Journal of Animal &Plant Sciences, 2013. Vol.19, Issue 1: 2864-2879
Publication date 2/9/2013, http://www.m.elewa.org/JAPS*; ISSN 2071-7024*

Effect of Bt cotton expressing Cry1Ac and Cry2Ab2 protein on soil nematode community assemblages in Mwea, Kenya.

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Keywords: Risk assessment, Renyi diversity, Bt cotton

1 SUMMARY

Objective: **The nematode community structure in soil cultivated with Bt cotton (containing Cry1Ac and Cry2Ab2 protein), isoline (non Bt cotton) and HART 89M (non Bt cotton) was evaluated in a field trial at Ndomba in the Central Province of Kenya.**
Methods and results: **The experiment was laid out in a completely randomized block design. Soil was collected for two seasons at 0, 30, 60, 90, 120, 150 and 180 days after planting (DAP). Presence of Bt protein in roots and soil was determined using ELISA and insect bioassays. Nematodes were extracted from soil using centrifugal-floatation method and identified to genus level using a compound microscope. ELISA analysis of soil samples indicated that Bt protein was present at 150 and 180 days after planting. Bacteriovorous nematodes were present in significantly ($P<0.05$) higher numbers in the Bt cotton (46.9%) than in isoline (42.1%) plots.**
Conclusion and application of findings: **Cry1Ac and Cry2Ab2 protein in Bt cotton (06Z604D) does not have a direct effect on nematode diversity. The results provide important biosafety data that will be useful in pre- and post release monitoring of potential negative impacts of Bt cotton cultivation in Kenya.**

2 INTRODUCTION

Biogeochemical cycling is a key ecosystem process and soil organisms contribute to this function through varied interactions (Beare *et al.,* 1995). Numerous ecosystem services are derived from nutrient cycling and are dependent on the status of the soil (De Groot *et al.,* 2002). Nematodes play a crucial role in nutrient cycling (Ingham *et al.,* 1985) by enhancing the release of nutrients immobilized in different organisms of the soil food web (Freckman, 1988; Ferris *et al.,* 2004). The nematode community structure can be used as a standard of measurement of ecosystem perturbations (Bongers and Bongers, 1998; Yeates and Bongers, 1999) and changes in different nematode trophic groups may be an indication of modification of the soil environment (Bongers and Bongers, 1998). Disturbances in the soil could be a result of different factors, including the quality of plant materials getting into the soil (de Deyn *et al.,* 2004). Release of root exudates and sloughed tissues at the rhizosphere influences the

availability of nutrients and makes this zone important in the overall soil biological activity. The biochemical composition, nutritional and physical aspects of Bt cotton may be different from that of non Bt cotton and this may influence the quality and availability of nutrients to nematodes. Nematodes that feed on microbes have a higher biomass in the rhizosphere of plants than other organisms (Griffths, 1990) and microbial processes at this zone are dependent on the interaction between the microbial community, plant roots, root exudates (Bonkowski *et al.,* 2000) and organic matter (Griffths, 1994). Resource quality is therefore critical since it could influence the below ground organisms and consequently affect the above ground components (Wardle *et al.,* 2004).

Bt crops may release proteins through different pathways posing a direct or indirect effect to soil organisms. The use of Cauliflower Mosaic Virus (CaMV) 35S promoter in expression of Bt protein in cotton results in constitutive expression of Cry protein in all parts of the plant throughout the growing season posing a risk to non target organisms (NTOs). The protein may persist and accumulate in tropical soils (Muchaonyerwa *et al*., 2004), and depending on the soil type they may bind to different constituents, remain insecticidal thereby posing a risk to non target soil biota (Crecchio and Stotzky 1998). Genetic transformation may also result in changes in the plant tissues and consequent modification of root exudates which may in turn influence the microbial structure in the rhizosphere (Liu *et al*., 2005).

Different Cry proteins have been shown to have nematicidal activity. Wei *et al.* (2003) demonstrated that Cry5B, Cry14A, and Cry21A, and Cry6A were toxic to four bacterial feeding nematode species. Cry1Ab protein has also been shown to affect reproduction in *Caenorhabditis elegans* (Hoss *et al*., 2004). However, there are differing reports on the effect of Bt proteins in transgenic crops on nematodes. Hoss *et al.* (2011) and Al-Deeb *et al.* (2003) found no effect of Cry3Bb1 in Bt maize on *C. elegans*. Bt eggplant (Cry3Bb1) did not have any effect on field nematode populations. Manachini and Lozzia (2003) reported low populations of *C. elegans* in Bt maize (Cry1Ab) fields. Saxena and Stotzky (2001b) and Manachini and Lozzia (2002) found no effect on nematode populations in Bt maize (Cry1Ab) fields. Shifts in nematode abundance and/or diversity as a result of cultivation of Bt maize (Cry1Ab) (Griffiths *et al*., 2005, 2006; Lang *et al*., 2006) and Bt canola (Cry1Ac) (Manachini *et al*., 2004) have been reported.

In addition to Bt protein causing changes in the soil fauna, pleiotropic effects resulting from insertion of genes during transformation of transgenic crops have also been shown to affect soil organisms (Donegan *et al*., 1995).

