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Evaluation of Some Jordanian Bt Strains against Two Species of Root-Knot Nematodes.

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ABSTRACT

Four Jordanian strains of *Bacillus thuringiensis*, (Bt) i.e. *Bt jordanica*, *Bt kurstak*i, *Bt pakistani*, *and Bt thuringiensis*, and two reference strains *Bt israelensis* and *Bt kurstaki* were tested against root knot nematodes, *Meloidogyne incognita* and *M. javanica*. Exposing freed eggs or eggs within eggmasses to 10^6 viable spores/ml of each local strain resulted in either complete inhibition or few J2s hatching of both nematode species. Lower concentrations (10^5 viable spores /ml) were less effective in reducing egg hatching. Depending on the local Bt strains, 100% mortality of J2 of both nematodes occurred either after 2, 4 or 6 days of exposure to the higher spore concentration. Lower concentration of Bt strains was less effective but with a significant increase of mortality of J2s of both nematodes.

KEYWORDS: Bt, *M. javanica*, *M. incognita*, mortality, hatching.

INTRODUCTION

Root Knot Nematodes (RKN) belonging to the genus *Meloidogyne* are major pests of economic plants. Throughout the world, root knot nematode causes average annual yield losses of about 5% (Sasser, 1987). Several control measures are employed to suppress this pest in infested areas. Chemicals include fumigants and non-fumigants are the most common methods used to suppress root knot nematodes (Minton *et al.*, 1980; Walker and Watchel, 1988; Lamberti *et al.*, 2000). Although fumigants and non- fumigant nematicides are highly effective against RKN, they are maybe hazardous to the environment, expensive, and have harmful effects on humans if used inappropriately. Therefore, minimal usage of chemicals and/or alternative control methods should be employed. Biological control is such an alternative method, and furthermore, it forms an important component of Integrated Pest Management program (IPM).

The bacterium *Bacillus thuringiensis* (Bt) is a promising bioagent against root-knot nematodes (Osman *et al.* 1988; Sharma, 1994). This bacterium produces endotoxins during sporulation that offers a remarkable alternative to chemical pesticides since they are not toxic to vertebrates, are most benign to the environment, and could be genetically engineered into crops to provide constant protection (Marroquin *et al*., 2000). However, strains of Bt varied in their nematicidal effects (Sharma, 1994; Leyns *et al.*, 1995; Carneiro *et al.*, 1998). In the present study, evaluation of four Jordanian strains of Bt (*Bt kurstaki* (J6), *Bt thuringiensis* (J23), *Bt jordanica* (J112) and *Bt pakistani* (J139)) for their ovicidal and juvenile toxicity was assessed on local populations of the most two common species of root knot nematode, i.e. *M. incognita* and *M. javanica.*

MATERIALS AND METHODS

Inoculum of *Meloidogyne incognita* was collected

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from an infected fig tree in Jarash area, while that of *M. javanica* was collected from an infested cucumber field in Al-baqa'a area. Pure cultures were established for each species on roots of tomato plants cv. GS12 grown in sterilized soil. Populations were maintained on tomato plant in pots in the greenhouse at the University of Jordan. Free eggs were obtained from handpicked egg masses by using 0.5% solution of sodium hypochlorite (Barker *et al.*, 1985). Second stage juveniles were obtained by incubating handpicked egg masses in water at 25°C for 24 hours (Stirling *et al.*, 1986).

Four Jordanian *Bacillus thuringiensis* (Bt) strains, viz. *Bt kurstaki* (J6), *Bt thuringiensis* (J23), *Bt jordanica* (J112) and *Bt pakistani* (J139), were used in this study (Table 1). Two reference strains,i.e. *B. thuringiensis israelensis* (H14) and *B. thuringiensis kurstaki* (H3a, 3b, 3c) were obtained from the Pasteur Institute, Paris (Khyami-Horani *et al.*, 1996; Khyami-Horani, 2002). *B. thuringiensis* cultures were grown on nutrient broth (beef extract 10.0 g/L, peptone 10.0 g/L, and sodium chloride 5.0 g/L) to aid sporulation. Cultures were incubated on a rotary shaker (200 rpm) at 30^{o}C for four days to ensure sporulation and cell lysis. The lyses were insured by observing the bacterial cultures using phase contrast microscope. The pellet (4-5 g/ liter) was washed with distilled water and diluted to obtain 10^5 –10^6 viable spores/ ml, to be used on the different experiments.

