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Biocontrol potential of *Bacillus altitudinis* AMCC1040 against root-knot nematode disease of ginger and its impact on rhizosphere microbial community

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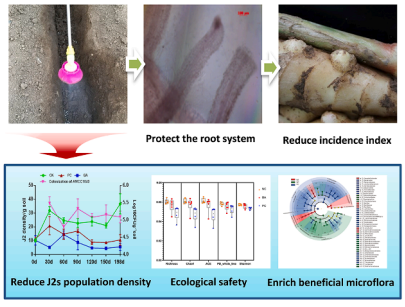
HIGHLIGHTS

- First described the occurrence of ginger bark cracking disease (GBCD) in detail.
- First report on the biocontrol potential of *B. altitudinis* on GBCD.
- Checking dynamic change of *B. altitudinis* by real-time PCR.
- Evaluated the impact of *B. altitudinis* on the rhizosphere microbial communities.

GRAPHICAL ABSTRACT



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ABSTRACT

Root knot nematodes are one of the most destructive hemibiotrophic root pathogens that can cause ginger bark cracking disease in ginger planting areas around the world. There is an urgent need for sustainable alternatives methods, such as biological control. The nematicidal activity of *Bacillus altitudinis* AMCC1040 has been previously shown *in vitro* and in pot experiments. Here, field experiments were conducted to evaluate the biocontrol potential of *Bacillus altitudinis* AMCC1040 against ginger bark cracking disease, and to evaluate its ability to colonize the rhizosphere. In addition, we also evaluated its impact on the rhizosphere microbial communities. The results of biocontrol experiments demonstrated that the application of *Bacillus altitudinis* AMCC1040 can significantly inhibit nematode reproduction, inhibit root galling and reduce the severity of ginger bark cracking disease. In addition, *Bacillus altitudinis* AMCC1040 colonized steadily in ginger rhizosphere soil ranging from 5.08 to 5.49 Log_{10} CFU g^{-1} soils. Compared with fosthiazate (One of the most commonly used nematicides in China), *Bacillus altitudinis* AMCC1040 performed better in terms of bio-safety. Meanwhile, the taxa enriched in *Bacillus altitudinis* AMCC1040 treatment were found in *Actinobacteria*, *Gemmatimonadetes* and *Firmicutes*. This study demonstrated that *Bacillus altitudinis* AMCC1040 have a strong potential to control ginger bark cracking disease

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in the field. These results provide strong support for further exploration of the application of *Bacillus* spp. as biocontrol agents to control root knot nematodes in many crops. Furthermore, the occurrence of ginger bark cracking disease described in this study will be of great help to future prevention and control measures.

1. Introduction

Root knot nematodes (*Meloidogyne* spp.) are highly successful and destructive animals that are hemibiotrophic root pathogens. Their worldwide distribution, their wide host range and their association with fungi, bacteria and viruses pose a complex problem (Trudgill and Blok, 2001). In fact, many growers are not aware of root knot nematodes because the above-ground symptoms and signs of the disease are indistinguishable from those of nutrient deficiency, such as incipient wilt, stunting and low yields (Loan et al., 2018; Coyne et al., 2018).

Ginger (*Zingiber officinale*) is widely planted in China, with an annual planting area of 630,000 ha and the highest production in the world (Qiao et al., 2012). In recent years, the ginger industry has suffered huge losses due to the occurance of various soil borne diseases (Stirling, 2004; Stirling et al., 2009). Ginger bark cracking disease caused by root-knot nematodes (*Meloidogyne* spp.) has long been consider as major obstacle to ginger yield and quality (Stirling et al., 2012). In addition to root knot nematode disease, typical symptoms of nematode-infected ginger include irregular shape, tumors, cracks or herpes on the surface.

Although many efforts have been made to control root-knot nematode disease, no suitable measures have been found (Sharma et al., 2018). In the past decades, various nematicides and soil fumigants have been widely used for root-knot nematode disease control; however, these highly toxic pesticides have caused serious problems for the environment and human health, as well as the multi-drug resistance in nematodes (Rajasekharan et al., 2020). A recent study showed the emergence of the fosthiazate-resistant phenotypes of *M. incognita* in tomato greenhouse (Huang et al., 2016). In some countries, some insecticides have been withdrawn from the market due to health hazards and environmental pollution issues. The above situation further emphasized the need for alternative strategies (Montiel-Rozas et al., 2019; Affokpon et al., 2011; Saxena, 2004). In the future, biological control will play an increasingly important role in the control of plant parasitic nematodes (Zhou et al., 2020).

Biological agents, including living organisms and their metabolites, are potential effective alternatives for root knot nematodes control (Loan et al., 2018). As an important natural enemy of nematodes, bacteria have been received more attention on developing into commercially available biological agents to control root-knot nematodes. According to their mode of action, nematode antagonistic bacteria can be divided into obligate parasitic bacteria, opportunistic parasitic bacteria, rhizobacteria, endophytic bacteria, parasporal Cry protein- forming bacteria and symbiotic bacteria (Tian et al., 2007).

In 2006, *Bacillus altitudinis* was first isolated and characterized from air samples (Shivaji et al., 2006). Due to its broad-spectrum antimicrobial activity such as *Phytophthora sojae*, *Streptomyces scabies*, *Magnaporthe oryzae*, *Colletotrichum gloeosporioides*, *Corynespora cassiicola*, *Fusarium verticillioides*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum*, this specie has since been considered as a potential factor for biological control (Li et al., 2019; Jin et al., 2012; Goswami and Deka, 2019; Lu et al., 2017). In addition, *B. altitudinis* A16 isolated from coal tar contaminated soil exhibited biodegradation of butachlor (Kaur and Goyal, 2020). However, studies have shown that *Bacillus altitudinis* is a plant pathogen that can cause soft rot in apples, pears and potatoes (Kumvinit and Akarapisan, 2019; Elbanna et al., 2014). This implies that attention should be paid to the ecological safety of this bacteria when using it as a biocontrol factor.

In our previous research, we obtained the bacteria strain AMCC1040 which has significant nematicidal activity against root-knot nematodes *in vitro* and pot experiments. According to the phylogenetic tree, morphological characteristics, and physiological and biochemical characteristics, this strain was identified as *Bacillus altitudinis* (Supplementary 1). Here, three purposes of this study were: (1) To evaluate the biocontrol effects of *Bacillus altitudinis* AMCC1040 on ginger bark cracking disease under field conditions; (2) To detect the colonization ability of the biocontrol strain in the rhizosphere soil by real-time PCR; (3) Assess its ecological safety based on its impact on the rhizosphere microbial communities.

