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Effects of *Bacillus thuringiensis* CRY1A(c) δ-endotoxin on growth, nodulation and productivity of beans [*Phaseolus vulgaris* (L.) and siratro (*Macroptilium atropurpureum* DC.)]

H. M. Makonde[1], F. K. Lenga[1], D. Masiga[2], S. Mugo[3] and H. I. Boga[1]*

[1]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000-00200 Nairobi.
[2]International Center for Insect Physiology and Ecology (ICIPE), P.O. Box 30772-00100 Nairobi, Kenya.
[3]International Maize and Wheat Improvement Center (CIMMYT), ICRAF House United Nations Avenue, P.O. Box 1041-00621 Village Market, Nairobi, Kenya.

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The recent introduction of Bt maize and Bt cotton transgenic crops into Africa has raised concerns on their potential short and long-term ecological effects on the environment. The effects of *Bacillus thuringiensis* (Bt) Cry1A(c) δ-endotoxin on the growth, nodulation and productivity of two leguminous plants grown in clay soil were evaluated. Bt Cry1A(c) δ-endotoxin from a local *B. thuringiensis* isolate (ICIPE L1-2) active against *Chilo partellus* (Swinhoe) was used. Beans (*Phaseolus vulgaris* L.) and Siratro (*Macroptilium atropurpureum* DC.) seedlings were grown in pots treated with Bt Cry1A(c) δ-endotoxin solution (100 µg/ml). Control experiments were treated with water. The plants were maintained in the greenhouse until nodulation (8 weeks) and maturity (14 weeks) stages when sampling was done for measurements of morphological, productivity and nodulation traits. Nodulation was observed in both plants species. Nitrogen content in treatments for both bean and siratro plants, with and without Bt-toxin not were significantly different. Leaf area, plant dry weight, number of pods per plants and number of seeds per pod observed in treatments with and without Bt-toxin for both bean and siratro plants were also not significantly different. This shows that Bt Cry1A(c) delta-endotoxin does not interfere with the host plant growth, nodulation and productivity in clay soil. Findings will provide researchers with data to design more robust experiments and will inform the decisions of diverse stakeholders regarding the safety of transgenic crops.

Key words: *Bacillus thuringiensis*, Bt Cry1A(c) δ-endotoxin, *Macroptilium atropurpureum* (DC.), nodulation.

INTRODUCTION

Genetic modification of living organisms, including plants and animals, to incorporate useful traits is a powerful technology for the future development of sustainable agricultural systems, through among others, insect resistance, herbicide resistance and improved quality of crop products. Cry proteins from *Bacillus thuringiensis* are by far the most common insecticidal proteins that have been engineered into plants (James, 2005).

Bt technology is an essential tool in agriculture, but it poses risks to the environment. Bt crops have codon modified genes from the soil dwelling *B. thuringiensis* that encode the production of insecticidal toxins into plants incorporated (Bohorova et al., 2001). Bt technology alleviates

*Corresponding author. E-mail: hboga@fsc.jkuat.ac.ke or hamadiboga@yahoo.com. Tel: +254-733926733.

Abbreviations: Bt, *Bacillus thuringiensis* toxin; **Cry1A(c),** crystalline protein 1A(c); **BTS,** Bt toxin solution; **CRD,** completely randomized design; **NBTW,** Non-Bt water control; **GM,** genetically modified.

many problems associated with the use of chemical pesticides, contributes to increased grain yields and a reduced need for insecticidal sprays. However, the potential impacts of Bt crops on the environment remain a topic of debate worldwide, calling for the assessment of Bt crops on the environment. Bt cry proteins are the only insecticidal proteins that are commercially used in genetically modified crops (James, 2005). Bt *cry* genes have been engineered into maize, cotton, potato, tomato, rice, eggplant, and oilseed rape (Ely, 1993; De Maagd, 2004) to impart insect resistance, and thereby reduce reliance on pesticides.

