NEMATICIDAL ACTIVITY OF TWO JORDANIAN STRAINS OF *BACILLUS THURINGIENSIS* ON ROOT-KNOT NEMATODES

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Summary. The effects of two local strains of *Bacillus thuringiensis*, *Bt. pakistani* (H13) and *Bt. entomocidus* (H6), on hatching and mortality of two root knot nematode species, *Meloidogyne incognita* and *M. javanica*, were studied under laboratory conditions. Two concentrations, 10^5 and 10^6 viable spores/ml, of each *Bt* strain were tested. Exposure of juveniles to 10^6 viable spores/ml caused 100% mortality of juveniles of both species of nematode. Similarly, significant reduction of hatch was observed from eggs within the gelatinous matrix or freed eggs exposed to 10^6 viable spores/ml of both *Bt* strains. At the lower concentration of *Bt* spores, significant reduction in hatch was detected with freed eggs of *M. incognita* but not with eggs of *M. javanica*. No reduction of hatch from egg masses of either species was found after exposure to 10^5 viable spores/ml.

In Jordan, there are two dominant species of root knot nematodes, *Meloidogyne javanica* (Treub) Chitw. and *M. incognita* (Kofoid *et* White) Chitw. *Meloidogyne incognita* is commonly found in the northern Jordan Valley and Jerash, while *M. javanica* exists predominantly in the middle and southern Jordan Valley. These two species cause an average annual loss of about 10-15 % of the vegetable crops grown in these regions (Abu-Gharbieh, 1994).

Chemical control is the most common method used to suppress root knot nematodes. Chemicals used in Jordan include fumigants such as methyl bromide, and non-fumigant nematicides such as fenamiphos, aldicarb, carbofuran, and oxamyl. However, due to the hazard posed by these chemicals to the environment and to humans, there is a global trend to find alternatives to these chemicals, using an integrated pest management (IPM) approach (Kerry, 1990). Biological control forms an integral part of IPM, and includes the use of fungi (Holland *et al.*, 1999) and bacteria (Davies *et al.*, 1988) to control plant parasitic nematodes. Among the bacteria used are the obligate endoparasitic bacterium *Pasteuria penetrans* (Davies *et al.*, 1988) and the free-living *Bacillus thuringiensis* (Zuckerman *et al.*, 1993).

Bacillus thuringiensis (*Bt*) is a gram-positive bacterium commonly found in natural soils and insect-rich environments. Upon sporulation, *Bt* produces one or more parasporal crystalline proteinaceous inclusions, the δ-endotoxins, (Schnepf *et al.*, 1998). Most of these crystal proteins are toxic to larvae of lepidopteran, dipteran, and coleopteran insects. *Bt* strains exhibiting toxicity against pathogenic protozoa, mites, and nematodes have also been reported (Schnepf *et al.*, 1998). The nematicidal activity of *Bt* strains has mainly been observed against eggs and, exceptionally, the first juvenile stage of animal parasitic nematodes (Meadows *et al.*, 1989) and free-living nematodes (Meadows *et al.*, 1990). *Bt* toxins offer a remarkable alternative to chemical pesticides since they are not toxic to vertebrates, are most benign to the environment, specific to single orders of insects, and could be genetically engineered into crops to provide constant protection (Marroquin *et al.*, 2000).

There are limited reports on the use of *Bt* against root-knot nematodes (Sharma, 1994; Carneiro *et al.*, 1998) but, in Jordan, no information exists about the use of such microbes to control these agronomically important nematode species. Therefore, the nematicidal activity of two Jordanian strains of *Bt* (J107, subsp. *pakistani*, and J115, subsp. *entomocidus*) was studied on local populations of *M. incognita* and *M. javanica*.

MATERIALS AND METHODS

Nematode cultures were established from single egg masses. *Meloidogyne incognita* was collected from a fig orchard in the Jerash area, whereas *M. javanica* was obtained from cucumber grown in plastic tunnel houses in the Baqa area. Both populations were propagated and maintained by periodic subculturing on tomato plants (*Lycopersicon esculentum* Mill.) in a glasshouse at the University of Jordan at 25 ± 5 °C. For *in vitro* studies, egg masses of *M. incognita* and *M. javanica* were collected from the stock cultures maintained on tomato plants.

