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Impact of transgenic cotton expressing *cry1Ac* and c*ry2Ab* genes on soil rhizosphere bacterial and fungal populations in soils of central Kenya

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The impact of 9 months cultivation of transgenic *Bacillus thuringiensis (Bt)* cotton on selected culturable bacterial and fungal populations in rhizosphere soil was investigated. The transgenic *Bt* cotton line (06Z604M), isoline (99M03) and a non-*Bt* cotton line (HART 89M) were planted in confined field site in Samuru-Thika where non-*Bt* cotton had been continuously cultivated for the past 2 years. Rhizosphere soil samples were collected at planting, maturity, flower and boll, and boll-opening stages and harvesting stages of cotton. Numbers of culturable soil microbial groups (bacteria, actinomycetes and fungi) involved in decomposition and nutrients recycling were measured at CFU and population levels. The proteins did not show effects on bacterial, actinomycetes and fungal counts and populations possibly as a result of adsorption of the proteins on soil particles, which could have rendered the proteins inaccessible for microbial utilization. Culturable microbial population and colony counts arranged in decreasing order were 06Z604D>99M03>HART89M, similar to the amounts of MBC and clay in the soils. Moreover, bacteria and fungi counts were higher at 110 DAS in 06Z604D than in 99M03 and HART89M plot soils. Our observations suggest that insecticidal proteins (*Cry1Ac* and *Cry2Ab2*) produced by Bollgard II *Bt* cotton could persist in tropical soils as a result of adsorption on soil clays but that there were no observable effect on the studied culturable microbial groups.The data presented here showed no consistent statistically significant differences (p<0.005) in the numbers of different groups of culturable bacteria, actinomycetes and fungi between rhizosphere soil of *Bt*, isoline and Hart 89M cotton lines in the same field, and no obvious trends in the numbers of the culturable bacteria and fungi with the increasing growth duration. Moreover, the studied culturable bacterial and fungal groups were positively correlated (p>0.001) with soil respiration and microbial biomass, which exhibited uneven trend with the treatments. Generally Soil from 06Z604D showed the slight higher microbial populations and CFU count, whilst HART 89M showed slight lower microbial count. This depicts the fact that slight variability in the treatments, quality and content of the root exudates might have a temporal or permanent shift in micro biota populations of a variety of crop studied.This study therefore suggests that a single-year cultivation of transgenic *Bt* cotton may not affect the functional bacterial and fungi populations in rhizosphere soil.

Key words: *Bacillus thuringiensis*, bacteria, population, colony forming units, fungi, *Bt* cotton.

INTRODUCTION

There is growing debate about the potential value of modern biotechnology, and in particular of transgenics, in helping to achieve Africa's development and food security goals. The challenge facing policymakers is not only to understand what the technology can do, or has done elsewhere, but also to establish what opportunities it presents to Africa. Currently, Africa's area under transgenic crop cultivation has witnessed a steady growth from 1.4 million hectares in 2006 (South Africa alone) to more than 3.5 million hectares in 2013 (South Africa, Egypt, Sudan and Burkina Faso) (Clive, 2014).

Transgenic crop plants modified to confer resistance against pests represent a potential environmentally safe tool to decrease the amount of chemical pesticides used in agriculture. Both field and laboratory studies showed that transgenic plants expressing *Bacillus thuringiensis* (Bt) cry proteins afford effective resistance to the larvae of a number of Lepidoptera species (Mapuranga et al., 2015). For example, *Bt* cotton plants are protected against the cotton bollworm, Helicoverpa armigera thus reducing the requirement for multiple insecticide treatments and the risk of pollution from chemical insecticide applications (Gutierrez et al., 2015). However, the release of genetically modified plants into the environment has become a public concern due to their potential environmental risks.

Environment risk assessment of transgenic plants has been mainly focused on (a) transfer of genes from the crops to wild relatives and related species (Snow and Palma, 1997; Hails, 2000), (b) resistance evolution to herbicide-tolerant crops, virus-resistant crops and insect-resistant crops and (c) impacts on non-target organisms and ecosystems (Schwember, 2008). The concerns of the impacts of transgenic plants on soil ecosystems were particularly over soil microorganism species, populations, and biodiversity (US EPA 2000; Kostov et al., 2014).

Inevitably, *Bt* toxin is introduced to soil primarily through the degradation of plant biomass remnants (Zhang et al., 2014), root exudates, through pollen deposition during tasseling, e.g., maize and by incorporation of plant residues after harvest (Zhang et al., 2015). There is no consensus about the persistence of the Cry proteins in soils. Some studies have shown that repeated and large scale use of transgenic *Bt* crops could lead to the accumulation and persistence of Bt proteins in soil (Saxena et al., 2010; Bakhsh et al., 2015). Bt toxin can quickly bind to clay minerals (Marutescu, 2012; Valldor et al., 2015) and humic acids in soil. The binding of *Bt* toxin onto soil particles reduces its bioavailability to microbes and consequently reduces its microbial degradation, but does not eliminate its insecticidal activity. Therefore, *Bt* toxin can be accumulated and kept toxic for over 234 days in soil (Marutescu, 2012).

