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Effect of transgenic cotton expressing Cry1Ac and Cry2Ab2 protein on infectivity of the entomopathogenic nematodes, *Steinernema karii* and *Heterorhabditis bacteriophora*

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Entomopathogenic nematodes are used in biological control of pests. The cry proteins from insect resistant cotton may affect their infectivity. The effect of *Bacillus thuringiensis* (Bt) cotton (06Z604D) on infectivity of *Heterorhabditis bacteriophora* and *Steinernema karii* was investigated in the green house. The nematodes were introduced into pots containing Bt cotton (06Z604D), isoline (99M03) and Hart 89M (local non Bt cotton cultivar). After 6 months, the nematodes were recovered from soil and their infectivity towards *Galleria mellonella* larvae was determined. The presence of Bt protein in roots and soil was determined at the end of the growing season by qualitative enzyme-linked immunosorbent assay (ELISA). Bt protein was present in the roots and soil of Bt cotton. No Bt protein was detected in HART 89M and isoline roots and soil. There was a significant species*time* treatment interaction and the nematodes collected from all the treatments caused >50% mortality.

Key words: *Bacillus thuringiensis,* entomopathogenic nematodes, virulence.

INTRODUCTION

Entomopathogenic nematodes (EPNs) belonging to the families Steinernematidae and Heterorhabditidae are used in biological control of soil insect pests. The nematodes are mutually associated with the bacteria genera *Xenorhabdus* and *Photorhabdus*, respectively. The dauer larva hosts the bacteria cells which reproduce and kill the insects while providing optimal conditions for nematode proliferation. The nematodes reproduce in the insect host and new larvae are released into soil where they exist until another host becomes available.

EPNs are ideal biological control agents due to their compatibility with different chemicals used in pathogen control and their synergistic action with some insecticides (Koppenhofer et al., 2000). Fitness costs of resistance to *Bacillus thuringiensis* (Bt) crops occur when insects with resistance alleles are less fit than susceptible insects. Different species of EPNs also magnify the fitness costs of resistance to Bt protein in some insect pests (Hannon et al., 2010). For instance, *Steinernema riobrave* increases the fitness cost of Bt resistance in the pink

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bollworm (Gassmann et al., 2008). Bt has been used together with EPNs for biological control of insects. Soil and foliage pests were controlled with Bt formulations integrated with *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* (Kaya et al., 1995).

EPNs have been identified in Kenya, and different species have been described in the central highlands, rift valley and coastal areas. These nematode species include *Steinernema yirgalemense*, *Steinernema weiseri* (Mwaniki et al., 2008), *Steinernema karii, H. bacteriophora* and H. *indica* (Waturu, 1998) and their efficacy against insect pests has been shown. EPN infectivity is affected by several biotic and abiotic factors. Their behavior is also influenced by the status of the host and the sex and age of the infective juvenile (Lewis et al., 2006). Cry proteins are among the compounds that affect nematodes by forming pores in their stomach wall. Once the toxin is ingested, it is activated by the enzymes in the gut which results in vacuole formation, degradation of the gut and death (Marroquin et al., 2000).

EPNs mainly attack insect pests in soil and cultivation of Bt cotton may result in accumulation of cry proteins in the soil which may affect their infectivity. Alterations in Bt cotton may also occur during genetic manipulation and this may cause changes in the compounds produced by the plants. These compounds may affect EPN behavior and ultimately their infectivity. For instance, the compound (E)-β-caryophyllene attracts the EPN *Heterorhabditis megidis* which is a natural enemy of the western corn rootworm (Degenhardt et al., 2009). The main objective was therefore to assess the effect of Bt cotton on infectivity of *H. bacteriophora* and *S. karii.*

MATERIALS AND METHODS

Greenhouse experiments using entomopathogenic nematodes

The plant material used in the experiment were Bt cotton (06Z604D), isoline (99M03) and HART 89M (local non Bt cotton cultivar). Bt cotton 06Z604D (Bollgard II) seeds were provided by Monsanto and they were results of retransformation of Bollgard I which contains Cry1Ac and Neomycin phosphotransferase type II (NPTII) selectable marker protein. In addition, Bollgard II produces beta-D-*glucuronidase* (GUS) marker protein (Monsanto, 2003).