2. Materials and methods

2.1. Description of experimental plot

From April to October in 2019, field trials were conducted in Weifang City, Shandong Province, China (36°48′ N, 119°18′ E, with an elevation of 9.83 m). The local cultivation mode was shown in Supplementary 2. The basic physical and chemical properties of the soils were as follows: pH, 6.77; total K, 22.89 g kg^{-1}; total P, 0.954 g kg^{-1}; total N, 0.83 g kg^{-1}; available K, 0.18 g kg^{-1}; available P, 112.40 mg kg^{-1}; available N, 61.37 mg kg^{-1}; organic carbon, 14.07 g kg^{-1}; electrical conductivity, 1385 μs cm^{-1}. There are seven years of ginger planting history in the experimental field, and was known to be heavily infested with root–knot nematodes as ginger bark cracking disease had occurred during the last planting. After the site being ready for planting, randomly collect soil samples (approximately 1000 g per sample) using five-spot-sampling method with a shovel from 5 to 30 cm deep every 20 square meters of the experimental area. The initial population density of root-knot nematode per gram of soil was determined to be 10.30–11.47 J2s using the Baermann funnel method (Jenkins, 1964; Botelho et al., 2019). The nematodes was identified as *Meloidogyne incognita* by molecular methods (Adam et al., 2007). Pre-plant presence *Bacillus altitudinis* was determined by real-time PCR (see details below), and no fluorescent signal were detected in 30 cycles.

2.2. Design of field experiments

Three treatments were arranged in the field experiment: (1) NC: negative control; (2) PC: positive control, treated with fosthiazate granules (effective content of 10%), totaling 6 kg per mu (Mu is the most commonly used unit for calculating land area in China, 1mu = 666.67 m^2), averagely divided into three periods (pre-planting, first and second soil hilling). (3) BA: biocontrol agent treatment. Before sowing ginger seeds, hand-pump sprayer to treat the gully floor with 100L per mu cell suspension of *Bacillus altitudinis* AMCC 1040. In order to prepare the cell suspension, the bacterial strains were activated on solid LB medium for about 18 h at 37 °C, and then a single colony was selected and inoculated into a 2000 mL flask containing 1000 mL BPY broth (Beef extract 5.0 g, Peptone 10.0 g, Yeast extract 5.0 g, Glucose 10.0 g, NaCl 5.0 g, ddH_2O 1000 mL, pH 7.0) and aerobic growth in a rotary shaker at 200 rpm and 37 °C for 36 h. After incubation, the cultures were centrifuged at 12000 rpm at 4 °C for 5 min. The filtrate was discarded and the remaining cells were suspended in sterile water. Live bacteria were counted by the gradient dilution coating method, and the cell concentration was adjusted to 10^8 CFU mL^{-1} with sterile water. The total area of each treatment is about 60 square meters, including three randomly distributed plots, totaling about 300 ginger seedlings. In order to prevent the surrounding area of the test site from being affected by the surrounding environment and avoid the marginal effects, there is a 1 m-wide protection line around each treatment.

2.3. Sampling and detecting

As shown in Fig. 1, samples were collected in six periods. Each sampling time each process was repeated six times and destructive sampling is used. Collect the tubers and roots carefully with a sterile stainless steel spatula, select soil loosely attached to the tubers and roots to measure the population densities of J2s, and collect the tightly attached soils with a sterile brush to measure rhizosphere colonization and its impact on rhizosphere microbial communities of *Bacillus altitudinis* AMCC 1040. Roots were rinsed thoroughly with clean water to count the number of penetrating nematodes and females. All samples were stored in ice bags at low temperature in the field, when transferred to the laboratory, roots were stored in the 4 °C refrigerator and soil samples were stored at −80 °C. Genomic DNA were extracted from soil samples using the using the E.N.Z.A.™ Soil DNA Kit (Omega, USA) according to the manufacturer's protocol instructions. The quantity and quality of DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and 1% agarose gel electrophoresis. DNA was stored at −80 °C until further analysis. Roots were stained using acid fuchsin method for counting the number of J2s and females then observed and photographed under Olympus BX51 optical microscope (Liu et al., 2008; Ramatsitsi and Dube, 2020). Because its pathological characteristics are similar to those of potato scabs, there is no relevant standard to evaluate the incidence level of ginger bark cracking disease. Therefore, the disease incidence, disease index and biocontrol efficacy were evaluated according to the references related to potato scab. In short, the severity of disease was given based on the 1–9 scale described by Shi et al. (2019). Use the following formulas to calculate disease incidence, disease index and biological control efficacy (Chen et al., 2017).

Disease incidence = number of diseased gingers/total number of gingers × 100%

Disease index = ∑(disease scale × number of corresponding disease scale gingers)/(the highest disease severity × total number of gingers)

Biocontrol efficacy = (disease index of control-disease index of treated)/disease index of negative control × 100%

2.4. Real-time PCR detection of Bacillus altitudinis AMCC1040

Specific primers were designed to amplify a 100-bp fragment of the *gyrA* gene from *Bacillus altitudinis* based on the comparative analysis of its *gyrA* gene sequence of other similar species (GenBank databases http://www.ncbi.nlm.nih.gov). Amplification specificity of the primer was determined by predicting with NCBI BLAST tools and the observation of a single band of the expected size through gel electrophoresis analysis (Almario et al., 2013). Primers used in this experiment were BA: 5′-GTCATACCAACAGCGATTCC-3′ and BF: 5′-AAGATAACTACGATGGCAGC-3′. All selected bacterial strains and sequences used in the experiments were listed in Supplementary 3 and Supplementary 4.

Real-time PCR was performed in CFX96 Real-Time System (Bio-Rad Hercules, CA, USA) using a SuperReal PreMix (SYBR Green) kit (TIANGEN, China) (Chen et al., 2017). Genomic DNA was extracted using the Fast TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. The plasmid with specific gene fragments were constructed using pGM-T Cloning Kit and transformed into *Escherichia coli* DH5α (TIANGEN, China). The recombinant plasmid was extracted with TIANprep Mini Plasmid Kit (TIANGEN, China), the purity and quality of plasmid standards were detected by ultramicro spectrophotometer. The real-time PCR assay used three technical replicates using 1 μL of DNA in a final volume of 20 μL containing 10 μL of SuperReal PreMix, 0.3 μM of each primer and achieved a final volume with sterile ultra-pure water. The thermocycling conditions were given as follows: 95 °C for 15 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s, a final melting cycle to produce a melting curve with a temperature gradient from 60 °C to 95 °C for quality control. Recombinant plasmids were serially diluted ten-fold in three separated series to obtain standards from 7.040×10^6 to 7.040×10^2 copies μL^{-1}. The standard curves were obtained by drawing the mean Ct value of the three replicates (per DNA concentration) against the log-transformed DNA concentration (Almario et al., 2013).