Bt toxin produced by *Bt* corn had been reported to have no long-term effects on total numbers of culturable bacteria, fungi, protozoa and nematodes (Saxena and Stotzky, 2001; Yang et al., 2014). In China, two of the three studied transgenic *Bt* cottons (Line 247 and Line 249) were reported to cause a transient increase in total bacterial and fungal population levels, but neither the third *Bt* (Line 81) cotton nor the purified *Bt* toxins had any significant effect on the total numbers of bacteria and fungi (Zhang et al., 2014). *Bt* tobacco gave a significant rise in the number of nematodes (Yang et al., 2014). It was reported that the rate of degradation of *Bt* toxin and its impacts on soil ecosystems were related to the types and concentrations of *Bt* toxin, *Bt* crop varieties, constitution of soil microbes, soil types as well as soil chemical and physical characteristics (Stotzky, 2010). Genetic manipulation of the plants produces a change in plant characteristics, aside from Bt toxin production, that could influence the growth and species composition of soil microorganisms (Tarafdar et al., 2012). A plant can modify its rhizosphere through ion uptake, root exudates, rhizo-deposition (carbon loss from roots) and changes in the acidity and alkalinity of the rhizosphere (Day et al., 2010). Therefore, studying the fate of *Bt* toxin in the rhizosphere and its impacts on soil culturable bacteria is an important aspect of the risk assessment of Bt toxin produced by Bt cotton to the tropical soil ecosystems of central Kenya.

MATERIALS AND METHODS

Experimental site

This experiment was conducted during both short (November-December) and long rain seasons (March-June) in a confined field site at the Kenya Agricultural Research Institute (KARI), Thika, (01° 01'S and 37° 06' E) at about 1000 m above sea level. The climate of Thika is semi-arid and subtropical and characterized by hot dry seasons and cold rainy seasons. The total precipitation during the study period was 431 mm, and average daily air temperatures ranged from 14.9 to 25.5°C. The soil (0 to 15 cm depth) was used in this experiment was collected from the rhizosphere of the three cultivars of the KARI research site. The soil reaction (pH) was acidic for crop growth with low organic matter content. The confined field trial (CFT) experiment duration was between November 2009 and July 2010.

Transgenic plant lines

The transgenic cotton cultivar 06Z604D (Bollgard II) carrying both

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cry1Ac and *cry2Ab2* genes developed by the Monsanto Ag Products LLC, 800 N Lindberg Blvd St. Louis Missouri, 63167, USA was used. Bollgard II cotton event 15985 was developed by Monsanto Company to produce the *Cry2Ab2* insect control protein, which provides effective season-long control of key lepidopteran insect pests. This product was produced by re-transformation of Bollgard® cotton event 531, which produces the Cry1Ac insect-control protein. Its Isoline (99M03) was obtained from the same source. The commercial cotton cultivar (HART 89M) originally used in Central Kenya was used as the conventional cotton. Seeds of all cotton cultivars were provided through the Kenya Agricultural Research Institute, KARI- Thika.

Treatments

This study included three treatments: *Bt* cotton (06Z6O4D), isoline (99M03) and conventional (HART 89M), with three blocks and completely randomized 18 plots. The plants were grown in a confined experimental site (25x25 m) in (5x25 m) blocks and in (3x5 m) blocks. The path width between plots was 0.5 m apart and the buffer zone/crop was 3 m around the experimental site. No chemical or organic manure was applied during the entire growing period. In each plot plants were maintained to maturity. Irrigation was applied during long droughts especially during early growth times. Insecticide sprays (Descis and Booster) were done equally/evenly in all plots. Since both the 99M03 and the HART 89M contained no *Bt* gene, it was assumed that any change in soil ecological functions were due to the result of the *Bt* gene/ protein/toxin.

Field design and sampling

Experiments were performed between December 2009 and July 2010 at Kari-Thika Samuru field trial site central province, in Central Kenya. The trial site had been cultivated with conventional cotton (HART 89M). Seeds of both the transgenic cotton (06Z604D), isoline (99M03) and the conventional cotton (HART 89M) were sown in a randomized block design in three blocks each on 18 plots with 3x5 m for each cultivar/treatment. Each time, rhizosphere soil of all the plots were sampled during the growing duration, and the plant were left undisturbed. Soil samples were collected at the planting, flowering, maturity, boll and boll-opening stages and harvesting (0, 64, 88, 120, 154 and 175 days) of cotton development. Soil samples were collected by digging out soil around the rhizosphere area (up to 20 cm from the plant). For each sampling, rhizosphere soil from 4 randomly selected plants per plot was mixed and used as a composite rhizosphere soil sample. The soil samples were analyzed at KARI NARL and KARI-Muguga of the Kenya Agricultural Research Institute in Nairobi and Muguga.

Sample collection processing and analyses

Soil samples were collected on six sample dates: 15 December 2009 (sample day 0); 17 February 2010 (sample day 64); 25 March 2010 (sample day 90); 14 April 2010 (sample day 110); 27 May 2010 (sample day 154); and 18 June 2010 (sample day 175). Soil samples (approx. 100-200 g) were collected with a 3 cm diameter soil coring device to a depth of 15 cm. For each sample date, four samples per plot within each treatment row was sampled. The 4 soil cores per plot were pooled into one bag. A total of 72 (4 cores per plot x 18 plots) pooled soil samples were collected at each sample day. A clean soil coring device was used for collecting soil cores from each sample plot. Collected samples were immediately transferred in cool box to KARI Muguga and NARL for laboratory

analysis the very and the next day. Analyses used for the soil samples, and the sample days on which they were performed and are given in Table 1.

