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ORIGINAL ARTICLE

A commercial formulation of *Bacillus subtilis* induces metabolomic changes in root exudates that invert the chemotactic responses of the nematode *Meloidogyne incognita* to host and non-host plants

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Abstract

Root exudates mediate plant interactions in the environment, and they are affected by physical, chemical and biological factors. Biocontrol agents can modify root exudates and influence plant–pathogen interactions. In this study, we showed that lettuce (*Lactuca sativa*), a host of the root-knot nematode *Meloidogyne incognita*, produced root exudates that attracted the second-stage juveniles (J2s) of this nematode and garlic (*Allium sativum*), an antagonistic plant, produced exudates that repelled them. However, the application of a commercial product containing *Bacillus subtilis* on lettuce roots made the exudates repellent to J2s of *M. incognita*, whereas treated garlic exudates were as attractive to the J2s as untreated lettuce. The repulsive behavior of *M. incognita* to exudates of roots colonized by biocontrol agents is common; however, the attractiveness of treated garlic root exudates was unexpected and not previously reported for non-host plants. Chemotaxis assays also showed that the commercial formulation of *B. subtilis* was repellent to J2s of *M. incognita*. The metabolomic analysis conducted on these samples unveiled a combined total of 34 compounds. There was an elevation in the levels of amino acids and peptides in samples that were inoculated with the commercial product. Additionally, certain metabolites appear to be connected to chemotaxis. These metabolic changes induced by the commercial product are interesting for field utilization in a dual control strategy, where lettuce is protected against the nematode due to its repellence and garlic becomes attractive, but is not infected by the nematode.

Keywords Root-knot nematode · *Lactuca sativa* · *Allium sativum* · Chemotaxis · Biological control

Introduction

Root exudates are a mixture of compounds from the primary and secondary metabolism released by plant roots in the rhizosphere and may be comprised of sugars, amino acids, peptides, enzymes, vitamins, organic acids, nucleotides, phenolics and other secondary metabolites. Root exudates play important roles in subterranean ecology (Haichar et al. 2014; Vives-Peris et al. 2020). The metabolites released into the soil mediate interactions between plants and the environment, including communications with beneficial microorganisms and soil-borne pathogens, such as plant-parasitic nematodes (Vives-Peris et al. 2020).

Plant-parasitic nematodes are able to infect almost all cultivated plants and are major threats to food production (Sasser et al. 1983). Root-knot nematodes in the genus *Meloidogyne* are responsible for most of the damage caused by plant-parasitic nematodes, with annual crop losses estimated in $US 157 billion (Coyne et al. 2018). Within this group, *M. incognita* stands out as the most important species worldwide. The only infective stage in the life cycle of *Meloidogyne* species is the second-stage juvenile (J2), which hatches from the eggs, locates the host and penetrates the roots to establish a feeding site (Sasser et al. 1983).

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Meloidogyne species use chemotaxis to perceive their surroundings and guide their movement to find a suitable host (Sikder and Vestergård 2020). Host roots release compounds collectively known as rhizodeposits, generating a gradient of chemical stimuli that diffuse through the soil and are used by J2s to locate host plants (Sikder and Vestergård 2020).

In addition to attractive signaling molecules, root exudates can also contain compounds repellent to the J2s of *Meloidogyne* species. Several bioactive plant compounds with this activity are known, such as polythienyls, isothiocyanates, glucosinolates, cyanogenic glycosides, polyacetylenes, alkaloids, lipids, terpenoids, sesquiterpenoids, diterpenoids, limonoid triterpenes, quassinoids, steroids, triterpenoids, simple and complex phenolic compounds, flavonoids, saponins, tannins, essential oils, and fatty acids (Sikder and Vestergård 2020). Root exudates can undergo qualitative and quantitative changes in their composition due to physical, chemical and biological factors, such as light intensity, extreme temperatures, water availability, phytohormones, nutrient concentration, plant age, allelopathy and beneficial and pathogenic microorganisms (Vives-Peris et al. 2020; Zhao et al. 2022).

Several species in the genus *Bacillus* are well-known as plant growth-promoting rhizobacteria, and some strains are biocontrol agents used in the management of *Meloidogyne* species in different crops (Cao et al. 2019). These biological control strains act against *Meloidogyne* by multiple mechanisms, including the production of nematicidal metabolites (antibiosis), disruption of plant–pathogen communication and induction of systemic resistance (Sikora et al. 2007; Migunova and Sasanelli 2021). Some *Bacillus* species, such as *B. subtilis* and *B. cereus*, can alter the production of root exudates or modify their composition after secretion, thereby affecting nematode chemotaxis (Gowda et al. 2022; Hu et al. 2017; Rekha et al. 2020). One of the main effects of microorganisms is to decrease the attractiveness of root exudates to *Meloidogyne* J2s (Hu et al. 2017; Zhao et al. 2022). These changes in root exudation may ultimately enhance plant tolerance to abiotic stresses and resistance to pathogens (Ansari et al. 2020).

In this study, our objectives were to investigate the changes in root exudates induced by *B. subtilis* in lettuce (*Lactuca sativa*), a suitable host to *M. incognita*, and in garlic (*Allium sativum*), an antagonistic plant to this nematode. Susceptible lettuce cultivars suffer heavy infections and losses of more than 80% when infected by *M. incognita* (Charchar and Moita 2005), whereas garlic cultivation reduces *M. incognita* populations (El-Saedy et al. 2014; Seman et al. 2020). Moreover, garlic is known to have nematicidal properties due to compounds found in its bulb, such as allicin and other sulfur compounds (Eder et al. 2021; Jardim et al. 2020), but no information is available on the antagonistic compounds present in its root exudates. Metabolomic analysis and chemotaxis assays with *M. incognita* J2s were used to study the changes in root exudates of these contrasting plant species.

