agronomy

MDPI

Article

Study of Novel Endophytic Bacteria for Biocontrol of Black Pepper Root-knot Nematodes in the Central Highlands of Vietnam

Thi Phuong Hanh Tran [1], San-Lang Wang [2,3,*], Van Bon Nguyen [4,*], Dinh Minh Tran [5], Dinh Sy Nguyen [5] and Anh Dzung Nguyen [5,*]

1 Department of Science and Technology, Tay Nguyen University, Buon Ma Thuot 630000, Vietnam; phuonghanh160183@gmail.com
2 Department of Chemistry, Tamkang University, New Taipei City 25137, Taiwan
3 Life Science Development Center, Tamkang University, New Taipei City 25137, Taiwan
4 Institute of Research and Development, Duy Tan University, Da Nang 550000, Vietnam
5 Institute of Biotechnology and Environment, Tay Nguyen University, Buon Ma Thuot 630000, Vietnam; minhdinh_chhcm@yahoo.com (D.M.T.); dinhsy2002@yahoo.com (D.S.N.)
* Correspondence: sabulo@mail.tku.edu.tw (S.-L.W.); bondhtn@gmail.com (V.B.N.); nadzungtaynguyenuni@yahoo.com.vn (A.D.N.); Tel.: +886-2-2621-5656 (S.-L.W.); Fax: +886-2-2620-9924 (S.-L.W.)

Received: 29 August 2019; Accepted: 31 October 2019; Published: 5 November 2019

Abstract: Black pepper is an industrial crop with high economic and export value. However, black pepper production in Vietnam has been seriously affected by the root-knot nematodes, *Meloidogyne* spp. The purpose of this study was to select active endophytic bacteria (EB) for the cost-effective and environmentally friendly management of *Meloidogyne* sp. Thirty-four EB strains were isolated. Of these, five isolates displayed the highest activity, demonstrating 100% mortality of J2 nematodes. These active EB were identified based on sequencing and phylogenetic analysis of the 16S rRNA gene; notably, all the potential endophytic bacterial strains belong to the genus of *Bacillus.* In greenhouse tests, *Bacillus megaterium* DS9 significantly reduced nematodes in the soil and pepper plant roots with great inhibition values of 81.86% and 73.11%, respectively, with the lowest rate of nematodes built up at 0.23. This active antinematodes strain also showed good effect on promoting pepper plant growth. Some enzymatic activities, including chitinase and protease activity related to the biocontrol of *Meiloidogyne* sp., were also detected. The results investigated in the current study suggested that these selected EB strains may be good candidates for biocontrol agents of *Meloidogyne* sp., and plant promoting effects. The results also enhanced the novel active antinematode endophytic bacterial communities.

Keywords: *Bacillus megaterium*; black pepper; endophytic bacteria; biocontrol

1. Introduction

Black peppercorn has long been considered a significant daily spice and among the most widely traded spices worldwide, amounting to 20% of all world spice imports [1,2]. This spicy plant has been cultivated widely in Vietnam, Indonesia, India, and Brazil. Of these, Vietnam is the largest producer and exporter of peppercorns with nearly 40% of the total 546,000 tons produced worldwide [3]. In Vietnam, black pepper trees have been mainly cultivated in the Central Highlands, the North Central Coast, Southeast Vietnam, and other southeastern areas. The Central Highlands and southeastern areas produce the largest amount of black peppercorns with about 124.5 hectares and production of 193.3 tons [4].

Black pepper cultivation has faced many problems, including the disease caused by the root-knot nematode, *Meloidogyne* sp. [5]. When crops infested by *Meloidogyne* sp. show stunted growth, yellowing, marginal and tip drying of leaves, wilting, and root-knot followed by the rotting of roots; finally, the plants may die [6–8]. Chemical nematicides have been used in a long history of root-knot nematode management; however, this treatment caused environmental problems and drug residues in agricultural products [9]. Thus, various alternative methods for root-knot nematode management have been investigated, such as soil organic amendment and antagonistic agents [10], chemicals and cultivars in cotton in a semi-arid environment [11], beneficial microbes [12], co-cultivation with various plants, resistant planting material, destroying infected plants [13], herbal extracts and natural compounds [14,15]. Of these, the utilization of microorganisms for the biocontrol of nematodes has received great attention due to its cost-effectiveness and environmental friendliness to agricultural products [10,12,16]. Microbes inhibit nematodes by their production of enzymes, volatiles, and some other natural compounds [17–20]. The use of endophytic bacteria has proven of great interest due to their reported promising multi-functions of antinematodes and plant growth promoting effect [21–23].

In the current report, *Meloidogyne* sp. was chosen as the target nematode since they are the main species seriously affecting black pepper plants in Vietnam [4]. Vinh Linh pepper, an industrial crop with high economic value, is largely cultivated in the Central Highlands of Vietnam and, as such, was selected as the plant model for testing. Based on the recent literature review, this study is the first to isolate, select, and identify endophytic bacteria in Vietnamese black pepper plant roots that possess potent anti-nematode activity for the environmentally friendly and cost-effective management of *Meloidogyne* sp. The potency of root-knot nematode management was evaluated via in vitro and in vivo tests (under greenhouse conditions). The enzymes related to antinematode activity were also tested.

2. Materials and Methods

2.1. Materials

TSB (trypticase soy broth) and TSA (trypticase soy agar) mediums purchased from Sigma Aldrich. The other chemicals used were of the highest grade available. *Meloidogyne* sp. and its eggs were obtained from the roots of black pepper plants cultivated in Buon Ma Thuot city (12°39′16.8″ N 108°02′06.2″ E). Nematode and its eggs were prepared according to the previous report by Khan et al., 2008 [24]. *Meloidogyne* sp. was originally collected from root-knot tissues of Vinh Linh black pepper cultivar (Figure 1A,B). The roots were washed by water to remove soil; the egg masses were then collected by hand using forceps. Masses of nematode eggs were washed with sterile water and then 0.5% sodium hypochlorite, finally agitated and rinsed with sterile water on a sieve (26 μm pores). The eggs were used for the assay of inhibition against egg hatch, and also incubated for 3–5 days to obtain second-stage juveniles (J2) [25]. The eggs and nematode J2 were used for all subsequent tests.

