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Bacterial Mixture from Greenhouse Soil as a Biocontrol Agent Against Root-Knot Nematode, *Meloidogyne incognita*, on Oriental Melon

Seo, Byoung Joo[1], V. J. Rejish Kumar[1], Rather Irfan Ahmad[1], Byung-Chun Kim[1], Wan Park[2], So-Deuk Park[3], Se-Eun Kim[1], Sang-Dal Kim[1], Jeongheui Lim[1], and Yong-Ha Park[1]*

[1]*Department of Applied Microbiology and Biotechnology, Yeungnam University, Kyongsan, Kyeongbuk 712-749, Korea*
[2]*School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Korea*
[3]*Agriculture Research and Extension Service Bureau, Gyeongsangbuk-do, Daegu 702-708, Korea*

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The biological control efficacy of a greenhouse soil bacterial mixture of *Lactobacillus farraginis*, *Bacillus cereus*, and *Bacillus thuringiensis* strains with antinematode activity was evaluated against the root-knot nematode *Meloidogyne incognita*. Two control groups planted in soil drenched with sterile distilled water or treated with the broad-spectrum carbamate pesticide carbofuran were used for comparison. The results suggest that the bacterial mixture is effective as a biocontrol agent against the root-knot nematode.

Keywords: Bacterial mixture, biocontrol, antinematode activity, *Meloidogyne incognita*

Root-knot nematodes (*Meloidogyne* spp.) are the causative organisms of root-knot disease, and one of the most destructive pests of a wide range of crops [16]. Six *Meloidogyne* species, *M. arenaria, M. incognita, M. hapla, M. javanica, M. hispanica*, and *M. cruciani*, have been recorded in Korea. Among them, the first three are major pests of greenhouse crops [7, 10]. Control of *Meloidogyne* spp. is carried out largely by a combination of methods including the use of nematicides and crop rotation [17]. Although chemical nematicides are effective to a certain extent, their use has been questioned in recent years because of increasing concern about environmental contamination and human health risks, high application costs, and dependence of nematicide action on soil conditions, as well as increasing governmental regulation [19]. There is now tremendous pressure on farmers to use methods of pest control that do not pollute or degrade the environment [1]. Nematode management through biocontrol is gaining importance globally. Nico *et al*. [18] reported the use of composted agro-industrial wastes for controlling root nematodes, while Ntalli *et al*. [20] evaluated the efficacy of a neem formulation in controlling root-knot nematodes.

Recently, there has been increasing interest in biological antagonists of nematodes, which include fungi of various genera that parasitize females and eggs of plant parasitic nematodes; for example, *Cylindrocarpon*, *Phoma*, *Fusarium*, *Gliocladium*, *Paecilomyces*, and *Pochonia* spp. [12, 24], antagonistic rhizobacteria [9, 23], and *Pasteuria penetrans*, a bacterial parasite of nematodes [11]. Bacteria and nematodes can interact in a variety of ways, the former producing metabolic by-products, enzymes, and toxins during the decomposition of organic matter in the soil, the collective action of which may make these organisms important natural antagonists of the latter [1, 3]. Other antagonists include arthropods, predatory nematodes, and a variety of other invertebrate organisms [21]. In the present study, we evaluated the biological control effect on *M. incognita* of a soil bacterial mixture isolated from an oriental melon greenhouse in Korea.

One hundred fourteen bacterial strains were isolated from greenhouse soil and screened for antinematode activity on *M. incognita* collected from a greenhouse cultivating oriental melon in Kyung-buk Province, Korea, and maintained in tomato (*Lycopersicon esculentum* Mill. var. Youngkwang) roots. Eggs of *M. incognita* were extracted from roots in 0.5% sodium hypochlorite solution and collected on a sieve with 25 mm pore openings. Freshly hatched J2s, not older than 3 days, were used in the experiment. To determine antinematode activity, 1 ml of a 24 h bacterial culture prepared in nutrient broth (NB) was transferred to a 96-well plate to which 1 ml of a suspension containing 20–25 freshly hatched juveniles was added, and the plate was incubated at room temperature.

**Corresponding author*
Phone: +82-53-810-3055; Fax: +82-53-813-4620;
E-mail: peter@ynu.ac.kr

Juveniles kept in sterile NB served as controls. Each treatment was replicated six times. After 24 h of exposure, the motility of the larvae was observed under a microscope. The nematodes were considered dead if they did not move on probing with a fine needle [6]. The experiment was repeated twice, and three bacterial isolates with antinematode activity were selected. The antinematode activity of the selected bacterial isolates was compared at different dilutions of the culture in nutrient broth.

The 16S rRNA genes of the selected isolates were amplified by PCR using the universal primers 27f and 1492r described by Lane [14] and were sequenced with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit v.3.1 on an ABI 3730XL capillary DNA Sequencer (Applied Biosystems, USA) at SolGent Co., Korea. The resultant sequences were assembled into a nearly complete 16S rRNA gene sequence with Vector NTI software (Invitrogen, USA). These sequences were compared with those in GenBank at the National Center for Biotechnology Information (NCBI) using the BLAST program.

