 70% of the variance among groups (Fig. 5 and Table S1). The analysis indicated that strain BMH alone was strongly related to the mortality of *M. incognita* J_2 by both cell-free supernatants and VOCs, whereas INV had the weakest influence on *M. incognita* J_2 mortality and shoot weight. The BMH + INV treatment was grouped as intermediate in terms of performance in all variables of the PCA. Taken together, these results indicate that strain BMH shows more beneficial effects than INV and BMH + INV.

3.5 *Bacillus* produce metabolites that may be putatively involved in their activity

A total of eight compounds belonging in the ketone and carboxylic acid classes were identified in strains BMH, INV and BMH + INV (Table 2). All VOCs, except for 2-pentanone, were produced by strain BMH. The combination BMH + INV had a VOC profile similar to strain INV, with the addition of isobutyric acid.

Strains BMH and INV had the same enzyme secretion profile, including cellulase, protease and lipase, but not chitinase. Additionally, both strains produced siderophores and were able to solubilize phosphate (Table S2).

4 DISCUSSION

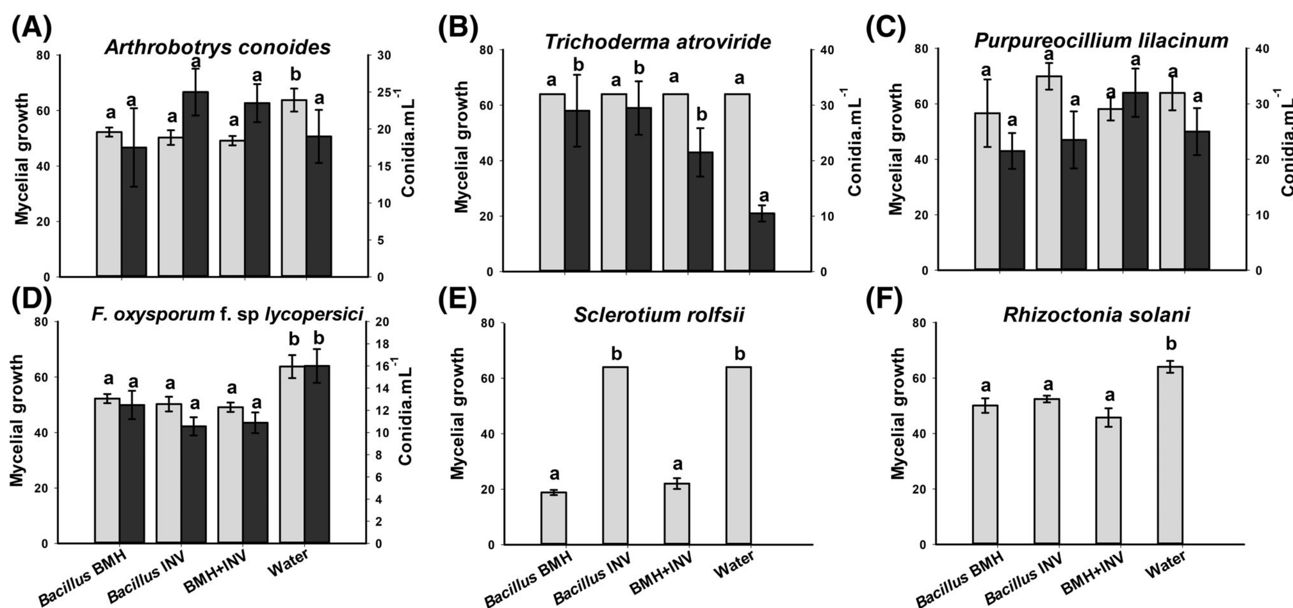
Considerable attention has been given to the use of antagonistic microorganisms capable of protecting plants against plant-parasitic nematodes.[62,63] In the present study, strains BMH and INV of *Bacillus* sp., alone and in combination, effectively killed *M. incognita* J_2 and decreased the number of galls and eggs in experiments performed *in vitro* and *in vivo* conditions. Additionally, these strains generally inhibited phytopathogenic fungi, while little or no significant effects were observed against fungi that are antagonistic to *M. incognita*. These two strains were previously tested for their compatibility *in vitro* and were shown to be incompatible and for this reason were not tested in experiments *in planta*.[38] Therefore, in this study, we further investigated whether this incompatibility affects their beneficial activities. The combination of strains BMH and INV had detrimental effects on plant growth and *M. incognita* suppression, but not on the biocontrol activity of other soilborne pathogens. The compatibility of strains in combinations to suppress plant pathogens is influenced by interactions with plant roots and native microorganisms, leading to different responses in *in vitro* and *in planta* assays. The mechanisms of activity of *Bacillus* are constantly influenced by root exudates and the potential against multiple pathogens is distinct according to the type of association.[64]

Sequence analysis of the gene16S rRNA indicated that the *Bacillus* strains BMH and INV are phylogenetically related and belong in the *B. velezensis* clade.[65] Species of this clade are known for their association with plants, especially with the rhizosphere, acting as agents of growth promotion and in the suppression of pathogens,[66] and for these reasons are frequently used in commercial formulations.[23,67] One of the advantages of this group of *Bacillus* is that they are not isolated in association with diseases in humans, other animals or plants as opposed to species such as *B. cereus* and *B. anthracis*.[68] The species in this clade include *B. amyloliquefaciens*, *B. velezensis* and *B. siamensis*, known to be beneficial and in some cases used as probiotics.[69]

The individual strains BMH and INV or their combination BMH + INV reduced the number of galls and eggs of *M. incognita* compared to the control, but the combination did not improve tomato

 © 2021 Society of Chemical Industry.

