

The first report of *Bacillus pumilus* influence against *Meloidogyne javanica* in Iran

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Abstract: In this study two *Bacillus pumilus* including ToIrFT- KC806241 and ToIrMA-KC806242 were obtained from tomato fields and characterized based on phenotypic and molecular properties. Their possibility to reduce root knot disease of tomato caused by *Meloidogyne javanica* was evaluated. The experiments were done in lab and under pot conditions. Application of the ToIr-MA against *M. javanica* reduced the number of galls and eggs. Preliminary results indicated that the ToIr-MA has an ability to produce proteolytic enzymes in lab conditions. It seems that the bacterial culture filtrates can suppress egg hatching and increase juvenile's mortality, but the role of these metabolites in disease suppression needs to be investigated. Significant enhancement in root and shoot length (33%) and dry root and shoot weight (64 and 67%) was also recorded over usage of strain ToIr-MA in comparison to untreated controls. Additionally, survival of bacterial strains in rhizosphere and increases in population density were seen using root colonization assay. To our knowledge, this is the first time that such *B. pumilus* strain with nematicidal activity against *M. javanica* has been reported in Iran which may suggest to manage disease and change microbial population dynamics in the rhizosphere.

Keywords: Iran, Root knot disease, Tomato

Introduction

The tomato root-knot nematode (*Meloidogyne javanica*) is an important pest in several major tomato growing areas of Iran (Mahdikhani Moghaddam *et al.*, 2003). Among management methods, chemical control very limited due to the high expenses of nematicide application and negative environmental impacts (Padgham and Sikora, 2006). Root-knot nematode resistant tomato varieties have been developed in some countries, but are not very popular due

to their lower yields (Terefe *et al.*, 2009). Therefore environmentally safe and economically feasible root-knot nematode control practices should be used. Biological control is free from residual and adverse environmental effects (Sumeet and Mukerji, 2000).

The rhizosphere is relatively rich in nutrients due to the loss of up to 40% of plant photosynthates from the root. Rhizosphere microorganisms utilize compounds released from the crop roots. So, this area supports large and active microbial populations capable of exerting beneficial, neutral or detrimental effects on plant growth (Siddiqui and Mahmood, 1999). Among the rhizosphere microorganisms, *Pseudomonas* and *Bacillus* spp. have been recognized as

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the dominant populations that are able to antagonize nematodes.

Bacillus species can act not only as 'antagonists' or 'killers' by inhibiting phytopathogen growth but also as 'spreaders' by facilitating root colonization and as 'immuno-stimulators' by reinforcing host resistance potential (Ongena and Jacques, 2007). Several studies have reported the influence of *Bacillus* species on managing *Meloidogyne* population and controlling disease of different agricultural crops including tomato. Biological control is an efficient way to reduce root knot disease of tomato. Several *Bacillus* species such as *B. cereus* (Oka *et al.*, 1993), *B. subtilis* (Krebs *et al.*, 1998; Siddiqui, 2002; Xia *et al.*, 2011), *B. nematocida* (Niu *et al.*, 2007), *B. firmus* (Mendoza *et al.*, 2008; Terefe *et al.* 2009), *B. thuringiensis* (Wei *et al.*, 2003; Salehi jousani *et al.*, 2008) and *B. megaterium* (Huang *et al.*, 2010) have been shown to effectively control *Meloidogyne* species. However, few reports have been published that document the ability of *Bacillus pumilus* to control plant disease (Huang *et al.*, 2012).

B. pumilus is one of the best known species in industrial production of protease and lipase which are widely used in the food, chemical, washing detergent and leather industries. In recent years due to its producing antifungal antibiotics and chitinases, *B. pumilus* is known for its biocontrol activity against pathogens (Ahmadian *et al.*, 2007; Akbulut *et al.*, 2013). To our knowledge there is little report about the effectiveness of *B. pumilus* in biocontrol of plant pathogenic nematodes (Siddiqui *et al.*, 2007). The present study intends to evaluate *B. pumilus* against the root-knot nematode *M. javanica* by testing the effects of the bacterium on nematode egg hatching, juvenile motility, gall formation and also evaluate its effect on tomato growth. The ability of the bacterium to colonize tomato roots was also determined. This is the first report of application *B. pumilus* as biocontrol agent against root knot nematode in Iran.