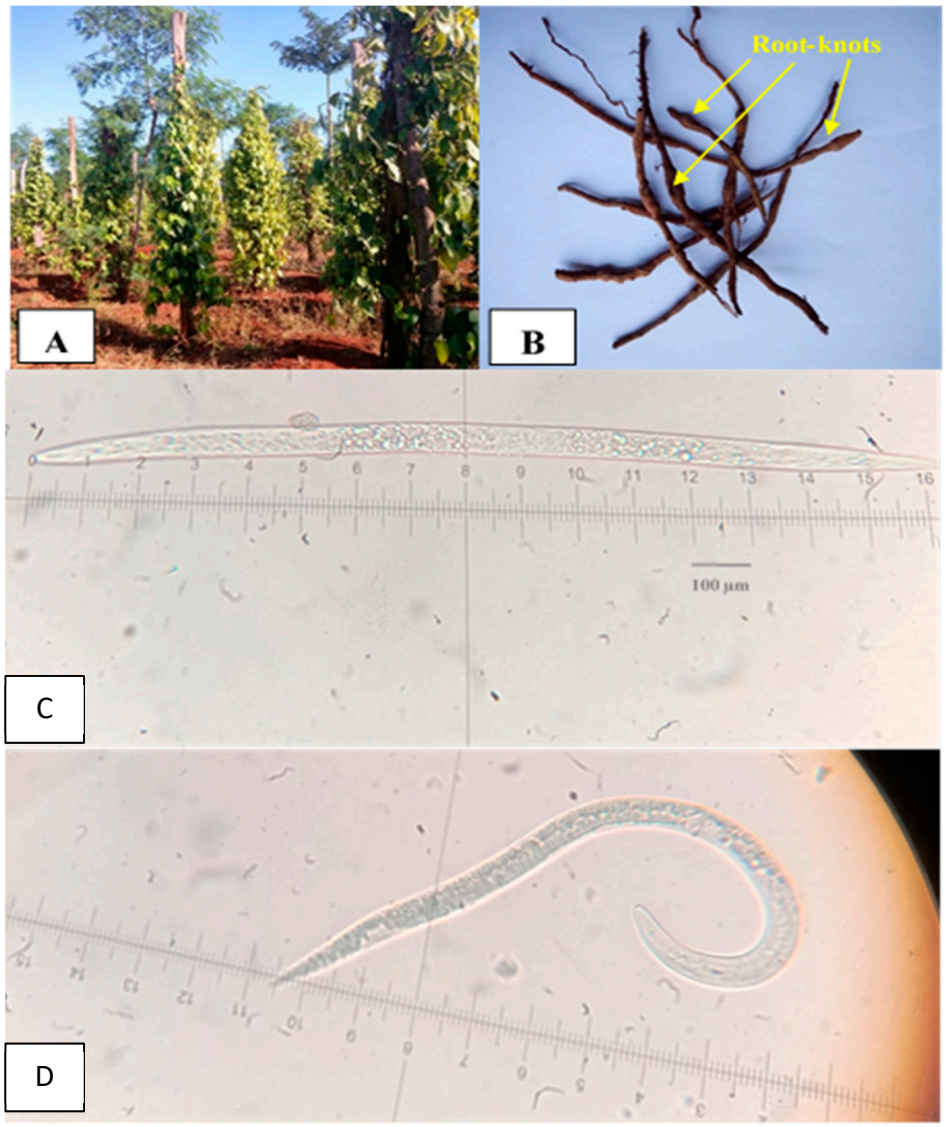

Figure 1. Vinh Linh black peppers plants field (**A**); root-knot tissues (**B**) collected from the roots systems of Vinh Linh black pepper; immobilized *J2* nematode (**C**) and mobilized *J2* nematode (**D**). Yellow arrows are root-knots which contain nematode eggs. The images of nematodes were recorded with the use of an Olympus optical microscope (model CH30RF200). Bar = 100 µm.

2.2. Isolation of Endophytic Bacteria

The black pepper plants roots were collected from healthy plants (3–7 years old) cultivated in Dak Nong province and kept in sterilized polyethylene bags. Endophytic bacteria were isolated according to the method described by Aravind [26]. The black pepper plant roots were washed with water, and then cut into pieces with the length of 1–2 cm. Tween 80 was used to treat the pieces for 10 min and sterilized water was then used for washing. Then 2% sodium hypochlorite solution was used for sterilizing the surface of the root samples for 10 min, and subsequently with 70% ethanol for 1 min. The samples were then washed six times with sterilized distilled water. The samples were mixed with phosphate buffer saline (PBS) pH 7·4, ground, and then centrifuged (600 rpm) at 5 °C for 2 min, and 100 µL of the suspension was inoculated into TSA medium and cultivated at 28 °C for 1–3 d. Single colonies were separated and sub-cultured on new TSA medium. All isolated strains were stored in 50% glycerol at −32 °C.

2.3. PCR Amplification, Sequencing, and Phylogenetic Analysis of the 16S rRNA Gene

The active bacterial strains were identified based on 16S rDNA gene sequencing. Genomic DNA from an overnight culture of each strain was extracted by the method described by Tran et al. 2018 [27]. The genomic DNA was used as a template for amplification by PCR. A nearly full-length segment of 16S rRNA gene nucleotides was amplified in a 100 µL reaction tube using the universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′). The 16S rRNA gene was amplified by iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using the following schedule: 94 °C for 5 min, repeated by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. The amplified products were then separated by electrophoresis on agarose gel (1.5%, *w*/*v*). The target bands in the agarose gel were cut out and purified using a QIA quick PCR purification (Promega Co., Madison, WI, USA). Sequencing reactions were carried out in a CEQ8000 Genetic Analysis System (Beckman Coulter Inc., Brea, CA, USA) using a CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., Brea, CA, USA).

The nucleotide sequences (from 1300 to 1440 bps) of the 16S rRNA genes were compared to known sequences in the DDBJ/Genbank/EMBL databases using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the taxonomic positions of the rhizobacteria isolates. The phylogenetic tree was made using Kimura's method and drawn using the Mega software version 6.0 after multiple alignments of the data by Clustal W [28,29].

