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Biocontrol Efficacy of *Bacillus thuringiensis* Strain 00-50-5 Against the Root-Knot Nematode *Meloidogyne enterolobii* in Pepper

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Abstract: The root-knot nematode *Meloidogyne enterolobii* is a major constraint to pepper production in tropical regions. In the long-term practice of root-knot nematode management, bacterial nematicides have attracted increasing attention as effective biocontrol agents. In this study, we evaluated the efficacy of *Bacillus thuringiensis* strain 00-50-5 (*Bt* 00-50-5) against *M. enterolobii* through in vitro, greenhouse and field trials. The cell-free supernatant of *Bt* 00-50-5 exhibited potent nematicidal activity against second-stage juveniles (J2s) of *M. enterolobii*, with mortality rates of 98.0% and 100% after 24 h and 36 h of exposure, respectively. In addition, *Bt* 00-50-5 showed inhibitory effects on the hatching of *M. enterolobii* eggs, resulting in a remarkable 96.6% reduction in the egg hatching rate after 6 days compared to the control. The pot trials showed that both pepper root galls and egg masses were reduced, and plant growth was improved after treatment with *Bt* 00-50-5. The field trials showed that the gall index was significantly reduced, with a 66.3% and 68.2% reduction in disease index in 2020–2021 and 2021–2022, respectively, and pepper yield was improved, with a 96.2% and 93.1% increase in yield in 2020–2021 and 2021–2022, respectively, compared to the control. These results indicate the potential use of *Bt* 00-50-5 as an effective biocontrol agent against *M. enterolobii.*

Keywords: *Bacillus thuringiensis*; root-knot nematode; *Meloidogyne enterolobii*; control efficacy; pepper

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1. Introduction

Root-knot nematodes (RKN, *Meloidogyne* spp.) are considered one of the most pathogenic plant-parasitic nematodes, threatening more than 3000 plants and causing an average yield loss of 10% in vegetable crops [1]. Among the nearly 100 described species of RKNs, *M. enterolobii* is considered to be one of the most damaging species due to its ability to infect and reproduce on host plants that are resistant to the major tropical RKN species, such as *M. incognita*, *M. javanica* and *M. arenaria* [2,3]. *M. enterolobii* was first described in 1983 from the roots of *Pacara earpod* trees on Hainan Island in China and is widespread across tropical regions in Africa, Europe and the Americas [4,5]. This nematode is particularly damaging to many important *Solanaceae* crops such as tomato, eggplant and pepper. In southern China, which belongs to the tropical regions, *M. enterolobii* is considered to be the major limiting factor in vegetable production and is now the most widespread and dominant RKN species in pepper growing areas of Hainan, China [6].

RKN has a wide host range, and the availability of resistant cultivars is limited, making it difficult to control. In recent decades, RKN control has relied primarily on chemical nematicides, such as soil fumigants, carbamates and organophosphates [7]. However, safety and environmental concerns have limited the use of chemical nematicides such as aldicarb in many countries due to their high environmental toxicity [8]. In addition, the

high cost of chemicals and the induction of pest resistance to these chemicals with repeated use are major concerns [9]. As a result, chemical nematicides are no longer considered to be an ideal way to control RKN. Meanwhile, biological control is attracting attention as a safer alternative to chemical nematicides. In recent years, beneficial microorganisms have gained considerable attention as biocontrol agents for pest management [10,11]. These include microbial insecticides, bactericides, agricultural antibiotics, and other similar agents. They are characterized by their specificity and selectivity [12–14]. Among these microbial pesticides, *Bacillus thuringiensis* (*Bt*), a Gram-positive soil bacterium that produces a variety of crystal proteins, is widely used to control a wide range of insect orders, nematodes, mites, protozoa and even human cancer cells [15]. It is also recognized as the most widely used microbial pesticide in the world. The toxicity of *Bt* to plant-parasitic nematodes was first demonstrated in 1972, with the thuringiensin (Thu) or β-exotoxin it produces having an inhibitory effect on *M. incognita* [16]. Subsequently, Cry proteins produced by some *Bt* strains, such as Cry5, Cry6, Cry13, Cry14, Cry21 and Cry55, were reported to be virulent against root-knot nematodes [17,18]. These studies suggest that Cry proteins are important virulence factors in the control of RKN. In addition, metalloproteinases and chitinases produced by *Bt* act in concert to enhance the virulence of the Cry proteins, making them an important component of virulence factors for *Bt* [19].

