농학석사학위논문

길항미생물을 이용한

*Meloidogyne hapla*의 생물학적 방제

Biological control of the root-knot nematode,

Meloidogyne hapla

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2013 년 02 월

서울대학교 대학원

농생명공학부 식물미생물학전공

박 지 영

A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Biological control of the root-knot nematode, *Meloidogyne hapla* with an antagonistic bacterium

BY
JIYEONG PARK

Department of Agricultural Biotechnology

The Graduate School of Seoul National University

February 2013

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Biological control of the root-knot nematode,

Meloidogyne hapla

with an antagonistic bacterium

UNDER THE DIRECTION OF
DR. YOUNG HO KIM

SUBMITTED TO THE FACULTY OF THE GRADUATE
SCHOOL OF SEOUL NATIONAL UNIVERSITY
BY JIYEONG PARK

MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

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APPROVED AS A QUALIFIED THESIS OF JIYEONG PARK
FOR THE DEGREE OF MASTER OF SCIENCE
BY THE COMMITTEE MEMBERS

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ABSTRACT

Biological control of the root-knot nematode, *Meloidogyne hapla* with an antagonistic bacterium

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Root-knot nematodes (*Meloidogyne* spp.) are distributed throughout the world and attack more than 2,000 plant species. In Korea, *Meloidogyne incognita*, *M. arenaria* and *M. hapla* are major root-knot nematodes. *M. incognita* and *M. arenaria* distribute in the warm regions especially damaging greenhouse crops; however, *M. hapla* favors rather cool temperature, widely distributing in crop fields such as ginseng and carrot. Up to this day, chemical control using nematicides has been often used for the control of the nematodes, which is not

enough to achieve a full control because of their soil-borne nature. Besides, chemical nematicides are very toxic to humans, animals and sometimes even to plants, and bring about soil and water pollution. Biological control is a good alternative for the chemical control, providing an efficient control value with no or little hazards to soil environments. Thus, the purpose of this study is to investigate biological control of the root-knot nematode (RKN), *M. hapla* that damages severely the root crops in fields using an antagonistic bacterium. Root-knot nematodes used in experiments were isolated from root galls of ginseng cultivated in Jinan, Korea and identified *Meloidogyne hapla* through 28S rDNA sequencing analysis. To find useful antagonistic bacteria against *M. hapla*, soil samples were collected from various regions and bacteria were isolated through the dilution plating method. Out of 523 bacterial isolates from soil samples and 19 *Paenibacillus* strains isolated from ginseng roots, a bacterial isolate C1-7 showed a very strong nematicidal activity *in vitro* against *M. hapla*. In pot experiment on tomato and carrot inoculated with the root-knot nematodes,

treatments of C1-7 bacterial culture (1×10^8 CFU/㎖) reduced root-galling with control values of 100 % and 90 % and egg mass formation with control values of 75 % and 100 %, respectively, compared to those on plants only with RKN inoculation, and the bacterial treatments alone showed no evident phytotoxicities to both plants. The bacterial isolate formed circular and flat colonies with undulate margin, and was rod shaped bacilli and gram-positive, which was identified *Bacillus cereus* by the morphological characters, and Biolog program, fatty-acid composition and 16S rDNA sequencing analyses. Population density of C1-7 were kept high for a long period of time and its nematicidal activity was strong when it was cultured in brain heart infusion broth, compared to other culture media such as nutrient broth, Luria Bertani broth and tryptic soy broth. Culture filtrate of C1-7 from 48-h culture with or without heat treatment showed strong nematicidal activity even at a concentration of 1 %. Light microscopy of RKN-infected root tissues treated with C1-7 showed the inhibition of intact giant cell formation, sometimes

accompanying dead nematodes located in the root tissues, while RKN alone induced fully matured giant cell formation with profound cell wall ingrowths. All of these results suggest the C1-7 bacterial isolate have a good potential to be developed as a biocontrol agent for the root-knot nematode, *M. hapla*.

KEYWORDS: *Bacillus cereus*, biological control, *Meloidogyne hapla*

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INTRODUCTION

In agriculture, the total damages caused by plant-parasitic nematodes are estimated to be US$100 billion per year around the world, among which the most important nematodes are root-knot nematodes, *Meloidogyne* spp. (Oka *et al.*, 2000). Root-knot nematodes are obligatory sedentary endoparasites and only infect roots of host plants at second juvenile stage. They are distributed through the world, attacking more than 2,000 plant species and thus reducing about 5 % of crop yield mainly due to the root-knot gall formation and nutritional deprivation (Sasser, 1977).

In Korea, *Meloidogyne incognita, M. arenaria, M. hapla* are major root-knot nematodes. *M. incognita* and *M. arenaria* distribute in the warm regions, damaging greenhouse crops, whereas *M. hapla* favor rather cool temperature and widely distribute in crop fields. *M. hapla* is a major cause of qualitative and quantitative production loss in field crops and root vegetables such as ginseng,

Codonopsis lanceolata and carrot in Korea (Park *et al.*, 1995; Park *et al.*, 1994; Kim *et al.*, 2001).

Root-knot nematodes are commonly controlled by chemical nematicides; however, these chemical nematicides, such as soil fumigant, soil disinfectant, and systemic nematicides, are not enough to achieve a full control because of their soil-borne nature. Besides, they are very toxic to human and animals and sometimes even to plants, and bring about soil and water pollution, for which new nematicide production is acutely needed (Osman and Viglierchio, 1981 & Oka *et al.*, 2000).

Biological control is a good alternative for the chemical control, providing an efficient control value with no or little hazards to soil environments (Noling and Becker, 1994). In soil, there are many natural enemies including bacteria, fungi, predatory nematodes, and mites antagonistic to nematodes. These natural enemies are usually found in nematode-suppressive soils, in which even when nematodes exist, their damages to plants are still below the economic threshold

levels. The microbes propagate and spread well for a long period of time in soil without permeation to host plants, and some microbes even enhance the plant growths. Therefore, application of antagonistic microbes from soil is expected to effectively control nematodes so that they can be useful as biological control agents for the root-knot nematodes (Oka *et al.*, 2000).

The purpose of this study was to investigate microbes from soil, which have a good potential to be developed as a biocontrol agent for the root-knot nematode, *M. hapla*. *In vitro*, selection of usful antagonistic bateria was conductied by examining their toxicity to second juveniles (J2) of *M. hapla* and, *in vivo*, suppression of root-galling and egg mass formation, and plant growths were assessed in comparison with those of untreated control. Also, biocontrol characteristics of the isolated bacterium were examined for providing information useful for its application for the control of the root-knot nematode.

MATERIALS AND METHODS

1. Preparation of nematode inoculum

Root-knot nematodes used in these experiments were isolated from root galls of ginseng cultivated in Jinan, Jeon-buk province, and identified as *Meloidogyne hapla* by the analysis of 28S rDNA sequences following the previous method (Oh *et al.*, 2009). Four-week-old tomato plants (*Lycopersicon esculentum* cv. Rutgers) grown in a growth chamber were inoculated with *Meloidogyne hapla* J2 and thereafter were cultivated at about 25±2 ℃ in a greenhouse. Seven weeks after inoculation, whenever inoculum is needed, tomato plants were uprooted and root systems were carefully washed with running tap water to remove adhering soil. Egg masses of *M. hapla* handpicked with the help of the forceps were placed on Baermann funnel for 3-5 days to obtain second stage juveniles hatched out (Southey, 1986). The nematode inoculum was used for *in vitro* and pot experiments, and inoculums density of J2 was adjusted to a

required density.