Materials and methods

Nematode inoculum

The population of *M. incognita* was established in tomato plants (*Solanum lycopersicum*) maintained in a greenhouse. Eggs were extracted and collected according to the method described by Hussey and Barker (1973) using 0.5% of sodium hypochlorite. To obtain the J2s, eggs were placed in a hatching chamber similar to a Baermann tray prepared in a plastic box filled with distilled water containing, at its top, two layers of Kleenex paper supported by a plastic screen. The egg suspension was poured onto the paper, and the J2s were collected after 24 h of incubation at 25 °C and counted under a microscope.

Collection of root exudates

Root exudate collection was based on the methods described by Čepulyte et al. (2018) and Liu et al. (2019). Lettuce and garlic plants with about 20 days of growth in autoclaved commercial substrate (Carolina Soil®, Brazil, comprised of peat, vermiculite, class A agro-industrial organic waste and limestone) were used. The roots were washed and placed inside a glass flask immersed in sterile distilled water with the aerial part out of the flask (Fig. 1a). To collect root exudates, the roots of 20 lettuce plants were submerged in 4 ml of sterile distilled water within the flask (approximately 0.2 g of roots per flask), and the roots of five garlic plants were submerged in 15 ml of sterile distilled water within the flask (approximately 5 g of roots per flask). These volumes of water were determined as the minimum amounts to cover the roots of each plant species. The identical arrangement was utilized to gather root exudates from lettuce or garlic treated with 100 µl per flask of the commercial product Biobaci® (Grupo Vittia; active ingredient *B. subtilis* BV09), which is recommended for controlling *M. incognita* in Brazil. The volume of water used to collect exudates from each plant species was different (4 ml for lettuce and 15 ml for garlic), but the amount of formulated commercial product added was the same (100 µl per flask). Therefore, it resulted in 40X dilutions of the product in lettuce and 150X in garlic exudates. The treatments, with 5 replicates each, were: treated and untreated root exudates of lettuce and garlic, and two controls, one composed of 100 µl of the commercial product diluted in 4 ml of sterile distilled water and the other with sterile distilled water only. The flasks were wrapped in aluminum foil to protect the roots from light, and the

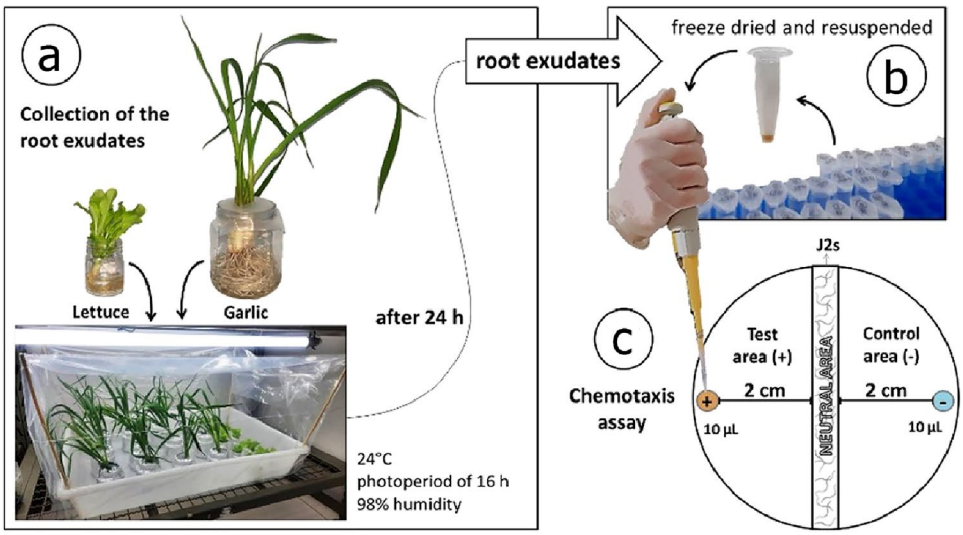

Fig. 1 Schematic representation of the procedure used in the collection of root exudates (**a** and **b**) and chemotaxis assays (**c**). The J2s were placed at the center (neutral area) of a 6-cm diameter Petri dish, root exudates were placed in the test area (+) and distilled water to the control area (-). The number of J2s in the test and control areas were determined and used to calculate the chemotaxis index (CI). The collection of root exudates was done according to Čepulyte et al. (2018) and Liu et al. (2019) and the chemotaxis assays according to Wang et al. (2021)

plants were placed inside a moist chamber with 98% humidity and incubated in a growth room for 24 h at 24 °C with a photoperiod of 16 h of light and 8 h of darkness. Two batches of root exudates were collected using the experimental setup described above. The first batch with 5 replicates was collected 24 h after the installation of the experiment, and the second batch was collected 24 h after the installation of another experiment, which took place the subsequent day. For each batch, 5 replicates of the exudates were collected per treatment, filtered through a 0.22 µm filter and, for each replicate (lettuce in 4 ml and garlic in 15 ml), 3 samples of 1 ml were collected in microcentrifuge tubes, totaling 30 microtubes per treatment, which included 15 microtubes for each batch. All samples, including the controls, were frozen at −80 °C, lyophilized and stored at −20 °C until use, when the sample in each microtube was used in the chemotaxis assays or in the metabolomic analysis (Fig. 1b). Therefore, at least 20 microtubes per treatment were used in the chemotaxis assays and 3 microtubes per treatment were used in the metabolomic analysis (see below).