Table(1):. Strains of endospore-forming bacilli, isolated from various sources in Jordan.

Bacillus thuringiensis **(Bt) strains**	**Serotype****	**Isolate**	**Source**	**Location**
Bt kurstaki (Btk)	H3a, H3b, H3c	J 6	Water	Al Khirbah Al- Samra
Bt thuringiensis (Btt)	H1	J 23	Chicken manure	Gawr Kabed, Jordan Valley
Bt jordanica (Btj)	H71	J 112	Soil	Jordan Valley
Bt pakistani (Btp)	H13	J 139	Water	Jordan Valley
Bt kurstaki (Btk)*	H3a, H3b, H3c		Reference strain *	
Bt israelensis (Bti)*	H1		Reference strain	

*Reference strains were obtained from WHO Collaborating Center for Entomopathogenic *Bacillus*, Institut Pasteur, Paris.

** Collection of *Bacillus thuringiensis* and *B. sphaericus* classified by H serotyping 1996. Unite des Bacteries Entomopathogenes Institut Pasteur International Entomopathogenic *Bacillus* Centre.

The ovicidal effect of Bt strains was conducted on free extracted eggs and on eggs within the egg masses. A total of 0.5 ml containing 100 extracted eggs were added to 4.5 ml of the tested aqueous bacterial suspension (10^6 and 10^5 viable spores/ml) of each local and reference Bt strains. Eggs with water alone were used as a control treatment. The treated and untreated eggs were incubated at 25˚C for six days. The number of hatched J2s was recorded every two days, and the percent of hatching was calculated.

The effect of Bt strains on egg hatching within egg masses was performed by placing five egg masses in 0.5 ml water and adding to 4.5 ml of each tested bacterial aqueous suspension (10^6 and 10^5 viable spores/ml). Egg masses with water alone served as a control treatment. The treated and untreated egg masses were incubated at 25˚C for six days. The number of hatched J2 was recorded every two days.

The effect of Bt on the mortality of J2s of root knot nematode was assessed by exposing J2s to two concentrations of Bt (10^6 and 10^5 viable spores/ml). Whereas a total of fifty J2s placed in 0.5ml water, were added to 4.5 ml of each tested aqueous bacterial suspension. J2s placed in 5 ml water served as a control treatment. Treated and untreated J2s were incubated for six days at 25 ºC. The number of dead J2s was recorded every two days. The percentage of J2 mortality was estimated.

Complete randomized design was used with four replicates for each treatment, analysis of variance (ANOVA) was performed. The Least Significant Difference (LSD) was estimated to separate means (Steele and Torrie, 1980).

RESULTS

Effect of Bt strains on J2 hatching from free eggs of *M. incognita*

All local and reference strains of *Bacillus thuringiensis*, Btk (J6), Btt (J23), Btj (J112), Btp (J139), Bti (Ref), Btk (Ref) reduced significantly the hatching of J2s from freed eggs at both concentrations at all tested exposure periods (Table 2). All strains completely inhibited the hatching when used at 10^6 at all incubation periods. After two days of egg exposure to 10^5 viable spores/ml Bt suspensions, the hatching percentages of J2 ranged from 6 to13. The most effective strain was the local strain Btk (J6) which caused a 94% reduction in hatching.

After four days of egg incubation with 10^5 viable spores/ml bacterial suspension, the percentages of hatching increased and ranged from 8 to 15. The local strain Btk (J6) with the lowest percentage of hatched J2s (8%) significantly differed from all other strains except for the local strain Btp (J139) which gave similar results.

The percentage of hatched J2s increased after six days of incubation with 10^5 viable spores /ml of Bt suspensions and ranged from 15 to 23.

