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Nematicidal activity of secondary metabolites of a plant growth promoting rhizobacterium, *Paenibacillus polymyxa*

Seon-Hye Son*, Zakaullah Khan*, Hyo Sun Moon*, Sang Gyu Kim*, Yilseong Moon, Dong-Ro Choi*** and Young Ho Kim***

***Department of Agricultural Biotechnology and Center for Plant Molecular Genetics and Breeding Research, Seoul National University, Seoul 151-921, Korea, e-mail: yhokim@snu.ac.kr**
****South Forest Research Center, Korea Forest Research Institute, Jinju 660-300, Korea,**
*****Department of Biology, National Institute of Agriculture Science and Technology, Suwon 441-853, Korea**

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Summary. Twelve strains of *Paenibacillus polymyxa* isolated from rotted ginseng roots were tested for their nematicidal activity against plant-parasitic nematodes. The secondary metabolites in the culture filtrates of all the tested strains of *P. polymyxa* caused mortality of plant-parasitic nematodes, i.e. *Meloidogyne incognita, Heterodera glycines, Pratylenchus pratensis* and *Bursaphelenchus xylophilus*, to varying degrees. Mortality increased with increasing concentrations of culture filtrates and exposure times. The 10% culture filtrate concentration of 3 strains, GBR-158, GBR-501, GBR-508, and 20% concentration of 5 strains, GBR-11; GBR-27; GBR-447; GBR-462 and GBR-477 caused 100% mortality of each tested nematode species after 24 h of exposure. Second-stage juveniles of *M. incognita* and *H. glycines* were more vulnerable than *P. pratensis* and *B. xylophilus* to the culture filtrates of each tested strains as their mortality rates were greater.

Key words: *in vitro* assays, mortality, *Paenibacillus polymyxa*, plant-parasitic nematodes.

Plant-parasitic nematodes are significant constraints in crop production. The cost to world agriculture of nematode parasitism was estimated recently to be US$ 125 billion annually (Chitwood, 2003). Use of chemical nematicides is one of the primary means of controlling plant-parasitic nematodes. However, the negative impact on the environment and human health has led to a ban or restricted use of some nematicides. Ecological and health concerns associated with the use of nematicides have resulted in intensified research for new biological or non-chemical agents for controlling plant-parasitic nematodes. The search for new microbial strains as source of biological nematicides is an important goal for reducing damage caused by plant-parasitic nematodes. Fungi and bacteria are two major groups of microbes that are abundant in soil and some of them have shown potential as biocontrol agents for nematodes (Stirling, 1991). Over the last decades, a great diversity of rhizospheric microorganisms has been described, characterized and in many cases tested for activity as biocontrol agents against soil pathogens. Such microorganisms can produce substances that may limit the damage caused by phytopathogens, e.g. by producing antibiotics, siderophores and a variety of enzymes. Plant growth promoting rhizobacteria are capable of providing substantial protection against nematode diseases (Siddiqui & Mahmood, 1999; Siddiqi & Shaukat, 2002, 2003).

Paenibacillus polymyxa (previously *Bacillus polymyxa*; Ash *et al.*, 1993) is a common soil bacterium belonging to plant growth promoting rhizobacteria (PGPR) and has been used to control several plant diseases (Mavingui & Heulin, 1994; Kim, 1995; Shishido *et al.*, 1996; Dijksterhuis *et al.*, 1999; Kharbanda *et al.*, 1999). Like other *Bacillus* spp., *P. polymyxa* showed antifungal activity and has suppressive effect on postharvest decay in onion bulbs in storage (Lee *et al.*, 2001). The bacterium also has inhibitory activity against human pathogenic microorganisms (Seldin *et al.*, 1999), and produces antimicrobial substances active against fungi and bacteria (Rosado & Seldin, 1993; Mavingui & Heulin, 1994;

