Assessment of Effects of *Bacillus thuringiensis* Cry1A(c) δ-endotoxin on nitrogen fixing bacteria and their host plants in clay soil

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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To all members of my family especially my parents Agnes and Gilbert Makonde. Thank you for all the support you gave me. You laid in me a great foundation that has successfully seen me through to this level of education.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

BNF	Biological Nitrogen Fixation
Bp	Base pairs
BLAST	Basic Local Alignment Search Tool
Bt	*Bacillus thuringiensis*
BTB	Bromothymol blue
CIMMYT	International Maize and Wheat Improvement Center
CIRAD	French Agricultural Research Centre for International Developement
DNA	Deoxyribonucleic acid
DRIP	Dissertation Research Internship Program
DTT	Dithiothrietol
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium Bromide
FID	Flame ionization detector
G+C	Guanine and Cytosine
GM	Genetically Modified
GMOs	Genetically modified organisms
HCl	Hydrochloric acid
IBR	Institute for Biotechnology Research
ICIPE	International Center of Insect Physiology and Ecology
ICP	Insecticidal Crystal Protein
IRMA	Insect Resistant Maize for Africa

JKUAT	Jomo Kenyatta University of Agriculture and Technology
KARI	Kenya Agricultural Research Institute
KEPHIS	Kenya Plant Health Inspectorate Services
KSTCIE	Kenya Standing Technical Committee for Imports and Exports
KU	Kenyatta University
m.e %	Milli equivalents
Mins	Minutes
ml	Milliliters
mM	Millimolar
NaCl	Sodium chloride
NARL	National Agricultural Research Laboratories
NCBI	National Center for Biotechnology Information
ng/g	Nanograms per gram
PCR	Polymerase chain reaction
P.p.m	Parts per million
TAE	Tris-Acetate-EDTA
CRD	Completely randomized design
RFLP	Restriction fragment length polymorphism
RRNA	ribosomal Ribonucleic Acid
RUFORUM	Regional Universities Forum for Capacity Building in Agriculture
SDS	Sodium dodecyl sulfate
µl	Microlitres

USDA	United State Department of Agriculture
UV	Ultra Violet
vol	Volume
wt	Weight

ABSTRACT

Farming is the backbone of the Kenyan economy and is important in the production of food crops for basic livelihoods and income generation in rural areas. However, yields have remained low owing to high disease, weed and pest incidences. Over the past 13 years, scientists have successful developed genetically modified (GM) crops (using genes from a soil bacterium, *Bacillus thuringiensis, Bt*) such as Bt maize and Bt cotton that are being introduced into Africa. Though seen as a promising technology, there is much debate about their potential short and long-term ecological effects on the environment. In addition, not many studies about their potential effects on beneficial soil microorganisms such as nitrogen fixing bacteria have been carried out in Kenya.

The aim of this project was to assess the effects of *Bacillus thuringiensis (Bt)* δ-endotoxin on nitrogen fixing bacteria in the soil, focusing on direct effects on diversity of nitrogen fixing bacteria (rhizobia), nitrogen fixation and host plant growth and productivity. In this study, Cry1A(c) δ-endotoxin from a local *B. thuringiensis* (ICIPE L1-2 isolate) active against *Chilo partellus* (Swinhoe) was used. Beans, *Phaseolus vulgaris* (L.) and Siratro, *Macroptilium atropurpureum* (DC.) seedlings were grown in potted soils that were treated with Bt toxin solution (100 µg/ml) and water as control. The plants were maintained in the greenhouse till nodulation and maturity stages when sampling was done for analysis. The results on the effects of Cry1A(c) δ-endotoxin on nitrogen fixation indicated comparable slow nitrogen fixing activity. However, there were no significant differences between the Bt toxin-treated and those treated with

water (control) samples. In addition, the high concentration of Bt toxin Cry1A(c) (100 µg/ml), reduced the diversity of rhizobium species in the test samples compared to the control samples as indicated from the RFLP profiles. Comparison of the sequences of the isolates in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website showed that the isolates shared sequence identity of between 93-100 % with known species from the genera *Bradyrhizobium* and *Rhizobium*. In conclusion, the presence of Bt δ-endotoxin in the soil does not interfere with host plant growth, nodulation, productivity and nitrogen fixation. However, Bt δ-endotoxin appears to reduce the diversity of *Bradyrhizobium species* and *Rhizobium species* in the Siratro and bean test samples respectively.

Key Words: GM crops, Bt maize, Bt cotton, *Bacillus thuringiensis,* Cry1A(c) δ-endotoxin, *Macroptilium atropurpureum* (DC.)

CHAPTER 1

1.0 INTRODUCTION

1.1 General Introduction

Poverty and malnutrition are major problems faced by developing countries including Kenya, necessitating a need for increased food production. Increased food production could emanate from intensification of farming i.e. increasing the crop yields on existing cultivable lands as increasing the area under cultivation is no longer an option. Maize is the staple food in large parts of eastern and southern Africa, produced and consumed by most farming households (Raemeker, 2001). Crop pests among other constraints including drought stress, low soil fertility and leaf diseases continue to be drawbacks to achieving greater crop yields.

Stem borers have been identified as the most important insect field pests of maize in Kenya causing yield losses variously estimated at 13% (Khan *et al.,* 1997). Control options for stem borers globally include cultural, chemical, and biological methods. Due to difficulties in the access and effectiveness of these methods globally, scientists have developed transgenic crops using the recombinant DNA technology, a process that allows the insertion of the codon-modified genes from the soil dwelling bacterium *Bacillus thuringiensis* (Bt) into the plant genome and encode for the production of insecticidal toxins called Bt δ-endotoxins. *B. thuringiensis* a naturally occurring soil bacterium produces proteins active against certain insects. It is a gram positive, aerobic, spore-forming rod shaped bacterium that produces a parasporal, proteinaceous,

and crystalline inclusion during sporulation. This inclusion may contain more than one type of insecticidal crystal protein (ICP) (Höfte and Whiteley, 1989; Schnepf *et al.*, 1998). Bt δ-endotoxin is solubilized and hydrolyzed in the midgut of larvae of susceptible insects. When ingested it releases polypeptide toxins that eventually cause death of the larvae (Höfte and Whiteley, 1989; Schnepf *et al.,* 1998). Bt δ-endotoxin binds to the insect larvae gut wall and induces histopathological changes (Lane *et al.*, 1989) that result in the formation of pores in the midgut epithelium. Within minutes of ingesting the toxin, the larva ceases to feed and dies over the next few days (Knowles and Ellar, 1987).

More than 3,000 isolates of Bt from 50 countries have been collected (Crickmore *et al.*, 1998; Schnepf *et al.*, 1998). Numerous distinct *Cry* genes that code for insecticidal proteins (Cry proteins) have been identified. The δ-endotoxins comprise a group of 292 Cry and 22 Cyt proteins classified into Cry1 to Cry46 and Cyt 1 to Cyt 2 according to the degree of amino acid sequence homology (Kurt *et al.,* 2005). The Cry proteins are specifically toxic to lepidoptera and diptera (CryIIA class), lepidoptera (CryIA class), coleoptera (CryIIIA class) and Diptera (CryIV) (Schnepf *et al.,* 1998). Some ICPs also exhibit activity against other orders of insects (such as; Homoptera, Hymenoptera, Orthoptera, Mallophaga), nematodes, mites, collembola and protozoa (Schnepf *et al.*, 1998).

Preparations of Bt sprays that contain a mixture of cells, spores, and parasporal crystals, have been used as insecticides for almost 40 years (Stotzky, 2001). Conventional methods of stem borer control that employ chemicals or biopesticides sprays including those based on Bt, have not always been effective due to the challenges of timing these application and the resulting difficulties in eradicating the pests once they have infested the crops (International Life Sciences Institute, 1998).

Due to difficulties in tackling the stem borer problems worldwide, scientists have transformed crop plants with Bt genes responsible for the production of insecticidal toxins into the plants' genome. A common example of the transgenic crop plants is the Bt maize. Stem borer larvae that penetrate the plant tissues are killed when they ingest the toxin produced in the Bt maize cells (Carl *et al.*, 2006).

Cereal stem borers attack maize, sorghum, sugarcane, millet, rice and wild grasses. Stem borers are the most important field pests of maize (Ransom, 2000; Mugo *et al.*, 2005). The five major maize stem borer species that occur in Kenya include; *Chilo partellus* Swinhoe (Crambidae), Coastal stem borer *Chilo orichalcociliellus* (Crambidae), pink stem borer *Sesamia calamistis* Hampson (Noctuidae), African stem borer *Busseola fusca* Fuller (Noctuidae) and sugarcane bore *Eldana saccharina* Walker (Crambidae) (Muhammad and Underwood, 2004).

Different species may have different occurrences among the various maize growing ecologies in Kenya. *B. fusca* occurs in all highland areas of Kenya up to 2600 m, and is dominant maize stem borer species in the Moist Transitional zone and Highlands Tropics. *C. partellus* is an exotic species that was first reported in Kenya in the 1950s (Nye, 1960), and has spread throughout the maize and sorghum growing areas of Kenya at elevations below 1500 m, and sometimes higher (Zhou *et al.,* 2001b; Songa *et al.,* 2002b). *C. partellus* dominate the Dry Mid-altitude and Dry Transitional zones. *S. calamistis* is a native stem borer species that occurs in low densities in all areas of Kenya up to 2400 m. *C. orichalcociliellus* is a native stem borer species found in lowland Tropics and in Dry Mid-altitude zone. *E. saccharina* is a pest of maize, sorghum and sugarcane in Western Kenya.

Strategies for stem borers control in Kenya include chemical control using synthetic pesticides such as Bulldock® (beta-cyfluthrin), biological control (gregarious larval parasitoid, *Cotesia flavipes* Cameron), push-pull system, conventional, and transgenic host resistance which is by incorporation of *B. thuringiensis* genes into maize genome.

The Bt technology is increasingly being adopted around the world as a solution to the problem of pests (Lövei, 2001). Genetically modified crops achieved several milestones in 2006: annual hectarage of transgenic crops exceeded 100 million hectares; for the first time, the number of farmers growing biotech crop (10.3 million) exceeded 10 million; the accumulated hectarage from 1996 to 2007 exceeded two

thirds of a billion hectares for the first time at 690 million hectares, making it the fastest adopted crop technology in recent history (James, 2008).

However, laboratory and field studies have demonstrated that Bt toxin is released into soil during growth (Saxena *et al.,* 1999; Saxena and Stotzky, 2000), upon harvest as corn trash (Hopkins and Gregorich, 2003; Zwahlen *et al.,* 2003b), and pollen falling down (Losey *et al.,* 1999). In soil, Bt toxin does not change its conformation (Lee *et al.,* 2003) and remains active, protected from microbial degradation by absorption to clays or linkage to humic acids (Crecchio and Stotzky, 2001; Chevallier *et al.,* 2003). Moreover, Bt toxin released through corn root exudates retains its activity for 180 to 234 days in both laboratory and soil experiments (Stotzky, 2000; Saxena and Stotzky, 2001), thus representing a potential risk for nontarget organisms and microorganisms (Zwahlen *et al.,* 2003a; Turrini *et al.,* 2004).

Therefore, if the Bt toxin released in the soil is not all degraded by microbiota and abiotic factors, the toxins could accumulate and constitute a hazard to non-target organisms such as the soil microbiota including beneficial insects in the environment that may enhance the control of target pests or constitute a hazard to non-target organisms such as the soil microbiota, beneficial insects (Flexner *et al.,* 1986; Addison, 1993; James *et al.,* 1993; Johnson *et al.,* 1995; Hilbeck *et al.,* 1998a, b; Losey *et al.,* 1999), and other animal classes. The accumulation and persistence of the toxins could also result in the selection and enrichment of toxin-resistant target insects (VanRie *et*

al., 1990; McGaughey and Whalon, 1992; Entwistle *et al.,* 1993; Tabashnik, 1994; Bauer, 1995; Ferre *et al.,* 1995; Tabashnik *et al.,* 1997).

Concerns are raised on the potential environmental risks posed by Bt crops such as the possibility of gene escape to other susceptible plant species and effects on non-target insect species (Seidler and Levi, 1994; Seidler *et al.,* 1997; Bergelson *et al.,* 1998; Watrud and Seidler, 1998; Donegan and Seidler, 1999; Hansen and Obrycki, 2000). Studies have been done on the potential impacts of insect resistant transgenic Bt maize on non-target arthropods in Kenya (Songa *et al.,* 2004), but data are limited on the effect of Bt toxins on soil bacteria.

