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Diversity and chemotaxis of soil bacteria with antifungal activity against *Fusarium* wilt of banana

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Abstract The chemotactic response of bacteria to root exudates plays an important role in the colonization of bacteria in the rhizosphere. In this study, 420 strains of antifungal bacteria against *Fusarium oxysporum* f. sp. *cubense* (Foc) were screened for chemotaxis based on a *che*A molecular diagnostic method. A total of 124 strains with antifungal efficiencies of 27.26–67.14 % generated a characteristic band of *che*A. The chemotaxis of 97 bacterial strains producing a *che*A band was confirmed using the drop assay and swarm plate assay using catechol, *p*-hydroxybenzoic acid, salicylic acid, and asparagine as the attractants. A phylogenetic analysis based on restriction fragment length polymorphisms (RFLPs) and 16S rDNA sequences indicated that the 124 chemotactic antagonists of Foc were affiliated with 18 species of *Paenibacillaceae*, *Bacillaceae*, *Streptomycineae*, *Enterobacteriaceae*, and *Pseudomonadaceae*. The chemical composition of banana root exudates were analyzed by GC–MS, and 62 compounds, including alkanes, alkenes, naphthalenes, benzenes, and alcohols, were evaluated. Five representative antagonists of Foc showed 1.76- to 7.75-fold higher chemotactic responses than the control to seven compounds in banana root exudates, as determination by capillary assays.

Keywords *Fusarium oxysporum* f. sp. *cubense* · Banana · Biological control bacteria · Chemotaxis · Root exudates

Introduction

Banana (*Musa* spp.) is one of the most important food crops in the tropical and subtropical countries of the world [19]. The worldwide banana production is under severe threat due to *Fusarium* wilt, a potentially devastating disease caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) [14, 28]. This pathogen is believed to have originated in Southeast Asia and was first reported in Australia in 1876 [25]. By 1950, it had spread to all of the banana-producing regions of the world, with the exception of some islands in the South Pacific, the Mediterranean, Melanesia, and Somalia [26]. Four races of this pathogen have been described to attack different banana cultivars; among these, a highly virulent form, tropical race 4 (TR4), attacks *Musa* (AAA group) 'Dwarf Cavendish' in addition to the hosts of races 1 and 2 [27].

Various control measures have been practiced to manage this disease, and biocontrol using antagonistic microorganisms is considered a viable strategy [9, 13, 16]. In greenhouses, the reintroduction of naturally occurring endophytes into tissue-culture banana plantlets led to a 67 % suppression rate of *Fusarium* wilt disease by the fifth month after inoculation on the plantlets [19]. Furthermore, banana plantlets pretreated with *Pseudomonas* spp. increased the systemic resistance of the plants and

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significantly reduced the disease severity [30]. Additionally, an isolate (BRIP 29089) of non-pathogenic *F. oxysporum* was reported to reduce the disease severity of *Fusarium* wilt in both Lady Finger and Cavendish cultivars [10], and an endophytic actinomycete, *Streptomyces griseorubiginosus*, isolated from banana roots inhibited the growth of Foc [6].

The ability of beneficial microorganisms to colonize, survive, and develop in the rhizosphere is thought to be important for the effective suppression of soil-borne pathogens [29] and increases in the growth promotion of crops [18]. It has been reported that well-colonized, antagonistic microorganisms are ideal for use as biocontrol agents against soil-borne diseases and in protecting plant health [7, 37, 39]. The rhizosphere represents a highly dynamic front for interactions between the roots and beneficial and pathogenic soil microbes, invertebrates, and the root systems of competitors [12], processes that involve root exudates and bacterial chemotaxis. Plant roots exude an enormous range of potentially valuable compounds into the rhizosphere during growth, and a large body of evidence has indicated that root exudates may play an important role in the plant–microbe interactions in the belowground ecosystem [3]. Bacteria are likely to locate plant roots through signals exuded from the roots, and root exudates, such as carbohydrates and amino acids, stimulate bacterial chemotaxis on root surfaces [31]. In addition, root exudates influence the flagellar motility of some rhizospheric bacteria, such as biocontrol and plant growth-promoting strains of *Pseudomonas* spp. [3]. Thus, chemotaxis appears to be important for the competitive colonization by chemotactic bacteria. Chemotaxis is a process by which cells sense changes in chemical concentration gradients present in the environment and move toward these gradients. At the molecular level, the minimal set of proteins that are required for the signal transduction systems controlling chemotaxis in bacteria includes chemoreceptors (methyl-accepting chemotactic proteins [MCPs], a histidine kinase [CheA], a receptor-coupling protein, and a response regulator [CheY]). MCPs detect environmental signals and modulate the activity of CheA, which, in turn, communicates the signal to the flagellar motors by phosphorylating CheY [5]. CheA is essential to chemotaxis, and organisms possessing the gene are expected to be chemotactic [5]. Accordingly, a molecular diagnostic tool based on the gene encoding the central regulator of bacterial chemotaxis (*che*A) has been developed to characterize and temporally track specific populations of native microbes with chemotactic potential in the rhizosphere [5].

On the basis of the above, it is hypothesized that biocontrol strains with the ability to sense and rapidly migrate toward roots exudates via chemotactic mechanisms will provide these organisms with a competitive advantage in rhizosphere colonization and a potentially effective ability to control their target pathogens. However, little is known about the complex system related to the chemotactic biocontrol microbes, root exudates, and pathogens of a specific crop. In this study, we (1) screened the chemotactic antifungal bacteria against Foc found in the agricultural soils of China by *che*A gene detection and plate assay, (2) determined the phylogenetic diversity of chemotactic antifungal bacteria against Foc, (3) analyzed the chemical composition of banana root exudates, and (4) discovered that a portion of the exudate compounds were attractants of chemotactic antifungal bacteria against Foc.