Comparisons were made between Bt cotton and its isogenic counterpart to test the effect of the Bt gene while HART 89M was compared with isoline to test for any varietal effects. The plants were grown in pots containing sterile soil (sand: loam, 1:1). Each treatment was replicated 4 times with 12 plants per replicate. The pots were arranged in a completely randomized design. One seed per treatment was sown at the centre of each pot after surface sterilization with 0.1% mercuric chloride for 2 min. After 14 days post germination, 5000 juveniles of freshly produced *H. bacteriophora* and *S. karii* (Source: Dr Waturu, EPN laboratory at KARI, Thika) were inoculated separately into the soil. At the end of the growing season (180 days after planting (DAP)), presence of Bt protein in soil and roots was determined using the enzyme-linked immunosorbent assay (ELISA).

The experiment was repeated once with temperature during Trial 1 being 26 ± 1°C and 29 ± 2°C in Trial 2. Nematode infectivity was determined using the method described by Gaugler and Boush (1978). Soil samples (50 cm^3) from each treatment were collected from the greenhouse and put into bowls. *G. mellonella* obtained from the Entomology Department, KARI, NARL were reared on an artificial diet at 27ºC in an incubator. Ten late instar larvae of *G. mellonella* were introduced into each bowl and mortality of the larvae was assessed after every 24 h until all the larvae died. Water (50 ml) was used as a control in the infectivity tests. *G. mellonella* was used due to its susceptibility to the 2 EPN species.

Qualitative Bt protein detection using ELISA

Rhizosphere soil and roots from Bt cotton, HART 89M and isoline treatments were collected at 180 DAP. One gram each of soil sample was used for analysis of Bt protein using ELISA. A qualiplate combo kit for Cry1A and Cry2A (AP 051) (EnviroLogix, Portland, ME, USA) was used. Extraction buffer (1000 µl) was added and vortexed for 1 min. For plant samples, 0.5 g was snap frozen and ground in 1 ml of extraction buffer. Extraction was allowed to take place overnight at 4°C. Quantification of Cry2Ab2 and Cry1Ac was determined using a spectrophotometer (Benchmark®, Bio-Rad, Hercules, CA). The results are reported as present or absent.

Statistical analysis

Treatment effects on nematode infectivity as shown by percentage mortality of *G. mellonella* were determined using Analysis of variance (ANOVA) (GenStat 12.1). A significance level of 0.05 was used for all statistical analysis. Comparisons were made between Bt cotton and its isogenic counterpart to test the effect of the Bt gene while HART 89M was compared with isoline to test for any varietal effects.

The ELISA results were interpreted according to the manufacturer's protocol where the mean optical density (OD) of the blank wells in the Cry1Ac and Cry2Ab part of the test was such that it did not exceed 0.15 and 0.35 respectively. The mean, blank-subtracted OD of the positive control wells was at least 0.2 and the coefficient of variance (CV) between the duplicate positive control wells did not exceed 15%. The positive control ratio was calculated by dividing the OD of each sample extract by the mean OD of the positive control wells. For Cry2Ab, if the positive control ratio calculated for a sample was less than 1.0, the sample did not contain Cry2Ab. In the Cry1Ac part of the test, if the positive control ratio was less than 0.5, the sample did not contain the protein. The results are reported as absence or presence of protein.