Bt toxins released through maize root exudates retain its activity for 180 to 234 days in both laboratory and field experiments (Saxena and Stotzky, 2001). Genetically engineered Bt *cry* genes in plants continue to be expressed during growth of the plants. Concerns have been raised that insect-resistant genetically modified (GM) crops expressing cry proteins from *B. thuringiensis* could harm susceptible non-target organisms (Donegan and Seidler, 1999; Sanvido et al., 2007). If the Bt toxins released are not all degraded by microbiota and abiotic factors, the Bt toxins could accumulate in the soil and constitute a hazard to susceptible non-target organisms such as the soil microbiota including beneficial insects in the environment (James et al., 1993; Johnson et al., 1995; Losey et al., 1999) and crucial organisms that provide important ecological and economic services within agricultural systems including decomposers, pollinators, parasites and nitrogen fixing bacteria.

Soil organisms regulate a number of processes in terrestrial ecosystems that are not only critical for productivity but are also essential for maintenance of ecosystem health (Brussard et al., 1997). Very few biological processes are mediated by individual species of biota; therefore, the successful functioning of most ecosystem processes requires a balance of biota interactions in the complex soil biota community (Gupta and Yeates, 1997). Microbial-faunal (microfauna, mesofauna and macrofauna) interactions play a critical role in a variety of biological functions both in the rhizosphere and near decomposing residues (Coleman and Crossley, 1995; Gupta and Yeates, 1997). The rhizosphere contains a large majority of the soil's biota populations and the plant microbe interaction in the rhizosphere is one of the major factors regulating the health, growth and productivity of plants.

It is widely acknowledged that root exudates govern which organisms reside in the rhizosphere (Lynch, 1994; Bardgett et al., 1999). Change to the quality of rhizosphere exudates will potentially modify the dynamics of the soil biota composition (biodiversity) and activity and may cause changes to both deleterious and beneficial microflora and microfauna (Losey et al., 1999).

One crucial function carried out by soil microorganisms is nitrogen fixation, which is the major source of nitrogen for many natural ecosystems. It is important primarily because nitrogen is often the limiting nutrient in many terrestrial ecosystems (Vitousek and Howarth, 1991). More over, nitrogen fixation is a function performed by a wide diversity of bacteria belonging to many different taxa (Young, 1992; Zehr et al., 2003). Symbiotic relationships between nitrogen fixing bacteria and their eukaryotic hosts bring mutual benefit to each participant. However, little is known about how rhizobia benefit from nodulating legumes. Studies on the legume-rhizobium interaction points to a potentially severe cost to rhizobia as a result of symbiotic nitrogen fixation. During nodulation, infecting rhizobia enter host meristem cells and differentiate into bacteroids. They can fix atmospheric dinitrogen only in this differentiated state (Caetano-AnolleÂs and Gresshof, 1991); however, it is reached through a terminal developmental event during which bacteroids lose the ability to reproduce (Zhou et al., 1985). Rhizobia might nonetheless benefit from nodulation via benefits conveyed to their free-living kin (Jimenez and Casadesus, 1989; Olivieri and Frank, 1994). Host plant roots excrete many compounds into the rhizosphere that increase rhizobial growth and nodulation may increase the rate of excretion into the rhizosphere (Boivin et al., 1991; Hartwig et al., 1991). The ability of a legume to fix nitrogen depends upon the presence of the appropriate bacterium, *Rhizobium*. Some species are very versatile with respect to the strain of *Rhizobium* that is suitable; other species are highly specific in their requirements. Similarly, some strains of *Rhizobium* are highly specific for certain legumes; others are capable of living in nodules of many leguminous species. Furthermore, some strains of *Rhizobium* are adapted to acid soils while others survive only on alkaline soils. Some examples of the leguminous species are *Cajanus cajan*, *Macroptilium atropurpureum*, *M. lathyroides*, *Stylosanthes guianensis* and all species of beans. Siratro (*Macroptilium atropurpureum* DC.) and bean (*Phaseolus vulgaris* L.) plants were used in this study. This is because of their ability to form a symbiotic relationship with rhizobia, their high nitrogen fixing ability and versatility with respect to the strain of *Rhizobium* that can interact with the plants. Determination of the potential effects of Bt Cry proteins on leguminous plants growth, nodulation and productivity is important to gather data on any potential risks that genetically modified crops may have on these plant species that form an important symbiotic relationship with rhizobia in the soil, which is a key natural process that replenishes the lost nutrients in the soil (Stewart, 1973). This study was aimed at assessing the effects of Bt Cry1A(c) δ-endotoxin on leguminous plants growth, nodulation and productivity in clay soil.

