Endophytic *Bacillus cereus* Effectively Controls *Meloidogyne incognita* on Tomato Plants Through Rapid Rhizosphere Occupation and Repellent Action

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Abstract

Root-knot nematodes (*Meloidogyne* spp.), which cause severe global agricultural losses, can establish a special niche in the root vascular cylinder of crops, making them difficult to control. Endophytic bacteria have great potential as biocontrol organisms against *Meloidogyne incognita*. Three endophytic bacteria were isolated from plant tissues and showed high nematocidal activity against *M. incognita* second-stage juveniles (J2) in vitro. The *gyrB* gene sequence amplification results indicated that the three isolates were *Bacillus cereus* BCM2, *B. cereus* SZ5, and *B. altitudinis* CCM7. The isolates colonized tomato roots rapidly and stably during the colonization dynamic experiment. Three pot experiments were designed to determine the potential of three endophytic bacterial isolates on control of root-knot nematodes. The results showed that the preinoculated *B. cereus* BCM2 experiment significantly reduced gall and egg mass indexes. The inhibition ratio of gall and egg mass was up to 81.2 and 75.6% on tomato roots and

significantly enhanced shoot length and fresh weight. The other two experiments with inoculated endophytic bacteria and *M. incognita* at the same time or after morbidity had lower inhibition ratios compared with the preinoculated endophytic bacteria experiment. The confocal laser-scanning microscopy method was used to further study the possible mechanism of endophytic bacteria in the biocontrol process. The results showed the localization pattern of the endophytic bacteria *B. cereus* BCM2-(*str'*)-pBCgfp-1 in tomato root tissues. Root tissue colonized by endophytic bacteria repelled *M. incognita* J2 infection compared with the untreated control in a repellence experiment. We isolated an endophytic *B. cereus* strain that stably colonized tomato and controlled *M. incognita* effectively. This strain has potential for plant growth promotion, successful ecological niche occupation, and *M. incognita* J2 repellent action induction. It plays an important role in endophytic bacteria against root-knot nematodes.

Root-knot nematodes (*Meloidogyne* spp.), which obtain their food exclusively from living cells in roots, are among the most detrimental parasitic nematodes in vegetables and field crops worldwide. Once invading the root, the juveniles migrate intercellularly into the vascular cylinder to establish a permanent feeding site and induce the growth of giant cells that result in the formation of root galls (Gheysen and Fenoll 2002). Root-knot nematodes attack roots and cause severe impairment to plant nutrient and water uptake (Griffiths 1986). The plant-parasitic nematodes caused an estimated \$157 billion losses annually (Abad et al. 2008). Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops in the world. *Meloidogyne incognita* usually causes severe yield losses of over 30% in highly susceptible vegetable crops (Sikora and Fernández 2005). Nematodes live in the soil and usually invade underground tissue to established highly specialized feeding structures and occupy the organism permanently, which makes the management and control of nematodes difficult (Radwan et al. 2012).

An endophyte is a kind of organism that colonizes the internal tissue of a plant at some stage during its life cycle without causing plant disease. Both endophytic bacteria and fungi have been shown to be important regulators of plant health (Backman and Sikora 2008), because they share the same niche with nematodes. Currently, there are several potential biocontrol microorganisms with inhibitory activities against nematodes that have been extensively studied. *Arthrobotrys oligospora*, *Paecilomyces lilacinus*, *Bacillus thuringiensis*, and *B. nematode* were systematically investigated in past studies (Niu et al. 2010; Wang et al. 2014; Zhang et al. 2012). *Myrothecium verrucaria*, *Pochonia chlamydosporia*, *B. firmus*, and *Pasteuria penetrans* have been successfully used to control nematodes in agricultural fields

(Butt et al. 2001; Kokalis-Burelle 2015; Trudgill et al. 2000; Xiong et al. 2015). *Pochonia chlamydosporia* is also a commercial biocontrol strain in China and Cuba. *B. firmus* is the only commercial *Bacillus* product currently in the market that has been developed in Israel as a biological nematicide agent (Keren-Zur et al. 2000). The evidence described above proves that microorganisms possess a powerful potential for biological control against the root-knot nematode.

Due to their negative impact on the environment and increasing concerns about food safety, many chemical nematicides with high toxicity have been withdrawn or totally forbidden in developed countries (Nico et al. 2004; Schneider et al. 2003). For food safety and human health interests, using biocontrol microorganisms to manage *M. incognita* is being systematically investigated (Mostafa 2001; Singh et al. 2013). Fast-growing endophytic bacteria are capable of colonizing the internal tissues of plants without causing disease (Ryan et al. 2008; Schulz 2006) and consistently occupying the ecological niche (Hallmann et al. 1997). This fact leads to competition for space and nutrition between endophytes and plant-parasitic nematodes. Complex endophytic bacteria-plant networks and co-occurrence relationships are formed in the root, which gives an advantage to the endophytic bacteria in such competitions with the root-knot nematode (Kerry 2000). Many studies have been conducted on endophytic bacteria for the biological control of plant-parasitic nematodes, which were compiled and reported by Sikora et al. (2007). The reports described above suggested that endophytic bacteria can be potential candidates for biocontrol of root-knot nematodes. In the present work, scientists focused on the endophytic bacterial mechanisms of biological control of nematodes, including niche competition, direct toxicity, plant growth promotion, changing exudates, systemic repelling of resistance, and induction (Hashem and Abo-Elyousr 2011; Sikora et al. 2007). Understanding endophytic bacteria interactions with host plants and root-knot nematodes will improve endophyte biocontrol efficiency.