RESULTS

Bt protein was present in roots and soil of Bt cotton at 180 DAP in both Trials. However, the insecticidal activity of the Bt protein was not determined through insect bioassays. No Bt protein was detected in Hart 89M and isoline plant tissues and soil. There was a significant nematode species*treatment*time interactions in mortality of *G. mellonella* between Bt and isoline ($F = 91.7_{[6, 69]}$; $P < 0.001$, Trial 1; $F = 66.9_{[6, 69]}$; $P < 0.001$, Trial 2). There were no significant differences in mean mortality of *G. mellonella* due to the infection with *H. bacteriophora* between Bt cotton and isoline in Trial 1. In Trial 2 *H. bacteriophora* from isoline caused slightly higher mean mortality than Bt cotton *S. karii* from Bt cotton caused significantly ($F = 102.9_{[2, 69]}$; $P < 0.001$, Trial 1; F =215. 9

Table 1. Mortality (%) of *G. mellonella* larvae caused by *H. bacteriophora* and *S. karii* collected from Bt cotton and its isoline.

Treatment	Time (h)	Trial 1		Trial 2	
		Mortality (%)		Mortality (%)	
		H. bacteriophora	*S. karii*	*H. bacteriophora*	*S. karii*
Water	48	36.6	38.4	36.8	38.3
	72	55.2	58.4	54.4	58.8
	96	66.0	74.1	66.5	74.2
	120	80.1	86.7	78.0	83.4
Mean		59.4	64.3	58.9	63.7
Bt cotton	48	31.6	36.7	32.2	36.8
	72	51.3	57.4	51.5	57.2
	96	65.3	72.5	62.8	72.4
	120	74.6	83.0	73.5	82.1
Mean		55.7	62.4	55	62.1
Isoline	48	34.1	37.2	35.3	37.2
	72	49.3	57.5	49.0	57.6
	96	66.1	71.2	62.7	71.1
	120	73.5	81.9	74.4	81.0
Mean		55.8	61.9	55.3	61.7
SEM		0.13		0.11	
LSD	Species	0.1*		0.09*	
LSD	Treatment	0.13*		0.11*	
LSD	Time	0.15*		0.13*	
LSD	Species*Treatment	0.18*		0.16*	
LSD	Species*time*	0.21*		0.19*	
LSD	Treatment*time	0.25*		0.23*	
LSD	Species*Treatment time	0.36*		0.32*	

*Significant effect at 0.05 level.

$_{[2,\ 69]}$; P < 0.001, Trial 2) higher mean mortality than isoline in both trials (Table 1).

Significant nematode species*treatment*time interactions in mortality of *G. mellonella* were also observed between isoline and HART 89M ($F = 98.6_{[6,\ 69]}$; P < 0.001, Trial 1; $F = 125.6_{[6,\ 69]}$; P < 0.001, Trial 2). The 2 nematode species collected from the HART 89M treatment caused a slightly higher but significant mean mortality than isoline in Trial 1 ($F = 44.3_{[2,\ 69]}$; P < 0.001). In Trial 2 *S. karii* from isoline treatment caused a slightly higher ($F = 127.4_{[2,\ 69]}$; P < 0.001) mortality than HART 89M (Table 2). Mortality caused by both species generally increased with time in all the treatments.

DISCUSSION

Bt cotton did not negatively affect the ability of *H. bacteriophora* and *S. karii* to infect *G. mellonella* larvae and in some instances infectivity of *S. karii* from the Bt cotton treatment was higher, although only moderately, than isoline. Mureithi et al. (2010) also reported that, Cry1Ac and Cry2Ab2 protein in Bt cotton did not affect the infectivity of *S. karii*. However, other studies have reported toxicity of Cry5, Cry6, Cry12, Cry13, Cry14, and Cry21 to free living nematodes (Wei et al., 2003).