Serial dilutions of soil suspension from the different cotton rhizospheric soils from the 18 plots were prepared. The inoculum from each dilution was deposited as a drop onto the surface of a solid growth medium from a calibrated dropping pipette. Each 20 µl drop was allowed to fall from the 2.5 cm height onto the surface of the well dried medium, where it spreads over 1.5 to 2 cm. Each of the (6) 18 plates received a single drop of the same dilution. Colony counts were made in the drop areas showing the largest numbers of discrete colonies. The mean counts of the (6) plates gives the viable counts per 20 µl of dilution and from this the viable count in the original sample was calculated (Miles and Misra drop plate method, 1985)

Soil samples from each plot were collected at 0, 64, 88, 110, 154, 175 days after sowing (DAS), and were used for microbial cultures, counts and evaluation of soil moisture content. Ten grams of freshly collected soil from each plot were thoroughly mixed for 5 min (tray shaker) with 90 ml of sterile water, and then diluted 10 fold to 10^{-6}. For fungi, dilutions 10^{-2}-10^{-3} were used and for bacteria 10^{-5}-10^{-6}. The resulting suspensions were then spiral plated (Spiral System) in the appropriate culture media. The selective medium used to isolate fungi from soil was MEA (Malt extract agar, Difco Co., amended with 100 mg L^{-1} tetracycline hydrochloride; 50 mg L^{-1} rose Bengal). Isolations of bacteria were done in 5% TSA (Tryptic soy agar Difco Co., amended with 50 mg L^{-1} benomyl). Number of colonies per plate was determined directly two and five days after inoculation for bacteria and fungi, respectively.

Twenty bacterial isolates from each replication were randomly picked with sterile tooth picks, grown in TSA, and maintained in small tubes (1.2 ml) with 1.0 ml of sterile water. Identification of bacterial isolates was based on analysis of fatty acid methyl-esters profiles (Stead, 1988). Bacterial samples were prepared as described by Kloepper et al. (1992), analyzed with a Hewlett-Packard Gas Chromatography (5890 II), and identified according to Sherlock Microbial Identification System software. Twenty fungal isolates from each replication were randomly picked and maintained in tubes with MEA. Identification of fungi was based on morphology and growth on MEA, PDA and Czapek Dox Agar (Difco Co.), and consulting appropriate literature (Domsch et al., 1980).

**Laboratory incubation for numeration of culturable bacteria**

A drop plate method was used to determine the numbers of the colony-forming units (c.f.u.) of culturable bacteria. To enumerate the three culturable microbial groups, rhizospheric soil from three plants (of a particular treatment) was sampled in triplicate in each plot on a monthly basis during growth stages and after harvest. The same sampling batches were used to determine the bacterial colonies. Cottons were last harvested on July 2010 and the plants were left in the field for post harvest studies. The rhizospheric soil samples collected within the same plot were then mixed thoroughly. An amount of about 10 g of the mixed soil sample was added into 100 ml of sterile water, shaken for 20 min, and then diluted to 10^{-5}. An amount of 0.1 ml of the suspension was evenly spread onto agar plate in triplicate, and they were then incubated at 30°C for 3 days for enumeration. The colonies were visually counted.

Determination of total bacterial and fungal population

To determine the total bacterial and fungal populations, rhizosphere soil were homogenized in a pestle to break the clods. Samples were prepared in sterile distilled water by suspending 1 g soil in 100 ml water and shaking the sample vigorously for 20 min on an orbital shaker at 250 rpm. The primary soil suspensions were serially

Table 1. Bacterial populations in soil rhizosphere of 06Z604D expressing both *cry1Ac* and *cry2Ac* genes at five different crop growth stages at Thika CFT site.

Treatment	Mean rhizosphere culturable bacterial population (Cells/g dry Soil)				
	Sampling time (DAS)				
	0	64	110	154	175
06Z604D	1.85×10^5	3.32×10^5	4.32×10^5	5.74×10^5	5.46×10^5
99M03	1.97×10^5	2.92×10^6	8.12×10^4	9.58×10^5	1.06×10^6
HART89M	1.78×10^5	3.56×10^5	8.91×10^4	5.21×10^5	6.23×10^5
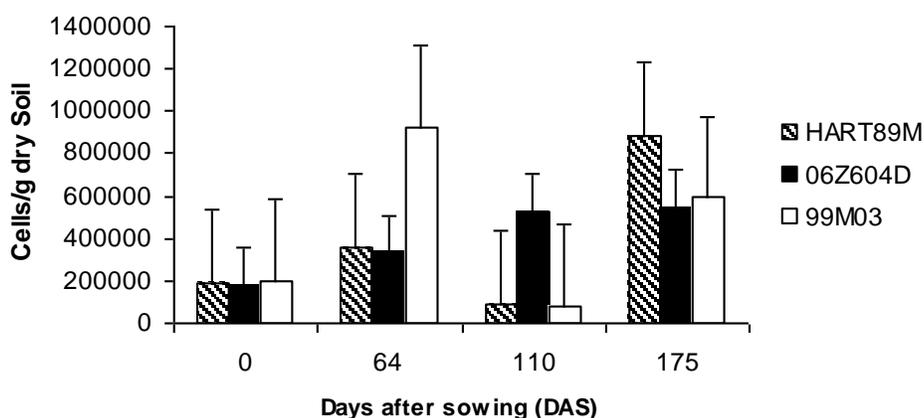
Figure 1. Bacteria populations in the three crop lines with growth time.