The objective of this study was to screen for an efficient endophytic bacteria strain contributing to reduction of the use of pesticides in the control of root-knot nematodes. We obtained three endophytic bacterial isolates to manage plant-parasitic nematodes on crops. We studied the colonization dynamics of endophytic bacteria BCM2, SZ5, and CCM7 as well as their biocontrol potential

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*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary videos are published online.

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on *M. incognita* in tomato. To confirm the potential mechanisms in the process of endophytic bacteria biocontrol of root-knot nematodes on tomato, green fluorescent protein (GFP)-tagged *B. cereus* BCM2 and repellent action experiments were conducted.

Materials and Methods

Sampling of nematocidal endophytic bacteria and *M. incognita*.

Isolate of endophytic bacteria strains. Endophytic bacteria were isolated from the fruit and plant tissues of strawberry, persimmon, chili, and tomato grown in Nanjing, Jiangsu Province, China. Different tissue parts were washed under tap water and surface sterilized by sequential immersion in 70% (vol/vol) ethanol and NaClO (1%) for 5 min (Suryanarayanan et al. 1998). The explants were washed five times in sterilized water to remove surface sterilization agents. These segments were then incubated on beef extract peptone agar medium (3 g of beef extract, 10 g of peptone, and 5 g of NaCl) for 1 to 5 days at 28°C. Single colonies were storage at -80°C in beef extract peptone medium containing 20% glycerol.

Preparation of second-stage juveniles of *M. incognita*. Dahong tomato Hezuo903 (*L. esculentum* Mill.) seed were obtained from the Tomato Institute of Shanghai. Tomato plants were grown under conditions of 28 and 20°C (day: 14 h and night: 10 h, respectively) and 75% humidity in pots (15 cm in diameter by 14 cm in height) which contained 1,000 g of a 1:1 substrate mixture of silica sand and potting soil. *M. incognita* MIJ53 was provided by Professor Hongmei Li from the Department of Plant Pathology of Nanjing Agriculture University. The *M. incognita* MIJ53 population was maintained on tomato plants. Egg masses were harvested from the root 8 weeks after inoculation. After treatment with 0.5% NaClO for 3 min and rinsing with distilled water, the egg masses were put on sieves with 25-μm openings suspended over deionized water in plastic bowls, and pre-parasitic second-stage juveniles (pre-J2) were hatched (Hussey and Barker 1973). The J2 were then collected using a modified Baerman funnel technique (Gray 1984).

Nematicidal activity of endophytic bacterial isolates in vitro. To determine the nematicidal activity of endophytic bacterial isolates, the purified bacterial strains were cultured in 100 ml of beef extract peptone medium with shaking at 160 rpm and 28°C for 60 h. Bacterial culture was centrifuged (8,000 rpm, 5 min) and cells were removed from the supernatant with a 0.22-μm Millipore filter (Aalten and Gowen 1998). Treatments consisted of (i) medium control and (ii) fermentation liquor of isolates. In total, 100 hatched pre-J2 were added to Eppendorf tube containing 1 ml of medium and isolate supernatant was incubated at 25°C. Each treatment was replicated 10 times. Mortality rates and different body morphologies of dead nematodes of J2 were recorded after 6, 12, and 24 h under a light microscope.

Bacterial identification by sequencing of the 16S ribosomal RNA and gyrase B gene. Three strains (BCM2, SZ5, and CCM7) were selected for amplification of the 16S rDNA and gyrase B (*gyrB*) genes. Genomic DNA was extracted with an Ezup genomic DNA extraction kit from Sangon Biology. 16S ribosomal RNA (rRNA) and *gyrB* were amplified by PCR with Takara Taq polymerase (Takara Biotechnology). Isolates were first identified by using 16S rRNA gene sequencing. Amplification of the 16S rRNA with the eubacterial universal primers 27F and 1492R was carried out (Lane 1991). The polymerase chain reaction (PCR) was performed by using a 5-min predenaturation at 95°C; followed by 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 90 s; a final extension step of 10 min at 72°C; then, the reaction was held at 4°C. The *gyrB* gene was amplified and sequenced by using the primers UP-f 5'-AGCAGGGTACGGATGTGCGAGC CRTCACRTGCTGCTAT-3' and UP-r 5'-GAAGTCATC ATGACCGTTCTGCAYGCNGGNGGNAARTTYGA-3' (Yamamoto and Harayama 1995) to identify three isolates. The PCR was performed by using a 4-min predenaturation step at 95°C; followed by 30 cycles of 98°C for 10 s, 62°C for 1 min, 72°C for 2 min; a final extension 72°C for 10 min; and a 4°C hold indefinitely. PCR products were purified using the AxyPrep DNA Gel Extraction kit, ligated into the pMD-19T vector, then transformed into *Escherichia coli* DH5α. Sequencing of the PCR products was conducted by Sangon Biology. The results were queried against the GenBank database

with BLAST, and MEGA 5.0 was used to construct a phylogenetic tree.

