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Efficacy of *Bacillus thuringiensis* Cry14 Toxin against Root Knot Nematode, *Meloidogyne javanica*

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Abstract

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Two *Bacillus thuringiensis* strains including ToIr65 and ToIr67 with nematicidal activity against hatched juveniles and eggs of *Meloidogyne javanica* were identified by phenotypic, microscopic, 16s rDNA sequencing and nematode *cry* gene specific PCR. Two forms of bacterial isolates including bacterial suspension (BS) and spore/crystal mixture (SCM) were tested in lab and pot conditions to evaluate their efficacy in *M. javanica* management. The BS of ToIr65 and ToIr67 showed 70% nematicidal activity in comparison to SCM *in vitro*. In pot experiments, two forms of ToIr65 significantly (by 51%) decreased number of gall over infested control and also increased growth parameters on tomato plants, but ToIr67 did not. Our results suggested that Bt-ToIr65 could be employed as a biocontrol agent for the management of *M. javanica*.

Keywords: cry toxins; Iran; nematicidal; nematode management; tomato

Plant-pathogenic nematodes are a highly destructive group of plant pathogens worldwide. Among them, root-knot nematodes (RKN) are significant parasites of a broad range of crops, especially vegetables, causing severe yield losses mainly in tropical and sub-tropical agriculture (Sikora & Fernandez 2005) and are extremely challenging to control. Nematicide application was reduced due to the high cost and environmental concerns (Noling & Becker 1994). Therefore as an alternative method, using microbial substances has been suggested elsewhere for the management of root-knot nematodes. The beneficial microorganisms have an acceptable potential to control plant pathogenic nematodes in a safe way.

In Iran, vegetable production is threatened by several pests and parasites, including the root knot nematode, *Meloidogyne javanica* (Mahdikhani Moghaddam *et al*. 2003; Shokoohi *et al*. 2004). The common control method is limited to nematicide application which creates a hazard potential for humans and the environment in the area. However, other low risk techniques consisting in plant extracts usage (Sadeghi *et al.* 2012) and microbial treatments (Tavakol-Norabadi *et al*. 2013) were applied as an alternatives on a small scale.

Among rhizobacteria, *Bacillus* species is considered as antagonist of the phytopathogen and also promotes host resistance potential (Ongena & Jacques 2007). *Bacillus thuringiensis* (Bt) is a beneficial bacterium which produces large crystal proteins which are toxic to some invertebrates, mostly insects and nematodes, during their sporulation process (Wei *et al.* 2003).

This microorganism is found in many different habitats, particularly soils, due to its spore-forming capacity (Schnepf *et al.* 2005). Widespread screening of Bt strains and *cry* gene sequencing has led to the identification of more than 700 *cry* gene sequences (Crickmore *et al*. 2011). These sequences were categorised based on their amino acid sequence similarity in at least 70 different *cry* gene groups among which Cry5, Cry6, Cry12, Cry13, Cry14, and Cry21 displayed nematicidal activity (Bravo *et al*. 2012).

Several *in vitro* and *in vivo* experiments have been conducted to test the effectiveness of Bt strains against

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the hatching, motility, penetration, development, and reproduction of RKN (Zuckerman *et al*. 1993; Sharma 1994; Carneiro *et al*. 1998). Zuckerman *et al*. (1993) applied an efficient Bt isolate significantly reducing root galls on tomato plants. Peng *et al*. (2011) evaluated the use of a mixture of three nematicidal crystal proteins (Cry6Aa, Cry5Ba, and Cry55Aa) against *M. incognita* to select the best toxin combination for its management. They observed that the combination of Cry6Aa and Cry55Aa increased synergistic toxicity five times more if compared to separate toxicity against this nematode.

Isolation of *Bacillus* spp. from Iranian tomato fields was done previously. The potential of the selected isolates was determined in prohibition of *M. javanica* egg hatch and juvenile mortality under *in vitro* and greenhouse conditions (Ramezani-Moghaddam *et al*. 2013). Additionally, isolates containing nematode-active *cry* genes were tested for bioassays on *M. incognita* (Salehi-Jouzani *et al*. 2008).

Keeping in view the importance of root knot nematodes of vegetables and also significance of Bt as a biological control agent, *in vitro* and pot experiment studies were performed to evaluate the nematicidal potential of Bt isolates containing Cry14 protein against *M. javanica* on tomato plants.