2. Effect of biological agents on the inhibition of *Meloidogyne hapla*

2-1. Collection of potential antagonistic microbes

Soil samples were collected from various areas and bacteria were isolated through the dilution plating method from the soil samples. A Total 523 bacteria isolated from soil and 19 *Paenibacillus* strains isolated from the four-year-old Korean ginseng roots with rot symptoms (Jeon *et al.*, 2003) were subjected to the screening for nematicidal activity test *in vitro*. The isolates were stored at -80 ℃ in sterilized distilled water with 15 % glycerol until used. They were screened for nematicidal activity against *M. hapla* after culturing in Brain Heart Infusion (BHI) (CONDA, Madrid, Spain) broth at 28 ℃ for 2 days in a shaking incubator (200 rpm).

2-2. *In vitro* screening of antagonistic bacteria for nematicidal activity against *M. hapla* J2

First screening

Five hundred and forty-two bacterial isolates were screened for nematicidal activity against *M. hapla* J2, which were grown in BHI broth in a shaking incubator (200 rpm) at 28 ℃ for 48 hours. The bacterial cultures were diluted with sterilized distilled water to concentrations of 2 % and 1 %, and then 100 ㎕ diluted bacterial culture were transferred to 96-well Microtest™ Tissue Culture Plate (Becton Dickinson Ladware, NJ, USA) into which 50-70 *M. hapla* J2 were placed. After 24 hours of exposure, dead *M. hapla* J2 were counted under a low power stereoscopic microscope. In this experiment, nematodes were considered dead if they were straight and stiffened under the microscope and did not move when probed with a fine needle (Cayrol *et al.*, 1989).

Second screening

Twenty-thee bacterial isolates, which showed high nematicidal activity at the first screening, were screened again for further selection of bacterial isolates with much higher nematicidal activity. The bacteria were incubated in BHI broth at 28 ℃ for 48 hours in a shaking incubator (200 rpm), which were centrifuged to collect bacterial cells by removing supernatant from bacterial cultures. The collected bacterial cells were suspended in sterilized distilled water (SDW) and diluted to proper concentrations. To know nematicidal activity of bacterial cells, 100 ㎕ of the bacterial suspensions at concentrations of 1×10^6 CFU/㎖ and 1×10^8 CFU/㎖ were transferred to 96-well Microtest™ Tissue Culture Plate into which 50-70 *M. hapla* J2 were placed. After 24 hours of exposure, dead *M. hapla* J2 were counted under a low power stereoscopic microscope.

3. Effect of antagonistic bacterium on the control of *M. hapla* in pot experiments

As the results of the previous experiments, isolate C1-7 had the strongest nematicidal activity against *M. hapla* J2. Isolate C1-7 was tested for biological control of *M. hapla* on host plants. The host plants used in this experiment were tomato (*Lycopersicon esculentum* cv. Rutgers) and carrot (*Daucus carota* subsp. *sativus* cv. Shinheukjeon-Ochon), respectively, susceptible to the root-knot nematode. Four-week-old seedlings were planted in plastic pots of 8 cm diameter which were filled with 130 g sterilized soil mixture (sand 1 : bed soil 1) and each plant was inoculated with J2 of *M. hapla*. The J2 suspension was dispensed with pipette at the rate of 5 ㎖ (200 J2/㎖) per pot and then, for biological control, 10 ㎖ bacterial culture of isolate C1-7 was poured around the rhizosphere. Plants with neither nematode inoculation nor bacterial treatment were served as control. Each treatment was replicated five times and the pot experiments were conducted under a greenhouse condition at 25±2 ℃.

Pots were watered sufficiently every other day. Four weeks after treatments, plants were carefully uprooted and root systems were gently washed with running tap water to remove adhering soil. Severity of root galling on plants infected with *M. hapla* was assessed on a 0-5 rating scale according to the percentage of galled tissues, in which 0=0-10% of galled roots; 1=11-20%, 2=21-50%, 3=51-80%, 4=81-90%, and 5=91-100% (Barker, 1985). Egg masses of *M. hapla* were handpicked with the help of the forceps from infected roots and egg mass index was assigned to each count using a rating scale of 0= no egg masses, 1=1-3 egg masses, 2=4-10 egg mass, 3=11-30 egg masses, 4=31-100 egg masses, 5= >100 egg masses per root system (Roberts *et al.*, 1990).

4. Characteristics of antagonistic bacterium

4-1. Identification of antagonistic bacterium

Isolate C1-7 was identified based on physiological and culture characteristics, 16S rDNA partial sequencing analysis using universal primers, 27mF (5'-GAGTTTGATCMTGGCTCAG-3') and 1492mR (5'-GGYTACCTTGTTACGACTT-3') (Osborne *et al.*, 2005). And to confirm the bacterial identity, Biolog and cellular fatty acid composition assays were also performed, which were analyzed by Korea culture center of microorganisms (KCCM). For examining the bacterial morphology, bacteria were mounted on copper grids, negatively stained with 2 % phosphotungstic acid (PTA), and then, examined under an energy-filtering transmission electron microscope (LIBRA 120, Carl Zeiss, Oberkochen, Germany) operated at an accelerating voltage of 120kV.

4-2. Growth of antagonistic bacterium on different culture media

To determine the optimal population density and culture media for the efficient application of isolate C1-7, growth curve of isolate C1-7 was observed at every 6 hours for 5 days after inoculation on four different media including Nutrient Broth (NB) (CONDA, S.A), Brain Heart Infusion (BHI) (CONDA, Madrid, Spain) broth, Luria-Bertani (DIFCO, USA) broth, and Bacto™ Tryptic Soy Broth (BD, France). The number of bacterial cells per micro-liter was counted at every 6 hours after inoculation by dilution plating method.

5. Biocontrol characteristics of the antagonistic bacterium

5-1. Nematicidal activity of antagonistic bacterium on different culture media

To know suitable media for nematicidal activity against *M. hapla*, four different non-selective media including NB, BHI, LB and TSB were tested. Each bacterium was cultured as described above and diluted with sterilized distilled water to various concentrations, and then 100 ㎕ diluted bacterial culture were transferred to 96 well Cell Culture Plate (F-Type) (SPL, Korea) into which 50-70 *M. hapla* J2 were placed. After 24 hours of exposure, dead *M. hapla* J2 were counted under a low power stereoscopic microscope.

5-2. Effect of bacterial culture filtrate on nematode mortality

To determine if isolate C1-7 culture filtrate also has nematicidal activity, the isolate was grown on BHI broth in a shaking incubator (200 rpm) at 28 ℃ for 18 hours and 48 hours. The culture time was based on growth curve; incubation time for 18 hours is just after the end of the exponential growth phase and that for 48 hours is in the stationary phase. Each bacterial culture supernatant was separated by centrifugation and filtered through a sterilized 0.22 ㎕ Millex Filter Unit syringe (Millipore, Bedford, USA). Obtained culture filtrate was applied to the nematodes at three heat treatment conditions including no heat treatment, heat treatment at 80 ℃ for 30 minutes and heat treatment at 121 ℃ for 20 minutes. Then, each sterilized culture filtrate was diluted with SDW to various concentrations and 100 ㎕ diluted culture filtrate was transferred to 96 well Cell Culture Plate (F-Type) into which 50-70 *M. hapla* J2 were placed. After 24 hours and 48 hours of exposure, J2 mortality was measured as above.

