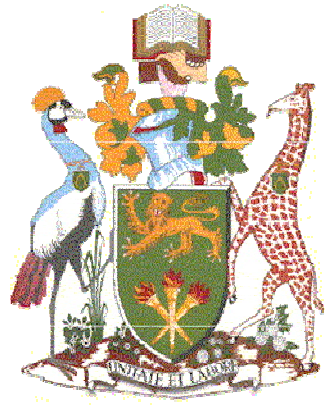
UNIVERSITY OF NAIROBI



DETERMINATION OF NATIVE *BACILLUS THURINGIENSIS* DELTA-ENDOTOXIN POTENCY AGAINST *PROSTEPHANUS TRUNCATUS* AND FUMONISIN PRODUCING *FUSARIUM VERTICILLIOIDES* ISOLATED FROM MAIZE GRAINS

By

AKELLO SUNDY OTIENO

(BSc. BIOTECHNOLOGY SC. HONS, JKUAT)

A thesis submitted in partial fulfillment for the award of the Degree of Master of Science in Biotechnology in the Centre for Biotechnology and Bioinformatics, University of Nairobi

JULY, 2013

DECLARATION

This is my original work and has not been presented for the award of any degree at any other University or educational institution.

Sundy Akello Otieno

Signature ……..

Date ……..

This thesis has been submitted for examination with our approval as the University supervisors.

Prof. J. O. Ochanda,

University of Nairobi,

Signature ……..

Date ……..

Prof. Sheila Okoth

University of Nairobi,

Signature ……..

Date ……..

Dr. V. Wangondu,

University of Nairobi,

Signature ……..

Date ……..

ACKNOWLEDGEMENTS

First of all, I would like to thank Prof. James Ochanda, the Director, Centre for Biotechnology and Bioinformatics (CEBIB), Prof. Sheila Okoth and Dr. Virginia Wang'ondu, School of Biological Sciences, for serving as my supervisors. During my two years of graduate study, they taught me skills necessary to succeed as a researcher. They have nurtured me academically, helped me explore interests beyond my research area, and encouraged me to accomplish my work independently.

I am grateful to Dr. Isabella Ocholla Oyier and Dr. Joel Ochieng' for offering generous help and support as members of my supervisory committee, and for reviewing an earlier draft of the proposal. I thank fellow centre for biotechnology graduate students for providing information relevant to my study. I would like to recognize several of my laboratory mates, Harry Ndung'u and Eric Mukundi with whom I have shared this past year. They offered their assistance and support whenever I needed it.

This research was supported by the School of Biological Sciences and the Centre for Biotechnology and Bioinformatics, University of Nairobi to which I am grateful to, for allowing me to use their laboratories and other facilities in carrying out my investigation.

DEDICATION

I wish to dedicate this thesis to my father, Dickson Otieno Achieng' and my mother Priscilla Aoko Otieno, for supporting my education and the rest of the family members for their encouragement, love, patience and understanding throughout the entire period of study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ACRONYMS AND ABBREVIATIONS

FAO – Food and agricultural organization

WHO – World health organization

FB1 - Fumonisins (B1)

IPM- Integrated pest management

HRP-Horseradish peroxidase

ITU-International Toxic Units

Spp – Species

KM- Kenya Machakos

KG-Kenya Gazi

var – Variety

Bt – Bacillus thuringiensis

KARI - Kenya Agriculture Research Institute

BSA – Bovine Serum Albumin

DDT-Dichlorodiphenyltrichloroethane

PBS- Phosphate Buffered Saline

u.v- Ultraviolet

v.v- Volume by volume

w.v- Wavelength by volume

Tris- Tris-(Hydroxy methyl)-amino methane

CEBIB-Centre for Biotechnology and Bioinformatics

UON-University of Nairobi

NARL-National Agricultural Research Laboratories

LIST OF UNITS AND SYMBOLS

UNITS	SYMBOLS
μm- micrometer	δ- Delta
μg – micrograms	
μl- microlitre	β- Beta
^{o}C – Degrees Centigrade	
min – minutes	
Hr/hr – hour	
nm – nanometer	
kDa – kilodalton	
Sec- Seconds	
cm- Centimeter	
g- gram	
M- Molar concentration	
ml- milliliter	
mM-millimolar	

ABSTRACT

Insect pests contribute to reduced maize grain yield pre and post harvest. They affect 46% of global maize growing area post harvest, 60% of this maize loss occurs in tropical regions. *Bacillus thuringiensis* has become vital in the pest management strategy, as an insecticide, biopesticide, fungicide and also in transgenic plants. In Kenya maize is the staple food. *Prostephanus truncatus* and fumonisin producing fungi *Fusarium verticillioides* are prevalent in tropical and sub tropical regions, and are hard to eradicate using pesticides. This study sought to investigate effects of native *B. thuringiensis* delta endotoxin against *P. truncatus* and fumonisin producing *F. verticillioides. F. verticillioides* was isolated from maize grains obtained from Makueni district in Kenya. VERT-1(5'-GCGGGAATTCCAAAGTGGCC-3') and reverse VERT-R (5'-CGACTCACGGCCAGGAAACC-3') which are species specific PCR primers for the identification of *F. verticillioides* were used after morphological methods of identification were carried out. A set of specific primers, VERTF-1, VERTF-2 and Fro-fum8-rev were used to analyze the isolated *F. verticillioides* for fumonisin production. Translation Elongation Factor TEF-1a gene was amplified using primers EF-1 and EF-2 and the resulting PCR product was used to carry out sequencing for confirmation of identity of the isolated species. Delta crystals of *B. thuringiensis* were bioassayed against the *F. verticillioides* and *P. truncantus*. Two species of *Fusarium* (*F. verticillioides* and *F. oxysporum*) were isolated from the maize grains from Makueni district, Eastern province. Sixty five percent of the *Fusarium* species isolated produced fumonisins. Novel *Bt.* Varieties used showed statistically significant differences on their effects on the maize pests, at a confidence level of 0.05. KG 20 showed antagonistic action against *P. truncantus*, varieties TEN and ISR showed best results in *F. verticillioides* control, while KG 411 was effective against both *P. truncantus* and *F. verticillioides*. This study shows that *F. verticillioides* is naturally present on maize grains in Makueni district; Eastern province in Kenya and *Bt.* toxins can be used successfully against both pests.

Keywords: *Bacillus thuringiensis*, delta crystals, fumonisin, *Fusarium, Prostephanus truncatus, Fusarium verticillioides*.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1General Introduction

Food security is a challenge faced worldwide. In Africa, where cereals serve as the staple diet, food insecurity is majorly caused by insects which indirectly contribute to fungal toxin contaminants on grains, both pre and post harvest.

Prostephanus truncatus (Horn), the larger grain-borer, is an invasive pre and post harvest pest, originating in Central America, and found prevalent in East African farms. *Prostephanus truncatus* causes damage on the grains, by drilling holes on them, used for entry into the kernel. Infestation of the grains results in rot and sometimes mycotoxin production. Insect pest and subsequent mycotoxin contamination of human food and feeds has over the years been reported to have become a more important feature than direct yield losses that occur regularly.

Fusarium verticillioides is one of the most important fungal pathogens of maize and is associated with maize ear rot. Toxigenic strains of this species produce fumonisins which is toxic and carcinogenic to humans and causes leukoencephalomalacia, pulmonary oedema, infertility, diarrhea, vomiting, anorexia, leucopenia, immunosuppression, skin and gastrointestinal irritation and hemorrhaging in animals. Fumonisin producing fungi *F. verticillioides* is found prevalent in tropical and sub tropical regions.

Prevention methods shown to alleviate fusarium ear rot include crop rotation and tillage. *Bacillus thuringiensis* (*Bt*.) has been employed in vital pest control strategies as biopesticide, against insect pests and through genetic engineering of plants. It has been shown to reduce mycotoxins in the plant through enhanced crop resistance mechanisms, against insect pests in developed countries. In Africa, not much has been carried out on the application of *Bacillus thuringiensis* (*B. thuringiensis*) as a biocontrol agent, although this could lead to enhanced quality and safety of maize for both animal and human consumption (Romies *et al*., 2011).

Bacillus thuringiensis strains have been evaluated using biochemical methods but gaps include investigation of the effects of their toxins on organisms such as fungi and insect pests not known to be susceptible to these toxins. This project aims to isolate *F. verticillioides* from maize grains, employ molecular techniques in carrying out analysis of the fumonisin producing *F. verticillioides* strains and to evaluate the antagonistic effects of native *B. thuringiensis* strains delta toxins against maize insect pest *P. truncatus* and the fungi *F. verticillioides* isolated from maize grains. The native strains of *B. thuringiensis* isolates KG 411, KG 12-0, KG 20, (from mangroves), and KM 31and KM 24 (from Machakos) in soil samples previously isolated was classified and characterized in terms of toxicity against the maize insect pests and fumonisin producing fungi *F. verticillioides.*

1.2 Literature review

1.3 *Bacillus thuringiensis*

1.3.1 Use of microbial pathogens such as *Bacillus thuringiensis* as agricultural biocontrol

Bacillus thuringiensis was first discovered in the year 1901 by Ishiwata Shigetane (Aizawa, 2001). Discovery and study of insecticidal bacteria and fungicidal bacteria has been going on for more than a century. Biopesticides have been applied for over 60 years in forestry, agriculture and vector-borne disease control (Federici, 2005). The use of microbial pathogens for control of pests in agriculture, started as early as the late 19th century where microbes used include *B. thuringiensis* as an insecticide (Höfte and Whiteley, 1989) , viruses such as baculoviruses in transgenic plants (Bedford, 1980) and fungi such as *Trichoderma* (Harman, 2006) .

1.3.2 *Bacillus thuringiensis* Taxonomy

Bacillus thuringiensis is a member of the *Bacillaceae* family and belongs to the *Bacillus cereus* group, which contains *B.cereus, B. thuringiensis, B. anthracis, B. mycoides, B. pseudomycoides,* and *B. weihenstephanensis* (Rasko *et al.*, 2005). *Bacillus thuringiensis* is however different from the other members due to the fact that it produces large crystalline inclusions that contain protoxins. Structurally, *Bt*. forms spore and crystal protein inclusion that consists of δ- endotoxins. The cry proteins have 3 domains. The first domain is responsible for gut insertion and pore formation, the second domain helps in binding to the receptors on the target organisms gut epithelial lining and finally the third is responsible for preventing cleavage of the endotoxin by gut proteases.

1.3.3 *Bacillus thuringiensis* insecticidal properties

The use of *Bt*. as an insecticide started in Europe in 1920s and this was later adapted in America to control lepidopterans that destroyed maize plants (Lord, 2005) It is preferred due to the fact that it isn't harmful to the environment; it is produced naturally and has no adverse effects on other non target organisms (López-Meza *et al*., 1996). *Bacillus thuringiensis* have mostly been known to produce delta endotoxins(Sick,2007), though it's recently been found that they also produce other toxins known as vegetative insecticidal proteins (Vips) which are expressed during the *Bacillus thuringiensis* vegetative growth phase unlike that of the delta endotoxin produced during the sporulation phase (Schnepf *et al*., 1998; Estruch, *et al.*, 1996). Recently *Bt*. Cry toxins have been used in the creation of transgenic plants, able to resist susceptible insects. This has been carried out in plants such as cotton, maize and others, though their effects on the environment and other non-target organisms are still being investigated (Tian *et al*., 2012).

1.3.4 *Bacillus thuringiensis* target organisms

Insects in the order Lepidoptera, Diptera and Coleoptera have been found to be susceptible to *Bt.* toxins (Van Frankenhuyzen *et al*., 1993). Biological control system using an endophytic bacterium *Bacillus subtilis* has been developed, that shows great promise for reducing mycotoxin accumulation during the endophytic (vertical) growth phase of *Fusarium moniliforme* in maize.

This bacterium occupies the identical ecological niche within the plant, and is considered an ecological homologue to *F. moniliforme* and the inhibitory mechanism, regardless of mode of action, operates on the competitive exclusion principle. Novel *Bt.* toxins have also been found to be active against other organisms other than insects such as nematodes, protozoan's, flukes, collembolans mites, moths and worms among others (Lee *et al*., 1996; Thompson *et al.*, 1991; Thompson *et al.*, 2004; Iriarte and Caballero, 2001). Amino acid sequences of the toxins can be used to determine their target organisms (Narva *et al*., 2007; Thompson, *et al*., 2004).