Persistence is enhanced when the Bt toxins are bound on surface-active particles like clays and humic substances, thereby, rendering it less accessible for microbial degradation but still retentive of toxic activity and become resistant to degradation by microorganisms (Stotzky, 1986; Saxena and Stotzky, 2000). These may be potential hazards to the soil communities especially soil microorganisms that may be exposed to Bt toxin over long periods (Hilbeck and Andow, 2004) and consequently affect soil microorganism dynamics.

Species diversity in soils is as great as in forests so that more than 90% of the biodiversity in agro-ecosystems is in the soil. Whether nutrients are made available for plant uptake or lost to the environment is dependent on microbial functioning in the

soil. Plant species and crop variety, can have enormous effects on rhizosphere microbial communities (Wardle, 2002; Kourtev *et al.,* 2003). Plant inputs drive the soil microbial community. As plants differ in their system inputs, the resultant microbial communities may differ. Changes in soil microbial diversity may adversely affect functional dynamics in the plant-soil system (Hilbeck and Andow, 2004).

Transgenic plants could affect the soil community in two ways. First, transgenic products could exude from roots and directly affect soil organisms (Saxena *et al.,* 1999). Second, transgenic crops could affect plant tissue quality (lignin or cellulose content) (Hopkins *et al.,* 2001) and, therefore, affect tissue decomposition rates. Effects on tissue quality are most likely to have important ecological consequences (Kowalchuk *et al.,* 2003). Some transgenic crops affect soil ecosystem by decreasing the species diversity of soil microorganisms (Donegan *et al.,* 1995; Donegan *et al.,* 1997; Griffiths *et al.,* 2000). However, the long-term significance of any of these changes is unclear (Wolfenbarger and Phifer, 2000). Decreasing species diversity of soil microbes in some cases can cause lower community diversity and above ground productivity (Van der Heijden *et al.,* 1998).

Microbial soil processes are vital for agricultural productivity. Little is known on how agricultural innovations including transgenic crop plants would affect prokaryotes diversity and function. The objective of this study was to evaluate the potential short-term effects of the Bt toxin on soil bacterial diversity and activity.

1.2 Justification

Bt technology is an essential tool in agriculture; however, it poses its own risks. This technology of incorporating codon modified genes from the soil dwelling *Bacillus thuringiensis* that encode the production of insecticidal toxins into plants alleviates many problems associated with the use of chemical pesticides, contributes to increased grain yields and a reduced need for insecticidal sprays. Bt maize, therefore, represents a promising technology for reducing losses from stem borer damage in Kenya. However, Bt toxin released through corn root exudates retains its activity for 180 to 234 days in both laboratory and soil experiments (Saxena and Stotzky, 2001). Again, when the genes that code for these toxins are genetically engineered into plants, the toxins continue to be synthesized during growth of the plants. If the Bt toxins released are not all degraded by microbiota and abiotic factors, the toxins could accumulate and constitute a hazard to non-target organisms such as the soil microbiota including beneficial insects in the environment.

Little is known about the potential impacts of Bt maize in Kenya. Considering that any natural or man made changes to the environment could lead to the interference with the number of microbial species, caution should be exercised when introducing new factors, and assessment of the impact such factors will have on soil microbial diversity is important. Bt maize technology should be deployed in a safer way and risk assessment is a prerequisite for sustainable use of the biotechnology in Kenya. This study was conducted as a part of the process of assessing the potential risks posed by transgenic crops prior to their release in the environment.

1.3 Hypotheses

The hypotheses were;

1. There is difference in host plant growth, nodulation and productivity between soils treated with *Bacillus thurungiensis* Cry1A(c) δ-endotoxin solution and the control not treated with the endotoxin
2. There is difference in diversity of nitrogen fixing bacteria between soils treated with *Bacillus thurungiensis* Cry1A(c) δ-endotoxin solution and the control not treated with the endotoxin.
3. There is difference in nitrogen fixation between soils treated with *Bacillus thurungiensis* Cry1A(c) δ-endotoxin solution and the control not treated with the endotoxin.

1.4 Objectives of the study

1.4.1 Main objective

To assess the effects of *Bacillus thurungiensis* Cry1A(c) δ-endotoxin on host plants and bacterial communities important for soil fertility in Clay soil.

1.4.2 Specific objectives

The specific objectives were to;

1. Determine the effects of *Bacillus thurungiensis* Cry1A(c) δ-endotoxin on host plant growth, nodulation and productivity in Clay soil.
2. Investigate the effects of *Bacillus thurungiensis* Cry1A(c) δ-endotoxin on nitrogen fixation in Clay soil.
3. Evaluate the effects of *Bacillus thurungiensis* Cry1A(c) δ-endotoxin on diversity of nitrogen fixing bacteria in Clay soil.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Importance of soil biota

The prokaryotes are by far the most abundant organisms inhabiting planet Earth. They are also phylogenetically the most diverse. They thus represent a large proportion of life's genetic diversity. Moreover, the prokaryotes are metabolically far more diverse than the eukaryotic organisms, and they are responsible for many of the key processes in the biogeochemical cycling on Earth (Madigan *et al.*, 2003).

Soil biota mediate/regulate a number of processes in terrestrial ecosystems that are not only critical for plant growth and productivity but are also essential for soil resource structure and maintenance of ecosystem health (Brussard *et al.*, 1997). The composition and metabolic capabilities of the soil microbial and faunal communities underpin the occurrence and rates of many soil processes. Microorganisms and microbial activity have a key role in stable aggregate formation. Water-stable aggregates are essential for good soil structure in all types of soils. Good soil structure is necessary to reduce soil erosion. Very few biological processes are mediated by individual species of biota. The successful functioning of most ecosystem processes, therefore, require a balance of biota interactions in the complex soil biota community (detritus food-web) (Brussard *et al.*, 1997).

Microbial-faunal interactions play a critical role in a variety of biological functions both in the rhizosphere i.e. the zone directly surrounding and influenced by plant roots, and the soil near decomposing plant residues (Coleman and Crossley, 1995; Gupta and Yeates, 1997). The rhizosphere contains a large majority of the soil's biota populations (>10-fold of that in the bulk soil) and the plant-microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of plants.

Genetically modified plants, through the products of introduced genes, modified rhizosphere chemistry, altered crop residue quality, could change the microbial dynamics, soil biodiversity and essential ecosystem functions such as nutrient mineralisation, disease incidence, carbon turnover and plant growth.

2.2 Adoption of Bt technology

With the advent of genetic engineering, genes that confer resistance to pest organisms have been inserted into various crop plants (USDA, 1995). Among the biological pesticides, bacteria have been the most successful group of organisms identified as a source of biological insecticide for commercial crops. Bt transgenic plants containing insecticidal proteins have featured prominently in agricultural systems in both developed and developing countries (James, 2008).

In 2007, the number of countries planting transgenic crops increased to 23, and comprised 12 developing countries and 11 industrial countries; they were, in order of hectarage, USA, Argentina, Brazil, Canada, India, China, Paraguay, South Africa,

Uruguay, Philippines, Australia, Spain, Mexico, Colombia, Chile, France, Honduras, Czech Republic, Portugal, Germany, Slovakia, Romania and Poland. Notably, the first eight of these countries grew more than 1 million hectares each – the strong growth across all continents in 2007 provides a very broad and stable foundation for future global growth of transgenic crops. The two new transgenic crop countries in 2007 were Chile producing over 25,000 hectares of commercial transgenic crops for seed export, and Poland growing Bt maize for the first time (James, 2008). The accumulated hectarage from 1996 to 2007 exceeded two thirds of a billion hectares for the first time at 690 million hectares. This very high adoption rate by farmers reflects the fact that transgenic crops have consistently performed well and delivered significant economic, environmental, health and social benefits to both small and large farmers in developing and industrial countries. The USA, followed by Argentina, Brazil, Canada, India and China continued to be the principal adopters of transgenic crops globally, with the USA retaining its top world ranking with 57.7 million hectares (James, 2008). Notably, 63% of transgenic maize, 78% of transgenic cotton, and 37% of all transgenic crops in the USA in 2007 were stacked products containing two or three traits that delivered multiple benefits. Stacked products are a very important feature and future trend, which meets the multiple needs of farmers and consumers and these are now increasingly deployed by ten countries – USA, Canada, the Philippines, Australia, Mexico, South Africa, Honduras, Chile, Colombia, and Argentina, with more countries expected to adopt stacked traits in the future (James, 2008).

2.3 The Future of GM crops

The number of transgenic crop countries, crops and traits and hectarage are projected to double between 2006 and 2015, the second decade of commercialization; in the developing countries, Burkina Faso and Egypt, and possibly Vietnam are potential candidates for adopting transgenic crops in the next one or two years. The lifting of the four-year ban on transgenic canola in late November 2007 in the states of Victoria and New South Wales was a very important development for the future of transgenic crops in Australia, where drought tolerant wheat is already being field-tested. By 2015, the number of farmers adopting transgenic crops could increase up from ten fold to 100 million (James, 2008).

2.4 Use of transgenic technology in Kenya

Kenya is considering the introduction of transgenic crops. The Insect Resistant Maize for Africa (IRMA) project, a collaborative effort between the CIMMYT and the Kenya Agricultural Research Institute (KARI), is developing genetically modified maize varieties suited to various Agro-ecological zones for Kenya by incorporating Bt genes for stem borer control (De Groote, 2003; Mugo *et al.*, 2004 a). Contained lab and greenhouse trials as well as confined field trials have been carried out with these crops in Kenya, including Bt maize for stem borer control. Other transgenic technologies in Kenya are; Bt cotton for bollworm control, transgenic sweet potato for feathery mottle virus control, as well as transgenic cassava for control of white flies.

Efforts to introduce Bt maize in Kenya are underway. CIMMYT acquired Bt genes from the private and public sectors, and has also synthesized other Bt genes with partners. Various Bt Cry genes (Cry1Ab, Cry1Ac, Cry1Ba, Cry1E, Cry1Ca, and Cry2Aa) have been used to develop constructs carrying the maize ubiquitin and rice actin promoters (Mugo *et al.,* 2005). Cry1Ab was obtained from the University of Ottawa in Canada, Cry1Ba and Cry1Ca were synthesized by CIRAD in France, while Cry2Aa was obtained elsewhere in Canada. These constructs were used to transform embryos from a CIMMYT maize hybrid (CML216xCML72), thereby developing various Bt maize events. Backcrosses were made to CML216 to develop inbred line carriers of the Bt genes, resulting in a number of useful Bt maize events, and the lines have shown high levels of resistance to various stem borers (Mugo *et al.*, 2005).

Through the IRMA project efforts to identify Bt genes and their Cry protein products that are effective against each of the target stem borer species in Kenya were made. Insect bioassays of maize leaves containing the different Cry genes were conducted (Mugo *et al.*, 2004 b). To carry out these bioassays, a specially constructed biosafety level II laboratory was built and approved by the Kenya Standing Technical Committee for Imports and Exports (KSTCIE) in 2001 (Mugo *et al.,* 2004 b). Likewise to test whole plants, a biosafety level II greenhouse and an open quarantine site were constructed.

2.5 Concerns about the Bt Maize Technology

Concerns have been raised on the potential environmental risks such as the possibility of gene escape to other plant species and effects on non-target insect species (Seidler and Levi, 1994; Seidler *et al*., 1997; Bergelson *et al.,* 1998; Watrud and Seidler, 1998; Donegan and Seidler, 1999). Studies done on the potential impact of insect resistant transgenic Bt maize on non-target arthropods in Kenya indicated no harmful effect. There are no studies in Kenya on the impact of the Bt toxins on soil bacterial and fungal communities.

2.6 Bt δ-endotoxin and its Impact on environment

More than 3,000 isolates of Bt from 50 countries have been collected (Crickmore *et al*., 1998; Schnepf *et al*., 1998). Numerous distinct crystal proteins Cry that code for insecticidal proteins (Cry proteins) have been identified. The δ-endotoxins comprise a group of 292 Cry and 22 Cyt proteins classified into Cry1 to Cry46 and Cyt1 to Cyt2 according to the degree of amino acid sequence homology (Kurt *et al*., 2005).