MATERIALS AND METHODS

Soil collection and physical and chemical analyses and processing

About 300 kg of the soil was collected from the Kenya Agricultural Research Institute - National Agricultural Laboratories (KARI-NARL) farm in Nairobi, an area where maize is predominantly grown in

Kenya. The standard soil analyses of the soil sample were performed at the National Agricultural Laboratories (NARL), Nairobi. The analyses involved both physical (texture, bulk density and particle size) and chemical (soil pH, % total Nitrogen, % organic carbon, phosphorous, potassium, calcium, magnesium, manganese, copper, iron, zinc and sodium) analyses.

Processing of the soil

The soil was pounded to break the large cramps, mixed thoroughly for homogeneity and then sieved through a 4 mm wire mesh. 1 Kg of the clay soil was put into polythene bags (10 cm in diameter) and pots (15 cm in diameter).

Microbial test for the *B. thuringiensis* isolate (ICIPE L1-2)

The local *B. thuringiensis* isolate (ICIPE L1-2) was retrieved from the ICIPE germplasm bank and cultured in KN-media (Corn starch 10 g, Soybeans flour 20 g, Peptone 8 g, Yeast extract 5 g, $MgSO_4$ 0.3 g and $CaCl_2$ 0.1 g) per litre (Lelmen et al., 2007). This was incubated in a shaker incubator (Controlled Environment Incubator Shaker, New Brunswick Scientific Co. INC. Edison, N.J. U.S.A.) at 32°C and at 250 rpm for 3 days when microscopic analysis of the isolate was done.

Cells were picked from the nutrient broth culture using a sterile wire loop and spread on 10 sterile slides. The slides were air-dried for 10 min and then 5 slides were gram-stained while the other five slides were stained using carbol fuchsin stain, which clearly stained the cells, crystals and spores. Microscopic examination done revealed that the isolate was gram positive. Rod shaped cells were observed with crystals and endospores of different shapes confirming the isolate ICIPE L1-2 as a *B. thuringiensis*.

Preparation of Cry 1A(c) Bt toxin

B. thuringiensis isolate (ICIPE L1-2) was cultured in KN-media and incubated in a shaker incubator as described by Lelmen et al. (2007). Harvesting and purification of the δ-endotoxins was carried out by centrifugation at 10,000 rpm x g for 10 min, at 4°C (Sorvall refrigerated centrifuge). The crystals and cells that settled at the bottom were washed 3 times with normal saline (0.85% NaCl). Solubilization was carried out by suspending the pellet in 50 mM Na_2CO_3.$NaHCO_3$ buffer (pH 9.5) containing 10 mM Dithiothreitol (DTT) for lysis of intact cells and incubated for 1 h at 37°C. The solubilized toxin was concentrated using polyethylene glycol for 30 min and then dialyzed in DTT free 50 mM Na_2CO_3 buffer (pH 9.5). Determination of protein concentration was performed using the Bicinchonicic Acid (BCA) protein assay method according to instructions supplied by the manufacturer (Pierce, Rockford, IL. USA). Enzymatic cleavage of the protoxin was carried out using trypsin in a ratio of 1:50 and incubated at 37°C for 30 min (Osir and Vundla, 1999). The concentration of the protoxin was found to be 96 mg/ml while that of the purified activated Bt endotoxin was 75 mg/ml. The activated Bt Cry1A(c) delta-endotoxin was diluted with distilled water to a concentration of 100 µg/ml that was used in experimental setup described below. Previous studies have indicated that purified Bt toxin amended in the soil up to a concentration of 100 µg/ml exhibits negative effect on non target organisms (Lelmen et al., 2007).

Pre-germination and planting of the leguminous bean and siratro seeds

Bean and Siratro plants were used in this study because of their ability to form a symbiotic relationship with rhizobia (Young, 1992; Zehr et al., 2003), their high nitrogen fixing ability and versatility with respect to the strain of *Rhizobium* that can interact with the plants. Furthermore, they are readily available, easy to manage and evaluation of the potential impact of Bt Cry proteins on leguminous plants growth, nodulation and productivity is important to gather data on any potential risks that Bt crops may have on these plant species that form an important symbiotic relationship with *Rhizobia* in the soil, which is a key natural process that replenishes the lost nutrients in the soil (Stewart, 1973).

