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Effects of seed treatment with rhizobacterium, *Paenibacillus* species on management of root-knot nematode-Fusarium wilt fungus disease complex in tomato plants

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Summary. Seed treatment with *Paenibacillus lentimorbus* GBR158-Rif and *P. polymyxa* GBR462-Rif had no adverse effect on seed germination but increased growth of tomato plants. However, the bacterial population densities recovered from GBR158-Rif+ treated seeds (hypocotyls) 7 days after treatment were much higher (5.9×10^4 CFU/cm hypocotyl) than those treated with GBR462-Rif+ (1.3×10^3 CFU/cm hypocotyl). The SEM observation showed that GBR158-Rif+ bacterial cells proliferated abundantly, covering the surface of seeds and hypocotyls. In pot experiments, *P. lentimorbus* GBR158 significantly reduced the symptom development of the disease complex caused by *Fusarium oxysporum* f. sp. *lycopersici* and *Meloidogyne incognita* and increased growth of tomato plants. The control effect of bacterial treatment on disease complex was about 92% compared with untreated control. The seed treatment inhibited giant cell formation as no or a few (1–3) small giant cells were formed in plants treated with GBR158, whereas in untreated controls root galls contained 6–14 giant cells.

Key words: Biological control, disease complex, *Fusarium oxysporum* f. sp. *lycopersici*, *Meloidogyne incognita*, *Paenibacillus lentimorbus*, *P. polymyxa*, seed coating.

Root-knot nematodes, *Meloidogyne* spp., are sedentary endoparasites, attacking a wide range of crops worldwide. The infection starts with root penetration of second stage juveniles (J2) hatched in soil from eggs encapsulated in egg masses laid by females on the infected roots (Barker *et al.*, 1985). Wilt fungi (*Fusarium* spp.), deutromycetous fungus, causes wilting of the infected plants that leads to mortality. Infection of roots by root-knot nematodes predisposes plants to infection by soil-borne root-infecting fungi, resulting in the development of root-rot and wilt diseases (Armstrong *et al.*, 1976). Disease complexes in soil caused by root-knot nematodes and soil-borne fungal pathogens often damage plants more severely and render the disease control more difficult than single pathogens alone. The significant role of nematodes in the development of diseases caused by soil-borne pathogens has been demonstrated in many crops throughout the world (Griffin, 1986; France & Abawi, 1994; Abawi & Barker, 1984; Suleman *et al.*, 1997). In fact, nematode influence on Fusarium-wilt expression is so profound that wilt control in a number of crops is based upon concomitant root-knot nematode control. This appears to be necessary even if the crop involved is one of a Fusarium wilt-resistant cultivar (Powell, 1971).

During the last few decades, plant disease control has been based largely on the use of chemicals. However, most of the synthetic pesticides are banned due to their adverse effect on human health, their phytotoxicity, the toxic residual problems causing environmental and ground water pollution and the deleterious effects on the beneficial microorganisms in soil (Stirling, 1991). It is therefore necessary to develop alternative strategies for the control of diseases. One such alternative is biological control, in which microorganisms are selected for their ability to antagonize pathogens. A variety of soil microorganisms have been used to control soil-borne plant pathogens. Over the last decades, a great diversity of rhizosphere microorganisms has been described, characterized, and in many cases tested for their activity as biocontrol agents against soil-born pathogens. Researchers have studied various plant growth-promoting rhizobacteria (PGPR) strains for their potentials to increase the plant growth and yield, and to control the plant

pathogens (Kloepper *et al.*, 2004; Leeman *et al.*, 1995; McSpadden-Gardener, 2004, Weller, 1988).

Paenibacillus spp. are common soil bacteria belonging to PGPR. The *Paenibacillus* genus is currently the subject of many studies and its antibiotic activity has been confirmed against a wide spectrum of microorganisms (Nielsen & Sorensen, 1997; Piuri *et al.*, 1998; Seldin *et al.*, 1999; Dijksterhuis *et al.*, 1999; Beatty & Jensen, 2002; von der Weid *et al.*, 2003; Son *et al.*, 2007). Some *Paenibacillus* strains have been used for the biocontrol of plant disease (Mavingui & Heulin, 1994; Kim, 1995; Shishido *et al.*, 1996; Dijksterhuis *et al.*, 1999; Kharbanda *et al.*, 1999; Khan *et el.*, 2008), and have gained importance as antifungal biocontrol agents because of their ability to degrade chitin, the major compound present in fungal cell walls (Budi *et al.*, 2000; Chung *et al.*, 2000).