Effect on J2 hatching from eggs within egg masses of *M. incognita*

All strains were more effective in reducing the number of hatched J2 from egg masses when used at 10^6concentration (Table 2). The strain Btj (J112) caused a complete inhibition of hatching of J2s from the exposed egg masses (up to six days of incubation).

The average number of J2s hatched from egg masses exposed for two days to the 10^5 concentration varied within strains and it ranged from 25 to 159. However, the number of these hatched J2s was significantly less than those from the untreated egg masses.

Except for Btp (J139), significant effects of the treated egg masses with the other five strains was observed when compared with the untreated egg masses. The lowest number of hatched J2s was recorded from those egg masses exposed to the strain Btt (J23) while the highest number was recorded from the local strain Btp (J139) (Table 2).

Table (2):. Effect of two concentrations of four Jordanian strains and two reference strains of *Bacillus thuringiensis* on hatching of J2s from free eggs and from eggs within egg masses of *Meloidogyne incognita* at different exposure period.

strain	concentration (viable spores/ml)	% hatched juveniles (J2s) from free eggs			Number of hatched juveniles (J2s) from eggs within egg masses		
		2 days	4 days	6 days	2 days	4 days	6 days
Btj (J112)	10^6	0 d	0 d	0 d	0 e	0 d	0 g
	10^5	10 b	15 b	15 c	49 cde	80 cd	113 defg
Btp (J139)	10^6	0 d	0 d	0 d	7 de	20 b	29 efg
	10^5	11 b	13 bc	16 c	159 b	242 ab	321 abc
Btt (J23)	10^6	0 d	0 d	0 d	14 de	23 d	25 efg
	10^5	12 b	15 b	19 bc	25 de	76 cd	191 bcdef
Btk (J6)	10^6	0 d	0 d	0 d	4 de	7 d	7 g
	10^5	6 c	8 c	20 bc	56 cde	97 cd	149 cdefg
Btk (Ref)	10^6	0 d	0 d	0 d	12 de	12 d	12 fg
	10^5	13 b	15 b	23 b	92 bcd	140 bcd	203 bcde
Bti (Ref)	10^6	0 d	0 d	0 d	55 cde	107 bcd	236 bcd
	10^5	10 b	14 b	19 bc	118 bc	193 bc	351 ab
Control	(water alone)	23 a	39 a	54 a	278 a	351 a	430 a
	LSD	4	4.7	6.1	88.8	143.1	180.6

Means within each column followed by same letters are not significantly different according to Duncan's multiple- range test (P= 0.05).

Effect of Bt strains on J2 mortality of *M. incognita*

All tested strains used at both concentrations significantly increased J2 mortality compared to the untreated J2s. There was variation in the mortality of J2 (35-100%) among the tested strains used at a concentration of 10^6 viable spores /ml. Btk (J6) and Btj (J112) caused a complete killing of J2s after two days of exposure. After four days of exposure, 100% mortality was observed for Btp (J139) and the reference strain Btk (Table 3).

The mortality of J2s exposed to the 10^5 viable spores/ml of Bt suspensions for four days ranged from 27-82%. The three strains Btj (J112), Btk (J6) and Btk (Ref) caused the highest J2s mortality and were significantly different to the other strains.

Increasing the exposure period of J2s for six days to the lower concentrations of Bt strains resulted in increases of mortality percentages and it ranged from 67 to 98. The mortality percentage in untreated J2s was 16.

Table (3): Effect of two concentrations of four Jordanian strains and two reference strains of *Bacillus thuringiensis* on mortality of J2s of *Meloidogyne incognita* at different exposure period

Strain	concentration (viable spores/ml)	% mortality of juveniles (J2s)		
		2 days	4 days	6 days
Btj (J112)	10^6	100 a	100 a	100 a
	10^5	58 b	76 b	89 c
Btp (J139)	10^6	94 a	100 a	100 a
	10^5	31 c	56 c	93 abc
Btt (J23)	10^6	86 a	99 a	100 a
	10^5	35 c	56 c	91 bc
Btk (J6)	10^6	100 a	100 a	100 a
	10^5	31 c	82 b	98 ab
Btk (Ref)	10^6	99 a	100 a	100 a
	10^5	50 b	71 b	87 c
Bti (Ref)	10^6	35 c	36 d	45 e
	10^5	15 d	27 de	67 d
Control	(water alone)	7 d	15 e	16 f
	LSD	13.7	13.2	6.9

Means within each column followed by same letters are not significantly different according to Duncan's multiple- range test (P= 0.05).