About 400 wholesome clean legume seeds of uniform size were selected by hand sorting for each seed type. They were put into clean bottle containing water and placed in a water bath set at 30°C for 24 h to break dormancy and allow for early germination. Two seedlings of the bean (*Phaseolus vulgaris* L.) and siratro (*M. atropurpureum* DC.) were planted in each pot, by digging holes using a fine spatula and carefully picking a seedling using fine forceps and inserting it into the hole and carefully covering to avoid damage. Each treatment required a total of 40 pots. Eighty (80) pots were, therefore, used for the bean and siratro plants, respectively. A week after planting the plants, thinning was done leaving one plant per pot to avoid excessive competition for nutrients.

Treatments and experimental design

A completely randomized design (CRD) was used with two treatments that were randomly assigned to the pots. The experiment was conducted in a greenhouse. The first treatment was Bt toxin solution (BTS), that is, Bt toxin solution - 100 µg/ml of Bt Cry1A(c) δ-endotoxin. The soil in the pots was treated to this solution twice a week for the entire experiment duration. This was assumed to maintain the Bt toxin concentration (100 µg/ml) at the rhizosphere and simulate the highest concentration that may occur in the soil when Bt crops exude the activated Bt toxin for the entire growth period of the crops. The second one was the Non-Bt water control (NBTW) (that is, Non-Bt treated water where the soil was sprayed with an equal volume of water twice a week).

The pots were watered with the respective treatment before planting the seedlings. The pots were labeled and positioned randomly by plot number that avoided bias in the positioning of the pots in the greenhouse. The plants were maintained in the greenhouse until nodulation (8 weeks) and maturity stages (14 weeks) when sampling and measurements were done.

The temperatures in the greenhouse were monitored daily and maintained at the mean maximum day temperature of 30.8°C and mean minimum night temperature of 16.4°C over the experimental period. The experiment was carried out twice.

Assessment of plant parameters

Host plant morphological and plant productivity traits

Several plant morphological traits including number of leaves (no.), shoot height (cm) and leaf area (mm^2), were measured on a per plant basis at the maturity stage of the plants. The plants were uprooted and shoot height measured. Five leaves per plant were plucked and leaf area measured using a 1 mm^2 graduated film paper. The leaf area average value was calculated and recorded.

The host plant productivity was assessed at the maturity stage with counts of the number of pods per plant, the number of seeds per pod and the nitrogen content of the leaves analyzed on per plant basis.

Evaluation of host plant nodulation

The host plants nodulation were assessed at the nodulation stage

Plate 1. Bean (*P. vulgaris* L.) plants at nodulation stage in the greenhouse. Scale Bar = 4 cm.

Plate 2. Siratro (*M. atropurpureum* DC.) plants at nodulation stage in the greenhouse. Scale Bar = 6.7 cm.

of the plants. Whole plants were uprooted to retrieve root nodules (Plates 1, 2 and 3). The soil around root nodules from every plant was removed carefully and the exposed root nodules were collected with sterile forceps, washed using running tap water, counted

Plate 3. Nodulation in Siratro (*M. atropurpureum* DC.) Bt toxic treated and control plants. Scale Bar = 6cm.

and stored in a vial.

Data analysis

The bean and Siratro plant morphological and plant productivity traits including nodulation data were subjected to a one-way analysis of variance (ANOVA) and the mean differences were compared by least significant deviations (LSD) test.

RESULTS

Soil physical and chemical properties data

The chemical analysis results indicated that the soil was deficient in nitrogen, zinc and organic matter (% C) (Table 1). However, the soil had adequate potassium, calcium, manganese, copper, iron and sodium. The soil pH was moderately acidic. The physical analysis data (Table 2) indicated that the soil had a clay texture grade.

Effect of Bt-endotoxin on morphological, productivity and nodulation traits on Beans (*P. vulgaris* L.)