Cell-free supernatants



VOCs

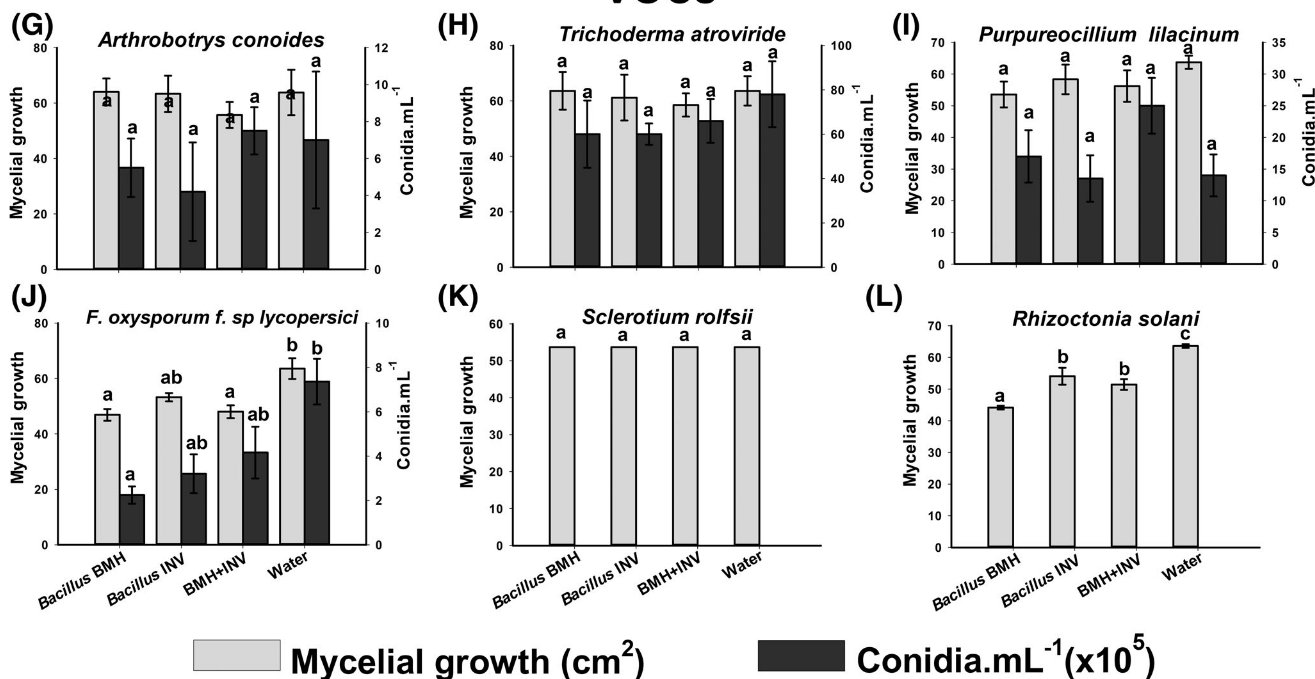


Figure 4. Effect of cell-free supernatants and volatile organic compounds (VOCs) on fungal growth and conidiation. Cell-free supernatants (A–F) were applied over the fungal mycelium in plates containing PDA medium. VOCs (G-L) produced by *Bacillus* were tested against the fungi in plates split into two compartments. The fungi tested were biocontrol agents (A–C and G–I): (A, G) *Arthrobotrys conoides*, (B, H) *Trichoderma viride*, (C, I) *Purpureocillium lilacinum*; and pathogens (D–F and J–L): (D, J) *Fusarium. oxysporum* f.sp. *lycopersici*, (E, K) *Sclerotium rolfsii*, (F, L) *Rhizoctionia solani*. Mycelial growth and the number of conidia were determined after the controls reached one of the edges of the plates or after 7 days. Mean values followed by the same letter are not significantly different according to Tukey's test at 5%. Error bars represent the standard error of the means. The results show two experiments combined.

growth. These results prompted us to study the possible mechanisms of activity of these strains either alone or in combination. An arsenal of mechanisms, such as improved plant nutrition, the production and regulation of phytohormones, and the suppression of disease-causing organisms, are among the strategies often employed by rhizospheric *Bacillus* spp.[70] These strains were able to decrease the motility and result in mortality of *M. incognita* J_2 by metabolites present in the cell-free supernatants and in the VOCs produced by each strain or their combination. Although these experiments were performed *in vitro*, it is expected that, at

 © 2021 Society of Chemical Industry.

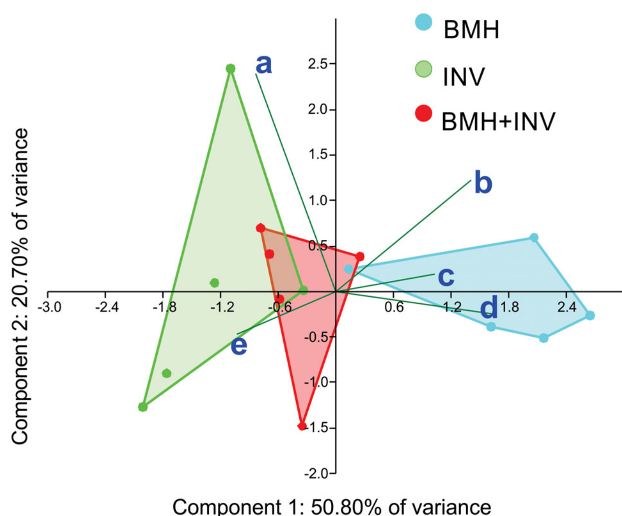


Figure 5. Principal components analysis (PCA) among all the results of experiments that showed a significant difference for the bacterial strains BMH and INV alone and combined (BMH + INV). (a) Conidia formation by *Fusarium oxysporum* f.sp *lycopersici* exposed to VOCs. (b) Tomato shoot weight. (c) Mortality of *Meloidogyne incognita* J_2 by cell-free supernatants. (d) Mortality of *M. incognita* J_2 by VOCs. (e) Mycelial growth of *Rhizoctonia solani* by cell-free supernatants. All the data used in the PCA were calculated in comparison with the control.

least partially, these VOCs cause some reduction in the infection in plant roots by killing *M. incognita* J_2 before root penetration, as already reported.[31,70-72]

The metabolite profiles of strains BMH and INV were similar for some enzymes, siderophores and the capacity to solubilize phosphate (Table S2), but not for the VOCs (Table 2). Additionally, through a PCR-based screening, we verified that the strains under study have the genes encoding for the synthesis of bacillomycin (Supplementary Text). The fact that these strains are closely related to *B. velezensis* corroborates the production of the antibiotic bacillomycin, which may be present in *B. velezensis*.[65] However, zwittermicin A has never been reported in *B. velezensis* and related species, but was found in *B. cereus*.[73] The lipopeptide bacillomycin has been shown to have activity against different fungi[74,75] and multiple plant-pathogens.[20,74–78] Furthermore, the potential showed by *Bacillus* spp. to synthesize a large number of compounds is one of the determining factors in their ability to suppress plant pathogens, promote plant growth and induce systemic defense responses.[79–81]

