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Reproduction of root knot nematode (*Meloidogyne incognita*) on Bt cotton expressing Cry1Ac and Cry2Ab2 protein

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ABSTRACT

Objective: The sedentary endoparasite *Meloidogyne incognita* is an important plant parasitic nematode that infects cotton causing significant yield losses. The objective of this study was to evaluate reproduction of *M. incognita* in Bt cotton (06Z604D), isoline (99M03) and HART 89M (local non-Bt cotton cultivar) under greenhouse conditions.

Methods and results: Plant height, number of squares/bolls, fresh shoot and root weight were determined before root knot nematode (RKN) screening at 90 and 180 days after planting (DAP). Galling severity, egg mass index, number of juveniles and the presence of Bt protein in roots and soil were also determined. The ELISA detected Bt protein in soil and roots of Bt cotton but not in HART 89M and isoline plant tissues and soil. Reaction of Bt cotton and isoline to *M. incognita* was different with the transgenic cotton being more susceptible to RKN. HART 89M was more resistant to RKN infection compared with the isoline.

Conclusion and application of findings: The study has demonstrated that Bt cotton (06Z604D) is susceptible to *M. Incognita*. The results indicate the importance of integrating nematode management practices such as the use of organic amendments and nematicides with other cultural practices in future Kenyan Bt cotton agroecosystems.

Keywords: *Bacillus thuringiensis,* Biosafety, root knot nematode, cotton

INTRODUCTION

The root knot nematode (RKN) *Meloidogyne incognita* is an important plant parasitic nematode that infects different plant species causing significant yield losses either through direct feeding of roots or indirectly through interactions with soil borne fungal pathogens. The infective juvenile (IJ) penetrates into the plant through the roots and migrates into the actively dividing plants cells (Srivastava, 1973). The development and reproduction of RKN is dependent on whether or not specialized feeding sites within the vascular tissues are induced. If the host is susceptible, the feeding sites are enlarged resulting in giant cells which arise due to repeated cell divisions. Galling occurs due to hypertrophy of cortical cells and within the root galls nematodes continually

undergo moulting to the mature female (Jenkins *et al.,* 1995). The changes that occur in the roots include disruption of the root xylem epidermal and cortical tissues development, which in turn affect water and nutrient uptake resulting in stunted growth (Kirkpatrick *et al.,* 1991). *Bacillus thuringiensis* commercial preparations have been used in the control of plant parasitic nematodes. Sharma (1994) reported 53 to 65% control of *M. incognita* in barley while Prasad *et al.* (1972) reported that purified exotoxin was toxic to *Meloidogyne* larvae and eggs. In other studies, hatching of *M. javanica* was reduced while the mortality was increased after application of *Bacillus* spp (Dawar, 2008). Application of *B. thuringiensis* as seed dressing resulted in an increase in seed germination, root length, root weight, shoot length, shoot weight and seed germination (Sheikh *et al.,* 2006). *Meloidogyne* spp in tomato and okra were also controlled by *B. thuringiensis* (Srivastava, 1973). Tomato and pepper plants had fewer galls and reduced populations of *M. incognita* after application of *Bacillus* formulations (Zuckerman *et al.,* 1993). Crops have also been genetically engineered for nematode resistance. Li *et al.* (2007) challenged tomato roots expressing Cry6a protein with *M. incognita* juveniles and compared different infection parameters. *M. incognita* ingested the toxic 54 KDA protein which resulted in a fourfold decrease in reproduction. Phap *et al.* (2010) reported that Cry1Ab protein in transgenic brinjal resulted in a decrease in the number of galls, egg masses and number of eggs per egg mass. Bt cotton has been evaluated for its reaction to parasitic nematodes. According to Senthirkumar *et al.* (2008), there was a significant reduction in nematode and egg production in the reniform nematode populations in Bt cotton. There was a delay in hatching and development to third stage juvenile (J3) in Bt cotton but fourth stage juvenile (J4) showed normal penetration. Colyer *et al.* (2008) reported that insect resistant cotton was more susceptible to *M. incognita* than non-transgenic cotton. The reason for the observed responses of Bt cotton to the parasitic nematodes may be due to the effect of Bt protein or changes in the plant makeup as a consequence of gene insertion. Different studies have shown that various genes are involved in feeding sites formation and resistance of crops to nematode infection. *Meloidogyne* induced cotton 3 (MIC 3) resistance genes in cotton do not affect RKN penetration into roots but they prevent the development of the juvenile into adults. The juveniles develop only to J4 stage and they do not form giant cells (Jenkins *et al.,* 1995). In resistant genotypes the J1 fails to establish and maintain a feeding site and those juveniles that are able to form a feeding site have a slower development process (Wubben *et al.,* 2008). The MIC gene family is involved in defence mechanisms in cotton and it is independent of lipid peroxidation and gossypol biosynthesis.