Application of these PGPR strains onto seed to enhance the seedling growth and to control the soil-borne pathogens is the most convenient and ideal method to introduce microorganisms to plant material instead of supplying greater amount of biomaterials to large area. Numerous instances can be listed showing the bacterial inoculation on plant seeds, which have achieved plant growth promotion and disease suppression (Turner, 1991). The efficacy of *Paenibacillus polymyxa* E681 for biocontrol and plant growth promotion was increased by sesame seed pellets compared to the treatment with strain E681 alone (Ryu *et al.*, 2006). The present study was aimed to evaluate the biocontrol potential of *P. polymyxa* GBR462 and *P. lentimorbus* GBR158 collected from rotted ginseng roots against the disease complex caused by *Meloidogyne incognita* and *Fusarium oxysporum* interactions in tomato.

MATERIAL AND METHODS

Nematode inoculum. Root-knot nematode, *M. incognita* race-1 was obtained from pure cultures maintained on tomato plants (*Lycopersicon esculentum*). Whenever nematode inoculum was required, plants were uprooted and 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. The eggs were incubated for 3-5 days using a modified Baermann funnel method to obtain second-stage juveniles (J2). The nematode inoculum was used for *in vitro* and pot experiments. The population density of nematodes was determined from five replications of 2 ml aliquots of the inoculum suspension and adjusted to the required density.

Fungal pathogen inoculum. Rice hull medium (rice hull:sand:distilled water, 1:4:1.5) was used for preparing inoculum of *F. oxysporum* f. sp. *lycopersici*. The medium was autoclaved for 20 min at 121°C. Mycelial plugs of *F. oxysporum* f. sp. *lycopersici* grown on potato dextrose agar (PDA; 26.5 g Difco potato dextrose broth, 18 g agar, 1 l sterile water) at 25°C for 7 days were inoculated into the rice hull medium. The inoculated medium was ground in a blender and used as the fungal pathogen inoculum.

Bacterial culture. Mutants of *P. polymyxa* GBR462 and *P. lentimorbus* GBR158 resistant to rifampicin, which were designated as GBR462-Rif+ and GBR158-Rif+, respectively, were obtained by culturing them on media supplemented with 100 ppm rifampicin for mutation. The mutants were cultured and stored at -70°C in sterilized distilled water with 20% glycerol until used. Each bacterium was grown in brain heart infusion (BHI) (CONDA, Madrid, Spain) broth at 28°C for 2 days with shaking at 200 rpm. Bacterial biomass was collected by centrifugation at 13000 g for 20 min, and the pellet was suspended again in distilled water and adjusted to 10^8 CFU ml^{-1} (OD_{600}= 0.8).

Seed treatment. The tomato seeds were soaked in a mixture of the bacterial suspension and 0.05% CMC (carboxymethyl cellulose sodium) for 1 h and air-dried for 12 h on filter paper at room temperature. Five seeds treated with GBR462-Rif+ and GBR158-Rif+ separately were placed on filter paper (Whatman № 2) on the bottom of Petri plate (11 cm diam.), which was poured with 5 ml sterile distilled water, and sealed with polypropylene wrap to maintain constant moisture inside the Petri plate. After 7 days of incubation at 28°C, the effects of seed treatments on seed germination and phytotoxicity were examined. Also population densities of GBR462-Rif+ and GBR158-Rif+ on germinated seeds were examined to know their establishment capacity on the plant root system. For this, hypocotyl segments of 1 cm from the tip were cut with a sterile surgical blade, macerated, and serially diluted in sterile distilled water. Colony forming units (CFU) on each of the hypocotyl segments were determined by plating the dilutions on the BHI agar added with 100 rifampicin for 2 days of incubation at 28°C.