The functional diversity of bacteria and fungi in the rhizosphere of transgenic cotton is different from non Bt cotton and this could result in differences in the nematode community (Donegan *et al.,* 1995; Rui *et al.,* 2005). Changes in soil enzyme activity and nutrient availability due to cultivation of Bt cotton may also affect nematodes. Soil urease, cellulase, invertase and acid phosphomonoesterase activities in soil grown with Bt cotton were stimulated (Sun *et al.,* 2007) and in other studies availability of P was enhanced and that of N constrained (Sarkar *et al.,* 2008). Although nematode communities may be influenced by other factors such as the crop species, plant age and environmental variables it is still important to assess the impact of Bt protein. Risk assessment of the impact of Bt cotton on nematodes is a prerequisite before introduction hence the need for the study. The objective of this study was therefore to examine the effect of Cry1Ac and Cry2Ab2 protein in Bt cotton on soil nematodes.

3 MATERIALS AND METHODS

3.1 Experimental site: The experimental site was set up at a confined area in Ndomba (KARI, Mwea), Central province, Kenya (0°35'S, 37°20'30"E). The soil contained 9% sand, 14% silt and 77% clay. The experiment was laid out in a completely randomized block design with 5x5m plots of Bt

cotton (06Z604D), isoline (99M03) and HART 89M (local non Bt cotton cultivar) separated by 3x3m strip. Each treatment was planted in five rows per plot and replicated four times. Maximum and minimum temperature and rainfall were recorded during the first (Dec 2009-June 2010) and second (July-Dec 2010) season.

3.2 Plant material: 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 Company 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.

3.3 Soil samples used in Enzyme-linked Immunosorbent Assay (ELISA) and insect bioassays: Rhizosphere soil was collected from the different treatments for two seasons at 0, 30, 60, 90, 120, 150 and 180 days after planting (DAP). Soil subsamples were collected randomly from plants in the inner rows of each plot. The plants were carefully dug out, and the soil adhering to their roots collected in plastic bags. The subsamples from each row were pooled into one composite sample. In the laboratory, plant and root residues were removed and the soil samples sieved. The soil samples were then kept at -20^{0}C until further use in ELISA and insect bioassays.

3.4 Qualitative Bt protein detection in soil and roots collected from the field: Roots and rhizosphere soil from Bt cotton, HART 89M and isoline treatments were collected as previously described. One gram each of soil and 0.5g of plant material was used in the analysis using a qualiplate combo kit for Cry1A and Cry2A (AP 051) (EnviroLogix, Portland, ME, USA) following the manufacturer's instructions. Quantification of Cry2Ab2 and Cry1Ac was determined using a spectrophotometer (Benchmark®, Bio-Rad, Hercules, CA). The results are reported as present or absent.

3.5 Insect bioassay: *Helicoverpa armigera* (African bollworm) larvae were collected from fields in different locations of Eastern Province, Kenya. The larvae were mostly in the fifth and sixth instar stage and were fed until pupation in the laboratory on natural and chickpea based artificial diet. (Gujar *et al.,* 2004). The adults emerging from pupae were offered a 10% (v/v) honey solution throughout their egg-laying period. Five pairs of adults were kept in each jar, which was covered with rough cotton cloth. The adult females laid eggs mostly on the cloth, which was sprinkled with water and then stored in separate jars. The experimental conditions were kept at 68+5% relative humidity, 25±1°C with a photoperiod of L:D 16:8.Upon hatching, the neonates were labeled as the F1 generation and were used in bioassays.

3.7 Standard (Cry1Ac) protein bioassay: To serve as a reference standard, standard mortality bioassays were done, by exposing neonate larvae to various concentrations of diet incorporated Cry1Ac protein that caused 0-100% mortality (Head *et al.*, 2002). Ten fold dilutions of pure Cry1Ac were prepared by mixing 1g of non Bt soil with 10 fold dilutions to make concentrations of 100, 10, 1, 0.1, 0.01 and 0.001 µg/g. One gram of non Bt soil was mixed with 4ml of each dilution and then brought up to 20ml using artificial diet (Head *et al.*, 2002). The soil-diet mixture was assayed with *H. armigera* by introducing a single larva into a petri plate covered with a soft paper towel. For each concentration, there were four replicates and for each replicate 100 insects were used. Mortality and larval growth inhibition (failure of neonates to reach 3rd instar or failure of 3rd instar to reach 4th instar) was assessed after 7 days.

3.8 Soil bioassay: To assay for Cry1Ac and Cry2Ab2 protein using *H. armigera,* soil samples were incorporated into the artificial diet and then presented to *H. armigera* neonates. The soil samples were collected in the first and second season from the Bt cotton, isoline and HART 89M experimental plots as described above. The treatments were replicated four times. Artificial diet with no soil sample was included as a control. One gram of soil from each sample was mixed with 4ml of water and brought up to a volume of 20ml using melted artificial diet (Gujar *et al.,* 2004). After the media cooled and solidified, 1 neonate of *H. armigera* was introduced into a petri plate covered with a soft paper towel. For each replicate in the respective treatments, 100 insects were used. Mortality and larval growth inhibition (failure of neonates to reach 3rd instar or failure of 3rd instar to reach 4th instar was assessed after 7 days.

3.9 Nematode analyses: Soil samples were collected before planting and after 30, 60, 90, 120, 150 and 180 DAP in the first (December 2009 to June

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2010) and second (July to December 2010) season. Soil subsamples were collected randomly from the rhizosphere of plants in the inner rows of each plot. To get one subsample, five core samples (each from a 30 cm deep and 8 cm diameter) were taken around the plant and mixed thoroughly. The subsamples from each row were pooled into one composite sample. From this mixture, 200 cm^3 of each soil sample was used for nematode extraction using centrifugal-floatation method (Jenkins, 1964). Nematode numbers were counted and identification to genus level was done under a compound microscope at a magnification of ×400 and ×1000. They were assigned to different trophic groups according to Yeates *et al.* (1993).

