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Plant growth-promoting rhizobacterium (*Paenibacillus polymyxa*) induced systemic resistance in tomato (*Lycopersicon esculentum*) against root-knot nematode (*Meloidogyne incognita*)

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ABSTRACT

Three strains of plant growth-promoting rhizobacteria, *Paenibacillus polymyxa* GBR-447, GBR-477 and GBR-501 were tested in potted soil under greenhouse conditions for their ability to induce systemic resistance in tomato (*Lycopersicon esculentum* Mill.) roots against root-knot nematode (*Meloidogyne incognita*). The application of bacterial suspensions of these strains of *P. polymixa* into potted soil at the rate of 5ml (10^8CFU/ml)/plant significantly reduced penetration rate of *M. incognita* juveniles (J2) into tomato roots and gall formation, inhibited giant cells formation and enhanced plant growth significantly. Strain GBR-447 was the most effective, as J2 penetration into tomato roots reduced by 46.4%, 52.5 and 55.8% after 5, 10 and 25 days of J2 inoculation, respectively. The lowest effect was observed in pots treated with GBR-501, wherein J2 penetration reduced by 15.2%, 17.7% and 26.5% after 5, 10 and 25 days of J2 inoculation, respectively. Application of bacterial suspension of GBR-447 and GBR-477 to one-half of the split root system caused systemic reduction in the number of J2 penetration in the untreated other half of the split root system and inhibited giant cells formation. Penetration of J2 was reduced by 38.3-44.2%, 45.7-52.5% and 51.3-54.3% after 5, 10 and 25 days of J2 inoculation, respectively. GBR-501 inhibited J2 penetration up to 22.8% on 25th day of inoculation.

Key words: Induced systemic resistance*, Meloidogyne incognita, Paenibacillus polymyxa*

Root-knot nematodes (*Meloidogyne* spp) are sedentary endoparasites of roots, causing damage to a wide range of crops, resulting in significant economic losses worldwide, (Sikora and Fernandez 2005). Control of root-knot nematodes has been accomplished primarily through nematicides, crop rotation and resistant cultivars where available. Environmental and health hazards associated with chemical pesticides and the recent loss of methyl bromide as a multipurpose soil fumigant have spurred research into alternative methods. Plant growth-promoting rhizobacteria (PGPR) have been identified as a biocontrol alternative to pesticide for disease suppression (Siddiqui 2006).

Genus *Paenibacillus* is gram-positive, aerobic, rod-shaped, endospore-forming bacteria, belongs to PGPR. *Paenibacillus* spp are known to suppress several plant pathogens (Akhtar and Siddiqui 2007, Haggag 2007, Haggag and Timmusk 2008, Khan *et al*. 2008, Kim *et al*. 2009, Son *et al*. 2007, 2008, 2009). Studies have demonstrated that some rhizobacteria can induce systemic resistance in the plants towards soil-borne fungi and plant-parasitic nematodes (Siddiqui and Shaukat 2002, van Loon and Glicj 2004, Munif *et al*. 2001, Hauschild *et al*. 2004). Haggag (2007) investigated that *P. polymyxa* induce resistance to crown rot disease on peanut, where he found the induction level of protein was high in treated roots than in untreated plants. Siddiqui and Shaukat (2004) reported that rhizobacteria mediated ISR against the root-knot nematode (*M. javanica*) on tomato (*Lycopersicon esculentum* Mill.) is independent of salicylic acid production. In view of above findings the present study was undertaken to investigate whether *Paenibacillus* strains can induce resistance to *M. incognita* in tomato plants.

MATERIALS AND METHODS

Present study was carried out in 2008 and 2009 at Seoul National University, Seoul, Korea. The *Paenibacillus polymyxa* strains used in the present experiments were isolated from the 4-year-old roots of rotten ginseng, which were collected from commercial markets and storage facilities.

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The isolated *P. polymyxa* strains were stored at –70 °C in sterilized distilled water with 20% glycerol. By comparing cultural and physiological characteristics with those of known *Paenibacillus* strains and analyses using Biolog system and gas chromatography of fatty acid methyl esters (GC-FAME), among which the *P. polymyxa* strains were confirmed by 16S rDNA sequencing (Jeon 2004). Each bacterial strain was grown in Brain Heart Infusion (BHI) broth at 28 °C for two days with shaking at 200 rpm in 250 ml Erlenmeyer flasks containing 100 ml of BHI broth. The cultures were centrifuged at 8 000 g for 10 min. to separate bacterial cells from culture media. After centrifugation, supernatants were discarded and pellets (bacterial cells) were washed twice with sterilized distilled water (SDW) by centrifugation. Bacterial cells were re-suspended in SDW and adjusted to 10^8 CFU/ml (OD_{600}= 0.8), which were used for experiments.