diluted further and 10^{-4} dilutions were plated on nutrient agar (NA) and Rose-Bengal supplemented potato dextrose agar (PDA) media in Petri plates to determine populations of bacterial and fungal populations respectively. The plates were incubated at 27 to 30°C for 3 and 7 days for bacteria and fungi respectively and observed for the appearance of colonies. The population count of the organisms was recorded after the stipulated incubation periods. The differences in the total bacterial and fungal populations in 06Z604D, 99M03 and HART 89M rhizosphere were determined using SAS package with analysis of variance (ANOVA).

Statistical analysis of data

The experiment encompassed triplicate experimental units and all data obtained were analyzed in respect of the statistical significance of the differences observed within treatments and between treatments. Response variables such as microbial populations and CFU numbers were analyzed using two-way analysis of variance (ANOVA) and principal components analysis (PCA). For ANOVA, the data were considered to be significantly different using the 5% P-value ($P < 0.05$) as the criterion.

RESULTS

Physio-chemical parameters

Variations of rainfall, soil temperature (at 0 to 20 cm depth) and percentage moisture content are presented in Figure 1. The highest rainfall was 209 mm in March 2010 while in December 2010 there was no rain at all. The total rainfall between December 2009 and July 2010 was 810 mm. The normal rain seasons in East Africa occur in Nov-Dec (short rains) and March to May (long rains). However, during the study period, the rains started in late February 2010 and continued through March to late July 2010. Soil temperature and the percentage moisture content ranged from 20 to 34.5°C and 15.8 to 33.3% respectively. Soil temperatures were higher during hot dry seasons and lower during the rain season while the percentage moisture contents were higher during cool rainy season and lower during hot dry season. However, the variations in values of both soil temperature and percentage moisture content depended on the time and weather the measurement was taken. The lowest soil temperature was recorded in April 2010 and the highest in December 2009. The lowest percentage moisture content was recorded in December 2009 and the highest was in February 2010.

Bacterial populations

Comparison between plots

The results of the bacterial population study are
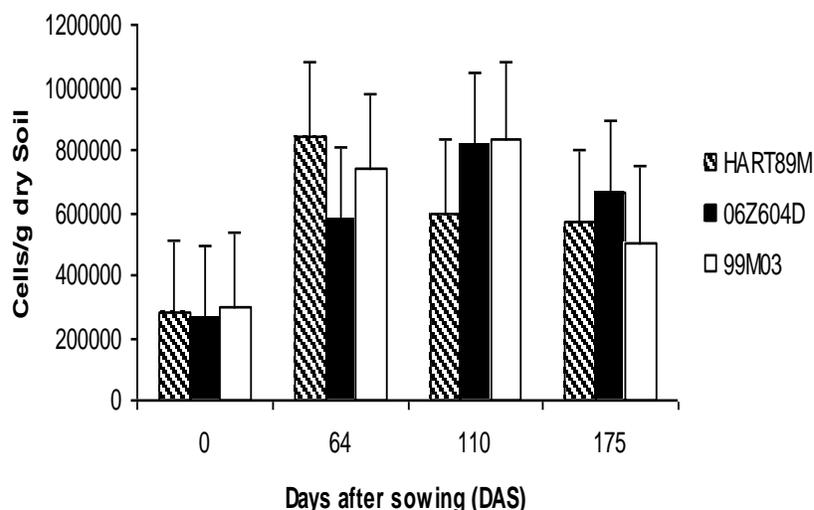
Figure 2. Actinomycetes population variation in the three crop lines with growth time.

presented in Table 1. The average population of bacteria from various plots with time are shown in Figure 1. Generally population of the two culturable bacterial groups varied considerably between treatments and among plots themselves. The overall populations between treatments arranged in decreasing order were 06Z604D > 99M03 > HART 89M. On average, HART plots had the highest population numbers (8.799×10^5 Cells/g dry Soil) followed by 99M03 plots (5.908×10^5 Cells/g dry Soil) and the least population numbers were from 06Z604D plots (5.464×10^5 Cells/g dry Soil). The highest bacterial population (1.556×10^7 Cells/g dry Soil) was measured within the 99M03 rhizosphere at 64 DAS, followed by HART 89M rhizosphere (1.8172×10^6 Cells/g dry Soil) at 110 DAS and the least numbers (1.01865×10^6 Cells/g dry Soil) was measured within 06Z604D rhizosphere at 110 DAS. Both bacterial and actinomycetes variations between plots and within the growth period were not significant different ($p > 0.05$) and ($p = 3249$) respectively. Generally bacterial population numbers varied considerably with time but tended to increase as growth duration increases. However there was no significant difference ($p = 0.5150$) between the plots with time. Populations of rhizosphere bacteria went on increasing from 64 DAS to 154 DAS in 06Z604D, 99M03 and HART 89M plants. Reason for this trend is not clear. However it might be possible that increased total root biomass with the passage of time, might be instrumental in supporting higher bacterial population (Table 1). No significant difference was however observed in the culturable bacterial population count between 06Z604D, 99M03 and HART 89M rhizosphere. Also no potential shift in the population levels of any type of bacterium was observed between the three treatments (Figure 3). The types and shapes of colonies of the three microbial groups which

grew from 06Z604D, 99M03 and HART 89M treatments were more likely similar.