When genes coding for these Bt toxins are genetically engineered into plants, the Bt toxins continue to be synthesized during the entire life of the plants. If inactivation and degradation is slow, the Bt toxins could accumulate and constitute a hazard to non-target organisms such as the soil microbiota, beneficial insects (Flexner *et al.,* 1986; Addison, 1993; James *et al.,* 1993; Johnson *et al*., 1995). The accumulation and persistence of the Bt toxins could also result in the selection and enrichment of Bt

toxin-resistant target insects (VanRie *et al*., 1990; McGaughey and Whalon, 1992; Entwistle *et al*., 1993; Tabashnik, 1994; Ferre *et al*., 1995).

The protein structure is not modified as a result of binding on clays (Tapp *et al.,* 1994). Studies done (Saxena and Stotzky, 2001a,b; Saxena *et al.,* 2002) indicated that active Bt toxin from transgenic maize biomass persisted in the soil for up to 350 days, the longest time studied. Other studies have also reported the persistence of purified Bt toxin in soil for up to 234 days, when the trials were terminated (Tapp and Stotzky, 1995a; Palm *et al.,* 1996; Tapp and Stotzky, 1998). These may be potential hazards to the soil communities especially soil microorganisms that may be exposed to Bt toxins over long periods (Hilbeck and Andow, 2004). Exposures to Bt toxins exuded from roots and released during the decay of Bt plant materials may affect soil microorganism dynamics (Losey *et al.,* 1999). Therefore, due to persistence of Bt toxins in soils, potential effects to the environment ought to be evaluated.

2.7 Genetically Modified Organisms and the Environment

When transgenic plants are planted in the field, they inevitably come into contact with many other species that are crucial to the ecological health of an agricultural ecosystem. These include plants, herbivores, and natural enemies of pests, pollinators, root symbionts and detritivores. Many of these actors participate in the ecological processes that are useful and necessary for the agricultural production termed ecosystem services (Costanza, 1997)

A number of researchers have hypothesized based on laboratory studies that repeated large scale use of Bt crops could lead to accumulation of the protein in the soil that could reach biologically active levels. However, results of current field studies indicate no unreasonable risks to non-target organisms (Pilcher *et al.,* 1997; Reed *et al.,* 2001). Moreover, Bt protein is not taken up from soil by non-target Bt corn, carrot, radish, or turnip plants grown in soil previously planted with Bt corn plants (Saxena and Stotzky, 2002).

Early studies have shown that force-fed pollen from transgenic maize caused mortality in the larvae of the monarch butterfly (*Danais plexipus*) both in the laboratory and field environments (Losey *et al*., 1999). In contrast to these results, a number of laboratory and field studies conducted in the U.S. and Canada demonstrated that Bt corn pollen posed no significant risk to monarch butterflies under actual field conditions (Pleasants *et al.,* 2001; Stanley-Horn *et al*., 2001; Zangerl *et al*., 2001). Although high concentrations of Bt corn pollen clearly have negative effects on monarch larvae, only a very small proportion of milkweed plants accumulate any Bt pollen, let alone pollen in sufficient concentration to have negative effects (Obrycki *et al*., 2001). A variety of studies have evaluated the effects of transgenic crops on the natural enemies of crop-feeding herbivores (Schuler *et al.,* 1999). There is direct evidence that Lacewigs (Hilbeck *et al*., 1998b) and Coccinellides (Birch *et al.,* 1999), which are natural enemies of the target pests, were adversely affected.

Plant species and crop variety, can have enormous effects on rhizosphere microbial communities (Wardle, 2002; Kourtev *et al*., 2003). Plant inputs drive the soil microbial community. As plants differ in their system inputs, the resultant microbial communities may differ. Changes in soil microbial diversity may adversely affect functional dynamics in the plant-soil system (Hilbeck and Andow, 2004). Changes induced by transgenic plants are generally small relative to the effects of plant community and ecosystem properties (Kowalchuk *et al*., 2003). Transgenic plant affects the soil community in different ways. First, transgenic products could exude from roots and directly affect soil organisms (Saxena *et al.,* 1999). Second, transgenic crops could affect plant tissues quality (lignin or cellulose content) (Hopkins *et al*., 2001) and, therefore, affect tissue decomposition rates. Effects on tissue quality are most likely to have important ecological consequences (Kowalchuk *et al*., 2003). However, because links between microbial community structure and functional consequences (such as C and N cycling) are only poorly understood (Wardle, 2002), these effects are difficult to evaluate.

Some investigations showed decrease in species diversity of soil microorganisms caused by some transgenic crops (Donegan *et al.,* 1995; Donegan *et al*., 1997; Griffiths *et al*., 2000). However, the long-term significance of any of these changes is unclear (Wolfenbarger and Phifer, 2000). Decreasing species diversity of soil microbes in some cases can cause lower community diversity and above ground productivity (Van der Heijden *et al*., 1998). Microbial soil processes are vital for agricultural productivity.

Other studies concluded that *B. thuringiensis* subsp. *kurstaki* endotoxin both the purified and those produced by the transgenic plant did not have a direct effect on populations of soil microorganisms, bacteria, fungi (Donegan and Seidler, 1999). Nonetheless, little is known on how agricultural innovations including transgenic crop plants would affect prokaryotes diversity and function.

2.8 Effects of Bt toxin on Nitrogen fixing organisms

Studies conducted (Yu-Kui *et al.,* 2005) indicated that fortification of pure Bt toxin into rhizospheric soil had no significant changes in the numbers of culturable functional bacteria, however, the numbers of nitrogen-fixing bacteria were reduced when the concentration of Bt toxin was higher than 500 ng/g. These studies concluded that Bt toxin was not the direct factor causing decrease of the numbers of bacteria in the rhizosphere, and other factors may be involved. Another study (Lamarche and Hamelin, 2007) on the impact of Bt white spruce on nitrogen- fixing communities showed no effect associated with the presence of the *B. thuringiensis* transgene was observed, which is in agreement with other earlier studies evaluating the impact on overall bacterial diversity (Blackwood and Buyer, 2004; Brusetti *et al.,* 2004; Baumgarte and Tebbe, 2005;. Liu *et al.,* 2005).

CHAPTER 3

3.0 GENERAL MATERIALS AND METHODS

3.1 Collection and analysis of soil sample

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

3.2. Physical and chemical analysis of soil

The standard soil analysis of the soil sample was 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.

The chemical analysis results indicated that the soil was deficient in nitrogen, zinc and organic matter (%C) (Table 3.1). However, the soil had adequate potassium, calcium, manganese, copper, iron and sodium. The soil pH was moderate acidic. The physical analysis data (Table 3.2) evidently indicated that the soil had a Clay texture grade.

Table 3.1 Soil chemical properties data.

Soil Analytical Data	
	Study site
	KARI-NARL
Sample description	**KNS**
SCP	**Average values**
Soil pH	5.82
Total Nitrogen %	0.09
Organic Carbon %	1.08
Phosphorous p.p.m	35
Potassium m.e %	1.19
Calcium m.e %	3
Magnesium m.e %	2.37
Manganese m.e %	1.1
Copper p.p.m	1.06
Iron p.p.m	24.05
Zinc p.p.m	7.5
Sodium m.e %	0.08

Key: ARS-Athi River Soil, KS-Kikuyu Soil, KNS-KARI-NARL Soil, SCP-Soil Chemical Properties, p.p.m-parts per million, m.e-Milli equivalents, and deficiencies are underlined.

Table 3.2 Soil physical properties data.

		Study site
		KARI-NARL
Sample description		**KNS**
Average texture class	%SAND	7
	%CLAY	84
	%SILT	9
Bulk density gcm^{-3}		1.3
Particle density gcm^{-3}		2.39
Texture Grade		Clay

3.3 Selection of the soil type

The soil sample from KARI-NARL was used for the greenhouse experiments. The soil properties (general fertility Table 3.1, high organic matter and water holding capacity i.e % Clay + % Silt, Table 3.2) were appropriate for this study.

3.4 Microbial test of the B. thuringiensis isolate (ICIPE L1-2)

Microbial analyses were performed to ascertain that the isolate (ICIPE L1-2) indeed was a *B. thurimgiensis*. The local *Bacillus 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. 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.

3.5 Microscopic analysis of isolate ICIPE L1-2

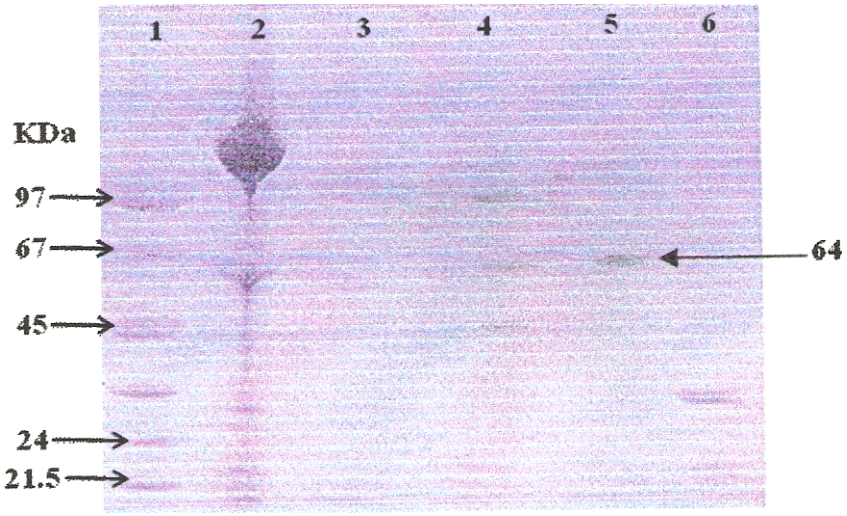
Cells were picked from the nutrient broth culture using a sterile wire loop and spread on 10 sterile slides. The slides were air-dried for 10 min and then 5 slides were gram-stained while the other five slides were stained using carbol fuchsin stain. Microscopic examination was done at × 2000 magnification. The results revealed that the isolate was gram positive. Road shaped cells were observed with some crystals and spores of different shapes. This indicated that the isolate ICIPE L1-2 was a *B. thuringiensis* that produced crystals and spores during sporulation.

3.6 Preparation of Cry 1A(c) Bt toxin

A local *Bacillus thuringiensis* isolate (ICIPE L1-2) obtained from the ICIPE germplasm bank was 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. 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. Harvesting and purification of the δ-endotoxins, was carried out by centrifugation at 10,000 rpm x g for 10 minutes, 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 1hour at 37 °C. The solubilized toxin was concentrated using polyethylene glycol for 30 minutes, and then dialyzed in DTT free 50mM 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 minutes (Osir and Vundla, 1999). The activated toxin (Figure 3.1) was used in the experiments.

Figure 3.1 Bt toxin at various stages of preparation.



Key: Electrophoresis of the Bt endotoxin separated by SDS-PAGE (4-20%). (1) Molecular weight standard (2) Crystal pellet (3) Solubilized toxin (4) Solubilized and dialyzed toxin (5) Trypsin activated toxin (6) Trypsin (enzyme).

CHAPTER 4

EFFECTS OF *Bacillus thuringiensis* CRY1A(c) DELTA-ENDOTOXIN ON HOST PLANTS GROWTH, NODULATION AND PRODUCTIVITY IN CLAY SOIL

Abstract

It is important that new technologies do not result in unfavorable effects on non-target organisms. Kenya is exploring the possibilities of using Bt maize for control of stem borer pests in maize production. However, concerns have been raised on the safety of transgenic crops such as Bt maize on the environment. The study aimed at assessing the effects of Bt Cry1A(c) δ-endotoxin on host plant growth, nodulation and productivity in Clay soil. Clay soil from KARI-NARL farm in Kabete, Kenya and Cry1A(c) δ-endotoxin from a local *B. thuringiensis* isolate (ICIPE L1-2) active against *Chilo partellus* (Swinhoe) were used. Beans, *Phaseolus vulgaris* (L.) and Siratro, *Macroptilium atropurpureum* (DC.) seedlings were grown in pots that were treated with Bt Cry1A(c) δ-endotoxin solution (100 µg/ml) and water as control. The plants were maintained in the greenhouse till nodulation and maturity stages when sampling was done for analysis. The growth parameters, nodulation and productivity of the plants were evaluated. Bt Cry1A(c) δ-endotoxin at a concentration of 100 µg/ml did not interfere with the host plant growth and productivity. No significant effect on host plant nodulation (nitrogen fixing activity) by rhizobia was observed (t_{78}, 1.990, $P>0.05$, $n=40$).