Colonization dynamic of endophytic bacteria in tomato root.

Endophytic bacteria BCM2, SZ5, and CCM7 colonization dynamics were tested by using the spread-plate method. Endophytes were inoculated by root stabbing methods: a 2-cm-wide iron ruler was thrust into the tomato root four to six times before pouring the bacterial suspension into the wound. The population of endophytes in tomato roots were determined at 1, 3, 5, 7, 9, 11, 15, 20, 25, 30, 35, 40, 45, and 50 days postinoculation (dpi). First, the isolates were selected and labeled with the streptomycin sulfate (*str'*) resistance gene (Kumar and Tyagi 1978) so that the isolates were able to grow on plates containing *str'* at 300 μg/ml. The bacteria cultures were then collected with centrifugation at 8,000 rpm for 5 min. Bacteria cells was diluted with sterile water and the concentrations of BCM2-*str'*, SZ5-*str'*, and CCM7-*str'* were 2.25×10^8 , 1.75×10^8 , and 1.5×10^8 CFU/ml and the optical densities at 600 nm of these suspensions were 0.480, 0.441, and 0.463, respectively. The suspension (10 ml) was watered onto each 1-month-old tomato plant. Three tomato plants were carefully taken from soil randomly at each dpi, of which 0.2 g of root was ground after surface disinfection with 70% (vol/vol) ethanol and NaClO (1%) for 5 min. The suspension (200 μl) was then diluted and spread onto three beef extract peptone agar mediums, each containing *str'* at 300 μg/ml to determine the population (CFU per gram of root) of three isolates on tomato roots. Each experiment was carried out twice.

Potential of plant endophytic bacteria to control *M. incognita* and the effect on tomato growth.

Three experiments were designed for testing the potential of endophytic bacteria BCM2, SZ5, and CCM7 for biocontrol of the root-knot nematodes and plant growth promotion under greenhouse. Tomato plants (30 days old) obtained from seedling substrates were transplanted into pots, each containing 1,000 g of soil (as described above). Treatments consisted of (i) 10 ml of sterile water as a control, (ii) 10 ml of Abamectin as a control, (iii) BCM2 suspension, (iv) SZ5 suspension, and (v) CCM7 suspension. The bacterial suspension density was approximately 10^8 CFU/ml. Each treatment was replicated 10 times. Abamectin (1.8%) was diluted 3,000-fold with sterile water (Sun et al. 2006). In experiment 1, tomato plants were preinoculated with 10 ml each of the three endophytic bacteria cell suspensions. Two weeks later, for its stable colonization of endophytic bacteria, 1,000 J2 in 2 ml of water were inoculated into 2-cm-deep holes around each tomato stem. A dilution plate counting method was used to calculate the population of endophytes in tomato roots. In experiment 2, tomato plants were inoculated with 10 ml of endophytic bacterial cell suspensions and J2 as described above at the same time. In experiment 3, the 1,000 J2 were inoculated for 10 days with gall formed in the plants; then, 10 ml of bacterial suspension was added into holes 2 cm deep around the stem, following a modified procedure previously reported by Radwan et al. (2012). After 50 dpi of *M. incognita* J2, the number of galls, egg masses per plant, stem height, fresh weight, and dry weight of shoot were determined (Adam et al. 2014). The inhibition ratio (IR) was calculated with the method previously reported (Niu et al. 2012) using the formula $IR = 100\% \times (CN - TN)/CN$, where CN is the number of galls or egg masses in untreated tomato roots and TN is the number of galls or egg masses in treated tomato roots.

Colonization pattern of endophytic bacteria on the tomato root.

Construction of isolate BCM2 tagged with GFP. To determine the ecological niche and pattern of endophytic bacteria in tomato roots, the *B. cereus*-BCM2 (*str'*) isolate with the best colonization potential among three isolates in this study was used for GFP tagging. Plasmid pBCgfp-1 was stored in our lab. The competent cells were prepared as previously described (Turgeon et al. 2006). Electroporation of *B. cereus* BCM2 was carried out as previously described (Cao et al. 2011) with a Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories) equipped with a Bio-Rad pulse controller. Plasmid (1 μg) was added to 100 μl of competent cells, and electroporation was carried out in 2-mm electroporation cuvettes (Bio-Rad Laboratories) with the conditions of 2.5 kV, 200 Ω, and 25 μF. The mixture was incubated

for 1 h at 37°C and 220 rpm; then, the proper amount was plated on Luria-Bertani (LB) agar medium containing kanamycin at 20 µg/ml, which was cultured at 37°C for 12 h. A single colony was observed under a fluorescence microscope (Imager A1; Zeiss). The growth curve was measured. Colonization amounts in tomato root of BCM2 (*str'*)-pBCgfp-1 were regulated to ensure that the *gfp*-tagged BCM2 produced a sufficiently bright fluorescent signal for single-cell detection without influencing the growth speed and invasion of the isolate.