Root-knot nematode, *M. incognita* race-1 was obtained from pure cultures maintained on roots of tomato plants in a greenhouse, at Seoul National University, Seoul, Korea. The entire root system was dipped in water to remove adhering soil. Egg masses of *M. incognita* were hand-picked with the help of forceps and were rinsed with sterile water. The egg masses were incubated for 2–3 days using modified Baermann funnel method to obtain second stage juveniles (J2), which were used as inoculum for experiments. The population density of J2 was determined from five replications of 1 ml aliquots of inoculum suspension for pot experiments. Tomato seeds, cultivar Seon-myeong (susceptible to root-knot nematode) were used in all experiments. The seeds were surface sterilized with 0.1% mercuric chloride for 2 min., rinsed three times with sterile water, and was sown in 4.5 cm × 4.5 cm plastic cell plug trays filled with Baroker Horticultural substrate. The seeded trays were kept in growth chamber at 25°C for three weeks, and watered daily with a sprinkler.

To evaluate effect of bacterial treatments on nematode penetration into tomato roots, gall formation and plant growth. Three-weeks old tomato seedlings were planted into 6 cm diameter plastic pots containing 500 cm^3 sterile mixture of sand: soil (1:1). Two days after plantation, bacterial suspension of *P. polymyxa* GBR 447, GBR 477 and GBR 501 were added to the potting soil around the roots at the rate of 5 ml/pot (10^8 CFU/ml) separately. After two days of treatments, each plant was inoculated with 2000 freshly hatched J2 of *M. incognita* in 10 ml suspension. Plants treated with tap water served as control. Pots were arranged in randomized block design in greenhouse with a mean temperature of 24°C, a regime of 12 hr light and 12 hr dark, and 60–70% relative humidity. Plants were watered daily to field capacity. The number of J2 penetration into tomato roots was determined after 5, 10 and 25 days of J2 inoculation. Root galls were counted and plant growth parameters (shoot and root) were measured at final harvest, i e 25 days after J2 inoculation. Each treatment having five pots and the experiment was repeated once. Plants were uprooted from pots after 5, 10 and 25 days of J2 inoculation and roots were cleaned by shaking in tap water. To determine J2 penetration, roots were stained in a 0.1% acid fuchsin solution and macerated at high speed in water in a blender for 10 sec., the J2 suspensions obtained were filled up to a volume of 100 ml, the numbers of J2 in two 5 ml aliquots were counted and the total number of J2/root system was calculated. Severity of root galling was assessed on scale of 0–5 (gall index, GI); 0 = no galls, 1 = 1–2, 2 = 3–10, 3 = 11–30, 4 = 31–100, and 5 = >100 galls per root system (Taylor and Sasser 1978).

To determine whether above mentioned *P. polymyxa* strains can induce systemic resistance in the tomato plants against root-knot nematode. Bacterial suspension and the *M. incognita* J2 were applied spatially separated in a split-root system to prevent direct contact of bacteria and nematode. The split-root system allows inoculation of the bacterium and nematode at separate locations on the root system. For that a split-root chamber composed of three pots was design as shown in Fig. 1. Each pot was filled with 500 cm^3 of a sterilized soil: sand mixture (1:1). Three-week-old tomato seedlings were planted into the upper pot, which consist two

Fig 1 Design for split root system: Two opening at base of the top pot (*a*) allowed roots to grow into two bottom pots (*b, c*). Roots in bottom pots spatially separated and inoculated individually with nematode (*b*) and bacteria (*c*)

openings at its bottom, which allowed the roots to grow into the two bottom pots. The roots in the two bottom pots were spatially separated and treated individually. Five days after planting, bacterial suspension of *P. polymyxa* GBR 446, GBR 477 and GBR 501 were added to one of the bottom pot (inducer) around the roots at the rate of 5 ml/pot (10^8 CFU/ml) separately. The roots in another bottom pot (responder) remained untreated. Plants treated with tap water in the inducer side served as controls. Two days after the treatment, *M. incognita* J2 suspension was dispensed with pipette at the rate of 2000 J2/pot around the roots in the responder pot that was not treated with bacterial suspension. Experimental design and conditions remained same as was in one pot experiment. The number of J2 penetration into the tomato roots was determined at 5, 10 and 25 day after J2 inoculation in the responder roots in the same way as described in above section. Each treatment has five pots and the experiment was repeated once.