4.0 Introduction

Genetic modification of organisms (plants, microbes and animals) to incorporate useful traits is a powerful technology for the future development of sustainable agricultural systems. Cry proteins from *Bacillus thuringiensis* are by far the most common insecticidal proteins that have been engineered into plants. They are the only insecticidal proteins that are commercially used in genetically modified crops (James, 2005). Bt *cry* genes have been engineered into a large number of plant species such as maize, cotton, potato, tomato, rice, eggplant, and oilseed rape (Shelton *et al*., 2002; Ely, 1993; De Maagd, 2004) to resist insects (to reduce reliance on pesticides) amongst other traits such as withstand specific herbicide application (better weed management), and to improve crop quality (nutritional value).

However, there are concerns that insect-resistant GM crops expressing Cry proteins from *B. thuringiensis* could harm organisms other than the pest(s) targeted by the toxin. One factor of particular interest in this respect is the potential effect of accumulated Bt Cry proteins on non-target organisms that provide important ecological and economic services within agricultural systems. These include beneficial bacteria (like the nitrogen fixing bacteria), parasitoids and predators that are of importance for natural pest regulation, pollinators, and butterflies.

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. 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). Therefore, any 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. Determination of the potential effects of Bt cry proteins on the host plant growth, nodulation and productivity is of paramount importance to gather more data on the risks that GM crops may have. Consequently, there is need for a case-by-case study to further evaluate the effects of transgenic plants on other non-targets organisms and soil ecosystem functions (Biao *et al.,* 2005). In this study, we evaluated the effects of Bt Cry1A(c) δ-endotoxin on host plant growth, nodulation and productivity in Clay soil.

The ability of a legume to fix nitrogen depends upon the presence of the appropriate bacterium, *Rhizobium*. This bacterium lives in special structures on legume roots, the root nodules. 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*) and bean (*Phaseolus vulgaris*) plants were used in this study. This is because of their high nitrogen fixing ability and versatility with respect to the strain of *Rhizobium* that can interact with the plants.

4.1 Objectives

The objectives of the study were to:

1. Evaluate the effect of Bt Cry1A(c) δ-endotoxin on host plant growth and productivity in Clay soil.
2. Assess the effects of Bt Cry1A(c) δ-endotoxin on host plant nodulation activities in Clay soil.

4.2 Materials and methods

4.2.1 Preparation of the Bt Cry1A(c) δ-endotoxin

The toxin used in this experiment was prepared as described in Chapter 3. The activated Bt Cry1A(c) delta-endotoxin was diluted with distilled water to a concentration of 100 μg/ml that was used for the test experiment. This concentration was used because previous studies using purified Bt toxin in varying concentrations up to 50 μg/ml have shown no negative effects on the non target organisms, however, high

concentration (100 μg/ml of Bt toxin) was found to reduce the percentage germination of spores and severely reduced the radius spread of the hyphae of arbuscular mycohorrizae (Lelmen, 2007).

4.2.2 Pre-germination of the leguminous seeds

About 400 wholesome clean seeds of reasonably 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^{o}C for 36 hours. This was done to break the dormancy stage and allow for early germination of the seeds.

4.2.3 Processing of the soil

The soil sample from KARI-NARL was used for the greenhouse experiments. The soil properties were appropriate for this study because research data have indicated that persistence of *Bacillus thuringiensis* toxins in the soil is enhanced when the toxins are bound on surface-active particles like clays and humic substances, thereby, rendering it less accessible for microbial degradation but still retentive of toxic activity (Stotzky, 1986; Saxena and Stotzky, 2000) and so this would allow to investigate its effects on nitrogen fixing bacteria. The soil was pounded to break the large cramps, mixed thoroughly for homogeneity and then sieved through a 4 mm wire mesh. One kilogram of the clay soil was put into planting polythene bag (pots).

4.2.4 Experimental Design

The experiment was conducted in a greenhouse and comprised of two treatments. A completely randomized design (CRD) was used with each experiment having 40 samples. The treatments were as follows:

1. BTS (Bt toxin treated with water - 100 μg/ml of Bt Cry1A(c) delta-endotoxin)

2. NBTW (Non-Bt treated with water – used as control).

4.2.5 Planting of leguminous seeds

The Bean, *Phaseolus vulgaris* (L.) and Siratro, *Macroptilium atropurpureum* (DC.) seedlings were pre-germinated overnight in the water bath at 30^{o} C. Two seedlings were planted in each pot, by digging holes using a fine spatula and carefully picking a seedling using a fine forceps and inserting it into the hole and carefully covering to avoid damage. Each treatment required a total of 80 pots and therefore, 160 pots were used for the two treatments. The experiments were conducted using the completely randomized design (CRD). The pots were watered with the right treatment for each pot before planting the seedlings. The pots were well labeled with indelible ink indicating the pot number and type of treatment to distinguish the pots. Randomization of the pots was done by writing the identities of the pots in 2 cm square plain papers, folding them and mixing them in a box from where random selection of the pieces of papers was done. This avoided bias in the positioning of the pots in the greenhouse. A week after planting the plants, thinning was done leaving one plant per pot to avoid excessive competition for nutrients. The plants were maintained in the greenhouse till nodulation (8 weeks) and maturity stages (14 weeks) when analyses were done.

4.2.6 Maintenance of plants in the greenhouse

The conditions in the greenhouse were monitored daily. The mean maximum temperature was 30.8^{o} C and mean minimum temperature was 16.4^{o} C over the experimental period. For the test experiment, the soil in the pots was treated with

activated Bt toxin at a concentration of 100 μg/ml 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 soil without the Bt toxin (control experiment) was sprayed with an equal volume of water. Watering of the soil in the pots was also done when necessary (whenever the soil was dry). Development of the plants was monitored throughout the period. However, no fertilizer application was done in soil. The experiment was repeated two times after a trial experiment had been carried out.

4.2.7 Evaluation of host plant growth parameters

The plant growth parameters (number of leaves, shoot height and leaf size) were analyzed at the maturity stage of the plants. The number of leaves per plant were counted and recorded. Similarly, the plant height for each plant was measured (centimeters) and the leaf size (mm^2) was determined and data recorded.

4.2.8 Evaluation of host plant productivity

The host plant productivity was assessed at the maturity stage of the plants. The number of pods per plant and seeds per pod were counted and recorded. Pod weight was determined. Nitrogen content of the leaves was also analyzed and results recorded.

4.2.9 Evaluation of Host plant nodulation

The host plants nodulation activities were assessed at the nodulation stage of the plants. The whole plants were excavated to retrieve root nodules (Plates 4.1, 4.2, and 4.3). The soil around root nodules was removed carefully and the exposed root nodules were collected with sterile forceps and washed using running tap water. All nodules from a single host plant represented one unit of collected material and were stored in the same vial and counted. Root nodules of plants of the same species from different pots were not combined because they represented different soil environments.

Plate 4.1 Bean plants, (*Phaseolus vulgaris*) at nodulation stage in the greenhouse.

Plate 4.2 Siratro plants, (*Macroptilium atropurpureum*) at nodulation stage in the greenhouse.

Plate 4.3 Uprooted Siratro, *Macroptilium atropurpureum* plants for both control and test samples showing nodulation.

4.3 Results

4.3.1 Impact of Bt-delta endotoxin on host Plant growth parameters

The bean, *Phaseolus vulgaris* (L.) plants planted in the pots showed nodulation in both treatments. The nodulation in the test experiment was not significantly different from the control (t_{78}, 1.990, $P>0.05$, $n=40$). The average number of nodules per plant was 30.8±3.47 and 31.7±3.13 for the control and test bean plant samples respectively. The average shoot height (cm) was 22.24±0.67 and 22.92±0.55 while the average leaf size (mm^2) was found to be 5315.9±39.99 and 5339.05±37.37 for the control and test bean

plant samples respectively. It is evident that the growth parameters (nodules per plant, shoot height and leaf area) determined were not significantly different between the control and test samples (t78, 1.990, P>0.05, n=40, Table 4.1). In addition, the number of pods per plant was found to be 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 test bean plant samples respectively. The percent nitrogen was found to be 3.21±0.17 and 3.11±0.19 for the control and test bean plant samples respectively. There was insignificance difference in the bean plant productivity (% nitrogen, number of pods per plant and seeds per pod) between the control and test bean samples (t78, 1.990, P>0.05, n=40, Table 4.1). In conclusion, Bt delta-endotoxin did not have any significant effect on the bean plant growth and productivity.

Table 4.1 Bean plant parameters determined at nodulation and maturity stages, after treatment with Bt toxin 100μg/ml (Test) or with distilled water (control).

	Plant parameters						
Treatment	**Nodules/ Plant**	**Leaves/ Plants**	**No. of pods/ plant**	**Seeds/ plant**	**Shoot Height (cm)**	**Leaf Size (mm^2)**	**Nitrogen %**
Distill H_2O	30.8±3.47a	5.8±0.33a	3.1±0.34a	3.6±0.42a	22.24±0.67a	5315.9±39.99a	3.21±0.17a
Bt toxin	31.7±3.13a	6.0±0.30a	3.0±0.36a	3.7±0.37a	22.92±0.55a	5339.05±37.37a	3.11±0.19a

Means with the same letter are not significantly different from each other by paired t-test (t78, 1.990, P > 0.05, n = 40).

For the Siratro, *Macroptilium atropurpureum* (DC.) plants, nodulation was observed in both treatments. Nodulation in treatments was not significantly different from each other (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 test Siratro plant samples respectively.

The average leaf size (mm^2) was found to be 2507±36.14 and 2522.43±42.10 while the number of seeds per pod was 12.5±0.31 and 12.7±0.26 for the control and test Siratro plant samples respectively (Table 4.2). Moreover, 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. The growth parameters (nodules per plant, seeds/pod, leaf area and % nitrogen) determined were insignificantly different between the treatments (t78, 1.990, P>0.05, n=40, Table 4.2). In conclusion, there was absence of evidence that Bt delta-endotoxin had any significance effect on Siratro plant growth parameters.

Table 4.2 Siratro plant parameters determined at nodulation and maturity stages, after treatment with Bt toxin 100µg/ml (Test) or with distilled water (control).

	Plant parameters			
Treatment	**Nodules/plant**	**Seeds/pod**	**Leaf size mm^2**	**Nitrogen %**
Distilled H2O	22.3±1.09a	12.5±0.31a	2507±36.14a	4.21±0.12a
Bt toxin(100 µg/ml)	22.9±1.05a	12.7±0.26a	2522.43±42.10a	4.24±0.21a

Means with the same letter are not significantly different from each other by paired t-test (t78, 1.990, P > 0.05, n = 40).

4.4 Discussion

The results from this study 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 despite the differences in the treatments. Nodulation of the bean plants did not differ significantly between the treatments, demonstrating that the

availability of the Bt Cry1A(c) δ-endotoxin in the soil did not seem to interfere with the plant-bacteria interaction and the nodulation activity of rhizobia at the rhizosphere of the bean plants. Furthermore, the formation of the root nodules was not affected by the presence of the Bt toxin in the soil. It was evident that the host plant growth parameters evaluated were insignificantly different between the control and test samples (t78, 1.990, P>0.05, n=40, Table 4.1). Moreover, no significance difference in the bean plant productivity (% nitrogen, number of pods per plant and number of seeds per pod) was observed. This indicated that Bt toxin had no influence on the host plants productivity despite its presence in the soil.

Similarly, there was insignificant difference 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 rhizobia on the Siratro plants. In addition, there was no evidence of significant differences in the Siratro plant growth parameters evaluated (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. 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 same case was observed for the Siratro plants that showed insignificant difference in percent nitrogen between the test and control samples. Notably, the percent nitrogen was slightly higher in Siratro plants (4.21±0.12 and 4.24±0.21) than in bean plants (3.21±0.17 and 3.11±0.19) for the control and test

samples, respectively (Table 4.1 and Table 4.2). However, the mean number of nodules per plant was higher in bean plants (30.8±3.47 and 31.7±3.13) than in Siratro plants (22.3±1.09 and 22.9±1.05) for the control and test samples respectively.

Currently, there is no data showing the effect of Bt delta-endotoxin on host plant growth. The results of this study, therefore, demonstrated that the presence of Bt Cry1A(c) δ-endotoxin had no significant effect on the host plant growth, nodulation and productivity (t_{78} 1.990, $P > 0.05$, $n = 40$).

4.5 Conclusion

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, more studies are still going on to assess the potential environmental impacts of GM crops. The findings of this study showed that Bt Cry1A(c) delta-endotoxin did not significantly interfere with the host plant growth, nodulation and productivity in Clay soil.

CHAPTER 5

EFFECTS OF *Bacillus thurungiensis* CRY1A(c) DELTA-ENDOTOXIN ON DIVERSITY OF RHIZOBIA AND NITROGEN FIXATION IN CLAY SOIL.