Chemotaxis index determination

The chemotaxis assay was performed based on the method described by Wang et al. (2021) with some modifications. Petri dishes of 6 cm in diameter containing 10 ml of 2% agar–water were used to observe the movement of *M. incognita* J2s toward or away from the exudates. Approximately 200 J2s were placed in the center of the Petri dish (neutral area, Fig. 1c), 10 µl of root exudates were added at the edge of the test area (+), and 10 µl of distilled water was added at the edge of the control area (-). Plates were incubated at room temperature in the dark. After 16 h, the number of J2s in the test and control areas was determined. The chemotaxis index (CI) was calculated as CI = (number of J2s in the test area—number of J2s in the control area)/ (number of J2s in the test area + number of J2s in the control area) (Wang et al. 2021). CI values ≥ 0.2 were considered highly attractive, CI ≥ 0.1 but < 0.2 as slightly attractive, CI ≥ −0.1 but < 0.1 as a patternless response, CI > −0.2 but < −0.1 as slightly repellent, and a CI ≤ −0.2 as highly repellent (Wang et al. 2019). The treatments were: treated and untreated root exudates of lettuce and garlic and two controls, one composed of the commercial product and the other one had only water at the edges of the test and control areas.

To test the effect of different batches of root exudates, an initial chemotaxis assay was performed with 5 replicates using exudates from the two batches separately. The final chemotaxis assays were installed with 10 replicates per treatment, where each of these replicates represented one of the microtubes obtained in Sect. "Collection of root exudates." The whole assay was performed twice (Experiments 1 and 2).

Sample preparation for metabolomic analyses

Three microcentrifuge tubes of each sample described above were extracted and concentrated by adding 100 µl of a H_2O:MeOH (8:2) solution with 2 µg ml^{-1} of the internal standard p-fluoro-DL-phenylalanine ($C_9H_{10}FNO_2$, Rt 2.22 min, m/z 184.076830 [M + H] +) added to the first microtube. This first microtube was homogenized in a vortex for 60 s and the content was transferred to the second tube, which was mixed in the same way and its content was transferred to the third tube that was mixed again and placed in an ultrasonic bath for 10 min at 25 °C. The samples were centrifuged at 5000 g for 10 min, and 80 µl of the supernatant was used for the analysis. The extraction control was composed of 100 µl of a H_2O:MeOH (8:2) solution with 2 µg ml^{-1} of

the internal standard p-fluoro-DL-phenylalanine without any of the other treatments.

Chromatographic method and mass spectrometer parameters

Chromatographic analyses were performed using a Dionex LC UltiMate 3000 liquid chromatograph coupled to a Q-Exactive Plus high-resolution mass spectrometer (Thermo Scientific). The chromatographic method used in the analysis was performed using the Thermo Syncronis C_{18} column 50 × 2.1 mm, 1.7 µm of particle size. Mobile phase A was water with 0.1% (v/v) formic acid, and mobile phase B was methanol with 0.1% (v/v) formic acid. The chromatographic separation was performed in gradient elution mode at a flow rate of 0.35 ml min^{-1} as follows: 0–9 min B 5–60%, 9–13 min B 60–98%, 13–16 min B 98%, 16.1–20 min B 5%. Sample injection volume was 8 µL, and the column oven temperature was 40 °C. Mass spectrometer data acquisition was performed in positive electrospray ionization mode. The ionization source conditions were: sheath gas: 45 arbitrary units (a.u.), auxiliary gas: 15 a.u., spray voltage: 3.9 kV, S-lens voltage: 60 V, capillary temperature: 320 °C, and source temperature: 400 °C. A full ion scan was performed in the m/z range of 100–1000 with a resolution of 70,000 FWHM (full width at half maximum), automatic gain control (AGC) target of 1e6 and maximum ion injection time (IT) 100 ms, combined with data-dependent acquisition DDA Top5 type at a resolution of 17,500 FWHM, AGC target of 1e5, maximum IT 50 ms and an isolation window of 1.2 Da.

Annotation of the compounds

Annotation of the compounds was performed by comparing the m/z values of the precursor ions obtained experimentally to the theoretical values calculated or available in the spectra libraries with an error smaller than 5 ppm (Table S1). Annotations were also based on similarities between the fragmentation spectra (MS/MS) obtained with data from spectral libraries. For the annotation of these compounds, the raw spectra (RAW) were submitted to processing in MS-DIAL v4.8 software using the following parameters: MS1 and MS2 tolerances: 0.005 and 0.05 Da, respectively; minimum peak height: 500,000; mass width: 0.05 Da; sigma window value for deconvolution: 0.4; 0.1 min and 0.005 Da tolerances for peak alignment; and similarity score higher than 80% between spectra. The libraries used were Mass Bank of North America (MONA, public) and NIST MSMS 2020 (commercial).

Statistical analysis

The normality of the chemotaxis index data was evaluated by the Shapiro–Wilk test, and the homogeneity of the variances was evaluated by the Levene test. The data were subjected to analysis of variance (ANOVA), and the means were compared with the *t* test at 5% significance. The comparisons were done between treated and untreated exudates of the same plant species. The R software was used to perform the statistical analyses with the *t* test and the boxplot figures (R Core Team 2019).

Results

Chemotactic responses of *M. incognita* to root exudates

The two different batches of root exudates did not differ from each other in their chemotactic responses to *M. incognita* J2s according to the *t* test at 5% probability ($p > 0.05$) (Fig. S1). Therefore, the effect of the different batches was not considered in subsequent experiments.