Actinomycetes population

The average population of actinomycetes from various plots with time are shown in Figure 2. Generally population of the two culturable bacterial groups varied considerably between treatments and among plots themselves. The overall populations between treatments arranged in decreasing order were 06Z604D > 99M03 > HART 89M. On average, 06Z604D plots had the highest actinomycetes population numbers (6.634×10^5 Cells/g dry Soil) followed by 99M03 plots (5.712×10^5 Cells/g dry Soil) and the least population numbers were from HART plots (5.061×10^5 Cells/g dry Soil). The highest actinomycetes population (1.7623×10^6 Cells/g dry Soil) was measured within the 06Z604D rhizosphere at 110 DAS, followed by 99M03 rhizosphere (1.7041×10^6 Cells/g dry Soil) at 110 DAS and the least numbers (1.434×10^6 Cells/g dry Soil) was measured within HART 89M rhizosphere at 64 DAS. Actinomycetes variations between plots and within the growth period were not significant different ($p > 0.05$) and ($p = 3249$) respectively. Generally actinomycetes population numbers varied considerably with time but tended to increase as growth duration increases (Table 2 and Figure 2). However, there was no significant difference ($p = 0.5150$) between the plots with time.

Fungal populations

The fungal population ranged between 1.709×10^7 cells/g dry soils and 2.8352×10^4 cells/g dry soil and did not differ

Table 2. Effect of 06Z604D expressing both *cry1Ac* and *cry2Ac* genes on soil rhizosphere actinomycetes population at five different crop growth stages at Thika CFT site.

Treatment	Mean rhizosphere culturable actinomycetes population (Cells/g dry Soil)				
	Sampling time (DAS)				
	0	64	110	154	175
06Z604D	2.77×10^5	5.74×10^5	8.16×10^5	8.91×10^5	7.06×10^5
99M03	2.98×10^5	7.36×10^5	8.38×10^5	9.06×10^5	5.71×10^5
HART89M	2.65×10^5	8.45×10^5	5.97×10^4	7.06×10^5	5.06×10^5

(a)   **(b)**

(c)
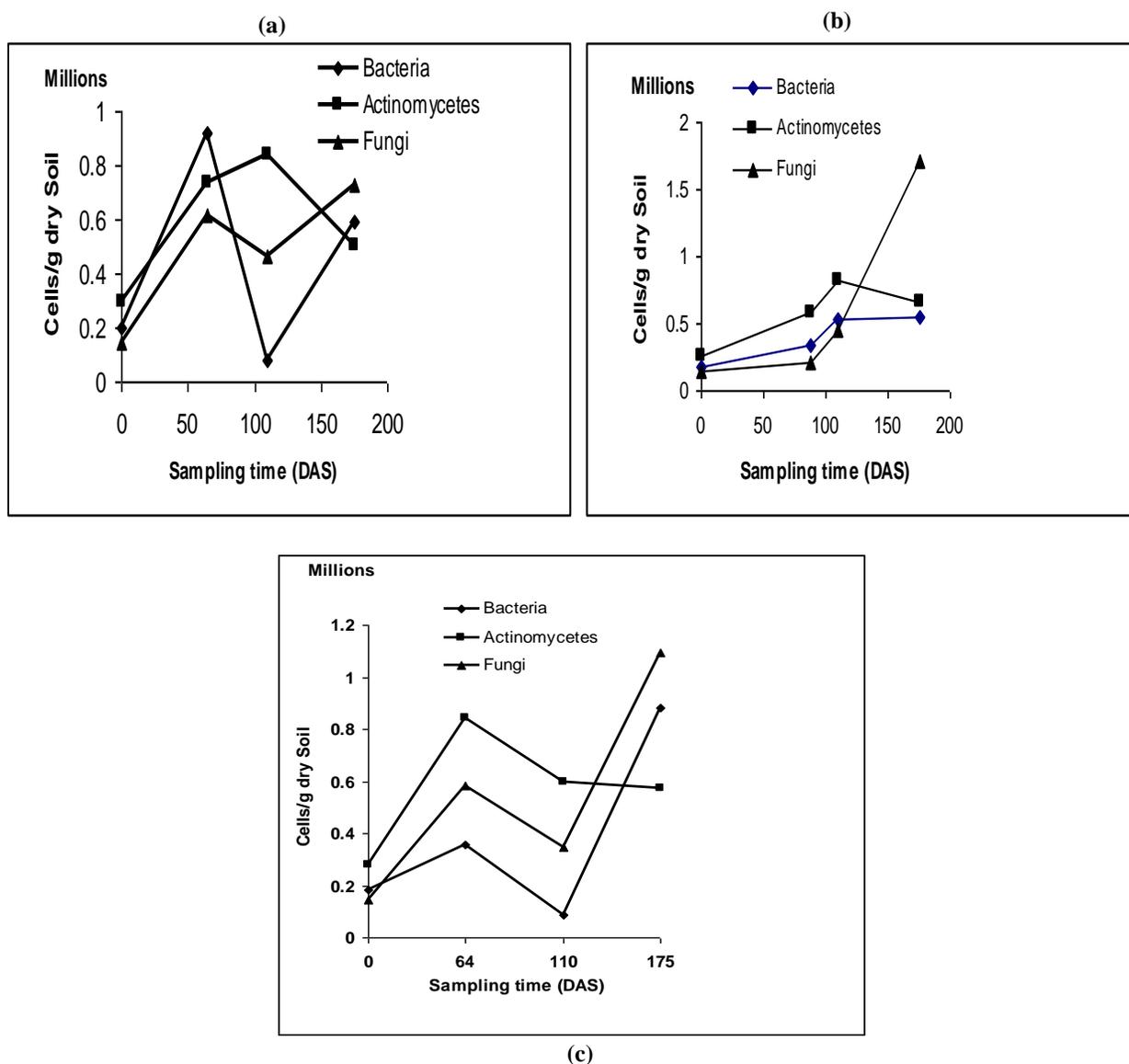
Figure 3. Mean variations of different microbial groups in 99M03 (a), 06Z604D (b) and HART89M (c) plots at different growth stages.