2.5. Illumina MiSeq sequencing and analysis

The conserved regions of the bacterial V3-V4 genes were amplified by PCR by using the universal primers 343F: 5′-TACGGRAGGCAGCAG-3′ and 798R: 5′-AGGGTATCTAATCCT-3′. Amplicon libraries were constructed and sequenced on a MiSeq PE250 sequencer (Illumina, USA). The original sequencing data has been submitted to the NCBI Sequence Read Archive (SRA) database, the accession number is: PRJNA6611900.

USEARCH v.10.0 software were used to process the original sequence, including merge, trim, filter, align and cluster by operational taxonomic units (OTUs) (Edgar, 2013). Sequences were clustered to the same OUT with 97% similarity using the UPARSE-OTU algorithm. The Alpha-diversity indices included PD_whole_tree, Chao1, ACE, Richness and Shannon were calculated by Mothur (version 1.34.4) (Schloss et al., 2009). Principal coordinate analysis (PCoA) based on weighted Bray-

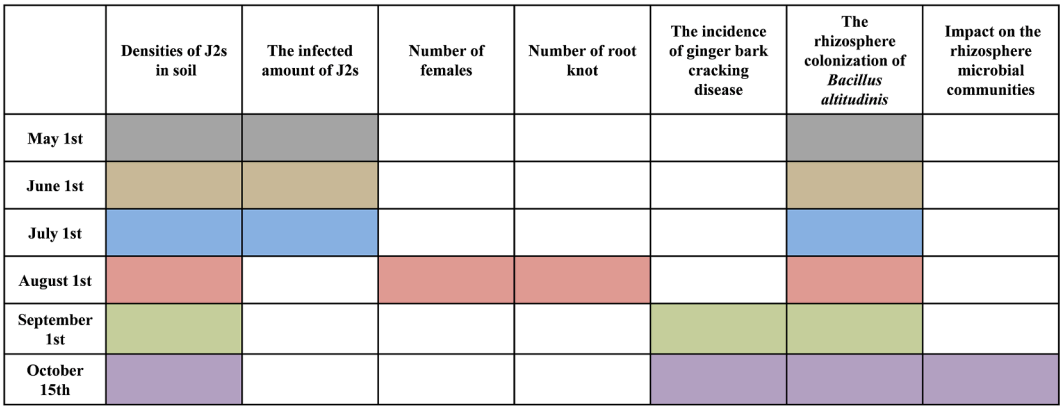

Fig. 1. Sampling time and testing items, coloring represents the detection of this item at this time node. Briefly speaking, monitor the dynamic change of population densities of J2s and the colonization number of *Bacillus altitudinis* AMCC1040 in rhizosphere soil on the entire ginger growth cycle; count the number of nematodes penetrating the roots of ginger in the early stage (about 30 days after planting); the number of female and root knot were counted at 60 and 90 days after planting; the bioassay of ginger bark cracking disease incidence, severity and biocontrol efficacy was performed at harvesting stage.

Curtis matrix was used to analyze β diversity (Zhang et al., 2020). To clarify the dominant bacterial groups of each treatment, the bacterial community composition of the soil samples was analyzed by phylogenetic analysis. The top 10 bacterial groups in the sample in terms of abundance were selected, and the remaining groups were combined and labeled as others groups and superimposed histograms were drawn; *t*-test were performed on the bacterial groups between treatments, and a histograms were drawn to analyze the differences in composition of the main phyla. Use the online interface Galaxy (http://huttenhower.sph.harvard.edu/lefse/) to perform the linear discriminant analysis (LDA) effect size (LEfSe) statistical analysis (Shi et al., 2019).

2.6. Statistical analysis

All statistical analyses were performed using analysis of variance with SAS statistical software. The results of the table are presented as the mean ± standard deviation. Significant differences between treatments were determined according to Duncan's multiple range test at $p < 0.01$.

3. Results

3.1. The development of GBCD in Shandong province

The occurrence of GBCD can be summarized as illustrated in Fig. 2. Thirty days after planting, the population densities of J2s in the soil increased by 209.32% (from 10.30 to 31.86 per g soil) compared to the initial phase. It was also observed that an average of 31.67 J2s per seedling roots (Figs. 3 and 4). The root knot was not discovered until 60 days. At this stage, the root knot was single and smaller, with only 1–2 females, but after 90 days, the shapes of string beads root knot with many females appeared. On the 120th days, nearly 81.16 root knot and 58.50 females per seedling roots were counted, and parts of the ginger bark cracking disease were observed at this sampling point. Almost all ginger has bark cracking disease during harvest (50%–90% of the surface area of ginger), and the densities of J2s in the soil were significantly increased by 2.6 times compared with the original period.

3.2. Biocontrol of GBCD by Bacillus altitudinis AMCC1040 in field experiments

Compared with the negative control, the nematode populations in the soil (Fig. 3) and roots (Fig. 4) were reduced by 84.48% and 93.68%. There were even 46.48% and 58.59% reductions, compared with the positive control (Table 1). In addition, the application of the bacterial strain significantly reduced the nematode reproduction index because it significantly reduced females in the roots (Table 1, Fig. 4). The lowest

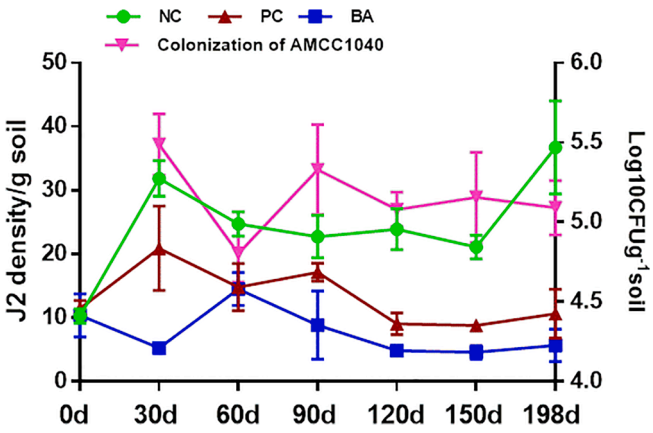

Fig. 3. Dynamic changes of J2s population density in soil and the real-time PCR detection of *Bacillus altitudinis* AMCC1040 at different stages. NC: negative control; PC: positive control; BA: biocontrol agent treatment.

single root knot or beaded root knot recorded from the BA control was reduced by 63.61% and 70.48%, respectively compared with NC. During the harvest period, differences in disease index between the treatments was shown in Table 1, Fig. 5. Compared with the negative control, the biocontrol efficacy of BA was 57.14%.