Scanning Electron Microscopy. Effects of seed treatment with *P. lentimorbus* GBR158 on its establishment capacity on plant roots system was observed under the scanning electron microscope (SEM). After 7 days of treatment, hypocotyl segments of 1 cm from the tip cut with a sterile

surgical blade were fixed with Karnovsky's fixative in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffers at pH 7.2 (Karnovsky, 1965) at 4°C. After 12 h, the fixed specimens were washed three times with 0.05 M cacodylate buffer solutions for 15 min each. The specimens were fixed again in 1% OsO_4 and 0.05 M cacodylate buffers (pH 7.2) at 4°C for 4 h. The specimens were briefly washed again three times with distilled water. Then specimens were dehydrated in an ethanol series of 30, 50, 70, 80 and 90% for 10 min in each. Final exposure to 100% ethanol was repeated three times. Then the specimens were rinsed twice with 100% isoamyl acetate at room temperature for 10 min, each time. The specimens were critical-point dried and coated with gold with a Sputter Coater (JFC-1110E, JEOL, Tokyo, Japan). The specimens were observed under a scanning electron microscope (JSM-5410LV, JEOL, Japan).

Suppression of Fusarium wilt-root-knot disease complex. The results of the previous experiments showed that *P. lentimorbus* GBR158 proliferated abundantly and aggregated on tomato seeds and hypocotyls after seed treatment. Therefore, this strain was used for biological control of the disease complex caused by *F. oxysporum* f. sp. *lycopersici* and *M. incognita* on tomato plants.

The test of seed treatment with GBR158 for suppression of Fusarium wilt-root knot diseases was determined under the glasshouse conditions. Plastic pots, 6-cm-diam., were filled with 500 g sterilized sand and potting mixtures. Three-week-old tomato seedlings cv. Seon-myeong (susceptible to root-knot nematode) treated with GBR158 were planted into pots. Each plant was inoculated with *F. oxysporum* f. sp. *lycopersici* and J2 of *M. incognita*. The fungus was grown for 15 days in rice hull medium and 20 g of this medium was mixed with the top 5 cm of soil. The J2 suspension was dispensed with pipette at the rate of 1000 J2/pot. Plants grown in untreated pots served as control. Each treatment was replicated five times and repeated. Pots were arranged in a randomized block design on a bench in a glasshouse at 25±2°C and pots were watered daily to field capacity.

Seven weeks after inoculation of pathogens, plants were carefully uprooted from pots and the soil adhering to the roots was removed by gentle agitation in water. Severity of root galling 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). Wilt severity was graded using a 0-5 scale, where 0= no visible symptoms; 1= epinasty and chlorosis/wilting primary leaves; 2= chlorosis/wilting of 2nd and 3rd leaves, primary leaves may be lost; 3= wilting above 3rd leaves, 2nd and 3rd leaves may be lost; 4= chlorosis/wilting of whole plant and 5= plant completely desiccated (Marley, 1996).

Effect on giant cell formation. Root-galls caused by *M. incognita* and *F. oxysporum* f. sp. *lycopersici* interactions were taken from plants treated with *P. lentimorbus* GBR158 and untreated control plants and fixed in Karnovsky's fixative in 0.01M cacodylate buffer (pH 7.0) for 24 h. The root-gall samples were post-fixed with 0.01M osmium tetroxide in the same buffer solution for 2 h, 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 (RMC, Inc., Tucson, AZ, USA). The sections were stained with 1% toluidine blue in 30% ethanol and observed under a compound light microscope (Axiophot, Zeiss, Germany).

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 final analysis. Analyses of variance were 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 $P \leq 0.05$.

RESULTS

Characteristics of seed treatment. Seed germination, phytotoxicity, and bacterial population densities recovered from germinated seeds (hypocotyls) were examined to know the relevance of seed treatment with the bacterial strains, GBR158-Rif+ and GBR462-Rif+ for biological control. Germination rates of tomato seeds treated with both bacterial strains were as high as of untreated control, and there was no symptom indicating phytotoxicic effect (Table 1). However, the bacterial population densities recovered from GBR158-treated seeds (hypocotyls) 7 days after treatment were significantly higher (5.9×10^4 CFU/cm hypocotyl) than those treated with GBR462 (1.3×10^3 CFU /cm hypocotyl) (Table 1). The SEM observation of seeds and hypocotyls showed that GBR158 bacterial cells proliferated abundantly, covering their surfaces (Figs 1, 2).