significantly among treatments. However, overall fungal populations were higher in 06Z604D soil and were the lowest in HART 89M soil (Table 3 and Figure 3). The overall evaluations of fungal populations between

Table 3. Effect of 06Z604D expressing both *cry1Ac* and *cry2Ab* genes on soil rhizosphere fungal population at five different crop growth stages at Thika CFT site.

Treatment	Mean rhizosphere culturable fungal population (Cells/g dry Soil)				
	Sampling time (DAS)				
	0	64	110	154	175
06Z604D	1.46×10^5	2.06×10^5	4.56×10^5	7.01×10^5	8.02×10^5
99M03	1.47×10^5	6.17×10^5	4.67×10^5	6.84×10^5	1.09×10^6
HART89M	1.46×10^5	5.84×10^5	3.48×10^4	5.16×10^5	1.03×10^6
CD (p=0.05)					

treatments arranged in decreasing order were 06Z604D > HART 89M > 99M03. On average, 06Z604D plots had the highest fungal population numbers (1.709×10^6 Cells/g dry Soil) followed by HART plots (1.091×10^5 Cells/g dry Soil) and the least population numbers were from 99M03 plots (7.293×10^5 Cells/g dry Soil). The highest fungi population (1.709×10^7 Cells/g dry Soil) was measured within the 06Z604D rhizosphere at 175 DAS, followed by HART 89M rhizosphere (3.465×10^6 Cells/g dry Soil) at 175 DAS and the least numbers (2.053×10^6 Cells/g dry Soil) was measured within 99M03 rhizosphere at 64 DAS. Rhizosphere fungal population declined at 88-110 DAS with the advancement of crop growth stage irrespective of the type of cotton grown (Table 3 and Figure 3). This could either be due to decline in the soil moisture content with the progress of the season or due to change in temperature regime. Fungi variations between plots and within the growth period were not significant different ($p>0.05$) and ($p=4549$) respectively. However there was no significant difference ($p=0.5150$) between the plots with time.

Colony forming units (CFUs)

The average CFU counts of bacteria, actinomycetes and fungi from various plots with time are shown in Table 4. Generally CFU counts of the three culturable microbial groups varied considerably between and among treatments and plots. The overall CFU counts of the three culturable groups are arranged in decreasing order were 99M03 > HART 89M > 06Z604D. On average, 06Z604D plots had the highest fungi CFU counts (5.61×10^5 colonies/g dry Soil), however, in contrary 99M03 plots had the highest culturable actinomycetes and bacterial CFU counts 5.98×10^5 and 4.48×10^5 (colonies/g dry Soil) respectively than both 06Z604D and HART89M. The highest CFU counts (17.09×10^5 colonies/g dry Soil) was measured within the 06Z604D rhizosphere at 175 DAS and the lowest CFU counts (1.46×105 colonies/g dry Soil) was measured at planting (0 DAS). The CFU counts between plots and within the growth period were not significant different ($p>0.05$) and ($p=3249$) respectively. Generally CFU counts varied considerably with time but

tended to increase as growth duration increases. However there was no significant difference ($p=0.5150$) between the plots with time.