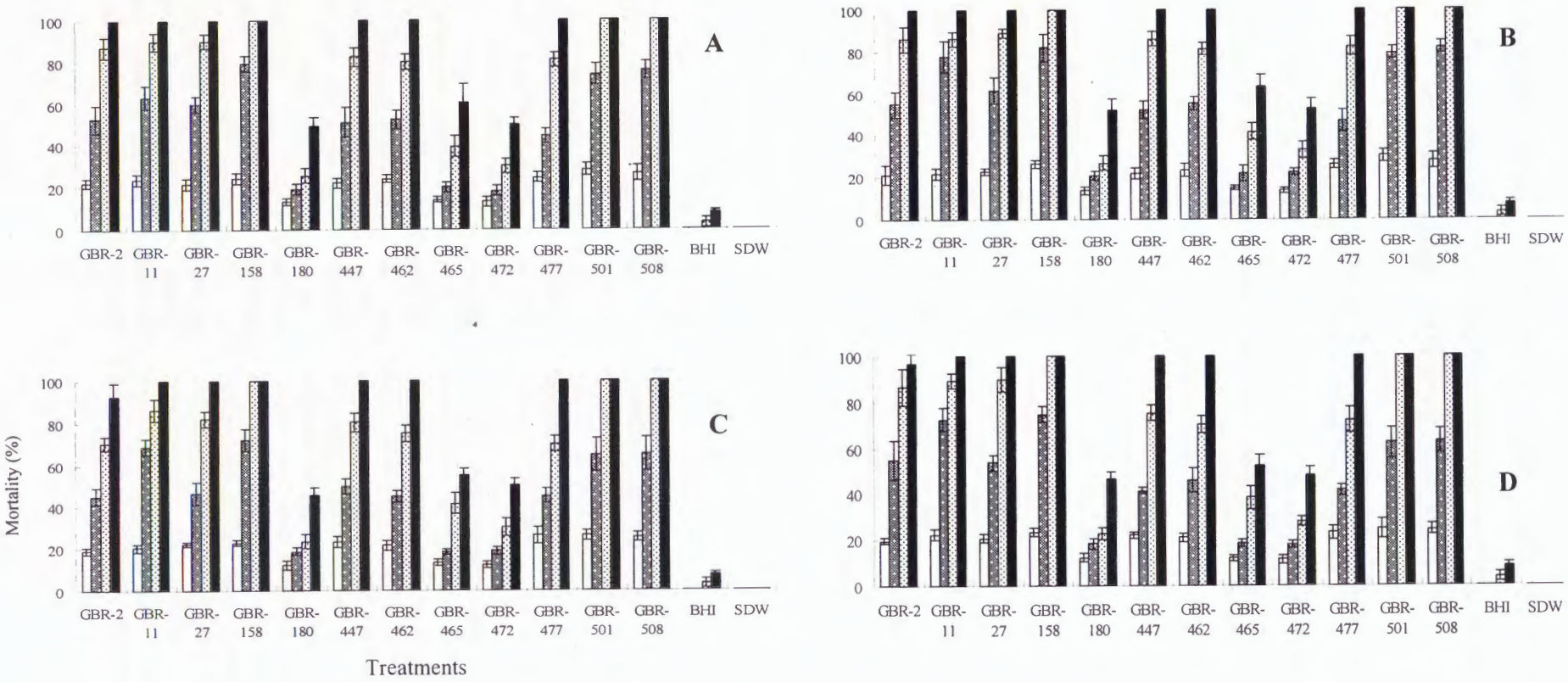

Fig. 1. Effect of concentrations of culture filtrates (▭ - 2%, ▩ - 5%, ▩ - 10% and ■ - 20%) of various strains of *Paenibacillus polymyxa* on plant parasitic nematodes mortality: A- *Meloidogyne incognita* J2; B- *Heterodera glycines* J2; C- *Pratylenchus pratensis*; D- *Bursaphelenchus xylophilus*. BHI= culture medium without bacterium; SDW= sterile distilled water. Standard error bars are attached to the means. The culture filtrate of each strain of *P. polymyxa* was obtained from BHI medium after 48h of growth.