Recently, some studies have shown that beyond crystal proteins, other virulence factors produced by *Bt* strains are toxic to RKN. Cyclic dipeptide produced by *Bt* strain MB751 showed significant nematicidal activity against *M. incognita* [20]. Cyclo (D-Pro-L-Leu) produced by bacterial culture supernatant and crude extract of *B. amyloliquefaciens* strain Y1 inhibited egg hatching and caused juvenile mortality against *M. incognita* in vitro and in vivo on tomato [21]. *Bt*7N crude suspension (CS) and cell-free supernatant (CFS) could reduce the number of egg masses and eggs of *M. incognita* in tomato plants compared to a control [22]. TAA from the *Bt* strain CT-43 exhibited lethal activity against *Caenorhabditis elegans*, *M. incognita* and soybean cyst nematode [23]. Thus, these strains, which have the ability to produce toxic compounds with different chemical structures and properties, offer a good direction for finding more biocontrol resources.

In our previous study, a *Bt* strain 00-50-5 (*Bt* 00-50-5), isolated from sunflower head extract, was found to be highly toxic to *lepidopteran* insects such as *Cochylis hospes* and *Trichoplusia ni* [24,25]. To further extend the applicability of this *Bt* strain against plant-parasitic nematodes, we evaluated its nematicidal activity both in vitro and under greenhouse and field conditions to determine its potential biocontrol efficacy in cell-free supernatant (CFS) and bacterial culture against *M. enterolobii*. This comprehensive approach provides valuable insights into the practical applicability of *Bt* 00-50-5 as a sustainable alternative to chemical nematicides, potentially transforming nematode management practices in tropical regions.

2. Materials and Methods

2.1. Bacteria Culture

Bacillus thuringiensis 00-50-5 was isolated from sunflower head extract in a previous study [25] and stored in glycerol at −80 °C. *Bt* 00-50-5 was inoculated and cultured on nutrient agar (NA) in a Petri dish for 24 h at 30 °C. Subsequently, it was transferred to 500 mL flasks containing 100 mL of liquid broth (LB) as the culture medium and incubated for 8 to 12 h at 30 °C on an incubator shaker. Cultures in the mid-logarithmic growth phase were then transferred to 400 mL of nutrient broth (NB) in 1 L flasks and shaken at 200 rpm in the dark for 3 days. At this stage, the concentration was approximately 2×10^8 CFU/mL. The cultures were then centrifuged at 12,000 rpm for 10 min. The supernatants were filtered through Whatman No. 6 filter paper, and a syringe filter (0.22 μm) was subsequently used to remove any remaining bacterial cells. This final product was considered to be 100% CFS of *Bt* 00-50-5.

2.2. Nematode Extraction and Hatching

M. enterolobii was first collected from Wenchang City, Hainan Province, China, identified in the laboratory, and cultured on tomato plants from a single egg mass. After 45 days, the roots of infected plants were carefully cleaned, cut into 1–2 cm slices and macerated in 0.05% sodium hypochlorite (NaOCl) solution. The mixture was stirred for 5 min using an electric blender to break up the egg mass matrix. The supernatant was then poured through a 30 µm diameter sieve mesh and rinsed several times with sterilized water. *M. enterolobii* eggs were obtained as described by Hussey and Barker [26]. The eggs were transferred to a funnel and allowed to hatch into second-stage juveniles (J2s), and freshly hatched J2s were used for experiments on the same day.