Two in vitro experiments were performed to evaluate the effects of lettuce and garlic root exudates on *M. incognita* J2 chemotaxis. In both experiments, the root exudates affected J2 migration since the J2s moved randomly in the water control, but were clearly influenced by the other treatments (Fig. 2). The J2s were weakly attracted by lettuce root exudates and weakly repelled by garlic root exudates. However, the application of *B. subtilis* commercial formulation inverted the behavior of the J2s in relation to the untreated root exudates of lettuce ($p = 0.00004$ in the first and $p = 0.00000071$ in the second experiment; t test) and garlic ($p = 0.0042$ in the first and $p = 0.0043$ in the second). This inversion was stronger for the host lettuce than for the non-host garlic. The commercial formulation containing *B. subtilis* was always repellent to the J2s of *M. incognita* (Fig. 2).

Metabolomic changes in root exudates

Metabolomic analysis was performed to identify the metabolites in lettuce and garlic root exudates, with and without the application of the commercial product containing *B. subtilis*. The number of compounds detected in each sample and their relative abundance were determined by examining the chromatograms of the mass spectrometer (Fig. 3; Table S1). A total of 34 metabolites were annotated (Table 1) and amino acids and peptides comprised the most numerous class of compounds, with a total of 20. The other classes contained one or two compounds and were comprised of fatty acids, nucleotides and nucleosides, organic acids, vitamins,

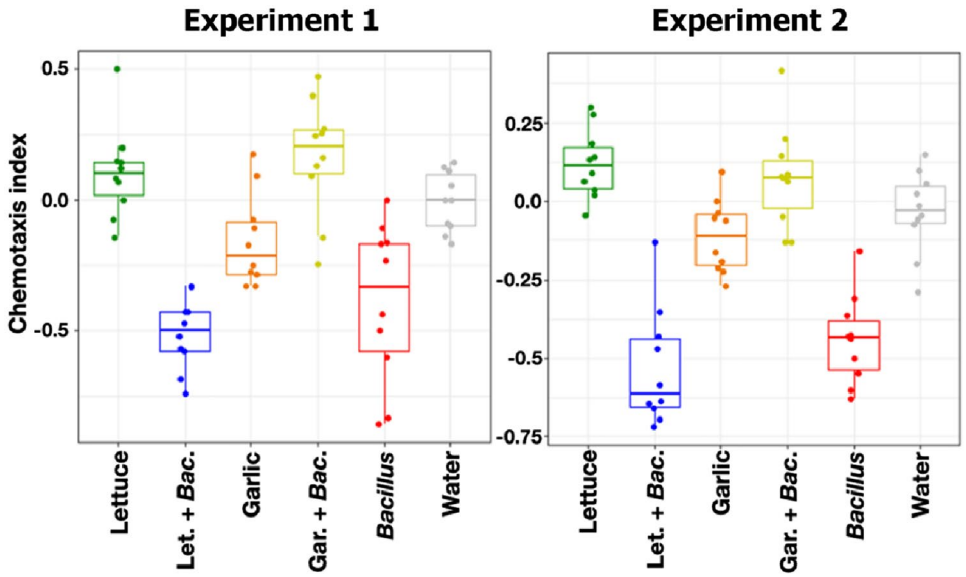

Fig. 2 Chemotaxis index (CI) of root exudates from roots of lettuce and garlic plants with and without the commercial product containing *Bacillus subtilis*. Chemotaxis assays were performed in 6-cm diameter Petri plates, and CI was calculated by determining the number of J2s in the control and test areas. Treatments were treated and untreated lettuce and garlic root exudates, and two controls, one with commercial product and the other one with water. *Bac.* and *Bacillus* represent the commercial product containing *B. subtilis* suspension; Let. and Gar. represent, respectively, lettuce and garlic. Both experiments were performed with 10 replicates per treatment and although the trend was the same, the values differed and therefore they were analyzed and are shown separately