Bt cotton expressing Cry1Ac and Cry2Ab2 may react differently to infection with *M. incognita* compared with the conventional cultivars due to direct effects of the Cry proteins on the nematodes or indirectly due to pleiotropic effects. The objective of this study was therefore to evaluate RKN reproduction in Bt cotton and its isogenic counterpart.

MATERIALS AND METHODS

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 a result 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.

Pots were filled with 20kg sterile soil (sand: loam, 1:1). The soil in each pot was infested with 6000 *M. incognita* eggs and/or juveniles collected from stock cultures of *M. incognita* maintained on tomato (*Lycopersicon esculentum*). Inoculum was prepared by washing the infected tomato roots and chopping them into 2cm segments. The galled root segments were processed in 0.05% Sodium Hypochlorite by agitated extraction. Eggs were then rinsed thoroughly and

placed in a water suspension. Ten milliliters of the egg suspension containing the appropriate number of eggs and juveniles was pipetted onto the soil surface in each pot and incorporated into the soil. Pots without *M. incognita* eggs/juveniles served as controls. Two seeds of the appropriate cultivar were planted into each pot immediately after infestation of the soil with the nematodes. Treatments were arranged in the greenhouse in a completely randomized design and replicated four times with each replicate consisting of 12 plants in separate pots. Seven days after planting, pots were thinned to one seedling per pot. Plant height and number of squares/bolls was determined before RKN screening at 90 and 180 days after planting (DAP). Data on fresh shoot and root weight was also recorded at 90 and 180DAP. The experiment was repeated once.

Galling severity per plant was rated according to the following scale: 0 =no galls; 1 = 1 to 2 galls; 2 = 3 to 10 galls; 3 = 11 to 30 galls; 4 = 31 to 100 galls; 5 = >100 galls per root system (Colyer *et al.,* 2008). Egg masses were stained using phloxine B (Holbrook *et al.*, 1983) and rated using a scale of 0-5 where, 0= no egg masses, 1=1-2, 2=3-10, 3=11-30, 4=31-100, and 5= >100 egg masses per root system (Kirkpatrick *et al*., 1991). The number of juveniles in soil was determined at the end of the experiment by taking 200cm^3 of soil from all the treatments and extracting the nematodes using the Whitehead and Hemming (1965) tray method. Rhizosphere soil and roots from Bt cotton, HART 89M and isoline treatments were collected at 90 and 180 DAP. One gram each of soil sample was used for analysis of Bt protein using the enzyme-linked immunosorbent assay (ELISA). A qualiplate combo kit for Cry1A and Cry2A (AP 051) (EnviroLogix, Portland, ME, USA) was used. Extraction buffer (1000µl) was added and vortexed for one minute. For plant samples, 0.5g 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).

Treatment effects on different growth parameters were determined using ANOVA (GenStat 12.1). Means were separated using Fischer's least significant difference test. Differences at $P<0.05$ level were considered statistically significant. 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. Results are reported as absence or presence of Bt protein.

RESULTS

Reaction of Bt cotton and isoline to *M. incognita* was different with the transgenic cotton being more susceptible to RKN in two greenhouse trials (Table 1, 2, 3 and 4). Plant growth parameters in both treatments were negatively affected by infection with *M. incognita* at 90 and 180DAP. Reduction in growth parameters was apparent at 90 and 180DAP. However, fresh root weight in isoline increased at 180DAP in both trials. The number of juveniles, galling and egg mass index were higher in Bt cotton than in isoline and the values were greater at 180DAP. There was a greater reduction in number of bolls in Bt cotton than in isoline. There were significant month*treatment interactions in both trials for plant height ($F=289.9_{[3, 21]}$; $P<0.001$; $F=24.9_{[3, 21]}$; $P<0.001$), fresh root weight ($F=220.2_{[3, 21]}$; $P<0.001$, trial 2), fresh shoot weight ($F= 26.9_{[3, 21]}$; $P<0.001$; $F=104.4_{[3, 21]}$; $P<0.001$), egg mass index ($F=71.5_{[1, 9]}$; $P<0.001$, trial 1), galling index ($F=51.9_{[1, 9]}$; $P<0.001$; $F=9.4_{[1, 9]}$; $P=0.01$) and number of juveniles ($F=88.7_{[1, 9]}$; $P<0.001$; $F=98.9_{[1, 9]}$; $P<0.001$). There were significant differences in the number of squares ($F=762.9_{[3, 9]}$; $P<0.001$; $F=472.6_{[3, 9]}$; $P<0.001$) and number of bolls ($F=1952_{[3, 9]}$; $P<0.001$; $F=161.6_{[3, 9]}$; $P<0.001$) between Bt cotton and isoline at 90 and 180DAP. A decrease in number of bolls in both treatments was recorded at 180DAP.