2.3. In Vitro Mortality Assay

M. enterolobii J2s were prepared in sterile distilled water. A suspension of 10 µL containing approximately 200 J2s, together with 1.3 mL of fermentation filtrate and NB medium, was added to each sterilized 1.5 cm diameter plastic dish. NB medium was used as a control. After 12, 24, and 36 h of treatment, the J2s were rinsed and transferred to distilled water, and 4 h later, J2 mortality was measured using a stereomicroscope (25×) (PXS-EX, Shanghai, China). Nematodes were considered dead if they remained straight, stiff and immotile when agitated with a fine hair needle [27]. Morphological images were captured using a light microscope (40×) (Nikon, Tokyo, Japan) after 24 h. The experiment was conducted in triplicate and each treatment was repeated three times. J2 mortality was calculated using Equation (1):

$$\text{J2 mortality } (\%) = \frac{\text{Number of dead J2s}}{\text{Total number of J2s}} \times 100 \quad (1)$$

2.4. In Vitro Inhibition of Egg Hatching Assay

M. enterolobii eggs were obtained from mature egg masses and purified using the centrifugal/sugar flotation technique [28]. The egg suspension was then washed thoroughly with sterilized water prior to use in the bioassay to remove any potential microbial contamination. Subsequently, 10 µL of the egg suspension (containing 100–300 eggs) and 1.3 mL of fermentation filtrate were added to a sterilized plastic dish with a diameter of 1.5 cm. The NB medium was used as a control. The plate was then covered with Parafilm (Bemis, Sheboygan Falls, WI, USA) and sealed. An inverted microscope (Ni-E, Nikon, Tokyo, Japan) was used to observe the hatching of the eggs after 2, 4, and 6 days. To assess the ovicidal properties of *Bt* 00-50-5 fermentation, unhatched eggs were placed in distilled water for further observation [29]. Each treatment was replicated three times to ensure the accuracy of the results. The cumulative hatching rate was calculated using Equation (2):

$$\text{Egg hatching rate } (\%) = \frac{\text{Number of hatched J2s}}{\text{Total number of eggs}} \times 100 \quad (2)$$

2.5. Pot Trials

The pot experiment was conducted with pepper plants (*Capsicum annuum* CV. Changfeng, susceptible to *M. enterolobii*). Four-week-old pepper seedlings were transplanted into 8 cm diameter plastic pots containing 500 g of soil, sand and vermiculite in a 2:1:1 ratio. Before use, the soil mixtures were dry-autoclaved at 180 °C for 120 min to eliminate indigenous microbes. Each treatment consisted of eight replicates, with each replicate containing one pepper plant. The pots were arranged in a randomized block design and maintained in a greenhouse under ambient light at 25 ± 2 °C.

One week after transplanting, four treatments were applied: bacterial fermentation broth (2×10^8 CFU/mL) at full strength (100%) and dilutions at 5-fold (20%) and 10-fold (10%) [30], and water serving as a control. Each plant received a dose of 50 mL via root irrigation. Five hours later, 500 freshly hatched *M. enterolobii* J2s were inoculated into a

1–2 cm deep cavity at the base of the stem for each plant. The plants were watered and fertilized as usual.

Forty-five days after transplanting, the plants were harvested by gently shaking the soil around the roots to obtain intact plant roots, followed by rinsing with water to remove any remaining soil from the root surface. The root fresh weight, shoot fresh weight and number of nematode galls and egg masses were measured.

2.6. Field Trials

Field experiments were performed over two consecutive years in a field naturally and severely infested with *M. enterolobii* in Wenchang City (Hainan, China) from October 2020 to March 2021 and from October 2021 to March 2022. The temperature during the experimental period ranged from 15 °C to 30 °C. The field was divided into nine blocks (8 m^2 each) arranged in a randomized block design with three replications. Each block consisted of 2 rows and 10 pepper plants planted per row, with a row spacing of 50 cm and plant-to-plant spacing of 80 cm.

Four-week-old healthy pepper seedlings were transplanted to the field. Three treatments were applied: (1) water (CK), (2) *Bt* 00-50-5 and (3) 1.8% abamectin. The untreated control was irrigated with water. For the *Bt* treatment, each pepper seedling was irrigated with a mixture of 100 mL *Bt* fermentation broth (2×10^8 CFU/mL) and 300 mL water. The first irrigation was conducted on the day of transplanting, followed by additional irrigations 7 days and 14 days after transplanting. For the abamectin treatment, the standard solution (800× dilution) was applied for the first time along the planting line after transplanting. Abamectin was applied three times at the same intervals as *Bt* 00-50-5. Sixty days after transplanting, the pepper plants were uprooted and the disease index and biocontrol efficacy were calculated. During this time, the fruit was harvested four times and the yield was measured. The gall index was scored on a 1- to 5-point scale: 1 = no galls; 2 = 1–25% of roots with galls; 3 = 26–50% with galls; 4 = 51–75% with galls; and 5 = over 75% with galls [30]. The disease index was calculated according to Equation (3). Biocontrol efficacy was calculated using Equation (4). Twenty plants were harvested 60 days after transplanting, and the rate of yield increase was calculated using Equation (5).