2.4. Bacterial Strains Preparation

Bacterial strains were cultured on liquid medium containing beef extract (7 g), peptone (7 g), NaCl (50 g) in 1000 mL distilled water, and an initial pH 7.0, at a shaking speed of 150 rpm at 28 °C for 24 h. Thereafter, each endophytic bacterial strain was harvested via centrifuge at 8000 rpm for 10 min to remove the culture supernatant before the bacterial mass was suspended in saline buffer. Each strain was then arranged by spectrophotometry to 10^7 CFU mL^{-1} at a wavelength of 600 nm prior to the in vitro and greenhouse tests.

2.5. Anti-Nematode Activity In Vitro Assays

The nematicidal activity in vitro assay: 250 µL sterile distilled water containing around 30 nematode J2 of *Meloidogyne* sp. was mixed with 100 µL bac solution (10^8 CFU/mL) in a 96-well tissue culture plate. The mixture was kept at 28 °C for 24 h before the mobilized nematodes and immobilized J2 were counted. Nematodes that did not move when touched with a fine needle were defined as inactive (immobilized nematodes) [30] (Figure 1C,D). The control experiment was conducted under exactly the same conditions as described above but without bacteria. All the testes were done with triplicates.

The effect of culture filtrates on nematode egg hatching: 100 µL culture filtrates were mixed with 250 µL sterile distilled water containing around 200 *Meloidogyne* sp. eggs in a 96-well tissue culture plate. The mixture was kept at 28 °C for three days; the hatched juveniles were then counted with the use of a low power stereoscopic microscope. The control experiment was conducted triplicates under exactly the same conditions as described above but 100 µL unfermented culture filtrate was used instead of fermented culture filtrates.

2.6. The Effect of Endophytic Bacteria on Reducing Nematodes and the Plant Promoting Effect for Black Pepper Trees in Plots Experiments

The most active anti-nematodes endophytic bacterial strains in the in vitro test were selected for the further bioassay in the greenhouse. Local Vinh Linh black pepper seedlings with five leaves were used for the plot experiments. The experiment was arranged according to the random distribution formula. Each experiment had six groups, including five experimental groups treated with selected bacterial strains and one control group (no bacteria treatment). Each group consisted of 30 pepper

seedlings, with one pepper seedling cultivated per pot. The distance between each group was 60 cm, with 50cm between each pot. The greenhouse conditions were set to 25–30 °C, 75–80% humidity, and a light intensity of 50–550 μmol m^2/s^{-1} (measured from 08:00 to 16:00). Five-leaf-old seedlings were transplanted in plastic pots containing about 0.5 kg of sterilized soil, sand, and organic fertilizer in a ratio of 2:1:1 (*v*/*v*), respectively. Two times 100 mL of endophytic bacterial suspension with a density of 10^7 CFU mL^{-1} as inoculated into each pot two weeks apart. After two weeks, each pot was inoculated with 300 nematode J2. The control group received the same treatment as described above but saline was used instead of bacteria. Tap water was used for irrigation. Each of these treatments was used to fill ten plastic pots arranged in a randomized block design on benches in a greenhouse. After three months of endophytic bacterial inoculation, the black pepper seedlings were carefully removed and washed from the soil. Nematode parameters as numbers of J2 in 10 g of soil and in 1 g roots were counted. The percentages of efficacy and the rates of nematode build-up were calculated. The growth parameters, such as increased shoot length, increased root length, number of leaves, and chlorophyll a+b contents.

2.7. Enzymic Activity Assays

Protease activity of the culture supernatants (CS) of *Bacillus megaterium* DS9 was measured according to the techniques described in the previous report [31], with slight modifications. In brief, 0.1 mL of CS was mixed with 1 mL casein solution (1% in 50 mM phosphate buffer). The mixture was incubated at 37 °C for 60 min. Trichloroacetic acid (TCA) solution (5%) was added to stop the reaction before the mixture was centrifuged at 4000 rpm for 15 min. A total of 0.75 mL of this solution was then mixed with 1.5 mL of NaOH 0.28 N and 0.48 mL of Folin's reagent. It was left to rest for 15 min before being measured at OD_{660} nm. Tyrosine was used as reference. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 μmol of tyrosine per min.

The chitinase activity of CS was measured according to the methods described in our previous report [32]. In brief, 50 μL of CS, 100 μL of pNPg (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) (1 mg/mL) and 500 μL of sodium acetate buffer (50 mM, pH 5.8) were incubated at 37 °C for 30 min. A total of 350 μL of sodium carbonate buffer (50 mM, pH 10.7) was then added to the mixture before the final solution was measured at OD_{415} nm. The chemical compound pNP (*p*-nitrophenol) was used as a reference. The amount of chitinase that produced 1 μM of pNP per min was defined as one unit of chitinase activity.

2.8. Statistical Analysis

For the statistical analysis of the results, the data on the experiments were subjected to analysis of variance (ANOVA), and means were separated ($p \leq 0.01$) by Duncan's multiple-range test using Statistical Analysis Software (SAS-9.4, SAS Institute Taiwan Ltd, Taipei, Taiwan).

3. Results

3.1. Isolation, Evaluation, and Identification of the Active Antinematodes Endophytic Bacteria in the In Vitro Test

Thirty-four endophytic bacterial strains were isolated from the 3–5 years black pepper roots in Dak Nong Province in the Central Highlands of Vietnam, and the antinematode effect was evaluated in the in vitro test (Table 1). The results illustrate that almost all of the newly isolated endophytic bacterial strains showed potent activity. Seventeen strains displayed the potential antinematode activity with the mortality higher than 85.56%. Of these, five strains: DS5, DS8, DS9, DR2, and DR10 demonstrated the most efficacious antinematode activity with the mortality of 100%. The active strains were identified as *Bacillus flexus* DS5, *Bacillus* sp. DS8, *Bacillus megaterium* DS9, *Bacillus* sp. DR10, and *Bacillus* sp. DR2, based on the 16s rRNA gene sequences analysis. Phylogenetic analysis of the identified strains

is illustrated in Figure 2, and the accession number of 16s rRNA genes of the selected endophytic bacterial strains is listed in Table 2.