5-3. Effect of antagonistic bacterium on giant cell formation by *M. hapla*

Root specimens inoculated with *M. hapla* alone, inoculated with *M. hapla* and treated BHI broth, and inoculated with *M. hapla* and treated with bacterial culture were taken from plants. Each root specimen was put into micro-tubes and fixed with Karnovsky's fixative consisting of 2 % glutaraldehyde and 2 % paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2) for 48 hours at 4 ℃ (Karnovsky, 1965). Each root specimen was washed in 0.05 M cacodylate buffer (pH 7.2) at 4 ℃ three times each for 10 minutes. Then, the specimens were post-fixed in 1 % osmium tetroxide in 0.05 M cacodylate buffer for 1.5 hours at 4 ℃ in a refrigerator and briefly rinsed with distilled water. After *en bloc* staining in 0.5 % uranyl acetate for 30 minutes at 4 ℃, the specimens were dehydrated in an ethanol series of 30, 50, 70, 80, 90 % each for 10 minutes and finally three times in an ethanol of 100 % for 10 minutes each. For complete dehydration, the specimens were treated with propylene oxide at room temperature two times each for 15 minutes. The specimens were infiltrated with

mixture of 2 to 1 ratio between propylene oxide and Spurr's epoxy resin for 4 hours, and then they were put in the 1:1 mixture overnight. Then they were infiltrated with 100 % Spurr's epoxy resin and kept in the desiccator with a vacuum for 4 hours. The specimens were carefully removed from micro-tubes and placed in a flat mould, which was placed at 70 ℃ for 8 hours in a dry-oven. The polymerized specimens were cut 1000 nm in thickness with a glass knife on an ultra-microtome (MT-X, RMC, Tucson, AZ, USA). The sections were stained with decuple diluted toluidine blue working solution consisting of 1 % toluidine blue in 2 % sodium borax and observed under a compound light microscope (Axiophot, Carl Zeiss, Germany).

RESULTS

1. *In vitro* screening of antagonistic bacteria for nematicidal activity against *M. hapla* J2

First screening

Under *in vitro* conditions, a total of 542 bacterial isolates cultivated in BHI broth for 48 hours and the bacterial cultures diluted with distilled water at concentrations of 2 % and 1 % were screened for nematicidal activity against *M. hapla* J2. Twenty-three isolates were showed high nematicidal activity (J2 mortality) even at bacterial culture of 1 %, compared to sterile distilled water and BHI broth diluted to the same concentrations (Fig. 1A).

Second screening

In second screening, three of the 23 bacterial isolates selected at the first screening showed strong nematicidal activity against *M. hapla* J2, when they were treated with the bacterial suspensions (1×10^6 CFU/㎖ and 1×10^8 CFU/㎖). Especially, isolate C1-7 showed the strongest nematicidal activity (Fig.1B, Table 1A, 1B). Therefore, this C1-7 bacterial isolate was finally selected for further studies on the biological control of *M. hapla*.

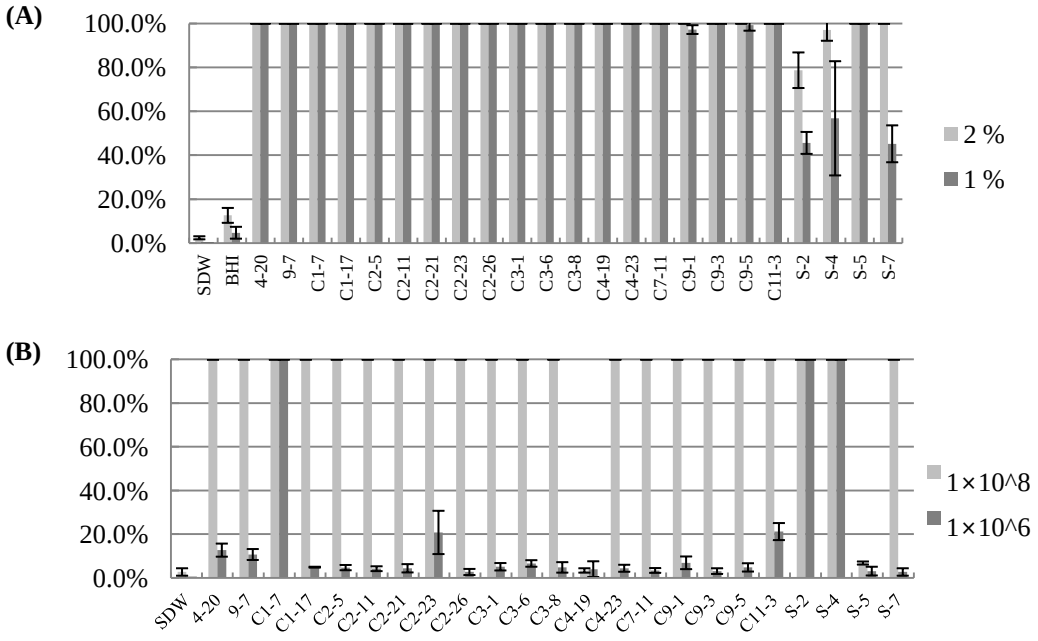

Fig. 1. Effect of bacterial isolates on mortality of *M. hapla* J2 (A) J2 mortality in different concentrations of bacterial cultures (B) J2 mortality in high (1×10^8 CFU/㎖) and low (1×10^6 CFU/㎖) densities of bacterial suspensions

Table 1. Effect of bacterial isolates on the mortality of *M. hapla* J2 at different concentrations of bacterial cultures (A) and different cell densities of bacterial suspensions (B)

(A)

	2 %	1 %
C1-7	**100.0±0.0 %**	**100.0±0.0 %**
S-2	78.7±8.1 %	45.6±5.0 %
S-4	97.1±5.0 %	56.8±26.0 %

(3 replications)

(B)

	1×10^{8} CFU/mℓ	1×10^{6} CFU/mℓ
C1-7	**100.0±0.0 %**	**100.0±0.0 %**
S-2	100.0±0.0 %	100.0±0.0 %
S-4	100.0±0.0 %	100.0±0.0 %

(3 replications)

2. Effect of antagonistic bacterium on the control of *M. hapla* in pot experiments

In the pot experiments under the greenhouse conditions, root-knot galls and egg masses formed on tomato and carrot by *M. hapla* were significantly suppressed by the treatment of C1-7 bacterial culture (1×10^8 CFU/㎖), compared with non-treatment control only inoculated with root-knot nematodes (Fig. 2, 3). The size of developed root-knot galls induced by the nematode was smaller in C1-7 treatment than in nematode inoculation alone, and the number of root galls and egg masses caused by the nematode were also significantly lowered in C1-7 treatment, compared to the non-treatment control (Data not shown).