3.3. The real-time PCR detection of Bacillus altitudinis AMCC 1040

In this experiment, the dynamic colonization of biocontrol bacteria in rhizosphere soil was detected by real-time PCR. The specificity of the primer was tested in PCR and agarose gel experiments. The first derivatives of melting curve analysis showed that the amplification of *Bacillus altitudinis* AMCC1040 produced a single peak at 83 °C, and at the same time, it was only one band of about 100 bp was visible on gel (Supplementary 4). The equation for obtaining the standard curves was as follows: $Y = -3.432X + 38.798$, $R^2 = 0.995$, $E = 95.6\%$ (Supplementary 5). "X" represented Ct value and "Y" represented the number of *Bacillus altitudinis* AMCC1040 (Log_{10} CFU). 30 days after ginger transplanting, the number of *Bacillus altitudinis* AMCC1040 per gram of soil in rhizosphere quantitatively analyzed by real-time PCR was 5.49 Log_{10} CFU, but, compared with the previous time, the colonization within 60 days was decreased by 4.78 times, because a broad-spectrum bactericide (Product name: Puerfu, the active ingredients are a mixture of hymexazol and metalaxyl) was used by farmers in order to prevent bacterial wilt. In the remaining time, the amount of *Bacillus altitudinis* AMCC1040 per gram of soil varied from 5.08 to 5.33 Log_{10} CFU, indicating that the

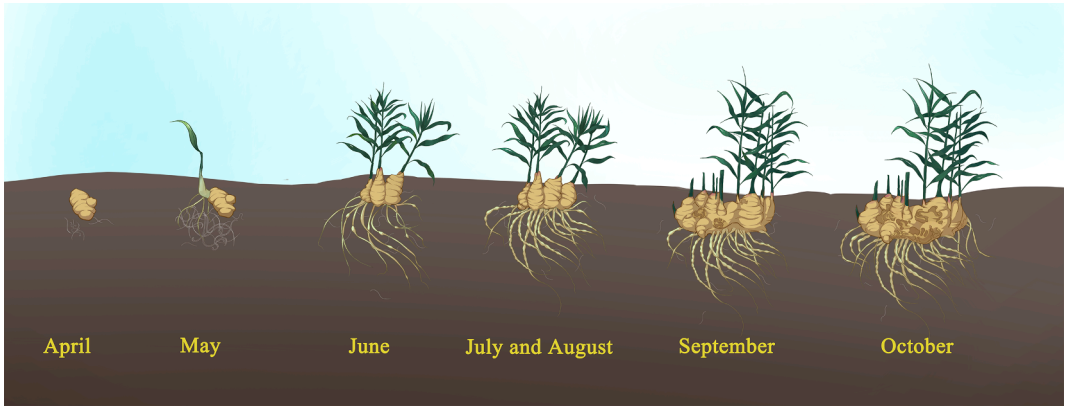

Fig. 2. The occurrence law of GBCD in Shandong Province. Ginger was planted in early April, since May, the density of the population densities of J2s in soil continued to increase. Single root knot began to appear in June, it developed into string beads root knot in July and August. In September, parts of ginger bark cracking disease were observed (Dark part of ginger surface), at harvest period, the lesion was enlarged to 50%–90% of the surface area.

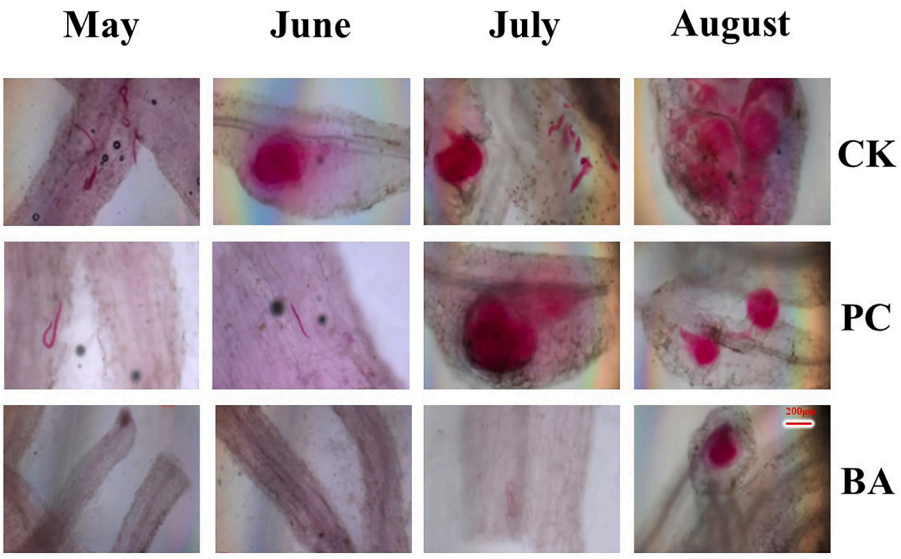

Fig. 4. Infection and development of *Meloidogyne incognita* in the roots of ginger. A brief description as below:In the negative control, a certain number of J2s were observed in May, females appeared in June and increased significantly in July and August. However, the development of root knot nematodes was delayed in positive control and biocontrol agent treatment for they provided significantly reduction of J2s and females in roots.

Table 1
The number infected J2s in May, females in July, root knot in August and the disease index at harvest time per ginger plant. NC: negative control; PC: positive control; BA: biocontrol agent treatment.

	Infection J2s	Females	Single root knot	String beads root knot	Disease index
NC	31.67 ± 2.72A	58.50 ± 4.51A	56.33 ± 4.94A	24.83 ± 3.17A	0.70
PC	4.83 ± 0.54B	16.83 ± 2.06B	32.50 ± 2.90B	10.83 ± 1.70B	0.23
BA	2.00 ± 0.37B	10.00 ± 0.97B	20.50 ± 1.67C	7.33 ± 0.76B	0.30

biocontrol microorganism could successfully survive in the rhizosphere soil of ginger (Fig. 3).