Table 1. Percentages of mortality of the 2nd-stage juveniles *of Meloidogyne* ssp. in vitro tests.

Isolates	Mortality (%) of Nematodes	Isolates	Mortality (%) of Nematodes
DS1	91.11 ^{c}	DR5	8.89 ^{jk}
DS2	63.33 ^{e}	DR6	5.56 ^{kl}
DS3	7.78 ^{jk}	DR8	7.78 ^{jk}
DS4	6.67 ^{jkl}	DR9	96.67 ^{ab}
DS5	100 ^{a}	DR10	100 ^{a}
DS6	57.78 ^{fg}	DR11	95.56 ^{b}
DS7	95.56 ^{b}	DR12	57.78 ^{fg}
DS8	100 ^{a}	DR13	61.11 ^{ef}
DS9	100 ^{a}	DM1	3.33 ^{ml}
DS10	96.67 ^{ab}	DM2	5.56 ^{kl}
DS11	91.11 ^{c}	DM3	15.56 ^{i}
DS12	85.56 ^{d}	DM4	6.67 ^{jkl}
DS13	96.67 ^{ab}	DM5	55.56 ^{g}
DS14	51.11 ^{h}	DM6	10.00 ^{j}
DR1	3.33 ^{ml}	DM7	95.56 ^{b}
DR2	100 ^{a}	DM8	0.00 ^{m}
DR3	96.67 ^{ab}	Control (no bacterial)	0.00 ^{m}
DR4	95.56 ^{b}		

Endophytic bacterial strains were cultivated in trypticase soy broth (TSB) at 28 °C for three days. Bacteria were harvested by centrifuged the cultured medium at 8000 rpm for 10 min. The bacterial solution was adjusted at 10^{7} CFU/mL and used for antinematode activity assay. The mortality values (%) of nematodes with the same letter are not significantly different, based on Duncan's multiple range test (alpha = 0.01), using SAS version 9.4, Statistical Analysis Software analysis. Coefficient of variation = 3.29.

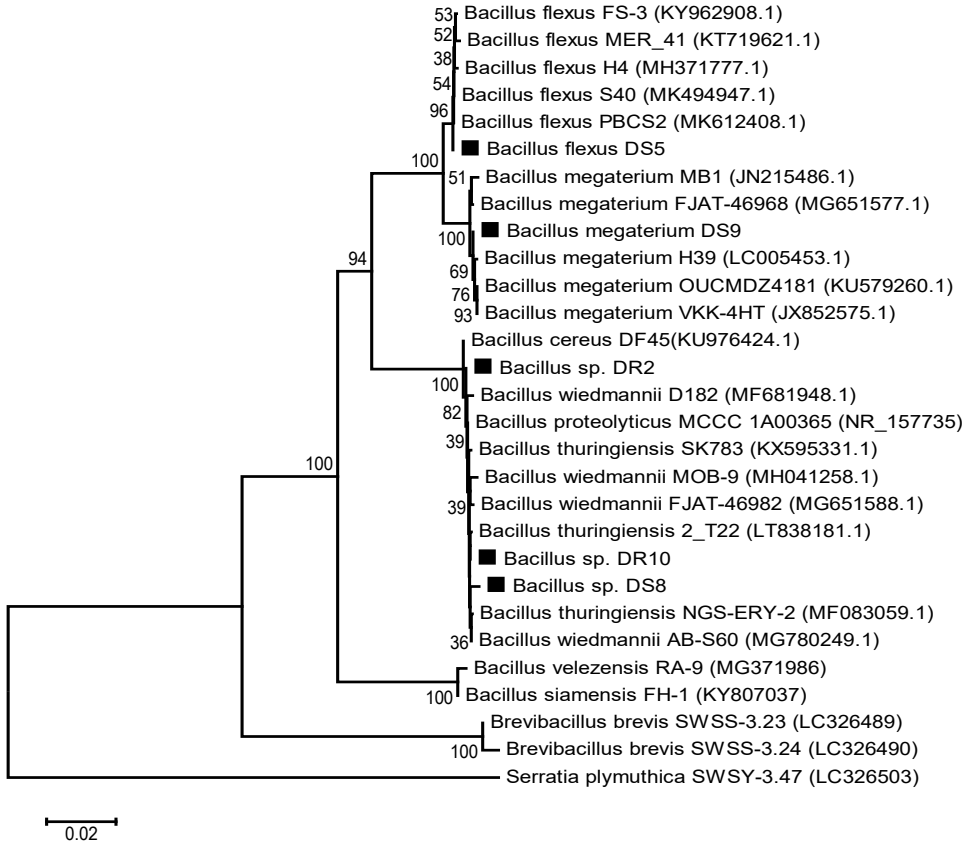

Figure 2. Phylogenetic analysis of the endophytic bacteria based on the 16s rRNA gene sequences.

The phylogenetic tree was made as per Kimura's method and created using Mega software version 6.0 after multiple alignments of the data by Clustal W. The numbers at the branches are bootstrap confidence percentages (%).

Table 2. The accession IDs of 16s rRNA genes of the selected endophytic bacterial strains.

No	Bacterial Name	Accession Number
1	*Bacillus* sp. DR2	LC496557
2	*Bacillus* sp. DR10	LC496558
3	*Bacillus flexus* DS5	LC496559
4	*Bacillus* sp. DS8	LC496560
5	*Bacillus megaterium* DS9	LC496561

3.2. Evaluation of Potent Biocontrol of Meloidogyne sp. by the Selected Endophytic Bacteria under Greenhouse Conditions

The five active *endophytic* bacterial strains: *Bacillus flexus* DS5, *Bacillus* sp. DS8, *Bacillus megaterium* DS9, *Bacillus* sp. DR10, and *Bacillus* sp. DR2 underwent greenhouse tests to evaluate their effect on nematode inhibition in soil and in roots, as well as their interaction with pepper plants. The results in Table 3 show that all the five tested strains demonstrate reduced nematodes in soil and in pepper roots with inhibition values of 56.33–87.34% and 38.89–76.44%, respectively. The rates of nematodes built up in all the plots treated with RB were lower than that of the control plot. Of these tested strains, DS9 showed the most potential in biocontrol of nematodes with great inhibition values of 81.86% and 73.11%, both in soil and in pepper roots, respectively, indicating the lowest rate of nematodes built up of 0.23.