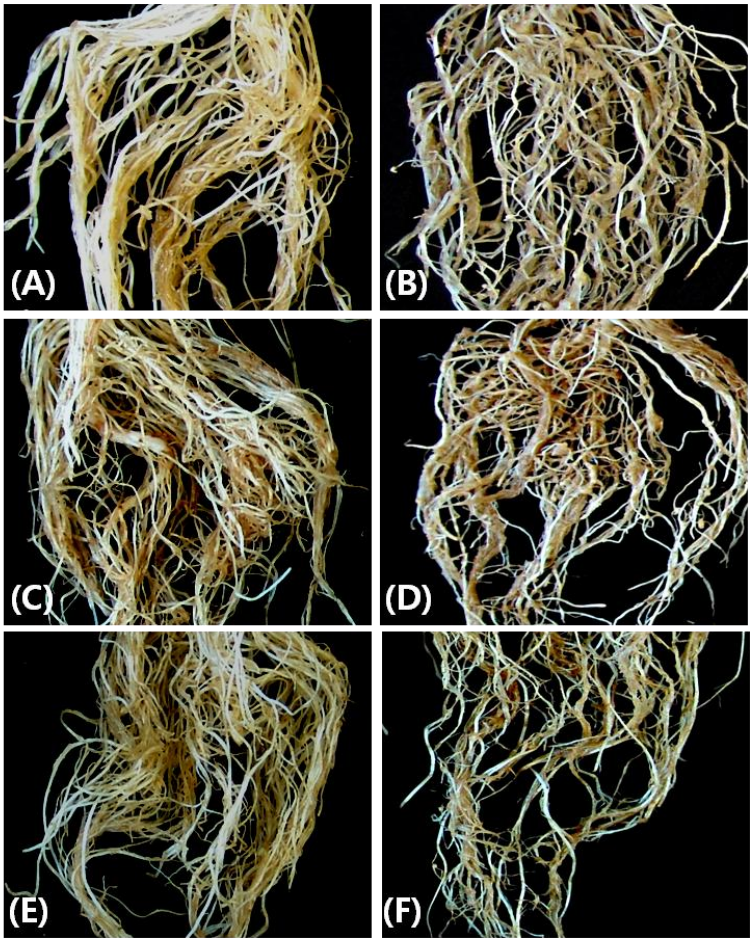

Fig. 2. Effect of C1-7 on root galling caused by *M. hapla* on tomato; (A) Control; no inoculation and no treatment, (B) RKN; only inoculated with *M. hapla* J2, (C) BHI; treated with BHI broth alone with no nematode inoculation, (D) BHI+RKN; inoculated with RKN and treated with BHI broth, (E) C1-7; treated with the antagonistic bacterium alone, (F) C1-7+RKN; inoculated with RKN and treated with antagonistic bacterium C1-7

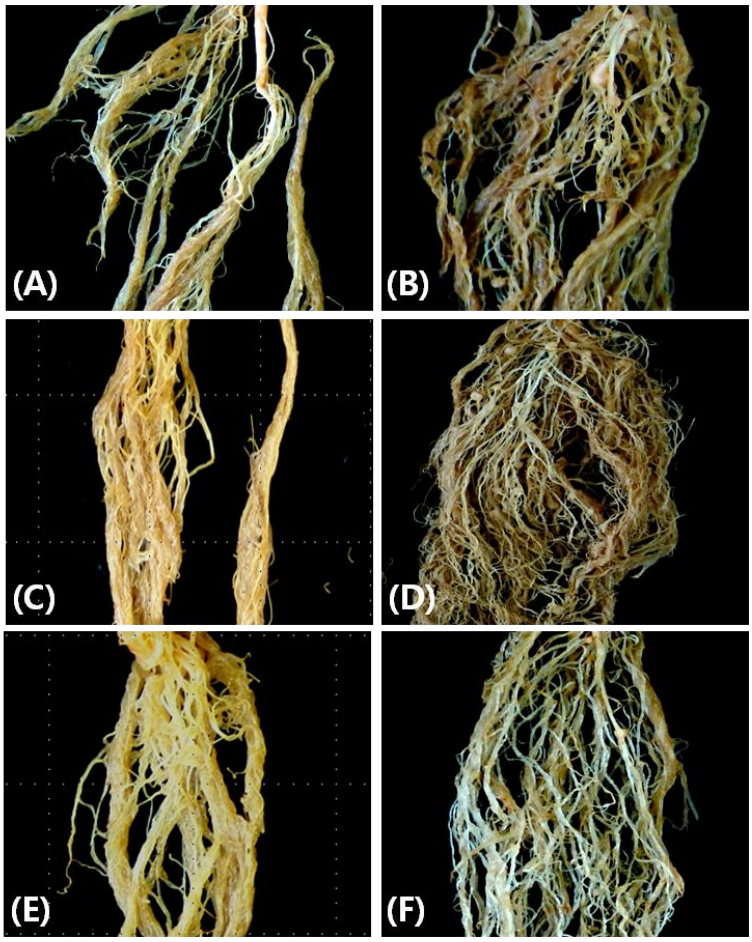

Fig. 3. Effect of C1-7 on root galling caused by *M. hapla* on carrot; (A) Control; no inoculation and no treatment, (B) RKN; only inoculated with *M. hapla* J2, (C) BHI; treated with BHI broth alone with no nematode inoculation, (D) BHI+RKN; inoculated with RKN and treated with BHI broth, (E) C1-7; treated with the antagonistic bacterium alone, (F) C1-7+RKN; inoculated with RKN and treated with antagonistic bacterium C1-7

On tomato, control effects of C1-7 were estimated to be 100 % and 75 %, respectively (Table 2). And its final populations were also greatly reduced by the treatment of C1-7 bacterial culture, compared with those only inoculated with the root-knot nematode. When inoculated with root-knot nematodes, root-galling and egg mass indices were 2.0 and 4.0, and final nematode populations were increased by 6.05 folds relative to the initial J2 population inoculated. Whereas, root-galling and egg mass indices on plants inoculated with root-knot nematodes and treated C1-7 bacterial culture were 0.0 and 1.0, and final nematode populations were reduced to 0.25 of the initial J2 populations (Table 2). And, plant growth was seemed to be enhanced by the treatment of C1-7 bacterial culture, compared to the no treatment control or root-knot nematode inoculation alone.

Table 2. Effect of C1-7 on root galling and egg mass formation caused by *M. hapla* on tomato

	Gall index[a]		Egg mass index[b]		Shoot height (cm)	Shoot weight (g)	Root weight (g)	Pf[c] of *M. hapla*	RK population increasing rate
	Index	CE[d] (%)	Index	CE (%)					
Control	-	-	-	-	22.0±4.0bc	5.35±1.46c	1.74±0.39ab	-	-
BHI	-	-	-	-	26.2±3.2a	10.94±1.93a	2.16±0.37a	-	-
C1-7	-	-	-	-	24.0±2.0abc	10.70±2.01a	1.99±0.40a	-	-
RKN	2.0±0.0a[*]	0	4.0±0.0a	0	17.2±0.6d	3.84±0.61c	1.38±0.23b	6051	6.05
BHI+RKN	2.0±0.0a	0	3.2±0.4b	20	20.7±2.1c	8.37±1.58b	1.77±0.35ab	1991	1.99
C1-7+RKN	0.0±0.0b	100	1.0±0.7c	75	24.2±2.4ab	10.91±0.71a	1.94±0.18a	248	0.25

(5 replications)

[*] Means followed by the same letters are not significantly different at *P=0.05* using the least significance difference test (LSD).