DISCUSSION

Physio chemical parameters

The total annual rainfall that occurred during the study period, November 2009- July 2010 was higher at (745.8 mm) than the annual rainfall reported in Thika in 2009 (KMD, 2010). The peak of the highest rainfall occurred in March 2010 at 209.0 mm higher than the one reported on the previous year (2009), which was 173.9 mm (KMD, 2009). The variation of rainfall affects soil temperature and percentage moisture content. The higher percentage moisture content values observed during rainy season were obviously due to increased water supply from rain. The low soil temperatures during the rainy season were undoubtedly due to the cooling effect of the rain water and low air temperatures which normally corresponds with rains. Furthermore, the finding that organic matter content of the experimental plots tended to be higher during rainy season than the dry season agrees with previous findings (Satrio et al., 2009). Satrio et al. (2009) reported values up to 97.69% higher during rainy season. Runoff, due to rains, brings organic material into the ecosystem (allochthonous), and would appear that the import of organic matter more than compensates for increased the mineralized rate (Sarkar et al., 2009). With respect to this study, (i) we are in agreement with this fact that the transient increases in the microbial populations in rhizosphere soil of the treatments are probably related to changes in root exudation and root characteristics (Figure 2 and Table 2). (ii) We are in agreement with this fact that the transient increases in the basal activity yields in rhizosphere soil of 06Z604D are related to changes in root exudation and root characteristics. Moreover, the soil microbes largely depends on the type of soil, temperature, moisture, plant growth, nutrients, pH, and many other factors which may vary between locations but also within a single plot and over very small distances (OECD, 2010).

Table 4. Comparisons of colony counts on soils from the rhizosphere of the three different cotton lines (Miles and Misra drop plate method).

Treatment	Bacteria	Actinomycetes	Fungi
06Z604D	2.38×10^5	4.81×10^5	5.61×10^5
99M03	4.48×10^5	5.98×10^5	4.91×10^5
HART 89M	3.77×10^5	5.72×10^5	5.43×10^5
CD (p=0.05)			

Microbial populations

The observed range of the number bacterial and fungal populations (10^4 to 10^7 Cells gram^{-1} dry Soil) in Thika CFT is within the range of previous findings (Martinez et al., 2014; Jason et al., 2014). Garder et al. (2014) reported that under optimal growing conditions, total microbial abundance in background soils can exceed about 10^6 to 10^8 colony forming units per gram (dry weight) of soil (cfu/g) for bacteria, 10^6 cfu/g for actinomycetes, and 10^5 cfu/g for fungi. Garder (2014) estimated that there were approximately 4×10^3 and 4×10^4 species per g of soil respectively. However, due to variations on both biological and physiochemical parameters between sites, microbial populations in soils can relatively vary. In this study, the slight shift in population numbers (from 0 DAS to 175 DAS) of bacterial and fungal populations in 06Z604D plots, 99M03 and HART 89M plots of this study (Tables 1, 2 and 3) could be due to the fact that the increase of exudates supply. Previous studies revealed that qualitative and quantitative differences in root exudation could strongly affect the structure of microbial communities in the rhizosphere (Mansouri et al., 2002; Oger et al., 2000). Moreover, Bhatt et al. (2001) observed that the soil pH of the study site could affect the microbial activity of both bacteria and fungi consequently their populations through retarding organic matter decomposition rates and hence substrate availability. Though there were significant differences in numbers of the bacterial and fungal populations in the rhizosphere between 06Z604D, 99M03 and HART 89M within each sampling date from December 2009 to July 2010. But no significant differences were found in the populations and CFU numbers of the bacteria, action-mycetes and fungi among three cultivars after the growth season. Furthermore, there were no correlations between *Bt* toxin levels and numbers of the culturable bacteria and fungi. Those results were also verified by the pure *Bt* toxin fortification experiment. *Bt* toxin may not be the direct factor influencing the micro flora in the rhizosphere. The difference in the numbers of the culturable bacterial and fungal population in the rhizosphere may be due to different crop cultivars with different root exudates and root characteristics. Root exudates have a profound qualitative and quantitative effect on the rhizosphere microflora (Philippot et al., 2013). The composition of microbial communities in the rhizosphere is governed mainly by the quality and quantity of carbon sources that are released as root exudates (Philippot et al., 2013; Churchland and Grayston, 2014).

This study has shown that *Bt* protein (*Cry2Ac*) slightly stimulate the increase on populations of culturable bacterial and fungal naturally occurring in the cotton rhizosphere. Furthermore, this work fills the information gap on *Bt* cotton on East African tropical cotton soils and supplements data presented by Muchaonyerwa et al. (2014) concerning the effects and persistence of *Bt* toxin on microorganisms in some Zimbabwean soils. No statically significant ($P < 5\%$) correlation was shown between *Cry1Ac/Cry2Ab2* and population of other microorganisms. In 06Z604D soil and 99M03 the population of fungal was around 4.56×10^5 cells/g dry soil to 5.6 4.56×10^5 cells/g dry soil and 5.56×10^5 cells/g dry soils respectively. A change induced through genetic manipulation of the plants had produced a change in plant characteristics aside from *Bt* toxin production that could influence the growth and species composition of the soil microorganisms (Zhang et al., 2014). It had also been reported that, there is precedent for unanticipated changes in plant quality occurring from insertion of genes which resulted in changes in root exudates. Thus, an altered composition of root exudates may induce a different community of rhizosphere microorganisms. Even small modifications, as may exist between different cultivars of the same plant species, can result in the occurrence of different microbial communities in the rhizosphere (Berendsen et al., 2012). Indirect effects of *Bt* toxin, such as due to differences in invertebrate pests and their impacts on plant physiology, may also affect the rhizosphere bacteria. Although the transitory reduction of functional bacteria populations in the rhizosphere of *Bt* cotton 06Z604D, 99M03 and HART 89M may not be of environmental concern, the accompanying change in microbial species composition, with a potential to impact soil processes, may be of ecological significance and warrants further investigation.