Table 3. Effect of endophytic bacterial strains on populations of 2nd-stage juveniles *of Meloidogyne* sp. under greenhouse conditions (after three months of treatments).

Treatments	Nematode J2 in Soil (Count)	Reduction of Population Density Ability in Soil (%)	Nematode J2 in Roots (Count)	Reduction of Population Density Ability in Roots (%)	Rate of Buildup (pf/pi)
DS5	69.00 ^{c}	56.33	91.67 ^{bc}	38.89	0.54 ^{b}
DS8	43.00 ^{d}	72.78	35.33 ^{e}	76.44	0.26 ^{cd}
DS9	28.67 ^{de}	81.86	40.33 ^{e}	73.11	0.23 ^{d}
DR2	20.00 ^{e}	87.34	77.67 ^{dc}	48.22	0.33 ^{c}
DR10	33.67 ^{de}	78.69	52.00 ^{de}	65.33	0.29 ^{cd}
Control	158.00 ^{a}	-	150.00 ^{a}	-	1.03 ^{a}
LSD	14.36		26.05		0.14
CV%	10.13		13.93		7.47

DS5, DS8, DS9, DR2, and DR10 are endophytic bacterial strains: *Bacillus flexus* DS5, *Bacillus* sp. DS8 *Bacillus megaterium* DS9, *Bacillus* sp. DR10 and *Bacillus* sp. DR2. Pots with nematode J2 only inoculation were treated as the control group. Values in the same column with the same letter are not significantly different, based on Duncan's multiple range test (alpha = 0.01) using SAS version 9.4, Statistical Analysis Software analysis. CV: Coefficient of variation; LSD: Least significant difference. (-): Not determined.

In the greenhouse tests, all the strains showed no negative effects on the pepper plants. In addition, they also demonstrated a significant effect on plant growth promotion, including the increased shoot length (22.64–29.81 cm), formation of new leaves (3.79–4.19 leaves) and increased root length (6.65–8.98 cm). These values are significantly higher than those of the untreated group (control), with increased shoot length (17.25 cm), new leaves forming (one leaf) and increased root length (5.56 cm). The contents of chlorophyll a+b in the tested bacterial groups also show higher rates than those of the control group (Table 4).

Table 4. Effect of endophytic bacterial strains and *Meloidogyne* sp. on growth parameters of black pepper seedling under greenhouse conditions (after three months of treatments).

Treatments	Increased Shoot Length (cm)	formed New Leaves	Increased Root Length (cm)	Chlorophyll a + b (mg/g LFW)
DS5	25.71 ^{ab}	3.92 ^{a}	6.65 ^{cde}	0.78 ^{d}
DS8	29.38 ^{a}	4.19 ^{a}	8.62 ^{ab}	1.76 ^{a}
DS9	29.81 ^{a}	3.99 ^{a}	8.98 ^{a}	1.52 ^{b}
DR2	28.68 ^{ab}	3.99 ^{a}	7.64 ^{abc}	0.92 ^{c}
DR10	22.64 ^{abc}	3.79 ^{a}	7.2 ^{bcd}	0.75 ^{d}
Control	17.25 ^{c}	1.00 ^{b}	5.56 ^{de}	0.57 ^{e}
CV%	12.52	12.38	17.10	5.38
LSD	7.62	1.06	1.06	0.07

Chlorophyll a+b (mg/g LFW): chlorophyll a + b contents in leaf fresh weight; DS5, DS8, DS9, DR2, and DR10 are endophytic bacterial strains: *Bacillus flexus* DS5, *Bacillus* sp. DS8 *Bacillus megaterium* DS9, *Bacillus* sp. DR10 and *Bacillus* sp. DR2. Pots with nematode J2 only inoculation were treated as the control group. Values in the same column with the same letter are not significantly different, based on Duncan's multiple range test (alpha = 0.01) using SAS version 9.4, Statistical Analysis Software analysis. CV: Coefficient of variation; LSD: Least Significant Difference; LFW: leaf fresh weight.

3.3. Chitinase, Protease Activities of Fermented Product by Bacillus megaterium DS9

Bacillus megaterium DS9 demonstrated active antinematodes activity both in vitro and in the greenhouse tests, and also good effect on plant growth promotion. Thus, this strain was chosen for further investigation of active components of its fermented product. To determine the anti-nematode agents produced by this selective strain, we tested the inhibition of supernatant fermented by DS9 against egg hatching and J2 nematodes. Chitinase and protease activity related to the biocontrol of *Meloidogyne* spp. and anti-nematode activity were also detected.

As shown in Table 5, the culture supernatants of *B. megaterium* DS9 demonstrated protease (0.69 IU/mL) and chitinase (2.72 IU/mL) activity when mediums were supplemented with casein and chitin, respectively; these culture supernatants also displayed antinematodes J2 activity (52.22–73.33%) and egg hatching inhibition (60–71.11%). The unfermented mediums were also tested activity showing no inhibition against eggs hatching and weak anti-nematode J2 (3.6–8.1%) (Table 5). After being treated at a high temperature (100 °C) for 60 min, none of the extracted enzymes showed any anti-nematode effect (Table 6). Enzymatic activity in the culture supernatants disappeared, but the anti-nematode effect still remained in the range of 21.11–30% and 30–37.78% against J2 nematodes and egg hatching, respectively (Table 7).

Table 5. The enzyme activity and anti-nematodes activity of culture supernatants of *B. megaterium* DS9.

Bacterial Strains	Supernatants of Fermented Medium	Enzyme Activity (IU/mL)		Anti-Nematodes Activity (%)	
		Chitinase	Protease	Nematode J2 Inhibition	Egg Hatching Inhibition
DS9	Medium containing casein	-	0.69 ± 0.06	73.33 ± 1.92	60.00 ± 8.85
	Medium containing chitin	2.72 ± 0.12	-	52.22 ± 4.0	71.11 ± 4.0
	Unfermented medium containing casein	-	-	3.6 ± 0.01	-
	Unfermented medium containing chitin	-	-	8.1 ± 0.05	-

IU/mL: International Units per millilitre; (-): No activity.