Materials and methods

Sample collection and bacterial isolation

To evaluate the resource of bacterial antagonists against Foc from agricultural soil, a total of 194 soil samples were collected randomly from rhizosphere with various plants including banana in 28 provinces of China. For a sample, about 1 kg of soil was collected at the top layer (2–15 cm) over an area of more than 5 m^2 around the plant. Soil samples were spread out to air dry at room temperature for 2–3 days and sieved through a 2-mm sieve, then stored in glass bottles at 4 °C until use. All the samples were used for bacterial isolation. The serially diluted soil samples were plated on King's MB (KMB) medium [22]. After incubation at 28 °C for 3–6 days, the bacterial strains were purified at random.

Screening of bacterial antagonists against Foc

The amended agar disk diffusion method [4] was used to qualitatively screen the antagonists. Briefly, four strains were streaked onto a potato dextrose agar (PDA) plate (square plate), and a PDA plug (5 mm in diameter) removed from an actively growing colony margin of Foc was placed in the center of the plate (Fig. 1a). After incubation for 7 days at 28 °C, the strains exhibiting antifungal activity were selected for an antagonistic bioassay using the Oxford cup method [35]. The candidate strains were cultured in NB medium (3 g beef extract, 10 g peptone, 5 g NaCl, 17 g agar, and 1,000 ml water, pH 7.2) at 28 °C, shaking at 180 rpm for 48 h. After centrifugation at 10,000 rpm for 10 min, 200 µl of a cell-free suspension was added into an Oxford cup (5 mm in diameter) that had been placed onto the center of a PDA plate. Two 5-mm PDA plugs removed from the actively growing colony of Foc were inoculated separately on the sides of the Oxford cup (Fig. 1b). An equivalent volume of NB medium in place of the suspension was used as a control. All of the treatments were performed

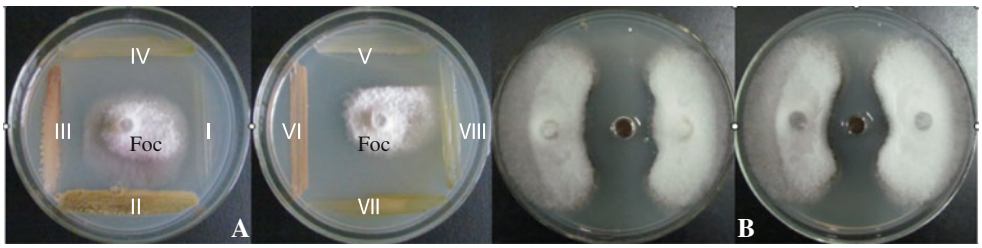

Fig. 1 Agar disk diffusion method (**a**) and Oxford cup method (**b**) used to screen the bacterial antagonists against *Fusarium oxysporum* f. sp. *cubense* (Foc). Strains of *III–VII* showed antifungal activity towards Foc, whereas *I*, *II*, and *VIII* were inactive

in triplicate. After incubation at 28 °C for 7 days, the antifungal efficiency (AE) was calculated using the following formula: AE = (DC − DT)/DC × 100 %, where DC and DT represent the colony diameters of Foc on the control and treatment plates, respectively.

Screening of chemotactic antifungal bacteria against Foc via *che*A gene detection and the petri dish assay

The bacterial DNA was extracted using a bacterial genomic DNA extraction kit (DP2001; BioTeke Corporation, China). The *che*A gene was amplified by PCR using primers P4P5.for and P4P5.rev [5]. The 25-μl reaction mixture consisted of 2 μl DNA template, 3.75 U Taq DNA polymerase (TaKaRa, Japan), 3 μl 10× PCR reaction buffer supplied with the enzyme, 1.25 μl 50 mM $MgCl_2$, 1 μl each 10 μM primer, 13 μl 2.5 mM dNTP (Vivantis, Malaysia), and 3 μl nuclease-free water (Promega, WI, USA). The reaction conditions were 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension step at 72 °C for 3 min. After purification using an Agarose gel DNA purification Kit (DP1502; BioTeke Corporation, China), the amplification products were detected by horizontal electrophoresis through 1 % Sigma type II agarose gels. The electrophoresis was performed at 120 V, 300 mA, and the image was photographed under UV illumination using Gel Doc (BIO-RAD) software.

The chemotaxis of the bacteria was tested using the drop assay and soft-agar swarm plate assay [23, 24], using catechol, *p*-hydroxybenzoic acid, salicylic acid, and asparagine as the chemotactic attractants. Briefly, bacteria were grown in NB medium at 28 °C, 180 rpm. At log phase (OD600 = 0.3), bacterial cells were harvested and washed three times with MM solution [24], then resuspended in MM solution at a concentration of about 1×10^8 CFU/ml. For soft-agar swarm plate assay, 5 ml of cell suspension was added into drop assay medium (MM solution containing 0.3 % agar) and poured into petri plates. In a plate, 0.1 g of tested compound was added in the center and chemotactic response was observed after incubation 3–4 h at room temperature (25 °C). For soft-agar swarm plate assay, the tested compounds (final concentration 0.2 mM) were added to the swarm plate medium (MM solution containing 0.16 % agar) before pouring into the plates. In a plate, 100 μl cell suspension containing 1 mM glucose was gently poured onto the center of the plate and incubated at room temperature (25 °C). All of the treatments above were performed in triplicate.

Phylogenetic analysis of antifungal bacteria having the *che*A gene

Genomic DNA was extracted using a bacterial genomic DNA extraction kit (DP2001; BioTeke Corporation, China), and the 16S rRNA genes were amplified by PCR using the 27f and 1492r primer pair [36]. The amplified products were purified with the kit (DP1502), as listed above. To select isolates for sequencing, the purification products were analyzed by restriction fragment length polymorphisms (RFLPs) using separate enzymatic digestions with *Msp*I (TaKara), *Hae*III (TaKara), and *Hha*I (TaKara) endonucleases. The digested DNA fragments were electrophoresed through 4.0 % agarose gels. After staining with ethidium bromide, the gels were photographed using an image-capture system, UVITEC DBT-08, and scanning image analyses were performed manually.