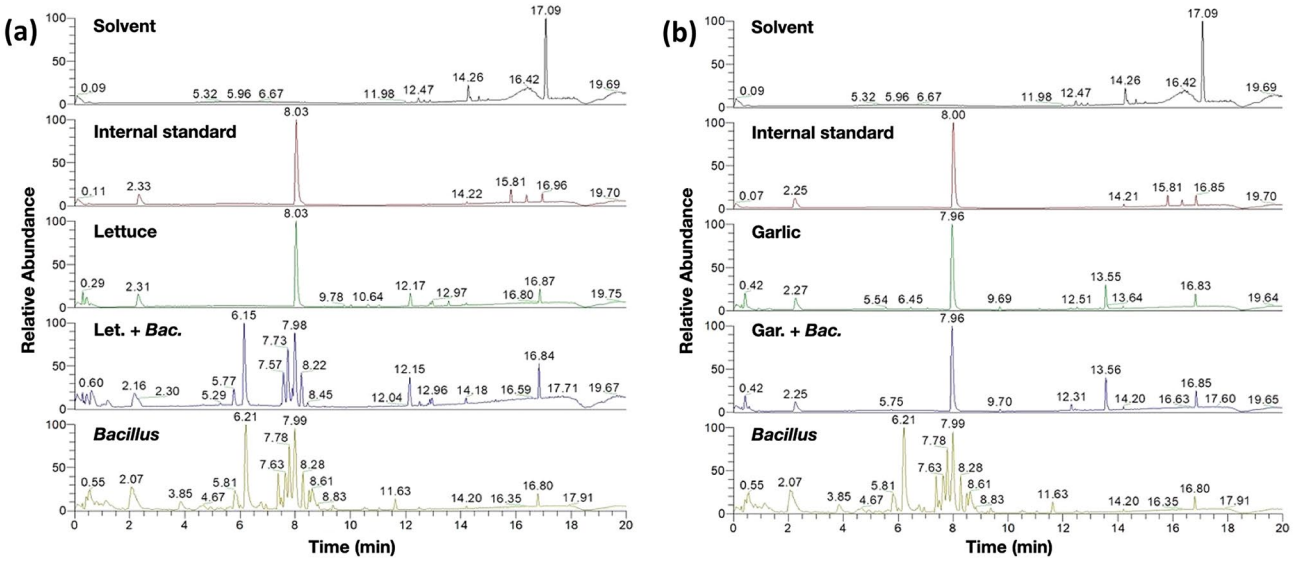

Fig. 3 Typical LC-HRMS chromatograms of lettuce (**a**) and garlic (**b**) root exudates with and without the application of the commercial product containing *B. subtilis*. Controls were solvent H_2O:MeOH and internal standard p-fluoro-DL-phenylalanine. In (**a**) the treatments were Lettuce root exudates and Let. + *Bac.* (Lettuce + *Bacillus*) and *Bacillus*. In (**b**) the treatments were Garlic root exudates and Gar. + *Bac.* (Garlic + *Bacillus*) and *Bacillus*. *Bac.* and *Bacillus* represent the commercial product containing *B. subtilis* suspension

alkaloids, phenolic compounds, phytohormones and other metabolites (Table 1). Most of them were classified as compounds from the primary metabolism, with the exception of alkaloids and phenolic compounds that are from the secondary metabolism and phytohormones and other metabolites that may be classified as from the primary and secondary metabolism at the same time.

The application of the commercial product containing *B. subtilis* on lettuce and garlic roots nearly doubled the number of metabolites in the exudates when compared to

Table 1 Compounds detected by LC-HRMS/MS in lettuce and garlic root exudates with and without the commercial product containing *B. subtilis*

Nº	Metabolite name	Metabolite class	Peak area				
			Lettuce	Let. + *Bac*	Garlic	Gar. + *Bac*	*Bacillus*
1	4-Guanidinobutyric acid	Organic acids	8.10×10^{7}	1.04×10^{9}	1.31×10^{8}	1.22×10^{9}	3.12×10^{8}
2	N,N-Diethyl-2-aminoethanol	Others	2.18×10^{6}	0	4.10×10^{7}	4.13×10^{7}	0
3	L-Valine	Amino acids and peptides	1.31×10^{8}	2.86×10^{8}	4.24×10^{8}	3.27×10^{8}	9.08×10^{8}
4	L-Pipecolic acid	Amino acids and peptides	6.39×10^{6}	5.30×10^{7}	0	8.37×10^{7}	2.16×10^{8}
5	Nε-Acetyl-L-lysine	Amino acids and peptides	1.27×10^{7}	5.35×10^{7}	0	0	3.23×10^{9}
6	His-Pro	Amino acids and peptides	0	1.10×10^{9}	0	4.36×10^{7}	9.37×10^{9}
7	L-Arginine	Amino acids and peptides	0	3.99×10^{9}	0	2.12×10^{9}	1.03×10^{10}
8	2-Pyrimidinylacetic acid	Organic acids	1.87×10^{8}	1.17×10^{8}	8.76×10^{7}	5.42×10^{7}	3.41×10^{7}
9	Niacin	Vitamins	1.08×10^{8}	1.33×10^{8}	1.11×10^{8}	7.21×10^{7}	1.76×10^{9}
10	Pyroglutamic acid	Amino acids and peptides	1.44×10^{8}	3.46×10^{8}	6.75×10^{8}	3.02×10^{8}	1.04×10^{9}
11	Adenosine monophosphate	Nucleotides and nucleosides	1.18×10^{8}	4.51×10^{8}	0	1.98×10^{6}	2.47×10^{7}
12	Adenosine	Nucleotides and nucleosides	2.13×10^{8}	7.15×10^{8}	4.43×10^{8}	4.07×10^{7}	0
13	Cyclo(glycylprolyl)	Amino acids and peptides	0	2.29×10^{9}	0	4.06×10^{8}	7.88×10^{9}
14	Pro-Asp	Amino acids and peptides	0	2.01×10^{8}	0	1.24×10^{8}	9.20×10^{8}
15	Cyclo-Ala-Pro-diketopiperazine	Amino acids and peptides	0	0	0	1.37×10^{8}	2.05×10^{10}
16	Pantothenic acid	Vitamins	0	0	4.88×10^{7}	3.60×10^{7}	1.52×10^{9}
17	Tyr-Pro	Amino acids and peptides	0	0	0	9.41×10^{7}	8.79×10^{8}
18	Pro-Val	Amino acids and peptides	0	0	0	7.25×10^{7}	6.29×10^{8}
19	PyroGlu-Val	Amino acids and peptides	0	7.11×10^{7}	2.36×10^{7}	0	8.28×10^{8}
20	Cyclo(L-prolyl-L-prolyl)	Amino acids and peptides	0	6.46×10^{8}	0	3.44×10^{7}	3.49×10^{9}
21	Thr-Leu	Amino acids and peptides	0	0	0	8.82×10^{6}	2.42×10^{8}
22	4-Hydroxyquinoline	Alkaloids	2.28×10^{7}	6.20×10^{7}	1.93×10^{7}	2.84×10^{7}	4.26×10^{8}
23	Cyclo(prolyltyrosyl)	Amino acids and peptides	0	2.38×10^{9}	0	7.31×10^{8}	9.85×10^{9}
24	1,3,8-Trimethyl-3,9-dihydro-1H-purine-2,6-dione	Alkaloids	1.34×10^{8}	4.72×10^{7}	2.00×10^{8}	8.09×10^{7}	7.96×10^{7}
25	Cyclo(alanylisoleucyl)	Amino acids and peptides	0	5.09×10^{8}	0	1.14×10^{7}	2.44×10^{9}
26	Ile-Pro-Ile	Amino acids and peptides	0	8.32×10^{6}	0	0	1.38×10^{9}
27	Cyclo(leucylprolyl)	Amino acids and peptides	0	3.06×10^{9}	0	4.29×10^{7}	1.51×10^{10}
28	DL-Prolylphenylalanine	Amino acids and peptides	0	8.00×10^{8}	0	4.78×10^{7}	6.00×10^{9}
29	3,5-Dimethylhexanoic acid	Fatty acids	1.44×10^{8}	3.94×10^{7}	4.94×10^{7}	4.50×10^{7}	7.84×10^{7}
30	Valylphenylalanine	Amino acids and peptides	0	2.97×10^{7}	0	0	1.98×10^{8}
31	(2E)-4-Hydroxynon-2-enoic acid	Fatty acids	1.11×10^{8}	7.46×10^{7}	1.96×10^{8}	3.72×10^{7}	4.60×10^{7}
32	1-(2-Hydroxy-4,5-dimethylphenyl)ethanone	Phenolic compounds	2.99×10^{8}	9.38×10^{7}	0	2.28×10^{7}	0
33	Tuberonic acid glucoside	Phytohormone	0	0	1.75×10^{8}	1.95×10^{6}	0
34	3,4-Dimethylbenzaldehyde	Others	2.49×10^{8}	9.78×10^{7}	4.99×10^{8}	3.18×10^{8}	1.15×10^{8}