Materials and Methods

Establishment of root-knot nematodes culture

Tomato plants infected with root-knot nematodes were collected from a vegetable farm in Khorasan Razavi province, Iran. Egg masses were picked off infected roots using forceps and a needle separately and then allowed to hatch. Juveniles were inoculated around the roots of tomato seedlings grown in sterilized soil in pots. Following nematode detection based on Jepson (1987), The *M. javanica* culture was raised on tomato plants cv. Early Urbana using a single nematode egg mass. This was sub cultured to maintain sufficient numbers of root-knot nematodes for subsequent experiments.

Bacterial isolates

The strains named ToIrFT- KC806241 and ToIrMA-KC806242 with significant nematotoxic activity were screened from a large collection of endospore forming rhizobacteria (Ramezani Moghaddam *et al.*, 2013) and identified as a *Bacillus pumilus* using a combination of phenotypic tests and phylogenetic analysis based on 16S rDNA sequences amplified by polymerase chain reaction (PCR) using two universal primers (Weisburg *et al.*, 1991). These two bacteria served as biocontrol agents in this study.

Preparation of cell free extract from rhizospheric microbes

Both isolated rhizobacterial strains were inoculated into 250 ml Erlenmeyer flasks containing 50 ml YPD (Yeast, Peptone, Dextrose) medium each and grown at 28°C on a rotary shaker at 220 rev min⁻¹ for 3 days. After centrifugation at 8500g for 15 min, the culture supernatants were collected and sterilized through 0.22 µm filter (Lian *et al.*, 2007). These were designated as undiluted standard cell free extracts which were used to study their effect on *M. javanica* eggs and juveniles.

Bioassay test of bacterial culture filtrate against *M. javanica* in lab conditions

Meloidogyne egg hatch and juvenile mortality in vitro assay was done according to Siddiqui

and Shaukat (2003). One ml of the filtrates were transferred into separate sterile Eppendorf tubes into which approximately 200 surface sterilized eggs or 50-100 juveniles were placed. Following 4 days exposure period, the hatched eggs were counted under a low power microscope (x10). The numbers of dead juveniles were also counted after 2-3 days. There were four replicates for each treatment. *Escherichia coli* and YPD medium served as controls. Whose ability to produce alkaline protease, protease and lipase were estimated according to Schaad et al. (2001).

Pot experiments under greenhouse condition

Seeds of tomato (cv. Early Urbana) were sown in sterile soil in plastic trays in the greenhouse at 25 ± 2 °C. A three-week old seedling was transferred to each pot, after one week, inoculation with bacterial suspension and nematode juveniles were done under greenhouse condition. The data were collected 45-days after treatment application. For preparation of nematode inoculums, the hatched juveniles were collected from the Petri plates every 24 h and fresh water was added to the Petri plates. The concentration of second stage juveniles of *M. javanica* in the water was adjusted so that each milliliter contained 100 ± 5 juveniles. Ten ml of both juveniles and bacterial suspension (10^6 cfu/ml) were inoculated into each 15 cm diameter pot around the tomato seedling (Siddiqui et al., 2007). Root gall severity was assessed on a 0-5 rating scale according to the percentage of galled tissue, in which 0 = 0 -10%, 1 = 11-20%, 2 = 21-50%, 3 = 51-80%, 4 = 81-90%, 5 = 91-100% (Barker, 1985).

Experimental design and statistical analysis

The experiment was carried out in a completely randomized blocked design. All statistical analyses were carried out using SPSS 16 software. Three replications were used for the growth chamber experiment. Critical differences were calculated at $P = 0.05$ and Duncan's

multiple range test was employed to test the significant differences between treatments.

Root colonization

Both ToIr-FT and ToIr-MA bacterial isolates are spontaneous rifampicin resistant. Root colonization was determined in non-sterile soil to screen their efficiency to survive on tomato roots. They were cultured on suitable growth media with 200 µg/ml rifampicin. Tomato seedlings were coated with bacteria by dipping the seedlings in 10^6 CFU ml⁻¹ bacterial suspensions. After one month, tomato roots inoculated with rhizobacteria were collected. Following that 1 g roots were crushed in sterile normal saline solution and 100 µl serially diluted extracts were plated on growth medium amended with 200 µg/ml rifampicin. After 2-3 days incubation, colonies falling within the 30–300 range on a Petri plate were selected and multiplied by a reciprocal dilution factor to obtain the bacterial colony number and represented as colony forming units (CFU) per g of root. Treatments and control were replicated four times.