Abstract

The recent introduction of *Bt* maize and *Bt* cotton transgenic crops into Africa has raised concerns about their potential short and long-term ecological effects on the environment. Few studies on the potential effects of Bt crops on beneficial soil microorganisms such as rhizobia have been done in Kenya. The study aimed at evaluating the effects of Bt δ-endotoxin on the diversity of rhizobia and nitrogen fixation in Clay soil. Beans, *Phaseolus vulgaris* (L.) and Siratro, *Macroptilium atropurpurum* (DC.) seedlings were grown in pots that were treated with Bt Cry1A(c) δ-endotoxin solution (100µg/ml) and water as control. The plants were maintained in the greenhouse till nodulation and maturity stages when sampling was done for analysis. Pure isolates were obtained from the root nodules of the leguminous plants. The diversity and species composition were assessed using DNA fingerprinting (RFLP) and sequencing of the 16S rRNA gene. The extracted Bt δ-endotoxin solution was applied to the test soil samples in hungate tubes at a concentration of 100 µg/ml while the controls were treated with distilled water. Subsequently, acetylene reduction assays were performed over 60 hr periods. The results indicated that there was conversion of acetylene to ethylene in both the test and control soil samples and the difference

between the treatments was insignificant (t48, 2.009, P>0.05, n=25). However, RFLP analyses data showed that Bt Cry1A(c) δ-endotoxin present at a concentration of 100 µg/ml in the soil influenced (reduced) the rhizobial diversity.

5.0 Introduction

There have been concerns on the effects of naturally occurring or genetically modified bacterial biopesticides and biofertilizers on other soil microbial communities. Specifically, there is little data on their interaction with nitrogen fixing bacteria, one of the most essential components of the soil microbial populations. Studies have shown that in the soil, Bt toxin has been detected at concentrations that can kill insects (Saxena and Stotzky, 2000). However, the long-term impact of this is not yet known (Lövei, 2001). Assessment of the effects of genetically modified (GM) plants on soil processes is important as maintenance of soil fertility is a biological process.

Molecular nitrogen (N_2) is the major component (78 %) of the earth's atmosphere. Nitrogen is an essential part of many of the chemical compounds, such as proteins and nucleic acids that are the basis of all life forms. However, N_2 cannot be used directly by biological systems to build the chemicals required for growth and reproduction and nitrogen fixation is, therefore, important.

Biological nitrogen fixation is the reduction of atmospheric N_2 gas to biologically available ammonium, exclusively mediated by prokaryotic organisms in symbiotic relationships, associative relationships, and under free-living conditions (Postgate,

1998). This process is considered to be an important nitrogen input in many terrestrial environments, particularly in those without any chemical fertilizer input such as forests, grasslands, and organic farming plots.

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 often is the limiting nutrient in many terrestrial ecosystems (Vitousek and Howarth, 1991). Moreover, nitrogen fixation is a function performed by a wide diversity of bacteria belonging to many different taxa (Young, 1992; Zehr *et al.,* 2003).

Nitrogen is the nutrient element most frequently found limiting to the growth of green plants. This results from the continual loss of nitrogen from the reserve of combined or fixed nitrogen, which is present in soil and available for use by plants. It is continually depleted by such processes as microbial denitrification, soil erosion, leaching, chemical volatilization, and most important, removal of nitrogen-containing crop residues from the land (Berkum and Bohlool, 1980). The nitrogen reserve of agricultural soils must, therefore, be replenished periodically in order to maintain an adequate level for crop production. This replacement of soil nitrogen is generally accomplished by the addition of chemically fixed nitrogen in the form of commercial inorganic fertilizers or by the activity of biological nitrogen fixation (BNF) systems (Stewart, 1973; Iwao, 2000).

It is estimated that BNF on a global scale may reach a value of 175 million metric tons of nitrogen fixed per year. The amount of nitrogen fixed in any given situation would depend upon the environmental conditions and the nature of biological system(s) present that are capable of nitrogen fixation. The significance of the contribution of any BNF system to the nitrogen economy in any situation is a function of the supply and demand of the biological community for nitrogen (Berkum and Bohlool, 1980).

The nitrogen fixing activity of free-living, non-photosynthetic, aerobic bacteria is strongly dependent on favorable moisture conditions, oxygen, and an organic food source. Nitrogen fixation is characteristically higher in environments such as tropical soils, where such factors as substrate availability, temperature and moisture are more favorable to the maintenance and activity of a high bacterial population (Murder, 1975).

It is known that any change to the quality of rhizosphere exudates will potentially modify the dynamics of the soil biodiversity and activity and may cause changes to the soil microbial communities (Losey *et al.*, 1999). However, little ecological consequences of the presence and persistence of Bt toxin in soils have been published; therefore, more empirical studies addressing these consequences are needed to provide much-needed information to evaluate the possibility of long-term effects on non-target organisms.

5.1 Objectives

The objectives of the study were to:

1. Evaluate the effects of Bt Cry1A(c) δ-endotoxin on the diversity of rhizobia in Clay soil.
2. Determine the effects of Bt Cry1A(c) δ-endotoxin on nitrogen fixation in Clay soil.

5. 2 Material and methods

5.2.1 Experimental design

The experimental design for this study was as described in chapter 4. The plants were treated and maintained in the greenhouse till nodulation stage when sampling was done. The root nodules were sampled as described in chapter 4. Rhizobia were isolated from the root nodules to assess their diversity.

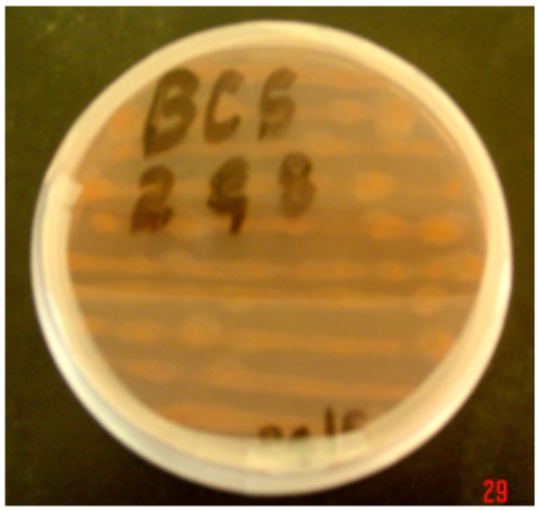
5.2.2 Isolation of Rhizobia from fresh nodule

Fresh root nodules of legume crops collected from pots were cleaned with running tap water to remove all soil and organic particles (Plate 5.1). Sterile forceps were used to hold the nodules, and the roots with attached nodules were cut, 2-3 mm on each side of the nodules. Intact and, undamaged nodules were immersed for 10 second in 95 % ethanol (to break the surface tension and to remove air bubbles from the tissue); transferred to a 3 % (v/v) solution of sodium hypochlorite and soaked for 4-5 min. The segments were then rinsed in five changes of sterile water using sterile forceps for transferring. Forceps were sterilized quickly by dipping in alcohol and flaming. The nodules were crushed in a sterile tube with sterile glass rod and sterile water. The slurry

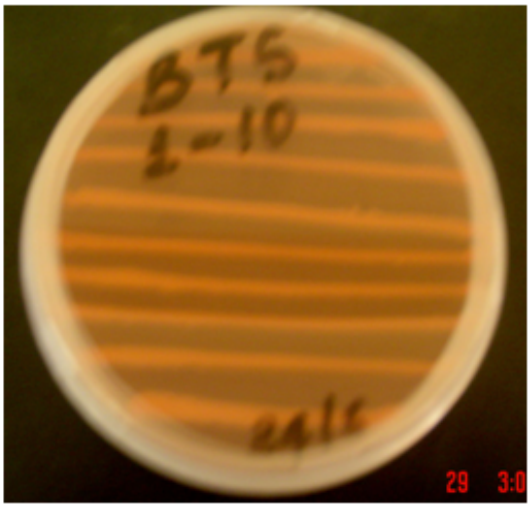
was diluted and then streaked on the surface of yeast extract mannitol (YEM) agar containing 0.0025 % (w/v) Congo red and YMA plates containing BTB. The inoculated Petri plates were incubated at 30 ^{o}C for 3 to 10 days, depending on the strain and species until colonies appear (Plate 5.2). Single colonies were selected and re-streaked on YEM agar for purity. The colony of rhizobia is mucoid, round and shows little or no Congo red absorption.

Plate 5.1 Isolated root nodules harvested at the nodulation stage.

Plate 5.2 Rhizobium species isolated from both control (BCS2&8) and test (BTS1-10) bean plant nodules.



(a) BCS 2 & 8



(b) BTS 1-10

5.2.3 DNA Extraction from Rhizobia isolates

DNA was extracted from the isolated rhizobia cultures. An isolate suspension of 500 µl was put into a 2 ml sterile eppendorf tube followed by 250 µl of solution A (50 mM Tris pH 8.5, 50 mM, EDTA pH 8.0 and 25 % sucrose). Then 7 µl of lysozyme (20 mg/ml) and 7 µl of ribonuclease A (20 mg/ml) were added and mixed gently. Incubation was done at 37^{o}C for 2 hours. A volume of 600 µl of Solution B (10 mM Tris pH 8.5, 5 mM EDTA and 1 % SDS) and 10 µl of Proteinase K (20 mg/ml) were added and mixed gently by inverting several times before incubation at 50^{o} C for 2 hours. The mixture was separated into two equal parts. DNA was extracted by adding equal volumes of Phenol:Chloroform and centrifuging for 15 minutes at 13,000 rpm. The aqueous phase, which contained the crude DNA was carefully pipetted out into a sterile eppendorf tube. The Phenol:Chloroform extraction step was repeated. An equal volume of Chloroform: Isoamylalcohol (24:1) was added to the aqueous phase and

spun at 13,000 rpm for 15 minutes. The aqueous phase was pipetted out into a sterile eppendorf tube and the extraction step repeated another round to remove all the phenol from the DNA. The aqueous phase was treated with an equal volume of isopropanol and 0.1 volumes of 3 M NaCl and kept at -20^{o} C overnight. The DNA sample was defrosted and then centrifuged at 4^{o} C for 30 minutes to pellet the DNA. The pellet was washed with 70 % ethanol, centrifuged at 13,000 rpm for 5 minutes and then ethanol pipetted out taking care not to dislodge the pellet. The wash step was repeated and the pellet air-dried at room temperature for 20 minutes. The pellet was dissolved in 100 µl of TE buffer pre-warmed at 55^{o} C and DNA stored at -20^{o} C for further application.

5.2.4 DNA Detection

Total DNA samples extracted from the isolates were detected using gel electrophoresis. A volume of 5 µl of each DNA sample was loaded on an ethidium bromide containing agarose gel (1 %) in 1X TAE buffer and run at 80 volts for 1 hour. Gel documentation was done using the Gel Logic 200 Imaging System (Sambrook *et al.*, 1989).

5.2.5 PCR amplification of bacterial 16S rDNA gene

The extracted total DNA from each sample was used as a template for amplification of the 16S rRNA gene. This was done using the GenScript kit (GenScript Corporation) according to the manufacturer's instructions. The 16S rRNA gene sequences were PCR-amplified using bacterial primer pair 27F forward 5'-GAG TTT G(AC)T CCT GGC TCA G-3') and 1492R reverse, 5'-TAC GG(CT) TAC CTT GTT ACG ACT T-3' (eurofins MWG GmbH). Amplification was performed using a model 9800 Fast Thermal Cycler from Applied Biosystems. Amplification was carried out in a 30 µl

mixture containing 3 µl of 10x PCR buffer, 4 µl of 2.5 mM dNTPs, 2.5 µl of 27F forward primer (5 pmol), 2.5 µl of 1492R reverse primer (5 pmol), 0.4 µl of 5U/ul Taq polymerase, 1.5 µl template DNA and 16.6 µl of PCR grade water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles: Initial denaturation at 94^{o} C for 5 minutes, 30 cycles of denaturation at 94^{o} C for 45 seconds, primer annealing at 55^{o} C for 50 seconds, chain extension at 72^{o} C for 90 seconds, and a final extension at 72^{o}C for 8 minutes. Amplification products (5 µl) of each DNA sample was loaded on an ethidium bromide containing agarose gel (1 %) in 1X TAE buffer and run at 80 volts for 1 hour. Gel documentation was done using the Gel Logic 200 Imaging System (Sambrook *et al*., 1989).