The bean (*P. vulgaris* L.) plants showed nodulation in both treatments; the average number of nodules per plant was not significantly different between the control (30.8 ± 3.47) and the Bt-treated plant (31.7 ± 3.13). The average shoot height was 22.24 ± 0.67 and 22.92 ± 0.55 cm, while the average leaf area was found to be 5315.9 ± 39.99 and 5339.05 ± 37.37 mm^2 for the control and test bean plant samples, respectively. Average plant dry weight (g) per plant was 14.7 ± 0.12 and 14.9 ± 0.21 for the control and test bean plant samples, respectively. The number of pods per plant was 3.1 ± 0.34 and 3.0 ± 0.36, while the number of seeds per pod was 3.6 ± 0.42 and 3.7 ± 0.37 for the control and Bt treatments, respectively. The percent nitrogen was 3.21 ± 0.17 and 3.11 ± 0.19 % for the control and the Bt treatments, respectively.

Effect of Bt-endotoxin on morphological, productivity and nodulation traits on Siratro (*M. atropurpureum* DC.)

The Siratro plants (*M. atropurpureum* DC.) nodulation was observed in both treatments, but nodulation in the two treatments was not significantly different (t78, 1.990, $P > 0.05$, n = 40). The average number of nodules per plant was 22.3 ± 1.09 and 22.9 ± 1.05 for the control and Bt treatment, respectively. The average number of pods per plant was 7.1 ± 0.30 and 7.1 ± 0.33, the number of seeds per pod was 12.5 ± 0.31 and 12.7 ± 0.26; while the plant dry weight (g) per plant was 24.3 ± 0.32 and 23.9 ± 0.37 for the control and the Bt treated, respectively. The average leaf size was found to be 2507 ± 36.14 and

Table 1. Soil chemical properties data of Soil from KARI-NARL, Nairobi.

SCP	KNS	Optimal
	Average values	Range values
Soil pH	5.82	5.5 – 7.5
Total nitrogen (%)	0.09	2 – 20
Organic Carbon (%)	1.08	7 – 10
Phosphorous (p.p.m)	35	10 – 50
Potassium (m.e %)	1.19	0.3 – 6.0
Calcium (m.e %)	3	1 – 3.5
Magnesium (m.e %)	2.37	0.2 – 3
Manganese (m.e %)	1.1	0.02 – 1.2
Copper (p.p.m)	1.06	1 – 75
Iron (p.p.m)	24.05	10 – 150
Zinc (p.p.m)	7.5	30 – 100
Sodium (m.e %)	0.08	0.16 – 0.24

KNS, KARI-NARL Soil; SCP, soil chemical properties; p.p.m, parts per million; m.e, milli equivalents, and deficiencies are underlined.
The optimal range value shows the levels expected when the soil is not deficient in the parameters measured.

Table 2. Soil physical properties data of Soil from KARI-NARL, Nairobi.

Sample description		KARI-NARL soil
Average texture class	% Sand	7
	% Clay	84
	% Silt	9
Bulk density g cm^{-3}		1.3
Particle density g cm^{-3}		2.39
Texture grade		Clay

2522.43 ± 42.10 mm^2, while the number of leaves per plant was 13.2 ± 0.31 and 13.2 ± 0.34 for the control and the Bt treated, respectively (Table 3). The percent nitrogen was found to be 4.21 ± 0.12 and 4.24 ± 0.21% for the control and test bean plant samples respectively.

DISCUSSION

Results showed that in all treatments, root nodules were observed in both host plant species, indicating that there was plant-bacterial interaction within the plant rhizosphere in both Bt and control treatments. Nodulation of the bean plants was not different between the treatments, demonstrating that the Bt Cry1A(c) δ-endotoxin in the soil did not interfere with the plant-bacteria interaction and the nodulation activity of rhizobia at the rhizosphere of the bean plants. The formation of the root nodules was also not affected by the presence of the Bt Cry1A(c) δ-endotoxin in the soil. Plant morphological and productivity traits measured were also not significantly different between the control and the Bt toxin (t78, 1.990, $P > 0.05$, n = 40; Table 2). No significant differences in the bean plant productivity, that is, number of pods per plant and number of seeds per pod was observed, indicating that Bt Cry1A(c) δ-endotoxin had no influence on the host plants productivity. Percent nitrogen did not differ significantly between the treatments for the bean plants, indicating that the presence of the Bt toxin in the soil did not interfere with the nitrogen fixing ability of the rhizobia. The results, however, are incongruent with a study by Ferreira et al. (2003) who concluded that inoculated *B. thuringiensis* and its crystal protein increased the nodule formation and growth of soyabean (*Glycine max*) var. Br 322 when compared with uninoculated plants. This could mean that Bt cry proteins in the soil may have some influence on plant growth traits depending on the plant species. Nonetheless, many studies indicate that Bt toxin present in the soil does not have harmful effect on non target organisms (Pilcher et al., 1997; Reed et al., 2001).