MATERIAL AND METHODS

Bacterial strains. Several *Bacillus* spp. were previously isolated and identified by morphological and biochemical features during a survey in 2010–2012 from the tomato rhizosphere in different fields of Khorasan Razavi province, northeast Iran (Ramezani-Moghaddam *et al*. 2013). Five bacterial isolates similar to *Bacillus thuringiensis* colonies and showing a considerable nematicidal activity in primarily bioassay test targeting *M. javanica* larvae and eggs were subjected to further studies in this article. They were characterised as *Bacillus thuringiensis* based on phenotypic tests as described by Logan and De Vos (2006) as well as microscopic observation.

The reference isolates of *Bacillus thuringiensis* subsp. *sotto* (PTCC1383), B. *thuringiensis* subsp. *darmstadiensis* (PTCC1373), and B. *thuringiensis* subsp. *galleriae* (PTCC1375) obtained from the Persian Type Culture Collection were included for comparison in different tests.

Molecular identification and evaluation of cry gene content. Total DNA extraction was achieved following the method of Welington *et al.* (2004). Briefly, overnight bacterial culture in LB broth was centrifuged at 3000 *g* for 5 min and resuspended in 500 µl of TEN buffer (100 mM EDTA, 150 mM NaCl, 100 mM Tris-HCl, pH = 8.0) containing 20 mg/ml lysozyme. The suspension was incubated at 37°C for 45 min and following that, 25 µl of 8.5% SDS was added and incubated at 75°C for 30 min before the addition of potassium acetate (5 M, pH = 5.2). After passing 20 min at 4°C, the suspension was mixed with chloroform-isoamylalcohol and centrifuged at 13 000 *g* for 5 minutes. The supernatant was precipitated with isopropanol and washed with 70% ethanol. In the final step, the pellet was dried and re-suspended in 100 µl of distilled water.

For each bacterial isolate, partial 16S rDNA sequences were amplified with the primers fD1/rD1 (Weisburg *et al*. 1991). PCR amplifications were carried out in a thermal cycler (Applied Biosystems 2720; Applied Biosystems, Foster City, USA). The PCR products were visualized after electrophoresis on 1.2% agarose gels and sequenced using an automated sequencer ABI 3730XL (Macrogen Inc., Seoul, South Korea). The specific cry gene primers were used in order to identify the nematicidal *cry* genes possessed by each Bt isolate (Ejiofor & Johnson 2002). Each amplification process was carried out in a 25 µl reaction mixture containing 2.5 µl of 10X PCR buffer, 2.5 mM of $MgCl_2$, 200 µM of deoxynucleoside triphosphates, 10 pmole of each primer, 2.0 U of Taq DNA polymerase (CinnaGen, Tehran, Iran), and 3 µl of extracted DNA. PCR amplification was carried out using thermal cycler based on the following program: 5 min of denaturation at 94°C followed by 25 cycles of amplification with a 1 min denaturation at 94°C, 45 s of annealing at 45°C, and 2 min of extension at 72°C. An extra extension step of 10 min at 72°C was also added for a complete extension.

Root-knot nematode preparation. *M. javanica* was isolated from infected tomato farms in Khorasan Razavi province, Iran. It was propagated by subculturing on tomato (*Solanum lycopersici* cv. Mobile) in the greenhouse at 22–28°C. Nematode eggs were harvested from the root knot of infected tomato and disrupted in 1% sodium hypochlorite for 2 min by the procedure described by Hussey and Barker (1973). The eggs were collected and rinsed with tap water on nested 150- and 25-µm-pore sieves. To collect the second-stage juveniles (J2) for use as inoculum, the eggs were placed in hatching dishes and incubated in moist chambers in sterile distilled water

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at 25°C. Then the J2 were collected using pore sieves once a day for 3 days and used for further experiments.