The metabolites were annotated according to experimental and theoretical values of *m/z* and fragmentation spectra in mass spectra libraries, and the metabolite classes were defined according to their function in plant metabolism. The peak area represents the abundance of the compound in each sample. Treatments were lettuce and garlic root exudates with and without the commercial product containing *B. subtilis*, and the control, which was the suspension of the commercial product. *Bac.* and *Bacillus* represent the commercial product containing *B. subtilis* suspension; Let. and Gar. represent, respectively, lettuce and garlic

untreated roots. Suspensions of the bacterium alone contained the highest number of metabolites, which coincided with the number detected in treated garlic root exudates (Fig. 4). Amino acids and peptides were the major chemical classes in all treatments, mainly in *Bacillus* alone, but the number and percentage of these compounds increased in treated lettuce and garlic exudates. Chemical compounds from the primary metabolism were found in all treatments. Compounds from the secondary metabolism, such as phenolics, were detected in all exudates, except for untreated garlic and *Bacillus* alone. The phytohormone tuberonic acid glucoside was only found in garlic root exudates, with a decreased concentration when *Bacillus* was applied (Tables 1 and 2; Fig. 4).

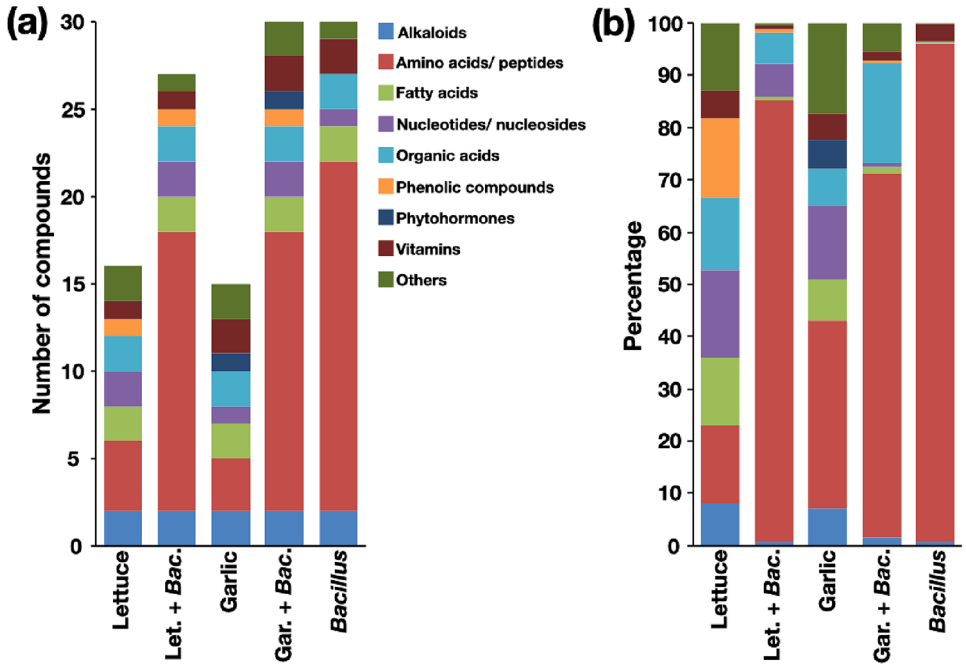

Fig. 4 Number of compounds and percentage of each metabolite chemical class in lettuce and garlic root exudates with or without the commercial product containing *Bacillus subtilis* determined by LC-HRMS/MS. The percentage of each metabolite chemical class was assessed by considering the number of metabolites of each chemical class to the total number of metabolites of each treatment. **a** A total of 34 compounds were detected in root exudates and these were classified in different classes according to their role in plant metabolism. **b** The percentage of each metabolite class was estimated by their relative abundance in the samples according to their LC-HRMS chromatograms. Treatments were lettuce and garlic root exudates with and without the commercial product containing *B. subtilis*, and the control, which was the suspension of the commercial product. *Bac.* and *Bacillus* represent the commercial product containing *B. subtilis* suspension; Let. and Gar. represent, respectively, lettuce and garlic