GFP-tagged *B. cereus* BCM2 (*str'*) colonization pattern. The colonization patterns of endophytic bacteria on tomato roots were tested with tissue sectioning and detected by confocal laser-scanning microscopy (CLSM) observation. The GFP-tagged *B. cereus* BCM2 (*str'*) was inoculated into tomato plants as described in the previous section. Two weeks later, for its stable colonization, different parts of the tomato roots were monitored by CLSM. The roots taken from soil were washed by tap water, then dipped in 0.05% NaClO for 3 min. Different root tissues were detected using a Nikon Ti A1 with 488- and 568-nm band-pass filters to capture the green fluorescence from GFP-tagged BCM2 (*str'*) and the red autofluorescence from the tomato, respectively (Chi et al. 2005).

Repelling effects of endophytic bacteria on *M. incognita* J2 in vitro. Tomato root fragments (20 mg) were placed on a 0.5% water agar plate to detect the repelling effect of the isolates on J2. Treatments consisted of (i) sterile water and (ii) endophytic bacteria BCM2, SZ5, and CCM7. Each treatment was repeated 10 times. Endophytic bacterial isolates were inoculated on 1-month-old tomato plants (which were grown in sterile soil) and for their stable colonization (2 weeks, in this experiment). Tomato plants were harvested, thoroughly washed under tap water, and surface sterilized with 0.05% NaClO for 3 min. Roots fragments treated with endophytic bacteria and sterile water were placed on a water agar plate (with 2 cm distance), and 100 *M. incognita* J2 in 20 µl of sterile water were added in the middle and incubated at 28°C in an electroheating standing-temperature cultivator. The movement and infection of J2 on the water agar plate was observed with an inverted microscope (Olympus IX 71). Fragments of tomato roots were pulverized and the number of J2 in the roots was recorded after 48 h under a light microscope (Nikon A1).

Statistical analysis. Data from the experiments were conducted with R software using the “multcomp” R package. Treatments (nematicidal activity in vitro, colonization dynamics, and biocontrol of *M. incognita*) were compared by one-way analysis of variance ($P \leq 0.05$). When a significant *F* value was determined, means were separated by Tukey’s multiple comparison test. For compare the repelling effects of three isolates, the *P* value was adjusted by

Dunnnett’s test. Data from colonization numbers were transformed to log *x* for analysis (Poonguzhali et al. 2008).

Results

Screening endophytic isolates with nematocidal activity. Twelve endophytic bacteria strains were isolated from the plant tissues. Based on the nematocidal activity ($P \leq 0.05$) and different appearances of dead nematodes in vitro after 6, 12, and 24 h of treatment with the isolate’s suspension, the mortality rate of the 12 isolates was above 90%. Three endophytic bacterial isolates (BCM2, SZ5, and CCM7) were used for further experiments (Table 1).

Identification of three isolates by sequencing of 16S rRNA and *gyrB* genes. The 16S rRNA gene was amplified and sequenced for species identification. Sequences of the 16S rRNA gene were comparatively analyzed by using the BLAST algorithm in the GenBank nucleotide sequence database. However, the alignment was not specific enough for identification. Therefore, we did more identifications by combinations of *gyrB* gene sequencing and biochemical experiments (data not shown). The results indicated that the isolates BCM2 and SZ5 fit to *B. cereus* and CCM7 fit to *B. altitudinis*. A phylogenetic neighbor-joining tree was constructed to determine the taxonomic relationships of the three isolates (Fig. 1). The GenBank accession numbers of BCM2, SZ5, and CCM7 were KT923659, KT923660, and KT923661, respectively.

Colonization dynamics of endophytic bacterial isolates in the tomato root. Three isolates rapidly proliferated in large quantities within 7 dpi on the tomato root. The population of endophytic bacteria in the roots was stable, with $\geq 10^3$ CFU/g fresh weight until 50 dpi (Fig. 2). Differences between the three isolates in tomato roots after inoculation were recorded. BCM2 was found to have the strongest colonization ability, and the maximum density of cells was 3.53×10^6 CFU/g fresh weight at 9 dpi on the tomato root; overall colonization was significantly higher than for SZ5 and CCM7 ($P \leq 0.05$).