3.10 Statistical analysis : 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. In the standard protein bioassay, the median lethal concentration (LC50) and median growth inhibition concentration (IC50), and their 95% confidence limits were determined through probit analysis (SPSS, 2004). Where the mortalities of the control were between 5 and 10%, the larval mortalities were corrected using Abbott's formula before analyses while those >10% were excluded (Abbott, 1925). In the soil bioassays, comparisons of treatment effects on mortality and larval growth inhibition (failure of neonates to reach 3rd instar or failure of 3rd instar to reach 4th instar) was done using ANOVA (GenStat 12.1).

Maximum and minimum temperature and rainfall data during the first and second season was analyzed using ANOVA. Genus richness index (d=(S-1)logN, where S=number of genera, N= total number of nematodes) and proportions of trophic groups in the different treatments were calculated (Bongers, 1990). To meet assumptions of normal distribution, abundances of nematodes were log-transformed [ln(x+1)] and proportions were arcsine transformed prior to analysis. Differences in genus richness and proportions of trophic groups among treatments were tested using repeated measures ANOVA (GenStat 12.1). Principal response curves (PRC) analysis, a multivariate technique was used to show treatment effects on specific nematode genera over time (Van den Brink and Ter Braak 1998), using the following statistical model:

$$Y_{d(j)tk} = y_{0tk} + b_k c_{dt} + \varepsilon_{d(j)tk}$$

where $Y_{d(j)tk,}$ is the abundance of species k in replicate j of treatment d at time t, y_{0tk} is the mean abundance of taxon k in month t in the control, b_k is the species weight and c_{dt} is the least-squares estimate of the coefficients, and $\varepsilon_{d(j)tk}$ is a random error term. Nematode counts were log transformed before PRC analysis and significance of the model was tested using the F-test statistic. The species weight is an indication of the response of each species in relation to the entire community response. The vegan package of R 2.12.0 software was used for the PRC analysis. Renyi diversity index was used (Tóthmérész, 1995) to evaluate diversity of nematode functional groups. Renyi index was calculated as:

$$HR(\alpha) = \frac{1}{1-\alpha} log \sum_{i=1}^{s} p_i^{\alpha}$$

where α, is the scale parameter, p_i is the relative abundance of the i th species, and S is the number of species. The scale parameter has the values, 0, 1, 2, 3, 4 and 5. A community is more diverse when the curve of the diversity profile is above that of another community on the whole range of a scale parameter. A community with higher values at scale 0 has greater diversity whereas those with higher values at larger scale parameter have a smaller dominance of the dominant species. Biodiversity-R program was used to generate the diversity profiles (Kindt and Coe, 2005).

4 RESULTS

Maximum and minimum temperature varied significantly (P<0.001) between the two seasons. The average minimum temperature was 17.3+1.90^{0}C and 15.6 +1.27^{0}C while the maximum temperature was 27+1.86^{0}C and 28.6+3.25^{0}C in the first and second season respectively. There was no significant difference in rainfall between the two seasons. Cry1Ac was detected at 30DAP in both seasons while Cry2Ab2 was present in roots throughout the growing period in the two seasons. There was a significant season*DAP interaction in the levels of Cry1Ac ($F=10_{[5,\ 33]}$; P<0.001) and Cry2Ab2 ($F=655_{[5,\ 33]}$; P<0.001) in roots. In rhizosphere soil, Bt protein was detected at 150 and 180 days after planting Bt cotton

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in both seasons. There was a significant season*DAP interaction ($F=249_{[1, 9]}$; $P<0.001$) in the levels of Cry1Ac. Cry1Ac was detected in the first and second season at 150DAP and 180 DAP. There was also a significant season*DAP interaction ($F=258_{[1, 9]}$; $P<0.001$) in the levels of Cry2Ab2. In the first and second season, no Cry2Ab2 was detected at 150DAP but it was present at 180DAP. No Cry1Ac or Cry2Ab2 was detected in HART 89M and isoline roots and soil. In the pure protein bioassay, dose mortality relationship from the probit analysis showed that the IC50 of pure Cry1Ac on the *H. armigera* population was 0.017 µg/g (95% confidence limit from 0.009 to 0.026 µg/g). The LC50 was 0.31 µg/g (95% confidence limit from 0.12 to 0.60 µg/g). There was a significant DAP*treatment interaction ($F=8.45_{[1, 117]}$; $P=0.004$) in the %3rd instars between the Bt cotton and isoline treatment. Soil samples from Bt cotton treatment at 150DAP allowed a higher number larvae to reach the 3rd instar on the seventh day of incubation than at 180DAP in both seasons. However, isoline had a higher number of larvae reaching the 3rd instar than Bt cotton (Table 1). There were no significant differences in % 3rd instar between isoline and HART 89M (Table 2). No significant differences were observed between the HART 89M, isoline and the artificial diet control.

Table 1. Number of 3rd instar *Helicoverpa armigera* larvae after 7 days of incubation in Bt and isoline treatments

Treatment	Season	Time (DAP)	% 3rd instar
Bt cotton	1	150	70.7
		180	59.3
	2	150	69.0
		180	63.7
Isoline	1	150	83.7
		180	83.4
	2	150	82.5
		180	82.9
SEM			2.03
[a] LSD	Season		2.84
LSD	DAP		2.84*
LSD	Treatment		2.84*
LSD	DAP*Season		4.02
LSD	Season*Treatment		4.02
LSD	DAP*Treatment		4.02
LSD	DAP*Season*Treatment		5.69*

[a] Least significant differences at $P<0.05$
* Significant differences at $P<0.05$

There was a significant treatment*trophic group ($F=5.10_{[4, 298]}$; $P=0.001$), season*trophic group ($F=4.0_{[3,298]}$; $P=0.009$), time*trophic group ($F=2.96_{[24,298]}$; $P<0.001$) and season*time*trophic groups ($F=1.97_{[18, 298]}$; $P=0.011$) interaction between Bt cotton and isoline. In the Bt cotton treatment, bacterial feeders were the most dominant trophic groups in both seasons (First season 47.6±1.5; Second season 46.4±1.5). Predators were the least dominant trophic group across all treatments. Plant parasitic nematodes were not abundant across all treatments, but the common genera were *Meloidogyne* and *Pratylenchus*.