Table 1: Effect of *Meloidogyne incognita* inoculations on plant growth of Bt cotton and its isoline (Trial 1)

Time	Treatment	Plant height (cm)	No of squares	No of bolls	Fresh shoot weight (g)	Fresh root weight (g)
90DAP	Control Bt cotton	45.1b	2.5a	Not collected	42.1bc	6.7a
	Control isoline	48.7d	5.9c	Not collected	43.7c	6.6a
	Bt cotton	38.5a	2.5a	Not collected	28.7a	5.3b
	Isoline	45.9b	4.0b	Not collected	39.2b	6.7a
180DAP	Control Bt cotton	64.3g	Not collected	17.99d	86.3f	10.0c
	Control isoline	61.5f	Not collected	14.9c	84.5f	9.5e
	Bt cotton	52.8e	Not collected	9.12b	53.4d	9.0d
	Isoline	46.8c	Not collected	8.65a	63.5e	10.5f
SEM		0.23	0.05	0.1	1.01	0.17

Means within the same column with the same letter are not different ($P < 0.05$) according to least significant difference test (LSD).

Table 2: Number of *Meloidogyne incognita* juveniles, egg mass and galling index of Bt cotton and its isoline (Trial 1)

Time	Treatment	[a]Galling index	[b]Egg mass index	No of juveniles (200cm^3)
90DAP	Bt cotton	2.5b	2.4b	201.6b
	Isoline	1.9a	2.0a	167.9a
180DAP	Bt cotton	4.0c	4.0c	349.0d
	Isoline	2.5b	2.6b	270.6c
SEM		0.05	0.04	1.68

Means within the same column with the same letter are not different ($P < 0.05$) according to least significant difference test (LSD).

[a]Based on a 0-5 scale, where 0 =no galls; 1 = 1 to 2 galls; 2 = 3 to 10 galls; 3 = 11 to 30 galls; 4 = 31 to 100 galls; 5 = >100 galls per root system (Colyer *et al.,* 2008).

[b]Based on a 0-5 scale, where, 0= no egg masses, 1=1-2, 2=3-10, 3=11-30, 4=31-100, and 5= >100 egg masses per root system (Kirkpatrick *et al*., 1991).

Table 3: Effect of *Meloidogyne incognita* inoculations on plant growth of Bt cotton and its isoline (Trial 2)

Time	Treatment	Plant height (cm)	No of squares	No of bolls	Fresh shoot weight (g)	Fresh root weight (g)
90DAP	Control Bt cotton	46.5c	4.4c	Not collected	47.3a	7.1a
	Control isoline	48.9d	4.5c	Not collected	49.0a	10.8d
	Bt cotton	33.4a	2.1a	Not collected	45.5a	6.8a
	Isoline	34.9b	2.4b	Not collected	48.3a	8.1b
180DAP	Control Bt cotton	62.0f	Not collected	16c	112.1e	14.0e
	Control isoline	61.7f	Not collected	13.51b	85.3d	10.3c
	Bt cotton	51.1e	Not collected	10.54a	63.6b	7.8b
	Isoline	46.9c	Not collected	10.34a	75.7c	10.7cd
SEM		0.37	0.06	0.21	1.39	0.21

Means within the same column with the same letter are not different ($P < 0.05$) according to least significant difference test (LSD).

Table 4: Number of *Meloidogyne incognita* juveniles, egg mass and galling index of Bt cotton and its isoline (Trial 2)

Time	Treatment	[a]Galling index	[b]Egg mass index	No of juveniles (200cm^3)
90DAP	Bt cotton	2.4b	2.5a	176.7b
	Isoline	1.4a	1.5c	121.8a
180DAP	Bt cotton	4.5d	4.5b	415.4d
	Isoline	3.0c	3.5d	312.8c
SEM		0.07	0.05	2.39

Means within the same column with the same letter are not different (P < 0.05) according to least significant difference test (LSD).

[a]Based on a 0-5 scale, where 0 =no galls; 1 = 1 to 2 galls; 2 = 3 to 10 galls; 3 = 11 to 30 galls; 4 = 31 to 100 galls; 5 = >100 galls per root system (Colyer *et al.,* 2008).