Some species of *Bacillus* have been shown to offer protection against root pathogens by forming endospores and their broad spectra antibiotics. *B. subtilis* has been shown to have biological control properties against *F. verticillioides* at the root level (Cavaglieri *et al*., 2005). *Bacillus thuringiensis* subspecies *Kurstaki* controls various types of lepidopteran insects (Wie *et al*., 1982) and has been used in most commercial applications in creation of biopesticide. *Bacillus thuringiensis* subspecies *israelensis* has been shown to be effective against mosquitoes, blackflies' and some midges (Wie *et al*., 1982). *Bacillus thuringiensis* subspecies *tenebrionis* is used effectively against certain beetle species chrysomelids and the boll weevil (De Maagd *et al*., 2001). *Bacillus thuringiensis* subspecies *japonensis* has been shown (De Barjac and Frachon, 1990) to be effective against many species of scarabid beetles (Brownbridge, 1989). *Bacillus thuringiensis* subspecies *aizawai* is used against wax moth larvae in honeycombs.

1.3.5 Characterization of *Bacillus thuringiensis* toxins

The gram-positive bacterium *Bt.* has been largely characterized using its spore forming characters. Biochemical methods have also been used in the classification and characterization of this *Bacillus* species (Sneath, 1986). It forms protein endotoxins during its stationary phase that vary in appearance due to their difference in shape. These shapes vary from bi-pyramidal, square, rhomboid and circular. Chimeric crystal proteins have been developed over recent years in a bid to increase toxicity levels. This applies gene fusion and chimeric protein construction methods, which may allow alteration of two or more of the amino acids involved (Marroquin *et al*., 2000). There are over 70 serotypes and 92 subspecies described to date (Galán-Wong *et al*., 2006).

Phenotypic methods used include H-serotyping, used to classify *Bt* strains into serovarieties on the basis of flagellar antigens (De Barjac and Frachon, 1990). There are currently 143 unique Cry toxins (Piggot and Ellar, 2007). This large number has been credited on transposable elements and conjugative transfer leading to creation of new toxins (De Maagd *et al*., 2001). This large number has led to the ability to compare sequences and the elucidation of elements important for both basic toxin function and insect specificity. Sequence analysis methods are applied in this case.

Standard methods such as bioassays for quantifying crystal protein content and determining the potency of *B. thuringiensis* crystal protein preparations and the use of the standardized techniques that measure in International Toxic Units (ITU/mg) of protein are generally applied, for characterization of *Bt*. toxins, through evaluation of their effects (Dulmage *et al*., 1971). This in turn leads to increased discoveries on the effects of the proteins.

1.3.6 Crystal proteins

Bacillus thuringiensis toxins are produced from endotoxins produced by the microorganism. These produce crystals (cry) which are parasporal inclusion proteins that have toxic effect on target organisms and cytolitic (cyt) proteins, which are parasporal inclusion proteins that exhibits hemolytic activity (Tyrell, 1981). There are a very large number of varieties of Cry proteins subgroups to date. This is due to efforts aimed at the isolation and characterization of novel strains of *Bt.* and toxins with new targets for control in agriculture and other fields. This crystal protein subgroups include Cry I-IX proteins and their various subgroups Cry I A(a), A(b,) A(c), B,C,D,E,F,G (Knowles, 1994). They are all pyramidal and 130-138 kDa and are used against lepidopterans. Cry II with subgroups A, B, C are cuboidal 69-71 kDa and affect lepidoptera and dipteral. Cry III subgroups A, B, C are flat / irregular 73-74 kDa and affect Coleoptera. Cry IV are A, B, C, D bipyramydal, 73-134 kDa and are used against dipterans. Cry V-IX they vary in shape, are 35-129 kDa and have a large number of target organisms (Knowles, 1994).

Earlier classification methods of the *Bt.* cry toxins used their target organisms Höfte and Whiteley, 1989). This was later changed due to the fact that some cry toxins showed dual activity. The type and number of the different protoxins in the crystalline inclusions are presently both used in classification.

1.3.6.1 Exotoxins

Exotoxins are usually produced during sporulation of *Bt.* (Levinson *et al*., 1990). They are insoluble crystalline inclusions and include I- exotoxinor thuringiensis, P-exotoxin and β-exotoxin (De Maagd *et al*., 2001).

1.3.6.2 δ -endotoxins

Spore from the soil bacterium are produced during the stationary phase. The spores contain crystals known as delta endotoxins (δ-endotoxins) that contain cry and cyt protein (Aronson *et al*., 1986).

1.3.7 Mode of Action of *Bacillus thuringiensis* toxins

The mode of action of *Bt.* δ- endotoxin comprises of multiple steps (Knowles, 1994). The toxin kills the target organism through colloidal osmotic lysis. The crystal proteins are ingested by the target organisms on the plant in its protoxin inert form, and transformed into a cytotoxic form, this leads to the death of the insect. Most insects that have been shown to be susceptible to the *Bt*. toxins have an alkaline gut condition, which further explains why it is not toxic to other insects and human beings with acidic conditions in their guts. The alkali condition leads to the Solubilization of the protoxin produced by the *Bt*. in the insect. This leads to proteolysis which converts the protoxin into an active toxin in the gut of the target insect (Höfte and Whiteley, 1989). Activation enables the toxin to bind to receptors found on the gut lining of the insect. Subsequently the toxin conjugates itself (Lee *et al*., 1996) into the cell membrane. Pore or ion channels form, due to increase in the permeability within the receptors on the membrane (Lorence *et al*., 1995). Subsequent germination of the *Bt*. spores in the insect, followed by cell lysis then results due to the allowed free flux of ions and liquids (Höfte and Whiteley, 1989). This results in the weakening and or death of the insect. The primary mechanism by which *B. thuringiensis* kills insects is through combining the delta-endotoxin associated with crystals and potentially vegetative growth and causing septicemia in the gut of the larvae.

1.3.8 Advantages of *Bacillus thuringiensis* toxins

Different strains of *Bt.* have been isolated from different environments and their toxins have been found to have effect on a small group of organisms (De Barjac and Frachon, 1990). *Bacillus thuringiensis* has become vital in the pest management strategy, as an insecticide, biopesticide, fungicide and also in transgenic plants. Many spore-crystal formulations have been developed from *B. thuringiensis* (Ninfa, 2009). This produces a suitable alternative to synthetic chemical insecticides because it is not harmful to the environment and it has negligible effect on non-target organisms (Sanahuj, 2011). It's stable over a long time during storage, and the *Bt.* crystals can be used together with other chemicals. It is safe to apply, and can be applied during any stage of the plant (Schnepf *et al.*, 1998).

1.3.9 Disadvantages of *Bacillus thuringiensis* toxins

Bacillus thuringiensis formulations, are highly specific, and thus act on a very narrow range of hosts at a time, it takes longer to kill pests, which might be perceived as it being less effective, it only acts after ingestion, which might be a factor contributing to its effectiveness and host range. It is easily destroyed by sunlight when topically applied.

1.3.10 *Bacillus thuringiensis* isolated in Kenya

Bacillus thuringiensis samples in Kenya have been isolated from a variety of regions and different sources of materials including, soil, dead insects etc. The regions documented in literature include Central Kenya (Brownbridge, 1989), the Rift Valley region, Western and Eastern provinces (Wang'ondu, 2001) and the coastal regions. This has been done to find varieties that are active against local pests affecting the region, but none has as yet, been found potent enough to be applied as an insecticide.

1.4 *Prostephanus truncatus*

This insect has been shown to be one of the causes of increase in mycotoxin production according to studies carried out in relation to their prevalence in tropical and sub-tropical areas. Adverse weather conditions, pests and diseases are factors contributing to low yield of maize (Mugo *et al.*, 2002). Insects have been shown to be the main course of maize related losses with *P. truncatus* being a principal cause of grain losses (Gwinner *et al.*, 1996). Studies by several scientists show that *Fusarium* is the main mould associated with rotting maize after its contamination by this insect pests in the field (Samson, 1991; Ominski *et al.*, 1994; Munkvold and Desjardins, 1997; Desjardins *et al.*, 2000; Orsi *et al.*, 2000; El-Shabrawy, 2001).

1.4.1 History of *Prostephanus truncatus*

Prostephanus truncatus is a dead wood and maize pest originally found in the Central part of the American continent and spread to other parts of the world such as Asia and Africa. The Larger Grain Borer *P. truncatus* (Horn) (Coleoptera: *Bostrichidae*) was accidentally introduced and has subsequently established itself in 16 countries in tropical Africa, and is said to have increased average farm-stored maize losses from 5% prior to its introduction, to 10% (Borgemeister *et al.*, 2003). In Africa, it was documented to have first been sighted in 1981 in Tanzania and to have later spread to other regions of the continent. It also affects cassava in African countries. Lack of a natural predator to offer significant control has allowed its populations to expand rapidly in the eastern and western region of Africa. It is both a pre and post harvest pest. The beetle thrives in conditions that are hot and humid especially the tropical areas. It spread into southern Kenya in 1983 from Tanzania where it was first introduced and became a pest of stored maize in coastal districts and in semi-arid areas inland (Hodges *et al.*, 1996). The pest spread slowly north during the 1980s. Strict regulations were imposed upon the movement of maize within Kenya in an effort to prevent this pest from spreading to the main maize-growing areas in the west of the country around the shores of Lake Victoria (Hodges *et al.*, 1996).

1.4.2 Classification of *Prostephanus truncatus*

It belongs to the family *Bostrichidae* most of which are wood borers. It is bout 3-4mm long, cylindrical and dark brown in color. It has been found to be similar to other beetles such as the Lesser Grain Borer *Rhyzopertha dominica*, and *Dinoderus spp* of the same *Bostrichidae* family (Farrell *et al*., 2002). It is distinguished from other Bostrichids by the presence of a square cut end. It's Name and Synonyms were first described by Horn (1878) as *Dinoderus truncatus* Horn *Stephanopachys truncatus Prostephanus truncatus* Larger grain borer Greater grain borer Taxonomic Description Horn, (Hodges, 1994).

1.4.3 Morphological characteristics and habitat of *Prostephanus truncatus*

Identification of characteristics of this insect enables effective management. Its wing ends are flattened and thus it creates an illusion of a sloping region. This sets it apart from other *Bostrichids* such as the Lesser Grain Borer, *Rhyzopertha dominica* and *Dinoderus spp*. It has ridges which presents a square appearance at the back. It is 3-4mm long, cylindrical and dark brown in color. Typical of grain feeding insects, it lays eggs.

The life span of *P. truncatus* is several months; a single adult *P. truncatus* can cause damage to an equivalent of five corn kernels (Demianyk and Sinha, 1988). It has been shown (Shires, 1979; Hodges, 1986) that optimal *P. truncatus* growth occurs at 32°C and 80% relative humidity while Haubruge and Gaspar in1990 showed that *P. truncatus* survived best at temperatures around 30°C, with relative humidity's of above 60%. Bostrichids are generally found to inhabit dead wood (Beiriger, *et al.* 1996). *Prostephanus truncatus* is considered a wood-boring species that has become adapted to stored commodities. Tubers and roots probably serve as the natural host. Nang'ayo *et al*. (2002) demonstrated that *P. truncatus* bred successfully on 27 species of woody plant.

1.4.4 *Prostephanus truncatus* host plants

The plant shown to be a main host to *P. truncatus* that has been recorded in literature is maize, *Zea mays* (Hodges, 1986). Harder, Maize grain varieties have been shown to be affected less in comparison to other varieties (Shires, 1979). In Africa, it has been shown that maize stored on cobs are, more affected than those that are shelled. Other plants affected by this pest include cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*), Teak seeds (*Tectona grandis*) and Jowar seeds. They have also been found in trees of families *Anacardiaceae* and *Burseraceae* according to literature (Borgemeister, 1997a; Fisher, 1950).

1.4.5 Symptom Description of *Prostephanus truncatus*

Infestations begin prior to harvest, when the moisture content of maturing maize may still exceed 40% and this continues during storage, especially in unshelled maize (Meikle, 1996). Respiratory and feeding processes by the insect contribute to accumulation of frass and thus agitate the situation by creating an environment suitable for fungal infestation and subsequent mycotoxin production (Demianyk and Sinha, 1987). *Prostephanus truncatus* causes damage and weight loss, in softer varieties of maize (Demianyk and Sinha, 1987). Tunnels, frass production and subsequent reduction of the weight of the maize grains are symptoms of *P. truncatus* presence in maize (Kingsolver, 1971).