3.4. The impact on the rhizosphere microbial communities of Bacillus altitudinis AMCC 1040

In order to assess the ecological safety of *Bacillus altitudinis* AMCC 1040, the rhizosphere microbial communities were detected during the ginger harvest period. For each sampled soil, the 16S rRNA gene amplicon library covering the bacterial V3–V4 region were sequenced on the MiSeq PE250 sequencer. Pyrosequencing generated a total of 3917 operational taxonomic units (OTUs) from the bacterial community. According to the results of α-diversity in Fig. 6, compared with NC and BA, the abundance of ACE and Chao1 were significant reduced in PC. PCoA was performed using the weighted Bray-Curtis metric to analyze the differences and similarities of microbial communities between treatments. The results showed that there were significant differences in the bacterial microbial community structures between three treatments, especially the comparison of PC with the other two treatments (BA-NC-PC: $R^2 = 0.38675$, $P = 0.001$***, BA-NC: $R^2 = 0.1669$, $P = 0.038$*, BA-PC: $R^2 = 0.28993$, $P = 0.006$**, NC-PC: $R^2 = 0.46569$, $P = 0.003$**, Fig. 7). As shown in Fig. 9, the top 10 phyla of abundance were the same in the rhizosphere soil of the three treatments, but the proportion of each phyla was different. According to the *t*-test results of the top 6 phyla, there in *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Gemmatimonadetes* were significantly different between the positive control and the other two treatments ($p < 0.01$). On the contrary, there was no significant difference between NC and BA except for *Actinobacteria* ($p < 0.05$) (Fig. 10). The above results showed that the application of fosthiazate can significantly affected the soil microecology, while the effect of *Bacillus altitudinis* AMCC1040 has a small effect on the structure of the soil microbial community. Linear discriminant analysis of effect size (LEfSe) with LDA scores > 3 confirmed significantly ($P < 0.05$) differentiated bacterial taxa in different treatments (Fig. 8). It should be noted that the taxa enriched in BA were found in *Actinobacteria*, *Gemmatimonadetes* and *Firmicutes*.

4. Discussions

Although *Meloidogyne* spp. species can attack ginger and affect not only its yields but also affect its quality, unfortunately, information on the regularity of root-knot nematode disease in ginger is limited. The severity of the disease directly depends on the initial population density of J2s in the soil (Moosavi, 2015). Therefore, before the field test, a survey was conducted to investigate the population density in several areas of Weifang city (data not shown). The results showed that the initial population densities of J2s in the soil were ranged from 1.08 to 11.47 per g soil, which means that even if the populations are insufficient, it may cause harm to ginger. Current research on the occurrence of GBCD demonstrated that nematodes infection can occur 30 DAP (days after planting), females and root knot appeared at 60 DAP, and at the same time, as the disease progressed, the above symptoms gradually increase at 90 to 120 DAP, but, ginger bark cracking disease were observed until 150 days. This means that root knot nematodes mainly damage the root system and the ginger bark cracking are far from obvious in the early stage, but in the later stage of ginger growth, the explosion of tumors, cracks or herpes occurred in a very short time. In addition, without any control measures, the population density of J2s in the soil increased by 2.6 times, which is obviously disastrous for the next round of planting.

In our study, the ability of *Bacillus altitudinis* AMCC1040 derived from inhibitory soil to control ginger bark cracking disease under field conditions was evaluated for the first time. According to previous study, *in vitro* and pot experiments, *Bacillus altitudinis* AMCC1040 showed the significant resistance to root-knot nematodes (Wang et al., 2020). In this study, the pre-planting application of *Bacillus altitudinis* AMCC1040 could significantly reduce J2 density in the soil, the number of J2 and females in root, root galling and the index of ginger bark cracking

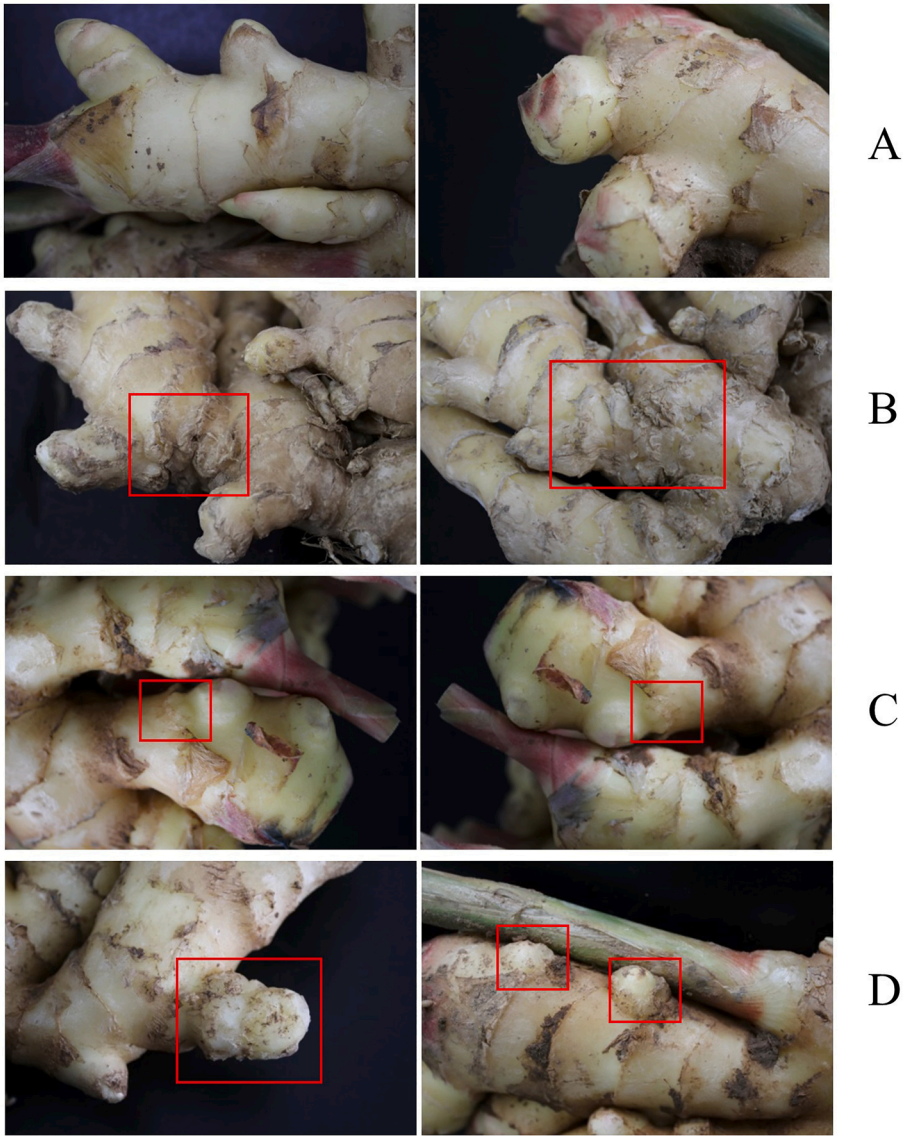

Fig. 5. Severity of ginger bark cracking disease among different treatments. The surface of normal growing ginger is smooth (A), however, when seriously infected by nematodes, tumors, cracks or herpes can occur extensively (B, negative control). Fewer bulges were found in positive control (C) and biocontrol agent treatment (D).