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Table 2: Number of 3rd instar *Helicoverpa armigera* larvae after 7 days of incubation in isoline and HART 89M treatments

Treatment	Season	Time (DAP)	% 3rd instar
Isoline	1	150	83.7
		180	83.4
	2	150	82.5
		180	82.9
HART 89M	1	150	83.5
		180	83.2
	2	150	84.5
		180	84.3
SEM			2.87
LSD	Season		4.02
LSD	DAP		4.02
LSD	Treatment		4.02
LSD	DAP*Season		5.69
LSD	Season*Treatment		5.69
LSD	DAP*Treatment		5.69
LSD	DAP*Season*Treatment		8.05

There was a significant treatment*trophic group ($F=3.19_{[4,\ 292]}$; P=0.014), season*trophic group ($F=2.55_{[4,\ 292]}$; P=0.04) and time*trophic group ($F=2.6_{[22,\ 292]}$; P<0.001), interaction between isoline and HART 89M treatment. Bacterial feeders were dominant in HART 89M (First season 46±4.1; second season 48±4.1) than in isoline treatment in both seasons. Thirty nine nematode genera composed of predators, omnivores, bacteriovores, fungivores and plant feeders were identified across the treatments (Table 3). Genus richness was not significantly different ($F=1.12_{[1,\ 81]}$; P=0.291) between the Bt cotton and isoline treatment but there was a significant effect of time ($F=7.42_{[6,\ 81]}$; P<0.001) and season ($F=66.7_{[1,\ 81]}$; P<0.001). In comparison, genus richness was significantly different ($F=5.94_{[1,\ 81]}$; P=0.017) between isoline and HART 89M treatment and there was also a significant effect of time ($F=4.62_{[6,\ 81]}$; P<0.001) and season ($F=84.0_{[1,\ 81]}$; P<0.001).

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Table 3: Nematode genera identified in Bt cotton, isoline and HART 89M treatments.

Genus	cp value	Trophic group
Cephalobus	2	Bacterial feeder
Heterocephalobus	2	Bacterial feeder
Prismatolaimus	3	Bacterial feeder
Eucephalobus	2	Bacterial feeder
Rhabditis	1	Bacterial feeder
Cervidellus	2	Bacterial feeder
Acrobeloides	2	Bacterial feeder
Acrobeles	2	Bacterial feeder
Drilocephalobus	2	Bacterial feeder
Tylocephalus	2	Bacterial feeder
Chiloplacus	2	Bacterial feeder
Plectus	2	Bacterial feeder
Wilsonema	2	Bacterial feeder
Geomonhystera	1	Bacterial feeder
Rhabdolaimus	3	Bacterial feeder
Aphelenchus	2	Fungal feeder
Tylencholaimus	4	Fungal feeder
Aphelenchoides	2	Fungal feeder
Prodorylaimus	5	Omnivore
Labronema	4	Omnivore
Eudorylaimus	4	Omnivore
Pungentus	4	Omnivore
Aporcelaimellus	5	Omnivore
Meloidogyne	3	Plant feeder
Scutellonema	3	Plant feeder
Paratylenchus	2	Plant feeder
Filenchus	3	Plant feeder
Pratylenchus	3	Plant feeder
Tylenchus	2	Plant feeder
Xiphinema	5	Plant feeder
Rotylenchus	3	Plant feeder
Rotylenchulus	3	Plant feeder
Hoplolaimus	3	Plant feeder
Helicotylenchus	3	Plant feeder
Tylenchorhynchus	2	Plant feeder
Longidorous	5	Plant feeder
Discolaimus	5	Predator
Mononchus	4	Predator
Tripyla	3	Predator

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The diversity of functional groups in both seasons was not influenced by the functional group richness but by dominance of specific trophic groups. The Renyi diversity profiles of plots in the first season before planting had a lower diversity of nematode functional groups in the Bt cotton treatment. At 180DAP, Bt cotton was less diverse than isoline. Bt cotton had a higher dominance of the dominant bacterial feeding functional group but was less diverse in terms of the rare functional group (predators). It was not possible to compare HART 89M and isoline treatments in terms of diversity due to crossing of their profiles (Figure 1). In the second season before planting, Bt cotton was less diverse than isoline. At 180 DAP, HART 89M was less diverse than Bt cotton and isoline. It was not possible to order isoline and Bt cotton treatment, however at higher values of the scale parameter isoline was more diverse while Bt cotton had high numbers of the dominant functional group (bacteriovores) (Figure 2). The PRC model showed no significant treatment effect on specific nematode genera over time in the first ($F = 2.4$; $P = 0.34$) (Figure 3) and second ($F = 2.3$; $P = 0.31$) (Figure 4) season.