Table 2 Differentially produced metabolites in root exudates of lettuce and garlic according to the presence/absence criterion and lack of production by the control of *B. subtilis* commercial formulation and changes in the concentration of the metabolites

	Lettuce	Garlic
[a] Presence induced by *B. subtilis* commercial formulation	–	1-(2-Hydroxy-4,5-dimethylphenyl) ethanone (32)
[a] Presence suppressed by *B. subtilis* commercial formulation	N,N-Diethyl-2-aminoethanol (2)	PyroGlu-Val (19)
[b] *B. subtilis* commercial formulation increased concentration	Adenosine (12)	–
[b] *B. subtilis* commercial formulation decreased concentration	1-(2-Hydroxy-4,5-dimethylphenyl)ethanone (32)	Adenosine (12); Tuberonic acid glucoside (33)

[a]Based on the presence/ absence criterion in untreated x *B. subtilis*-treated samples and lack of production by the *B. subtilis* commercial formulation

[b]Based on increased or decreased concentrations of the compound and lack of production in the *B. subtilis* commercial formulation

There were 13 compounds differentially produced in treated versus untreated lettuce root exudates as defined by their presence and absence without considering their relative abundances or concentrations (Table 1). From these, only N,N-diethyl-2-aminoethanol (2) was suppressed in treated roots and was not produced by commercial formulation of *B. subtilis* alone, whereas all the other 12 compounds were detected in both *B. subtilis*-treated lettuce roots and in *B. subtilis* commercial formulation (Tables 1 and 2). In *B. subtilis*-treated versus untreated garlic root exudates, there were 17 differentially produced compounds. From these, only the phenolic 1-(2-hydroxy-4,5-dimethylphenyl) ethanone (32) was not produced in untreated roots and by commercial formulation of *B. subtilis* alone, but was

induced in treated roots, whereas PyroGlu-Val (19) was suppressed in treated roots although produced by *B. subtilis* commercial formulation. All the other 15 compounds were detected in both treated garlic roots and *B. subtilis* commercial formulation (Tables 1 and 2). The concentrations of compound 1-(2-Hydroxy-4,5-dimethylphenyl)ethanone (32) were reduced in *B. subtilis*-lettuce, while adenosine (12) and 3,4-dimethylbenzaldehyde (33) were reduced in garlic treated root exudates. Finally, adenosine (12) increased in *Bacillus*-treated root exudates (Table 2).

Discussion

Plants use their root exudates to interact with soil microorganisms. Juveniles (J2s) of *Meloidogyne* spp. move through the soil to find their hosts by chemotactically responding to plant root signals contained in exudates (Haichar et al. 2014; Wang et al. 2021). Host plants produce root exudates that attract nematodes, whereas non-host plants such as crown daisy (*Chrysanthemum coronariun*) and castor bean (*Ricinus communis*) release repellent exudates (Dong et al. 2014; Torto et al. 2018). Microorganisms are able to modify the secretion patterns and composition of host root exudates and turn them repellent to *Meloidogyne* J2s (Hu et al. 2017; Zhao et al. 2022). Among the diverse mechanisms of action of the biocontrol agents, they can influence the host root exudates profile, inducing the production and secretion of substances that affect *M. incognita* infection of the host (Gu et al. 2023; Li et al. 2019; Yin et al. 2021).

Our chemotaxis assays showed that the commercial product containing *B. subtilis* repelled J2s of *M. incognita* and turned lettuce exudates repellent as expected and demonstrated many times in the literature. Biocontrol agents may induce a repulsive response in *M. incognita* (Zhao et al. 2022), as well as induce modifications in exudates of host plants and make them repellent to nematodes (Sikora et al. 2007; Hu et al. 2017). However, the repellent garlic root exudates reversed to attractant after treatment with the commercial product containing *B. subtilis* and this unexpected phenomenon has not been previously reported. It is not known if this phenomenon also occurs with other non-host plants, such as crown daisy and castor bean, nematode species different from *M. incognita* and other species and isolates of bacteria, as chemotaxis was shown to be highly dependent on nematode (Oota et al. 2020) and bacterial species (Sikora et al. 2007; Hu et al. 2017; Wang et al. 2019; Zhao et al. 2022). Further research on these aspects is certainly warranted. Yet, another component that deserves further investigation is the response of nematode host and non-host plant root exudates to bacteria that attract nematodes, such as *Paenibacillus polymyxa* KM25021-1 and *Streptomyces plicatus* G (Cheng et al. 2017; Wang et al. 2019).

In this study, the commercial product was used instead of the isolate of *B. subtilis*. Future studies should focus on purified and quantified bacterial isolates of *B. subtilis*. The concentrations of the commercial product used in this study were different for each plant species. Nevertheless, it still invoked chemotactic responses from J2s of *M. incognita*. It will be interesting to study the effects of different concentrations of the commercial product and purified bacterial isolates on root exudates of host and non-host plants and their chemotactic responses to *M. incognita* and other nematode species.