Root galls caused by *M. incognita* were taken from responder pots of each treatment in split root system as well as root samples from untreated pots and fixed in Karnovsky's fixative in 0.01M cacodylate buffer (*p*H 7.0) for 24 hr. The samples were post-fixed with 0.01M osmium tetroxide in the same buffer solution for two hr, and then rinsed with the buffer three times. The samples were dehydrated in ethanol series and embedded in Spurr's epoxy resin. Nematodes could be seen clearly through the embedding materials. The embedded samples were sectioned (800 nm in thickness) with glass knife on an MT-X ultramicrotome. The sections were stained with 1% toluidine blue in 30% ethanol and observed under a compound light microscope.

All experiments were performed twice. A two-way analysis of variance (ANOVA) was performed on the data followed by mean separation with Fisher's protected least significant difference (PLSD, *P*=0.05) to compare between treatments at *P*=0.05. All statistical analysis was done using Analyse-it software for Microsoft Excel. Since no significant difference existed between the repeated experiments, therefore, data from duplicate tests were pooled for analysis.

RESULTS AND DISCUSSION

Applications of *P. polymyxa* GBR 447 and GBR 477 and GBR 501 reduced the penetration of *M. incognita* J2 into tomato roots compared with untreated plants (*P*=0.001) (Table 1). However, the inhibitory effect of above three strains on J2 penetration was variable, the highest inhibition was observed in plants treated with GBR 447, it inhibited J2 penetration in to plant roots by 46.4%, 52.5% and 55.8% after 5, 10 and 25 days of nematode inoculation, respectively. Similarly, GBR 477 treatment had reduced J2 penetration by 39.3%, 48.1 and 50.6% after 5, 10 and 25 days of nematode inoculation, respectively. There was no significant difference (*P*=0.05) between the penetrations rates of J2 treated with GBR 447and GBR 477 (Table 1). However, treatment with GBR 501 had the lowest inhibitory effect on nematode infection, as only 15.2%, 17.7% and 26.5% reduction in J2 penetration was noticed after 5, 10 and 25 days of inoculation, respectively. Shoot and root growth parameters of tomato plants were enhanced and root galling was reduced significantly (*P*=0.05) by the treatments of all the three bacterial strains tested compared to untreated controls (Table 2). Root gall formation by *M. incognita* was reduced by 78%, 72% and 48% in pots treated with GBR 447, GBR 477 and GBR 501, respectively compared with untreated control.

Addition of *P. polymyxa* GBR 447, GBR 477 and GBR 501 to one half of the split-root system (inducer side) caused a significant systemic reduction in J2 penetration in another

Table 1 Effect of treatments with three strains of *Paenibacillus polymyxa* on penetration rate of *Meloidogyne incognita* J2 into tomato roots

Treatment	Nematode penetration after inoculation (J2/root system)[x]					
	5DAI		10DAI		25DAI	
	J2/ root	CE (%)	J2/ root	CE (%)	J2/ root	CE (%)
Untreated control	112a[y]		158a			170a
GBR 447	60c	46.4	75cd	52.5	75cd	55.8
GBR 477	68c	39.3	82c	48.1	84c	50.6
GBR 501	95b	15.2	130b	17.7	125b	26.5

[x]Values are average of five replicate plants. [y]Values in rows followed by the same letters are not significantly different according to Fisher's protected least significant difference at *P*=0.05. DAI, Days after inoculation, CE, control effect

Table 2. Effect of treatments with three strains of *P. polymyxa* on root gall formation in tomato roots caused by *M. incognita* and on plant growth after 25 days of J2 inoculation

Treatment	Gall severity		Shoot length (g)	Shoot weight (g)	Root (g)
	RGI[y]	CE (%)			
Untreated with nematode	5.0a[z]		195.1d	2.6c	3.1a
GBR 447	1.1c	78.0	415.5a	14.6a	2.4b
GBR 477	1.4c	72.0	390.9a	13.1a	2.3b
GBR 501	2.6b	48.0	308.8b	9.2b	2.8ab
Untreated without nematode	0.0d		284.5bc	6.5bc	2.0b

[y]Severity of root galling was assessed by counting the number of galls/root system and a scale of 0–5 root index was used as follows: 0= no galls, 1= 1–2, 2= 3–10, 3= 11–30, 4= 31–100, 5= >100 galls/root system.

[z]Values in a columns followed by the same letters are not significantly different according to Fisher's protected least significant difference at *P* = 0.05. CE, control effect

Table 3 Effect of treatments with three strains of *P. polymyxa* on penetration rate of *M. incognita* J2 into tomato roots of untreated side (responder) in split root system

Treatments	Nematode penetration after inoculation (J2/root system)[x]					
	5DAI		10DAI		25 DAI	
	J2/ root	CE (%)	J2/ root	CE (%)	J2/ root	CE (%)
Untreated control	120a[y]		175a		197a	
GBR 447	67c	44.2	83c	52.5	90c	54.3
GBR 477	74c	38.3	95c	45.7	98c	51.3
GBR 501	105ab	12.5	140b	20.0	152b	22.8

[x]Values are average of 5 replicate plants. [y]Values in columns followed by the same letters are not significantly different according to Fisher's protected least significant difference at P=0.05. DAI, Days after inoculation; CE, control effect.

half of the split-root system (responder side) (P=0.05) (Table 3). Like in one pot experiment, GBR 447 was found to be the most effective strain this experiment also, as after 5, 10 and 25 days of J2 inoculations, nematode penetration into the responder root section treated with GBR 447 was reduced by 44.2%, 52.5% and 54.3%, respectively. Application GBR 501 was least effective, it reduced J2 penetration into roots by 12.5%, 16.6% and 22.8% after 5, 10 and 25 days of J2 inoculation.