$$\text{Disease index} = \frac{\sum(\text{Number of diseased plants in each rating} \times \text{scale})}{(\text{Total number of plants investigated} \times \text{the highest scale})} \quad (3)$$

$$\text{Biocontrol efficacy}\ (\%) = \frac{(\text{Disease index of control} - \text{Disease index of treatment})}{\text{Disease index of control}} \times 100 \quad (4)$$

$$\text{Yield increase}(\%) = \frac{(\text{Average yield of treated group} - \text{Average yield of control})}{\text{Average yield of pepper of control}} \times 100 \quad (5)$$

2.7. Statistical Analysis

Statistical analyses and graphs were generated using OriginPro 2021 software version 9.8. The data were checked for normality and homogeneity of variance before performing the ANOVA. If the assumptions of normality and variance were not met, the data were log-transformed. One-way analysis of variance (ANOVA) was performed, and significant differences were identified using Fisher's least significant difference (LSD) test, with significant differences indicated by lowercase letters ($p < 0.05$). All data are presented as the mean ± standard error.

3. Results

3.1. Nematicidal Activity of Bt 00-50-5 Against M. enterolobii J2s In Vitro

The 100% CFS of *Bt* 00-50-5 had a significant effect on the mortality of J2s, with mortality rates gradually increasing over time, reaching 82.9%, 98.0% and 100% at 12, 24 and 36 h, respectively (Figure 1A). At 24 h, the morphology of the control J2s appeared

alive and curved (Figure 1C), whereas those treated with *Bt* 00-50-5 were stiff and dead (Figure 1B). *Bt* 00-50-5, thus, had strong nematicidal activity against *M. enterolobii*.

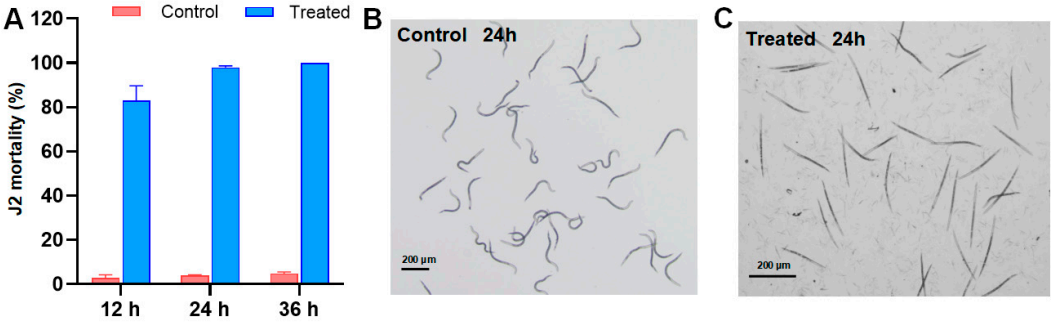

Figure 1. Effect of 100% CFS of *Bt* 00-50-5 on J2 mortality in vitro. (**A**) Mortality rates of J2s treated with 100% CFS of *Bt* 00-50-5 at different times; (**B**) morphology of J2 treated with NB medium control after 24 h; (**C**) morphology of J2 treated with 100% CFS of *Bt* 00-50-5 after 24 h. Scale bars indicate 200 μm.