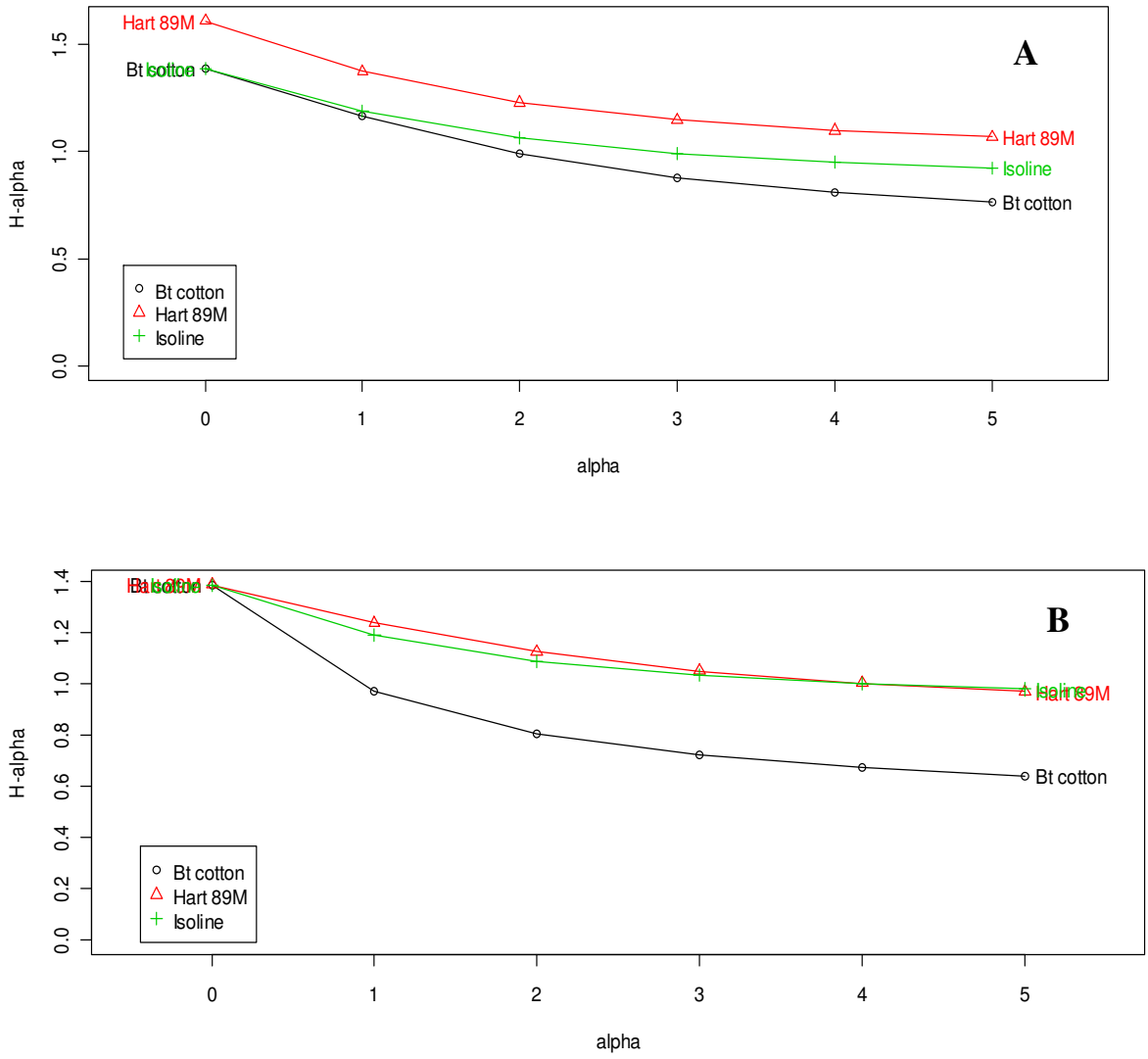

Figure 1: Renyi diversity profiles of nematode communities in Bt cotton, HART 89M and isoline treatments in the first season, before planting (A) and 180 days after planting (B). Scale parameter, alpha with values 0, 1, 2, 4 and 5 are shown.