One to three isolates of each unique RFLP type were submitted to Beijing Huada Biological Company for sequencing. The acquired sequences were compared with those available in GenBank by using the BLAST program to determine their approximate phylogenetic affiliation and 16S rRNA gene sequence similarities [2]. Only sequences confirmed as not being chimeras and with similarity to 16S rRNA genes in the BLAST database were included in the phylogenetic analysis. The sequences were aligned with representative bacterial sequences from GenBank using ClustalX [35] and were then manually adjusted. The distance matrices and phylogenetic trees were calculated using the Kimura two-parameter model [17] and neighbor-joining algorithms in the MEGA program (version 4) by bootstrap analysis of 1,000 replications [33]. The partial 16S rDNA sequences of the representatives were deposited in GenBank (Table 1).

Preparation of banana root exudates

Root exudates from banana (*Musa acuminata* AAA Cavendish cv. Brazil) were acquired according to the method of Sood [32]. Briefly, 3-month-old banana seedlings cultured at 30 °C in a greenhouse were removed from their pots and carefully washed with sterile water until free of soil. The plants were immediately placed in a beaker (250 ml) with the roots submerged in 100 ml of aerated 0.5 mM $CaCl_2$ solution containing 0.05 g l^{-1} rifampicin and 0.025 g l^{-1} tetracycline and incubated for 2 h to reduce any bacterial contamination. The bacterial contamination was monitored by periodically sampling 0.2 µl of the solution during the exudate collection and plating of the dilutions on BPY medium (10 g casein, 5 g yeast extract, 4 g K_2HPO_4, and 18 g of agar in 1 l water). The plates were incubated at 28 °C for detection of bacterial contamination after 72 h. After antibiotic pretreatment, the roots were rinsed with 250 ml of sterile 0.05 mM $CaCl_2$ solution and immediately submerged in 100 ml of sterile 0.5 mM $CaCl_2$ solution for 22 h in the dark at 25 °C. The exudates obtained were concentrated from 100 to 10 ml by rotary evaporation at reduced pressure and then filtered through a 0.22-µm Millipore filter and stored at 4 °C until use.

Chemical analysis of root exudates

The root exudates were analyzed by gas chromatography–mass spectrometry (GC–MS) following the procedure described by Xu et al. [38]. Briefly, the concentrated root exudates were extracted with the same volume of methylene chloride. A 1-µl aliquot was injected into an Agilent 6890N GC equipped with an HP-5 capillary column (30 m × 0.25-mm inner diameter × 0.25-µm phase thickness) coupled to a 5,975 detector operated in the positive chemical-ionization mode with ammonia as the reagent gas at a pressure of 10^{-3} Torr and an electron energy of 170 eV. The MS source and quadrupole temperatures were set at 230 eV and 150 °C, respectively. The GC column temperature was set at 50 °C for 1 min, increased by 5 °C min^{-1} until 220 °C, and kept at this temperature for 1 min, then increased at 10 °C min^{-1} until 300 °C. The compounds were identified by comparison of their mass spectra with those in the mass spectra NIST08.L library.

Chemotaxis of chemotactic antifungal bacteria toward root exudates and compounds in the exudates

Five representative chemotactic antifungal bacteria, *Paenibacillus jamilae* NXHG29, *Brevibacillus brevis* HNHa2, *Bacillus sonorensis* ZJXBC21, *Pseudomonas aeruginosa* BJSS14, and *Providencia rettgeri* GZLB11, were selected for the chemotaxis assays. The chemotactic experiments of the chemotactic antifungal bacteria against Foc to chemoattractants were performed using a capillary assay [32]. Briefly, a glass tube of 0.5 cm internal diameter and 2 cm long was cemented near one end of a microscopic glass slide (7.5 × 2.5 cm), parallel to the long axis. Capillary micropipettes (10 µl, Dade Division, American Hospital Supply Corporation, USA) were sealed at one end and filled with the root exudates, compounds (20 mM), or the control buffer (pH 6.8). The open end of the capillary micropipette was inserted into a glass chamber containing 0.2 ml of bacterial suspension (1×10^7 cell ml^{-1}). After 1 h of incubation at room temperature (approximately 25 °C), the capillary tube was removed, and the exterior was washed with a jet of sterile distilled water. The tube was crushed in 10 ml of sterilized 0.9 % NaCl solution. This was diluted, and 0.2 ml of the suspension (10^{-2} and 10^{-3} dilutions) was spread over petri dishes containing BPY agar medium. The plates were incubated at 28 °C, and the bacterial colonies were counted after 3–4 days. All of the treatments were performed in triplicate. The value of the relative chemotactic response (VRCR) was the ratio of the cell number of bacteria in the capillary tubes containing the attractant to the cell number of the bacteria in the capillaries containing phosphate buffer; this value was used to represent the chemotactic capabilities of each bacterium.

Statistical analysis

The data were subjected to analysis of variance using SPSS software. The mean values among the treatments were compared by the least significant difference (LSD) test and Duncan's multiple range test at a 5 % level of significance ($P = 0.05$).

Results

Candidates of chemotactic antifungal bacteria of Foc from agricultural soil

A total of 2,270 bacterial strains were isolated from 194 agricultural soil samples using KMB medium. Of them, 420 isolates (18.50 % of the total) were found to exhibit antifungal activity against Foc, as detected by an agar disk diffusion method. The antifungal efficiencies (AE) of the 124 antifungal bacteria with a *che*A band varied in their abilities to suppress the mycelial growth of Foc, and the AE values ranged from 27.26 to 67.14 %. Among these antagonists, 34 and 18 strains respectively displayed AE values under 40 % and more than 60 %, whereas most of them (72 strains, 58.07 % of the total) showed AE values ranging from 40 to 59.9 % (Fig. 2). By amplification using

Table 1 Similarity of 16S rDNA sequences of representative Foc-antagonistic bacteria with those of culturable strains in the NCBI GenBank database, antifungal efficiency to Foc, and chemotaxis