Bacillus thuringiensis Berliner strains *Bt. pakistani* De-Barjac *et al.* (H13) and *Bt. entomocidus* Heimpel *et* Angus (H6) (assigned numbers J107 and J115, respectively) were isolated from tomato fruits and lentil seeds, respectively (Khyami-Horani, 2002). The strains were grown in nutrient broth supplemented with 1 ml/l of mineral salt solution (sodium chloride) to aid sporulation. Cultures were incubated on a rotary shaker at 200 rpm and 30 °C for four days to ensure sporulation and cell lysis. Spores and crystals were harvested by centrifugation at 10,000

rpm for 10 min at 4 °C. The pellets were washed with deionized water and then spore-crystal suspensions were diluted to 10^5 and 10^6 viable spores/ml. These dilutions of both *Bt* strains, J107 and J115, were used in the following experiments.

Nematicidal activity of the spore-crystals of both *Bt* strains was determined by adding 4.5 ml aliquots of the different bacterial dilutions (10^5 and 10^6 viable spores/ml) to glass wells containing 0.5 ml of aqueous suspensions of 50 second stage juveniles (J_2s). Control wells were supplemented with 4.5 ml of water. The number of dead J_2s was counted every 48 hours up to six days and the percentage of mortality was determined.

Egg masses were washed in distilled water before exposure to the bacterial suspensions. Three egg masses from each nematode species were exposed to 5 ml of each spore concentration. Egg masses placed in 5 ml water served as controls. The number of hatched J_2s was recorded every 48 hours for a total of six days.

A total of 100 eggs from each nematode species were exposed to 5 ml of each bacterial spore suspension for six days. Eggs were released from the gelatinous matrix by using sodium hypochlorite (Barker *et al.*, 1985). One hundred eggs placed in 5 ml water served as a control. Numbers of hatched J_2s were recorded every 48 hours for a period of six days and the proportions of hatched eggs determined.

All experiments were done at 25 ± 1 °C using completely randomized designs with three replicates for each treatment. Analysis of variance (ANOVA) was performed and the least significant difference (LSD) was used to separate the means (Steel and Torrie, 1980).

RESULTS

Two days exposure of J_2s of both *M. incognita* and *M. javanica* to 10^6 viable spores/ml of the two bacterial strains caused 100% mortality (Fig. 1A and 1B). Ninety and 92% of J_2s of *M. incognita* were killed when exposed for two days to 10^5 viable spores/ml of *Bt entomocidus* (J115), and *Bt pakistani* (J107), respectively. Mortality reached 97% after four days and did not change at six days of incubation with *Bt* strain J115, while mortality of J_2s remained at 92% after six days when exposed to *Bt* strain J107 (Fig. 1A). Similarly, 95% and 97% of J_2s of *M. javanica* were killed after two days of exposure to 10^5 viable spores/ml of strain J107 and J115, respectively, but no further increase in mortality was observed by the time the experiment was terminated (Fig. 1B). Microscopic examination of the dead J_2s that were exposed to *Bt* strains of both nematode species revealed that these J_2s exhibited degenerated intestinal morphology.

The hatching of eggs was completely inhibited when egg masses of *M. incognita* were exposed to the higher concentration of strain J107, while only six eggs hatched when exposed to strain J115 throughout the duration of the experiment (Fig. 2A). Greater numbers of eggs hatched when egg masses were incubated in the lower concentration of both *Bt* strains (Fig. 2A). At two days after exposure, the number of hatched eggs from egg masses treated with 10^5 spores/ml was not significantly different from those hatched from untreated egg masses, but at four and six days after exposure significantly more juveniles hatched from exposed eggs than from the control.