1.4.6 Statistics on *Prostephanus truncatus* prevalence in the world

Insect pests affect 46% of the global maize growing area (Likhayo *et al*., 2002) and cause 10% world loss annually. The larger Grain Borer threatens maize which is an integral crop, an estimated loss of 15 million U.S. dollars is recorded yearly (Pierce and Schmidt, 1992). An enormous damage of 40% and 70% weight loss had been reported in maize and cassava, respectively (Mugo and Gichuki, 2009) due to this pest. This has also been documented in countries like Togo (Pantenius, 1988). Sixty percent of the losses incurred by this pest are in the tropics, this has led to use of hundreds of millions of worth of insecticides annually. According to Keil (1988), dry weight losses of 17.4 % and 41.2% have been reported as a result of this pest in Tanzania after 6 months storage.

1.4.7 Control measures applied against *Prostephanus truncatus*

Spaces alongside the lines of maize on cob, enables the insect to attach to the maize cobs, furrowing tunnels and thus infesting the grains as a result (Hodges, 1986; Bell and Watters, 1982).

High losses of stored maize resulting from *P.truncatus* attack (Dunstan & Magazini, 1981; Harnisch and Krall, 1984; Farrell *et al.,* 1996b) triggered a number of emergency control programmes such as shelling of maize from cobs, treatment using organophosphate/synthetic pyrethroid dust before storage which has adverse effects on the environment (Golob, 1988). The beetle *Teretriosoma nigrescens* has been used as control for *P. truncatus.* Despite release into areas with high prevalence of *P. truncatus*, presence of damage as a result of *P. truncatus* infestation was present (Meikle *et al*., 2002). This method was applied in Togo in 1991 (Biliwa *et al.*, 1992) and Kenya in May 1992 but results on this proved inconclusive. In Togo sharp decline of *P. truncatus* was observed and some decline was observed in Kenya (Nang'ayo, 1996).

1.5 *Fusarium*

Fusarium is filamentous fungus found commonly on soil and plants. It belongs to the Subkingdom-Dikarya, Phylum-Ascomycota, Subphylum-Pezizomycotina, Class-Sordariomycetes, Order-Hypocreales, Family-Nectriaceae. *Fusarium* is both a plant pathogen and a food contaminant, and is ranked 3rd after *Candida* and *Aspergillus* in causing systemic infections in transplant patients (Morrison *et al*, 1993). This has also led to concerns involving food safety and impacted the grading of grains in the world market. Studies show *Fusarium* to be the most important field fungi of maize (Cahagnier, 1995), within this genus, there are a variety of species that produce mycotoxins. They cause diseases like wilts and ear rot in plants. In Kenya *Fusarium* has been documented as an ardent attacker of the wheat crop and the kernels of maize, resulting in headblight in areas like Nakuru district (Muthomi *et al.,* 2002). This contributed further to contamination with the mycotoxin deoxynivalenol, DON. In Kenya's Taita district *Fusarium* causes the root rot of both maize varieties at all stages of growth (Okoth and Siamento, 2011).

1.5.1 *Fusarium verticillioides*

Fusarium verticillioides (Sacc.) Nirenberg was previously known as *Fusarium moniliforme* Sheldon. *Fusarium verticillioides* is found under section Liseola. It has been estimated to be the most common fungus infecting grains of maize worldwide (Charles *et al*., 2001; FAO, 2002). Characteristics of *F. verticillioides* include its production of microconidia in long chains, and their purplish mycelial color on (Potato Dextrose Agar) PDA media when stored in the dark for a period of 7-10 days. The major mycotoxins produced by this species are estrogenic fusariotoxins such as trichothecens, zearalenone, and fumonisins widely produced by both *F. proliferatum* and *F. verticillioides*. This toxin is associated with high oesophageal cancer levels in humans, through cereals like corn and corn based products. Fumonisins are divided into 15 different toxin groups which mainly include Fumonisin B1 (FB1), Fumonisin B2 (FB2) and Fumonisin B3 (FB3). Some *Fusarium* species account for more than 95% of human infections and this includes *Fusarium verticillioides* (Guarro and Gené, 1992; Mohamed, 2009).

In the World areas associated with the spread of cancer through FB1 contamination includes China, (Groves, 1999; Ueno *et al*., 1997; Yoshizawa *et al*., 1994). High cancers prevalence has been shown to be connected to FB1 production in areas like the western and eastern region of Kenya. *Fusarium verticillioides* causes ear rot and cob-rot, which gradually translates into the production of fumonisins during the pathogenic state in maize. Secondary metabolites produced by *Fusarium* include fumonisins. Fumonisins produced by *F. verticillioides* have been shown to have adverse effects on the immune system. Fumonisin B1 is produced by both maize contaminants *F. verticillioides* and *F. proliferatum.* Some studies show that *F. napiforme, F. anthophilum, F. dlamini* and *F. nygamai* are able to produce FB1 (Environmental Health Criteria (EHC), 2000 and National Toxicology Program (NTP) 1999).

1.5.2 History of *Fusarium verticillioides*, Discovery and Classification

There are over 100 species of *Fusarium* described; there is however a discrepancy since different researchers use different methods for classification. The *Fusarium* taxonomy was first outlined by Link in1809 (Snyder & Toussoun, 1965). There are a variety of other classification methods (Wollenweber (1913); Snyder and Hansen (1940); Nelson *et.al*., 1983).

Gerlach and Nirenberg in 1982 introduced 78 species in the genus by using their differences, and not similarities. Other scientists have over the years come up with other methods of classification, though this has led to problems in agreeing on one system of classification of *Fusarium* internationally. *F. verticillioides* is found in section Liseola. It is a genus of hyphomycetes (Nelson *et al*., 1983).

1.5.3 *Fusarium verticillioides* Distribution

Fusarium verticillioides has been documented around the world in different geographic areas and also in soil, air and plants (Booth, 1971; Nelson *et al*., 1983). Most are in the tropical regions, and a few others in the colder regions (Burgess *et al*., 1994). It has been documented in plants ranging from corn, bananas, legumes, wheat, paprika, rice and tomatoes (Szczech, 1999) in different continents around the world (Burgess *et al*., 1991).

1.5.4 *Fusarium verticillioides* Habitat

Infection of maize with *Fusarium* and subsequent Fumonisin production is influenced by a variety of factors, namely; environmental, agricultural post and pre harvest practices, the variety of the maize and biotic factors such as storage insects and fungal interactions. Most are found in soil, and produce dormant structures known as chlamydospores to enable this. They are found prevalent in tropical and sub-tropical areas, where the weather conditions prevailing are moist, humid and warm.

1.5.5 Symptoms of *Fusarium verticillioides* in plants

It causes ear rot, head blight in wheat and maize varieties (Muthomi *et al*., 2002). It contributes to the yellowing of the bean leaves, stunting of the plant, rotting of the roots and eventually leads to death if adverse (Okoth and Siamento, 2011).

1.5.6 Mycotoxins produced by *Fusarium*

Mycotoxins include fumonisins produced by *Fusarium* spp. and others produced by *Aspergillus* species (Marasas, 2001; Hendricks, 1999; Wang *et al*., 2003). Control and testing regulations have been put in place by international organizations such as FAO, and analytical tests should also be carried out to check for the presence of mycotoxins in both the processed and unprocessed foods. Though mycotoxins are mostly known to affect the liver, it also can affect other systems of the body, though this appears different in each animal. Mycotoxins are formed both in the field on the plant and in the stores. Availability of moist, humid and hot weather promotes the production of fumonisins. Mycotoxins produced by *Fusarium* are important since they cause contamination in food and feeds. The different mycotoxins are grouped in classes such as Deoxynivalenol (DON), which has been associated with *F. graminearum* and *F. subglutinans* and leads to feed refusal, reduced weight gain, diarrhea, and vomiting in farm animals. Trichothecene (T-2 toxin, HT-2 toxin) is the other class, produced by the *Fusarium* spp. *F. graminearum*, *F. culmorum* and *F. Poae*. This mycotoxin shows in contaminated foods and causes ailments such as alimentary toxic aleukia, necrosis, hemorrhages and oral lesion in broiler Chickens (Nigerian Stored Products Research Institute (NSPRI), 2010).

Fumonisins were first described by both Gelderblom *et al*., 1992; Bezuidenhout *et al*., 1988). Some fumonisins appear as polar mycotoxins (NTP, 1999). They are produced in *F. verticillioides and F. proliferatum* and in smaller quantities in other *Fusarium* species which produce FB1 (Nigerian Stored Products Research Institute (NSPRI) 2010; EHC, 2000; NTP, 1999; Marasas *et al*., 2001; Rheeder *et al* 2002). They are secondary metabolites and are prevalent in cereals especially maize plants. Eight are known to date, A1 and A2 and B1, B2, B3, and B4 (Pittet, *et al*., 1992; Fandohan *et al*., 2003).

1.5.7 Effects of fumonisins on other organisms

The Food and Agricultural Organization estimates that 25-50% of grains are contaminated annually worldwide by *Fusarium* (FAO, 2002). This leads to reduced nutritional value, discoloration of the seed and mycotoxin production (Dutton,1985). Fumonisins cause diseases such as Equine leukoencephalomalacia and porcine pulmonary edema. *Fusarium oxysporum* has been claimed to produce Fumonisin C1 and C2 (Larkin *et al*., 1993). They are suspected to be carcinogenic and this has been documented in Asia and Africa (East and South) (Dutton, 1966). According to (Marasas *et al*., 1966) FB1 accounts for 70-80% of total fumonisins produced, and FB2 15-20%.

1.5.8 Methods used in identification of *Fusarium* species

Sequenced based methods are currently being applied for the identification of *Fusarium* species (Balajee, 2009). Molecular methods have been applied to help identify specific differences in fungal varieties. This is due to the fact that numerous studies demonstrate the inadequacies involved in only applying morphological procedures in carrying out species identification (Hennequin *et al*., 1997; Morrison *et al.*, 1993). Comparative sequence identification is the new molecular "gold standard" (Balajee, 2009) for identification of fungi. It involves carrying out PCR on a selected region of the genomic DNA in question and then subsequently carrying out sequencing, and blasting this sequence against a database.

Conventional methods of identification include colony description on suitable media and microscopically identifying the conidia and conidiophores. Primary characteristics involve morphological characterization, of their spores which include microconidia, macroconidia and chlamydospores (Nelson *et al*., 1983). The macroconidia appear crescent /fusiform and septate. Secondary characteristics include pigmentation, growth rates and secondary metabolites produced.

1.5.9 Fumonisin infection/ contamination in plants and animals

Fusarium species are capable of producing a variety of fungal toxins such as zearalenols, trichothecene, fumonisins, fusarins, and moniliformin, which threaten the health of humans and animals that consume them. Fumonisins B1and B2 have been reported in black oats feed in Brazil (Sydenham *et al*., 1992) and forage grass in New Zealand (Mirocha, 1992). In rural areas of South Africa, FB1 and FB2 have been found in subsistence crops locally consumed (Rheeder, 1992). It has been found in corn in the US and in Brazil where it's been shown to cause porcine pulmonary edema (PPE) (Sydenham *et al*., 1992; Ross *et al*., 1991). Low levels of FB1 have also been reported in cornflakes breakfast cereals in Switzerland, the USA and South Africa (Pittet *et al*., 1992; Sydenham *et al*., 1991). Fumonisins B1, B2 have been reported to be present in animal feed, and fecal samples and also in trace amounts in the milk from goats and cows in South Africa (Rheeder, 1992). *Fusarium* species cause plant diseases, like vascular wilts, root, stalk and cob rots, collar rot of seedlings, and rots of tubers, bulbs and corms (Agarry, 2005). Some pathogenic species produce mycotoxins that contaminate grain.

1.5.10 Advantages of *Fusarium* species

Fusarium oxysporum is a species of *Fusarium* that has been reported to increase plant growth rates (Pung *et al.,* 1992) and to suppress plant disease (Larkin *et al*., 1993). It has also been shown to produce cutinase, (Tatiana *et al.*, 2008) which is a versatile enzyme showing several interesting properties for application in industrial processes.

Fusarium verticillioides has been shown to be an effective entomopathogen against grasshoppers (Pelliza *et al*., 2010). It has also been shown to prevent opportunistic saprotrophs such as *Aspergillus* from spreading in maize kernels, and it may contain much lesser amount of toxin than fungi such as *Aspergillus* in such instances.