The number of VOCs produced by the combination of strains was reduced, resembling the profile shown by strain INV. A lower number of VOCs coincided with the reduced capacity of strain INV to kill *M. incognita* J_2, but does not provide a satisfactory explanation for the significantly higher activity of the combination of strains in killing *M. incognita* J_2 when compared with strain INV alone, unless the VOC isobutyric acid is considered. Additionally, VOCs may act in concert or synergistically to deliver a given effect. In one study, isobutyric acid inhibited egg hatching of *M. incognita* by 63%,[82] but no information was provided on its effect on mortality of the J_2. These studies will certainly be worth pursuing in the future. For the same reasons as mentioned above, it is difficult to correlate the reduced capacity of the combination of BMH + INV to promote tomato shoot growth only by looking at the profile of VOCs produced by the strains. Although strain INV produced the lowest number of VOCs when compared to BMH and BMH + INV, its capacity to promote shoot growth did not differ from strain BMH when applied alone.

The VOCs and the metabolites in cell-free supernatants generally had a stronger negative effect against the fungal plant pathogens than against the beneficial fungi. These results are interesting when the application of other beneficial microbes in combination with *Bacillus* is considered. These results also reaffirm the antagonistic capacity of *Bacillus* against numerous plant pathogens, including *M. incognita*.[33,62,67,83–87]

The combination of strains is an interesting strategy as it may expand the effects, performance and spectrum of activity of biological products in agricultural systems.[88,89] The combination of compatible bacterial strains frequently enhances plant growth and antagonistic activity by adding multiple mechanisms.[38] However, in our study, combined incompatible strains as determined in *in vitro* assays did not completely lose their beneficial properties, although negative changes occurred in metabolic profiles, in the suppression of RKN and in tomato growth promotion. Although we did not perform an extensive analysis and based on these two strains, it seems that *in vitro* assays are not enough to confirm the incompatibility when there is an interest in combining bacterial strains.

5 CONCLUSIONS

The combination of *Bacillus* sp. strains BMH and INV affected biocontrol activity against *M. incognita* as measured by the reduction

Table 2. Volatile organic compounds (VOCs) identified in *Bacillus* sp. strains BMH and INV analysed alone or in combination (BMH + INV) by SPME -GC-MS

	Compound	RI Exp.†	RI Lit.‡	BMH	INV	BMH + INV
1	2-pentanone	709	700	nd§	+	+
2	3-methyl-2-pentanone	754	743	+	nd	nd
3	Isobutyric acid	777	775	+	nd	+
4	5-methyl-2-hexanone	847	832	+	nd	nd
5	Isovaleric acid	858	868	+	+	+
6	2-heptanone	890	889	+	+	+
7	6-methyl-2-heptanone	954	954	+	nd	nd
8	5-methyl-2-heptanone	963	–	+	nd	nd

† Experimental retention indices calculated by injecting a homologous series of alkanes.
‡ Theoretical retention INDICES according to the literature.
§ Not detected.

of the numbers of galls and eggs in tomato roots, but not the capacity to kill *M. incognita* J_2 and to inhibit mycelial growth and conidiation of pathogenic fungi *F. oxysporum*, *R. solani* and *S. rolfsiii*. The combination also negatively affected the growth of tomato shoots and caused a decrease in the number of VOCs produced, which could partially explain the detrimental performance in tomato plants. Overall, these results show that the combination BMH + INV is an intermediate treatment in terms of performance, but both strains suppressed *M. incognita*, reduced mycelial growth of other plant pathogens and had little or no effect on beneficial fungi.

ACKNOWLEDGEMENTS

The authors thank the financial support of CNPq – Brazil (Grant 409611/2018-2) and CAPES – Brazil.

AUTHOR CONTRIBUTIONS

VCM, RAG, JCPS, AFF and MPP performed experimental work. VCM, RAG, JCPS, AFF and PASM designed the experiments. VC-M, RAG and JCPS discussed and interpreted the results. VCM, RAG, JCPS designed the research. MPP, VPC, PASM, FHVM and JTS contributed with financial support and scientific advice. VCM, RAG, JCPS and JTS wrote the manuscript. FHVM and JTS reviewed the manuscript. All authors reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. The accession numbers of the 16S rRNA sequences of Bacillus sp. BMH and Bacillus sp. INV can be found in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genbank/), under the accession numbers (KU207996 and KU207997)

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

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

2 Khanna K, Jamwal VL, Kohli SK, Gandhi SG, Ohri P, Bhardwaj R *et al*., Role of plant growth promoting bacteria (PGPRs) as biocontrol agents of *Meloidogyne incognita* through improved plant defense of *Lycopersicon esculentum*. *Plant Soil* **436**:325–345 (2019). https://doi.org/10.1007/s11104-019-03932-2.

3 Viljoen JJF, Labuschagne N, Fourie H and Sikora RA, Biological control of the root-knot nematode *Meloidogyne incognita* on tomatoes and carrots by plant growth-promoting rhizobacteria. *Trop Plant Pathol* **44**:284–291 (2019 Jun 15). https://doi.org/10.1007/s40858-019-00283-2.

4 Jones JT, Haegeman A, Danchin EGJ, Gaur HS, Helder J, Jones MGK *et al*., Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol Plant Pathol* **14**:946–961 (2013). https://doi.org/10.1111/mpp.12057.

5 DiLegge MJ, Manter DK and Vivanco JM, A novel approach to determine generalist nematophagous microbes reveals *Mortierella globalpina* as a new biocontrol agent against *Meloidogyne* spp. nematodes. *Sci Rep* **9**:1–9 (2019). https://doi.org/10.1038/s41598-019-44010-y.

6 Saucet SB, Van Ghelder C, Abad P, Duval H and Esmenjaud D, Resistance to root-knot nematodes Meloidogyne spp. in woody plants. *New Phytol* **211**:41–56 (2016). https://doi.org/10.1111/nph.13933.