Preparation of cell free extract and spore/crystal mixture. The five *B. thurigiensis* strains including ToIr65, ToIr67, ToIr73, ToTr75, and ToTr76 were grown at 30°C in 70 ml of Bt culture medium (Bactopeptone 7.5 g, glucose 1 g, KH_2PO_4 3.4 g, K_2HPO_4 4.35 g, distilled water to 1 l) with shaking at 250 rpm. Following autoclave at 120°C for 20 min, two salt solution including $MgSO_4 \cdot 7H_2O$ 2.46 g, $MnSO_4 \cdot H_2O$ 0.04 g, $ZnSO_4 \cdot 7H_2O$ 0.28 g, $FeSO_4 \cdot 7H_2O$ 0.4 g, distilled water to 100 ml, and $CaCI_2 \cdot 2H_2O$ 3.66 g, distilled water to 100 ml were first sterilised through a 0.22 µm filter, then added to the medium (Leyns *et al*. 1995). After 72 h, one fraction of bacterial suspension was centrifuged at 8500 *g* for 15 min, the culture supernatants were collected. The spore crystal mixtures were prepared according to Lecadet and Dedonder (1971).

In vitro bioassay. One ml of spore-crystal mixtures (SCM) of five Bt isolates plus reference strain PTCC1383 were transferred separately into glass cavity slides into which approximately 300 surface sterilised eggs or 100 juveniles per ml were placed. After 96 and 48 h the numbers of non-hatched eggs and dead juveniles were counted respectively. Assessment of each preparation involved four replicates. In the control slides, sterile water was added. *Bacillus thuringiensis* subsp. *sotto* (PTCC1383), which carries the *cry*14 gene, was applied in all tests.

Greenhouse bioassay. The experiment was conducted in a completely randomised block design in 20 cm diameter pots filled with steam sterilised sandy loam soil at 25 ± 2°C in greenhouse. Three weeks old tomato seedlings cv. Mobil were transplanted to the new pots for further experiments.

Ten ml of BS and SCM mixtures of two selected strains in addition to PTCC1383 were inoculated into each pot around the tomato seedlings one week after transplanting. After two days, each pot was inoculated with approximately 5000 nematode eggs by pouring the egg suspension into holes made 2–4 cm below the soil surface around the base of the plants. In the control pots, sterile water was added. Each treatment was done with four replicates per treatment. The experiments were evaluated 50 days after application of Bt isolates. Data on plant growth parameters including fresh and dry weight, root and shoot length of tomato plants were determined. Root gall severity was assessed according to Barker *et al*. (1985). The data were analysed statistically using SPSS 16 software. The treatments were grouped using Duncan's multiple range tests at the 0.05 significance level.

RESULTS

Identification of** Bacillus thuringiensis **isolates. Among the collection of *Bacillus* spp. (Ramezani Moghaddam *et al*. 2013), five bacterial isolates including ToIr65, ToIr67, ToIr73, ToTr75, and ToTr76 were characterised as *Bacillus thuringiensis* based on colony morphology, biochemical tests, microscopic analysis, and molecular methods. In all assays their features were compared to reference isolates. Microscopic studies showed that strains ToIr65 and ToIr67 have bipyramidal and cuboidal forms of parasporal inclusions and the remaining strains carry bipyramidal and rhomboidal ones. All the tested strains were also confirmed based on 16S rDNA sequencing. The nucleotide sequences published in this paper had been submitted to GenBank and assigned as accession numbers KF765934-38.

Establishment of the nematicidal effect of Bt isolates against** M. javanica **in vitro. As our previous data confirmed the *Bacillus* species, in the primary bioassay (Table 1) we examined the nematicidal activity of five spore/crystal mixture (SCM) of isolates against the juveniles and eggs of *M. javanica* in order to screen the most potent isolates. The first concentration of bacterial suspensions to make SCM was adjusted to 10^8 CFU/ml. In this assay, strains ToIr65 and ToIr67 SCM showed better efficacy in comparison to the three other isolates and controls

Table 1. Effect of spore-crystal mixtures of *Bacillus thuringiensis* isolates on juveniles mortality (%) and egg hatch prohibition (%) of *M. javanica in vitro* after 2 and 4 days, respectively

Isolates	Juveniles mortality	Nonhatched eggs
ToIr65	52.3 ± 2.2[a*]	43.2 ± 1.8[a]
ToIr67	43.4 ± 2.5[b]	32.7 ± 2.7[b]
ToIr73	23.5 ± 1.8[cd]	26.4 ± 1[cd]
ToIr75	25.4 ± 0.7[c]	28.8 ± 2.7[c]
ToIr76	21.3 ± 1.5[d]	25.5 ± 0.8[d]
PTCC1383	41.5 ± 2.3[b]	32.5 ± 1.3[b]
Sterile water	20.3 ± 1.0[d]	24.4 ± 1.8[d]