1.5.11 Disadvantages of *Fusarium* species

Grain and feed quality is a topic of extreme importance to animal producers. Mould growth on feedstuff not only leads to an altered nutrient profile, it can also lead to mycotoxin formation. United Nations experts at the Food and Agriculture organization (FAO) estimate over 25% of the world's foodstuff having been lost as a result of mycotoxin contamination, with the *Fusarium* species contributing substantially to food contamination (Chelkowski, 1998). They cause diseases and economic losses. Most common described pathogen *F. solani* and *F. oxysporum*; others have also been described to cause infection in humans (Alastruey-Izquierdo *et al*., 2008). They are highly resistant to anti fungal treatments both in plants and in animals.

1.5.12 Prevalence of *Fusarium verticillioides* in Kenya and the world

In Kenya *F. verticillioides* has been documented in the western, central, eastern and coastal parts of the country to a large extent (Muthomi *et al*., 2002; Okoth and Siamento, 2011). Studies show that in Africa, *F. verticillioides* is the most prevalent fungus of maize (Marasas *et al*., 1988; Allah Fadl, 1998; Baba-Moussa, 1998; Kedera *et al*., 1999). Fumonisins have been documented to be prevalent all around the world, both in the developed and developing countries (Dunstan and Magazini, 1981; Desjardins, 2000; Bacon, 2000).

1.5.13 Methods of eradication of *Fusarium verticillioides*

Elimination of fungi using most conventional methods proves difficult due to the fact that fungi like *F. verticillioides* are a systemic. Applications of systemic fungicides are impossible during later stages of plant growth, and because the fungus is a systemic seed-borne infection, conventional fungicides as seed treatments are also ineffective. Prevention methods that can be used to alleviate the fumonisins contamination at the field level are crop rotation and tillage, recommended to control plant contamination with *Fusarium* spp. (Sutton, 1982). Fertilizers also can be used because it can alter the rate of residue decomposition, act on the rate of plant growth and change the soil structure and its microbial activity. This will affect *Fusarium* spp. contamination of crops.

Few contamination symptoms were observed when urea was used instead of ammonium nitrate (Martin *et al*., 1991). *Bacillus subtilis* and *B.cereus* have also been used as against *F. solani* (El-Hamshary *et al*.,2008). Genetic engineering (Duvik, 2001), can be used to reduce mycotoxins in the plant by enhanced insect resistance procedures e.g. transgenic *Bt*. Crops or induction of a detoxification pathway that inhibit the production of mycotoxin in the grain and increase the resistance of the plant to infection.

Harvest and post harvest control of fumonisins include carrying out procedures like , controlling the humidity levels before and after storage, Physical treatment of contaminated grains**,** and cleaning the external surface of grains and eliminating insects, and physically insect damaged kernels can minimize or avoid spore containing material and reduce the infection of healthy grains by contaminated ones (Balzer, 2004). Biological treatments have also been employed whereby enzymes from a filamentous saprophytic fungus growing on maize that is capable of degrading fumonisins have been isolated and the corresponding genes cloned and transferred in transgenic maize (Duvik, 2001).

Most studies which have been carried out in Europe show that transgenic *Bt*. maize consistently decreases the level of fumonisin both directly and indirectly, through doing away with both insect pests and fungi (Munkvold *et al*., 1999). The effect of *Bt*. maize on the prevalence of mycotoxins in stored maize in Africa is currently being studied, but not much has been carried out on the application of biocontrol agents in Kenya and its ability to eradicate both pests in maize. It appears this could enhance the quality and safety of maize for both animal and human consumption (Romies *et al*., 2011).

Recent advances in agricultural biotechnology highlight the need for sound scientific judgment on the use of biopesticide and application of other technologies, their acceptance, risks and benefits to society. Environmental benefits have been noted on use of biopesticide, and beyond this consumers stand to benefit by development of food crops with increased nutritional value or that whose value is maintained and not affected by presence of antinutrients and toxic factors.

Biopesticide use thus provides a powerful tool to enhance the production of plants with higher potential value to society. One of the most publicized applications of biotechnology in agriculture is the modification of plants to express proteins produced by the common soil bacterium, *B. thuringiensis (Bt.*). Organic farmers have been using *Bt*. as an insecticidal spray for over 40years. *Bacillus thuringiensis* expresses a class of insecticidal proteins against certain insects but harmless to humans, mammals and birds.

Using transformed fungi, it's also been demonstrated that during the life cycle of the fungus within the host tissue the endophytic hyphae of the fungus responds to abiotic stress imposed on the plant e.g. drought and flooding and under this two stresses, synthesis of the fumonisins increases. Plants maintained under uniform moisture produces very little fumonisins.

In previous studies, use of gamma irradiation treatment was shown not to be an effective method of eliminating fumonisins (Sreenivasa *et al*, 2009). Preservatives such as camphor , volatile compounds formic acid, and propionic acid , Aniline, benzaldehyde and formaldehyde can also suppress fumonisin B1 (Narasimha *et. al*., 2009). Tissue Culture techniques have been applied for bananas and plantains in East Africa as a method to increase productivity of bananas constrained by a range of pests including the fungus *Fusarium* spp. and bacteria (Wambugu, 2001).

Control of mycotoxin production can be employed by traditional management of agricultural practices both pre and post harvest (Julian, 1995). These control strategies for curbing production of mycotoxins are being investigated and include elimination of the residue on the field soil through deep tillage, irrigation during drought stress, breeding for pathogen and insect- resistance, and genetic engineering. Others include control using iodine based products (Yates *et al*., 2000) endophytic bacterium (e.g. *Bacillus mojavensis*) as a biological control agent on maize seed (Bacon and Hinton, 2000). Using non-producing strains of *F. verticillioides* aiming to minimise fumonisin levels in maize has also been employed (Plattner *et al*., 2000).

Okoth and Siamento in 2011 documented the use of *Trichoderma* spp. to suppress the occurance of *Fusarium* when intercropping maize and beans, this and the use of inorganic fertilizers and cow manure were applied as a means of managing the fertility of the soil in order to assess for alternatives to fungicides. The results obtained showed positive results, especially when the inorganic fertilizers were used. *Saccharomyces cerevisiae* has also been used as a biocontrol against *Fusarium* moulds, and the level of production of FB1 (Mohamed, 2011).

Transgenic maize varieties which have *Bt.* engineered into them have been shown to have decreased *Fusarium verticillioides* effects on the maize grains and to have reduced fumonisin concentration. Studies have been carried out to determine whether *Bt.* toxins can be used effectively to counter effects of *F. verticillioides* (Kheseli *et al*., 2011) and *P.truncatus* (Likhayo *et al*., 2002). The results obtained indicated some success in the control of the fungi, and very little success in controlling *P.truncatus*.

1.6 Problem Statement

Challenges to food security attainability in Africa, are contributed to by factors that lead to significant food crop loss. Poor handling and storage further increase the post harvest losses. *P. truncatus* (Horn) (Coleoptera: Bostrichidae) is a main storage insect pests in Kenya which infest maize crop in the field just before harvesting and in storage, causing weight loss of up to 30% (Farrell and Schulten, 2002; Gueye *et al*., 2008). Insect attacks that bore into the ear of the produce, produces an ideal breeding ground for fungi. Fungi are important as they rank 2nd as the cause of deterioration both pre and post harvest (Ominski *et al*., 1994). FAO estimates that 25% to 50% of the world's food crops are contaminated by mycotoxins yearly (Mannon and Johnson, 1985). Mycotoxin production might subsequently result due to rotting of the corn ears. Damage to stored maize by this insect pest is a daunting challenge and requires the use of novel technology. Elimination of fungi using most conventional methods proves difficult due to the fact that fungi like *F. verticillioides* are systemic. Thus there is a need to come up with a biocontrol method suitable to eradicate these pests effectively.

1.7 Justification of the study

Pre and Postharvest maize grain loss due to pests is substantially high in developing countries due to inadequate control measures against the pests. This is aggravated by the climate conditions that are naturally suitable for the prevalence of these pests. One main storage insect pest in Kenya is the larger grain borer, *P. truncatus* which aids in propagating the spread of fumonisin producing *F. verticillioides*. It has been reported by Kassou and Aho (1993), that under favorable conditions, 50% to 80 % of damage on farmers' maize during the storage period is caused by fungi. In Kenya, adverse effects have been documented, leading to loss of lives and food insecurity. Fungicides and insecticides currently present are not able to effectively eradicate these pests, leading to lower grading of crops, and diseases in man and animals. *Bacillus thuringiensis* has become vital in the pest management strategy, as an insecticide, biopesticide, fungicide and also in transgenic plants. This calls for effective local solutions, tailored to meet the requirements of the local farmer, and to consider the safety of the consumer.

1.8 Objectives

1.8.1 Broad objective

To investigate the effects of native *Bacillus thuringiensis* delta endotoxin on *Fusarium verticillioides* and *Prostephanus truncatus* affecting maize grains.

1.8.2 Specific objectives

1. To isolate *Fusarium verticillioides* strains from maize.

2. Identify toxigenic strains of *Fusarium verticillioides* isolates using molecular methods.

3. To carry out bioassays of *Bacillus thuringiensis* delta toxins against *Fusarium verticillioides* and *Prostephanus truncatus*.

1.9 Hypothesis

Native *Bacillus thuringiensis* strains prevent infestation of maize kernels by *Prostephanus truncatus* and reduce the growth of *Fusarium verticillioides* and fumonisin production on maize kernels.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Source of *Bacillus thuringiensis* samples

The native *Bt.* Strains (Kenya Machakos and Kenya Gazi) KM 24, KG 20, KG12-0, KG 411, KM 31 and standard *Bt.* strains, *Bt.* var *tenebrionis*, var *israelensis* and var *kurstaki* samples used were obtained from the biotechnology and bioinformatics lab at CEBIB-UON and maintained in culture at -20^{0}C. They had originally been isolated from the Eastern and Coastal provinces of Kenya, and archived for 3years.

2.2 Rearing and maintenance of *Prostephanus truncatus* in the laboratory

Adult *P. truncatus* (Plate 1A; Plate 1B) were obtained from KARI-NARL and maintained using autoclaved maize grains (Plate 1C) at temperatures of 27±2^{0}C and relative humidity levels 65±5% humidity.

2.3 Source of *Fusarium verticillioides* and maintenance in the laboratory

Fusarium verticillioides used was isolated from maize grains (Plate 2D) obtained from Makueni District in Eastern province. The isolates were maintained in culture at -20^{0}C.

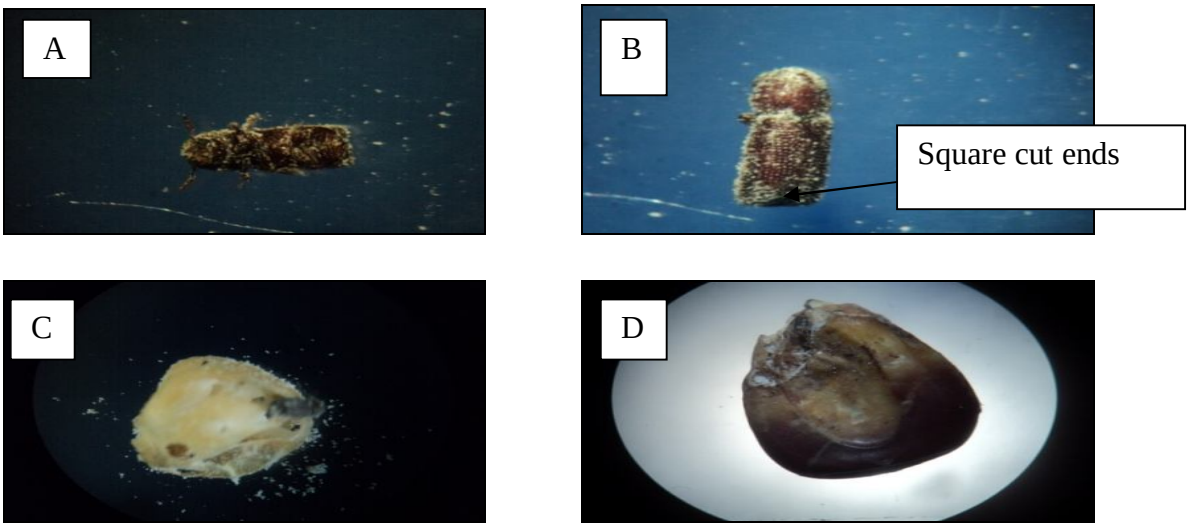


Plate 1: Maize pests **A:** Adult *Prostephanus truncatus* ;**B:** Underside of the *Bostrichidae* insect; **C:** Maize grains infested and damaged by *Prostephanus truncatus*; **D:** Fungal mould.