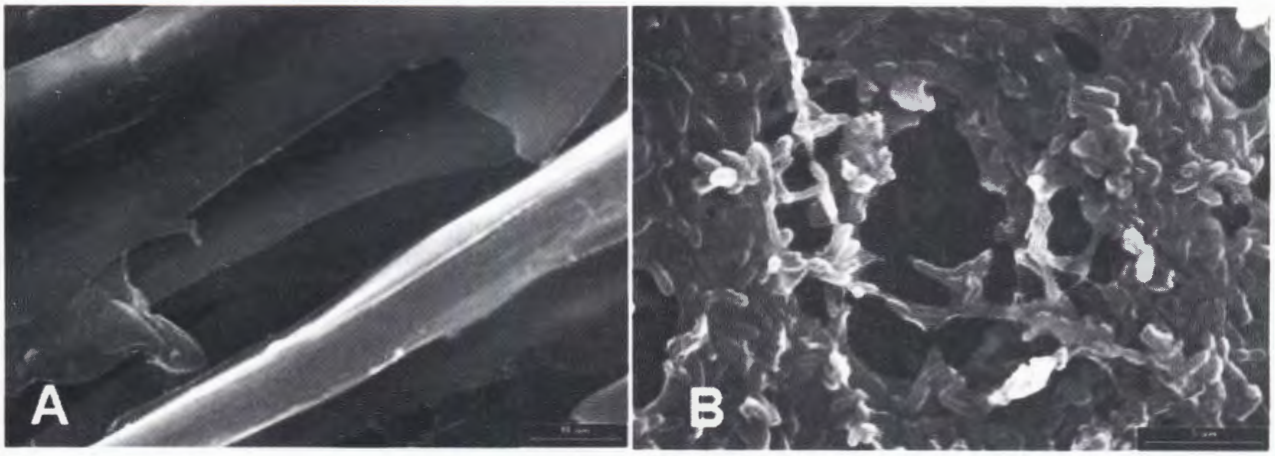

Fig. 1. Scanning electron micrographs of tomato seeds. A: untreated control; B: treated with *Paenibacllus lentimorbus* GBR158-Rif+, showing bacterial cells attachment.

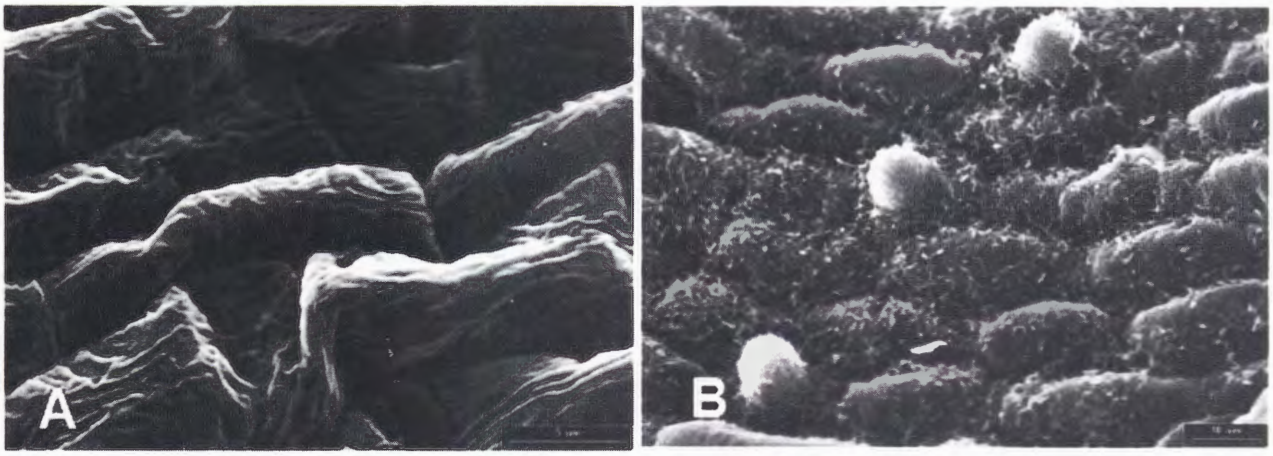

Fig. 2. Scanning electron micrographs of hypocotyls of tomato seeds. A: untreated control; B: treated with *Paenibacllus lentimorbus* GBR158-Rif+, showing bacterial cells attachment.