disease. *Bacillus* spp. has extensively studied its effects on their biocontrol potential against root-knot nematodes such as *Bacillus amyloliquefaciens* (Abdel-Salam et al., 2018), *Bacillus licheniformis* (Siddiqui and Mahmood, 1999), *Bacillus megaterium* (Padgham and Sikora, 2007), *Bacillus thuringiensis* (Gao et al., 2015), *Bacillus subtilis* (Rao et al., 2017), *Bacillus coagulans* (Serfoji et al., 2010), *Bacillus toyonensis*, *Bacillus simplex*, *Bacillus weihenstephanensis*, *Bacillus safensis*, *Bacillus mojavensis* (Ni et al., 2017), *Bacillus aryabhattai* (Zhao et al., 2020). As far as we know, there has never been a report of using *Bacillus altitudinis* to control ginger bark cracking disease. This study provides strong support for the further exploration of the use of *Bacillus* spp. as the biocontrol agents to control root knot nematodes in many crops. It is worth noting that although compared with the positive control, *Bacillus altitudinis* AMCC1040 showed better in reducing population density, inhibiting nematode reproduction, and suppressing root galling, but the disease index was higher at harvest. This phenomenon may be related to the soil hills. At this stage, the ridges will slowly become gullys. On the contrary, the gullys will gradually become higher to prevent the rhizome from being exposed to the ground as they expand. In this experiment, a one-time application of biocontrol agents to the gully floor can effectively reduce the population density of J2s at the bottom of furrow, but it is not effective for the ridge. Therefore, it can protect the bottom of ginger from nematode infection, but it cannot effectively prevent the damage to the upper part of ginger caused by J2s from ridge. Instead, fosthiazate was used in the first and second soil hilling. The result reminds us that it is important to combine the application methods of biocontrol factors with local agronomic measures. Another important point here is the high cost, which is the main obstacles to the application of biocontrol agents. Therefore, it is very important to discuss how to optimize the production process and establish application technical specification to reduce costs.

A large number of previous studies have shown that although biocontrol strain are proven effective in pots, its performance is not so effective in field experiments (Tian et al., 2007; Meyer, 2003). To a large extent, it is because strains were usually affected by a variety of unfavorable factors in field, such as climatic adaptability, physical and chemical properties of soil and the competition of other microorganisms (Affokpon et al., 2011; Sharon et al., 2007; Harman, 2006). Successful biological control depends on a thorough understanding of the population dynamics of pathogens and natural enemies. Therefore, it is very important to evaluate the persistence and traceability of inoculated biocontrol strains, because it is the basis for understanding behavior of

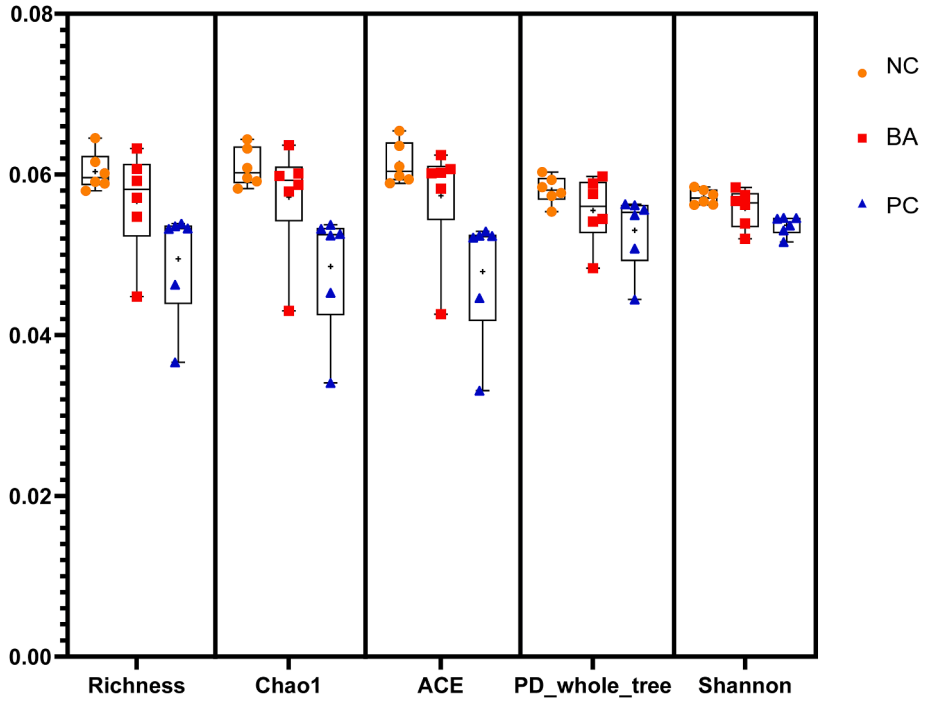

Fig 6. The results of Alpha-diversity indices included PD_whole_tree, Chao1, ACE, Richness and Shannon calculated by Mothur.