5.2.6 RFLP analysis

PCR products were digested with MspI restriction enzyme (Promega Corporation Madison, USA). This was performed in 30 µl of a restriction enzyme mixture containing 21.3 µl sterile distilled water, 3 µl of 10× restriction enzyme buffer, 0.2 µl of acetylated BSA (10 µg/µl), 5 µl of the template and 0.5 µl of restriction enzyme (10 U/µl). The digestion was performed for 3 hours at the optimum temperature (37 ^{o} C). RFLPs were analyzed in 1.5 % agarose gel in TAE buffer at 80 volts for 1 hour. Gel visualization was done using the Gel Logic 200 Imaging System (Sambrook *et al*., 1989).

5.2.7 DNA extraction and purification from agarose gel

The PCR amplicons from seven isolates were excised from the gel and purified using quickClean 5M gel Extraction kit (GenScript Corporation, 120 Centennial Ave, Piscataway, NJ 08854) according to the manufacturer's instructions.

5.2.8 Phylogenetic data analysis

Direct sequencing of purified PCR products was performed using Macrogen machine (Macrogen 3730XL1-1403-007) in South Korea. Sequences data were edited manually in chromous. The 16S rRNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity to sequences in the Genebank database (Shayne *et al*., 2003). The 16S rRNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.,* 2007). Bootstrap for 1000 replicates was performed to attach confidence estimates for the tree topologies (Felsentein, 1985).

5.2.9 Effects of Bt Cry1A(c) δ-endotoxin on nitrogen fixation

The experiment comprised of two treatments in a completely randomized design. Each experiment had 25 samples treated with the following treatments:

1. BTS (Bt toxin-treated solution – 100 μg/ml of Bt toxin)

2. NBTW (Non-Bt treated water - Control)

The activated Cry1A(c) Bt toxin was used for the test experiments while non-Bt treated water used for the control experiments.

5.2.10 Determination of nitrogen fixing activities in the soil

Activity of nitrogen-fixing bacteria in Clay soils from the different treatments was studied by measuring the rate of acetylene reduction to ethylene (Dilworth, 1966). The Clay soil from KARI-NARL farm was used. Each treatment had 25 samples. A total of 50 samples were used for the two treatments. Fresh soil was weighed (10 g) separately and then air-dried, put into hungate tubes (16 ml volume) and sprayed with Bt toxin solution (100 µg/ml) to reach the water content of the soil. The tubes were then sealed with rubber stoppers. A volume of 1.6 ml of air was removed from each tube using a gas-tight syringe and replaced with 1.6 ml acetylene to create 10 % acetylene atmosphere. The samples were incubated at room temperature until the required time for analyses.

5.2.11 Measurement of acetylene reduction

The rate of acetylene reduction to ethylene at times 0 hr, 22 hr, 36 hr, 42 hr and 60 hr were measured using Gas Chromatograph (GC) fitted with a Flame Ionization Detector (FID) (Knowles, 1980). A volume of 100 µl of gas was removed from the sample tube with a gas-tight syringe and injected into the GC. The GC analysis was done at the following GC conditions; 120^{o} C column temperature, 220^{o} C injection and detection temperatures and 50 ml/minute gas flow rate. The initial time (t_o) was recorded.

Ethylene standards were run as well and they were ranging between 10.28-19.36 nL/g.hr. The experiment was repeated two times after a trial had been done.

5.3 Results

5.3.1 Isolation of bacteria from host plant root nodules

Pure isolates of rhizobia were isolated from the root nodules of both leguminous plant species. For the bean plant samples, forty pure isolates of were obtained from both the control and test samples. Likewise, for Siratro plant samples, forty pure isolates of rhizobia were isolated from the root nodules of both the control and test samples.

5.3.2 Morphological Characterization of isolates from bean samples

Morphological studies of the 80 isolates of rhizobia were done using the dissecting micropscope (×16). All the 80 isolates grew well on Yeast Extract Mannitol (YEM) agar media. Growth on the media was slow (4 to 7 days) to fast (1 to 3 days) for most of the isolates (Table 5.1). Most of the isolates had similar colony appearance for both the control and test samples.

Table 5.1 Morphological characteristics of the isolates from bean plant samples as observed under dissecting microscope (×16)

	Characteristics	
Isolate	**Colony appearance**	**Growth form**
BTS1	purple milky translucent, shinny, raised, watery	Fast
BTS2	purple milky translucent, shinny, raised, watery	Fast
BTS3	purple milky translucent, shinny, raised, watery	Fast
BTS4	purple milky translucent, shinny, raised, watery	Fast
BTS5	purple milky translucent, shinny, raised, watery	Fast
BTS6	purple milky translucent, shinny, raised, watery	Fast
BTS7	purple milky translucent, shinny, raised, watery	Fast
BTS8	purple milky translucent, shinny, raised, watery	Fast
BTS9	purple milky translucent, shinny, raised, watery	Fast
BTS10	purple milky translucent, shinny, raised, watery	Fast
BTS11	purple milky translucent, shinny, raised, watery	Fast
BTS12	purple milky translucent, shinny, raised, watery	Fast
BTS13	purple milky translucent, shinny, raised, watery	Fast
BTS14	purple milky translucent, shinny, raised, watery	Fast
BTS15	purple milky translucent, shinny, raised, watery	Fast
BTS16	purple milky translucent, shinny, raised, watery	Fast
BTS17	purple milky translucent, shinny, raised, watery	Fast
BTS18	red, raised, shinny and mucoid	Fast
BTS19	red, raised, shinny and mucoid	Fast
BTS20	red, raised, shinny and mucoid	Fast
BCS1	red, raised, shinny and mucoid	Fast
BCS2	red, raised, shinny and mucoid	Fast
BCS3	milky, shiny, raised	Moderate
BCS4	milky translucent, shiny, flat and watery.	Slow
BCS5	red, raised, shinny and mucoid	Fast
BCS6	red, raised, shinny and mucoid	Fast
BCS7	red, raised, shinny and mucoid	Fast
BCS8	red, raised, shinny and mucoid	Fast
BCS9	red, raised, shinny and mucoid	Fast
BCS10	red, raised, shinny and mucoid	Fast
BCS11	milky translucent, shiny, flat and watery.	Slow
BCS12	red, raised, shinny and mucoid	Fast
BCS13	milky, shiny, raised	Moderate
BCS14	red, raised, shinny and mucoid	Fast
BCS15	yellow suspensions, translucent margin, shiny, raised	Slow
BCS16	red, raised, shinny and mucoid	Fast
BCS17	yellow suspensions, translucent margin, shiny, raised	Slow
BCS18	yellow suspensions, translucent margin, shiny, raised	Slow
BCS19	red, raised, shinny and mucoid	Fast
BCS20	milky translucent, shiny, flat and watery.	Slow

5.3.3 Morphological Characterization of isolates from Siratro samples

Morphological studies of the 80 isolates were done using the dissecting microscope (×16). All the 80 isolates grew well on Yeast Extract Mannitol (YEM) agar media. Growth on the media was slow to fast for most of the isolates (Table 5.2). Most of the isolates had similar colony appearance for both the control and test samples.

Table 5.2 Morphological characteristics of isolates from Siratro plant samples as observed under dissecting microscope (×16).

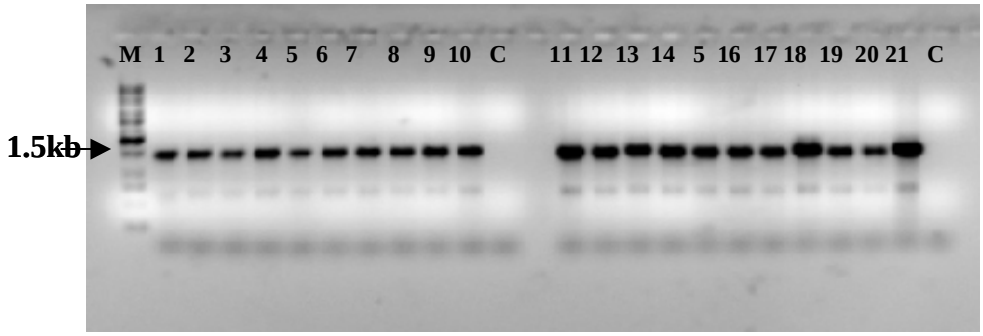
	Characteristics	
Isolate	**Colony appearance**	**Growth form**
STS1	pink, shiny, raised surface and regular margin	Fast
STS2	pink, shiny, raised surface and regular margin	Fast
STS3	pink, shiny, raised surface and regular margin	Fast
STS4	pink, shiny, raised surface and regular margin	Fast
STS5	pink, shiny, raised surface and regular margin	Fast
STS6	pink, shiny, raised surface and regular margin	Fast
STS7	pink, shiny, raised surface and regular margin	Fast
STS8	pink, shiny, raised surface and regular margin	Fast
STS9	pink, shiny, raised surface and regular margin	Fast
STS10	transparent, dome shaped, tiny colonies	Slow
STS11	transparent, dome shaped, tiny colonies	Slow
STS12	pink, shiny, raised surface and regular margin	Fast
STS13	pink, shiny, raised surface and regular margin	Fast
STS14	pink, shiny, raised surface and regular margin	Fast
STS15	pink, shiny, raised surface and regular margin	Fast
STS16	pink, shiny, raised surface and regular margin	Fast
STS17	pink, shiny, raised surface and regular margin	Fast
STS18	pink, shiny, raised surface and regular margin	Fast
STS19	transparent, dome shaped, tiny colonies	Slow
STS20	transparent, dome shaped, tiny colonies	Slow
SCS1	transparent, dome shaped, tiny colonies	Slow
SCS2	transparent, dome shaped, tiny colonies	Slow
SCS3	transparent, dome shaped, tiny colonies	Slow
SCS4	transparent, dome shaped, tiny colonies	Slow
SCS5	transparent, dome shaped, tiny colonies	Slow
SCS6	transparent, dome shaped, tiny colonies	Slow
SCS7	transparent, dome shaped, tiny colonies	Slow
SCS8	transparent, dome shaped, tiny colonies	Slow
SCS9	transparent, dome shaped, tiny colonies	Slow
SCS10	transparent, dome shaped, tiny colonies	Slow
SCS11	transparent, dome shaped, tiny colonies	Slow
SCS12	transparent, dome shaped, tiny colonies	Slow
SCS13	transparent, dome shaped, tiny colonies	Slow
SCS14	transparent, dome shaped, tiny colonies	Slow
SCS15	transparent, dome shaped, tiny colonies	Slow
SCS16	transparent, dome shaped, tiny colonies	Slow
SCS17	transparent, dome shaped, tiny colonies	Slow
SCS18	transparent, dome shaped, tiny colonies	Slow
SCS19	transparent, dome shaped, tiny colonies	Slow
SCS20	transparent, dome shaped, tiny colonies	Slow

5.3.4 Phylogenetic charcterisation of isolates from Bean plants

5.3.4.1 RFLP Phylotypes

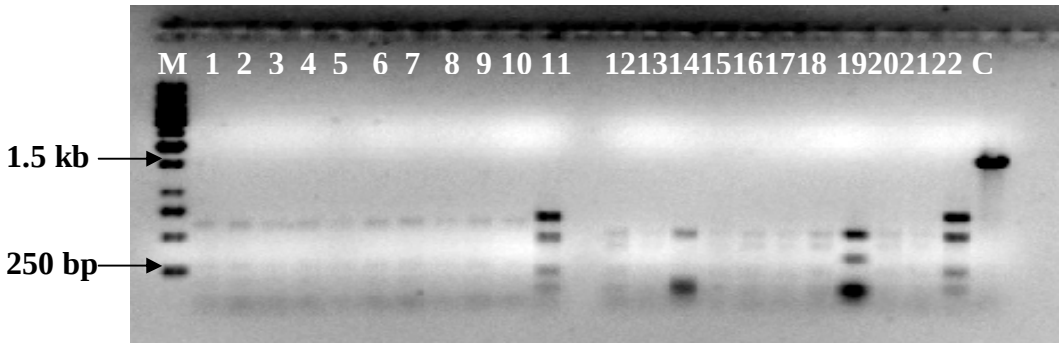
PCR amplification of the 16S rRNA gene was positive for all samples analyzed. Clear DNA bands of molecular weight 1.5 kb were observed for the bean samples (Figure 5.1). RFLP results indicated a high diversity of the rhizobia isolates for the bean samples (Figure 5.2). Five RFLP profiles were observed (Table 5.3). Five different rhizobia species were isolated from the root nodules of bean plants samples as represented by the RFLP profiles. One *Rhizobium species* was common in both root nodules obtained from Bt treated and Non-Bt treated bean samples. Only species from the genus *Rhizobium* were isolated from both the test and control bean samples.