3.2. Inhibition of *M. enterolobii* Egg Hatching by *Bt* 00-50-5 In Vitro

After 2, 4 and 6 days, the egg hatching rates in the 100% CFS of *Bt* 00-50-5 were 0.11%, 0.58% and 3.24%, respectively, while the control group exhibited rates of 38.5%, 63.7% and 94.5%, respectively (Figure 2A). This indicates that the CFS of *Bt* 00-50-5 significantly inhibits egg hatching. The morphological structures of the eggs treated with 100% CFS of *Bt* 00-50-5 were damaged, resulting in stagnation of embryo development at the J1 stage; J1 was not observed in these eggs. In contrast, the control eggs were well developed after 2 days, the embryo inside the egg was fully formed, and after 4 days, the embryo had progressed to the first-stage juvenile, with its outline still clearly visible at this time (Figure 2B,C). The hatching of *M. enterolobii* eggs was significantly suppressed by *Bt* 00-50-5.

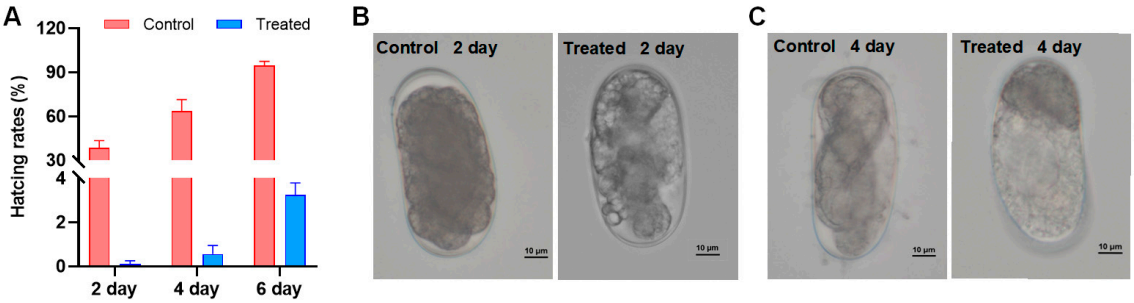

Figure 2. Effect of 100% CFS of *Bt* 00-50-5 on egg hatching in vitro. (**A**) Hatching rate of eggs treated with *Bt* 00-50-5 on different days; (**B**) morphology of eggs treated with 100% CFS of *Bt* 00-50-5 and NB medium control after 2 days; (**C**) morphology of eggs treated with 100% CFS of *Bt* 00-50-5 and NB medium control after 2 days. Scale bars indicate 10 μm.

3.3. The Biocontrol Efficacy of *Bt* 00-50-5 Against *M. enterolobii* in the Greenhouse

Plants treated with different dilutions of *Bt* 00-50-5 fermentation broth showed better growth compared to the control. The fresh shoot weight of plants treated with 100% *Bt* 00-50-5 fermentation broth increased by 25% compared to the control, but the positive effect gradually decreased with increasing dilution of the fermentation broth (Figure 3A). The root weight of plants treated with different concentrations of *Bt* 00-50-5 was also higher than that of the control, and 100% *Bt* 00-50-5 increased the fresh root weight by 18%, although this increase was not statistically significant compared to the control (Figure 3B). The number of root galls in plants treated with different dilutions of *Bt* 00-50-5 was lower than in the control (Figure 3E–H), with reductions of 64%, 41% and 37%, respectively (Figure 3C). Similarly, the 100% *Bt* 00-50-5 treatment resulted in a significant reduction ($p < 0.05$) in egg

masses compared to the control, with the reductions in the three dilutions (100%, 20% and 10%) being 70%, 37% and 14%, respectively (Figure 3D).