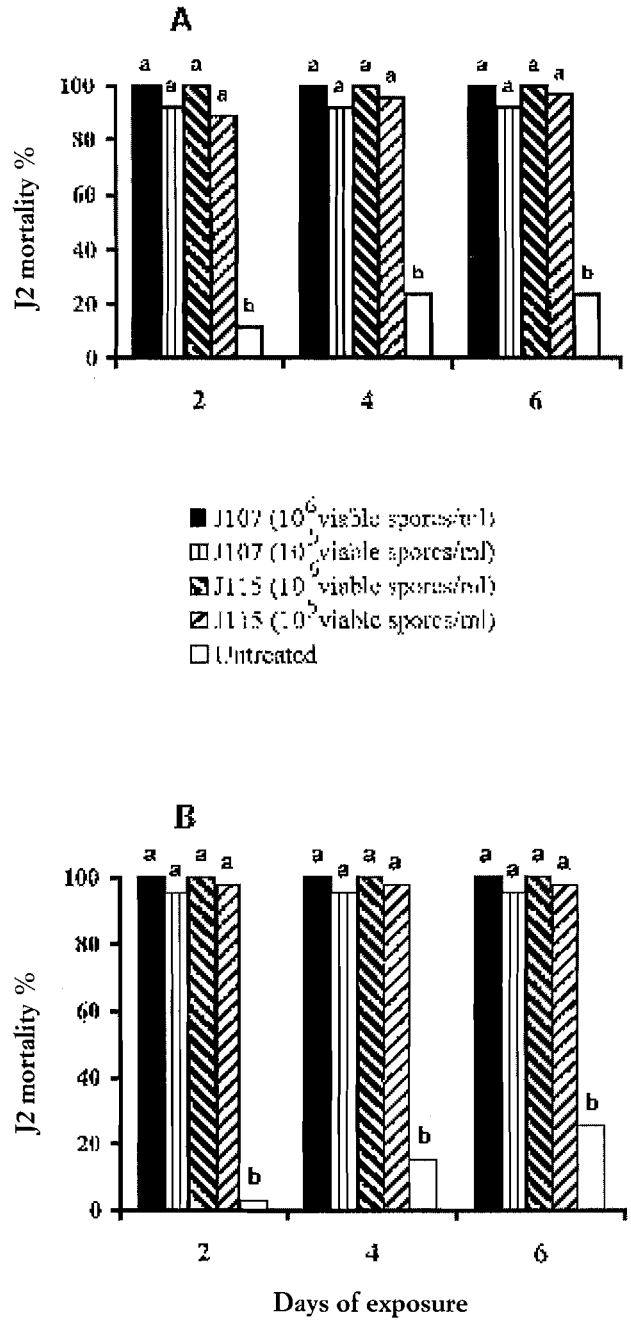

Fig. 1. Effect of two concentrations (10^6 and 10^5 viable spores/ml) of two Jordanian strains of *Bacillus thuringiensis*, *Bt entomocidus* (J115) and *Bt pakistani* (J107) on J_2 mortality (%). A, *Meloidogyne incognita*. B, *M. javanica*. Bars labelled with the same letter do not differ significantly (P = 0.05).

In contrast to *M. incognita*, hatch of *M. javanica* was observed from egg masses exposed to the higher concentration of both *Bt* strains but the number of hatched J_2s was significantly lower than from the untreated controls (Fig. 2B). The use of the lower concentration of the two *Bt* strains did not result in a significant reduction of *M. javanica* J_2 hatch compared to untreated controls but the higher concentration did (Fig. 2B).

Complete inhibition of hatch was observed from free eggs of *M. incognita* exposed to 10^6 viable spores/ml of *Bt* strain J115 over the period tested (Fig. 3A). Similarly, significant inhibition was found with strain J107 at this concentration, with only 1% hatch over the six days tested (Fig. 3A). Significant reduction of hatch was also detected at the lower concentration for both *Bt* strains, but the effect was less than for the higher concentration (Fig. 3A).

Similar to its effect on *M. incognita*, *Bt* strain J115 at 10^6 spores/ml caused complete inhibition of hatch of *M. javanica* J2s from free eggs (Fig. 3B). Also, very few J_2s hatched (0.3% and 2%) from eggs exposed to 10^6 spores of *Bt* strain J107 after four and six days of exposure, respectively (Fig. 3B). At the lower concentration, strain J115 significantly decreased the hatch of J_2s after two and four days of exposure but not at six days. The use of the lower concentration of the strain J107 did not result in a significant decrease in the hatch rate during the three periods tested (Fig. 3B).

DISCUSSION

The two Jordanian strains of *Bt* used in this study were very effective against J_2s of both *M. incognita* and *M. javanica*. Complete kill or more than 90% mortality of J_2s was observed after two days of exposure to 10^6 and 10^5 viable spores/ml, respectively. Similar results were obtained by Abu-Dhaim (2002), who reported that four Jordanian strains of *Bt*, *Bt jordanica* (J112), *Bt kurstaki* (J6), *Bt pakistani* (J139), and *Bt thuringiensis* (J23) caused 99, 100, 94, and 86% mortality of J2s of *M. incognita* after two days of exposure to 10^6 viable