Similarly, there were no significant differences in nodulation between the treatments of the Siratro plants, indicating that the presence of the Bt Cry1A(c) δ-endotoxin in the soil did not interfere with the nodulation activity of

Table 3. Morphological, productivity and nodulation traits of bean and Siratro after treatment with Bt toxin or with distilled water (control).

Treatment	Morphological and plant productivity traits of Bt toxin- treated plants and controls (average values)							
	Nodules/ Plant	Seeds/ pod	% Nitrogen	Leaves/ Plant	Plant dry wgt (g)	No. of pods/ Plant	Shoot height (cm)	Leaf size (mm^2)
Bean plants								
Distilled H_2O	30.8 ± 3.47a	3.6 ± 0.42a	3.21 ± 0.17a	5.8 ± 0.33a	14.7 ± 0.12a	3.1 ± 0.34a	22.24±0.67a	5315.9±39.99a
Bt toxin	31.7 ± 3.13a	3.7 ± 0.37a	3.11 ± 0.19a	6.0 ± 0.30a	14.9 ± 0.21a	3.0 ± 0.36a	22.92±0.55a	5339.05±37.37a
Siratro plants								
Distilled H_2O	22.3 ± 1.09a	12.5 ± 0.31a	4.21 ± 0.12a	13.2 ± 0.31a	24.3 ± 0.32a	7.1 ± 0.30a	nd	2507±36.14a
Bt toxin	22.9 ± 1.05a	12.7 ± 0.26a	4.24 ± 0.21a	13.2 ± 0.34a	23.9 ± 0.37a	7.1 ± 0.33a	nd	2522.43±42.10a

Data are mean values ± SD.
Means with the same letter are not significantly different from each other by paired t-test (t78, 1.990, P > 0.05, n = 40). nd = not determined.
Results from the two repeat experiments have been averaged in the table.
There was also no significant difference between the treatments when statistical analysis was done for the two experiments separately.

rhizobia on the Siratro plants. There were also no significant differences in the measured Siratro plant growth morphological traits (t78, 1.990, P > 0.05, n = 40). This showed that Bt toxin in the soil did not affect the growth and productivity of the Siratro plants. No significant differences were observed in percent nitrogen for the Siratro plants between the Bt and control treat-ments. Though much work has been done on the environmental effects of transgenic crops, little has been done on their effect on other beneficial crops such as leguminous crops. In fact, effect of *B. thuringiensis* and its crystal protein on plant growth and on functional groups of microorganisms is not well understood (Ferreria et al., 2003).

Clay soils are expected to bind Bt toxins more closely than sandy and loamy soils and Bt toxins from exudates are likely not to affect legumes under intercropping situations. Further, legumes grown in rotations following Bt maize may also not suffer as Bt toxin concentrations are likely to reduce below those maintained in this trial. Similar tests need to be done with other types of soil e.g. sandy and loam soils on which Bt maize might be grown.

Conclusions

Many experimental studies conducted to date indicate that transgenic plants have no adverse effects on non-target organisms. In addition, there is no scientific evidence as yet that the commercial cultivation of GM crops has caused environmental impacts beyond the impacts that have been caused by conventional agricultural management practices. Nonetheless, studies are still on-going to assess the potential environmental impacts of GM crops. The results of this present study show that Bt Cry1A(c) delta-endotoxin does not interfere with the host plant growth, nodulation and productivity in clay soil. However, similar tests needs to be done with other types of soil e.g. sandy and loam soils on which Bt maize might be grown.

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