Suppression of Fusarium wilt-root-knot nematode disease complex. In a pot experiment under the glasshouse conditions, seed treatment with *P. lentimorbus* GBR158 reduced wilt severity caused by Fusarium wilt and root-knot nematode disease complex and root gall formation by *M. incognita*, and increased tomato plant growth as compared to the untreated control, especially showing significantly more shoot growth than untreated control as well as uninoculated healthy plants (Table 2 & Fig. 3). Wilt severity index was 3.6 and root gall index was 2.5 in tomato seedlings caused by combined infections of both pathogens in untreated control. However, those values were reduced to 0.3 and 0.2 when seeds were treated with GBR158, of which the control effects were estimated to be 91.7% and 92.0%, respectively. Untreated tomato plants infected by both pathogens showed vascular bundle decay of the stem, but treated plants did not show vascular decay.

Effect on giant cell formation. Root-galls caused by *M. incognita* and *F. oxysporum* f. sp. *lycopersici* in untreated controls and treated with *P. polymyxa* GBR158 were observed after 7 weeks of inoculation. The seed treatment inhibited giant cell formation as no or a few (1-3) small giant cells were formed in plants treated with GBR158 (Fig. 4A). By contrast, in untreated control root galls contained 6-14 giant cells in the stele, occurring mainly in the xylem area (Fig. 4B). The giant cells were characterized by hypertrophied cells and dense cytoplasm.