[a] Severity of root galling on *M. hapla* was assessed on a 0-5 rating scale according to the percentage of galled tissue, in which 0=0-10% of galled roots; 1=11-20%; 2=21-50%; 3=51-80%; 4=81-90%; and 5=91-100% (Barker,1985).

[b] Egg mass index was assigned to each count using a rating of 0=no egg masses, 1=1-3 egg masses, 2=4-10 egg masses, 3=11-30 egg masses, 4=31-100 egg masses, and 5=>100 egg masses per root system (Roberts,1990).

[c] Pf = final population of root-knot nematode.

[d] CE = control effect.

On carrot, when inoculated with root-knot nematodes alone, root-galling and egg mass indices were 2.0 and 1.0, respectively; however, in those inoculated with root-knot nematodes and treated with C1-7 bacterial culture, root-galling and egg mass indices were 0.2 and 0.0, respectively, showing that its control effects were estimated to be 90 % and 100 %, respectively (Table 3).

Table 3. Effect of C1-7 on root galling and egg mass formation caused by *M. hapla* on carrot

	Gall index[a]		Egg mass index[b]		Root
	Index	CE[c] (%)	Index	CE (%)	weight (g)
Control	-	-	-	-	0.74±0.32a
BHI	-	-	-	-	0.46±0.08a
C1-7	-	-	-	-	0.74±0.44a
RKN	2.0±0.0a[*]	0	1.0±0.0a	0	0.84±0.35a
BHI+RKN	1.6±1.1a	20	0.6±0.5a	40	0.85±0.47a
C1-7+RKN	0.2±0.4b	90	0.0±0.0b	100	0.76±0.15a

(5 replications)

[*] Means followed by the same letters are not significantly different at *P=0.05* using the least significance difference test (LSD).

[a] Severity of root galling on *M. hapla* was assessed on a 0-5 rating scale according to the percentage of galled tissue, in which 0=0-10% of galled roots; 1=11-20%; 2=21-50%; 3=51-80%; 4=81-90%; and 5=91-100% (Barker,1985).

[b] Egg mass index was assigned to each count using a rating of 0=no egg masses, 1=1-3 egg masses, 2=4-10 egg masses, 3=11-30 egg masses, 4=31-100 egg masses, and 5=>100 egg masses per root system (Roberts,1990).

[c] CE = control effect.

3. Characteristics of antagonistic bacterium

3-1. Identification of antagonistic bacterium

C1-7 formed large circular, cream-colored, flat colonies with undulate margin on BHI agar for 3 days (Fig. 4). It was gram positive, rod-shaped, and had more than 4 peritrichous flagella (Fig. 5). Analysis of 16S rRNA gene sequences for C1-7 showed the maximum similarity of 100 % to those of *Bacillus* species including *B. cereus* strains with accession no. of KC113613.1, JX848326.1, JX994144.1, and JX994107.1 and *B. thuringiensis* strains with accession no. of JX841105.1, JX994146.1, JX994125.1 and JX994097.1 in NCBI, respectively. In carbon source assimilation of bacterial isolate examined in Biolog assay showed that it utilized 15 carbon sources including glycerol but not 34 carbon sources including sorbitol, revealing the highest similarity of 92.6 % to *B. cereus*. Also, cellular fatty acid composition assay of the isolate C1-7 showed higher similarity to *B. cereus* (similarity index of 0.277) than *B. thuringiensis* (similarity index of 0.240), confirming its identity to be *B. cereus* (Table 4).

Fig. 4. Colony morphology of *Bacillus cereus* C1-7 on BHI agar

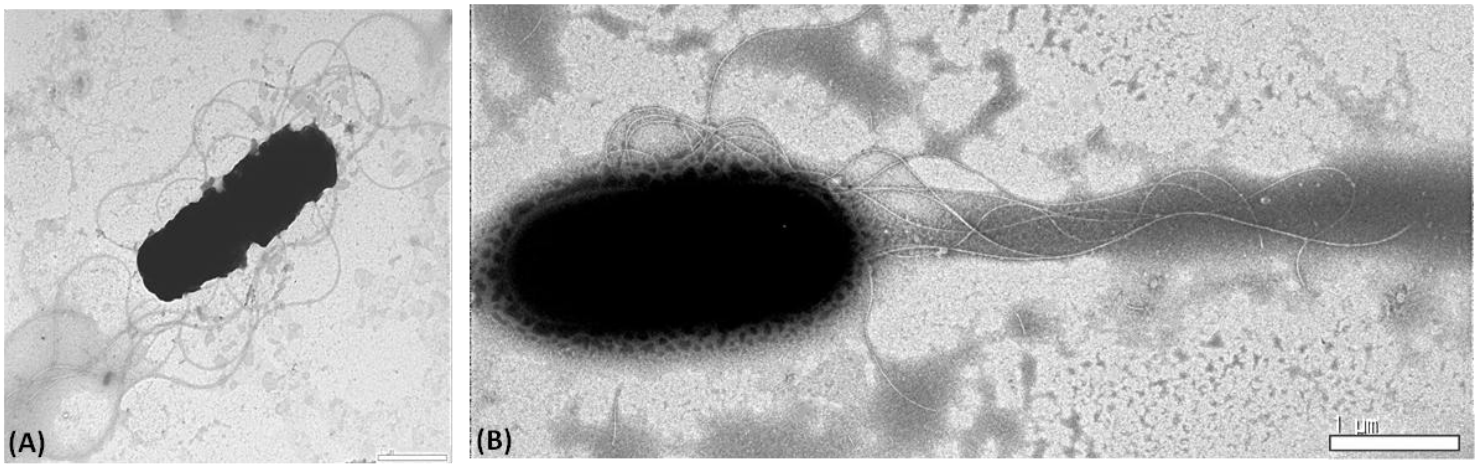

Fig. 5. Energy-filtering transmission electron microscopy (FE-TEM) of *B. cereus* C1-7

Table 4. Identification of C1-7 bacterial isolate based on colony morphology, 16S rRNA gene sequencing, Biolog and cellular fatty acid composition (MIDI) analyses

Strain	Colony morphology	Gram stain	16S rRNA gene sequencing	Biolog program	(%)	MIDI	SI*	Final identification
C1-7	Circular form, flat with undulate margins	+	*Bacillus cereus/ B. thuringiensis*	*Bacillus cereus*	92.5	*Bacillus cereus*	0.277	***Bacillus cereus***

* SI; Similarity index.

3-2. Growth of antagonistic bacterium on different culture media

Bacillus cereus C1-7 showed a similar growth pattern on NA, BHI, LB and TSB agar until 18 hours after inoculation (until the exponential growth phase), when its density reached between 1×10^8 CFU/㎖ and 1×10^9 CFU/㎖. On BHI, LB and TSB agar, in the stationary phase, cell density was maintained at about 1×10^7 CFU/㎖ and 1×10^8 CFU/㎖, except on NA. Among them, on BHI agar medium, the bacterial population was kept over 1×10^8 CFU/㎖ until 60 hours after inoculation (Fig. 6).