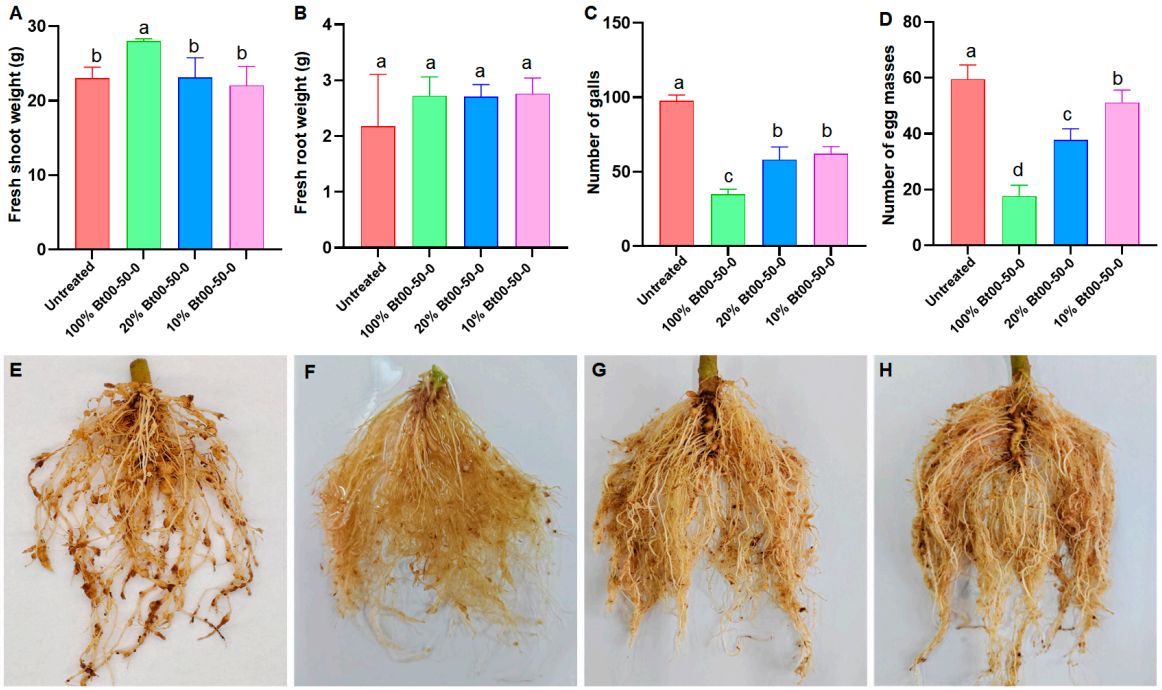

Figure 3. Biocontrol of *Bt* 00-50-5 against *M. enterolobii* in greenhouse. (**A**–**D**) Effects of different concentrations of *Bt* 00-50-5 fermentation on shoot weight, root weight, gall number and egg mass number. Statistical comparisons between treatments were performed using Fisher's LSD test at $p < 0.05$. Means with same letter are not significantly different. (**E**–**H**) Root symptoms treated with water, 100% *Bt* 00-50-5, 20% *Bt* 00-50-5 and 10% *Bt* 00-50-5 after 45 days.

3.4. The Biocontrol Efficacy of Bt 00-50-5 Against M. enterolobii in the Field

Sixty days after transplanting, the disease index of peppers treated with *Bt* 00-50-5 was 31.0% and 30.5% in the 2020–2021 and 2021–2022 seasons, respectively, which was significantly different from the untreated control (Table 1). The biocontrol efficacy of *Bt* 00-50-5 was also higher than that of abamectin, with efficacies of 66.1% and 68.2% versus 59.5% and 63.6% in the two growing seasons, respectively (Table 1). *Bt* 00-50-5 improved pepper yield compared to the untreated control, with increases of 96.2% and 93.1% in the 2020–2021 and 2021–2022 seasons, respectively (Table 1), compared to 77.2% and 69.4% for abamectin. The difference in yield between *Bt* 00-50-5 and abamectin was significant (Table 1). The *Bt* 00-50-5 strain showed high nematode control efficacy, thus reducing pepper yield losses in the field.

Table 1. Biocontrol efficacy of *Bt* 00-50-5 against *M. enterolobii* in the field.

Year	Treatment	Disease Index *	Control Efficiency (%)	Yield (kg/ha) *	Increase in Production Rate (%)
2020–2021	Untreated	92.0 ± 4.6 a	-	4595.2 ± 122.8 c	-
	Bt 00-50-5	31.0 ± 0.6 b	66.1	9017.2 ± 312.5 a	96.2
	Abamaectin	37.1 ± 2.1 b	59.5	8140.9 ± 61.3 b	77.2
2021–2022	Untreated	96.0 ± 2.3 a	-	4698.8 ± 172.5 c	-
	Bt 00-50-5	30.5 ± 0.5 b	68.2	9074.2 ± 234.6 a	93.1
	Abamaectin	35.5 ± 3.9 b	63.6	7961.0 ± 249.4 b	69.4

* Significant differences are indicated by lowercase letters ($p < 0.05$) according to Fisher's LSD test. All data are presented as mean ± standard error.