2.4 Media Preparation

2.4.1 Media for growth, and maintenance of *Bacillus thuringiensis*

2.4.1.2 Nutrient Agar Plates

The standard method described by Poinars and Thomas (1978), together with manufacturer's instructions was used to prepare the media. Nutrient Agar is composed of: Meat extract, peptone, Yeast extract, Sodium Chloride and Agar; pH7.1 (±0.2); Twenty eight grams was weighed and suspended in 1000ml of distilled water. The suspension was boiled while stirring until completely dissolved. The mixture was then autoclaved at 121^0C and 1 Bar (pressure) for 15 minutes. The agar was left to cool to 45-50^0C, mixed well and 20ml poured into sterilized petri dishes under aseptic condition. It was then left to cool and stored in a refrigerator at 4^0C. This media was used for the growth of *Bt*. cultures for use in the bioassays

2.4.1.3 Nutrient Broth

Nutrient broth composed of tryptone, meat extract and sodium chloride was prepared. Thirteen grams of nutrient broth composed of 'Lab- Lemco' powder and yeast extract was added to one litre of distilled water in a conical flask, and at pH6.8 (±2) or adjusted to neutral. The media was then mixed thoroughly and distributed into 250ml conical flasks, covered with cotton wool and aluminum foil and autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was then allowed to cool for preservation at 4^0C to be used in the culturing and preservation of the obtained *B. thuringiensis* isolates. This media was used for maintenance of *Bt.* cultures for the bioassays.

2.4.2 Media for isolation, growth, characterization, and maintenance of *Fusarium* species

2.4.2.1 Media for isolating *Fusarium*

2.4.2.2 Peptone PCNB Agar (PPA/ Nash-Snyder Medium)

This media is composed of sugar-free basal medium, supplemented by antibiotics and fungicides. The basal media was made up of Peptone15g, Potassium phosphate1g, magnesium sulphate0.5g, Pentachloronitrobenzene (PCNB) 750mg and agar 20g which was added to 1L distilled water to prepare this agar. The pH was adjusted to 5.5-6.6. This was mixed well and covered using cotton wool and foil and autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was left to cool to 45-50^0C, under aseptic condition, 1g of streptomycin sulfate and 0.12g of neomycin sulfate were then added and mixed till they dissolved in the media 20ml of the media was then poured into sterilized petri dishes. This media allows for only growth of *Fusarium* species, as it inhibits the growth of most other Fungi and bacteria.

2.4.3 Media for growing and identifying *Fusarium*

2.4 3.1 Potato Dextrose Agar (PDA)

PDA is a carbohydrate rich media composed of agar, and dextrose, and white potato broth. This was prepared as per manufacturer's instructions, and as described by Nelson *et al*., (1983). 37grams of PDA was added to 1000ml of distilled water this was mixed well and covered using cotton wool and foil and autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was left to cool to 45-50^0C, and 20ml poured into sterilized petri dishes under aseptic condition. This media was used for observing colony morphology, pigmentation and growth rates of *Fusarium* cultures, which are secondary criteria for identification of *Fusarium* species. The color of the mycelia of the colony and its pigmentation was classified using a color chart (Anon, 1969).

2.4.3.2 Carnation Leaf Agar (CLA)

This media was prepared as instructed by Nelson *et al*., (1983). One litre of this media was composed of 20 grams of pure agar, added to one litre of distilled water in a conical flask. This was mixed well and covered using cotton wool and foil and autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was left to cool to 45-50^0C, and 20ml poured into sterilized petri dishes under aseptic condition. A few sterile pieces (5-6 per plate) of carnation leaf 3-5mm^2 were placed on the surface of the 2% water agar as it was solidifying on petri dishes. This media was used to enhance sporulation and for production of macroconidia that are uniform in size and form.

2.4.3.3 Synthetic Nutrient Agar (SNA)

This media was prepared as instructed by Gerlach and Nirenberg (1982). One litre of the media constituted of Potassium phosphate 1g, Potassium nitrate1g, Magnesium sulphate, and Potassium chloride at 0.5g each, Glucose 0.2g, Saccharose 0.2g and Agar 20g. All the constituents were put in a conical flask, and one litre of water was added. This was mixed and covered using cotton wool and foil and autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was left to cool to 45-50^0C, mixed well and 20ml poured into sterilized petri dishes under aseptic condition. It was then left to cool. Pieces of sterile filter paper (5-6 pieces of 1cm square each) were added on the surface of the agar after it had solidified. The role of this media was for identification and maintenance of *Fusarium* strains it enhances sporulation for identification of the fungal species, and subsequent examination of microconidia.

2.4.3.4 Potato Dextrose Broth (PDB)

This media was composed of dextrose and broth from white Potatoes (Nelson *et al*., 1983). ½ PDB was made using, 26 grams of PDB consisting of dextrose and potatoes broth was added to 1000ml of distilled water. This was mixed well and covered using cotton wool and foil and autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was left to cool to 45-50^0C, and 20ml poured into sterilized 250ml conical flasks under aseptic condition. This media was used for obtaining mycelia for use during extraction of DNA from the *Fusarium* species for molecular identification.

2.4.4 Supplementary Identification media for *Fusarium*

2.4.4.1 Water Agar (WA)

Pure Agar media was used to create solidified media for fungal growth, by adding 15grams of the agar to one litre of distilled water in a conical flask. This made 1.5% water agar media. The conical flask was covered using cotton wool and foil, and then autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was left to cool to 45-50^0C, mixed well and 20ml poured into sterilized petri dishes under aseptic condition. It was then left to cool and stored in a refrigerator at 4^0C to be used in subculturing for single development of pure *Fusarium* cultures. This is because, hyphal growth is sparse on this medium, and thus individual hyphal tips can be isolated, to initiate new colonies.

2.4.4.2 Potassium chloride (KCl) Agar

4-8grams per litre KCl was added to 1.5 % WA prepared by adding 15grams of pure agar to one litre of distilled water in a conical flask. The conical flask was covered using cotton wool and foil, and then autoclaved at 121^0C, 15 lb pressure for 15 minutes. It was left to cool to 45-50^0C, mixed well and 20ml poured into sterilized petri dishes under aseptic condition. It was then left to cool and stored in a refrigerator at 4^0C. This media was used to enhance the *Fusarium* cultures production of more and longer conidial chains, easier to observe and with fewer droplets in the aerial mycelia (Leslie and Summerell, 2006).

2.5 Isolation of *Fusarium* species from maize grains.

Direct plating method was used to isolate *Fusarium* species from 50 maize grains six times. Five grains were used per plate, in 10 replicates. This was carried out 6 times. The maize grains used for isolation were observed for any signs of contamination and the results of this observation were recorded. The procedure was carried out under a sterile environment. Five contaminated grains per plate were surface sterilized using 2.5% sodium hypochlorite (NaOCL) for one minute and then rinsed three times in sterile distilled water before placing them on PPA medium. A separate petri dish was used for each sample in each case, prepared in replicates, and incubated at 25-26ºC. A total of 300 maize grains were used for the isolation.

Observations were made daily, for up to ten days, for mycelial growth and the number of infected grains and the characteristics of the colonies growing were recorded. Isolation was carried out six different times, to obtain more isolates.

Fusarium isolates were obtained from the PPA media by cutting a small piece off the edge of the *Fusarium* culture and transferring it into PDA media on plates under aseptic condition for recovery. It was then kept in the dark at 25^0C for 5 days, and then transferred into SNA at 25^0C for 5-7 days to enhance sporulation. Sterile double distilled autoclaved water was used to obtain 5-10 spores per drop suspension using a dissecting microscope, under low power magnification and then transferred into WA, for single development. The sterile suspension loops, were used to pick up a drop of the sterile, double distilled water, with 5-10 spores per drop. This was spread on SNA plates for growth.

2.6 Single sporing of *Fusarium* species.

This was carried out using a dissecting microscope. The SNA plates were put under a dissecting microscope, and single strands of hyphae were picked from the plates using a sterile wire loop, and placed in the WA media under aseptic conditions. The WA cultures were stored at 25^oC for 15 hours. Using a dissecting microscope, single strands visible in the plate, were cut out using sterile forceps and placed on quarter strength PDA, with antibiotics. The single-spore cultures obtained were stored in SNA media slants at -20 ^{o}C, in screw cap bottles.

2.7 Identification of *Fusarium verticillioides*

Isolates obtained were transferred into a variety of media to carry out identification of the various species of *Fusarium* obtained. The identification of the *Fusarium* cultures was used to classify them taxonomically and the frequency of each observed species was noted. After identification the single spore cultures were preserved in SNA medium slants as well as in sterilized soil, all in screw cap bottles and stored at 4^oC.

2.7.1Identification of *Fusarium* isolates based on Morphological characters

This was carried out after storing PDA culture in the dark at 25 ^{o}C, for 7days. Colony characters observed included growth rates, aerial mycelium and colony reverse. The rates helped distinguish between slow growing species in some sections, intermediately growing species, and more rapidly growing species in different sections. Aerial mycelia were used to distinguish between varieties through observing their textures, whether sparse or abundant. Lack of aerial mycelium could be attributed to slower growth rates. The color of the mycelia was also used to classify the *Fusarium* cultures obtained. The color of the backside of the colony was used in further morphological identification, the absence or presences of some colors helped classify some sections of the *Fusarium* and thus identify the species of *Fusarium* obtained, the presence and absence of diffusate, and their color, was also used. Morphological characters were used to identify *Fusarium* sections present, and to compare their different characteristics.

2.7.2 Identification of *Fusarium* Isolates Based on Microscopic characters

A drop of lactophenol was placed on a glass slide. A small block of the mycelia with little or no media from the CLA or SNA media plates was cut, and the mycelia was placed on the drop of lactophenol on the slide, covered using a cover slip, and heat fixed slightly, so any little amount of media is melted off. This was carried out in a fume chamber, since lactophenol is a carcinogen. The spores and mycelia could then be observed using a standard light microscope (Magnification ×1000).

Observation was made on 6-10 day old cultures grown under fluorescent lamps. The SNA media was used to mainly identify microconidia, and check for the presence of chlamydospores. Relative abundance of microconidia in the aerial mycelium, their presence in chains, or heads, microconidial shape, and presence of conidiophores in the aerial mycelium were observed. On CLA media, macroconidia was identified. The size and shape of the macroconidia, the mode of formation of microconidia on hyphae (i.e. in polyphialides, or monophialides) their presence of false heads, chains, and chlamydospores presence.

The microscopic characters were then used to key the different species of *Fusarium* according to sections and species. Microscopy was carried out by placing a drop of lactophenol blue stain on a glass slide, cutting a small piece of the *Fusarium* from the media, then, heat fixing the *Fusarium* on the glass slide. Observation was then carried out using a compound microscope.

2.7.3 DNA extraction for confirmation of *Fusarium verticillioides*

A small block of mycelium was cut from the edge *Fusarium* cultures on PDA plates and inoculated into 500 ml of sterile potato dextrose broth in 2 ml microfuge tubes. The microfuge tubes were incubated for 5 – 7 days at room temperature and then used to isolate genomic DNA. The isolation of genomic DNA from fungal strains involved sieving the media and isolation of the mycelia which was collected on muslin cloths. The broth was then discarded. The mycelium was resuspended in the lysis buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, pre-heated at 65ºC) and crushed using fused micropipette tips. The microfuge tubes was heated at 65ºC for 20 min and centrifuged at 3000 rpm for 5 min. An equal volume of phenol: chloroform (1:1) solvent was added and then again centrifuged at 3000 rpm for 5 min. The supernatant was transferred to new tubes and an equal volume of isopropyl alcohol added. The microfuge tubes was kept at -20º C for 2 h and then centrifuged at 9000 rpm for 8 min. The pellets with DNA obtained was dried at 37º C for 5 min and resuspended in 20 ml of nuclease free water for PCR analyses. DNA sequencing was done to validate the results obtained.