Results

Characterization of *Bacillus pumilus* strains

The sequences for 16S rDNA of two strains presented 99% similarity with the 16S rDNA sequences of *B. pumilus* in NCBI. Sequence data of ToIrFT and ToIrMA, obtained in this study have been deposited in GenBank under accession numbers KC806241 and KC806242 respectively. The strains are maintained in nutrient broth amended with 40% glycerol in -80 °C.

Nematicidal activity of *Bacillus pumilus* strains

As shown in Table 1, strains ToIr-MA and ToIr-FT, which were identified as *Bacillus pumilus*, showed a considerable nematicidal activity in this test targeting *M. javanica*. In the bioassays, about 93.33 and 81.33 % of the tested nematode juveniles were killed within 48 h by ToIr-MA and ToIr-FT, respectively.

However, on the control with non-pathogenic *E. coli* and YPD medium, about 82% of the juveniles were still mobile after four days. Inhibition of nematode egg hatch was also recorded 83.3 and 80.6% for ToIr-MA and ToIr-FT, respectively and 13.2% for *E. coli*. The strain ToIr-MA showed significant juveniles mortality in comparison to ToIr-FT. It also was positive in production three types of enzymes including alkaline protease, protease and lipase, while ToIr-FT only produced protease and lipase.

Effect of *B. pumilus* on nematode infestation and growth of tomato plants in the greenhouse

Two tested strains significantly inhibited root knot nematode from producing disease in tomato plants grown in greenhouse experiments. The incidence of root knot disease in tomato plants (Table 1) treated with ToIr-MA and ToIr-FT was significantly

lower than the incidence of galls in control pots (Only nematode). In the untreated plants root gall severity was 3.33 whereas, significantly lower ($P = 0.05$) root gall severity was recorded in the plants treated with ToIr-MA and ToIr-FT isolates. Treatments with both isolates promoted tomato growth parameters including plant length and plant dry weight (Table 2) in comparison to untreated controls in the pot experiments (Table 2). Significant enhancement in root and shoot length (33%) and dry root and shoot weight (64 and 67%) was recorded over application of strain ToIr-MA in comparison to untreated control. Based on our data, in the case of infected control, the fresh weight of root is low. Negative effect of nematode on plant growth and production of small galls in pilot scale in comparison to field may be the possible reasons.

Table 1 Effect of culture filtrates of *Bacillus pumilus* on *M. javanica* in lab and greenhouse conditions.

Isolates	Mean of egg hatch (%)	Mean of juveniles mortality (%)	Root galling index	Egg mass
ToIr-MA	16.67	99.33	0	0
ToIr-FT	19.33	83.33	0.67	2
YDP medium	85.11	18.2	0	0
<i>E.coli</i>	86.89	19.32	nd*	nd
Infected control	nd	nd	3.33	75

*: Not determined

Table 2 *Bacillus pumilus* inhibition of tomato root knot disease (greenhouse experiments).

Treatments	Shoot weight (g)		Root weight (g)		Length (cm)	
	Fresh	Dry	Fresh	Dry	Root	Shoot
ToIr-FT	28.33 ^b	31.4 ^b	1.98 ^b	13.43 ^b	2.95 ^c	21.83 ^b
ToIr-MA	34.33 ^a	39.21 ^a	3.03 ^a	18.37 ^a	4.7 ^a	28.57 ^a
Healthy control	26.21 ^c	29.33 ^c	1.84 ^b	12.58 ^c	2.8 ^c	19.6 ^c
Infected control (Nematode only)	21.67 ^d	20.67 ^d	1.42 ^c	9.57 ^d	1.22 ^d	10.33 ^d

The data represent mean values of five independent measurements. Duncan's multiple range tests at $P = 0.05$ is used.

Root colonization

The results of the present study demonstrated that ToIr-MA and ToIr-FT strains could colonize and survive in tomato rhizosphere with the density of 2×10^8 and 0.7×10^7 CFU/ g of root respectively after one month. The strain ToIr-MA showed significantly better colonization ability.

Discussion

Native populations of *Bacillus* spp. occur abundantly in most agricultural soils and can promote crop health in a variety of ways. For example, they can suppress plant pathogens and pests by producing antibiotic metabolites, or can directly stimulate plant host defenses prior to infection and promote plant growth and health. Increased understanding of the nematotoxic mechanism of these antagonistic populations in the soil could potentially enhance the value of these species as effective biocontrol agents (Morton *et al.*, 2004).