*means with the same letters are not significantly different from each other

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Table 2. Effect of bacterial suspension (BS) and spore-crystal mixtures (SCM) of *Bacillus thuringiensis* isolates on juveniles mortality and egg hatch prohibition of *M. javanica in vitro* after 2 and 4 days, respectively (in min)

Isolates	Juveniles mortality	Nonhatched eggs
ToIr65-BS	88.3 ± 1.5[a*]	73.7 ± 1.0[a]
ToIr65-SCM	52.3 ± 2.2[c]	43.2 ± 1.8[c]
ToIr67-BS	74.3 ± 0.9[b]	55.8 ± 1.7[b]
ToIr67-SCM	43.4 ± 2.5[d]	32.7 ± 2.7[d]
PTCC1383-BS	70.2 ± 1.6[b]	57.2 ± 0.8[b]
PTCC1383-SCM	41.5 ± 2.3[d]	32.5 ± 1.3[d]
Sterile water	20.3 ± 1[e]	24.4 ± 1.2[e]

*means with the same letters are not significantly different from each other

(P = 0.05). So we designed the second test which evaluated the efficiency of bacterial suspensions (BS) of ToIr65 and ToIr67 in addition to SCM. The BS of two selected strains showed more efficacies in comparison to SCM (Table 2). Application of BS showed 88.3 and 74.3% juveniles mortality, while that of SCM showed 52.3 and 43.4% juveniles mortality of *M. javanica* for ToIr65 and ToIr67 within 48 h, respectively. The effectiveness of the reference strain PTCC1383 was lower than that of the two representative strains. Application of BS and SCM of PTCC1383 caused 70.2 and 41.5% juveniles mortality, respectively.

Analysis of cry gene composition of Bacillus thuringiensis isolates. Only two strains (ToIr65 and ToIr67), which were isolated from Baghoon-abad village (Ghochan, north-east Iran), that were highly infected with *M. javanica*, harboured a *cry*14 gene while others did not give any PCR product when assayed with primers of the nematode-active genes. Strain PTCC1383 was used as positive control.

Greenhouse experiments. Based on previous steps, only two Bt isolates harbouring the *cry*14 nematicidal gene were potentially effective against nematode juveniles and eggs. So the influence of BS and SCM of ToIr65 and ToIr67 isolates was evaluated on growth parameters of tomato under greenhouse conditions (Table 3). After 50 days the SCM and BS of ToIr67 and PTCC1383 did not show significant difference at 5% probability, while the ToIr65-BS promoted root and shoot length of tomato by 28 and 25%, respectively, over untreated controls. In pot experiments usage of BS and SCM of ToIr65 caused enhancement in root dry weight (66 and 49%) and shoot dry weight (75 and 50%) respectively in comparison to controls. Isolate ToIr65 decreased gall index positively in comparison to other treatments.

DISSCUSSION

In this study, we evaluated two Bt isolates harbouringof *cry*14 gene for their nematicidal efficacy on *M. javanica* juveniles and eggs from tomato fields. Their possible effect on tomato growth parameters was also determined. Management of plant-parasitic nematodes involves reduction in nematode populations in the soil and roots without adverse effect on the plant yield (Salehi-Jouzani *et al.* 2008).

Many studies confirmed the toxicity of crystal proteins of *B. thuringiensis* against plant pathogenic and

Table 3. Influence of bacterial suspension (BS) and spore-crystal mixtures (SCM) of *Bacillus thuringiensis* isolates on growth parameters of tomato under greenhouse conditions