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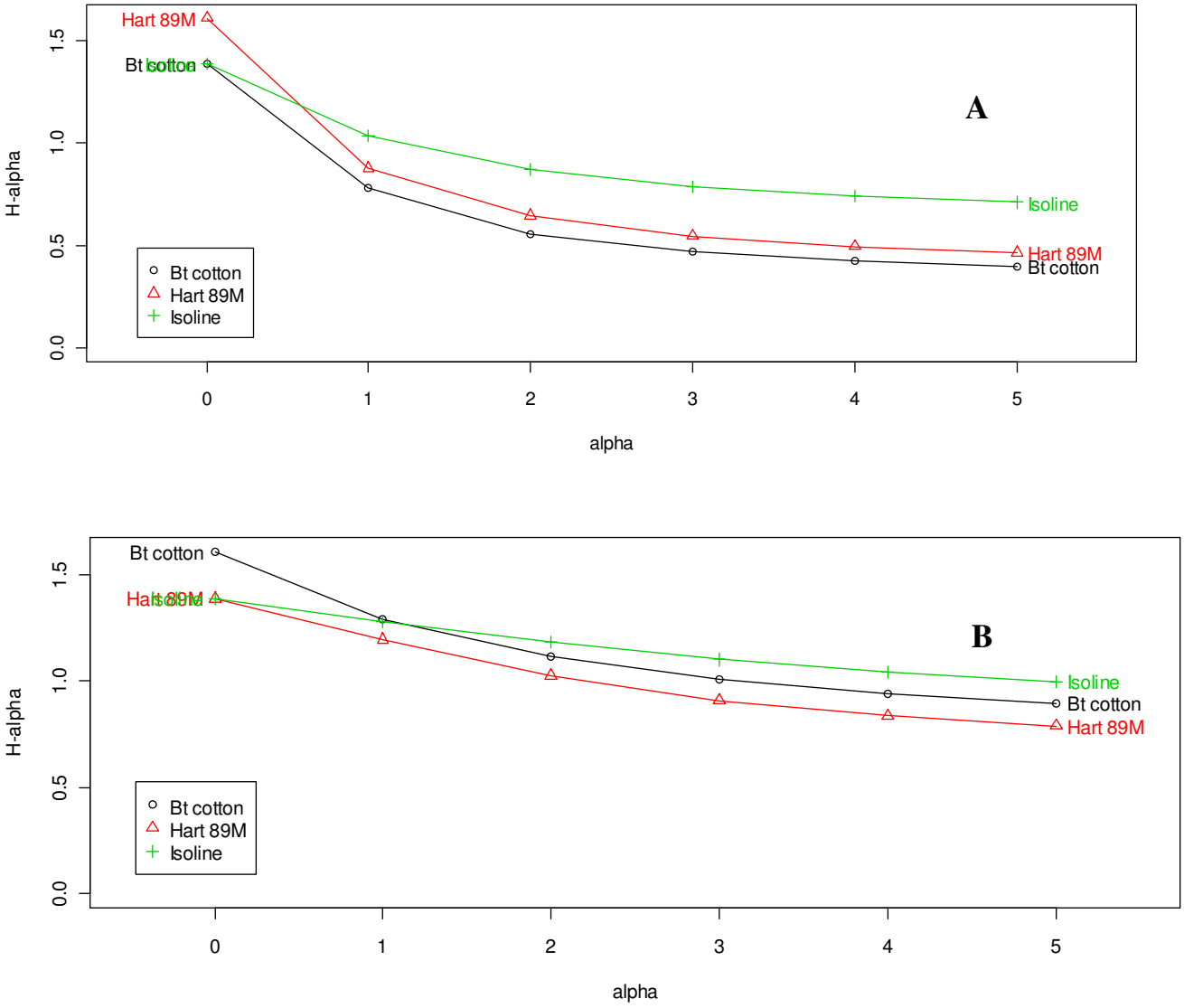

Figure 2: Renyi diversity profiles of nematode communities in Bt cotton, HART 89M and isoline treatments in the second season, before planting (A) and 180 days after planting (B). Scale parameter, alpha with values 0, 1, 2, 4 and 5 are shown.

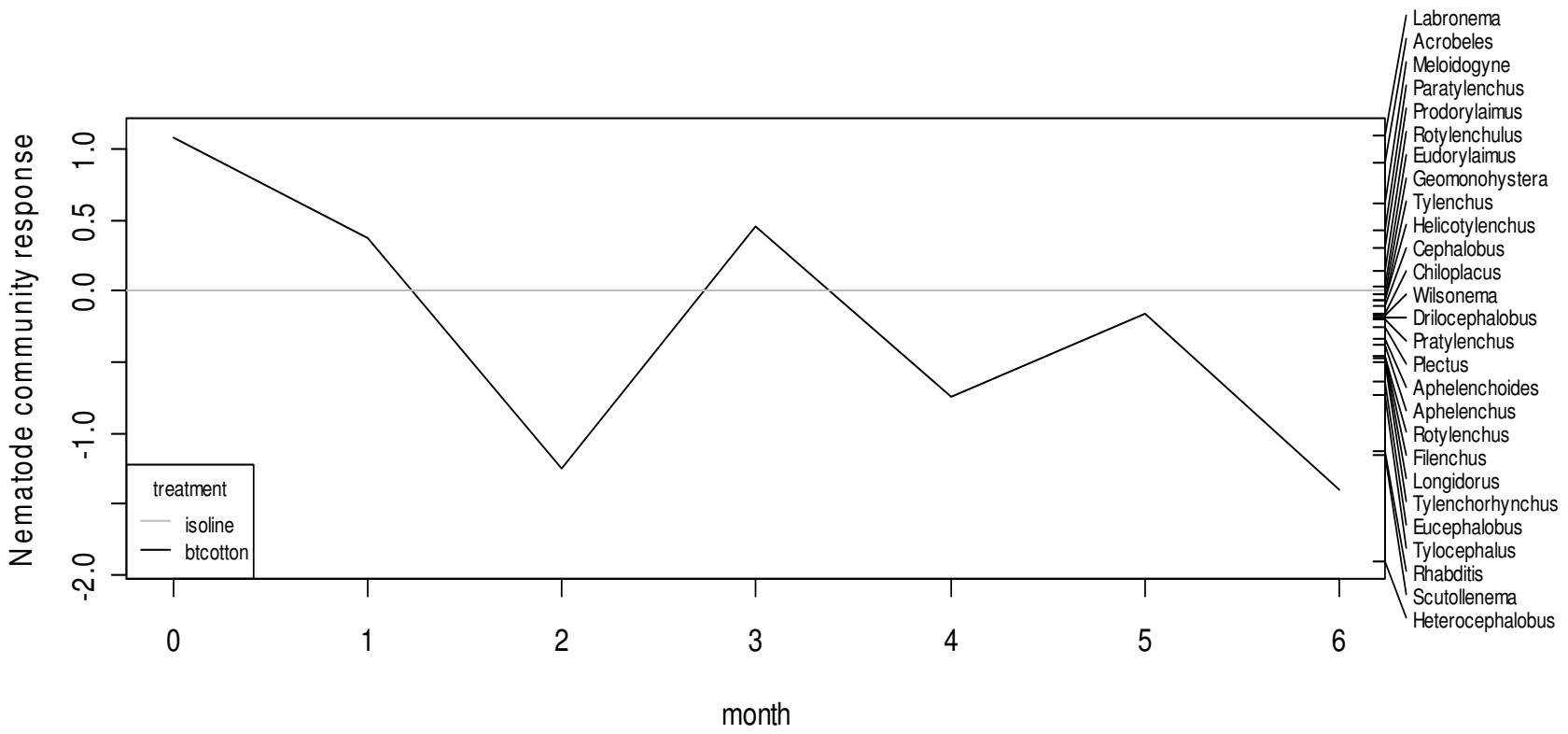

Figure 3: Principal response curves of nematode genera showing the effects of Bt cotton compared to the isogenic non Bt cotton.
The y axis represents the first principal component of the variance explained by treatment. The x axis represents sampling months during the first season. All taxa identified in Bt cotton and isoline plots are shown on the right. The horizontal line at 0 shows the response of the isoline nematode community. PRC model was not statistically significant (F =2.4; P=0.34).