Field validation was carried out at Seongju Fruit Vegetable Experiment Station, Korea. Oriental melon, *Cucumis melo* L. cv. "Geumssaragi-euncheon," grafted on Shintozoa (*Cucurbit maxima* × *Cu. moschata*), was used as the host crop in the experiment. To examine the efficacy of the bacterial consortium, 2 m × 1 m microplots were prepared in a 400 m^2 greenhouse, where the soil (sand-silt-clay, 70:19:11) was infested with *M. incognita*. Within a microplot, 42-day-old "Geumssaragi-euncheon" oriental melon seedlings were transplanted in 40 cm intervals. The soil ridge was mulched with black plastic film (0.02 mm thickness). Herbicide was not applied. The row was framed with iron wire and covered with clear plastic film (0.02 mm thickness). During the night, an additional blanket (thickness=400 g/m^2) was used over the plastic film to preserve heat. The row was drip irrigated (dripper flow = 1.49 l/h; Netafim Co., Korea).

An antinematode bacterial mixture was prepared with the three isolates that exhibited antinematode activity on *in vitro* screening by mixing equal quantities of these bacteria cultured in NB at a cell concentration of 10^6 CFU/ml. The bacterial mixture (BM) was applied to the crop by drenching in one of two quantities, 1 l/ha (BM 1×) or 2 l/ha (BM 2×). Both BM 1× and 2× were applied four times during the experiment, once just before planting and three times after planting with a gap of 2 weeks. Soil drenched with sterile distilled water was used as a negative control. Plants treated with 1.5 kg/ha carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate), a broad-spectrum carbamate pesticide, were also kept as a positive control for comparison. Plants were harvested on days 30 and 60 of the experiment (three plants from each group). The experiment was terminated after 60 days. All experiments were conducted in a randomized complete block design with three replicate blocks.

After terminating the experiment, roots were carefully dug out and washed gently under running tap water to remove all soil particles. Galled and healthy roots were separated and weighed. Galled roots were sectioned to ca. 1 mm thickness, and nematodes embedded within root tissues were stained using the bleach-stain method [5] and observed under a microscope. Egg masses in the galled roots were analyzed [3]. After terminating the experiment, the fresh weight of shoots and roots was recorded. Plants were randomly selected per plot and inspected for phytotoxicity by observing wilting, discoloration, leaf shedding, stunting of terminal buds, and death of the plant.

All of the experiments were conducted in triplicate. The data were analyzed for mean separations by Duncan's multiple range tests and significant differences were judged at $P<0.05$ with the Statistical Analysis System ver. 7.0 (SAS Institute, Cary, NC, USA). The coefficient of variation was also calculated for each mean value. The one-way analysis of variance was performed to test the significant difference between different treatments.

On screening 114 bacterial isolates from greenhouse soil, only three were observed to have antinematode activity. They were identified based on 16S rRNA gene sequences as *Lactobacillus farraginis*, *Bacillus cereus*, and *Bacillus thuringiensis* with GenBank nucleotide accession numbers GU471755, GU471752, and GU471753, respectively. During preliminary screening of the isolates, *Lactobacillus* sp. exhibited the greatest activity even with cultures at a 1:200 dilution, whereas *Bacillus* sp. showed activity at dilutions of up to 1:100.

After 30 days of the field experiment, a significant difference ($P<0.05$) in the % survival of nematodes was observed in carbofuran- and BM 2×-treated plants compared with the control (Table 1). The consistency of the treatment was comparable in both cases as evidenced by the coefficient of variation (CV) (%). Carbofuran showed a suppression of 45.4%, followed by BM 2× at 22.2%, whereas only 0.3% was achieved with the BM 1× treatment. After 60 days, both the bacterial mixture and carbofuran treatments exhibited significant control of the nematode ($P<0.01$). Carbofuran treatment showed a high level of control at 60.98%, followed by 52.1% with BM 2× and 38.7% with BM 1× ($P<0.05$). However, the consistency of treatment was greater in BM 2×-treated plants with a low CV of 15.87%.

On completing the experiment, the number of nematode egg masses/plant were found to vary, as shown in Fig. 1. The average number of egg masses/plant was 142 in the control plants, and 103 and 96 in the BM 1×- and BM 2×-treated plants, respectively, whereas only 52 egg masses were observed in the plants treated with carbofuran. The control effect of the carbofuran, BM 2× and BM 1×

Table 1. Suppression effect of bacterial consortium and carbofuran treatments on *Meloidogyne incognita* in oriental melon after 30 and 60 days of the experiment.