[b]Based on a 0-5 scale, where, 0= no egg masses, 1=1-2, 2=3-10, 3=11-30, 4=31-100, and 5= >100 egg masses per root system (Kirkpatrick *et al*., 1991).

Isoline and HART 89M also reacted differently to *M. incognita* infection, with isoline being more susceptible in two greenhouse trials (Table 5, 6, 7and 8). A reduction in growth parameters was observed for both treatments. Significant month*treatment interactions were recorded in both trials for plant height ($F=33.7_{[3, 21]}$; P<0.001; $F=13.1_{[3, 21]}$; P<0.001), fresh shoot weight ($F=349.7_{[3, 21]}$; P<0.001; $F=64.3_{[3, 21]}$; P<0.001), fresh root weight ($F=141.2_{[3, 21]}$; P<0.001; $F=71.7_{[3, 21]}$; P<0.001), number of juveniles ($F=7.7_{[1, 9]}$; P=0.02; $F=48.5_{[1, 9]}$; P<0.001), egg mass ($F=120.8_{[1, 9]}$; P<0.001) and galling index ($F=_{[1, 9]}$; P=0.005). There were significant differences in the number of squares ($F=979.5_{[3, 9]}$; P<0.001; $F=1110_{[3, 9]}$; P<0.001) and number of bolls ($F=556.1_{[3, 9]}$; P<0.001; $F=79.9_{[3, 9]}$; P<0.001) between isoline and HART 89M at 90 and 180DAP. The qualitative ELISA detected Bt protein in roots of Bt cotton at 90 and 180DAP in both trials. The protein was detected in soil at 180DAP in both trials. No Bt protein was detected in HART 89M and isoline plant tissues and soil.

Table 5: Effect of *Meloidogyne incognita* inoculations on plant growth of isoline and HART 89M (Trial 1)

Time	Treatment	Plant height	No of squares	No of bolls	Fresh shoot weight (g)	Fresh root weight (g)
90DAP	Control isoline	48.7c	6.0d	Not collected	43.8b	6.5a
	Control HART 89M	50.2d	2.0b	Not collected	44.4b	7.6b
	Isoline	45.9b	4.1c	Not collected	39.2a	6.7a
	HART 89M	35.1a	0.9a	Not collected	37.9a	6.8ab
180DAP	Control isoline	61.5f	Not collected	14.9c	84.6d	9.5c
	Control HART 89M	71.9g	Not collected	12.7b	143.5f	18.9f
	Isoline	46.9b	Not collected	8.7a	63.5c	10.5d
	HART 89M	58.9e	Not collected	8.9a	125.4e	18.1e
SEM		0.41	0.07	0.13	1.36	0.27

Table 6: Number of *Meloidogyne incognita* juveniles, egg mass and galling index of isoline and HART 89M (Trial 1)

Time	Treatment	Galling index	Egg mass index	No of juveniles (200cm^3)
90DAP	Isoline	1.9a	2.0a	167.9b
	HART 89M	2.0a	2.0a	144.4a
180DAP	Isoline	2.5c	2.6c	270.6d
	HART 89M	2.4b	2.5b	234.7c
SEM		0.06	0.11	2.22

Table 7: Effect of *Meloidogyne incognita* inoculations on plant growth of isoline and HART 89M (Trial 2)

Time	Treatment	Plant height	No of squares	No of bolls	Fresh shoot weight (g)	Fresh root weight (g)
90DAP	Control isoline	49.0d	4.5d	Not collected	49.0a	10.8c
	Control HART 89M	57.3e	1.5b	Not collected	54.4a	8.5b
	Isoline	34.9a	2.4c	Not collected	48.3a	8.1b
	HART 89M	43.9b	1a	Not collected	52.5a	6.9a
180DAP	Control isoline	61.7f	Not collected	13.5d	85.3c	10.3c
	Control HART 89M	75.9g	Not collected	12.5c	135.2e	17.2d
	Isoline	46.9c	Not collected	10.3b	75.7b	10.7c
	HART 89M	57.1e	Not collected	9.4a	99.7d	17d
SEM		0.59	0.05	0.22	2.06	0.42

Table 8: Number of *Meloidogyne incognita* juveniles, egg mass and galling index of isoline and HART 89M (Trial 2)

Time	Treatment	Galling index	Egg mass index	No of juveniles (200cm^3)
90DAP	Isoline	1.4a	1.5a	121.8b
	HART 89M	1.6a	1.6a	99.7a
180DAP	Isoline	3.0c	3.5c	312.8d
	HART 89M	2.6b	2.4b	262.3c
SEM		0.08	0.05	2.04