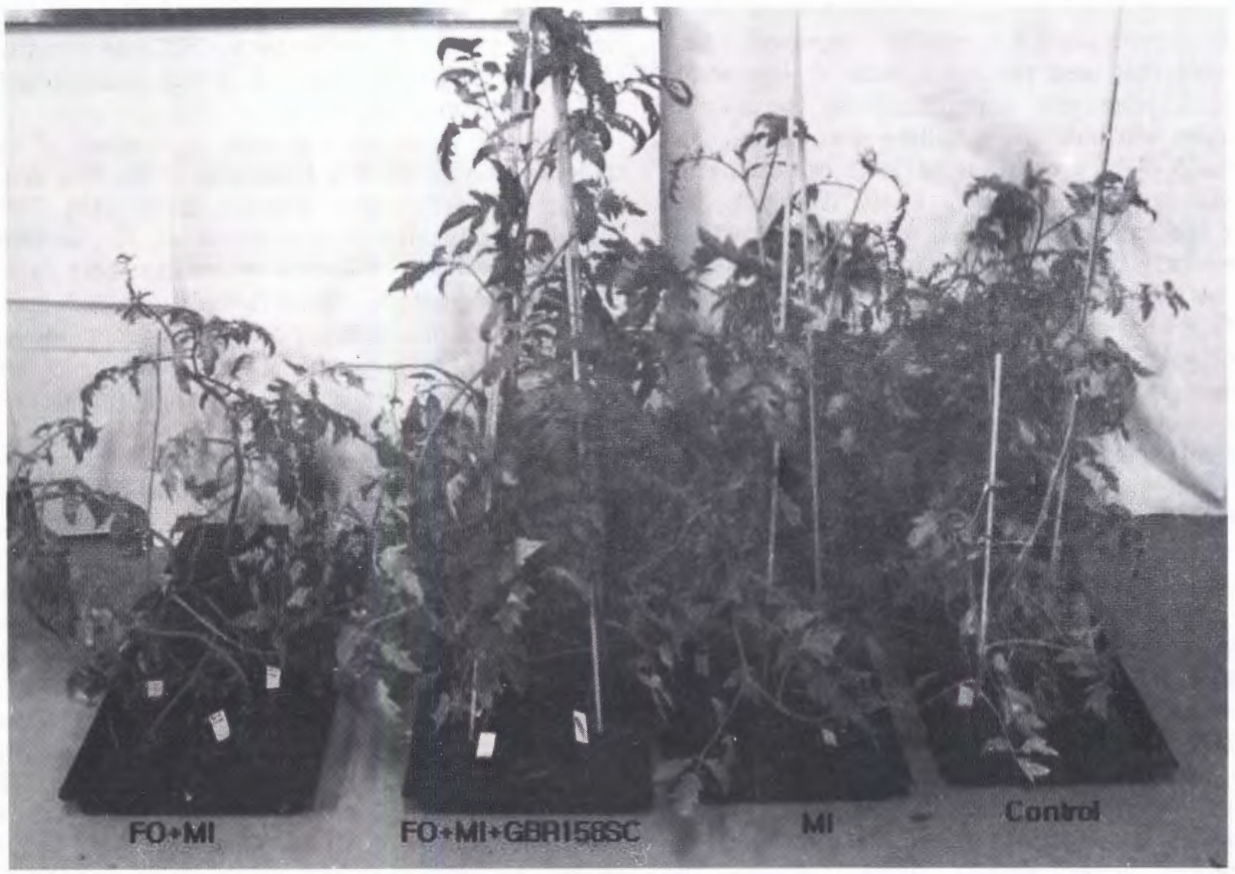

Fig. 3. Effect of seed treatment with *Paenibacillus lentimorbus* GBR158 on tomato seedlings infected with *Fusarium oxysporum* f. sp. *lycopersici* (FO) and *Meloidogyne incognita* (MI). FO+MI: inoculated with both pathogens; FO+MI+GBR158: inoculated with both pathogens and treated with *P. lentimorbus* GBR 158; Control: uninoculated and untreated control.

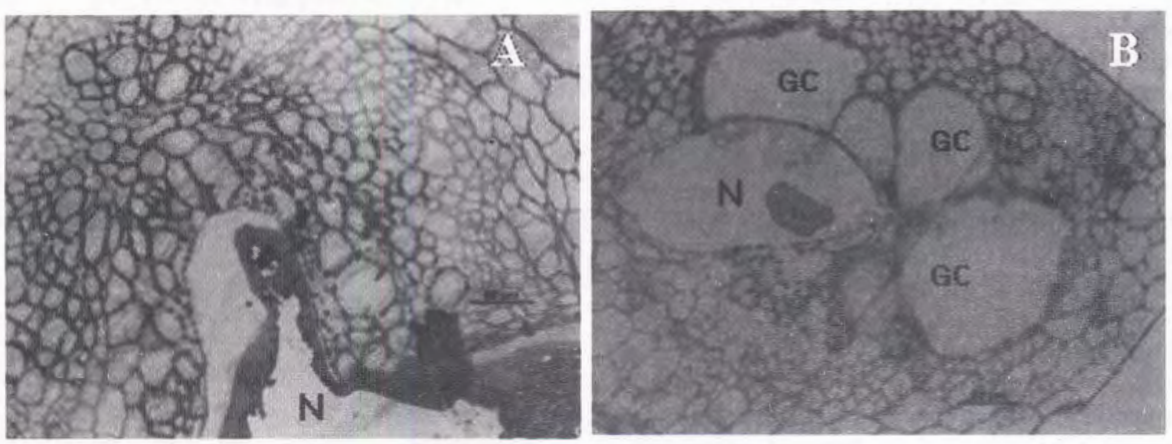

Fig. 4. Photomicrographs of giant cells induced in tomato roots by *Melodogyne incognita* (N) and *Fusarium oxysporum* f. sp. *lycopersici*. A: treated with *Paenybacillus lentimorbus* GBR158; B: untreated control, showing extensive giant cells.

DISCUSSION

The experimental results reported here indicated that seed treatment with *P. lentimorbus* GBR158 effectively suppressed the severity of Fusarium-wilt and root galling on tomato and increased plant growth. Root galls caused by *M. incognita* and *F. oxysporum* f. sp. *lycopersici* had fewer and smaller giant-cells when treated with *P. lentimorbus* GBR158 compared with untreated controls. From *in vitro* to pot experiments, GBR158 has been found to be a promising biocontrol agent, as well as an efficient promoter of plant growth. However, we could not define the exact mechanism of disease protection by GBR158 in these experiments. It can be hypothesized that the reduction of the disease complex may be attributed to direct effects of metabolites that inhibit hyphal growth and hatch of J2 and induce mortality of hatched J2. Alternatively, treatment may have enhanced the host defence mechanism in roots that reduces invasion and consequent infection by pathogens and inhibition of giant cell formation. The production of antibiotics and the competition for iron by the release of siderophores have been shown to be active mechanism for control of root infecting fungi (Weller, 1988). *Paenibacillus* strains produce antimicrobial substances active against fungi, bacteria and nematodes (Rosado & Seldin, 1993; Kajimura & Kaneda, 1996; Jung *et al.*, 2002, 2003; Son *et al.*, 2007). Earlier reports have shown that bacterization with *P. lentimorbus* B-30488R protected chickpea against subsequent inoculation with *F. oxysporum* f. sp. *ciceri*. In the presence of *F. oxysporum* f. sp. *ciceri*, bacterization resulted in significantly enhanced seed germination and decreased seedling mortality compared with nonbacterized seeds (DasGupta, 2006). Seed treatment with *P. plymyxa* strain reduced pre- and post-emergence damping off (Ryu *et al.*, 2006). A *P. lentimorbus* strain showed high level of insecticidal activity against some species of scarab beetle larvae (Yokoyama *et al.*, 2004). Spraying a suspension of CBCA-2, isolate of *P. lentimorbus,* on pruning wounds before inoculation with pycnidiospores of *Botryosphaeria dothidea* significantly reduced infection compared with the unsprayed, inoculated controls (Chen, 2003). There are reports where production of metabolites by rhizosphere bacteria causes lysis of nematode eggs (Wescott & Kluepfel, 1993), reduces hatching of J2 (Oostendrop & Sikora, 1989), affects vitality of J2 (Becker *et al.*, 1988; Son *et al.*, 2007) and degrade specific root exudates resulting in reduced attraction and penetration (Oostendrop & Sikora, 1990). Root colonization by rhizosphere bacteria has also been reported to reduce nematode invasion (Schroth & Hancock, 1981; Oostendrop & Sikora, 1989; Siddiqui & Ehteshamul-Haque, 2000; Siddiqui *et al.*, 2003).