Table 6. The anti-nematodes activity of extracted enzymes of *B. megaterium* DS9 before and after treatment at high temperature (100 °C) for 60 min.

Crude Enzymes	Antinematodes Activity (%) of Enzymes Not Treated at 100 °C		Antinematodes Activity (%) Enzymes Treated at 100 °C	
	Nematode J2 Inhibition	Egg Hatching Inhibition	Nematode J2 Inhibition	Egg Hatching Inhibition
DS9 protease	44.58 ± 6.44	42.22 ± 3.75	-	-
DS9 chitinase	15.63 ± 1.74	25.56 ± 1.94	-	-

Table 7. The enzyme activity and antinematodes activity of culture supernatants of *B. Bacillus megaterium* DS9 after treatment at high temperature (100 °C) for 60 min.

Bacterial Strains	Supernatants of Fermented Medium	Enzyme Activity (IU/mL)		Anti-Nematodes Activity (%)	
		Chitinase	Protease	Nematode J2 Inhibition	Egg Hatching Inhibition
DS9	Medium containing casein	-	-	30.00 ± 1.92	30.00 ± 5.09
	Medium containing chitin	-	-	21.11 ± 2.93	37.78 ± 2.22

4. Discussion

4.1. Isolation, Evaluation of the Active Antinematodes Endophytic Bacteria

Beneficial microbes have been widely used in nematode management and to promote plant growth [10,12,16,21–23]. In this study, beneficial bacteria were isolated then used as environmentally friendly, cost-effective nematode management agents. Unlike previous studies, we focused on isolating bacteria living in the roots of black pepper plants rather than other resources. We chose black pepper fields which suffered heavily from nematodes, and only the roots of healthy black pepper trees surrounded by sick pepper trees (yellow trees affected by root-knot nematodes) were collected for endophytic bacteria isolation. In sick pepper fields cultivated with the same Vinh Linh pepper seedlings, grown under the same conditions, some trees still remain healthy and unaffected by nematodes. This may be due to beneficial microbes in the soil surrounding the roots or living in the roots themselves. Our goal, therefore, was to isolate these microbes. In the current study, we focused on the isolation of endophytic bacteria. Five endophytic strains, DS5, DS8, DS9, DR2, and DR10, demonstrated high anti-nematode activity with 100% mortality. Notably, all of these potentially endophytic bacterial strains belonged to the genus of *Bacillus* (Figure 2). Various *Bacillus* species are reported to show anti-nematode activity, such as *B. subtilis*, *B. cereus*, *B.pumilus*, and *B. megaterium* [20,22,23,33,34]. However, there are few available reports on the anti-nematode activity of *B. flexus*. Based on recently reviewed literature, this is the first record of endophytic *Bacillus* species isolated and identified from the Vinh Linh pepper roots in the Central Highlands of Vietnam.

It is interesting to note that all selected endophytic bacteria had a positive effect on black pepper growth (Table 4). Beneficial bacteria may enhance plant growth via several ways, including the inhibition of pathogens and deleterious rhizosphere microorganisms (indirect mechanisms), or supplying growth agents like hormones and nutrients (direct mechanisms) [2,35–38]. In this study, *Bacillus* species displayed potent pathogen inhibition. This bacterial genus has also shown vast beneficial effects, including phosphate solubilization, nitrogen fixation and the production of indole acetic acid, cytokinin, and gibberellin [36]. These may cause the plant growth promotion effect of the selected endophytic bacteria in this study.

To date, vast amounts of genus *Bacillus* including *B. megaterium* have been applied for biocontrol and plant growth promotion agents for huge crops [20,22,23,33,34]. However, few studies on *B. megaterium* applied management of black pepper nematodes as well as their effect on this plant growth promotion.

4.2. Primary Determination of Active Components of Fermented Product by Bacillus megaterium DS9

The culture supernatant of *B. megaterium* DS9 displayed both enzymatic activity (protease and chitinase) and inhibition against J2 nematodes and egg hatching. As such, these tested enzymes may play an important role in anti-nematode activity. To clarify, the crude enzymes were extracted via dialysis, precipitated by ethanol and then tested for anti-nematode activity. As shown in Table 6, the crude enzymes contributed to anti-nematode activity, however this activity disappeared after treatment at high temperature (100 °C) for 60 min. This confirms the enzymes were closely related to anti-nematode activity. These results are consistent with those of previous reports [10,18]. Although the crude extracted enzymes of this bacterium displayed anti-nematode activity (Table 6), inhibition values were lower than those of the original culture supernatants. This may be due to the enzyme extraction process. To confirm this assumption, the culture supernatants were treated at 100 °C for 60 min to denature the enzymes, and then tested for enzyme and anti-nematode activity. The enzymatic activity of the culture supernatants disappeared but the anti-nematode effect remained (Table 7) with low values of 21.11–30% and 30–37.78% against J2 nematodes and egg hatching, respectively. These results prove that the anti-nematode effects were due to enzymes and some other unknown active agents. The culture supernatants showed higher activity than the extracted enzymes due to the other active components. To date, several studies have proven that anti-nematode agents are natural compounds [18–20]. Of these, some volatiles are reported as active agents for the control of nematodes [18]. In the current report however, the culture supernatants still maintained anti-nematode activity even after heating. As such, besides enzymes, the major anti-nematode agents must be natural compounds with high thermal stability. Further research is needed to isolate, identify and clarify the role of the major metabolites produced by *B. megaterium* DS9.

5. Conclusions

This is the first report on the isolation and selection of endophytic bacteria and utilization for cost-effective control of pepper nematodes in the Central Highlands of Vietnam. Five isolates displayed potent activity with mortality of J2 nematodes value at 100%, and were identified based on 16S rDNA gene sequencing. Of these selective strains, *Bacillus megaterium* DS9 demonstrated significant antinematode activity in both in vitro and green house tests. This active strain also showed good effect on the promotion of pepper plant growth. The major metabolites produced by *Bacillus megaterium* DS9 were identified and its enzymatic activities related to the antinematodes effect (chitinase and protease) was also tested. The results suggest that these selected EB strains may be good candidates for the biocontrol of *Meloidogyne* sp.