Effect of Bt strains on J2 hatching from free eggs of *M. javanica*

All local and reference strains of Bt, used at both concentrations, reduced significantly the hatching of J2 from freed eggs at all exposure periods (Table 4). Except the local strain Btp (J139), a complete inhibition of J2 hatching occurred at all incubation periods when eggs were treated with 10^6 viable spores /ml of the other five strains.

When the lower concentrations of the strains were used, hatching of J2s occurred and the percentage of hatching ranged from 3 to 27 at all incubation periods. The most effective strain after two days of exposure was Btj (J112) which reduced significantly hatch to 3%.

The number of hatched J2 increased with time; after four days of exposing the eggs to concentration of 10^5 viable spores /ml the hatching percentages ranged from 7 to 16 (Table 4). The reference strain Bti caused the lowest hatching percentage 7% with a significant difference, when compared with both Btt (J23), and Btp (J139) local strains. The hatching of J2 continued to increase after six days of incubating eggs with 10^5 viable spores /ml. The reference strain Bti caused the lowest hatching (7%) with a significant difference from all local strains (Table 4).

Results showed that the Bti (Ref) strain was the most effective while Btp (J139) was the least effective strain at the three incubation periods.

Effect of Bt on J2 hatching from eggs within egg masses of *M. javanica*

The application of 10^6 viable spores/ml of bacterial

suspension of all strains resulted in a significant reduction in the number of hatched J2s at each incubated period (ranged from 6-43%) (Table 4).

The average number of hatched J2s from treated egg masses with 10^5 viable spores /ml for two days ranged from 44 (Bti) to 92 (Btj (J112)) compared with 152 hatched J2s from untreated egg masses (Table 4). After four days of incubation, the number of hatched J2 increased and ranged from 109 (Bti) to 214 Btj (J112). Both Btp (J139), and the Bti reference strains significantly reduced the number of hatched J2s compared to those hatched from untreated egg masses (Table 4). After six days of incubation the average number of hatched J2s continued to increase and ranged from 172 to 346 in treated egg masses with a concentration of 10^5 viable spores /ml; while 382 J2s hatched from untreated egg masses (Table 4).

Table (4): Effect of two concentrations of four Jordanian strains and two reference strains of *Bacillus thuringiensis* on hatching of J2s from free eggs and from eggs within egg masses of *Meloidogyne javanica* at different exposure period.

Strain	concentration (viable spores/ml)	% hatched juveniles (J2s) from free eggs			Number of hatched juveniles (J2s) from eggs within egg masses		
		2 days	4 days	6 days	2 days	4 days	6 days
Btj (J112)	10^6	0 d	0 e	0 e	17 de	30 de	35 c
	10^5	3 cd	12 bc	25 b	92 b	214 ab	346 a
Btp (J139)	10^6	3 cd	3 de	4 de	14 e	21 e	43 c
	10^5	10 b	16 b	27 b	57 bcd	139 bc	212 b
Btt (J23)	10^6	0 d	0 e	0 e	8 e	20 e	25 c
	10^5	7 bc	16 b	19 bc	67 bc	159 abc	197 b
Btk (J6)	10^6	0 d	0 e	0 e	6 e	10 e	12 c
	10^5	9 b	12 bc	18 bc	78 bc	172 abc	281 ab
Btk (Ref)	10^6	0 d	0 e	0 e	20 de	30 de	30 c
	10^5	7 bc	10 bcd	13 cd	83 bc	187 abc	274 ab
Bti (Ref)	10^6	0 d	0 e	0 e	12 e	18 e	20 c
	10^5	4 bcd	7 cde	7 de	44 cde	109 cd	172 b
Control	(water alone)	22 a	35 a	52 a	152 a	226 a	382 a
	LSD	4.0	7.9	9.0	41.6	83.7	112.9

Means within each column followed by same letters are not significantly different according to Duncan's multiple- range test (P= 0.05)

Effect of Bt strains on J2 mortality of *M. javanica*

All local and reference strains used at 10^6 and 10^5 viable spores /ml, caused significant increase in J2s mortality at each incubation period (Table 5).