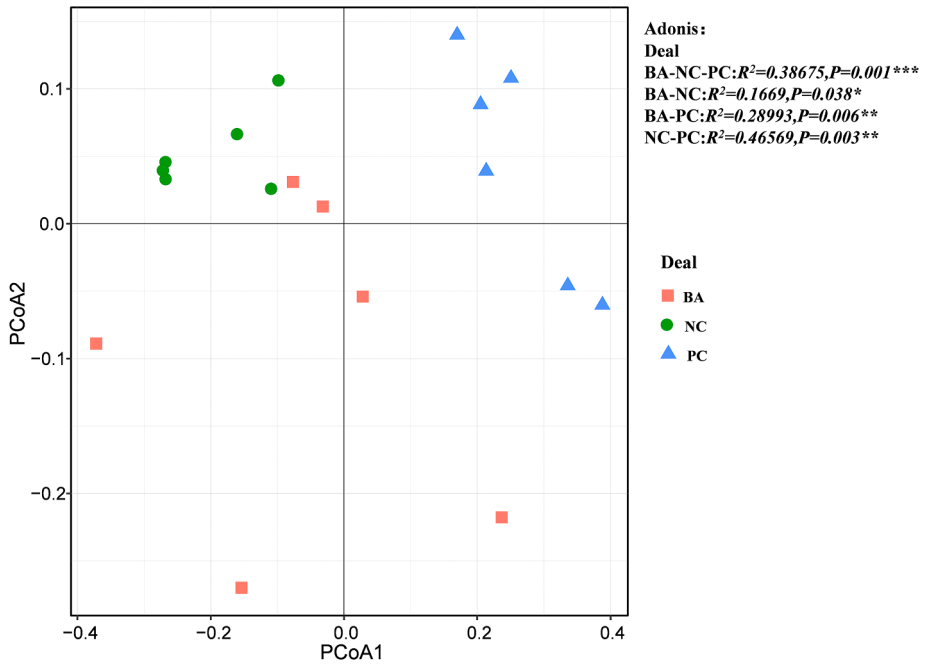

Fig 7. PCoA analysis of bacterial community structure calculated with the weighted Bray-Curtis matrix (red: BA; green: NC; blue: PC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biocontrol strains in the rhizosphere environment of crops (Pornthip and Pongrawee, 2020; Viaene et al., 2006). In the current study, the population of *Bacillus altitudinis* AMCC1040 per gram of soil varied from 5.08 to 5.49 Log_{10} CFU throughout the ginger period, indicating that this strain can successfully survive in rhizosphere soil. The results are consistent with previous similar studies. For example, the biocontrol agent *B. laterosporus* AMCC100017 can stably survive in the potato rhizosphere (10^6–10^5 CFU per g soil), and significantly reduced the incidence of potato scabs (Chen et al., 2017). It is worth mentioning that in order to prvent bacterial wilt, a broad-spectrum bactericide was used within about 45 days, which led to a reduction in biocontrol bacteria and a rebound in the population density of J2s. This result suggests that only when the number of inoculated biocontrol strains reached a certain level, root knot nematodes in the soil can be suppressed. Moreover, it should be pointed out that in the application of biological control methods, some human interference cannot be ignored, because to a certain extent, it plays a key role in determining success.

The rhizosphere microbial communities are one of the important indicators of soil health and soil function (Ferris and Tuomisto, 2015; Dong et al., 2018). Larkin and Honeycutt observed that several

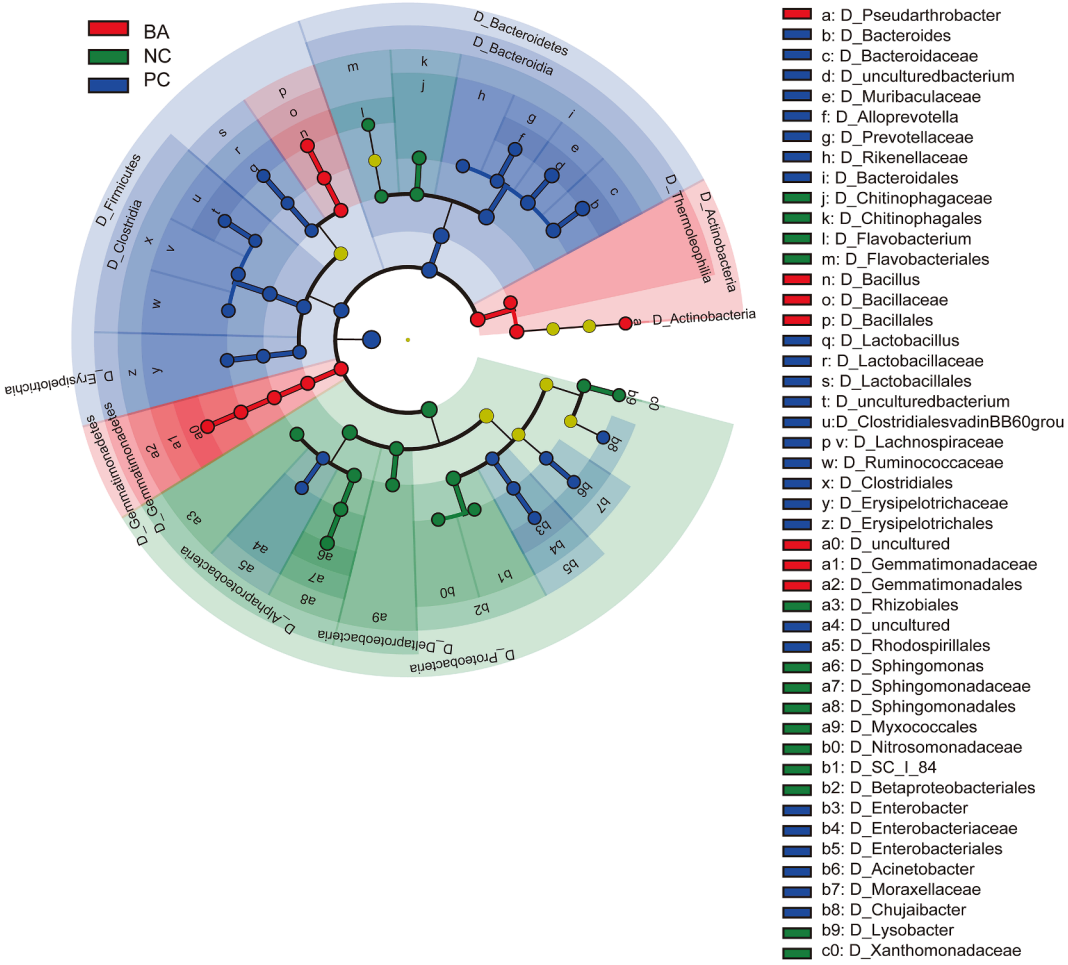

Fig 8. Significantly ($P < 0.05$) differentiated bacterial taxa between three treatments as evaluated by the LEfSe with LDA scores > 3. The colour represents the significantly ($P < 0.05$) higher relative abundance of the genera in the corresponding group (red: BA; green: NC; blue: PC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

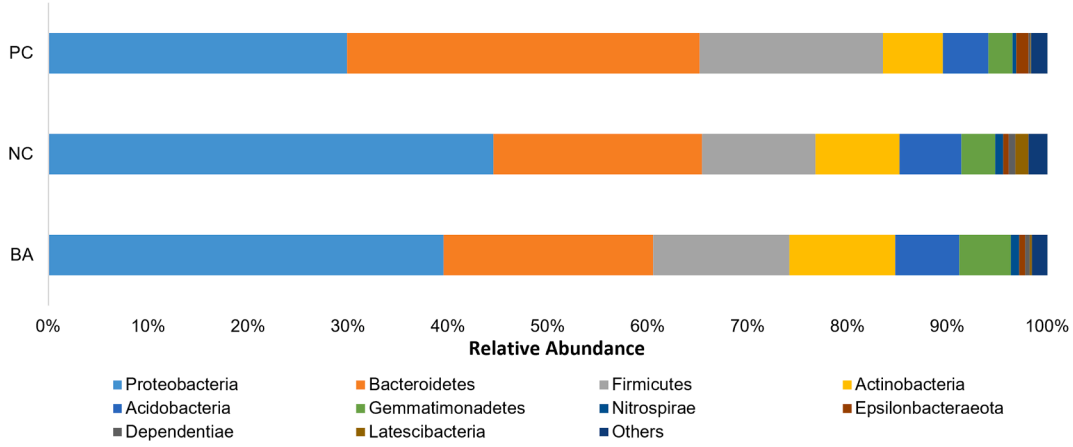

Fig. 9. Bacterial community composition at phylum levels in soil samples from different treatments.