7 Silva JCP, Nunes TCS, Guimarães RA, Pylro VS, Costa LSAS, Zaia R *et al*., Organic practices intensify the microbiome assembly and suppress root-knot nematodes. *J Pest Sci* **348**:1–13 (2021). https://doi.org/10.1007/s10340-021-01417-9.

8 Huang WK, Sun JH, Cui JK, Wang GF, Kong LA, Peng H *et al*., Efficacy evaluation of fungus *Syncephalastrum racemosum* and nematicide avermectin against the root-knot nematode *Meloidogyne incognita* on cucumber. *PLoS One* **9**:e89717 (2014). https://doi.org/10.1371/journal.pone.0089717.

9 Singh B, Devindrappa HKK, Singh U and Gupta S, Eco–friendly management of *Meloidogyne javanica* in chickpea (*Cicer arietinum* L.) using organic amendments and bio–control agent. *J Clean Prod* **257**: 120542 (2020). https://doi.org/10.1016/j.jclepro.2020.120542.

10 d'Errico G, Marra R, Crescenzi A, Davino SW, Fanigliulo A, Woo SL *et al*., Integrated management strategies of *Meloidogyne incognita* and *Pseudopyrenochaeta lycopersici* on tomato using a *Bacillus firmus*-based product and two synthetic nematicides in two consecutive crop cycles in greenhouse. *Crop Prot* **122**:159–164 (2019). https://doi.org/10.1016/j.cropro.2019.05.004.

11 Hu Y, Li J, Li J, Zhang F, Wang J, Mo M *et al*., Biocontrol efficacy of pseudoxanthomonas japonensis against *Meloidogyne incognita* and its nematostatic metabolites. *FEMS Microbiol Lett* **366**:fny287 (2019). https://doi.org/10.1093/femsle/fny287.

12 Ongena M and Jacques P, *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol* **16**:115–125 (2008). https://doi.org/10.1016/j.tim.2007.12.009.

13 Li B, Wang B, Pan P, Li P, Qi Z, Zhang Q *et al*., *Bacillus altitudinis* strain AMCC 101304: a novel potential biocontrol agent for potato common scab. *Biocontrol Sci Technol* **29**:1009–1022 (2019). https://doi.org/10.1080/09583157.2019.1641791.

14 Grayston SJ, Wang S, Campbell CD and Edwards AC, Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol Biochem* **30**:369–378 (1998). https://doi.org/10.1016/S0038-0717(97)00124-7.

15 Verma P, Yadav AN, Kumar V, Singh DP and Saxena AK, Beneficial plant-microbes interactions: biodiversity of microbes from diverse extreme environments and its impact for crop improvement, in *Plant-Microbe Interactions in Agro-Ecological Perspectives*. Springer, Singapore, pp. 543–580 (2017). https://doi.org/10.1007/978-981-10-6593-4_22.

16 Compant S, Samad A, Faist H and Sessitsch A, A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J Adv Res* **19**:29–37 (2019). https://doi.org/10.1016/j.jare.2019.03.004.

17 Chandrasekaran R, Revathi K, Senthil-Nathan S, Kalaivani K, Hunter WB, Duraipandiyan V *et al*., Eco-friendly formulation of wild *Bacillus thuringiensis* secondary metabolites through molecular characterization against the lepidopteran pest. *Physiol Mol Plant Pathol* **101**:93–104 (2018). https://doi.org/10.1016/j.pmpp.2017.09.002.

18 Harwood CR, Mouillon J-M, Pohl S and Arnau J, Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. *FEMS Microbiol Rev* **43**:341–361 (2019) https://academic.oup.com/femsre/advance-article/doi/10.1093/femsre/fuy030/5061627?searchresult=1.

19 Raza W, Wang J, Wu Y, Ling N, Wei Z, Huang Q *et al*., Effects of volatile organic compounds produced by *bacillus amyloliquefaciens* on the growth and virulence traits of tomato bacterial wilt pathogen *Ralstonia solanacearum*. *Appl Microbiol Biotechnol* **100**:7639–7650 (2016). https://doi.org/10.1007/s00253-016-7584-7.

20 Xu Z, Shao J, Li B, Yan X, Shen Q and Zhang R, Contribution of bacillomycin D in *bacillus amyloliquefaciens* SQR9 to antifungal activity and biofilm formation. *Appl Environ Microbiol* **79**:808–815 (2013). https://doi.org/10.1128/AEM.02645-12.

21 Shafi J, Tian H and Ji M, *Bacillus* species as versatile weapons for plant pathogens: a review. *Biotechnol Biotechnol Equip* **31**:446–459 (2017). https://doi.org/10.1080/13102818.2017.1286950.

22 Dunlap C, *Phylogeny and Taxonomy of Agriculturally Important Bacillus Species*. Springer, Cham, pp. 143–150 (2019). https://doi.org/10.1007/978-3-030-15175-1_8.

23 Choi SK, Jeong H, Kloepper JW and Ryu CM, Genome sequence of *Bacillus amyloliquefaciens* GB03, an active ingredient of the first

 © 2021 Society of Chemical Industry.