Potential of endophytic bacteria in biocontrol of *M. incognita* and plant growth promotion effect in tomato. Pot experiment results indicated that inoculation of *B. cereus* BCM2, *B. cereus* SZ5, and *B. altitudinis* CCM7 in tomato roots in different experiments reduced the number of galls and egg masses compared with sterile water at 50 dpi (Table 2). BCM2 was shown to have the highest IR with respect to galls and egg masses on tomato roots by up to 81.2 and 75.6%, higher than the positive control abamectin at 73.8 and 60% ($P \leq 0.05$). Where endophytic bacteria and *M. incognita* were inoculated at the same time, the bacteria also had reduced numbers of galls and egg masses; however, the effect on the IR of galls and egg masses was lower compared with the preinoculation of endophytic bacteria ($P \leq 0.05$). Preinoculation with *M. incognita* before bacteria inoculation did not show a solid effect of the three bacterial

Table 1. Endophytic bacterial isolates screened in this experiment and nematocidal activity of *Meloidogyne incognita* in vitro^x

Ser. ^y	Mortality rate			Source	Tissue	Symptom ^z
	6 h	12 h	24 h			
BCM2	87.0 ± 5.4 a	91.3 ± 5.2 a	100 ± 0.0 a	<i>Fragaria ananassa</i>	Fruit	Seepage
CCM3	21.2 ± 9.1 cd	86.9 ± 3.0 ab	99.2 ± 0.8 ab	<i>F. ananassa</i>	Fruit	Seepage
CCM7	78.9 ± 1.8 ab	90.2 ± 1.2 a	100 ± 0.0 a	<i>F. ananassa</i>	Fruit	Degradation
CCM9	71.8 ± 4.6 b	86.9 ± 2.4 ab	100 ± 0.0 a	<i>F. ananassa</i>	Fruit	Degradation
DP1	72.2 ± 4.4 b	85.9 ± 2.6 ab	97.4 ± 1.8 ab	<i>Lycopersicon esculentum</i>	Fruit	Rigidity
DP8	9.6 ± 2.5 de	31.6 ± 7.4 d	98.2 ± 1.7 ab	<i>L. esculentum</i>	Fruit	Rigidity
DP10	11.3 ± 1.9 de	71.4 ± 2.4 b	100 ± 0.0 a	<i>L. esculentum</i>	Fruit	Seepage
DP12	7.7 ± 3.1 de	43.9 ± 4.4 cd	90.9 ± 4.5 b	<i>L. esculentum</i>	Fruit	Rigidity
DP24	7.4 ± 7.4 de	52.4 ± 5.9 c	99.4 ± 0.6 ab	<i>L. esculentum</i>	Fruit	Rigidity
SZ5	71.5 ± 5.1 b	94.1 ± 6.2 a	100 ± 0.0 a	<i>Diospyros kaki</i> L.f	Fruit	Rigidity
XG32	19.2 ± 6.7 cd	46.9 ± 8.4 c	98.4 ± 1.1 ab	<i>Capsicum annuum</i> Linn.	Root	Rigidity
YC1	28.4 ± 2.2 c	41.8 ± 7.3 cd	92.1 ± 2.6 b	<i>C. annuum</i> Linn.	Root	Rigidity
CK	0 ± 0 e	0.9 ± 0.4 e	1.6 ± 1.1 c	...	...	...

^x Data are represented as the mean ± standard deviation (*n* = 10). Within each column, values followed by different letters are significantly different ($P \leq 0.05$) according to one-way analysis of variance and Tukey’s multiple comparison test.

^y Serial number.

^z Symptom of death.

isolates on the reduction of the galls and egg masses compared with other treatments mentioned before ($P \leq 0.05$), and the level was only half that of the abamectin control (Table 2). The results above indicate that precolonization of endophytic bacteria appeared to be able to reduce the number of galls and egg masses in the tomato, and the IR of the endophytic bacteria to the galls and egg masses was almost the same as with the chemical nematicide. Moreover, there is a close correlation between the biological control effect and the colonization of the bacteria population in the roots.

The growth parameters presented in Table 2 revealed that the three endophytic bacterial isolates have different effects on plant growth. All of them increased shoot length of tomato but, among the three isolates tested, only the preinoculated treatment with *B. cereus* BCM2 enhanced shoot length and fresh weight significantly ($P \leq 0.05$) (Table 2).

Construction of GFP-tagged strain BCM2. The strong fluorescence signal of GFP-tagged *B. cereus* BCM2 was detected under a fluorescence microscope (Fig. 3A), and the plasmid was stable for 6 months. The growth curve of *B. cereus* BCM2-*str'*-*gfp* was the same as wild-type *B. cereus* BCM2 cultured in 100 ml of LB within 108 h (Fig. 3B). *B. cereus* BCM2-*str'*-*gfp* reached 3.95×10^5 CFU/g at 7 dpi, indicating that carriage of the GFP gene did not change the colonization ability of *B. cereus* BCM2.