2.7.4 PCR amplification

All genomic DNA used was tested for their suitability for PCR amplification using, forward primer VERT-1(5'-GCGGGAATTCCAAAGTGGCC-3') and reverse VERT-R(5'-CGACTCACGGCCAGGAAACC-3') which are species specific PCR primers for the identification of *F. verticillioides*. The expected amplicon size was 1016 bp (O'Donnell *et al*., 1998).

The set of primers specific for fumonisin production used for detection of 'FUM gene' of *Fusarium* species included (VERTF-1(5'-GCGGGAATCAAAAGTGGCC-3') and VERTF 2 (5'GAGGGCGCGAAACGGATCGG-3') and Fpro-fum8-rev (5'-GTAGTGAGAGCATCATAGTATG-3') the expected amplicon size was 425 to 450 bp. Amplification reactions was carried out in volumes of 25 µl containing 2 µl of template DNA, 1 µl of each primer (20 pmol), 0.5µ l of Taq DNA polymerase (3 U/ml), 2.5 µl of 10X PCR buffer, 2.5 µl of $MgCl_2$, 15 µl nuclease free water and l µl of 2 mM dNTPs .

The PCR conditions for VER and FUM 1 regions included 94ºC for 2 min for initial denaturation, followed by 35 cycles of denaturation at 94ºC for 45 sec, primer annealing at 58ºC for 45 sec, and primer extension at 72ºC for 1 min. The final extension was set at 72ºC for 5 min, then 4 ºC until turned off. The translation elongation factor 1a gene, TEF-1a, was amplified with primers EF-1and EF-2 (O'Donnell *et al*., 1998) and the resulting PCR product was used in carrying out sequencing. The results obtained were used to confirm the presence of *F.verticillioides* species and the production of fumonisins by both this species and the other isolates the results obtained were observed and recorded.

2.7.5 Gel electrophoresis

Amplification products were checked by electrophoresis on 1.5% agarose ethidium bromide gels, in 1X Tris-acetate EDTA buffer. The gels were then illuminated and documented using a Biorad UV Transilluminator.

2.7.6 Sequencing of the *Fusarium* isolates PCR products

PCR products sequenced using geneious program were obtained. The sequences were first cleaned up using *MEGA* version 5 (Tamura *et al*., 2011) and MUSCLE (Edgar and Robert, 2004) to generate multiple alignments of the nucleotide sequences. Sequence based comparative analysis among closely related fungal species was carried out through blasting the DNA sequence using BLAST (Altschul *et al*., 1990).

2.8 Maintenance of *Bacillus thuringiensis*

The *Bt.* isolates used were maintained stored in the laboratory at -20°C, in Nutrient Agar slants. Sporulating cultures of *Bt.* isolates were obtained by culturing the *Bt* in nutrient agar plates, then incubating them at 30°C for 72 hours. The Sporulating cultures were used to inoculate 50ml of Nutrient Broth (Lab-Lemco powder in 250ml fluted Erlenmeyer flask. The inoculated flasks were incubated in a shaker for 96hrs at 30^0C and 200 rpm. At the end of the incubation period the spores and endotoxins were harvested by centrifugation of the Nutrient Broth at 4000 rpm for 10min. The supernatant was discarded and pellet washed three times by centrifugation in sterile 0.85% saline air-dried and weighed. The pellet was then suspended in 5 ml saline for storage at -20^0C (Wang'ondu, 2001). The purity of the crystals was ascertained by microscopy.

2.8.1 Ascertaining purity of *Bacillus thuringiensis* Isolates Based on Microscopy and Staining

This was carried out by checking for morphological characteristics of *B. thuringiensis* isolates based on the method by Poinar and Thomson (1978), while staining of parasporal bodies was as outlined by Wang'ondu (2001).

2.8.1.1 Gram Staining

This was done to ascertain the gram stain reaction of the cultures after 24hrs and 48hrs. Thin smear of bacterial cells were made on a clean slide by first air-drying and heat fixing for one second. The primary stain (crystal violet) was added and the slide kept for one minute before washing it with distilled water. The slide was then covered with a mordant (iodine solution) for one minute and washed off with alcohol until no more colour came out of the smear. Each smear was then flooded with a counter stain (Safranine) for a minute. Excess counter stain was drained off using absorbent capillarity method. Each slide was blot dried and observation done using a standard light microscope (Magnification ×1000). Gram positive bacteria such as *B. thuringiensis* were supposed to appear dark violet.

2.8.1.2 Smirnoff Staining

This was done for identification of parasporal crystal bodies as outlined by Wang'ondu (2001). A few drops of solution A containing 1.5g Amido black dissolved in 50 parts of 90% methanol, 40 parts distilled water 40 parts acetic acid was added to the heat fixed slides of single colony isolates and washed off after 70 seconds. A solution (B) containing

1g of basic fuschin dissolved in 10ml of 95% of ethanol and 5g of phenol dissolved in 90ml of distilled water was added to each slide and left for 20 seconds before draining it off using absorbent capillarity technique and; dried on a filter paper. Observation was made using a standard light microscope with an oil immersion objective lens, a standard filter being used. Various isolates had parasporal Crystals with different shapes.

2.8.1.3 Schauffer-Fulton Staining

Schauffer-Fulton stain was carried out using 5% of malachite green stain, prepared using 0.5g of malachite green, added to10ml double distilled water, then filtered to remove residual crystals, to give a total volume of 10ml of the stain. A thin smear of bacterial cells were made on a clean slide by first air-drying and heat fixing for one second. The fixed smear was then flooded with a solution of malachite green and placed over boiling water for 5minutes.Rinsing was then carried out using water, and then the smear was counterstained using safranine. Mature spores stained green, whether free or in the vegetative spores. Vegetative cells and spores stained red. The role of this stain was to help distinguish between spores and the vegetative cells.

2.9 Growth of *Bacillus thuringiensis* Isolates for Inhibition Test

The growth of *B. thuringiensis* isolates was obtained in nutrient broth, at 28±2ºC and 200 rpm, until 90% of cell lyses was achieved after 72 hours. The culture was then centrifuged at 5000 rpm, 5ºC, for 15 min, and the isolates obtained in form of pellets washed with phosphate buffer (0.1M $NaH_2PO_4.H_2O$ + 0.1M NaCl, pH 6.0).

2.10 Protein Estimation

The UV protein determination method was used whereby Bovine Serum Albumin (BSA) was used as a standard, at a concentration of one mg/ml. A standard curve was obtained, which was used to determine the protein concentration of *Bt* isolates for the bioassays. Total protein was determined using the Lowry procedure (Lowry *et al.,* 1951).

2.11 Solubilization of the crystals

The purified crystals (0.5mg) were transferred with 50Mm Na_2CO_3.HCl pH 9.5 and 10mm DTT. The mixture was incubated in a waterbath at 37º C for 14hours using a modification of Haider *et al.,* 1986 protocol. Insoluble materials were removed by centrifugation (4000xg, 10 min, 4º C) in a microfuge. The insoluble protoxin fraction was washed in PBS by centrifugation (4000xg, 10min, 4º C) to remove the Na_2CO_3, HCl and DTT. Protein estimation was carried out on both the solubilized protoxin and insoluble protoxin fractions as described above. Both fractions were stored at -20 ºC. Absorbance changes were monitored at 750nm.

2.12 Protease Treatment

The solubilized protoxin was dialysed in 0.1 M Tris - HCl , pH 8.0 at 4º C for 24 h , while the insoluble protoxin fraction was washed thrice in the above buffer by centrifugation (4000xg , 10 min , 4 ºC) . Each of the above samples was treated using bovine pancreatic trypsin and chemotrypsin (Serva , Heidelberg , Germany) and partially purified trypsin like protease from *Prostephanus* species midgut . Crude gut homogenate (adult *P. truncatus*) was prepared by dissecting out the guts; homogenizing (1 min) them in an ice cooled hand homogenizer containing ice cold 0.1M Tri-HCL, pH8. This is followed by centrifugation (12,000x g, 10min, 4^o C in a microfuge. The trypsin activity of supernatant solutions was determined using the chromogenic, α-N-benzoyl-DL-arginine-p-nitroanilide (BApNA) (Wamunyokoli, 1992). The assay consisted of 0.1M Tris-HCI, pH 8.0 (860µl) and the sample supernatant solution (40µl). The mixture was incubated in a waterbath set at 30^{o}C for 3min. The reaction was initiated by addition of 2mM of BApNA dissolved in 66% 9(w.v) dimethylformamide (100µl). Change in absorbance was monitored at 750nm using a spectrophotometer after every treatment.

The amount of substrate hydrolysed was determined from the change in molar extinction at 750nm. The protoxins (35µg) was treated with both trypsin and α- chymotrypsin (165µg). Protoxin (20µg) was treated with gut homogenates (*P. truncatus*), 40µl; partially purified trypsin from *Glossina* midgut, 10µl). The enzyme /protoxin mixtures were incubated in a waterbath at 37^0C for 60min. The enzyme treated samples were then kept at 4ºC.

2.13 Screening Assays

All *Bt.* endotoxin screening bioassays were carried out using adult *P. truncatus* (not less than 30day old), and 7day old fumonisin-producing *F. verticillioides* cultures.

2.13.1 Screening Bioassays using *Fusarium verticillioides*

2.13.1.1 *In vitro* plate assay for evaluation of antagonistic activity using well diffusion method

To test the activity of *B. thuringiensis* against *F. verticillioides*, 5 wells were made into the PDA, around the periphery of the plate, using the method by Iqbal *et al.*, (1999) with slight modifications. A plug of fumonisin producing *F. verticillioides* was placed on surface of the media in the middle of the plate. The *B. thuringiensis* toxin was poured into the PDA plate wells about 5cm apart from each other and the plates were assessed for about 10 days, for appearance of a zone inhibition (Perez, 1990; Abeysinghe, 2006). Three replicate plates were used for each *Bt*. toxin per fungal isolate.

2.13.1.2 *In vitro* plate assay using *Bacillus thuringiensis* toxin in culture

1ml of the *B. thuringiensis* toxins was added to PDA, before it solidified on the petri dishes. *F. verticillioides* disks, positive for fumonisin production was cut from the edge of 7day old culture, and placed in the centre of the Petri plates containing PDA media and the toxins from *B. thuringiensis*. The plates was cultured for 7days at 26^0 C. Fungal growth was measured and compared to the control growth where *Bt*. toxins use was substituted by sterile distilled water (Nour *et al.*, 2010).

2.13.2 Screening Bioassays using *Prostephanus truncatus*

Insecticidal activity was first tested using both spore-crystal mixture, and the pure toxin. For each treatment, 2 ml of *B. thuringiensis* toxin and spore-crystal mixture was used in liquid form. The toxins suspension was mixed with 10g of 3 different forms of toxins delivery diet; Maize Grain (MG), Maize Particles (MP) and Maize Flour (MF). Maximum absorption of the toxins was allowed when applied in liquid form by allowing a drying period of 24 hours at 30^0C. Ten adult *P. truncatus* (not less than 30 days old) were placed n each petri dish in 3 replicates and destructive sampling was done daily for a period of 21 days.

This process was followed for each of the *Bt.* isolates. Isolates with toxin causing deaths of the pests, after the experiment were recorded. The experiment was conducted 3 times (21 days per set of experiment), thus, mortality for *P. truncatus* was recorded for a period of approximately 63 days.

2.14 Data Analysis

Data collected included, counts of contaminated grains, the number of isolates obtained, and their toxicity. This data was analyzed using both SPSS and Excel. Mortalities of adult *P. truncatus* exposed to delta-endotoxins from the *B. thuringiensis* strains were subject to ANOVA. Antibiosis was analyzed by analysis of variance. Mean was compared by Post hoc ANOVA test to determine the significant difference between control and co-cultures. SPSS and Excel were both used in the analysis and the reproduction of the data obtained.

CHAPTER THREE

3.0. RESULTS

3.1 isolation of *Fusarium* species from maize grains

During Isolation of *Fusarium* species, the contaminated or mouldy grains per replicate appeared fewer than those non-contaminated (Table 1). *Fusarium* was however isolated from both maize grains appearing clean and uninfected on the surface, and those that appeared contaminated (Fig1; Plate2; Table 1; Table 2). Sixty one percent of the maize grains used for isolation appeared clean, while thirty one percent appeared contaminated or spoilt (Fig 2). Maize grains used were observed and their conditions were recorded (Table 1). The *Fusarium* isolates obtained were characterized by sparse and slow growth on the isolation media. Colonies formed as observed did not appear distinctive on the isolation media, they however had mycelial growth and a few spores could be observed on the surface of the petri plates (Plate 2). The contaminated maize grains appeared to be covered by *Fusarium* mycelial growth faster than the uncontaminated grains after the same amount of period, growth of *Fusarium* on the clean grains though slower, was observed (Plate 2A; 2B).