There were significant differences in infectivity of *H. bacteriophora* and *S. karii* and this may be due to the differences in biological characteristics of the nematodes and the bacteria they are associated with. The EPNs, *H. bacteriophora* and *S. karii* have different genetic constitution as revealed by restriction fragment length analysis (RFLP) of the ITS region of the rDNA repeat unit (Waturu et al., 1997). Interspecific variation in the bacteria associated with the nematodes results in differences in the compounds that they produce and consequently infectivity of respective nematode species (Forst and Nealson, 1996). The foraging behavior of the different EPNs species may also have caused the differences in infectivity. *S. karii* may be a sit and wait strategist with low motility while *H. bacteriophora* is a widely foraging strategist that utilizes most of its foraging time to host finding and it is characterized by high motility (Hazir et al., 2003).

Table 2. Mortality (%) of *G. mellonella* larvae caused by *H. bacteriophora* and *S. karii* collected from isoline and HART 89M.

Treatment	Time (h)	Trial 1		Trial 2	
		Mortality (%)		Mortality (%)	
		H. bacteriophora	*S. karii*	*H. bacteriophora*	*S. karii*
Water	48	36.6	38.4	36.8	38.3
	72	55.2	58.4	54.4	58.8
	96	66.0	74.1	66.5	74.2
	120	80.1	86.7	78.0	83.4
Mean		59.5	64.4	58.9	63.7
Isoline	48	34.1	37.2	35.3	37.2
	72	49.3	57.5	49.0	57.6
	96	66.1	71.2	62.7	71.1
	120	73.5	81.9	74.4	81.0
Mean		55.8	62.0	55.3	61.7
HART 89M	48	31.6	37.8	31.9	37.7
	72	49.0	56.8	49.7	56.4
	96	65.3	71.0	64.1	70.8
	120	77.5	82.9	76.2	81.2
Mean		55.9	62.1	55.5	61.5
SEM		0.16		0.11	
LSD	Species	0.13*		0.09*	
LSD	Treatment	0.16*		0.11*	
LSD	Time	0.18*		0.12*	
LSD	Species*treatment*	0.23*		0.15*	
LSD	Species*time*	0.26*		0.17*	
LSD	Treatment*time	0.32*		0.21*	
LSD	Species*treatment*time	0.45*		0.3*	

*Significant effect at 0.05 level.

Temperature, soil physical and chemical properties natural enemies and competition from other organisms are other factors that influence infectivity. Reproduction, development and infectivity of EPNs are affected by temperature (Molyneux, 1985). Temperature variations between Trials 1 and 2 may have caused the differences in larval mortality for each of the species tested. Jagdale and Gordon (1998) observed that, infectivity of EPNs was enhanced at temperatures of 25°C, but temperature above 35°C affected the nematodes negatively due to the increase in metabolism which causes energy reduction (Schmiege, 1963). Genetic differences between the EPNs also affect the sensitivity to different temperature levels with each having an optimal temperature for infectivity and reproduction (Grewal et al., 1994). *Xenorhabdus* and *Photorhabdus* which are bacteria species associated with the nematodes also have different optimal temperatures (Rahoo et al., 2011). Apart from temperature, other factors in the field such as fertilizers and metal ions may also affect EPN infectivity. Manganese and magnesium cations have been shown to enhance EPN infectivity in different insects while fertilizers have a negative impact (Brown et al., 2006).

The differences in infectivity of the 2 EPN species from Bt cotton and isoline treatments were numerically small and the ecological significance is not clear. Such differences in infectivity of EPNs have also been shown to occur as a result of variations in chemical and temperature gradients in the environment (Lewis et al., 2006). The study has revealed that Bt cotton does not negatively affect *H. bacteriophora* and *S. karii* infectivity in the greenhouse trials. However, the infectivity of the 2 nematode species should be evaluated under field conditions where different environmental factors, cultural and agronomic practices may interact with the Bt trait and affect infectivity.

Conclusion

The infectivity of *H. bacteriophora* and *S. karii* was not negatively affected by Bt cotton. This may hold promise

in their use as biological control agents in Bt cotton agroecosystems and further reduce the use of pesticides. The possibility of using the EPNs in Bt cotton refuges to increase the fitness cost of Bt resistance should also be investigated.

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