commercial biological control product. *Genome Announc* **2**:e01092–e01014 (2014). https://doi.org/10.1186/1471-2164-9-75.
24 McSpadden Gardener BB, Ecology of *bacillus* and *Paenibacillus* spp. in agricultural systems. *Phytopathology* **94**:1252–1258 (2004). https://doi.org/10.1094/PHYTO.2004.94.11.1252.
25 Hossain MJ, Ran C, Liu K, Ryu C-M, Rasmussen-Ivey CR, Williams MA *et al*., Deciphering the conserved genetic loci implicated in plant disease control through comparative genomics of *Bacillus amyloliquefaciens* subsp. *plantarum*. *Front Plant Sci* **6**:631 (2015). https://doi.org/10.3389/fpls.2015.00631.
26 Gardener BBM and Fravel DR, Biological control of plant pathogens: research, commercialization, and application in the USA. *Plant Health Prog* **3**:17 (2002). https://doi.org/10.1094/PHP-2002-0510-01-RV.
27 Zaccardelli M, Sorrentino R, Caputo M, Scotti R, De Falco E and Pane C, Stepwise-selected *Bacillus amyloliquefaciens* and *B. subtilis* strains from composted aromatic plant waste able to control soil-borne diseases. *Agriculture* **10**:30 (2020). https://doi.org/10.3390/agriculture10020030.
28 Cui L, Yang C, Wei L, Li T and Chen X, Isolation and identification of an endophytic bacteria *Bacillus velezensis* 8-4 exhibiting biocontrol activity against potato scab. *Biol Control* **141**:104156 (2020). https://doi.org/10.1016/j.biocontrol.2019.104156.
29 El-Nagdi WMA and Abd-El-Khair H, Application of *Bacillus* species for controlling root-knot nematode *Meloidogyne incognita* in eggplant. *Bull Natl Res Centre* **43**:1–10 (2019).
30 Crow WT, Effects of a commercial formulation of *Bacillus firmus* I-1582 on golf course bermudagrass infested with belonolaimus longicaudatus. *J Nematol* **46**:331–335 (2014). https://doi.org/10.1186/s42269-019-0187-6.
31 Xiong J, Zhou Q, Luo H, Xia L, Li L, Sun M *et al*., Systemic nematicidal activity and biocontrol efficacy of *Bacillus firmus* against the root-knot nematode *Meloidogyne incognita*. *World J Microbiol Biotechnol* **31**:661–667 (2015). https://doi.org/10.1007/s11274-015-1820-7.
32 Zhang J, Li Y, Yuan H, Sun B and Li H, Biological control of the cereal cyst nematode (*Heterodera filipjevi*) by Achromobacter xylosoxidans isolate 09X01 and *Bacillus cereus* isolate 09B18. *Biol Control* **92**:1–6 (2016). https://doi.org/10.1016/j.biocontrol.2015.08.004.
33 Ramezani Moghaddam M, Mahdikhani Moghaddam E, Baghaee Ravari S and Rouhani H, The nematicidal potential of local *Bacillus* species against the root-knot nematode infecting greenhouse tomatoes. *Biocontrol Sci Technol* **24**:279–290 (2014). https://doi.org/10.1080/09583157.2013.858100.
34 Gilbert GS, Parke JL, Clayton MK and Handelsman J, Effects of an introduced bacterium on bacterial communities on roots. *Ecology* **74**:840–854 (1993). https://doi.org/10.2307/1940810.
35 Correa OS, Montecchia MS, Berti MF, Fernández Ferrari MC, Pucheu NL, Kerber NL *et al*., *Bacillus amyloliquefaciens* BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities. *Appl Soil Ecol* **41**:185–194 (2009). https://doi.org/10.1016/j.apsoil.2008.10.007.
36 Dugassa A, Alemu T and Woldehawariat Y, In-vitro compatibility assay of indigenous *Trichoderma* and *Pseudomonas* species and their antagonistic activities against black root rot disease (*Fusarium solani*) of faba bean (*Vicia faba* L.). *BMC Microbiol* **21**:115 (2021). https://doi.org/10.1186/s12866-021-02181-7.
37 Amooaghaie R, Mostajeran A and Emtiazi G, The effect of compatible and incompatible *Azospirillum brasilense* strains on proton efflux of intact wheat roots. *Plant and Soil* **243**:155–160 (2002). https://doi.org/10.1023/A:1019914715054.
38 Barbosa LO, Lima JS, Magalhães VCVC, Gava CATCAT, Soares ACFACF, Marbach PAS *et al*., Compatibility and combination of selected bacterial antagonists in the biocontrol of sisal bole rot disease. *BioControl* **63**:595–605 (2018). https://doi.org/10.1007/s10526-018-9872-x.
39 Sezonov G, Joseleau-Petit D and D'Ari R, *Escherichia coli* physiology in Luria-Bertani broth. *J Bacteriol* **189**:8746–8749 (2007). https://doi.org/10.1128/JB.01368-07.
40 Paraszkiewicz K, Bernat P, Kuśmierska A, Chojniak J and Płaza G, Structural identification of lipopeptide biosurfactants produced by *Bacillus subtilis* strains grown on the media obtained from renewable natural resources. *J Environ Manage* **209**:65–70 (2018). https://doi.org/10.1016/j.jenvman.2017.12.033.
41 Wright HD, The preparation of nutrient agar with special reference to pneumococci, streptococci and other gram-positive organisms. *J Pathol Bacteriol* **39**:359–373 (1934). https://doi.org/10.1002/path.1700390210.
42 De Souza JT, Silva ACM, de Jesus Santos AF, Santos PO, Alves PS, Cruz-Magalhães V *et al*., Endophytic bacteria isolated from both healthy and diseased *Agave sisalana* plants are able to control the bole rot disease. *Biol Control* **157**:104575 (2021). https://doi.org/10.1016/j.biocontrol.2021.104575.
43 Leite HAC, Silva AB, Gomes FP, Gramacho KP, Faria JC, de Souza JT *et al*., *Bacillus subtilis* and *Enterobacter cloacae* endophytes from healthy *Theobroma cacao* L. trees can systemically colonize seedlings and promote growth. *Appl Microbiol Biotechnol* **97**:2639–2651 (2013). https://doi.org/10.1007/s00253-012-4574-2.
44 Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ, Basic local alignment search tool. *J Mol Biol* **215**:403–410 (1990). https://doi.org/10.1016/S0022-2836(05)80360-2.
45 Kumar S, Stecher G and Tamura K, MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**:1870–1874 (2016). https://doi.org/10.1093/molbev/msw054.
46 Parte AC, Carbasse JS, Meier-Kolthoff JP, Reimer LC and Göker M, List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol* **70**:5607–5612 (2020). https://doi.org/10.1099/ijsem.0.004332.
47 Terra WC, Campos VP, Pedroso MP, da Costa AL, Freire ES, de Pinto IP *et al*., Volatile molecules of *Fusarium oxysporum* strain 21 are retained in water and control *Meloidogyne incognita*. *Biol Control* **112**:34–40 (2017). https://doi.org/10.1016/j.biocontrol.2017.06.004.
48 Boneti J and Ferraz S, Modificação do método de Hussey & Barker para extração de ovos de *Meloidogyne exigua* de raízes de cafeeiro. *Fitopatol Bras* **6** (1981) Available from: http://www.sidalc.net/cgi-bin/wxis.exe/?IsisScript=CAFE.xis&method=post&formato=2&cantidad=1&expresion=mfn=015467.
49 Magalhães VC, de Barbosa LO, Andrade JP, ACF S, de Souza JT and PAS M, *Burkholderia* isolates from a sand dune leaf litter display biocontrol activity against the bole rot disease of *Agave sisalana*. *Biol Control* **112**:41–48 (2017). https://doi.org/10.1016/j.biocontrol.2017.06.005.
50 Mandels M and Reese ET, Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. *J Bacteriol* **73**:269–278 (1957).
51 Teather RM and Wood PJ, Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol* **43**:777–780 (1982). https://doi.org/10.1128/aem.43.4.777-780.
52 Dunne C, Crowley JJ, Moënne-Loccoz Y, Dowling DN, De Bruijn FJ and O'Gara F, Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity. *Microbiology* **143**:3921–3931 (1997). https://doi.org/10.1099/00221287-143-12-3921.
53 Roberts WK and Selitrennikoff CP, Plant and bacterial Chitinases differ in antifungal activity. *Microbiology* **134**:169–176 (1988). https://doi.org/10.1099/00221287-134-1-169.
54 Sierra G, A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie Van Leeuwenhoek* **23**:15–22 (1957). https://doi.org/10.1007/BF02545855.
55 Schwyn B and Neilands JB, Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**:47–56 (1987). https://doi.org/10.1016/0003-2697(87)90612-9.
56 Katznelson H and Bose B, Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere, and non-rhizosphere soil. *Can J Microbiol* **5**:79–85 (1959). https://doi.org/10.1139/m59-010.
57 Sylvester-Bradley R, Asakawa N, La TS, Magalhães FMM, Oliveira LA and Pereira RM, Levantamento quantitativo de microrganismos solubilizadores de fosfatos na rizosfera de gramíneas e leguminosas forrageiras na Amazônia. *Acta Amaz* **12**:15–22 (1982). https://doi.org/10.1590/1809-43921982121015.
58 Adams R, *Identification of Essential Oil Components by Gas Chromatography/Mass Spectorscopy*, Vol. **456**. Allured Publishing Corporation, Carol Stream, IL, p. 811 (2007). https://doi.org/10.1016/j.jasms.2005.07.008.
59 NIST Mass Spectrometry Data Center, 'Retention Indices' in NIST Chemistry WebBook, NIST Standard Reference Database Number 69, Eds. PJ Linstrom and WG Mallard, *National Institute of Standards and*