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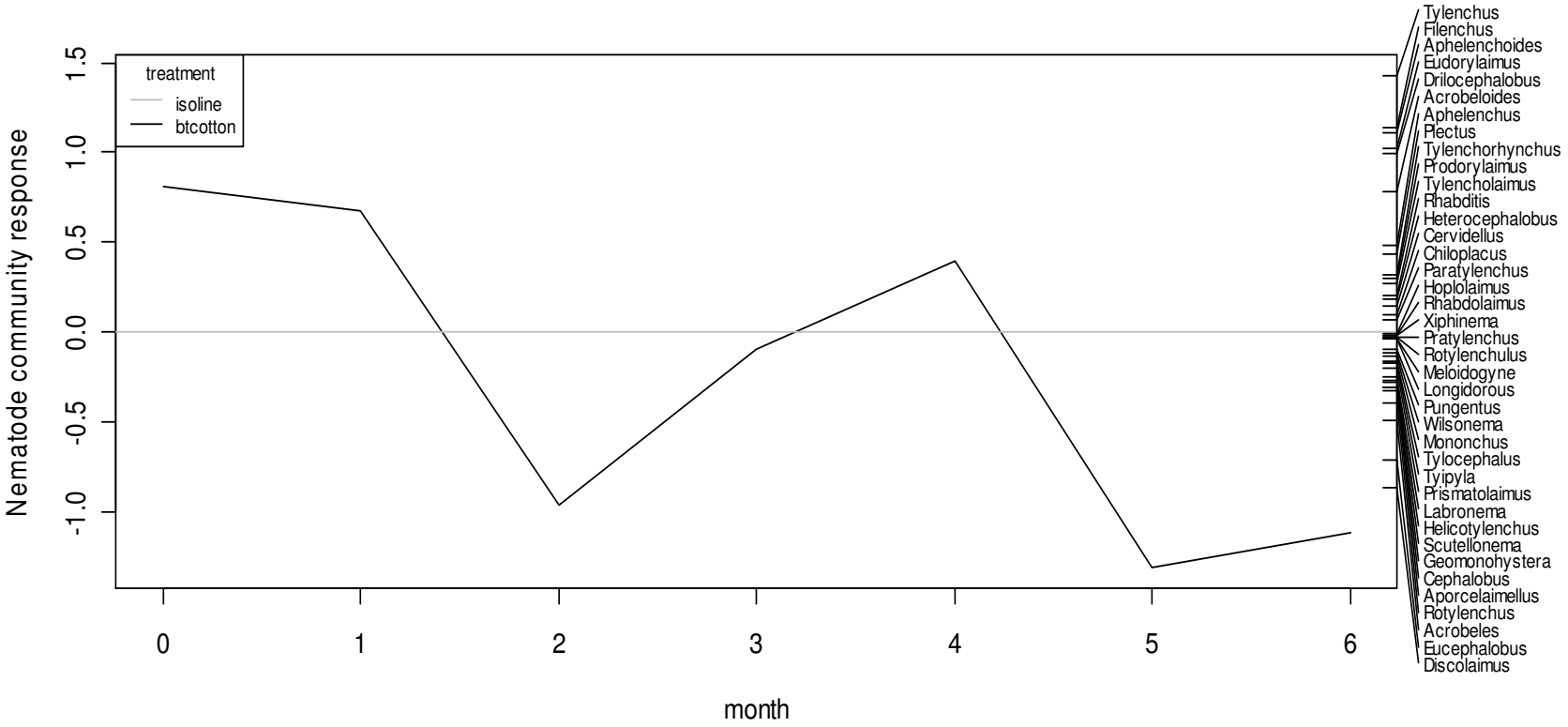

Figure 4: Principal response curves of nematode genera showing the effects of Bt cotton compared to the isogenic non Bt cotton.
The y axis represents the first principal component of the variance explained by treatment. The x axis represents sampling months during the second season. All taxa identified in Bt cotton and isoline plots are shown on the right. The horizontal line at 0 shows the response of the isoline nematode community. PRC model was not statistically significant (F =2.3; P=0.31).