DISCUSSION

The present study demonstrated that Bt cotton expressing Cry1Ac and Cry2Ab2 protein was more susceptible to *M. incognita* than its isoline. HART 89M was moderately resistant compared with isoline, probably due to varietal differences. Otipa *et al*. (2009) also reported the same level of resistance in Kenyan cotton (*Gossypium hirsutum*) cultivars. At 180DAP, number of juveniles, galling and egg masss index were higher than at 90DAP due to the longer nematode reproduction time. *M. incognita* lays about 300-400 eggs and it completes its lifecycle in 33-38 days (Banu, 2007). Galling index in cotton is a measurement of its response to infection by *M. incognita* and it is correlated to yield reduction in susceptible cultivars (Zhang *et al.,* 2006). Large values of egg mass and galling index had a negative effect on plant height number of squares/bolls, plant, shoot and root weight across all treatment. However, fresh root weight in isoline increased at 180DAP. The increase in root weight in the infected plants was also observed by Setty and Wheeler, (1968) and they attributed this to the large amounts of tryptophan and other amino acids that are produced following infection. At the end of the growing season, Bt cotton had higher numbers of juveniles,

galling and egg mass index compared with its isoline and this may be because isoline permitted penetration of infective juveniles and development of feeding sites but the nematodes did not develop into adult females resulting in a reduction of RKN reproduction (Jenkins *et al.,* 1995). The high RKN reproduction in Bt cotton as shown by the galling index reduced cotton growth resulting in shorter plants which also weighed less than the isoline treatment. Hao *et al*. (2009) similarly reported that plant height and weight were negatively correlated with the galling index. Shoot weight of inoculated lettuce was reduced up 32% after infection with *M. hapla* (Wong and Mai, 1973).

Stephan (1983) also reported a reduction in plant height, shoot and root weight in tomato after inoculation with different *Meloidogyne* species. Infection with *M. incognita* in Bt cotton may have resulted in a decrease in water movement from the roots to the leaves due to vascular disruption by giant cells. In a severe RKN attack, the vascular system is completely disrupted and numbers of rootlets are reduced thereby affecting nutrients and minerals uptake resulting in wilting and stunted growth (Kirkpatrick *et al.,* 1991). Other secondary effects of RKN infection include reduced light interception which results in a reduction in photosynthetic efficiency.

Various studies have reported nematicidal activity of Bt proteins (Wei *et al*., 2003; Hoss *et al*., 2004). However, this was not observed in the current study despite the fact that Bt protein was detected in soil (at 180DAP) and roots (at 90 and 180DAP). The susceptibility of transgenic cotton to RKN has also been reported by Colyer *et al.* (2008) .. In other studies, purified Bt toxin did not have any toxic effect on RKN and inoculated plants had a higher galling index than the uninoculated plants (Devidas and Rehberger, 1997). Other authors have reported toxic effects of Bt protein on *Meloidogyne* spp. Chahal and Chahal (1993) reported a reduction in reproduction of *M. javanica* and *M. incognita* following application of *Bacillus* formulations. Dhawan *et al*. (2004) demonstrated that *Bacillus* spp could reduce the mobility of *M. incognita* juveniles. Similarly, root galling and reproduction of *M. incognita* race 3 on chick pea was reduced after treatment with *Bacillus* species (Siddiqui and Mahamood 1983) and inhibition of nematode penetration in tomato was also observed (Oka *et al.,* 1993).

Cry proteins or pleiotropic effects resulting from genetic transformation may have been responsible for the susceptibility of Bt cotton to *M. incognita* since alterations in the host plant may change the level of resistance, nematode attraction and feeding behaviour. Bendezu and Starr (2003) identified two types of RKN resistance in plants including preinfection resistance which is due to the presence of compounds in the roots that inhibits penetration of RKN, and the post infection resistance where the nematodes enter the roots but they do not develop into mature females. Resistant plants show an upregulation of the 14KDA polypeptide MIC3 defence genes which accumulates within the immature galls (Callahan *et al.,* 1997). The upregulation of these genes may explain the moderate resistance observed in HART 89M.

The observed reaction of Bt cotton in the greenhouse should be confirmed in the field since *M. incognita* may be affected by other environmental factors. According to Vrain (1977), *Meloidogyne* infectivity is influenced by soil texture, temperature, moisture, aeration and density and it is also a function of the distance that the juvenile has to travel in order to penetrate the roots. The study has however demonstrated that Bt cotton is susceptible to *M. incognita* and it would be important to integrate nematode management practices such as the use of organic amendments and nematicides with other cultural practices in Kenyan Bt cotton agroecosystems.

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