The most obvious change in metabolic profiles was an increase in the number of compounds detected in treated versus untreated roots and for the most part these compounds coincided with the metabolites produced by the control suspensions of the commercial product containing *B. subtilis*. These compounds can, at least partially, explain the repellence induced in treated lettuce roots because the commercial product itself was repellent, but cannot explain the attractiveness induced in treated garlic roots. The most interesting compounds to explain these changes in chemotactic behavior are those that are differentially produced in untreated versus treated roots, either by the presence/absence criterion or by relative abundance, and at the same time are not produced by control suspensions of the commercial product. For example, the repellence of treated lettuce root exudates may be related to the absence of N,N-Diethyl-2-aminoethanol, lower concentration of 1-(2-Hydroxy-4,5-dimethylphenyl)ethanone (32) and slight increase in adenosine (12). By the same token, the attractiveness of treated garlic root exudates may, at least in part, be explained by the presence of 1-(2-Hydroxy-4,5-dimethylphenyl)ethanone (32), absence of PyroGlu-Val (19) and by the lower concentrations of adenosine (12) and tuberonic acid glucoside (33) (Table 2). Further chemotaxis assays should be performed with the purified compounds to confirm their involvement in the attractiveness or repellence of exudates to *M. incognita* J2s. These chemotaxis assays should consider different concentrations of the compounds being tested as responses were shown to change in a concentration-dependent manner (Kirwa et al. 2018; Shivakumara et al. 2018; Zhai et al. 2018). For example, the compound quercetin was found to be attractive at low concentrations and repellent at high concentrations (Kirwa et al. 2018).

The only compound found in this study that was previously tested for chemotaxis of *M. incognita* was the vitamin niacin or nicotinic acid (9), which was found to attract J2s (Kuang et al. 2020). This compound was produced in all our treatments and does not fit as a differentially produced compound nor does it appear to have any obvious involvement in the chemotactic responses of *M. incognita*. Nevertheless, this compound may act in the reduction of J2 penetration due to its nematicidal activity (Montasser 1990). It should

also be considered that this metabolite and many others occur and act in complex mixtures, thereby influencing the chemotactic responses of *M. incognita*. For example, different complex mixtures containing salicylic acid attracted *M. incognita* J2s, but its removal from these mixtures rendered them less attractive according to the mixture (Murungi et al. 2018). Similar responses occurred with mixtures containing the repellent compound thymol (Kihika et al. 2017). These results illustrate the complexity of pointing out the compounds responsible for chemotactic responses in root exudates.

'Amino acids and peptides' were the most common class of metabolites detected in treated lettuce and garlic roots. However, it is possible that these compounds were directly produced by the bacterium using root exudates as a food source. Specific types of amino acids and peptides are important molecules in plant–microbe interactions, participating in the plant's defense mechanisms or the interactions with beneficial microorganisms, shaping the plant microbiome (Moormann et al. 2022). Amino acids and peptides may act directly on J2s and decrease penetration due to their antimicrobial and nematicidal properties (Selim et al. 2019; Sun et al. 2021) and indirectly by enhancing plant resistance against nematodes (Shaimaa et al. 2021; Pandey et al. 2019; Waqas et al. 2015). Some amino acids, such as arginine (7), lysine (5), and leucine (21), may affect *M. incognita* J2 attraction, as well as reduce hatching and increase J2 mortality (Fleming et al. 2017; Jiang et al. 2022; Nazzal and Yaas 2023).

Adenosine (12), besides fitting the criterion of a differentially produced compound and possibly contributing to the repellence of lettuce and the attractiveness of garlic, and tuberonic acid (33), possibly involved in the attractiveness of treated garlic, are markers for increased defense responses (Aprile et al. 2022; Misaghi et al. 1975). Pantothenic acid (16) can be involved in plant defense response against *M. incognita*, and it can affect the development of J2 in host roots (Zhang et al. 2024). Pipecolic acid (4), pyroglutamic acid (10) and 4-guanidinobutyric acid (1) are also markers for defense amplification, positive regulation of salicylic acid and induction of priming (Návarová et al. 2012; Eloh et al. 2016; Liu et al. 2020), although they apparently cannot be directly linked to chemotactic responses. Our metabolomic analysis, although considered sensitive in the detection of a wide range of compounds, might not have been able to detect compounds with low affinity with water and compounds that occur at very low concentrations in the exudates.

The product used in this study is commercially distributed and is able to decrease penetration, galling and reproduction of *M. incognita* in the field (L.L. de Paula, Lavras Federal University, Lavras, Brazil, 07/12/2022, personal communication). The responses of both host and non-host plants we observed in this study, especially their combination in the same planting area, need to be further investigated in field experiments as they can be useful in the management of *M. incognita*. Indeed, a recent study showed that intercropping lettuce (host) and garlic (non-host) in pots led to a reduction in root-knot nematode populations in addition to an improvement in lettuce growth (Cavalcanti et al. 2023). The application of the commercial formulation of *B. subtilis* may further improve nematode management by disrupting chemotaxis, but this hypothesis remains to be proven in the field. While the host plant exudates colonized by the isolate of *B. subtilis* carried in the commercial product will possibly decrease the penetration of J2s by repelling them, the non-host plant will possibly attract the J2s and these will not infect due to the lack of compatibility. Therefore, theoretically, it is possible to decrease the penetration of *M. incognita* by a dual control strategy, where both host and non-host plants colonized by *B. subtilis* or other biocontrol agents would contribute.

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Data availability All data generated or analyzed during this study are available from the corresponding author upon request.

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