Isolates	Accession numbers	RFLP types	Closest NCBI library strain and its accession no.	Similarity (%)	AE (%)	*che*A	Chemotaxis analyzed by drop assay/and swarm plate assay			
							A	B	C	D
HLJQZ12	HQ844438	R1	*B. atrophaeus* JCM 9070T (AB021181)	97.53	33.79 ± 0.87	+	+/+	+/+	+/+	+/+
GZPGL11	HQ844436	R2	*B. sonorensis* NRRL B-23154T (AF302118)	98.05	43.48 ± 1.23	+	+/+	+/+	+/+	+/+
ZJHD14	HQ844512	R3	*B. tequilensis* NRRL B-41771T(EU138487)	98.09	51.65 ± 1.24	+	+/+	+/+	+/+	+/+
ZJXBC21	HQ844446	R3	*B. tequilensis* NRRL B-41771T(EU138487)	97.12	57.25 ± 0.89	+	+/+	+/+	+/+	+/+
HNHA2	HQ844439	R4	*Bre. brevis* NBRC 15304T(AB271756)	99.01	59.62 ± 1.24	+	+/+	+/+	+/+	+/+
GSLS11	HQ844458	R5	*Bre. formosus* NRRL NRS-863T(D78460)	97.92	31.11 ± 0.83	+	+/+	+/+	+/+	+/+
SZLY111	HQ844499	R5	*Bre. formosus* NRRL NRS-863T(D78460)	97.54	43.07 ± 2.19	+	+/+	+/+	+/+	+/+
BJSS14	HQ844447	R6	*Pa. polymyxa* IAM 13419T(D16276)	96.37	66.56 ± 1.23	+	+/+	+/+	+/+	+/+
DLDG26	HQ844448	R7	*Pa. jamilae* CECT 5266T(AJ271157)	99.45	27.26 ± 2.07	+	+/+	+/+	+/+	+/+
HLJFQ25	HQ844466	R8	*Pa. peoriae* DSM 8320T(AJ320494)	99.45	53.68 ± 0.94	+	+/+	+/+	+/+	+/+
HLJFQ23	HQ844465	R9	*Pa. brasilensis* PB172T(AF273740)	98.04	50.00 ± 0.82	+	+/+	+/+	+/+	+/+
GZLB11	HQ844461	R10	*Pr. rettgeri* DSM 4542T(AM040492)	98.83	43.48 ± 2.54	+	+/+	+/+	+/+	+/+
JSYM27	HQ8444400	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	98.29	39.29 ± 0.91	+	+/+	+/+	+/+	+/+
SZQPS28′	HQ844502	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	99.16	46.85 ± 1.32	+	+/+	+/+	+/+	+/+
XZPG11	HQ844508	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	99.24	58.47 ± 1.23	+	+/+	+/+	+/+	+/+
SCTS33	HQ844441	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	98.55	48.44 ± 1.23	+	+/+	+/+	+/+	+/+
QHFQ21	HQ844495	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	99.38	64.00 ± 0.82	+	+/+	+/+	+/+	+/+
NXHG29	HQ844486	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	98.61	67.14 ± 1.64	+	+/+	+/+	+/+	+/+
NYHS11	HQ844488	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	98.82	51.27 ± 0.90	+	+/+	+/+	+/+	+/+
ZJHD11	HQ844445	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	98.61	45.17 ± 0.85	+	+/+	+/+	+/+	+/+
ZJHG29	HQ844513	R11	*Ps. aeruginosa* LMG 1242T(Z76651)	99.17	45.26 ± 0.90	+	+/+	+/+	+/+	+/+
FJYM11	HQ853017	R12	*Ps. otitidis* MCC10330T(AY953147)	98.12	38.30 ± 2.49	+	+/+	+/+	+/+	+/+
FJWHG1	HQ844452	R13	*S. albiflaviniger* NRRL B-1356T(AJ391812)	97.11	50.65 ± 1.24	+	×	×	×	×
HuBZM22	HQ853021	R14	*S. cellostaticus* NBRC 12849T(AB184192)	98.61	34.14 ± 0.84	+	×	×	×	×
DLHG12	HQ844449	R15	*S. eurocidicus* NRRL B-1676T(AY999790)	98.12	37.29 ± 0.92	+	×	×	×	×
HLJQZ117′	HQ844475	R16	*S. globosus* LMG 19896T(AJ781330)	98.88	56.46 ± 1.23	+	×	×	×	×
GXSS21	HQ844464	R17	*S. racemochromogenes* NRRL B-5430T(DQ026656)	98.24	40.45 ± 0.41	+	×	×	×	×
HLJLJ17	HQ844468	R18	*S. rectiviolaceus* NRRL B-16374T(DQ026660)	97.69	38.60 ± 0.43	+	×	×	×	×

A, B, C, and D respectively represent the chemotactic compounds catechol, p-hydroxybenzoic acid, salicylic acid, and asparagine. "+" under the "*che*A" column indicates that the strains producing a 500 bp of *che*A characteristic band; "+" under the "Drop assay/swarm plate assay" column indicates that the strains showed chemotaxis after detection by plate assay, whereas "×" means that no chemotaxis was detected. *B.* = *Bacillus*, *Bre.* = *Brevibacillus*, *Pa.* = *Paenibacillus*, *Pr.* = *Providencia*, *Ps.* = *Pseudomonas*, *S.* = *Streptomyces*

the P4P5.for and P4P5.rev primer pair, 124 of the 420 strains produced a characteristic *che*A band (approximately 500 bp), whereas the remaining 296 strains produced no *che*A fragment. Of the 124 chemotactic candidates which produced the *che*A band, chemotactic responses of 97 bacterial strains towards four attractants were tested by the