Picard *et al.*, 1995; Kajimura, 1996; Lebuhn *et al.*, 1997; Nielsen & Sorensen, 1997; Dijksterhuis *et al.*, 1999). However, nothing is known about the nematicidal properties of *P. polymyxa*. The objective of the present study was to evaluate the effect of in vitro culture filtrates of twelve strains of *P. polymyxa* on plant parasitic nematodes.

MATERIAL AND METHODS

Nematode inoculum. Four nematode species, representing different trophic groups *viz.*, sedentary endoparasitic, root-knot nematode (*Meloidogyne incognita*) and soybean cyst nematode (*Heterodera glycines*), migratory endoparasitic, root-lesion nematode (*Pratylenchus pratensis*), and mycetophagus or phytoparasitic, pine wood nematode (*Bursaphelenchus xylophilus*) were used in the tests. Second-stage juveniles of *M. incognita* and *H. glycines* were obtained from galled tomato roots and cysts on soybean roots, respectively, using standard extraction methods (Hussey & Barker, 1973; Ayoub, 1980). Mixed stages of root lesion nematode, *P. pratensis,* were obtained from roots of infested chrysanthemum plants, and mixed stages of *B. xylophilus* were obtained from Petri dish cultures with *Botrytis cinerea* growing on potato dextrose agar (PDA).

Bacterial culture. The strains of *Paenibacillus polymyxa* were isolated from four-year-old roots of Korean ginseng with rot symptoms, which were collected from commercial markets and storage facilities (Jeon *et al.*, 2003). The isolated strains were stored at -70°C in sterilized distilled water with 20% glycerol until used. Culture filtrates with secondary metabolites were obtained by growing bacteria in 250 ml Erlenmeyer flasks containing 100 ml of brain heart infusion (BHI) (CONDA, Madrid, Spain) broth at 28°C for 2 days on a shaker at 200 rpm. The cultures were centrifuged twice at 8,000 g for 10 min to separate the bacteria from the media. After centrifugation, pellets were discarded and the supernatant was allowed to pass through two layers of Whatman No.1 filter paper. The resulting filtrate was considered as standard, and further dilutions were prepared by adding sterile distilled water.

Screening of bacterial strains for toxic metabolite activity to *M. incognita* juveniles. Twelve strains of *P. polymyxa* were randomly selected from our collection and screened for nematicidal activity against *M. incognita* J2. Sterile 50 ml flasks were filled with 9 ml sterile distilled water and 10 ml standard culture filtrates of each strain separately, and 2000 J2 in 1 ml water were pipetted into each flask. At each exposure time (1, 2, 4, 8, 12 and 24 h), a 2-ml sub sample was transferred into counting dish and examined under a dissecting microscope (60x magnification). Numbers of live and dead nematodes were counted. Nematodes were considered dead if they did not move when probed with a fine needle (Cayrol *et al.*, 1989). Each treatment had 5 replicates, and the experiment was repeated once.

Dose response on plant-parasitic nematodes. To determine the effect of concentration of bacterial culture filtrates on mortality of four species of plant-parasitic nematodes, 1 ml filtrates of various concentrations (Fig. 1) were transferred to 24-well Tissue Culture Testplate (SPL, Life Sciences, Pocheon city, Korea). One hundred surface sterilized individuals of *M. incognita, H. glycines, P. pratensis* and *B. xylophilus* were then added separately to each well. Control treatments consisted of equivalent concentrations of non-inoculated BHI liquid medium and sterile distilled water. Nematodes in culture filtrate solutions were kept in the dark at 25±2°C. Immobilized/dead nematodes were counted after 24 h of incubation. Each treatment was replicated 5 times and the experiment was repeated once.

Statistical analysis. All experiments were performed twice. Analysis showed no significant interaction between the two tests run for any of the treatment. Therefore, results from duplicate tests were combined for the final analysis. Analysis of variance was carried out using Statistix 7.0 (NH Analysis software, Roseville, MN). Duncan's multiple range tests was employed to test for significant difference between treatments at $P \geq 0.05$.