Treatments	Length (cm)		Root weight (g)		Shoot weight (g)		Root galling index
	root	shoot	fresh	dry	fresh	dry	
ToIr65-BS	35.2 ± 1.2[a]	33.0 ± 0.8[a]	16.0 ± 0.5[a]	2.9 ± 0.1[a]	25.5 ± 0.3[a]	3.5 ± 0.6[a]	2.1 ± 0.9[d]
ToIr65-SCM	33.4 ± 0.9[ab]	30.5 ± 1.1[ab]	15.4 ± 0.4[ab]	2.6 ± 0.1[b]	24.0 ± 0.8[ab]	3.0 ± 0.4[ab]	2.6 ± 0.3[cd]
ToIr67-BS	30.2 ± 1.4[b]	28.7 ± 1.0[b]	14.5 ± 0.2[b]	2.2 ± 0.09[c]	22.5 ± 0.3[b]	2.7 ± 0.1[b]	3.0 ± 2.3[b]
ToIr67-SCM	27.5 ± 1.2[bc]	26.0 ± 1.6[bc]	14.0 ± 0.6[b]	1.8 ± 0.4[d]	20.7 ± 0.2[bc]	2.0 ± 0.8[c]	3.3 ± 0.7[b]
PTCC1383-BS	30.4 ± 1.6[b]	27.4 ± 0.9[b]	14.2 ± 0.5[b]	2.0 ± 0.09[c]	21.5 ± 0.7[b]	2.4 ± 0.3[b]	2.9 ± 2.6[c]
PTCC1383-SCM	28.2 ± 0.8[bc]	26.0 ± 6.2[bc]	14.0 ± 0.6[b]	1.6 ± 0.8[d]	20.3 ± 0.2[bc]	1.9 ± 0.4[c]	2.8 ± 0.4[cd]
Healthy control	27.4 ± 1.0[bc]	26.2 ± 1.0[bc]	13.6 ± 0.3[bc]	1.74 ± 0.9[d]	20.3 ± 0.2[bc]	2.0 ± 0.3[c]	0.0 ± 0.0[e]
Infested control	20.7 ± 1.8[c]	22.6 ± 1.5[c]	14.7 ± 0.9[b]	2.1 ± 0.3[c]	11.3 ± 0.7[c]	1.4 ± 0.4[d]	4.3 ± 0.6[a]

[a–d]values followed by different letters in a column were significantly different (P = 0.05); numbers are average of four replications; root galling index was also determined

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free-living nematodes (Carneiro *et al*. 1998; Wei *et al.* 2003; Salehi-Jouzani *et al*. 2008). Cry protein exerts its effects by forming lytic pores in the cell membrane of gut epithelial cells. After ingestion of the toxin by target nematode larvae, the crystals dissolve within the gut of the nematode, and this is followed by proteolytic activation (Crickmore 2005). Recent study (Zhang *et al*. 2012) presented that some crystal protein can be able to enter the nematodes through the stylet. However, the toxicity mechanism may differ according to types of toxin and nematode species.

Two *B. thuringiensis* strains (ToIr65 and ToIr67) with nematicidal activity against hatched juvenile and eggs were identified by phenotypic, microscopic, and molecular methods. Remaining isolates (ToIr73, ToIr75, and ToIr76) did not amplify any bands with specific nematode cry primers. These isolates may contain other cry genes, as they all are characterised as *B. thuringiensis* based on 16S rDNA sequencing. We applied two fractions of ToIr65 and ToIr67 isolates including BS and SCM in lab and pot conditions to evaluate *M. javanica* management. The BS of ToIr65 and ToIr67 effect is evidenced by strong nematicidal activity in comparison to SCM. In accord with our data, some researches indicated that bacterial supernatant of Bt isolates can be able to kill free-living nematode *Caenorhabditis elegans* (Devidas & Rehberger 1992) and freshly hatched J2 of *M. javanica* within 24–48 h (Carneiro *et al*. 1998) and emphasised the effectiveness of Bt cell free extracts rather than crystal proteins. However others presented that the spore/crystal proteins of a few Bt strains could be effective against *M. incognita* (Mohammed *et al.* 2008; Salehi-Jouzani *et al.* 2008). In our survey, ToIr65-SCM also caused 52% larvae mortality and 43% prevention of egg hatch. So it seems that both exotoxin and endotoxin fraction should be considered in nematicidal studies.

The greenhouse obtained data demonstrated that the application of ToIr67 spore/crystal mixtures did not significantly improve the growth parameters of tomatoes. While two forms of ToIr65 application (BS and SCM) significantly increased growth promoting effects on tomato plants. Direct stimulation of Bt strains may include providing plants with fixed nitrogen, iron, soluble phosphate, and other nutrients, and the ability to produce right amounts of the plant hormones such as indole-3-acetic acid (Raddadi *et al*. 2007).

It seems that Bt-ToIr65 can reduce the nematode population through killing juveniles and prohibition of egg hatching. Our results revealed that ToIr65 strain can decrease number of gall significantly over the infested control. It may also have the potential as a biostimulator that could promote the plant growth simultaneously. Our results suggested that Bt-ToIr65 could be employed as a biocontrol agent for the control of the root-knot nematode *M. javanica*.

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