Colonization pattern of GFP-tagged endophytic bacteria BCM2. The GFP-tagged *B. cereus* BCM2 was tested for endophytic bacterial colonization patterns in tomato roots. Roots were examined by monitoring the green fluorescent cells of GFP-tagged colonizers around the tissue of tomato roots containing the epidermis, cortex, and vascular cylinder (Fig. 4). The fluorescent bacteria preferentially colonized around the lysed plant cells and root hair in the root epidermis (Fig. 4B and E). Laser three-dimensional scanning technology was applied to examine the strong fluorescent signal of younger root interiors (Fig. 4A). The GFP-tagged *B. cereus* BCM2 gathered in large numbers in lateral roots and junctions (Fig. 4B and C). They developed either as single cells or small clusters within intercellular spaces from cortex to vascular cylinder (Fig. 4C to E). More GFP-tagged cell clusters were detected between the giant cells caused by *M. incognita* in the roots of diseased tomato plants (Fig. 4F) as compared with the pattern in nematode-free tomato roots (Fig. 4G).

Repellent action of endophytic bacteria colonized in the tomato root during *M. incognita* invasion. Most J2 migrated to roots containing no endophytic bacteria at the same distance as in treated roots (inoculated with the endophytic bacteria). At 24 h after placement of J2 on the plate, under a light microscope, we detected that J2 were attacking the roots not inoculated with endophytic bacteria (Fig. 5B). A low number of J2 was recovered from roots which were inoculated with three endophytic bacterial isolates 48 h after *M. incognita* J2 were placed on the water agar plate compared with the sterile water control (Fig. 5C). The attraction assay revealed that most

J2 preferred the untreated plant roots. The results indicate that *M. incognita* J2 appeared to respond to repelling action on the roots treated with endophytic bacteria (Fig. 5).

Discussion

Antagonistic bacteria are often considered to be effective microorganisms for the biocontrol of root-knot nematodes (Adam et al. 2014; Hashem and Abo-Elyousr 2011; Xiong et al. 2015), as well as plant growth-promoting bacteria (Munif et al. 2000; Sturz and Kimpinski 2004; Vetrivelkai et al. 2010). Nevertheless, few studies were performed on the possibility that *B. cereus* and *B. altitudinis* as endophytic bacteria could provide biocontrol of *M. incognita*. Root colonization of endophytic bacteria is a prerequisite for a successful biocontrol agent to be able to establish efficient protection (de Weert and Bloemberg 2006; Weng et al. 2013). In this work, we reported three endophytic bacteria which possess strong nematocidal activity against *M. incognita* based on successful colonization.

As previously reported, biocontrol efficiency was closely related to the colonization density and degree to which niche competition was the main mode for endophytes within the root system (Sikora et al. 2007). However, low levels of G12 colonization in potato and *Arabidopsis* roots were related to a high level of biocontrol effectiveness in their experiment (Hallmann et al. 2001; Hasky-Günther et al. 1998). In our experiment, endophytic *Bacillus* spp. which occupied the ecology niche rapidly increased in population up to 3.53×10^6 CFU/g of root 9 dpi. Two weeks later, the population of endophytic bacterial colonization stabilized in tomato roots and was maintained at 10^3 CFU/g of root for 50 days. The bacterial endophytes in our experiment demonstrated a large ability to establish and persist

Fig. 2. Population of *Bacillus cereus* BCM2 (*str'*), *B. cereus* SZ5 (*str'*), and *B. altitudinis* CCM7 (*str'*) on tomato roots. Population is expressed in log x (CFU/g) of roots (y axis) and days (x axis).

Fig. 1. Neighbor-joining phylogeny depicting phylogenetic relationships of *Bacillus cereus*, *B. altitudinis*, and other allied genera based on combined analysis of *gyrB* gene sequences. Bootstrap values above 50% are given above branches.

either on the surface (data not shown) or in the root, and the amount of colonization was higher than previous observations of endophytic bacterial population in roots of 10^3 to 10^5 cells/g fresh weight (Hallmann et al. 1997; Vetrivelkai et al. 2010). In our opinion, in order to efficiently protect the root system against the attack of phytopathogenic microorganisms, a biocontrol strain has to establish itself in the rhizosphere. It has also been considered that effective root colonization is a crucial step for subsequent biocontrol (de Weert and

Table 2. Endophytic bacteria *Bacillus cereus* BCM2 and SZ5 and *B. altitudinis* CCM7 in biological control potential of *Meloidogyne incognita* and effect on the growth parameters of tomato plants infected with *M. incognita* in three treatments of pot experiment^x