Figure 5.1 1% agarose gel showing DNA bands after PCR amplification of 16S rRNA gene of the isolates visualized after ethidium bromide staining.



*Key: Lanes: (**M**) 1kb DNA ladder used as a molecular marker; (**1-10**) Bean test samples isolates (BTS); (**10-21**) Bean control samples isolates (BCS); (**C**) PCR-negative control sample.*

Figure 5.2 Agarose gel electrophoresis result after restriction digestion of 16S rRNA gene with MspI.



*Key: Lanes: (**M**) 1kb DNA ladder used as a molecular marker; (**1-11**) Bean test isolate samples (BTS); (**12-22**) Bean control isolate samples (BCS); (**C**) Restriction digestion negative control sample.*

Table 5.3 RFLP profiles with their representative sample identities and next relatives

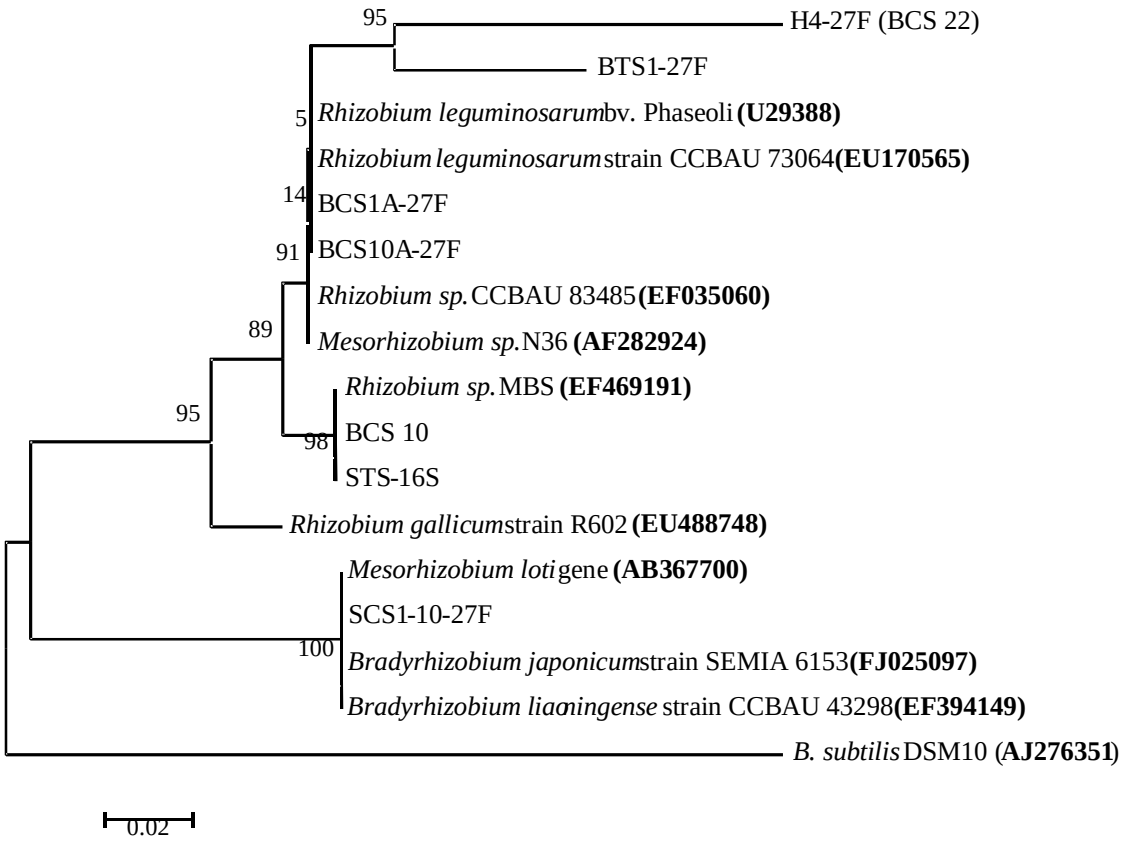
RFLP profile	Representative sample ID	Next relative
RFLP A	BTS 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10	*R. leguminosarum* bv. *trifolii* WSM 2304
RFLP B	BTS 11 and BCS 22	*R. leguminosarum* bv. *phaseoli*
RFLP C	BCS 12, 13,15, 16, 17, 18, 20, and 21	*Rhizobium gallicum* R602
RFLP D	BCS 14	*Rhizobium etli* CIAT 652
RFLP E	BCS 22	*Rhizobium sp.* CCBAU 83485
RFLP F	STS 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10	*Rhizobium tropici* 77
RFLP G	STS 11, 12 and SCS 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, and 23	*Bradyrhizobium japonicum* SEMIA 6153

5.3.4.2 Phylogenetic identification of isolates from bean plants

The phylogenetic tree shows two main clusters (Figure 5.3). The isolates from bean samples shared sequence identity of between 93-100 % with known rhizobium species (Table 5.4). The bean isolates were in the second cluster that comprised of the genus

Rhizobium. The isolate BCS 10 (Representing RFLP C) from bean control sample clustered together with *Rhizobium sp.* MBS (Accession EF469191) with a bootstrap value of 98 % and sequence identity of 100 %. The other isolates BCS10A-27F (Representing RFLP E) and BCS1A-27F (Representing RFLP D) from bean control samples clustered together with *Rhizobium sp.* CCBAU 83485 (Accession EF035060) with a bootstrap value of 91 % and sequence identity of 100 % while BTS 1-27F (Representing RFLP B) from bean test samples and BCS 22 (Representing RFLP B) from bean control sample clustered together with *R. leguminosarum* bv. Phaseoli (Accession U29388) with a bootstrap value of 95 % and sequence identity of 94 %. Generally, the isolates seem to have some relationship with known species from the genus *Rhizobium* in the public database.

Figure 5.3 phylogenetic positions of isolates.



The scale bar indicates approximately 2 % sequence difference. The 16S rDNA sequence of Bacillus subtilis was used as an outgroup.

Table 5.4 NCBI blast results showing sample identities, next relatives and % similarities from bean and Siratro plants.

Sample ID	RFLP Type	Accession No.	Next relative	% Similarity
BCS10	RFLP C	EU488748	*R. gallicum* R602	93 %
		EU488745	*R. tropici* 77	93 %
		EU074200	*R. lusitanum* strain CCBAU 03301	93 %
BCS1A-27F	RFLP D	EU618029	*R. leguminosarum* bv. Strain CCBAU 65673	99 %
		AF282924	*Mesorhizobium sp.* N36	99 %
		EF035060	*Rhizobium sp.* CCBAU 83485	100 %
BTS1-27F	RFLP B	CP001191	*R. leguminosarum* bv. Trifolii WSM 2304	89 %
		EJ188348	*R. leguminosarum* bv. Viciae isolate MJV12	89 %
		EF152481	*Rhizobium sp.* STM 4044	89 %
STS1-16S	RFLP F	EU488745	*R. tropici* 77	99 %
		U89831	*R. leguminosarum* bv. Viciae	98 %
		EF469191	*Rhizobium sp.* MBS	100 %
SCS1-10-27F	RFLP G	FJ025097	*Bradyrhizobium japonicum* SEMIA 6153	100 %
		AB367700	*Mesorhizobium loti* gene	100 %
		EF394149	*Bradyrhizobium liaoningense* strain CCBAU 43298	100 %
BCS10A-27F	RFLP E	CP001074	*Rhizobium etli* CIAT 652	96 %
		EU170565	*R. leguminosarum* strain CCBAU 73064	96 %
		EF221632	*Rhizobium sp.* JR020	97 %
H4 (BCS 22)	RFLP B	CP001074	*Rhizobium etli* CIAT 652	94 %
		U29388	*R. leguminosarum* bv. *Phaseoli*	94 %
		AM236080	*R. leguminosarum* bv. Viciae	94 %

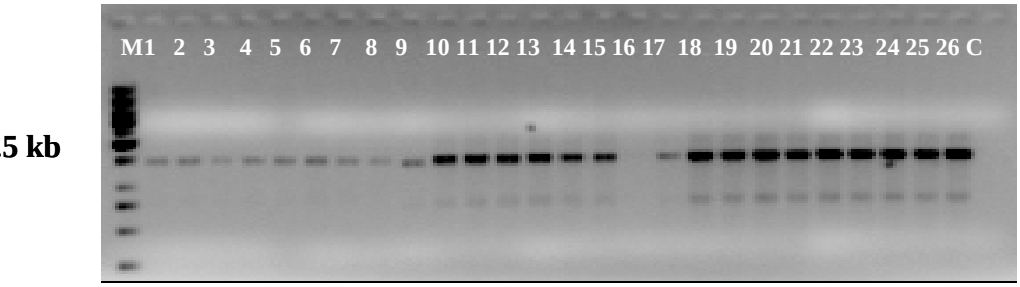
5.3.5 Phylogenetic characterization of isolates from Siratro plants

5.3.5.1 RFLP Phylotypes

PCR amplification of the 16S rRNA gene was successful for all Siratro samples analyzed. Clear DNA bands of molecular weight 1.5 kb were observed for the Siratro samples (Figures 5.4). Diversity of the rhizobia isolates from Siratro was observed as depicted by the RFLP profiles (Figure 5.5). Two RFLP profiles were observed from Siratro isolates (Table 5.3). Root nodule samples from both Bt and non-Bt treated Siratro samples had one common rhizobia species (Samples 11, 12, 22 and 23 of Figure

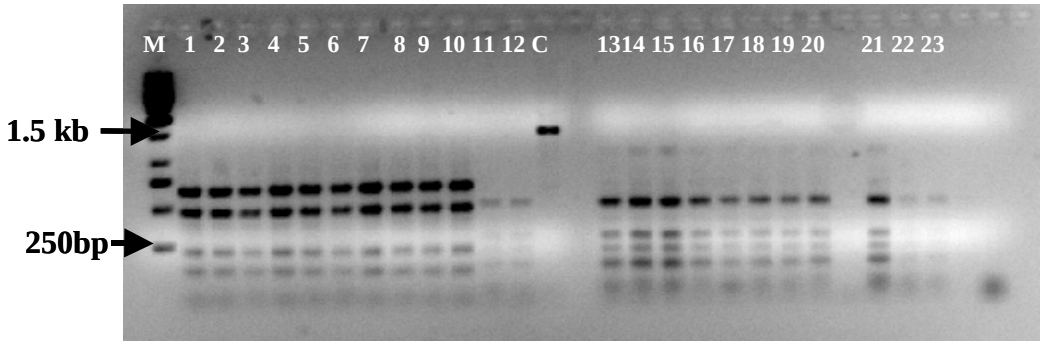
5.5). Notably, two different *Rhizobium species* were isolated from the root nodules of Siratro plants according to the RFLP profiles. *Rhizobium* species was isolated from the test Siratro samples (Table 5.5). *Bradyrhizobium* species was isolated from both the control and test Siratro samples.

Figure 5.4 1% agarose gel showing DNA bands after PCR amplification of 16S rRNA gene of the isolates visualized after ethidium bromide staining.



*Key: Lanes: (**M**) 1kb DNA ladder used as a molecular marker; (**1-10**) Siratro test samples isolates (STS); (**11-26**) Siratro control samples isolates (SCS); (**C**) PCR negative control sample.*

Figure 5.5 Agarose gel electrophoresis result after restriction digestion of 16S rRNA gene with MspI.