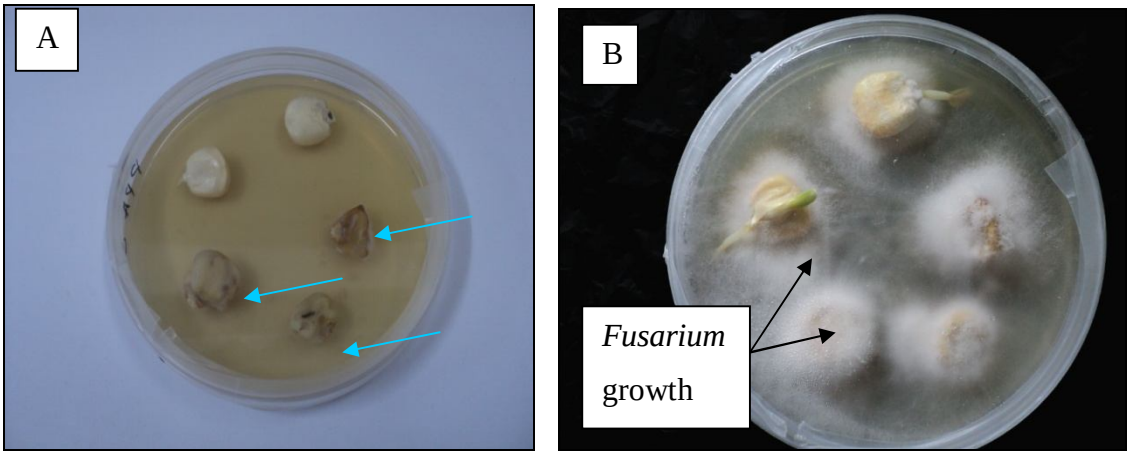


Plate 2: *Fusarium* isolation on peptone PCNB agar; **A:** arrows show some grains appear infected during plating; **B:** day 7 white mycelial growths indicate the growth of the *Fusarium* isolates.

Table 1: Condition of maize grains used to isolate *Fusarium* species on PCNB media

Plate No.	Number of infected or contaminated grains per plate for each replicate						
	Replicates used for the isolation						
	I	II	III	IV	V	VI	Average number of infected grains
PPA 1	4	5	5	5	0	5	4
PPA 2	3	2	4	5	1	3	3
PPA 4	0	0	0	0	0	0	0
PPA 5	0	3	0	2	4	3	2
PPA 6	1	1	2	2	3	3	2
PPA 7	0	2	3	1	4	4	3
PPA 9	0	0	4	0	5	3	2
PPA 10	0	0	0	0	0	1	0
PPA 11	5	3	3	4	5	5	4
PPA 12	0	0	0	0	0	0	0

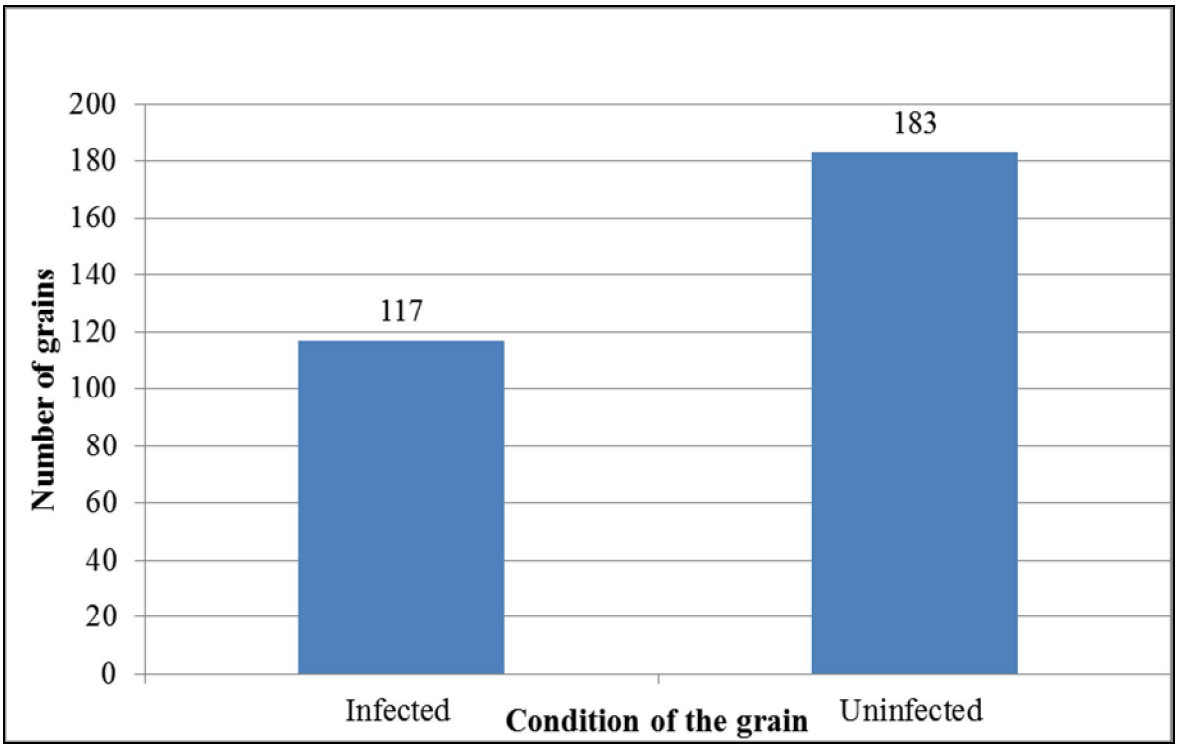


Fig 1: Condition of maize grains used in isolation of *Fusarium* species

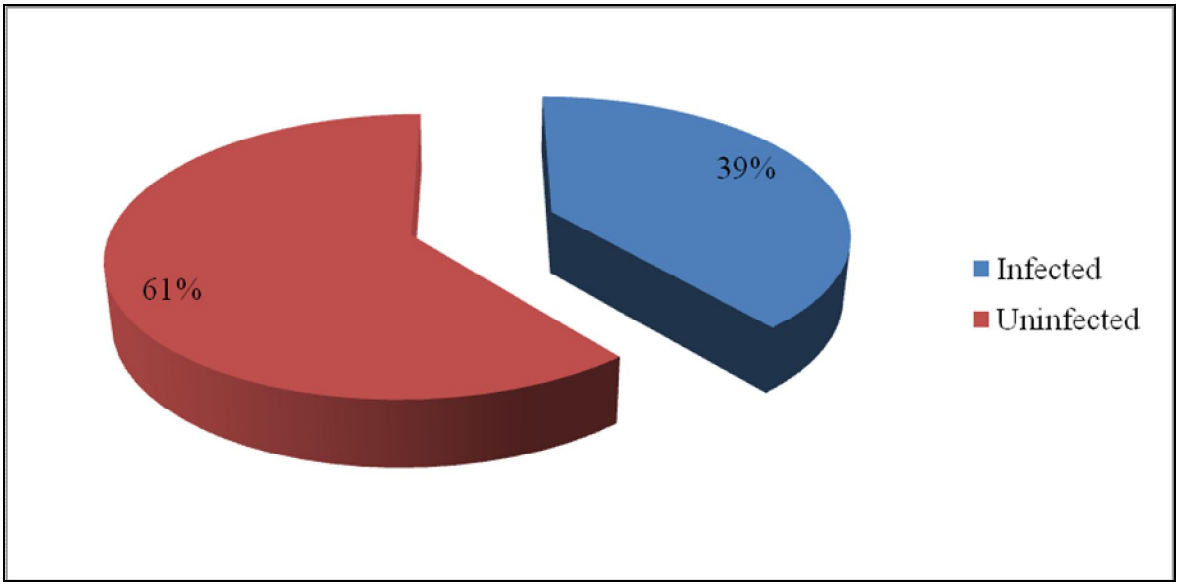


Fig 2: Frequency of contamination of maize grains used in isolation of *Fusarium* species

Table 2: *Fusarium* spp. isolated on Peptone PCNB Agar (PPA) media

Plate No.	*Fusarium* species isolated per plate (5 grains each) for six replicates					
	Replicates used for the isolation					
	I	II	III	IV	V	VI
1	*F.vert*	*G.monill*	*F.oxyspo*	*G.monill*	*G.monill*	*G.monill*
PPA 2	*F.vert*	*G.monill*	*G.monill*	*G.monill*	*G.monill*	*G.monill*
PPA 4	*F.vert*	*G.monill*	*F.oxysporum*	*G.monill*	*G.monilliformis*	*G.monilliformis*
PPA 5	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*
PPA 6	*G.monilliformis*	*G.monilliformis*	*F.oxysporum*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*
PPA 7	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*
PPA 9	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*
PPA 10	*F.verticillioides*	*G.monilliformis*	*F.oxysporum*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*
PPA 11	*F.verticillioides*	*G.monilliformis*	*F.oxysporum*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*
PPA 12	*F.verticillioides*	*G.monilliformis*	*F.oxysporum*	*G.monilliformis*	*G.monilliformis*	*G.monilliformis*