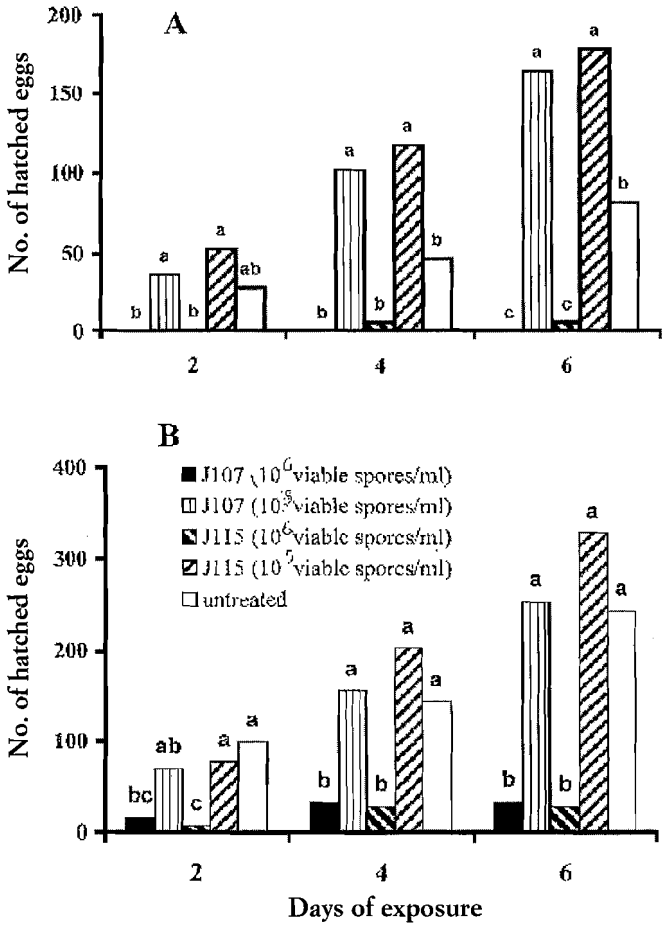

Fig. 2. Effect of two concentrations (10^6 and 10^5 viable spores/ml) of two Jordanian strains of *Bacillus thuringiensis*, *Bt entomocidus* (J115) and *Bt pakistani* (J107) on hatching of eggs from egg masses. A, *Meloidogyne incognita*. B, *M. javanica*. Bars labelled with the same letter do not differ significantly (P=0.05).

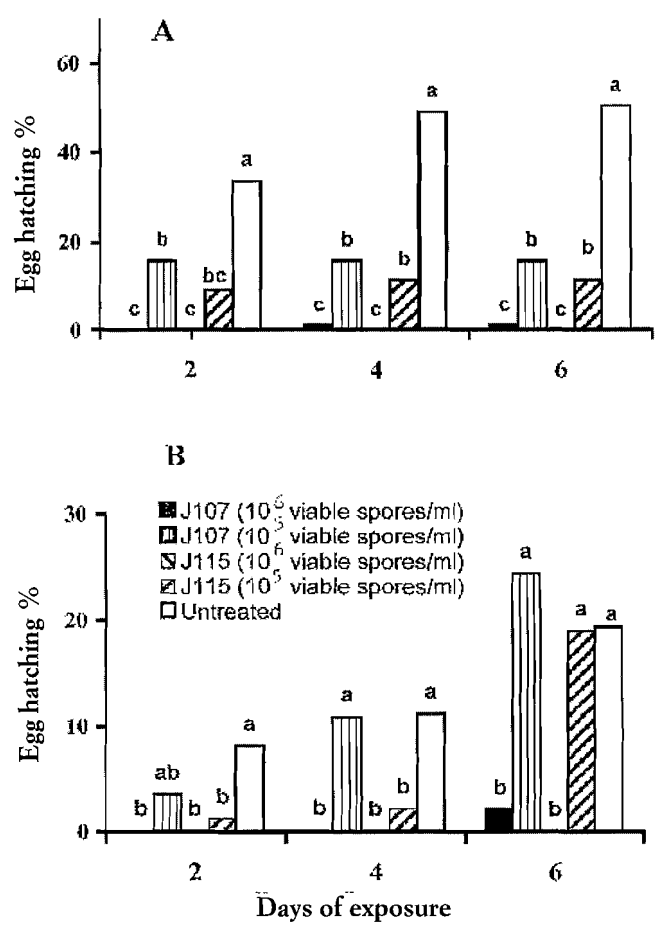

Fig. 3. Effect of two concentrations (10^6 and 10^5 viable spores/ml) of two Jordanian strains of *Bacillus thuringiensis*, *Bt entomocidus* (J115) and *Bt pakistani* (J107) on hatching of eggs (%). A, *Meloidogyne incognita*. B, *M. javanica*. Bars labelled with the same letter do not differ significantly (P=0.05).