*Key: Lanes: (**M**) 1kb DNA ladder used as a molecular marker; (**1-12**) Siratro test isolate samples (STS); (**13-23**) Siratro control isolate samples (SCS); (**C**) Restriction digestion negative control sample.*

Table 5.5 Isolated species from Siratro samples

***Rhizobium species* isolated**	
Bt toxin treated Siratro plants (Test samples)	Water treated Siratro plants (Control samples)
Rhizobium species *Bradyrhizobium species*	*Bradyrhizobium species*

5.3.5.2 Phylogenetic identification of isolates from Siratro plants

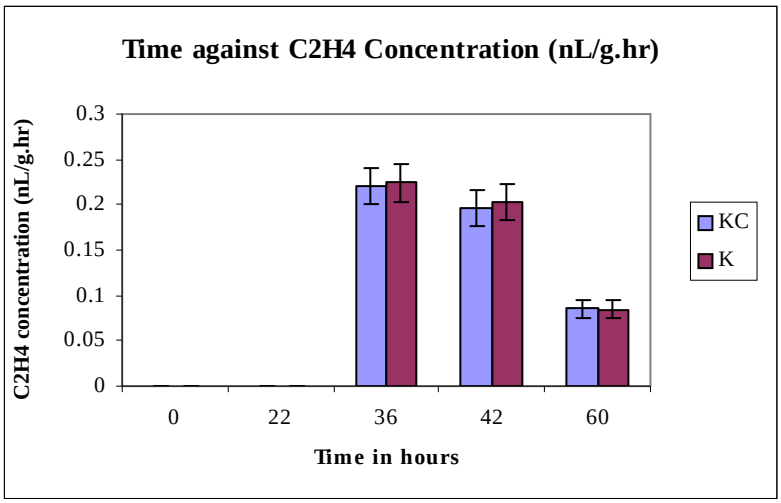
The isolates from Siratro samples shared sequence identity of between 98-100 % with known rhizobium species (Table 5.4). Isolate SCS1-10-27F (Representing RFLP G) from Siratro control samples was in the first cluster that comprised the genus *Bradyrhizobium* and clustered together with *Bradyrhizobium japonicum* SEMIA 6153 (Accession FJ025097) and this was supported by a bootstrap value of 100 % with a sequence identity of 100% (Figure 5.3) This shows that the isolate could be a very close relative of *B. japonicum* SEMIA 6153. Isolate STS1-16S (Representing RFLP F) from Siratro test samples clustered together with *Rhizobium sp.* MBS (Accession EF469191) in the second cluster with a bootstrap value of 98 % and sequence identity of 100 % (Figure 5.3). It is evident that the isolates seem to have some similarity with known species from the genera *Bradyrhizobium* and *Rhizobium* in the public database (Table 5.4).

5.3.6 Effects of Bt delta-endotoxin on nitrogen fixation in Clay soil

Nitrogen fixation in between treatments for the KARI-NARL soil samples were not significantly different from each other (t_{48}, 2.009, $P>0.05$, $n=25$). Interestingly, there

was a lag phase (a period of no activity) of about 22 hours before acetylene reduction to ethylene commenced (Figures 5.6). The mean values for acetylene reduction to ethylene for the samples were highest at the 36th hr (0.220±0.02 and 0.224±0.02) for the control and test samples respectively and lowest at the 60th hr (0.085±0.01and 0.084±0.01) for the control and test samples respectively (Figure 5.6).

Figure 5.6 Rate of ethylene production by the nitrogen-fixing bacteria in relation to time (Hrs) for both controls (KC) and test (K) samples from KARI-NARL farm.



5.4 Discussion

Most studies to date suggest that transgenic plants that have been released cause minor changes in microbial community structures that are often transient in duration (Biao *et al.*, 2005). In this study, experiments to specifically test the effects of Bt Cry1A(c) δ-endotoxin on diversity of rhizobia and nitrogen fixation in clay soil were carried out. The results showed minor changes in the rhizobial diversity as depicted by the RFLP profiles (Figures 5.2 and 5.5) that indicated some changes in the diversity of rhizobium

species between the Bt treated and non-Bt treated plant species. Notably, the control bean samples had four seemingly different rhizobium species compared to the test bean samples that had two rizobium species, one that was present in the control bean samples and another one that was missing (Figure 5.2). The four rhizobium species isolated from root nodules of the control bean samples were different from the ones isolated from the test bean samples except in one case (Samples 11 and 22 of Figure 5.2). Nonetheless, this scenario occurred in only one sample for each case that could mean the plant-bacterial interaction was not effective for that particular rhizobium species. The control Siratro samples had one rhizobiun species that was missing in the test samples; however, both the control and test samples shared one rhizobium species (Figure 5.5). Interestingly, the diversity of the rhizobia was pronounced in the control bean samples than in control Siratro samples. This observation could have been caused by such possibilities as; the rhizobium species in the studied soil being plant specific and or the presence of less rhizobium species that could interact with the Siratro plant species at the right stage of nodulation.

These findings are in agreement with other studies where differences in the microbial community structure, between transgenic and non-transgenic plants, have been observed, as in the cases of transgenic canola (*Brassica napus*) (Dunfield and Germida, 2001; Gyamfi *et al.,* 2002), and Bt and non-Bt corn plants (Castaldini *et al.,* 2005) observed some differences in aerobic culturable bacteria. Another study showed that transgenic potato lines producing *Galanthus nivalis* agglutinin and *Brassica napus*

resistant to the herbicide glyphosate modified the composition and diversity of soil and rhizospheric microbial communities (Siciliano and Germida, 1999; Griffiths *et al.*, 2000). Other studies have revealed insignificant differences, such examples being transgenic potato expressing GUS and Barnase (Lukow *et al.*, 2000), Bt white spruce, which constitutively expresses the Cry1Ab insecticidal toxin (Lamarche and Hamelin, 2007). However, the results of this study demonstrated that the presence of Bt delta-endotoxin modified (reduced) the populations of rhizobia in the soil as indicated by the DNA fingerprinting patterns (Figures 5.2 and 5.5)

Nonetheless, the culture-dependent method used for the rhizobium diversity study captured only the aerobic culturable bacteria, making it impossible to know the fate of the other unculturable nitrogen-fixing bacteria in presence of the toxin. This is important because different effects of transgenic plants on soil microorganisms is known to occur, mainly at the rhizosphere level, where root exudates directly affect the composition of microbial soil communities, in terms of both structure and function (Chelius and Triplett, 2001; Lynch *et al.*, 2004; Mansouri *et al.*, 2002; Schmalenberger and Tebbe, 2002). In addition, the use of leguminous plants for this study limited evaluation of the Bt toxin only to rhizobial communities.

Nitrogen fixation data indicated that there was conversion of acetylene to ethylene for both treatments by the nitrogen-fixing bacteria in the soil. There was a lag phase of 22 hours before nitrogen fixation commenced for both treatments. This is congruent with

the Broadbalk experiment (Day *et al.,* 1975) that reported a considerable lag phase before ethylene production in acetylene reduction assay began. Though the rate of the acetylene reduction was comparably slow for both treatments, the difference was insignificant (t48, 2.009, P>0.05, n=25). The reduction in ethylene production in all cases might have been contributed by some other factors such as; inhibitory effect from other gases (high partial pressure of oxygen, high levels of combined nitrogen in the soil), temperature and pH of the soil (Day *et al.,* 1975), and reduction in the concentration of acetylene and or increasing concentration of ethylene that inhibited the activity of the nitrogenase activity. In addition, it has been established that the rate of acetylene reduction increased exponentially with linear increase in soil moisture. Likewise, nitrogenase activity in wetland grasses has been reported to be much higher than that of plants growing in mesic or dry soils (Day *et al.,* 1975). However, our findings demonstrated that the presence of Bt Cry1A(c) δ-endotoxin (100 μg/ml) in the soil had no significant effect on nitrogen fixation activity. This is in agreement with work by Biao *et al.* (2005) who observed no deleterious effects of Bt Cry1A(c) toxin on microbial activity.

Plant root microflora is very sensitive to environmental factors, evident in the small differences found between cultivars that often select for different microbial rhizosphere populations (Gomes *et al.,* 2001; Smalla *et al.,* 2001; Smit *et al.,* 2001). It is known that Bt maize has a higher lignin content than its non-transgenic counterpart (Saxena and Stotzky, 2001), potentially affecting the rate of maize tissue degradation in soil,

and transgenic Cry protein has been found to be released in the root exudates (Saxena *et al.*, 1999, 2002; Saxena and Stotzky, 2000). This may have potential influence on the composition and activity of the soil microbial community.

5.5 Conclusion

The risks of GM crops for the environment, and especially for biodiversity, have been extensively assessed worldwide over the past 13 years of commercial cultivation of GM crops. GM crops have been found to have significant effects on soil populations of non-target bacteria and fungi (Donegan *et al.*, 1995, 1999), soil enzyme activities and the structure of microbial community (Griffiths *et al.*, 2000; Dunfield and Germida, 2001). Some of the effects caused by transgenic plants on soil microorganisms are transient and temporary (Donegan *et al.*, 1995) and occur only at certain growth stages of transgenic plants. In this study, it was found out that Bt Cry1A(c) δ-endotoxin had no effect on nitrogen fixation, however, it reduced the number of rhizobia and modified the diversity of rhizobia nodulating the beans and Siratro plants.

CHAPTER 6

6.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 General discussion

The rapid development of agricultural biotechnology and release of new transgenic plants for agriculture has provided many economic benefits but also raised concerns over their potential impact on the environment. Considerable research has been conducted on the effects of transgenic plants on soil microorganisms. These effects include unintentional changes in the chemical compositions of root exudates, and the direct effects of transgenic proteins on non-target species of soil microorganisms (Dunfield and Germida, 2001; Gyamfi *et al.,* 2002). The effect of transgenic plants on the rhizosphere community has been the subject of many studies. It has been shown that root exudates play an important role in microflora selection, and selectively influence microbial growth. Studies have indicated that qualitative and quantitative differences in root exudation can strongly affect the structural and functional diversity of the rhizosphere population (Savka and Farrand, 1997; Oger *et* a*al.*, 1997, 2000; Mansouri *et al.,* 2002). Zwahlen *et al*. (2003a) concluded that Bt maize expressing Cry1Ab δ-endotoxin apparently posed minimal risks to earthworms as far as growth and reproduction is concerned. The findings of this study on the impact of Bt delta-endotoxin on host plant growth parameters indicated absence of significant effects on the host plant growth parameters showing that Bt delta-endotoxin does not interfere

with growth of the host plants. In addition, no adverse effects were observed on the host plants nodulation. This demonstrates that the presence of Bt delta-endotoxin in the soil poses no effects on nodulation activities. Moreover, our study revealed no significant effect on the host plant productivity in the presence of Bt toxin in the soil. However, we detected some minor changes on the rhizobial diversity when Bt delta-endotoxin (100 μg/ml) was present in the soil, indicating that the presence of Bt delta-endotoxin has negative influence on rhizobial diversity as shown by the RFLP phylotypes (sections 5.3.4.1 and 5.3.5.1). This is in agreement with other studies, which concluded that the presence of Bt toxins modified the composition and diversity of soil and rhizospheric microbial communities (Siciliano and Germida, 1999; Griffiths *et al.*, 2000). Nonetheless, our study also showed that the presence of Bt delta-endotoxin in the soil has no impact on the nitrogen fixation by free living organisms. Case-by-case studies, however, need to be carried out to understand the impact of GMOs on the environment.

6.2 General conclusions

The results of our investigation demonstrated that Bt toxin does not adversely affect the activity of nitrogen fixing bacteria, but instead slightly modified their diversity in the rhizosphere, leading to the following conclusions:

1. There was no evidence of Bt Cry1A(c) δ-endotoxin effect on the host plant growth, nodulation and productivity.
2. Bt Cry1A(c) δ-endotoxin did not affect nitrogen fixation by the bacteria in soils.

3. The presence of Bt Cry1A(c) δ-endotoxin at 100 μg/ml concentration reduced the number of rhizobia nodulating the bean and Siratro plants in the soil.
4. Bt Cry1A(c) δ-endotoxin modified the diversity of rhizobia nodulating the bean and Siratro plants in the soil.

6.3 Recommendations

There has been a strong debate on the safety of genetically modified organisms ever since the introduction onto the market of plant products derived from transgenic organisms. The increasing concerns are mainly due to the insufficient knowledge available on biological systems, and by the potential danger that specific genetic manipulations could give unexpected effects (Schubert, 2002).

The results from this work will provide a baseline for more work to be done in this field. The major recommendations derived from the study carried out were:

1. Further investigations should be done with other soil types (Sandy and loamy).
2. Transgenic Bt maize plants should be used in a similar study in order to establish the overall impact of the Bt Cry proteins on the populations and activities of nitrogen fixing bacteria in the soil.
3. Research should be done to evaluate the long-term potential impact of GM crops on other soil microbial communities.

4. Further investigations should be set in a field environment, where other factors such as exudates from plants, variations in temperature, humidity, soil factors among others may interact with the toxin as well as with the bacteria.

Biodiversity is interdependent and consequently, there is need to protect each and every organism in the environment. Assessment of such modifications is consequential in finding out the likely adverse effects of the transgenic products. This information is of paramount importance to protect the environment and guide the policy makers on decision-making regarding the adoption and use of GMOs.

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