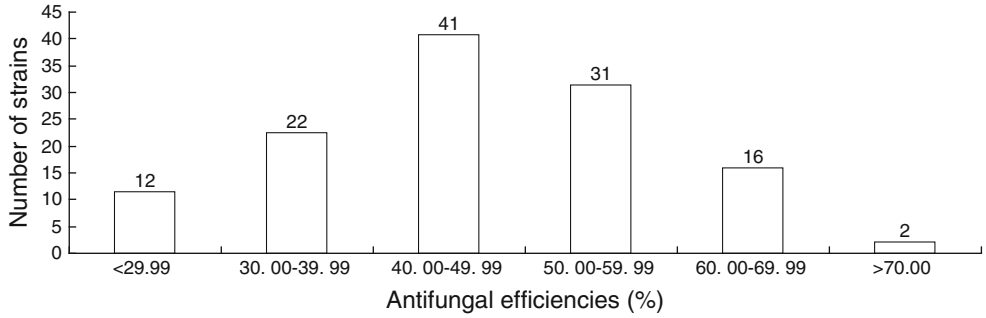

Fig. 2 Distribution of the 124 bacterial antagonists with different efficiencies in suppressing the mycelial growth of *Fusarium oxysporum* f. sp. *cubense*, tested by the Oxford cup method

drop assay and swarm plate assay; the remaining 27 species of *Streptomycineae* were not included in this experiment because of difficulties in preparing a suspension of spores or single cells. Results indicated that the 97 candidates could be taken as chemotactic strains, because all of them exhibited chemotaxis toward the tested chemotactic compounds, catechol, *p*-hydroxybenzoic acid, salicylic acid, and asparagine (Fig. 3).

Phylogeny of antifungal bacteria having the *che*A gene

For the phylogenetic analysis, all of the 124 strains of antifungal bacteria producing a characteristic *che*A band were analyzed first by RFLPs with *Msp*I, *Hae*III, and *Hha*I endonucleases, which resulted in 18 different RFLP patterns. On the basis of these RFLP patterns, 28 isolates (at least one from each unique pattern) were selected for 16S rDNA sequencing. A phylogenetic tree was constructed to illustrate the relationships (Fig. 4). The 124 strains were assigned into the following five bacterial families: *Bacillaceae*, *Enterobacteriaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, and *Streptomycineae* (Fig. 4; Table 1).

Pseudomonadaceae was the dominant group which contained 45 strains (36 % of the total). These strains were represented by 2 RFLP patterns (R11–R12) and were associated with the species of *Pseudomonas aeruginosa* (39 strains) and *Pseudomonas otitidis* (6), with similarities of 98.12–99.38 % and AE values of 38.30–67.14 %.

The *Paenibacillaceae* group included 31 strains (25 % of the total) represented by 6 RFLP patterns (R4–R9). Of them, 18 strains (R6–R9) were assigned into the genus *Paenibacillus*, with similarities of 96.37–99.45 %. These strains showed different antifungal efficiencies against Foc, with AE values of 27.26–66.56 %, and consisted of *Paenibacillus polymyxa*, *Paenibacillus jamilae*, *Paenibacillus peoriae*, and *Paenibacillus brasilensis*. There were 13 strains (R4–R5) with AE values of 31.11–59.62 % associated with the species *Brevibacillus brevis* and *Brevibacillus formosus*, with similarities of 97.54–97.01 %.

The *Streptomycineae* group contained 26 strains (20.97 % of the total) represented by the RFLP types R13–R18. Members of this group were phylogenetically associated with 6 species of the genus *Streptomyces*, with similarities of 97.11–98.88 % and AE values of 31.14–56.46 %: *S. albiflaviniger*, *S. cellostaticus*, *S. eurocidicus*, *S. globosus*, *S. racemochromogenes*, and *S. rectiviolaceus*.

The *Bacillaceae* group included 18 strains (14.52 %) represented by 3 RFLP types (R1–R3). These strains were phylogenetically associated with the species of *Bacillus sonorensis*, *Bacillus tequilensis*, and *Bacillus atrophaeus*, with similarities of 97.12–98.09 % and AE values of 33.79–57.25 %.

The remaining group, *Enterobacteriaceae*, contained 4 strains (R11) which all belonged to the species *Providencia rettgeri*, with similarities of 96.53–99.12 % and AE of 32.14–54.46 %.

Chemical characterization of banana root exudates

The analysis of the extracted banana root exudates by GC–MS produced 85 peaks (Fig. 5). On the basis of the comparison of the retention times in a mass spectra library, 62 compounds were found to be represented in the banana root exudates, including alkanes, alkenes, naphthalenes, benzenes, and alcohols. These compounds were identified as acetophenone, 1,4-bis(trimethylsilyl)-1,3-butadiyne, 2-bromododecane, 2-bromotetradecane, 2-butyl-1-octanol, 1-chlorooctadecane, 1-chlorooctadecane, cyclohexyldimethoxymethylsila, decane, 2,4-di-*tert*-butylphenol, 2,6-di-*tert*-butyl-4-methylphenol, 2,2′-dimethylbiphenyl, 1,5-dimethyl-2, 2,4,6,6-pentamethylheptane, 3,3′-dimethylbiphenyl, di-*tert*-dodecyldisulfide, 5,6-dimethylundecane, 2,4-dimethylheptane, 4,7-dimethylundecane, 2,4-dimethylbenzeneacetonitrile, 3,5-dimethylbenzaldehyde, 1,4-dimethylnaphthalene, 2,6-dimethylnaphthalene, eicosane, 3-ethyl-5-(2-ethylbutyl)octadecane, heneicosane, hentriacontane, heptadecane, hexadecane, hexatriacontane, 1-hexadecanol, 2-isopropyl-5-methyl-1-hexanol, 1-iodotridecane, 5-methylundecane,