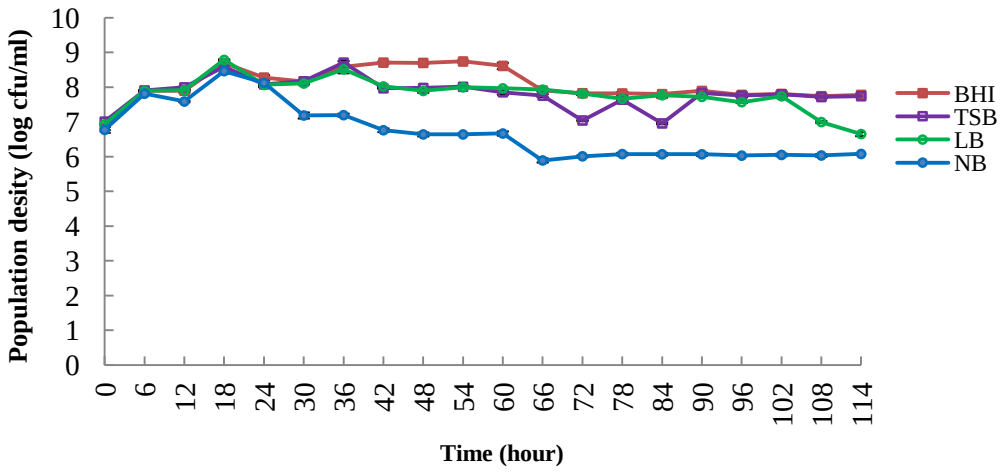

Fig. 6. Population changes of *B. cereus* C1-7 on different culture media

4. Biocontrol characteristics of the antagonistic bacterium

4-1. Nematicidal activity of antagonistic bacterium cultured on different media

Bacillus cereus C1-7 cultured on NA showed no nematicidal activity at any concentrations of 0.5 ~ 5.0 %, and on LB showed nematicidal activity only at the highest concentrations of 5.0 % (Table 5). However, high nematicidal activities were showed at 1.0 % and 2.0 % until 114 hours after treatment by the bacterial isolate cultured on BHI and TSB, respectively (Table 5.)

Table 5. Nematicidal activity of *B. cereus* C1-7 cultured on different media

		0	6	12	18	24	30	36	42	48	54	60	66	72	78	84	90	96	102	108	114
NB	5 %	++*	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	2 %	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	1 %	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	0.5 %	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
BHI	5 %	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	2 %	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	1 %	--	++	++	++	++	++	++	++	++	++	++	++	++	+	+	++	++	+	++	++
	0.5 %	--	--	--	--	--	--	--	-	-	--	--	--	-	--	--	--	--	--	--	--
LB	5 %	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	2 %	-	-	--	--	++	+	--	--	--	-	--	+	-	--	--	--	--	--	--	--
	1 %	-	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	0.5 %	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
TSB	5 %	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	2 %	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	1 %	-	++	++	++	++	++	+	+	++	++	--	--	-	-	--	--	--	-	--	--
	0.5 %	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

* Nematicidal activity was assessed according to the percentage of J2 mortality, in which -- = 0~24 % of mortality, - = 25~49 %, + = 50~74 %, ++ = 75~100 %

4-2. Nematicidal activity of bacterial culture filtrate with different culture time, heating temperature and treatment time

The bacterial culture filtrate showed much higher nematicidal activity against *M. hapla* J2 than BHI and control (SDW) regardless of its culture time, degree of heating temperature and treatment time for its activity to the nematode (Table 6). The culture filtrate of the bacterial isolate with culture time of 18 hours showed no significant difference in nematicidal activity against *M. hapla* J2 between treatment times of 24 hours and 48 hours regardless of heating temperatures, showing nearly total nematode mortality at the concentrations of not more than 2 %. However, nematicidal activity of the bacterial culture filtrate with the culture time of 48 hours was higher in the treatment time of 48 hours than 24 hours, showing that total nematode mortality was observed at its concentration of 1 % in the treatment time of 48 hours, but at its concentration of 2 % in the treatment time of 24 hours, although there was no significant difference in its nematicidal activity among the heating temperatures (Table 6).

Table 6. Nematicidal activity of culture filtrate C1-7 at various concentrations depending on culture time, treatment time and heating temperature against *M. hapla* J2

	Treatment			Concentrations					
	Culture time (hr)	Treatment time	Heating temperature*	50 %	10 %	5 %	2 %	1 %	0.5 %
C1-7	18	24 hr	-	94.7±3.5	100±0.0	100±0.0	100±0.0	4.0±3.0	1.5±1.5
			80 ℃	94.3±3.4	100±0.0	100±0.0	100±0.0	5.0±1.4	1.4±1.3
			121 ℃	94.9±1.4	100±0.0	100±0.0	100±0.0	3.0±2.8	0.6±1.0
		48 hr	-	100±0.0	100±0.0	100±0.0	100±0.0	15.9±10.0	18.6±4.4
			80 ℃	100±0.0	100±0.0	100±0.0	100±0.0	17.9±3.8	15.2±3.7
			121 ℃	100±0.0	100±0.0	100±0.0	100±0.0	24.3±5.1	16.4±4.9
	48	24 hr	-	100±0.0	100±0.0	100±0.0	100±0.0	6.1±2.5	2.9±1.0
			80 ℃	100±0.0	100±0.0	100±0.0	100±0.0	5.0±3.9	2.9±2.2
			121 ℃	100±0.0	100±0.0	100±0.0	100±0.0	4.3±1.0	1.1±1.0
		48 hr	-	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	17.1±2.2
			80 ℃	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	18.2±1.9
			121 ℃	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	15.5±3.6
BHI		24 hr		100±0.0	100±0.0	100±0.0	80.6±17.3	5.5±2.1	1.0±1.8
		48 hr		100±0.0	100±0.0	100±0.0	100±0.0	24.2±6.0	18.6±3.6
SDW		24 hr				3.4±1.5			
		48 hr				20.1±4.0			

(3 replications)

* Culture filtrate C1-7 was treated three conditions that no treated, treated at 80 ℃ for 30minutes and at 121 ℃ for 20minutes

4-3. Effect of antagonistic bacterial treatment on the formation of giant cells induced by *M. hapla*

On tomato, numerous large giant cells were formed in the tissues of only RKN-infected roots and RKN-infected roots treated BHI broth only (Fig. 7A, B, C) forming numerous nuclei with an enlarged nucleolus in the middle of giant cells and profound cell wall ingrowths adjacent to xylem vessels (Fig. 7E). However, no giant cell was formed in the root tissues infected with RKN and treated with C1-7, showing only a dead looking nematode body with a large hollow (Fig. 7D, F). On carrot also, intact and functional giant cells were formed in the root tissues infected with RKN alone, characterized by feeding plugs in the nematode feeding sites and profound cell wall ingrowths adjacent to xylem vessels (Fig. 8A, B, C, E), whereas, RKN-infected root tissues treated with C1-7 showed the formation of a few degenerated and nonfunctional giant cells characterized by degenerated nuclei (indicated by chromatin materials dispersed in cytoplasm) and no or little cell wall ingrowths (Fig. 8D, F).