5 DISCUSSION

Cry2Ab2 protein was present in transgenic cotton roots throughout the growing period in both seasons, but it was only present in soil at 150DAP and 180DAP. Constitutive expression of Cry1Ac and Cry2Ab2 (Monsanto, 2003) in all parts of the cotton plant explains the presence of Cry protein in the Bt cotton roots. There are various reports on exudation of Cry proteins from Bt cotton. Gupta and Watson (2004) estimated that about 4,900 to 18,700ppb of Bt protein was exudated from roots while Saxena and Stotzky (2001a) could not detect any Bt protein in roots. In this study, the presence of Bt protein in roots was confirmed, however, it is not clear if the Bt protein found in soil resulted from root exudates or a different pathway. The Bt protein in soil on the fifth and sixth month after planting transgenic cotton may have been from the decaying plant parts, the extensive cotton root system, sloughed off epidermal cells, or from root exudates as previously observed (Knox *et al*., 2007; Knox *et al*., 2008). Cry1Ac protein in the current study was present in soil up to 30 days from the first detection at 150DAP in both seasons. The study was done for 180 days which represents the cotton growing season. Persistence of Bt protein in soil for 234 days has been documented (Tapp and Stotzky, 1998) and Cry1Ac protein in Bt cotton has been shown to remain in soil upto 140 days (Palm *et al*., 1996). Toxins from Bt cotton may be retained in tropical soils through adsorption to different soil constituents such as clay particles (Muchaonyerwa *et al*., 2006). Organic matter in soil may also influence adsorption of toxins (Pagel-Wieder *et al*., 2007), leading to accumulation of the protein in soil (Crecchio and Stotzky, 2001). In this study, rainfall did not vary between seasons but temperature did and it may have been an important factor contributing to the seasonal differences in Bt protein levels in roots and soil.

The IC50 of 0.009-0.026µg/g Cry1Ac for the *H. armigera* population in this study was in the range reported by other authors (Wu *et al.,* 2002; Kranthi *et al.,* 2005). The LC50 was similar to that reported for *H. armigera* populations from Asia and Europe (Kranthi *et al.,* 2001). However, no mortality was observed in insect bioassays with soil samples, an indication of the low levels of protein in the soil. The IC50 and LC50 of Cry2Ab2 could not be determined due to the unavailability of the toxin. In the nematode community analysis at the trophic group level, there were significant differences ($P<0.05$) in nematode numbers in the Bt and isoline treatments in both seasons. Bacterial feeders were dominant to a lesser extent in Bt cotton than in isoline. This is in contrast with other studies on the effect of transgenic crops on nematodes that have reported an increase in fungivorous nematodes in fields containing Bt crops (Manachini and Lozzia, 2002; Manachini *et al*., 2003). However, the Bt crops and Cry proteins in these studies were different from those used in the current study and various Bt proteins have been shown to have specific toxicity to different nematode taxa (Bottjer, 1985; Borgonie*et al*., 1996; Wei *et al*., 2003). The Bt protein in soil was present at low levels and may not have caused the differences in nematode trophic groups. The differences may have been due to changes in Bt cotton during genetic transformation. Wei *et al.* (2003) and Hoss *et al.* (2004) demonstrated that Cry proteins are toxic to bacterial feeding nematodes and therefore if Bt protein was present at high concentrations in soil then the bacteriovores would have been in low abundance. Pleotropic effects in Bt cotton may have caused the observed differences. The insertion of Bt gene into cotton has been shown to cause changes in the amount of amino acids and soluble sugars and pleotropy has been reported to influence soil microbial communities in different Bt cotton events (Donegan *et al*., 1995). There were no differences in genus richness between Bt cotton and isoline. However, differences were observed between the isoline and HART 89M which could be attributed to varietal differences. This result is corroborated by Wang and Feng (2005) and also Griffiths *et al.* (2007). Varietal differences between isoline and HART 89M may have also caused the differences in nematode trophic group composition. The differences in trophic groups and genus richness in different months and seasons could be as a result of changes in temperature and moisture levels. Seasonal differences in soil microorganisms were also reported by Griffiths *et al.* (2007) in Bt maize fields. The influence of environmental factors on the protein levels and nematode indices indicate that their effect may be more important than even the Bt treatment. Specific nematode functional groups respond in different ways to disturbances. The Renyi profile in the first season at 180 DAP showed that Bt cotton had a lower diversity of functional groups but the dominance of the bacterial feeders was evident in both seasons. The differences in diversity of functional groups in both seasons were influenced by nematode abundance in the trophic groups and not by their richness. There were also differences in functional group diversity between isoline and HART 89M. Differences in functional group diversity may be due to various

reasons including varietal effects of the crop (Griffiths *et al.,* 2007), changes in the soil microbial structure (Cowgill *et al*., 2002) due to changes in enzyme activity (Mina *et al.,* 2011; Sun *et al.,* 2007) and availability of different nutrients (Sarkar *et al*., 2008), changes in soil properties (Sarkar *et al*., 2009; Chen *et al.,* 2011), fluctuations in soil moisture and temperature (Papatheodorou *et al.,* 2004) and pleiotropic effects in the genetically modified crop (Vauramo *et al*., 2006). Nematode genera have different levels of susceptibility to Cry proteins and this was investigated in the PRC. The PRC showed no significant treatment effect on specific nematode genera in the Bt and isoline treatment. This result is in agreement with Hoss *et al.* (2011) who reported a lack of effect of Cry3Bb1 in rhizosphere soil of MON88017 on the bacterial feeding nematode *C. elegans*. Griffiths *et al.* (2005) and Al deeb *et al.* (2003) also found no effect of Bt protein on specific nematode taxa. Although shifts in nematode trophic groups were observed, the biological significance is not clear and it may be important for further work to be done to study nematode communities in other Bt cotton agroecosystems probably with different soil properties in order to reveal any long term effects in nematode communities that may be of ecological importance.

6 ACKNOWLEDGEMENTS

This is paper No 30 of the BiosafeTrain project funded by the Danish International Development Agency (DANIDA). The authors thank the Kenya Agricultural Research Institute, National Agricultural Research Laboratories (KARI-NARL) for providing laboratory space and equipments. We acknowledge Mette Vesteergard for assistance in nematode identification,Maurice Okomo and Mary Ndunguli for technical support and Elias Thuranira for assistance in data analysis.

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