© 2021 Society of Chemical Industry.

Technology, Gaithersburg MD, 20899, USA. https://doi.org/10.18434/T4D303

60 Hammer DAT, Ryan PD, Hammer Ø and Harper DAT, Past: paleontological statistics software package for education and data analysis. *Palaeontol Electron* **4**:178 (2001) Available from: http://palaeo-electronica.orghttp//palaeo-electronica.org/2001_1/past/issue1_01.htm.

61 FigTree v1.4.4. Available from http://tree.bio.ed.ac.uk/software/figtree/

62 Engelbrecht G, Horak I, Jansen van Rensburg PJ and Claassens S, Bacillus-based bionematicides: development, modes of action and commercialisation. *Biocontrol Sci Technol*, **28**:629–653 (2018). https://doi.org/10.1080/09583157.2018.1469000.

63 Abd-Elgawad MMM, Plant-parasitic nematodes and their biocontrol agents: current status and future vistas, in *Management of Phytonematodes: Recent Advances and Future Challenges*. Springer, Singapore, pp. 171–203 (2020). https://doi.org/10.1007/978-981-15-4087-5_8.

64 Chowdhury SP, Hartmann A, Gao XW and Borriss R, Biocontrol mechanism by root-associated *bacillus amyloliquefaciens* FZB42 - a review. *Front Microbiol* **6**:780 (2015). https://doi.org/10.3389/fmicb.2015.00780.

65 Silva FDJ, Ferreira LC, Campos VP, Cruz-Magalhães V, Barros AF, Andrade JP *et al*., Complete genome sequence of the Biocontrol agent *bacillus velezensis* UFLA258 and its comparison with related species: diversity within the commons. *Genome Biol Evol* **11**:2818–2823 (2019). https://doi.org/10.1093/gbe/evz208.

66 Borriss R, Use of plant-associated *bacillus* strains as biofertilizers and biocontrol agents in agriculture, in *Bacteria in Agrobiology: Plant Growth Responses*. Springer, Berlin Heidelberg, pp. 41–76 (2011). https://doi.org/10.1007/978-3-642-20332-9_3.

67 Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I *et al*., Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *bacillus amyloliquefaciens* FZB42. *Nat Biotechnol* **25**:1007–1014 (2007). https://doi.org/10.1038/nbt1325.

68 Ceuppens S, Boon N and Uyttendaele M, Diversity of *Bacillus cereus* group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. *FEMS Microbiol Ecol* **84**:433–450 (2013). https://doi.org/10.1111/1574-6941.12110.

69 Wu Y, Wang Y, Zou H, Wang B, Sun Q, Fu A *et al*., Probiotic *bacillus amyloliquefaciens* SC06 induces autophagy to protect against pathogens in macrophages. *Front Microbiol* **8**:469 (2017). https://doi.org/10.3389/fmicb.2017.00469.

70 Wang Z, Zhong T, Chen K, Du M, Chen G, Chen X *et al*., Antifungal activity of volatile organic compounds produced by *Pseudomonas fluorescens* ZX and potential biocontrol of blue mold decay on postharvest citrus. *Food Control* **120**:107499 (2021). https://doi.org/10.1016/j.foodcont.2020.107499.

71 Niu Q, Huang X, Zhang L, Lian L, Li Y, Li J *et al*., Functional identification of the gene bace16 from nematophagous bacterium *bacillus nematocida*. *Appl Microbiol Biotechnol* **75**:141–148 (2007). https://doi.org/10.1007/s00253-006-0794-7.

72 Jansen-Girgan C, Claassens S and Fourie H, In vitro evaluations to determine the effect of *Bacillus firmus* strains on the motility of *Meloidogyne incognita* second-stage juveniles. *Trop Plant Pathol* **41**:320–324 (2016). https://doi.org/10.1007/s40858-016-0100-x.