RESULTS

The secondary metabolites in the culture filtrates of all the tested strains of *P. polymyxa* isolated from ginseng roots caused mortality of *M. incognita* J2 to varying degrees and mortality increased with the increase of exposure time ($P \geq 0.05$) compared with BHI culture medium without bacterium and sterile distilled water (Table 1). Culture medium, BHI without bacterium also caused J2 mortality ($P \geq 0.05$) after 6 h of exposure compared with the water control. Strain GBR-158 was the most effective, resulting in 100% mortality within a 4 h exposure time. Five other strains, GBR-11, GBR-27, GBR-462, GBR-501 and GBR-508 were also highly effective, requiring 6 h to cause 100% mortality of J2. Three strains, GBR-2, GBR-447 and GBR-477 resulted in complete nematode kill after 12 h. The remaining three strains, GBR-180, GBR-465 and GBR-472 failed to kill all nematodes even after a 24 h exposure.

Table. 1. Effect of exposure time to culture filtrate of various strains of *Paenibacillus polymyxa* on mortality of *Meloidogyne incognita*.

Paenibacillus polymyxa strains	Exposure duration (h)					
	1	2	4	6	12	24
GBR-2	10.6g	51.4f	76.0e	90.4b	100a	100a
GBR-11	15.2f	59.8dc	80.4dce	100a	100a	100a
GBR-27	15.4fe	56.0de	78.2de	100a	100a	100a
GBR-158	25.4a	72.2a	100a	100a	100a	100a
GBR-180	6.0b	11.8h	24.8h	40.8	59.4c	70.8b
GBR-447	18.4cd	60.4c	69.8f	87.8b	100a	100a
GBR-462	17.4fde	55.0fe	83.2c	100a	100a	100a
GBR-465	6.0h	11.6b	20.2i	40.6e	63.8b	72.0b
GBR-472	5.8h	10.4h	21.8ih	45.8d	65.8b	73.2b
GBR-477	18.2cde	40.4g	65.2g	84.0c	100a	100a
GBR-501	22.4b	59.1dc	81.2dc	100a	100a	100a
GBR-508	20.8bc	65.6b	88.0b	100a	100a	100a
BHI	0.0i	0.0i	0.0j	3.8f	6.6d	9.6c
SDW	0.0i	0.0i	0.0j	0.0g	0.0e	0.0d

The culture filtrate of *P. polymyxa* strains were obtained from BHI broth after 48h of growth. BHI= culture medium without bacterium; SDW = Sterile distilled water.

The secondary metabolites in culture filtrate of various strains of *P. polymyxa* at different concentrations (2, 5, 10 and 20%) caused significantly ($P \geq 0.05$) greater mortality of plant-parasitic nematodes, *M. incognita, H. glycines, P. pratensis* and *B. xylophilus* compared with BHI medium without bacterium and sterile distilled water (Fig. 1, A-D). Strains GBR-158, GBR-501 and GBR-508 induced 100% mortality of each tested nematode species at 10% concentrations, whereas GBR-11, GBR-27, GBR-447, GBR-462 and GBR-477 caused 100% mortality at 20% concentrations. At 20% concentration, GBR-2 caused 92.6 and 90.6% mortality of *P. pratensis* and *B. xylophilus,* respectively, but killed 100% juveniles of *M. incognita* and *H. glycines.* Strains GBR-180, GBR-465, GBR-472 showed lower toxic effect as they caused <60% mortality of nematodes at 20% concentration. Nematicidal activity of culture filtrates obtained from various strains of *P. polymyxa* differed between the nematode species tested. J2 of the sedentary endoparasitic nematodes, *M. incognita* and *H. glycines,* appeared to be more vulnerable to secondary metabolites of *P. polymyxa* strains, as their mortality rates were greater ($P \geq 0.001$) than those of *P. pratensis* and *B. xylophilus* in culture filtrates of each tested strain. The nematicidal activity of the culture filtrates decreased at higher dilutions (Fig. 1). Thus, concentration and exposure time directly affected nematode mortality.