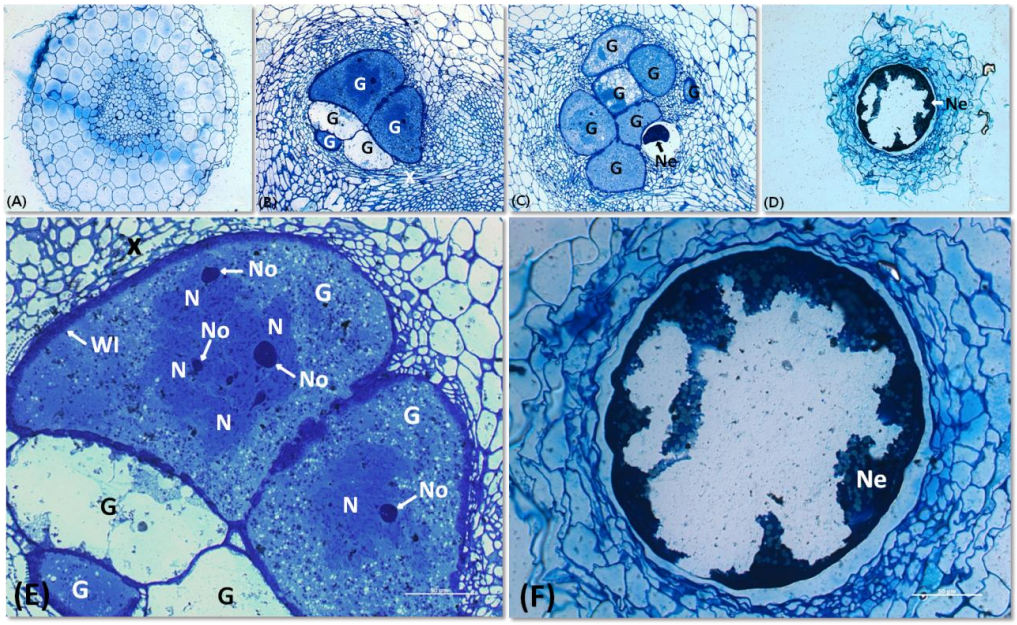

Fig. 7. Light microscopy of giant cells in tomato root induced by *M. hapla*; (A) control, (B) only inoculated with root-knot nematodes, (C) inoculated with root-knot nematodes and treated with BHI broth, (D) inoculated with root-knot nematodes and treated with C1-7 (× 200) (E) magnified view of (B), (F) magnified view of (D) (× 400) X: xylem vessel, G: giant cell, Ne: nematode, WI: cell wall ingrowths, N: nucleus, No: nucleolus.

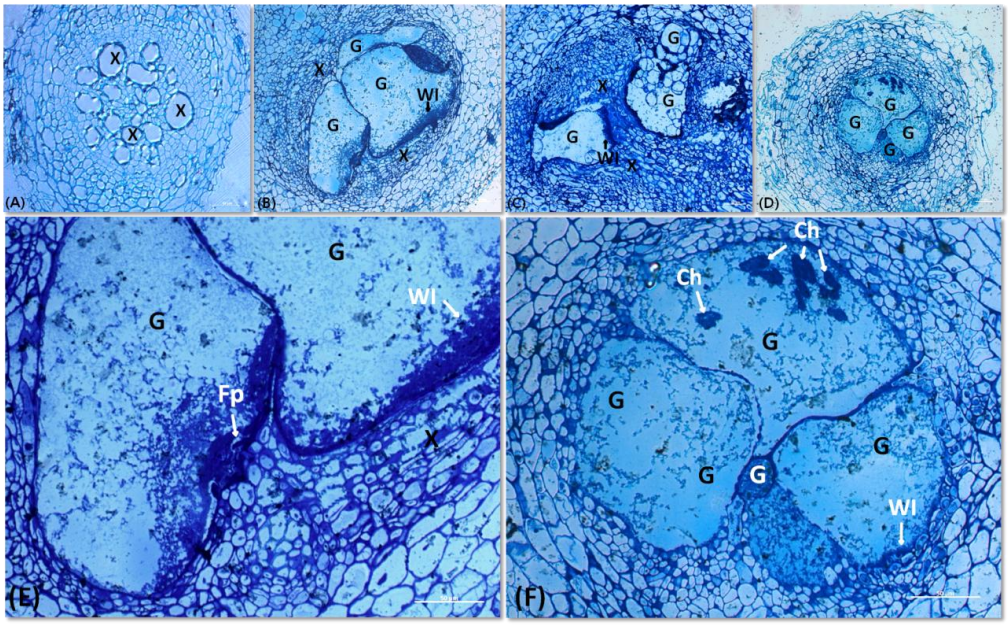

Fig. 8. Light microscopy of giant cells in carrot root induced by *M. hapla*; (A) control, (B) only inoculated with root-knot nematodes, (C) inoculated with root-knot nematodes treated BHI broth, (D) inoculated with root-knot nematodes and treated C1-7 (× 200) (E) magnified view of (B), (F) magnified view of (D) (× 400) X: xylem vessel, G: giant cell, WI: cell wall ingrowths, Fp: feeding plug, Ch: chromatin materials.

DISCUSSION

The root-knot nematode, *Meloidogyne hapla* is one of the major root-knot nematodes in Korea, which is major cause of damages to qualitative and quantitative production of field crops and root vegetables such as ginseng and carrot (Kim, 2001). However, it has been studied little on its biological control, compared to other root-knot nematodes, because propagation of *M. hapla* is fastidious under the greenhouse conditions.

Numerous microbes such as *Bacillus* spp., *Paecilomyces lilacinus*, *Verticillium chlamydosporium* have been tested for the control of *M. hapla*, as they suppressed root galls, egg masses formation, egg hatching and survival of second-stage juveniles (J2) (Bonants *et al.*, 1995; Chen *et al.*, 2000; De Leij *et al.*, 1993; Liu *et al.*, 2007; Mennan *et al.*, 2006; Viaene and Abawi, 2000; Townshend *et al.*, 1989).

In this study, out of 523 bacterial isolates from soil and 19 *Paenibacillus*

strains isolated from ginseng roots were tested for inhibitory activity against *M. hapla*, and 23 isolates showed nematicidal activity *in vitro*. Especially, among them, C1-7 isolate showed higher toxicity to *M. hapla* J2 than any other bacterial isolates tested. In pot experiments, addition of C1-7 into soil suppressed root galling and egg mass development on tomato and carrot, and also it seemed to promote plant growth on tomato. Root galls caused by *M. hapla* were smaller and fewer in C1-7 treatment, compared with those in no treatment control (inoculated with root-knot nematode alone). Treatments of C1-7 bacterial culture (1×10^8 CFU/㎖) reduced root-galling with control values of 100 % and 90 % and egg mass formation with control values of 75 % and 100 % on tomato and carrot, respectively, compared to those on the plants only with RKN inoculation. The bacterial treatment alone showed no evident phytotoxicity to both plants. These kind of antagonistic bacteria produce metabolites, enzymes and toxins that are not only directly lethal to the nematodes but also suppress juvenile survival, reproduction, and egg hatching

(Siddiqui and Mahmood, 1999; Siddiqui and Shaukat, 2003). C1-7 was identified as *Bacillus cereus* through the analyses of 16S ribosomal DNA sequencing, Biolog program and cellular fatty acid composition. *B. cereus* is gram positive, rod-shaped and genetically close to *B. thuringiensis* which is commercially used as biological insecticide. Generally strains of *B. cereus* are known to be saprophytic and even beneficial as probiotics, having a good potential as biological control agent against plant-pathogenic fungi and nematode (Ryan and Ray 2004; Silva *et al.*, 2004; Chang *et al.*, 2007; Handelsman *et al.*, 1990; Huang *et al.*, 2005; Oka *et al.*, 1993). Some *B. cereus* strains are harmful to humans and animals; however, they are not contagious but self-limiting. The plant health-promoting rhizobacterium *B. cereus* S18 was tested for the control of *Meloidogyne incognita, M. javanica*,and, and *M. arenaria* on tomato and it reduced root galls and egg masses induced by root-knot nematodes together with significant increases of plant growths including shoot height and weight, compared with the untreated control (Mahdy *et al.*,