Bt corn was reported to have no effects on numbers of culturable microorganisms (Saxena and Stotzky, 2001; Hannula et al., 2014), while transient increases in culturable microorganisms were observed with *Bt* cotton (Zhang et al., 2014) and Bt potato (Hu et al., 2013). *Bt* corn had been reported to have no impact on microbial

biomass, activity, or community structure (Turrini et al., 2015). Certain methanogenic archaeabacteria were inhibited by *Cry* proteins (Han et al., 2013), and Bt-rice straw had transient effects on microbial numbers and activity in a flooded rice paddy soil (Schmidt et al., 2015). Because the structure of the soil microbial community is an important component of soil quality and health, soil microbiological properties could be early and sensitive indicators of anthropogenic effects on soil ecology in both natural and agricultural ecosystems (Habig and Swanepoel, 2015). In the last decade many reports on potential impacts of transgenic crops on the structure and functioning of the soil microbial community have been published. Two of three transgenic *Bt* cotton lines caused a transient increase in total bacterial and fungal population levels; in contrast, neither the third transgenic *Bt* cotton line nor the purified *Bt* toxins affected the total numbers of bacteria and fungi (Zhang et al., 2014). There were no significant differences in the numbers of culturable bacteria, actinomycetes, fungi, protozoa, and nematodes in the rhizosphere of *Bt* vs non-*Bt* corn or in soil amended with biomass of Bt vs. non-Bt corn (Lu et al., 2010). Despite the detection of *Cry1Ab* protein in the rhizosphere soil of MON810 maize, the bacterial community structure was less affected by the *Cry1Ab* protein than by other environmental factors, such as plant age or field heterogeneity (Baumgarte and Tebbe 2005). However, farming practices and crop types may increase populations of beneficial microorganisms by soil amendments have the advantage that no improved methodology is needed to facilitate microbial growth and survival. Indigenous microorganisms, in general, have higher competence for survival in their own habitat than introduced ones. Other cultural methods, such as mulching and crop rotation, also induce microbial increases in soil (Yuliar et al., 2015).

We have found very little effect of *Cry1Ac*/*Cry2Ab2* gene expression on rhizosphere and soil microbial communities. In the Thika CFT experimental site, there was little large soil type effect. The bacterial and fungal population profiles were slightly affected by Cry gene expression in the Central Kenyan soil. Thika-Samuru soils is higher in clay content and Cry protein is known to persist longer in clay soils (Valldor et al., 2015), which may explain why this effect has also manifested in Kenyan soil. The strongest difference found so far was in the bacterial population, my results are generally similar to reports from other researchers (Hu et al., 2013; Hannula et al., 2014; Zhang et al, 2014). However, longer-term studies are needed, and newer methods may reveal effects not previously seen.

Conclusion

Our findings demonstrated that the fate of 06Z604D expressing both *cry1Ac* and *cry2Ab2* genes on culturable soil bacterial, actinomycetes, fungal populations and their CFU counts versus 99M03 and HART 89M had transient or no any adverse effect. However, knowledge of the impact of 06Z604D residues on red Kenyan soil microbial ecology is essential for understanding the long-term agronomic and environmental effects of genetically modified crops and for developing appropriate management practices for minimizing potential negative impacts.

Moreover, differences in the culturable bacterial and fungal population between rhizosphere soil of 06Z604D, 99M03 and HART 89M cotton in the same trial site were either transient or absent. The major conclusions from this study are: (1) Cultivation of 06Z604D expressing both *Cry1Ac* and *Cry2Ab2* protein did not result in significant change in the overall numbers of culturable bacterial and fungal populations; and (2) transgenic 06Z604D had no clear effect on the number of culturable bacterial and fungal populations in the rhizosphere within one growing season. These results suggest that cultivation of *Bt* crops over multiple years probably poses little ecological or environmental risk.

Overall the soils exposed to *Bt* did not show significant variation in the bacterial and fungal populations. The effect of transgenic plants on soil populations of non-target bacteria and fungi could be either transient or do not have any effect at all. Dunfield and Germida (2003) concluded that the changes in the microbial community structure associated with genetically modified plants were temporary and did not persist into the next field season. In the present study, the soil bacterial and fungal populations were comparable between the soils surrounding 06Z604D event MON 531 and event MON 15985 expressing both Cry 1Ac and Cry2Ab2 protein and the non *Bt* counterparts (99M03 and HART 89M). This observation indicates that 06Z604D, does not have adverse effect on culturable soil bacterial and fungal populations. Measurement of microbial activity is normally through the presence of culturable microbes in the soil.

The conclusion of this study is in agreement with other findings in the fact that, the insecticidal toxin released from *Bt* crops had no short-term deleterious effects on soil biological communities, but the potential long-term effects due to accumulation and persistence of the toxin on soil biodiversity have not been evaluated extensively (Donegan et al., 1995; Betz et al., 2000; Saxena and Stotzky, 2001a; Head et al., 2002; Zwahlen et al., 2003; Buiatti et al., 2013; Xiaogang and Liu, 2013; Malviya et al., 2014).

Conflict of Interests

This statement is to certify that all Authors have seen and approved the manuscript being submitted and declare no conflict of interests. We warrant that the article is the

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