Induced resistance can also be a result of root colonization by PGPR (Alstrom, 1991; Wei *et al.*, 1991, 1996). Recently Siddiqui & Shaukat (2003) have demonstrated that a strain of *P. fluorescens* induced systemic resistance in tomato roots against root-knot nematode. Some isolates of pseudomonas have shown biocontrol potential of root rot-root knot disease complex caused by root-knot nematodes and fusarium wilt fungi (Siddiqui & Ehteshamul-Haque, 2000; Siddiqui *et al.*, 2001).

The present study revealed that the seed treatment with bacterium had no adverse effect on seed germination but promoted plant growth. The plant growth promotion may be an indirect effect of the production of antibiotics through the suppression of plant disease. But there is also the possibility that GBR-158 may also have directly promoted plant growth by producing plant mimic hormone, as *Paenibacillus* species have been reported to produce many plant growth stimulators, including auxin, cytokinin and 2,3-butanediol (Lebuhn *et al.*, 1997; Nakashimada *et al.*, 2000; Ryu *et al.*, 2003; Timmusk *et al.*, 1999). Thus, the protective and nutritional properties of *P. lentimorbus* strains make it as useful tool to reduce deleterious impact of disease complex caused by root-knot nematode and fusarium wilt on plant growth, especially in organic farming system, where plant nutrition and disease control, including nematodes, are the main limiting factor.

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Seon-Hye Son, Khan Z., Sang Guy Kim, Young Ho Kim. Обработка семян ризобактериями *Paenibacillus* как эффективное средство борьбы с поражением томатов комплексом "галлообразующие нематоды – фузариоз".

Резюме. Обработка семян томата бактериями *Paenibacillus lentimorbus* GBR158-Rif и *P. polymyxa* GBR462-Rif не оказывает подавляющего воздействия на прорастание семян, а стимулирует рост растений. Бактериальные популяции на гипокотилях семян обработанных бактериями GBR158-Rif+, через 7 дней после обработки были достоверно выше (5.9×10^4 CFU/см гипокотиля), чем на растениях обработанных штаммом бактерий GBR462-Rif+ (1.3×10^3 CFU /см гипокотиля). Исследование в сканирующем электронном микроскопе показало акивное размножение бактерий штамма GBR158-Rif+, которые покрывали всю поверхность семян и гипокотиля. В экспериментах, проведенных в отдельных контейнерах с почвой, бактерии *P. lentimorbus* GBR158 существенно снижали проявление симптомов комплексного поражения томатов *Fusarium oxysporum* f. sp. *lycopersici* и *Meloidogyne incognita,* а также стимулировали рост растений. Общий эффект по сравнению с необработанным контролем составлял около 92%. Обработка семян подавляла развитие галлов, поскольку развитие гигантских клеток, индуцированных галловыми нематодами, снижалось до 1-3 или полностью прекращалось в растениях, обработанных GBR158, тогда как у необработанных растений галлы содержали по 6-14 гигантских клеток..