Treatment	Nematode population before treatment (no./plant)	Nematode population after 30/60 days (no./plant)		Survival of nematodes (%)		Mean survival (%)		Control effect of the treatments (%)		Coefficient of variation (%)	
		30d*	60d*	30d	60d	30d	60d	30d	60d	30d	60d
BM 1×	184.7±49.7	324±103	3,346±167	174±12	1,889±443	174.1[b]	1,889.0[a]	0.3	38.7	6.7	23.4
BM 2×	251.66±42	336±55	3,669±461	136±32	1,477±234	135.9[ab]	1,477.2[a]	22.2	52.1	23.6	15.8
Carbofuran	296±158	267±138	2,518±518	95.4±23	1,202±1,051	95.4[a]	1,202.5[a]	45.4	61	24.2	87.5
Control	209±46	345±41	6,185±1,564	175±66	3,082±1,066	174.7[b]	3,081.9[b]	0	0	37.6	34.6

30d, 60d: after 30 and 60 days.
*P<0.01 compared with control.
Means within a column followed by the same letter are not significantly different (P<0.05) according to Duncan's multiple range test.

treatments was 63.3%, 32.2%, and 27%, respectively. Fresh weights of shoots (g) in BM 1× and BM 2× treatments (89.4±6.8 and 99.33±10) were high compared with those of the carbofuran-treated and control groups (81.2±8.67 and 60.53±13.22). The plant root weight was significantly high in the control plant (16.3±2.06) because of the nematode population, whereas in the BM 1× and BM 2× and carbofuran treatments the weights were 10.87±0.77, 9.33±0.97, and 8.13±1.95 g, respectively. No phytotoxicity was noted in the plants treated with the bacterial mixture at 1× and 2×.

In the present study, we selected three potent antinematodal species, *Lactobacillus farraginis*, *Bacillus cereus*, and *Bacillus thuringiensis*, based on *in vitro* activity. The results of the field activity testing showed significant suppression of the nematode population compared with the control for both carbofuran and the bacterial mixture treatment at 2×. After 60 days of the experiment, the nematode control effect was more significant for both of these treatments than at 30 days. This finding indicates that long-term treatment with the bacterial mixture can be effective in controlling root-knot nematode. Of the members of the bacterial mixture, *Lactobacillus* sp. exhibited high activity followed by *Bacillus* sp. The latter are free-living rhizobacterial strains widely studied as plant growth-promoting biocontrol agents [8], and *Bacillus thuringiensis* is extensively used as a bioinsecticide for the control of many agricultural insect pests [22]. The incidence and severity of black rot *Xanthomonas campestris* pv. *campestris* of a cabbage cultivar were significantly reduced when antagonistic bacilli strains were applied through the roots [15]. Baysal *et al*. [4] reported the inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp. *radicis-lycopersici*. There are few reports of lactic acid bacteria being used as biocontrol agents. Takei *et al*. [25] reported that microcapsules of plant growth-promoting lactic acid bacteria are effective in the removal of root-knot nematodes.

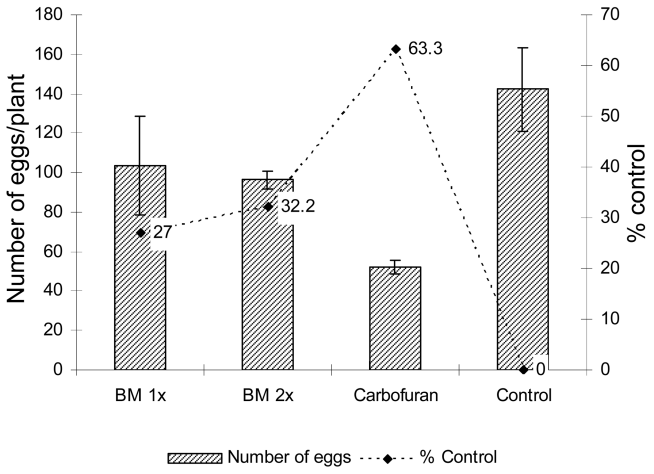

Fig. 1. Number of egg masses/plant and percentage control effect of a bacterial mixture and carbofuran on *Meloidogyne incognita* in oriental melon after 60 days of the experiment (P<0.05).

The bacterial mixture was effective on long-term treatment in reducing nematode egg masses, which are usually embedded in roots, and the mixture was effective in promoting growth without any phytotoxicty. Although the percentage of nematode suppression was lower compared with that obtained with carbofuran treatment, the mixture showed great potential as a biocontrol agent considering plant and environmental health. Antagonistic rhizobacteria have been repeatedly shown to be promising microorganisms for the biological control of plant-parasitic nematodes [9]. The addition of a plant growth-promoting rhizobacterium, *Paenibacillus polymyxa* strain GBR-1, to potted soil was reported to suppress root-knot nematode and root galling on tomato and to increase plant growth [13]. Akhtar and Siddiqui [2] studied the effect of *Pseudomonas putida*, *Pseudomonas alcaligenes*, and a *Pseudomonas* isolate (Ps28) on the root–rot complex of chickpea caused by the combination of *M. incognita* and *M. phaseolina* and

reported reduced *M. incognita* hatching and root penetration *in vitro*.

In the present study, the nematode population was reduced to a greater extent and with positive impact on the plant on long-term treatment with the bacterial mixture. However, further studies are required to understand the plant-nematode–bacterial mixture interaction. This approach will lead to strategies for early suppression of nematodes by the mixture, so that crops can develop without any adverse effect on growth and yield.

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