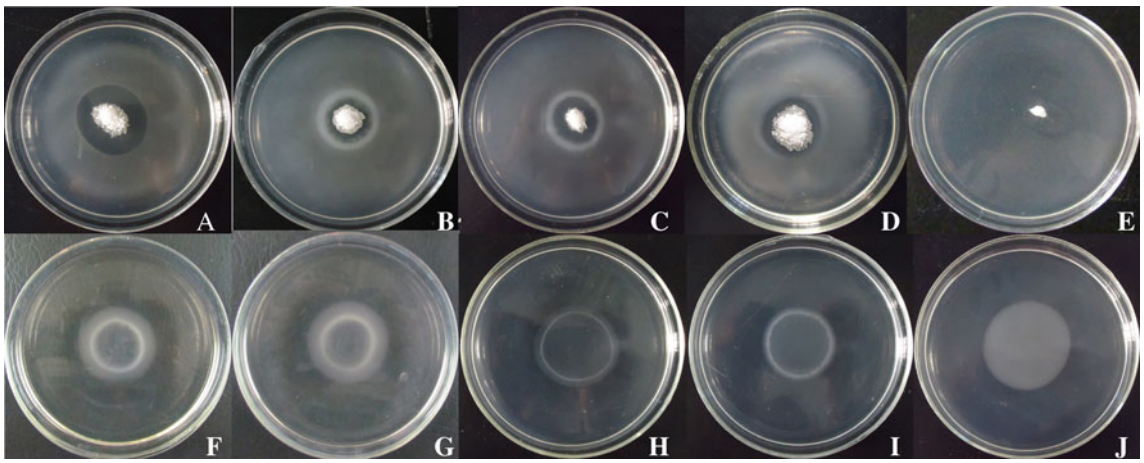

Fig. 3 Chemotactic response of *Pseudomonas aeruginosa* BJSS14 toward catechol (**a**, **f**), *p*-hydroxybenzoic acid (**b**, **g**), salicylic acid (**c**, **h**), and asparagine (**d**, **i**), as analyzed by the drop assay (**a**–**e**) and swarm plate assay (**f**–**j**) using MM solution as the control (**e**, **j**)

10-methylnonadecane, 6-methyldodecane, 3-methylheptane, methoxyacetic acid, 1-nonene, octadecane, 1-octadecanesulfonyl chloride, 7-oxabicyclo[4.1.0]heptane, 1,1′-oxybisoctane, pentacosane, phenanthrene, tetracontane, tetracosane, tetradecane, 2-tetradecylester, tridecane, 2,6,10,14-tetramethylhexadecane, tritetracontane, 2,4,6-trimethyloctane, 2,6,10-trimethylpentadecane, 1-tridecene, 2,6,10-trimethyldodecane, 1,4,6-trimethylnaphthalene, 2,3,6-trimethylnaphthalene, 2,3,5-trimethylnaphthalene, 2,3,6-trimethylnaphthalene, 1,4,5-trimethylnaphthalene, 2,3,5-trimethylpyrazine, and undecane.

Chemotactic response of chemotactic antifungal bacteria against Foc

The root exudates and seven compounds (1-bromododecane, 2,6-di-*tert*-butyl-4-methylphenol, eicosane, hexadecane, 2,4-di-*tert*-butylphenol, octadecane, and octacosane) found in the root exudates with higher peak areas (peaks 1–7 in Fig. 5) were used in the chemotactic assay with five representative chemotactic antifungal bacteria against Foc, namely *Bacillus tequilensis* ZJXBC21, *Brevibacillus brevis* HNHA2, *Paenibacillus polymyxa* BJSS14, *Providencia rettgeri* GZLB11, and *Pseudomonas aeruginosa* NXHG29 (Table 2). The results showed that the chemotactic response of all of the tested bacteria to the banana root exudates was significantly stronger ($P = 0.05$; 1.76- to 7.9-fold) than that of the control (phosphate buffer) (Table 2). Concerning the bacterial chemotaxis toward the compounds in the root exudates, all of the tested attractants were able to attract a significant number of cells of chemotactic antifungal bacteria against Foc ($P = 0.05$) in the capillary tubes, except 2,4-di-*tert*-butylphenol and 1-bromododecane with the *B. tequilensis* ZJXBC21 and *P. rettgeri* GZLB11 strains (Table 2). The chemotactic bacterial species varied significantly ($P = 0.05$) in their chemotactic ability toward the root exudates and specific chemical attractants (Table 2). For example, for hexadecane, the VRCRs of *P. polymyxa* BJSS14 (4.31) and *B. brevis* HNHA2 (4.15) were significantly higher than those of *B. tequilensis* ZJXBC21 (3.42), *P. aeruginosa* NXHG29 (2.78), and *P. rettgeri* GZLB11 (2.41) ($P = 0.05$). But for 2,6-di-*tert*-butyl-4-methylphenol, the VRCRs of *P. polymyxa* BJSS14 (5.23) and *P. rettgeri* GZLB11 (5.51) were similar, and both were significantly higher than the VRCRs of *P. aeruginosa* NXHG29 (4.41), *B. brevis* HNHA2 (3.73), and *B. tequilensis* ZJXBC21 (2.31). Additionally, *P. polymyxa* BJSS14 exhibited a significant difference in its cheomotactic response to different chemical attractants with a concentration of 20 μg ml^{-1} ($P = 0.05$), e.g., octadecane was the strongest attractant (VRCR = 7.38), followed sequentially by 2,6-di-*tert*-butyl-4-methylphenol (5.23), octacosane (4.69), hexadecane (4.31), eicosane (3.62), 1-bromododecane (2.55), and 2,4-di-*tert*-butylphenol (2.42).

Discussion

It was previously reported that certain bacterial species can act as biocontrol agents to effectively manage *Fusarium* wilt of banana. For example, the combined application of the biocontrol agents *Pseudomonas fluorescens* Pf1 and *Bacillus subtilis* TRC 54 with a botanical fungicide (Wanis 20 EC) reduced the wilt incidence significantly under greenhouse (64 %) and field conditions (75 %) [1]. These two antifungal bacteria also were reported to suppress the mycelial growth of Foc by 41 % [34]. The reintroduction of naturally occurring endophytes into banana tissue culture plantlets suppressed *Fusarium* wilt by nearly 67 % in