Histopathological studies were made on root galls caused by *M. incognita* in untreated and treated with *P. polymyxa* GBR 447, GBR 477 and GBR 501. Large giant cells were formed in the untreated control, while small size numerous giant cells were formed by *M. incognita* in the stele of the swollen roots (root galls) treated with GBR 501 (Fig 2 A-B). On the contrary, no or few (1–4) small size giant cells were formed in plant roots treated with GBR 447 and GBR 477 (Fig 2 C-D) that revealed the inhibition of giant cell formation. Female nematodes were usually established adjacent to the stellar region, protruding the anterior part of the nematode body into the inside of the stele. Infection sites of root (root galls) with *M. incognita* had giant cells, characterized by hypertrophied cells and dense cytoplasm. The giant cell walls were stained reddish or purple as were primary cell walls of parenchyma cells, while those of xylem vessels and phloem fibres stained light blue.

In the present study, using standardized assay with tomato plants, treated with rhizobacterial suspension of GBR 447, GBR 477 and GBR 501 reduced *M. incognita* J2 penetration into tomato roots in both one-pot and split-root experiments, inhibited giant cell formations and enhanced plant growth. This means inhibition of J2 penetration into tomato roots was not only due to the direct effects of the bacteria on nematode, because the bacteria and nematode were spatially separated in split root system. The results clearly revealed

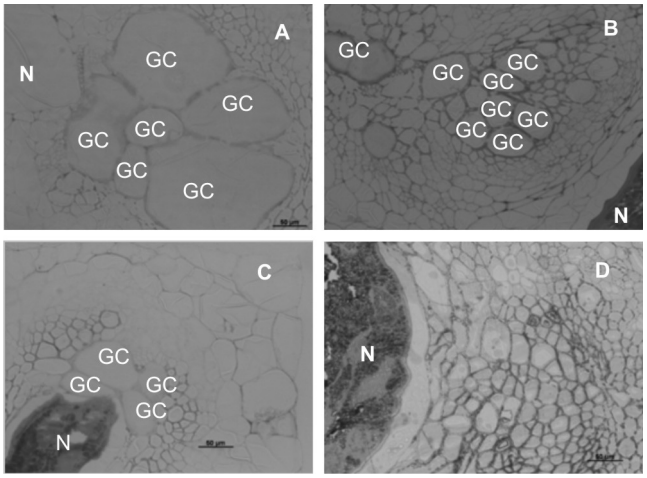

Fig 2 Giant cells induced by *M. incognita* in tomato roots. (*A*) untreated showing extensive giant cells around nematode neck; (*B*) treated with GBR 501 showing numerous but smaller giant cells; (*C*) treated with GBR 477 showing fewer and smaller giant cells; (*D*) treated with GBR 447 showing no giant cell formation; Bars=50µm and N=nematode.

that the induced systemic resistance was the main component of the mode of action of *P. polymyxa* strains (GBR 447 and GBR 477) on tomato plant roots or the key factor affecting J2 penetration into the roots. Siddiqui and Shaukat (2002) have demonstrated that a PGPR, *P. fluorescens* induced systemic resistance in tomato roots against a root-knot nematode, *M. javanica*. Haggag (2007) reported that seed treatment with *P. polymyxa* induced systemic resistance against crown rot disease of peanut.

Although, GBR 501 caused significant reduction in J2 penetration into the tomato roots as compared to untreated plants, but remained less effective in both the experiments when compared with GBR 447 and GBR 477. Results indicated that inoculation of selected strains of *P. polymyxa*, i e GBR 447 and GBR 477 can protect tomato plants against root-knot nematode by disrupting J2 penetration and inhibition of giant cell formation through induced systemic resistance. The histopathological modifications or suppression in giant cell formation after the nematode penetration suggest the effect of bacterial treatment on disease suppression may be more of post-infectional rather than pre-infectional defense mechanisms. The research reported here establishes *P. polymyxa* as a promising candidate for the management of root-knot nematode. It also has been shown that not all strains possess nematicidal or nematode suppressive properties.

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