Author Contributions: Conceptualization: V.B.N., S.-L.W., T.P.H.T. and A.D.N.; methodology: V.B.N.; software: V.B.N.; validation: S.-L.W., T.P.H.T., D.M.T., D.S.N. and A.D.N.; formal analysis: V.B.N.; investigation: T.P.H.T.; resources: V.B.N., S.-L.W., T.P.H.T. and A.D.N.; data curation: S.-L.W.; Writing—Original draft preparation: V.B.N. and T.P.H.T.; Writing—Review and editing: V.B.N. and S.-L.W.; visualization: T.P.H.T.; supervision: V.B.N. and S.-L.W.; project administration: T.P.H.T., A.D.N. and S.-L.W.

Funding: This study was supported in part by a grant from Ministry of Education and Training, Vietnam: Biotechnology application for sustainable black pepper production in the Central Highlands (B 2017–2019), and a grant from the Ministry of Science and Technology, Taiwan (MOST 106-2320-B-032-001-MY3).

Acknowledgments: We express great thanks to Institute of Biotechnology and Environment, Tay Nguyen University, 567 Le Duan Str., Buon Ma Thuot, Vietnam for the kind provision of some analysis tools for this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ravindra, H.; Sehgal, M.; Manu, T.G.; Murali, R.; Latha, M.; Narasimhamurthy, H.B. Incidence of root-knot nematode (Meloidogyne incognita) in black pepper in Karnataka. *J. Entomol. Nematol.* **2014**, *6*, 51–55.
2. Nguyen, V.B.; Wang, S.L.; Nguyen, T.H.; Nguyen, T.H.; Trinh, T.H.T.; Nong, T.T.; Nguyen, T.U.; Nguyen, V.N.; Nguyen, A.D. Reclamation of rhizobacteria newly isolated from black pepper plant roots as potential biocontrol agents of root-knot nematodes. *Res. Chem. Intermed.* **2019**, *45*, 5293–5307. [CrossRef]

3. Pepper (piper spp.), Production/Crops. Food and Agriculture Organization of the United Nations: Statistical Division (FAOSTAT). Available online: http://www.wikiwand.com/en/Black_pepper (accessed on 4 December 2018).
4. Thuy, T.T.T.; Yen, N.T.; Tuyet, N.T.A.; Te, L.L.; Waele, D.D. Population dynamics of Meloidogyne incognita on black pepper plants in two agro-ecological regions in Vietnam. *Arch. Phytopathol. Plant Protect.* **2012**, *45*, 1527–1537.
5. Trudgill, D.L.; Blok, V.C. Apomictic, polyphagous root-knot nematodes: Exceptionally successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopathol.* **2001**, *39*, 53–77. [CrossRef] [PubMed]
6. Prabhakaran Nair, K.P. *The Agronomy and Economy of Turmeric and Ginger*; Elsevier: Amsterdam, The Netherlands, 2013; pp. 139–157. [CrossRef]
7. Sikora, R.A.; Fernández, E. Nematode parasites of vegetables. In *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*, 2nd ed.; Luc, M., Sikora, R.A., Bridge, J., Eds.; CABI Publishing: Wallingford, UK, 2005; pp. 319–376.
8. Wiratno, M.S.; Ankardiansyah, P.P.; Ahmed, I.A.Y. Biological control of root-knot nematode (*meloidogyne* spp.) in pepper plants utilizing endophytic bacteria *Pseudomonas* sp. AND *Micrococcus* sp. *J. Pepper Ind.* **2018**, *9*, 11–22.
9. Josef, J.; Katarína, K. *Nanopesticides: Preparation, Targeting and Controlled Release*; Academic Press & Elsevier: London, UK, 2017; Volume 10, pp. 81–127.
10. Nguyen, V.N.; Kim, Y.J.; Oh, K.T.; Jung, W.J.; Park, R.D. The role of chitinase from *Lecanicillium antillanum* B-3 in parasitism to root-knot nematode *Meloidogyne incognita* eggs. *Biocontrol. Sci. Technol.* **2007**, *17*, 1047–1105. [CrossRef]
11. Wheeler, T.A.; Siders, K.T.; Anderson, M.G.; Russell, S.A.; Woodward, J.E.; Mullinix, B.G., Jr. Management of *Meloidogyne incognita* with chemicals and cultivars in cotton in a semi-arid environment. *J. Nematol.* **2014**, *46*, 101–107.
12. Mokbel, A.A. Impact of some antagonistic organisms in controlling *Meloidogyne arenaria* infecting tomato plants. *JOLST* **2013**, *1*, 69–74. [CrossRef]
13. Boraha, B.; Ahmed, R.; Hussaina, M.; Phukon, P.; Wann, S.B.; Sarmah, D.K.; Bhau, B.S. Suppression of root-knot disease in *Pogostemon cablin* caused by *Meloidogyne incognita* in a rhizobacteria mediated activation of phenylpropanoid pathway. *Biol. Control* **2018**, *119*, 43–50.
14. Mervat, A.A.; Shawky, S.M.; Shaker, G.S. Comparative efficacy of some bioagents, plant oil and plant aqueous extracts in controlling *Meloidogyne incognita* on growth and yield of grapevines. *Ann. Agric. Sci.* **2012**, *57*, 7–18. [CrossRef]
15. Regaieg, H.; Ciancio, A.; Raouani, N.H.; Grasso, G.; Rosso, L. Effects of culture filtrates from the nematophagous fungus *Verticillium leptobactrum* on viability of the root-knot nematode *Meloidogyne incognita*. *World J. Microbiol. Biotechnol.* **2010**, *26*, 2285–2289. [CrossRef]
16. Nguyen, A.D.; Huang, C.C.; Liang, T.W.; Nguyen, V.B.; Pan, P.S.; Wang, S.L. Production and purification of a fungal chitosanase and chitooligomers from *Penicillium janthinellum* D4 and discovery of the enzyme activators. *Carbohydr. Polym.* **2014**, *108*, 331–337. [CrossRef] [PubMed]
17. Nguyen, V.N.; Oh, I.J.; Kim, Y.J.; Kim, K.Y.; Kim, Y.C.; Park, R.D. Purification and characterization of chitinases from *Paecilomyces variotii* DG-3 parasitizing on *Meloidogyne incognita* eggs. *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 195–203. [CrossRef]
18. Vicente, P.C.; Renata, S.C.D.P.; Eduardo, S.F. Volatiles produced by interacting microorganisms potentially useful for the control of plant pathogens. *Ciênc. Agrotecnol. Lavras* **2010**, *34*, 525–535.
19. Abdelnabby, H.M.; Mohamed, H.A.; Abo Aly, H.E. Nematode-antagonistic compounds from certain bacterial species. *Egypt. J. Biol. Pest Control* **2011**, *21*, 209–217.
20. Gao, H.; Qi, G.; Yin, R.; Zhang, H.; Li, C.; Zhao, X. *Bacillus cereus* strain S2 shows high nematicidal activity against Meloidogyne incognita by producing sphingosine. *Sci. Rep.* **2016**, *24*, 28756. [CrossRef]
21. Elhady, A.; Giné, A.; Topalovic, O.; Jacquiod, S.; Sørensen, S.J.; Sorribas, F.J.; Heuer, H. Microbiomes associated with infective stages of root-knot and lesion nematodes in soil. *PLoS ONE* **2017**, *12*, e0177145. [CrossRef]
22. Basyony, A.G.; Abo-Zaid, G.A. Biocontrol of the root-knot nematode, Meloidogyne incognita, using an eco-friendly formulation from Bacillus subtilis, lab. and greenhouse studies. *Egypt. J. Biol. Pest Control* **2018**, *28*, 87. [CrossRef]