73 Khurana H, Sharma M, Verma H, Lopes BS, Lal R and Negi RK, Genomic insights into the phylogeny of *Bacillus* strains and elucidation of their secondary metabolic potential. *Genomics* **112**:3191–3200 (2020). https://doi.org/10.1016/j.ygeno.2020.06.005.

74 Olfa T, Antonio DG, Sana A, Imen BS, Salem E, Mohamed Najib A *et al*., Synergistic fungicidal activity of the lipopeptide bacillomycin D with amphotericin B against pathogenic *Candida* species. Calderone R, editor. *FEMS Yeast Res* **15**:fov022 (2015). https://doi.org/10.1093/femsyr/fov022.

75 Moyne A-L, Shelby R, Cleveland TE and Tuzun S, Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *J Appl Microbiol* **90**:622–629 (2001). https://doi.org/10.1046/j.1365-2672.2001.01290.x.

76 Liu J, Zhou T, He D, Li X, Wu H, Liu W *et al*., Functions of lipopeptides bacillomycin D and fengycin in antagonism of *Bacillus amyloliquefaciens* C06 towards *Monilinia fructicola*. *J Mol Microbiol Biotechnol* **20**:43–52 (2011). https://doi.org/10.1159/000323501.

77 Kumar A, Saini S, Wray V, Nimtz M, Prakash A and Johri BN, Characterization of an antifungal compound produced by *Bacillus* sp. strain a $_5$ F that inhibits *Sclerotinia sclerotiorum*. *J Basic Microbiol* **52**:670–678 (2012). https://doi.org/10.1002/jobm.201100463.

78 Gu Q, Yang Y, Yuan Q, Shi G, Wu L, Lou Z *et al*., Bacillomycin D produced by *Bacillus amyloliquefaciens* is involved in the antagonistic interaction with the plantpathogenic fungus *Fusarium graminearum*. *Appl Environ Microbiol* **83**:1075–1092 (2017). https://doi.org/10.1128/AEM.01075-17.

79 Cao Y, Pi H, Chandrangsu P, Li Y, Wang Y, Zhou H *et al*., Antagonism of two plant-growth promoting *Bacillus velezensis* isolates against *Ralstonia solanacearum* and *Fusarium oxysporum*. *Sci Rep* **8**:1–14 (2018). https://doi.org/10.1038/s41598-018-22782-z.

80 Athukorala SNP, Fernando WGD and Rashid KY, Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. *Can J Microbiol* **55**:1021–1032 (2009). https://doi.org/10.1139/W09-067.

81 Kloepper JW, Ryu CM and Zhang S, Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**:1259–1266 (2004). https://doi.org/10.1094/PHYTO.2004.94.11.1259.

82 Bansal RK and Bajaj A, Effect of volatile fatty acids on embryogenesis and hatching of *Meloidogyne incognita* eggs. *Nematol Mediterr* **31**:135–140 (2003) Available from: https://journals.flvc.org/nemamedi/article/view/86738.

83 Xiang N, Lawrence KS, Kloepper JW, Donald PA, McInroy JA and Lawrence GW, Biological control of *Meloidogyne incognita* by spore-forming plant growth-promoting Rhizobacteria on cotton. *Plant Dis* **101**:774–784 (2017). https://doi.org/10.1094/PDIS-09-16-1369-RE.

84 Lee YS and Kim KY, Antagonistic potential of *Bacillus pumilus* L1 against root-knot nematode, *Meloidogyne arenaria*. *J Phytopathol* **164**:29–39 (2016 Jan). https://doi.org/10.1111/jph.12421.

85 Zhou L, Yuen G, Wang Y, Wei L and Ji G, Evaluation of bacterial biological control agents for control of root-knot nematode disease on tomato. *Crop Prot* **84**:8–13 (2016). https://doi.org/10.1016/j.cropro.2015.12.009.

86 Jamal Q, Cho J-Y, Moon J-H, Munir S, Anees M and Kim KY, Identification for the first time of Cyclo(d-pro-l-Leu) produced by *Bacillus amyloliquefaciens* Y1 as a Nematocide for control of *Meloidogyne incognita*. *Molecules* **22**:1839 (2017). https://doi.org/10.3390/molecules22111839.

87 Abbasi MW, Tariq M, Qasim Khan M and Zaki MJ, Assessment of extracellular metabolites from *Bacillus* species against root-knot nematodes and root-infecting fungi in *Abelmoschus esculentus* (L.) Moench. *Pak J Bot* **49**:289–294 (2017).

88 Dimkić I, Živković S, Berić T, Ivanović Ž, Gavrilović V, Stanković S *et al*., Characterization and evaluation of two *Bacillus* strains, SS-12.6 and SS-13.1, as potential agents for the control of phytopathogenic bacteria and fungi. *Biol Control* **65**:312–321 (2013). https://doi.org/10.1016/j.biocontrol.2013.03.012.

89 Lim JH and Kim SD, Biocontrol of phytophthora blight of red pepper caused by phytophthora capsici using *Bacillus subtilis* AH18 and *B. licheniformis* K11 formulations. *J Appl Biol Chem* **53**:766–773 (2010). https://doi.org/10.3839/jksabc.2010.116.

 © 2021 Society of Chemical Industry.

Supplementary Material for

Pest Management and Science

The combination of two *Bacillus* strains suppresses *Meloidogyne incognita* and fungal pathogens, but does not enhance plant growth

Valter Cruz-Magalhães[1], Rafaela Araújo Guimarães[1], Julio Carlos Pereira da Silva[2,†], Amanda Flausino de Faria[1], Márcio Pozzobon Pedroso[3], Vicente Paulo Campos[1], Phellippe Arthur Santos Marbach[4], Flávio Henrique Vasconcelos de Medeiros[1] and Jorge Teodoro De Souza[1,†]

[1]Department of Phytopathology, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil.

[2]Departament of Phytosanitary Defense, CCR, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul, Brazil.

[3]Department of Chemestry, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil.

[4]Center for Agricultural, Biological and Environmental Sciences, Federal Universidade Federal do Recôncavo da Bahia, Cruz das Almas, Bahia, Brazil.

[†] Corresponding authors:

Julio Carlos Pereira da Silva
julio.c.silva@ufsm.br

Jorge Teodoro de Souza
jorge.souza@ufla.br

Keywords: Biocontrol**;** Root-knot nematodes; *Bacillomycin*; Metabolites; Volatile organic compounds.