microbial parameters were related to disease incidence and crop yield in the potato cropping system study (Larkin and Honeycutt, 2006). Shi et al. confirmed that the community composition and function of the soil microbiome are related to the severity of common potato scab (Shi et al., 2019). Evaluating the effects of biological control agents on the structure and function of soil microbial community is critical to understanding the activity and potential of these biocontrol agents and their potential impact on the environment (Klose et al., 2006). Liu et al. reported that compared with the use of Dazomet or Fostiazate alone, the combination of *P. lilacinum* with nematicides has a higher microbial diversity (Liu et al., 2014). Our results indicated that the use of *Bacillus altitudinis* AMCC1040 has little impact on the micro-ecological characteristics of ginger rhizosphere. Qiao et al. investigated the effect of *Bacillus subtilis* PTS-394 on the rhizosphere microbial community using

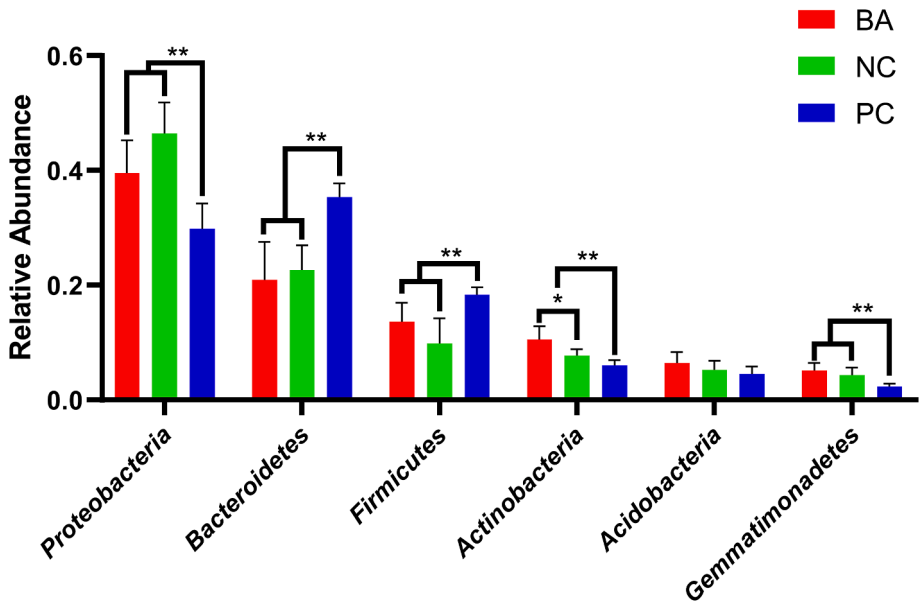

Fig. 10. Difference analysis of bacterial community distribution at phylum level among different treatments, * represents $p < 0.05$; ** represents $p < 0.01$.

Roche 454 pyrosequencing of 16S rRNA and ITS partial sequences, and they also found that *Bacillus subtilis* PTS-394 is an environmentally compatible plant protective agent with nopermanent effect on rhizosphere microbial community (Qiao et al., 2017). In our study, significant differences were observed in the positive control. This finding is consistence with the previous views that chemical pesticides posed a potential threat to the environmental micro-ecology (Ibekwe et al., 2001).

It is well known that disease suppressive soils has the ability to reduce the incidence or severity of disease compared with the surrounding soils (Weller et al., 2002). The functional basis of this inhibition is the synergistic activities of specific microbial groups, which have been extensively proven through soil sterilization and transplantation (Gómez et al., 2017; Garbeva et al., 2004; Mazzola, 2002; Scher and Baker, 1980; Mendes et al., 2011). In our previous studies, we explored the nematicidal bacteria that can be cultivated in suppressive soils in the region of Shandong Province, China. The results indicated that *Bacillus* was most abundant, accounting for >70% of the total, indicating that *Bacillus* played an important role in the functional of suppressive soils (The 16S rDNA sequence of the 110 obtained bacteria has been submitted to NCBI under the accession numbers MN826343-MN826452). In this study, the LEfSe method was used to analyze difference in taxonomic abundance between treatments. The taxa enriched in BA were mainly found in *Actinobacteria*, *Gemmatimonadetes* and *Firmicutes*. It has been reported in previous studies that some genus belonging to these three taxa are well known for their ability to inhibit soil-borne pathogens, such as *Bacillus* spp., *Pseudomonas* spp. and Actinomyces (Ni et al., 2017; Pornthip and Pongrawee, 2020; McSpadden-Gardener, 2007). Therefore, we hypothesized that *Bacillus altitudinis* AMCC1040 can help transform non-inhibitory soil into inhibitory soil by affecting the structure of the microbial communities, especially by improving the beneficial microflora. This finding further demonstrates that *Bacillus* occupies a very important position in the suppressive soils.

5. Conclusions

In conclusion, the purpose of this study was to evaluate the potential of field *Bacillus altitudinis* AMCC1040 for biological control of ginger bark cracking disease caused by root knot nematodes under field conditions in Shandong Province. At the same time, the occurrence of ginger bark cracking disease was described in detail. It seems that root knot nematodes damage the roots in the early stage of ginger and the symptoms of tumors, cracks or herpes were formed in the later stage. In this study, the pre-planting application of *Bacillus altitudinis* AMCC1040 could successfully survive in rhizosphere soil and significantly reduced root knot nematodes damage to ginger. The rhizosphere microbial communities were less affected by *Bacillus altitudinis* AMCC1040, while the fosthiazate had a great unfavorable influence. Moreover, *Bacillus altitudinis* AMCC1040 might help the soil to change from non-suppressive to suppressive type by enriching beneficial microflora.

CRediT authorship contribution statement

Jian-Yu Wang: Data curation, Software, Writing - original draft. **Cheng Guo:** Software, Visualization. **Peng Zhao:** Data curation. **Feng-Yuan Yu:** Data curation. **Jian-Ping Qu:** Software. **Jian-Long Wang:** Validation. **Rong-Shan Lin:** Writing - review & editing. **Bing Wang:** Writing - review & editing. **Zheng Gao:** Conceptualization, Formal analysis. **Zheng-You Yang:** Conceptualization, Writing - review & editing. **Bo Zhou:** Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2021.104598.

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