The local strains varied in their effects; after two days of exposure to the 10^6 viable spores /ml of Btp (J139) suspension, a complete kill of J2 occurred. While the strains Btj (J112), Btk (J6), Btk (Ref), Btt (J23), and the Bti (Ref) caused 98, 94, 90, 87, and 87% kill of J2, respectively. After four days of incubation, a complete kill occurred when J2 were treated with the higher concentration of Btj (J112), Btt (J23), and Btk (J6). After six days of exposure, the J2 mortality reached 100% in all strains.

When 10^5 viable spores /ml were used for each tested strain, the percentages of J2s mortality ranged from 10 to 15 after two days of incubation. After four days of exposure to 10^5 viable spores /ml, the J2s mortality increased, and ranged from 17 to 54. The highest percentage of J2 mortality 54% was obtained by the treatment with Btp (J139) local strain. The mortality percentage of J2s continued to increase after six days of exposure to the lower concentration of Bt suspension and ranged from 33 to 74.

Table(5): Effect of two concentrations of four Jordanian strains and two reference strains of *Bacillus thuringiensis* on mortality of J2s of *Meloidogyne javanica* at different exposure period.

strain	Concentration (viable spores/ml)	% mortality of juveniles (J2s)		
		2 days	4 days	6 days
Btj (J112)	10^6	98 ab	100 a	100 a
	10^5	12 e	36 d	70 b
Btp (J139)	10^6	100 a	100 a	100 a
	10^5	11 e	54 b	74 b
Btt (J23)	10^6	87 d	100 a	100 a
	10^5	10 e	44 c	74 b
Btk (J6)	10^6	94 bc	100 a	100 a
	10^5	11 e	52 bc	69 b
Btk (Ref)	10^6	90 cd	99 a	100 a
	10^5	14 e	17 e	33 c
Bti (Ref)	10^6	87 d	99 a	100 a
	10^5	15 e	46 c	70 b
Control	(water alone)	1 f	4 f	11 d
	LSD	6.3	8.2	9.7

Means within each column followed by same letters are not significantly different according to Duncan's multiple- range test (P= 0.05) .

DISCUSSION

In this *in vitro* study, all local and reference isolates significantly reduced the hatching of J2s of both *M. incognita* and *M. javanica* from free eggs. Bottjer *et al.* (1985) reported that treatment of the ruminant nematode *Trichstrongylus colubriformis* eggs with *Bt israelensis* toxin killed most hatched and unhatched larvae. Similar results were recorded by Bone *et al.* 1986 where no J2 hatched from eggs treated either with spores (14µg total proteins/ml) or with crystals (20 µg total proteins/ml) of *Bt israelensis*. Osman *et al.* (1988) reported that the application of the two Bt insecticides (0.05 gm / kg soil), SAN 415 and Dipel, reduced the percent of J2 hatching of the two nematodes *M. javanica* and *Tylenchulus semipenetrans*.

The results showed that the local strain Btk (J6) was more effective than the reference Btk strain in reducing the hatching of J2 of *M. incognita* at two and four days of incubations (Table 2). This might be explained by either that the local population of *M. incognita* is more susceptible to the local strain rather than the reference Btk strain, or the local strain of Btk (J6) is more virulent than the reference strain.

The effect on eggs within the gelatinous egg mass was studied because the eggs are naturally found in the soil or on infected roots within the gelatinous egg mass. Moreover, in this investigation, it was interested to test if the bacterial strains have an effect on the gelatinous matrix itself. When egg masses of both nematode species were treated with 10^6 viable spore/ml of local strains either completely inhibit or allow few juveniles to hatch occurred (Tables 2,4). Bt might affect the eggshell, embryonic development and/or the gelatinous matrix. Spiegel and El-Cohn (1985) reported that the gelatinous matrix of *Meloidogyne* species have chitin. Since the Bt strains produced chitinase, thus these bacteria were able to penetrate the gelatinous matrix and expose the eggs to the Bt toxins.