23. Beneduzi, A.; Ambrosini, A.; Passaglia, L.M. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genet. Mol. Biol.* **2012**, *35*, 1044–1051. [CrossRef]
24. Khan, Z.; Kim, S.G.; Jeon, Y.H.; Khan, H.U.; Son, S.H.; Kim, Y.H. A plant growth promoting rhizobacterium, *Paenibacillus polymyxa* strain GBR-1, suppresses root-knot nematode. *Bioresour. Technol.* **2008**, *99*, 3016–3023. [CrossRef]
25. Southey, J.F. *Laboratory Methods for Work with Plant and Soil Nematode*; Ministry of Agriculture Fisheries and Food, HMSO: London, UK, 1986; p. 202.
26. Aravind, R.; Kumar, A.; Eapen, S.J.; Ramana, K.V. Endophytic bacterial flora in root and stem tissues of black pepper (*Piper nigrum* L.) genotype: Isolation, identification and evaluation against *Phytophthora capsici*. *Lett. Appl. Microbiol.* **2009**, *48*, 58–64. [CrossRef] [PubMed]
27. Tran, M.D.; Sugimoto, H.; Nguyen, A.D.; Watanabe, T.; Suzuki, K. Identification and characterization of chitinolytic bacteria isolated from a freshwater lake. *Biosci. Biotechnol. Biochem.* **2018**, *82*, 343–355. [CrossRef] [PubMed]
28. Tamura, K.; Stecher, G.; Peterson, D.; Fillipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725. [CrossRef] [PubMed]
29. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947. [CrossRef]
30. Cayrol, J.C.; Djian, C.; Pijarowski, L. Study of the Nematocidal properties of the culture filtrate of the nematophagus fungus *Paecilomyces lilacinus*. *Revue Nematol.* **1989**, *12*, 331–336.
31. Nguyen, V.B.; Nguyen, Q.V.; Nguyen, A.D.; Wang, S.L. Screening and evaluation of α-glucosidase inhibitors from indigenous medicinal plants in Dak Lak Province, Vietnam. *Res. Chem. Intermed.* **2017**, *43*, 3599–3612. [CrossRef]
32. Tran, T.N.; Doan, C.T.; Nguyen, V.B.; Nguyen, A.D.; Wang, S.L. The isolation of chitinase from Streptomyces thermocarboxydus and its application in the preparation of chitin oligomers. *Res. Chem. Intermed.* **2019**, *45*, 727. [CrossRef]
33. Al-Rehiayani, S.; Hafez, S.L.; Thorton, M.; Sandararaj, P. Effects of *Pratylenchus neglectus*, *Bacillus megaterium*, and oil radish or rapeseed green manure on reproductive potential of *Meloidogyne chitwoodi* on potato. *Nematropica* **1999**, *29*, 37–49.
34. Terefe, M.; Tefera, T.; Sakhuja, P.K. Effect of a formulation of Bacillus firmus on root-knot nematode Meloidogyne incognita infestation and the growth of tomato plants in the greenhouse and nursery. *J. Inverteb. Pathol.* **2009**, *100*, 94–99. [CrossRef]
35. Walker, T.S.; Bais, H.P.; Grotewold, E.; Vivanco, J.M. Root exudation and rhizosphere biology. *Plant Physiol.* **2003**, *132*, 44–51. [CrossRef]
36. Akinrinlola, R.J.; Yuen, G.Y.; Drijber, R.A.; Adesemoye, A.O. Evaluation of *Bacillus* strains for plant growth promotion and predictability of efficacy by in vitro physiological traits. *Int. J. Microbiol.* **2018**, *2018*, 5686874. [CrossRef] [PubMed]
37. Trinh, T.H.T.; Wang, S.L.; Nguyen, V.B.; Tran, M.D.; Doan, C.T.; Vo, T.P.K.; Huynh, V.Q.; Nguyen, A.D. A potent antifungal rhizobacteria Bacillus velezensis RB.DS29 isolated from black pepper (Piper nigrum L.). *Res. Chem. Intermed.* **2019**, *45*, 5309–5323. [CrossRef]
38. Nguyen, A.D.; Wang, S.L.; Trinh, T.H.T.; Tran, T.N.; Nguyen, V.B.; Doan, C.T.; Huynh, V.Q.; Vo, T.P.K. Plant growth promotion and fungal antagonism of endophytic bacteria for the sustainable production of black pepper (Piper nigrum L.). *Res. Chem. Intermed.* **2019**, *45*, 5325–5339. [CrossRef]