Supplementary Table S1. List of the oligonucleotides used in this study to detect antibiotic genes by PCR screening, amplify the Zwittermicin-A resistance gene and 16S rDNA gene in the *Bacillus* strains.

Antibiotic	Primer	Primer sequence (5' – 3')	Length (Bp†)	Reference
Bacillomycin	BACC1F BACC1R	GAAGGACACGGCAGAGAGTC CGCTGATGACTGTTCATGCT	875	Ramarathnam et al[1]
Phenazine	PHZ1 PHZ2	GGCGACATGGTCAACGG CGGCTGGCGGCGTATTC	1408	Delaney et al[2]
Pyrrolnitrin	PRND1 PRND2	GGGGCGGGCCGTGGTGATGGA YCCCGCSGCCTGYCTGGTCTG	786	Souza and Raaijmakers[3]
2,4-Diacetylphloroglucinol	BPF2 BPR4	ACATCGTGCACCGGTTTCATGATG CCGCCGGTATGGAAGATGAAAAAGTC	535	McSpadden Gardener et al[4]
ZwittermicinA Resistance gene	677 678	TAAAGCTCGTCCCTCTTCAG ATGTGCACTTGTATGGGCAG	1000	Raffel et al[5]
16S rRNA	8F 1492R	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	1440	Leite et al[6]

†Base-pairs number of respective PCR amplicons that were sequenced and compared to database entries

Supplementary Table S2. PCA of the pathogenic and non-pathogenic variables.

PCA eigenanalyses between groups		Pathogenic variables			Non-pathogenic varaibles		
		PC 1	PC 2	PC 3	PC 1	PC 2	PC 3
Variance-covariance values	Eigenvalue	122.185	0.391	0.3522	220.022	127.872	0.085
	% variance	94.260	30.226	27.174	61.719	35.869	24.115
PC coeficients	J_2 Mortality by filtered	-0.416	-0.38823	0.0737	-	-	-
	J_2 Mortality by VOC	-0.4703	0.29794	-0.0613	-	-	-
	Galls	0.310	0.4678	-0.276	-	-	-
	Eggs	0.312	0.41986	0.0413	-	-	-
	Fusarium growth by filtered	0.504	-0.40797	-0.355	-	-	-
	Rhizoctonia growth by filtered	0.124	0.25215	0.396	-	-	-
	Sclerotium growth by filtered	0.199	-0.040421	0.776	-	-	-
	Fusarium growth by VOC	0.0954	-0.0705	0.0826	-	-	-
	Rhizoctonia growth by VOC	0.304	-0.358	0.136	-	-	-
	Root weight	-	-	-	-0.507	-0.0431	0.441
	Arthobotrys growth by filtered	-	-	-	0.431	-0.748	-0.249
	Purpureocillium growth by filtered	-	-	-	0.329	0.542	-0.215
	Arthobotrys growth by VOC	-	-	-	0.306	0.358	-0.0257
	Trichoderma growth by VOC	-	-	-	0.181	-0.105	0.655
	Purpureocillium growth by VOC	-	-	-	0.567	0.056	0.514

Supplementary Table S3. Secretion of enzymes, siderophores and activity of phosphate solubilization *in vitro* by *Bacillus* strains. All tests were done in Petri plates with indicative growth media. Positive (+) and negative (−) reactions were scored when a clear halo around the bacterial colony was present or absent, respectively.

Strains	Hidrolitic Activity and Biocontrol Traits†					
	Cellulase	**Chitinase**	**Protease**	**Lipase**	**Siderophores**	**Phosphate solubilization**
BMH	+	-	+	+	+	+
INV	+	-	+	+	+	+

†All tests were done in Petri plates with indicative growth media as indicated in the Material and Methods section.

Supplementary Table S4. Presence (+) or absence (-) of genes involved in the production of antibiotics (bacillomycin, phenazine, pyrrolnitrin, 2,4-diacetylfloroglucinol) and zwittermycinA resistance.

Strains	Antibiotics / Gene Resistance				
	***bamC*†**	***phzFAI*‡**	***prnD*§**	***phlD*¶**	***zmaR*¥**
BMH	+	-	-	-	-
INV	+	-	-	-	-

†Bacillomycin biosynthesis pathway gene.
‡ Phenazine Biosynthesis Pathway gene.
§ Pyrrolnitrin biosynthesis pathway gene;
¶ 2,4-dicetylphloroglucinol biosynthesis pathway gene.
¥Zwittermycin resistance gene.

References

1. Ramarathnam, R, Bo S, Chen Y, Fernando WGD., Xuewen G, and de Kievit T. Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. *Can. J. Microbiol.* **53**, 901–911. doi:10.1139/W07-049. 2007.

2. Delaney SM, Mavrodi DV, Bonsall RF, and Thomashow LS (2001). phzO, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. *J. Bacteriol.* **183**:318–327. doi:10.1128/JB.183.1.318-327. 2001.

3. Souza JT, and Raaijmakers JM. Polymorphisms within the prnD and pltC genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia* spp. *FEMS Microbiol. Ecol.* **43**:21–34. doi:10.1111/j.1574-6941.2003.tb01042.x. 2003.

4. McSpadden GBB, Schroeder KL, Kalloger SE, Raaijmakers JM, Thomashow LS, and Weller DM. Genotypic and phenotypic diversity of phlD-containing Pseudomonas strains isolated from the rhizosphere of wheat. *Appl. Environ. Microbiol.* **66**:1939–1946. doi:10.1128/AEM.66.5.1939-1946.2000. 2000.

5. Raffel SJ, Stabb EV, Milner JL, and Handelsman J. Genotypic and phenotypic analysis of zwittermicin A-producing strains of Bacillus cereus. *Microbiology* **142**:3425–3436. doi:10.1099/13500872-142-12-3425. 1996.

6. Leite HAC, Silva AB, Gome, FP, Gramacho KP, Faria JC, de Souza JT, and Loguercio LL *Bacillus subtilis* and *Enterobacter cloacae* endophytes from healthy Theobroma cacao L. trees can systemically colonize seedlings and promote growth. Applied microbiology and biotechnology **97**:2639-2651. 2013.