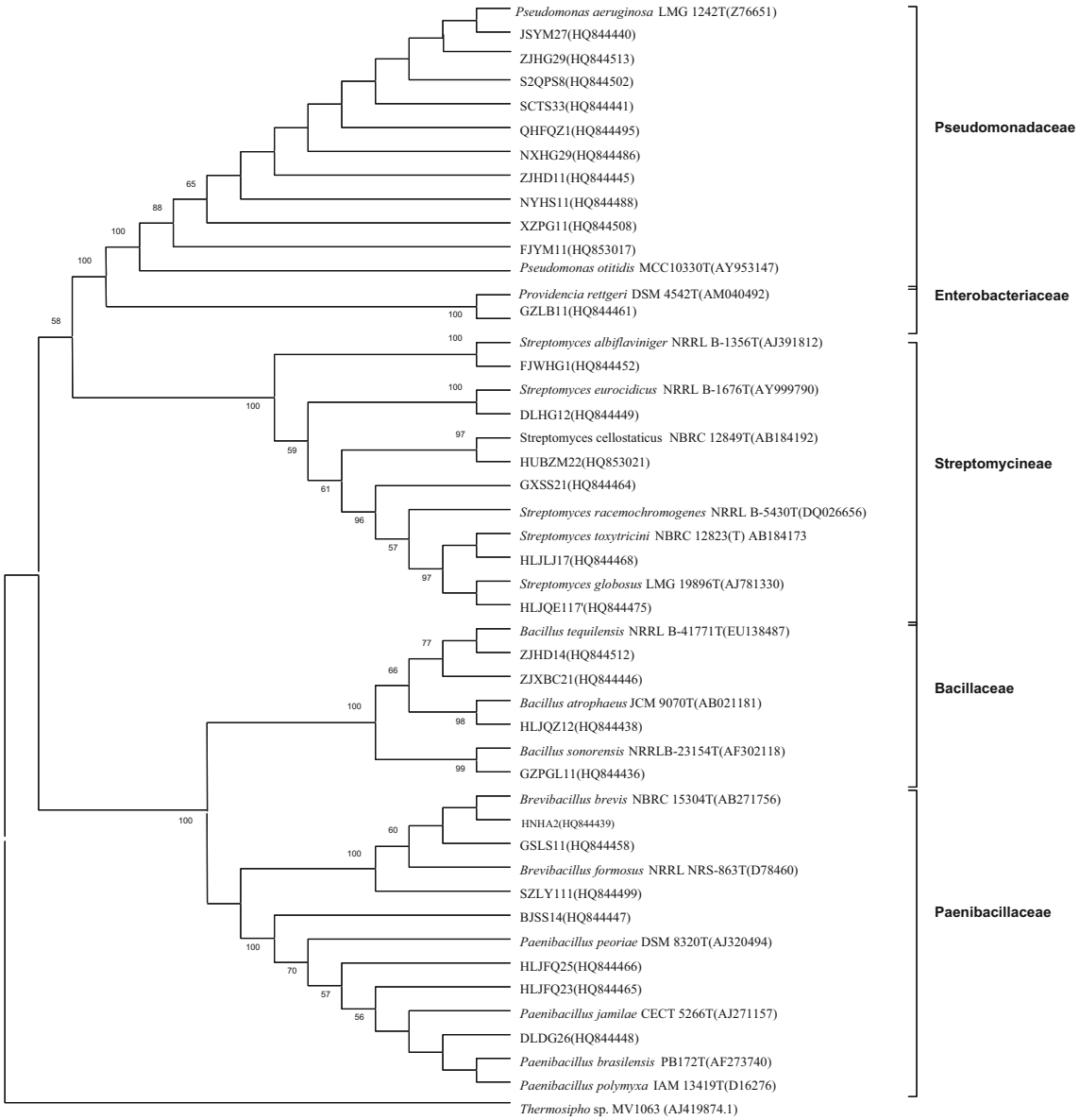

Fig. 4 Neighbor-joining phylogenetic tree of 16S rRNA gene sequences of chemotactic antagonists against *Fusarium oxysporum* f. sp. *cubense* and their closest phylogenetic relatives

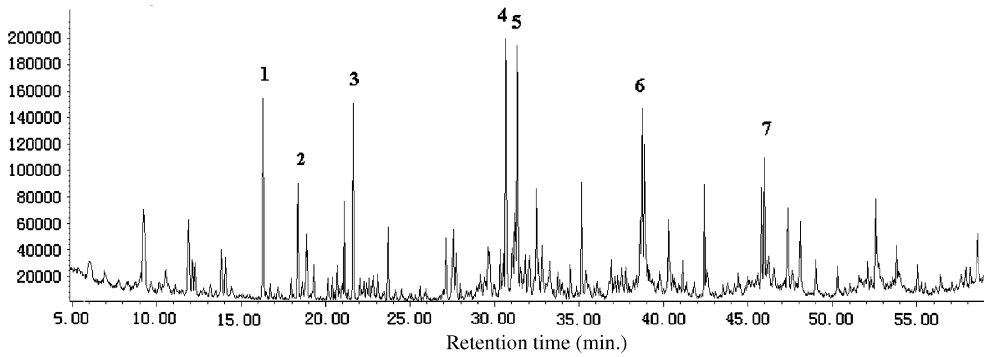

Fig. 5 Spectral analysis of the banana root exudates by GC–MS. *Peaks 1–7*, with relatively higher peak areas, were hexadecane, octacosane, 2,6-di-*tert*-butyl-4-methylphenol, 2,4-di-*tert*-butylphenol, octadecane, eicosane, and 1-bromododecane, respectively

Table 2 Relative chemotactic response of chemotactic antifungal bacteria against Foc to root exudates and compounds present in the root exudates of banana