Treatment	Inhibition rate (%) of ^y		Stem ^z		
	Gall	Egg mass	Length	Fresh weight	Dry weight
Experiment 1					
Sterile water	...	...	30.3 ± 4.6	6.6 ± 1.3	0.7 ± 0.1
Abamectin	73.8 b	60.0 ab	32.1 ± 5.1	6.7 ± 2.5	0.7 ± 0.3
BCM2	81.2 a	75.6 a	35.2 ± 3.0*	9.3 ± 2.1*	0.9 ± 0.3
SZ5	72.6 b	43.5 ab	31.0 ± 4.8	6.5 ± 1.8	0.8 ± 0.2
CCM7	70.1 b	53.0 ab	34.1 ± 3.3	8.3 ± 3.1	0.7 ± 0.3
Experiment 2					
Sterile water	...	...	30.7 ± 5.2	6.3 ± 1.6	0.6 ± 0.2
Abamectin	78.8 b	66.8 ab	31.0 ± 2.3	6.6 ± 1.2	0.7 ± 0.2
BCM2	77.8 b	66.8 ab	32.0 ± 4.7	7.3 ± 2.0	0.7 ± 0.2
SZ5	68.0 ab	62.6 ab	33.3 ± 4.3	7.3 ± 2.4	0.7 ± 0.2
CCM7	56.6 ac	40.4 ab	32.6 ± 4.3	6.9 ± 1.5	0.6 ± 0.2
Experiment 3					
Sterile water	...	...	31.6 ± 2.6	6.7 ± 2.3	0.7 ± 0.3
Abamectin	61.9 bc	63.2 ab	32.1 ± 5.5	7.0 ± 2.9	0.8 ± 0.4
BCM2	32.0 c	38.1 ab	33.8 ± 2.6	7.5 ± 1.6	0.7 ± 0.2
SZ5	42.7 bcd	38.3ab	32.1 ± 5.5	7.3 ± 3.6	0.8 ± 0.5
CCM7	35.0 cd	29.5 b	30.9 ± 4.1	6.8 ± 2.3	0.7 ± 0.2

^x Data are represented as the mean ± standard deviation ($n = 10$).

^y Values followed by different letters are significantly different ($P \leq 0.05$) according to one-way analysis of variance and Tukey's multiple comparison test.

^z Values followed by an asterisk (*) are significantly different in random design tests at $P \leq 0.05$ under Tukey's multiple comparison test; values followed by nothing indicate that the result was not significantly different ($P \leq 0.05$).

Bloemberg 2006; Weng et al. 2013). This finding is supported by our results that *B. cereus* BCM2 established in tomato root rapidly, with a strong potential to reduce root galling and egg mass production by *M. incognita* under greenhouse conditions. There is a corresponding low biological control effect with low numbers of SZ5 and CCM7 cells in roots. This indicates that an increase in competitive colonization ability could result in improved biocontrol ability (Barahona et al. 2011). There is a close relationship between the biological control effect and colonization population of endophytic *B. cereus*.

The results from the pot experiments testing *B. cereus* BCM2 and SZ5 and *B. altitudinis* CCM7 provided evidence of biocontrol of *M. incognita*, as well as of plant growth promotion. Many bacterial endophytes have been reported to promote growth and health and inhibit nematode pests of crop plants (Chi et al. 2005; Munif et al. 2000; Sikora et al. 2007; Sturz et al. 2000; Vetrivelkai et al. 2010). However, *B. cereus* and *B. altitudinis* endophytes have been poorly studied as biocontrol agents. Comparing the efficiency of bacterial endophyte biocontrol of *M. incognita* in three experiments, infection by the root-knot nematode *M. incognita* was significantly reduced by endophyte precolonization. In this work, preisolations were able not only to biocontrol *M. incognita* in tomato but also to promote plant growth. According to the report of Vos et al. (2013), bacterial endophytes triggered plants to develop a defense response after colonization, which activated the tomato roots more efficient or rapid defense mechanisms when they were subsequently attacked by root-knot nematodes. This provided an explanation of root-knot nematode management by endophyte bacteria.

Beneficial bacteria could induce systemic resistance against *M. incognita* in tomato plants (Adam et al. 2014). The bacterial induction of systemic resistance (ISR) of the host plant has been discovered and researched (Backman and Sikora 2008). Previous research used the split-root design of tomato roots to detect induced resistance (Mwangi 2002; Racke and Sikora 1992). ISR might explain how endophytic bacteria biologically control the plant-parasitic nematode (Sikora et al. 2007). In recent works, researchers proposed that endophytes might enhance the activity of defense-related biomolecules and enzymes against *M. incognita* (Singh et al. 2013; Vos et al. 2013). There is still no final conclusion about the details of biocontrol mechanisms of endophytic bacteria modifying root exudates and ISR, which should be further studied.

Endophytic bacteria *Bacillus* spp. often colonize the primary root surface, which is the zone of differentiation, elongation, and lateral root junctions (Cao et al. 2011). Around lateral root junctions, *Sinorhizobium meliloti* 1021 gathered through entry into the root interior

Fig. 3. A, Fluorescence micrograph (Zeiss A1) observe of green fluorescent protein (GFP)-tagged *Bacillus cereus* BCM2. *B. cereus* BCM2-*str'*-*gfp* after incubation in Luria-Bertani medium (LB) for 12 h. The bar represents 10 μm. **B,** Population of *B. cereus* BCM2-*str'* and *B. cereus* BCM2 (*str'*)-pBCgfp-1 represented by optical density at 600 nm observed during 1 to 108 h after incubation in LB.