DISCUSSION

Our results demonstrate that exposure to culture filtrate of various strains of *P. polymyxa* induced mortality in plant-parasitic nematodes to varying degrees, depending upon the concentration of culture filtrate and exposure time. Bacterial antibiotics and other toxic compounds present in the metabolites might be responsible for the nematode mortality. The bacterium has long been known for its ability to produce antibacterial compounds, i.e. polymyxin, and has been extensively studied (Storm *et al.*, 1977). In addition, *P. polymyxa* produces a variety of secondary metabolites such as antibiotics, siderophores, HCN and a variety of enzymes. The antimicrobial compounds may include fusaricidin A, a depsipeptide characterized by Kajimura & Kaneda (1996), which was produced by *P. polymyxa* strains isolated from garlic plants suffering from basal rot caused by *F. oxysporum.* Cell wall degrading enzymes such as chitinase and glycanase (Mavingui & Heulin, 1994; Nielsen & Sorensen, 1997) may also play a role in causing nematode mortality. Chitinase produced by *P. illinoisis* KJA-424 caused the lysis of *M. incognita*

egg shell and resulted in the inhibition of hatching *in vitro* (Jung *et al.*, 2002). Siddiqi & Shaukat (2003) also reported that a strain of *Pseudomonas fluorescens* produced metabolites such as 2, 4-diacetylphloroglucinol and HCN with nematicidal activity against *Meloidogyne* J2.

The nematicidal potential of all tested strains of *P. polymyxa* decreased at higher dilutions, suggesting the presence of a nematicidal substance. The different degree of mortality observed between the nematode species may be related to nematode stage tested or different species may have different level of tolerance against nematicidal substances produced by the *P. polymyxa*. Only second-stage juveniles of *M. incognita* and *H. glycines* were tested, whereas all juvenile stages and adult nematodes of *P. pratensis* and *B. xylophilus* were used. This study clearly shows that strains of *P. polymyxa* have nematicidal properties against *M. incognita*, *H. glycines*, *P. pratensis* and *B. xylophilus* that may be exploited in a biocontrol program to manage plant-parasitic nematodes. Siddiqi & Shaukat (2002) reported that bare root-dip treatment and soil drench with *P. fluorescens* and *P. aeruginosa* strains substantially reduced *M. javanica* penetration into tomato roots under glasshouse conditions. Such studies are needed to evaluate the biocontrol potential of the tested *P. polymyxa* strains for the management of plant-parasitic nematodes under glasshouse and field conditions.

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S. Son, Z. Khan, H. S. Moon, S. G. Kim, Y. Moon, D.-R. Choi, Y. H. Kim. Нематицидная активность вторичных метаболитов ризобактерии *Paenibacillus polymyxa*, стимулирующей рост растений.

Резюме. Двенадцать штаммов *Paenibacillus polymyxa,* изолированных из гниющих корней женьшеня, были испытаны на нематицидную активность. Вторичные метаболиты, полученные из культуральных фильтратов всех испытанных штаммов *P. polymyxa,* вызывали разные уровни смертности фитопаразитических нематод *Meloidogyne incognita, Heterodera glycines, Pratylenchus pratensis* и *Bursaphelenchus xylophilus*. Смертность нематод возрастала при увеличении концентрации культуральных фильтратов и увеличении времени воздействия. Фильтраты 10%-й концентрации трех штаммов: GBR-158, GBR-501, GBR-508 и 20%-й концентрации от пяти штаммов: GBR-11; GBR-27; GBR-447; GBR-462 и GBR-477 вызывали 100% смертность каждой из этих нематод после 24 ч экспозиции. Личинки второй стадии *M. incognita* и *H. glycines* были более чувствительны к воздействию культуральных фильтратов, чем личинки *P. pratensis* и *B. xylophilus*, а показатели их смертности были выше.