spores/ml, respectively. These strains caused 97, 94, 100, and 87% mortality of J_2s of *M. javanica* after two days of exposure to the same concentration of spores. Carneiro *et al.* (1998) tested 21 strains of *Bacillus* spp. against J_2s of *M. javanica* and found that strains of *Bt brasiliensis* and *B. laterosporus* caused high mortality, whereas *B. circulans, Bt aizwai,* and *Bt kurstaki* caused immobilization only. In addition, the efficacy of three strains of *B. thuringiensis* (289A, 958B, and 18247) against juveniles and adults of *Caenorhabditis elegans* has been reported at concentrations of 10^8 and 10^9 particles/ml, but not at 10^7 particles/ml (Leyns *et al.*, 1995).

In our experiments, J_2s killed by exposure to the *Bt* spores had distorted intestines. Similar observations have been reported for various species of nematodes exposed to *Bt* spores or toxins. Bottjer and Bone (1987) reported degenerated intestinal cells in dead juveniles of the ruminant nematode *Trichostrongylus colubriformis* treated with *Bt israelensis*. Marroquin *et al.* (2000) also observed degeneration, pitting and vacuolation of intestines of *C. elegans* two days after inoculation with *Bt* endotoxins. These endotoxins were described as Cry5B or Cry14A protein (Griffitts *et al.*, 2001). However, *Cry5* genes were detected in the Jordanian strains of *Bt* by the PCR technique (Khyami-Horani, unpublished).

Our preliminary work showed that death of J2s was not observed before two days while it has been reported that juveniles of *C. elegans* died after 24 hours of *Bt* treatment (Leyns *et al.*, 1995). This could be explained if the toxin of *Bt* is taken in more quickly through the relatively wide bucal cavity in *C. elegans* (Marroquin *et al.*, 2000) whereas, in *Meloidogyne,* the *Bt* might attach and enter the J_2 through the natural openings or through the cuticle. Spiegel and McClure (1995) indicated that the surface coat of nematodes is involved in the attachment of bacteria and fungi that parasitize them, and both strains of *Bt* were able to produce proteases and chitinases (Khyami-Horani, unpublished).

At the higher concentration (10^6 viable spore/ml), *Bt* strains were more effective against eggs within the egg mass, suggesting that at this concentration *Bt* has an efficient chitinase activity. Spiegel and Cohn (1985) reported that chitin is present in the gelatinous matrix of *Meloidogyne* species. Since the *Bt*s tested produced chitinase, it is therefore conceivable that the bacteria were able to penetrate the gelatinous matrix and expose the eggs to the *Bt* toxins.

In this study, the lower concentrations of the two bacterial strains were not effective in reducing J_2 emergence from eggs within egg masses and the number of hatched J_2s sometimes exceeded those hatched from the untreated egg masses. This may be because the lower concentration of *Bt* does not result in sufficient chitinase activity to expose the eggs to the toxin. In fact, J2s hatched before the relatively lower concentration of the *Bt* would have reached the eggs within the egg mass, and the results of the second experiment on free eggs, where both *Bt* strains used at the higher dose completely inhibited or significantly reduced egg hatch, support the above explanation. Nevertheless, the use of a lower dose (10^5 viable spores/ml) of both *Bt* strains resulted in a significant reduction of egg hatch of *M. incognita.*

The results of this study indicate that the efficacy of *Bt* (expressed as percentage hatch) varies from one *Bt* strain to another and depends on the nematode species used. Similarly, Osman *et al.* (1988) reported that the two commercial *Bt* insecticides, SAN 415 and Dipel, reduced the percentage of eggs hatching of the two plant parasitic nematodes *M. javanica* and *Tylenchulus semipenetrans*. The percentage of eggs hatch of *M. javanica* was 63, 66 while for *T. semipenetrans* it was 59, 66 for eggs treated with SAN 415 and Dipel, respectively.

Several reports have concluded that the inhibition or reduction of J_2 hatching from eggs treated with *Bt* might be due to the effect of *Bt* on both eggshell and the embryonic development inside the eggs. Bottjer and Bone (1987) investigated the changes in morphology of *T. colubriformis* eggs caused by *B. thuringiensis israelensis* and found that the treated eggs were not viable after 24 hours; the embryos did not develop, and were condensed with numerous refractive granules visible within the degenerated embryo.

Our results indicate that *Bt* is highly toxic against root-knot nematodes. The next step would be to test the efficacy of *Bt* as a nematode control agent when applied as a drench under controlled conditions.

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