In nematophagous fungi, it is believed that extracellular serine proteases are involved in several steps of the infection: releasing nutrients for pathogenic growth, facilitating penetration by degrading proteins of the cuticle, and digesting the host tissue. The bacterial proteases can also digest nematode cuticle (Ahman *et al.*, 2002) or degrade nematode juveniles and also reduce egg hatch (Mendoza *et al.*, 2008). Studies on the mode of infection and the function of protease enzyme from bacterium *Brevibacillus laterosporus* strain G4 which is involved in infection against nematode were done at molecular level (Tian *et al.*, 2006). It is proved that bacteria penetrate the nematode cuticle by enzymatic degradation and not by mechanical action as in fungi (Tian *et al.*, 2009). Bacterial culture of *B. megaterium* could significantly inhibit the hatch of eggs and reduce infection of the nematode through production of nematicidal volatiles (Huang *et al.*, 2010). Similar effects have been shown for secondary metabolites of this bacterium that reduced hatching of *Meloidogyne graminicola* in rice plants (Padgham and Sikora 2006).

The wide distribution of the serine cuticle-degrading protease belonging to *B. nematocida* (Niu *et al.*, 2005), *Bacillus* sp. (Lian *et al.*, 2007) and a neutral protease Npr219 from *Bacillus* sp. (Lian *et al.*, 2007) in nematophagous *Bacillus* suggested that proteases could serve as an important nematicidal factor in balancing nematode populations in different cultivations.

Two *Bacillus pumilus* strains (ToIr-MA and ToIr-FT) which were previously screened from a large collection of endospore forming bacteria (Ramezani Moghaddam *et al.*, 2013), have considerable inhibitory effect, in bioassay tests, on nematode juveniles and eggs. Preliminary results indicated that the ToIr-MA has an ability to produce proteolytic enzymes in lab condition. It seems that the bacterial culture filtrate can suppress egg hatching and increase juvenile's mortality, but the role of these metabolites in disease suppression needs to be investigated further. According to our findings, Application of the ToIr-MA against *M. javanica* reduced the number of galls, eggs and enhanced tomato growth in greenhouse condition. Additionally, survival of bacterial strain in rhizosphere and increases in population density was seen using root colonization assay. Increased understanding of the mechanism of action of *Bacillus pumilus* against nematodes could potentially enhance the value of the species as effective nematicidal agents that can be applied to manage diseases and change microbial population dynamics in the rhizosphere. To our knowledge, this is the first time that such *B. pumilus* strain with nematicidal activity against *M. javanica* has been reported in Iran.

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اولین گزارش از تأثیر *Bacillus pumilus* علیه *Meloidogyne javanica* در ایران

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چکیده: در این مطالعه دو جدایه از *Bacillus pumilus* به نام‌های KC806241-ToIrFT و ToIrMA-KC806242 از مزارع گوجه‌فرنگی استان خراسان رضوی جمع‌آوری و براساس ویژگی‌های فنوتیپی و مولکولی شناسایی شدند. توانایی این دو جدایه در کاهش بیماری ریشه گرهی در شرایط آزمایشگاه و گلخانه ارزیابی شد. کاربرد ToIr-MA علیه *M. javanica*، تعداد گال و تخم نماتد را در مقایسه با شاهد کاهش داد. آزمایشات اولیه توانایی قابل توجه ToIr-MA را در تولید آنزیم‌های پروتئولیتیک در شرایط آزمایشگاه مشخص نمود. به نظر می‌رسد متابولیت‌های مترشح از باکتری، توانایی جلوگیری از تفریح تخم و افزایش مرگ‌ومیر لارو نماتد را دارند. اما در ارتباط با نقش آنها در توقف بیماری، مطالعات بیشتری لازم است. افزایش چشمگیر طول ریشه و اندام هوایی (۳۳٪) و وزن خشک ریشه و اندام هوایی (۶۴ و ۶۷٪) به‌واسطه کاربرد ToIr-MA در مقایسه با شاهد دیده شد. علاوه بر آن در آزمون کلونیزاسیون، بقای جدایه مذکور در ناحیه ریزوسفر و افزایش جمعیت آن مثبت ارزیابی شد. براساس اطلاعات موجود، این اولین گزارش از فعالیت نماتدکشی *Bacillus pumilus* علیه *Meloidogyne javanica* در ایران می‌باشد. یافته‌های ما این جدایه را برای مدیریت بیماری و تغییر جمعیت میکروبی ریزوسفر پیشنهاد می‌کند.

واژگان کلیدی: ایران، بیماری ریشه گرهی، گوجه‌فرنگی