Attractants	VRCR of isolates towards compounds				
	BJSS14	HNHA2	ZJXBC21	NXHG29	GZLB11
Control (phosphate buffer)	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Root exduates	7.00 ± 0.46^{f}	1.79 ± 0.16^{b}	1.76 ± 0.13^{b}	2.70 ± 0.10^{b}	2.70 ± 0.10^{b}
Hexadecane	$4.31 \pm 0.30^{d\ A}$	$4.15 \pm 0.36^{e\ A}$	$3.42 \pm 0.23^{d\ B}$	$2.78 \pm 0.36^{b\ C}$	$2.41 \pm 0.15^{b\ C}$
Octacosane	$4.69 \pm 1.02^{d\ A}$	$3.47 \pm 0.31^{d\ B}$	$2.04 \pm 0.10^{b\ C}$	$3.62 \pm 0.37^{c\ B}$	$2.83 \pm 0.10^{b\ C}$
2,6-Di-*tert*-butyl-4-methylphenol	$5.23 \pm 0.42^{e\ A}$	$3.73 \pm 0.37^{d\ C}$	$2.31 \pm 0.05^{b\ D}$	$4.41 \pm 0.35^{d\ B}$	$5.51 \pm 0.15^{d\ A}$
2,4-Di-*tert*-butylphenol	$2.42 \pm 0.63^{b\ B}$	$2.80 \pm 0.18^{c\ A}$	$1.26 \pm 0.24^{a\ C}$	$1.24 \pm 0.18^{a\ C}$	$1.17 \pm 0.10^{a\ C}$
Octadecane	$7.38 \pm 0.97^{f\ A}$	$5.82 \pm 0.21^{f\ B}$	$2.26 \pm 0.06^{b\ D}$	$3.18 \pm 0.30^{c\ C}$	$3.40 \pm 0.10^{c\ C}$
Eicosane	$3.62 \pm 0.77^{c\ B}$	$3.43 \pm 0.15^{d\ B}$	$2.87 \pm 0.11^{c\ C}$	$7.75 \pm 0.62^{e\ A}$	$3.43 \pm 0.09^{c\ B}$
1-Bromododecane	$2.55 \pm 0.78^{b\ A}$	$2.78 \pm 0.33^{c\ A}$	$1.41 \pm 0.08^{a\ C}$	$2.25 \pm 0.17^{b\ B}$	$1.18 \pm 0.08^{a\ C}$

Data are means of three replicates. Values in a column followed by the same superscripted small letters and values in a line followed by the same superscripted capital letters are not significantly different according to Duncan's multiple range test at $P = 0.05$

the greenhouse [19]. *Streptomyces violaceusniger* strain G10 exhibited strong antagonism towards *F. oxysporum* f. sp. *cubense* races 1, 2, and 4 by producing extracellular antifungal metabolites [11]. Treating banana plantlets with a G10 suspension significantly reduced the disease severity index for leaf symptoms (47 %) and rhizome discoloration (53 %). Other bacterial species antagonistic to Foc include *Pseudomonas aeruginosa*, *Serratia marcescens*, *Burkholderia glumae*, *Erwinia chrysanthemi*, *Paenibacillus polymyxa*, *Streptomyces griseocarneus*, and *Bacillus licheniformis* [15, 16, 36, 38, 40]. The Foc suppression mechanisms of these bacteria involved the production of siderophores, antibiotics, and extracellular enzymes or the induction of systemic resistance [1]. Our study characterized the diversity profiles of chemotactic Foc-antagonistic bacteria from agricultural soils and included 124 strains of 18 species. With the exception of *Pseudomonas aeruginosa* and *Paenibacillus polymyxa*, the remaining 16 species and the new antagonists of Foc confirmed here are *Paenibacillus jamilae*, *Paenibacillus peoriae*, *Paenibacillus brasilensis*, *Brevibacillus brevis*, *Brevibacillus formosus*, *Bacillus sonorensis*, *Bacillus tequilensis*, *Bacillus atrophaeus*, *Streptomyces albiflaviniger*, *S. cellostaticus*, *S. eurocidicus*, *S. globosus*, *S. racemochromogenes*, *S. rectiviolaceus*, *Providencia rettgeri*, and *Pseudomonas otitidis*.

Root exudation includes the secretion of ions, free oxygen and water, enzymes, mucilage, and a diverse array of carbon-containing primary and secondary metabolites [20]. Root exudates are often divided into two classes of compounds. Low molecular weight compounds, such as amino acids, organic acids, sugars, phenolics, and other secondary metabolites, account for much of the diversity of root exudates, whereas high molecular weight exudates, such as mucilage (polysaccharides) and proteins, are less diverse but often compose a larger proportion of the root exudates by mass [3]. Xu et al. [38] first characterized the chemical composition of banana (*M. acuminata* AAA Cavendish cv. Brazil) root exudates using a GC–MS method, and 41 compounds, including hydrocarbons, terpenoids, alcohols, acids, esters, and aldehydes, were reported. In our banana root exudates sample, 62 compounds were identified of which 2,6,10-trimethylpentadecane, octadecane, eicosane, heneicosane, heptadecane, hexadecane, methoxyacetic acid, and 2-tetradecylester were also reported by Xu et al. [38]. The composition of the plant root exudates changed according to various factors, such as the cultivar, growth phase, culture condition, and infecting microbe. The current analytical approaches for exudate analysis mainly rely on spectroscopic databases [8]; however, alternative approaches are required to deduce the complete structures for unknown compounds and those not represented in the databases.

Buchan et al. [5] first developed and applied a molecular diagnostic tool based on the *che*A gene encoding the central regulator of bacterial chemotaxis to characterize and temporally track specific populations of native microbes with chemotactic potential. In the present study, we initially screened the chemotactic bacteria using primers specific for *che*A and found that 97 bacterial strains produced the characteristic *che*A band and all of them exhibited chemotaxis in our petri dish assays. This result indicated that the *che*A gene is a reliable molecular diagnostic method for bacterial chemotaxis. As alternatives of chemical pesticides, biological control agents (BCAs) have played an important role in agricultural practices; however, to control soil-borne pathogens effectively, a prerequisite for BCAs is that the beneficial microorganisms should be grown well to establish a considerable population after being applied to the soil. Soil is an extremely complex

milieu in which a number of factors, including the soil texture, cation exchange capacity, organic matter content, pH, moisture, and the presence of a viable soil microflora, can influence the persistence and/or efficacy of BCAs. It has been reported that most BCAs could not grow normally in soils due to soil fungistasis [21], thus reducing their efficacy. Root exudates represent an important source of nutrients for microorganisms in the rhizosphere and seem to participate in early colonization by inducing chemotactic responses of rhizospheric bacteria [3], and the chemoattractant characteristics of bacteria allow them to outcompete indigenous species without chemotaxis [20]. In our study, the number of cells of the five tested chemotactic antifungal bacteria against Foc in the capillary tubes containing the compounds in the root exudates was higher (1.76- to 7.75-fold) than in the control. In future studies, it should be clarified whether the chemotactic biocontrol bacteria show colonization advantages in the rhizosphere and increase their control efficiencies to targeted soil-borne pathogens with the application of attractant chemicals under natural conditions.

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