Fig. 4. Confocal laser-scanning microscopy analysis of endophytic bacteria *Bacillus cereus* BCM2 (*str*)-pBCgfp-1 in tomato root tissue after inoculated for 7 days. The bar indicates 200 μ m. **A**, Distribution of green fluorescent protein (GFP)-tagged BCM2 in new root; **B**, distribution of GFP-tagged BCM2 in lateral root; **C**, GFP signal was gathered in lateral root junctions; **D**, GFP-tagged BCM2 gathered small clusters to intercellular spaces from cortex to vascular cylinder; **E**, endophytic bacteria occupied the space of root hair and epidermal cells in tomato roots; **F**, more clusters were detected in giant cells in the root of disease tomato caused by *Meloidogyne incognita*; and **G**, tomato root uninoculated.

Fig. 5. *Meloidogyne incognita* second-stage juvenile (J2) repelling action when tomato roots were treated with three endophytic bacteria. **A**, Endophytic bacteria-treated and sterile water-treated tomato roots on water agar plate; **B**, 100 *M. incognita* J2 in 20 μ l of water; **C**, more *M. incognita* J2 invaded tomato roots not inoculated with endophytic bacteria; and **D**, the number of *M. incognita* J2 in different tomato roots; asterisks (**) indicate significantly different ($P \leq 0.001$, Dunnett test, $n = 10$) from control (no endophytic bacteria).

between displaced epidermal cells (Chi et al. 2005), which was also detected in this work. As previously reported (Prieto et al. 2011), root hairs also play a key role in endophytic bacterial colonization of their host. It has been reported that GFP-tagged bacteria occupied intercellular spaces of epidermal cells in the roots of tomato (Poonguzhali et al. 2008). In this work, GFP-tagged *B. cereus* BCM2 gathered in large numbers at the lateral root and junctions, and also developed as either single cells or small clusters in intercellular spaces from cortex to vascular cylinder. As previously reported, root-knot nematodes invading the plant added a competition site of endophytic bacterial colonization (Hallmann et al. 1998). We observed this phenomenon by GFP-tagged *B. cereus* BCM2 on CLSM for the first time; between the cells of hyperplasia and abnormal tissues, more clusters of endophyte cells were detected.

Endophytic *B. cereus* colonized the root surface or in intercellular space. The most direct effect was related to the production of metabolites and modulation of root secretion (Singh et al. 2013) to change nematode infection. It has been remarked that bacteria are promising biocontrol agents because of the direct effects on metabolites to induce mortality in J2 or enhance host defense mechanisms in roots (Hashem and Abo-Elyousr 2011). As previously reported, *B. megaterium* caused repellence of *M. graminicola* in rice roots (Padgham and Sikora 2007). Our results were also consistent with reports (Dababat 2007; Dababat and Sikora 2007) which demonstrated that *M. incognita* mostly chose the control plants lacking treatment with *Fusarium oxysporum*. Olfactory chemotaxis toward food-associated odors is among the most common behaviors of nematodes (Niu et al. 2010). From our in vitro experiment results, roots which have been colonized by endophytic bacteria had a lower probability of invasion by *M. incognita* J2. It is hypothesized that the reduction of the invasion index might be attributed to endophytic bacterial colonization, which changes the metabolites of host plant roots. It has been recognized that endophytes alter root exudate patterns and most likely influence the egg hatching, recognition, and attraction behavior of root-knot nematodes (Sikora et al. 2007), suggested as an explanation of repellent action in our experiment. Functional genomics could uncover the reason for systemic changes in roots after treatment with endophytic bacteria that led to their reduced attractiveness to nematodes (Backman and Sikora 2008). It has also been reported that endophytes have the potential to modulate the metabolism of host plants and, accordingly, the nutrient values of different lettuce varieties (Cardinale et al. 2015).

This study explored the potential of endophytic bacteria isolated from plants to biocontrol *M. incognita* in tomato plants under greenhouse conditions. Three potential isolates (*B. cereus* BCM2 and SZ5 and *B. altitudinis* CCM7) were applied to study the colonization dynamics in tomato roots. Our results showed that endophytic bacteria colonized roots in large numbers, and stability in tomato plants was a precondition of biocontrol of *M. incognita* under greenhouse conditions. It was also noted that endophytic bacteria precolonized tomato roots with more biological control potential of *M. incognita* in the pot experiment. The GFP-tagged strains and CLSM were introduced to detect the ecological niche of endophytic bacteria after invading the plants. We obtained direct evidence of the endophytic *Bacillus* sp. pattern in tomato roots. Endophytic bacteria colonized in tomato roots repelled *M. incognita* J2 on water agar plates. This indirect evidence suggests that endophytic bacteria modify the root exudates of plants.

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