4. Discussion

The use of antagonistic microorganisms to control pests is receiving increasing attention, and some bacterial *Bacillus* spp. have been used as biocontrol agents to control plant-parasitic nematodes [31,32]. In this study, the bacterium *B. thuringiensis* strain 00-50-5 exhibited a direct effect on *M. enterolobii* J2, resulting in high mortality rates and the suppression of egg hatching upon treatment with the bacterial fermentation supernatant. In addition, the *Bt* 00-50-5 strain significantly reduced nematode galls and egg masses and promoted pepper plant growth in the pot trials, and increased pepper yield in the field. Therefore, the *Bt* 00-50-5 strain showed great potential for controlling *M. enterolobii* in pepper production.

The CFS of the *Bt* 00-50-5 strain showed high nematicidal activity against *M. enterolobii* in vitro. A 100% concentration of CFS of *Bt* 00-50-5 over 24 h can result in 98% mortality of J2s. To date, several nematicidal mechanisms of *Bt* have been reported to contribute to biological control, including the RNA polymerase inhibition-like mechanism of thuringiensin, the degradation mechanism of proteases and/or chitinases, and the perforation mechanism of crystal protein [33]. More recently, a study reported that the protease enzyme secreted by *Bt* strain B79 resulted in mortality against *M. enterolobii*, and SEM showed that dead J2s treated with this strain were shrunken and wrinkled, with cuticle abnormalities [34]. Similarly, previously published studies have shown that protease secreted by *B. cereus* and *B. firmus* causes severe damage to the nematode cuticle and intestinal tissue of *M. incognita* [35,36]. However, further research is needed to identify and characterize the nematicidal compounds of *Bt* 00-50-5.

Bt 00-50-5 fermentation broth also showed good control of *M. enterolobii* both in the greenhouse and in the field, showing potential for use in practical production. In the pot experiments, different dilutions of *Bt* 00-50-5 significantly reduced nematode-induced root damage compared to the controls. In addition, 100% *Bt* 00-50-5 fermentation broth also increased the fresh root and shoot weight of pepper compared to the untreated control. Many studies have reported the growth-promoting properties of *Bacillus* spp. on plants, and the properties of plant growth-promoting bacteria are correlated with the genus of bacteria rather than the host plant species or plant part [37]. In natural field experiments, *Bt* 00-50-5 was even more effective than the chemical nematicide abamectin. It has been reported that abamectin is unstable in response to light and may be degraded by soil microorganisms, resulting in a low residues in soil or on crops, which may limit its efficacy against RKN in the field [38].

The main challenges for microbial nematicides in field applications are durability and efficacy against nematodes. Unlike chemical nematicides, which typically require only one application during one growing season, microbial nematicides usually require multiple applications to achieve the desired control efficacy. For example, *Bt* treatment applied twice per season reduced nematode density under field conditions in a citrus nematode-infested private orchard in Nubaria [31]. Three applications of 50 mL of Bt KYC culture broth to tomato plants at 1, 2 and 3 weeks after transplanting can significantly reduce the number of egg masses [32]. In this study, *Bt* 00-50-0 was applied three times by root irrigation at the early seedling stage of pepper and showed good control of *M. enterolobii* in the field. Undoubtedly, the application timing and concentration of *Bt* 00-50-0 could be optimized to further improve its control efficacy. Multi-location field trials are also needed to validate the efficacy of *Bt* 00-50-0 before it can be recommended for commercial application.

5. Conclusions

In the present study, we comprehensively evaluated the efficacy of *Bt* 00-50-5 against *M. enterolobii*. The results confirmed that the fermentation of *Bt* 00-50-5 resulted in high mortality of J2s of *M. enterolobii*, and strongly inhibited egg hatching. This strain also gave satisfactory results under both greenhouse and field conditions. *Bt* 00-50-5 as a nematicidal strain effectively controlled *M. enterolobii* and improved pepper yield, making it a promising alternative bionematocide.

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