The lower concentration was less effective than the higher one. The number of hatched J2 exposed to the lower concentration of Bt increased with time of incubation, however, the local strains varied in their efficacy at each incubation period and also varied with the nematode species. The gelatinous matrix might act as a barrier for this low concentration to reach the eggshell.

All local Bt strains increased the mortality of J2 of both studied nematodes when used at both concentrations. 100 % mortality occurred when the

higher concentration of Bt strains was used. However, local Bt strains varied in their efficacy complete mortality with both nematode species and the exposure period. The lower concentration was also effective but less than the higher concentration. Similarly, Al Banna and Khyami-Horani (2004) indicated that exposure of J2s to 10^6 viable spores/ml of *Bt. pakistani* (H13) and *Bt. entomocidus* (H6) caused 100% mortality of J2s of both nematode species *M. javanica* and *M. incognita*, of nematode. In another study, Khyami-Horani (2002) reported that Jordanian Btt, Btj, and Btp caused 87, 84 and 84% mortality of laboratory populations of *Drosophila melanogaster* after 24 hours, respectively. On the other hand, the two reference strains Btk and Bti caused 100 and 86% mortality after two days of exposure, respectively.

The dead J2s inoculated with the Bt strains had distorted intestine. Similarly, Marroquin *et al.* (2000) reported that the inoculated *Caenorhabditis elegans* with *B. thuringiensis* toxin exhibited degenerated intestine.

Nematicidal endotoxins have been identified as Cry5B and Cry14A (Borgonie *et al.*, 1996; Marroquin *et al.*, 2000; Griffitts *et al.*, 2001). The *cry* genes of the local strains were investigated using PCR, and *cry*5 was found in all tested Bt strains (Khaymi- Horani, personal communication), and thus assured the efficacy of these strains against nematodes.

The local strains of Bt were effective against the two studied nematode species, however, certain strains were more effective either against eggs, egg masses or J2s at 2, 4, or/and 6 days of exposures. These observations suggested that strains varied in their virulence (ovicidal and J2s toxicity). Furthermore, the two tested species of root knot nematodes varied in their responses to the local strains of Bt, which ensures that either one nematode species is more susceptible to certain Bt strains or that Bt strains are more virulent on this nematode species.

Results of this *In vitro* study were promising and interesting. However, further studies are undertaken currently to investigate the ecological relationships, formulation, and application of these Bt strains under greenhouse and field conditions.

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تقييم تأثير بعض سلالات أردنية من *Bacillus thuringiensis* (Bt) على نوعين من نيماتودا تعقد الجذور

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ملخـص

أجريت تجارب مخبرية لتقييم مدى فعالية أربع عزلات أردنية وعزلتين مرجعيتين من البكتيريا *Bacillus thuringiensis* على عمليتي فقس و موت الطور اليرقي الثاني في نوعين من نيماتودا تعقد الجذور *Meloidogyne incognita* و *M. javanica*. العزلات الأردنية المستخدمة هي*Bt kurstaki* و*Bt thuringiensis* و*Bt jordanica* و*Bt pakistani*، أما العزلات المرجعية فهي *Bt kurstaki* و *Bt israelensis*. أظهرت النتائج أن تعريض البيض الحرأو أكياس البيض من نوعي النيماتودا للتركيز المرتفع (10^6 جراثيم حية /مل) من جميع العزلات أدى إلى تثبيط عملية الفقس. وكان التركيز المنخفض (10^5 جراثيم حية /مل) اقل فعالية .تفاوتت العزلات المحلية في زمن(2- 6 أيام) لإحداث الموت بنسبة 100% ليرقات الطور الثاني عند تعرضها للتركيز المرتفع من المعلق. التركيز المنخفض للعزلات كان اقل كفاءة ولكن بحدوث زيادة معنوية في نسبة الموت.

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