2000). The population density of *B. cereus* C1-7 was kept high for a long period of time and its nematicidal activity was high when it was cultured in BHI broth, compared to other culture media such as NA, LB and TSB, indicating that BHI broth is a suitable medium for culturing *B. cereus* C1-7 for applying this bacterium for efficient biological control against *M. hapla*. The culture filtrate of C1-7 from the 48-hour culture showed strong nematicidal activity even at a concentration of 1 % regardless of heat treatment. Based on these results, it was suggested that *B. cereus* C1-7 may produce heat-stable secondary metabolites toxic to *M. hapla*. Most *Bacillus* spp. secrete toxoproteins which into the nematode body and act on their guts for the nematodes to be killed or immobilized (Carneiro *et al.*, 1998; Zhang *et al.*, 2012). These proteins are usually deprived of their toxicity due to denaturation of the protein by heat shock (Leyns *et al.*, 1995). On the other hand, some *Bacillus* strains produce non-protenaceous thermostable micromolecular compounds extremely toxic to the root-knot nematodes, which are synthesized at the stationary stage of the

bacterial growth (Yu *et al.*, 2012). These heat-stable characteristics of the bacterial isolate in this study may provide some advantages for its formulation and practical application for the biocontrol of the root-knot nematode. Light microscopy of only RKN-infected root tissues showed the formation of numerous large giant cells with profound cell wall ingrowths that increase the transfer of water and nutrients between cells by the expansion of surface area. On the contrary, RKN-infected root tissues treated with C1-7 showed the formation of relatively fewer and smaller giant cells and no or few cell wall ingrowths. Also chromatin materials derived probably form the rupture of nuclei were observed in the giant cells formed in RKN root tissues treated with C1-7, suggesting the giant cells degenerate early so as to inhibit the nematode feeding (Kim *et al*., 2010) as indicated by the hollowed nematode body in the root tissue treated with C1-7. Therefore, it is suggested that *B. cereus* C1-7 have a systemic effect against the nematode by inhibiting the formation of intact giant cells and/or accelerating their degeneration to hinder the nematode

feeding and to preclude the nematode from living in roots to the end of its life cycle. In this study, the modes for the nematicidal activity of *B. cereus* C1-7 is not examined in detail; however, it can be hypothesized that the reduction of root galling and egg mass formation by *M. hapla* may be attributed to direct effects of secondary metabolites that ill the nematode juveniles or indirect effects that enhance host plant defense mechanisms to prevent the nematodes from infection like the plant growth-promoting rhizobacteria *Paenibacillus* strains (Son *et al.*, 2009). In conclusion, *Bacillus cereus* C1-7 is a good potential biological control agent for solving problems caused by *Meloidogyne hapla* in field crop cultivation.

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길항미생물을 이용한

*Meloidogyne hapla*의 생물학적 방제

박 지 영

국 문 초 록

뿌리혹선충은 전세계적으로 분포하며 2,000여종이 넘는 식물을 가해한다. 우리나라에 피해를 많이 주고 있는 주요 뿌리혹선충으로는 *Meloidogyne incognita, M. arenaria, M, hapla*로, 이 중 *M. hapla*는 비교적 서늘한 지역에 분포하고 인삼이나 당근, 더덕 등 주로 노지작물에 많은 피해를 주고 있으며 국내에 존재하는 다른 뿌리혹선충에 비해 대량증식이 어려워 연구가 많이 이루어져 있지 않다. 오늘날까지 선충을 방제하기 위해 주로 화학살선충제들이 사용되고 있으나 이러한 화학살선충제들은 토양병원성인 뿌리혹선충에 있어서 완전한 방제가 어

려우며, 사람과 가축에게 유해하고 식물에 약해를 유발하기도 하며 환경오염 등의 문제로 이를 대체할 방법으로 생물학적 방제가 대두되고 있다. 특히, 인삼이나 당근과 같은 연작작물이나 근채 작물에 있어서 비교적 효율적이며 친환경적인 작물의 생산이 가능한 생물학적 방제법이 필요하다. 이에 따라 본 연구는 노지작물에 많은 피해를 주고 있는 *Meloidogyne hapla*의 효율적인 생물학적 방제제를 찾기 위해 다양한 지역의 토양으로부터 분리한 세균과 저장 중 인삼의 뿌리썩음병으로부터 분리한 plant growth promoting rhizobacteria (PGPR) 그룹에 속하는 세균들을 테스트하였고, 이 중 살선충력이 뛰어난 균주를 선발하여 방제 효과를 알아보고 이의 활용을 위한 실험들을 수행하였다. 실험에 사용된 *M. hapla*는 전북 진안에서 뿌리혹 피해를 받은 인삼으로부터 분리하여 28S rDNA sequencing을 통해 동정되었다. 총 542개의 균주 중 *in vitro*상에서 C1-7균주가 *M. hapla*의 2령충에 대해 가장 강한 살선충성을 보였고, 이를 가지고 당근과 토마토에 C1-7 배양액을 처리하여 방제 실험을 수행한 결과, 배양액을 처리한

것이 처리하지 않은 것보다 형성된 뿌리혹의 크기가 작고 그 수도 적었으며, 각각의 작물에서 뿌리혹 형성 정도는 100%와 90%의 방제 효과를 보였으며 알집 형성 정도는 75%와 100%의 방제 효과를 보였다. 또한, 선충의 증식률도 감소되었을 뿐 만 아니라 식물의 생육도 증가한 것을 확인하였고, 식물에서 약해도 나타나지 않았다. 이 C1-7균주는 간균 모양의 그람양성균으로 16S rDNA sequencing analysis, Biolog assay와 cellular fatty acid composition assay를 통하여 *Bacillus cereus*로 동정되었다. 균주의 효율적인 활용을 위해 다양한 기본 세균 배지에서 생장을 측정한 결과 다른 배지들 보다 Brain Heart Infusion broth에서 높은 수준의 개체 밀도를 가장 오래 유지하였으며 제일 강한 살선충성을 보였다. 또한, C1-7균주를 48시간 동안 키운 배양여액만을 처리하였을 때도 멸균처리 유무에 상관없이 동일하게 높은 살선충성이 관찰되었다. 뿌리혹선충에 감염된 식물체 뿌리 조직을 광학현미경으로 관찰 하였을 때, 뿌리혹선충만 처리한 것에서는 거대세포 (giant cell)가 크고 많이 형성된 것이 관찰

되었으나, C1-7균주를 함께 처리한 것에서는 거대세포가 비교적 작고 비정상적으로 형성되었으며 심지어 다 자라지 못하고 뿌리체내에 죽어있는 선충도 관찰되었다. 이러한 결과들을 바탕으로 *Bacillus cereus* C1-7은 *M. hapla*의 생물학적 방제제로서의 가능성이 있다고 사료된다.

중요어: *Bacillus cereus*, *Meloidogyne hapla*, 생물학적 방제

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