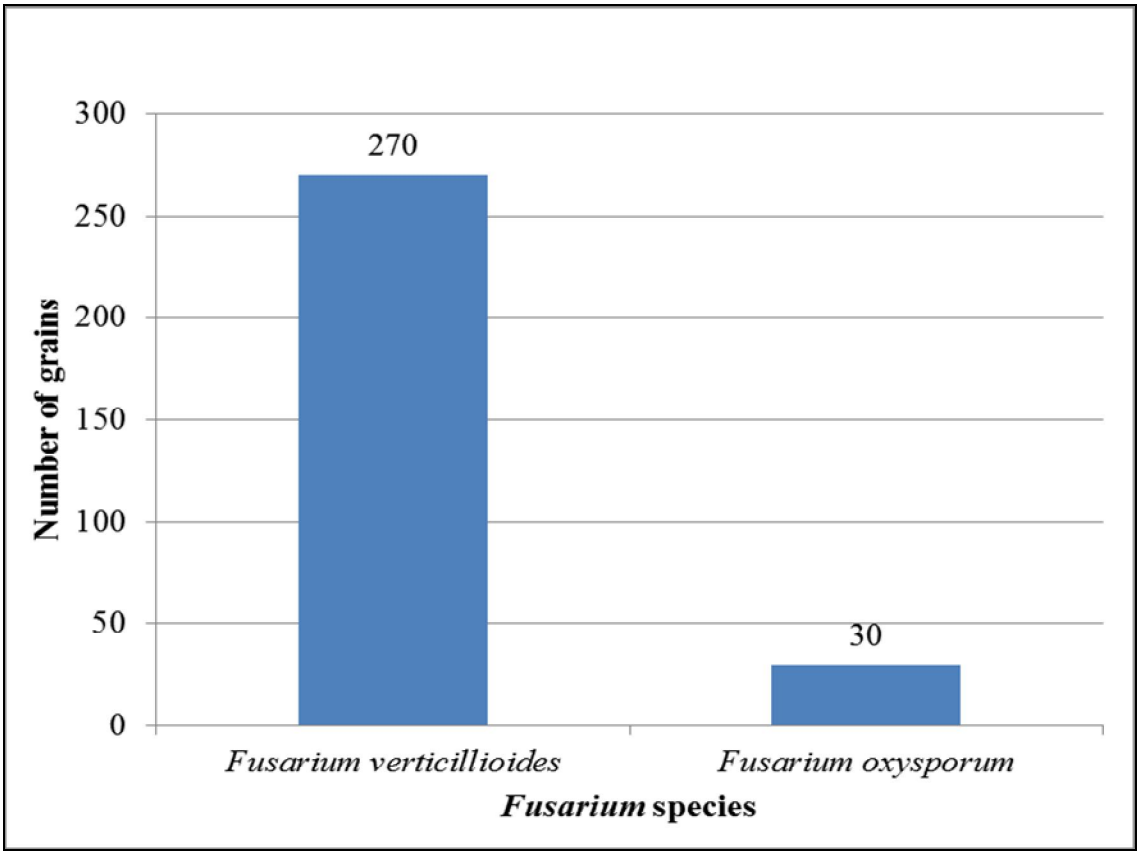


Fig 3: *Fusarium* species isolated from maize grains

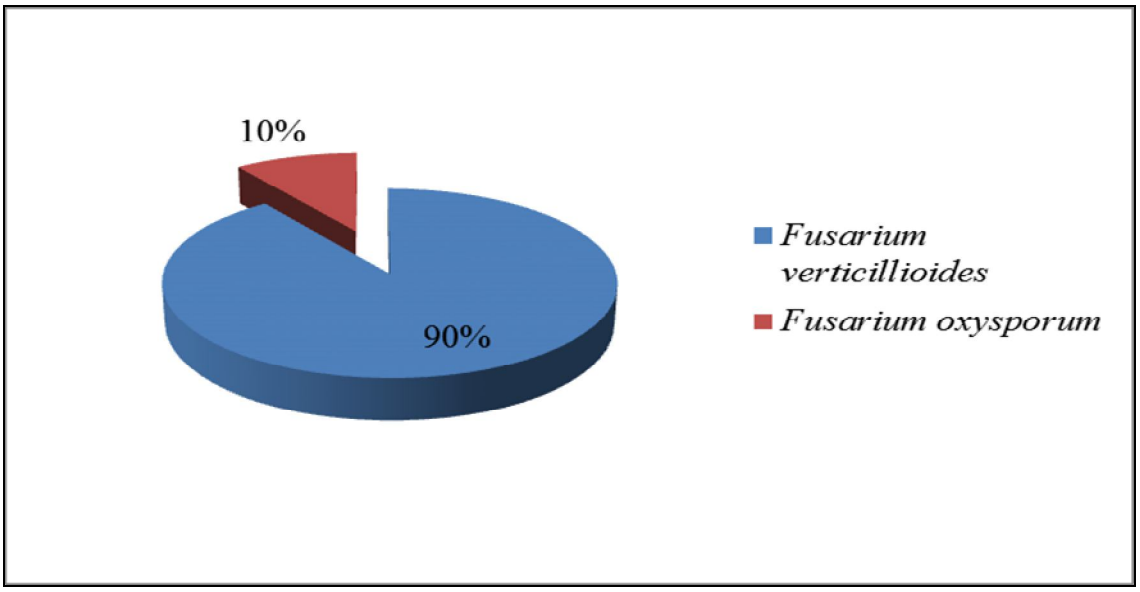


Fig 4: Frequency of isolation of *Fusarium* species from maize grains

3.2 Identification of *Fusarium* isolates obtained

3.2.1 *Fusarium verticillioides*

Taxonomy: Genus-*Fusarium*, Species- *Fusarium verticillioides* (Sacc.) Nirenberg, (1976) Section *Liseola*.

The color of the reverse section helped classify this isolates as those of section *Liseola*. Isolates obtained were mostly identified as *F. verticillioides*. The growth rates of most isolates in this species was moderate , at about 7cm, after 7days, but some varieties were fast growers, as they were 8.6cm, after 7 days of growth in the dark at 26°C.

Their mycelia were white initially, and then they turned pink with age, their bottom color in PDA ranged in color from orange to purple with age. Pigmentation formed in agar in most isolates in agar that ranged from orange to vinacious in color, while other isolates did not have pigmentation in agar. While some produced exudates in PDA cultures, some isolates did not. The isolates did not appear to develop sclerotia in most cultures. Sporodochia was discretely formed in this species, and this appeared tan to orange in color.

Macroconidia were long and straight (Plate 3F). Macroconidia formation however required the presence of UV, for their formation and subsequent observation to be made. Their macroconidia were relatively long and slender, tin walled and falcate shaped. The apical cells appeared curved and tapered (Plate 3F). While the basal cells appeared notch or foot shaped. The isolates had macroconidia that had 3-5 septate. Most isolates had few macroconidia.

Microconidia were distinct in the aerial mycelia, smaller, and with less septation than macroconidia observed on Synthetic Nutrient Agar (SNA) and Carnation Leaf Agar (CLA). The microconidia were produced both singly as glistening tips on conidiogenous cells as observed above, and as long chains. Microconidia were produced in chains in aerial mycelium. Microconidia in this isolates, ranged in length from approximately 6-18 spores in many cases. They were oval to club shaped, and 0-septate.

The isolates had a lot of the aerial mycelia and abundant spores, both in chains and singly in the aerial mycelia. All isolates obtained were monophialide, and some were branched. There were also a lot of false heads present on this isolates. Some monophialides were occasionally paired, giving rabbit ear appearance. There was a complete absence of chlamydospores. There was formation of monophialides only and no formation of polyphialides.

Two reproductive isolates were observed, the anamorph *Fusarium verticillioides*, and its telemorph *Gibberella moniliformis*. The difference between the two reproduction stages were however only discerned after carrying out molecular analysis of the isolates DNA and sequencing of their PCR products(Plate 14, 15), and comparing the sequences obtained(Appendix X) to those sequences of the fungi present in the databases. Production of fumonisins by this isolate was also observed and used in further identification of this species using molecular methods.

3.2.2 *Fusarium oxysporum*

Taxonomy: Genus-*Fusarium*, Species- *Fusarium oxysporum.* Schlecht. Emend, Snyder and Hansen. Section: *Elegans*

Observed morphological characters and microscopic characters enabled identification of this species and classification into section *Elegans*. The special form of the isolates obtained were however not determined,

The isolates were classified as one which occurs in temperate regions and causes disease in maize. The morphological characters varied widely for the isolates in this species, some aerial mycelia on PDA, were sparse and some abundant. Rate of growth after 7 days at room temperature of 26°C in the dark, was intermediate, at an average of 6.8cm. The aerial mycelia appeared white to deep pink in some isolates, and the bottom color of the cultured isolate ranged in color from orange to dark brown or rust as it aged. This isolates produced abundant pale orange microconidia that could be observed on the mycelia on PDA cultures. While most isolates produced brown diffusate, some did not have any diffusate produced.

On CLA and SNA media, there appeared to be an abundance of macroconidia produced in Sporodochia that appeared pale orange in color. The macroconidia from this isolates ranged in length from short to medium, straight, thin walled, and all were 3 septate. The macroconidia had tapered ends, and slightly pointed. The apical cells were short and slightly hooked, and the basal cells were notched or foot shaped.

Macroconidia was formed from monophialides on branched conidiophores as observed above, both on hyphae and to a larger extent in sporodochia. Phialides formed appeared short and were present in the aerial mycelium. There was a large number of monophialides formed, in Sporodochia and fewer in hyphae aerial mycelium. Microconidia observed were both oval and reniform in shape, and were abundantly found on false heads and also on short and plump monophialides. The microconidia observed all appeared to be 0-septate.

Chlamydospores were formed quickly in the media, and they appeared abundant in this isolates, although most appeared to be formed singly or in pairs, there were few formed in short chains, intercalary in aerial mycelium. They appeared both smooth and rough walled in the different isolates obtained. When the isolates were checked for production of fumonisins using molecular methods, they were negative for the Fum gene.

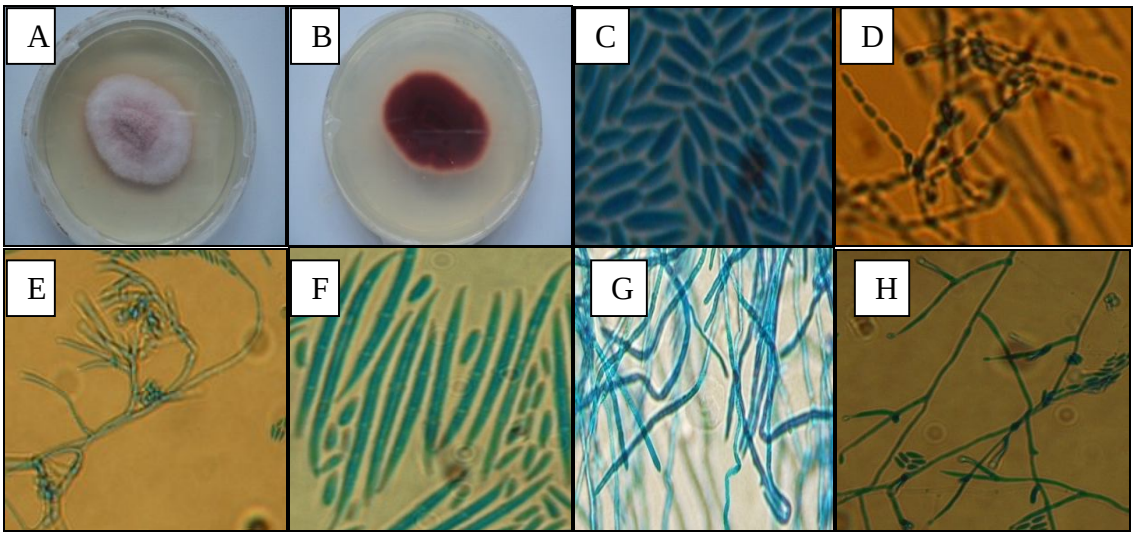


Plate 3: *Fusarium verticillioides* **A-B:** Isolates top and bottom colony characteristics; **C-D:** Microconidia appear oval single celled, and in long chains; **E:** Branched monophialides with false heads; **F:** Macroconidia; **G:** Abundant aerial mycelia; **H:** Monophialides with false heads. Magnification X1000.

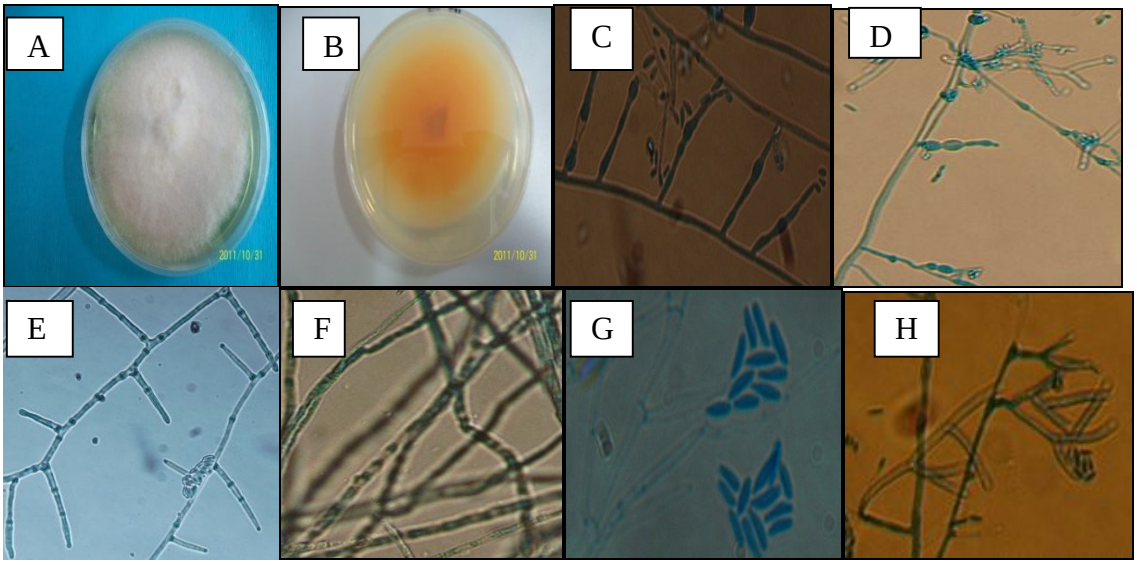


Plate 4: *Fusarium oxysporum* **A-B:** Isolates top and bottom colony characteristics; **C-D:** Chlamydospores in pairs and in simples; **E:** Short phialides; **F:** Aerial mycelium; **G:** Microconidia appear both oval and comma shaped; **H:** Short and plump monophialides in the aerial mycelium with false heads. Magnification X1000.

3.3 Comparison of *Fusarium* Isolates obtained

Fourteen isolates were used to carry out comparisons of the isolates obtained after isolation. Two *Fusarium* isolates were obtained from plate PPA 12 (Table 3).

Table 3: Colony morphology of *Fusarium* species on isolation media

Isolation of *Fusarium* spp. on maize grains directly on Peptone PCNB Agar (PPA) media

Plate No.	No. of infected grains	Characteristics of the colonies growing				
		Top				Bottom
		Elevation	Margin	Configuration	Color	Color
PPA 1	4grains	Convex	Wooly	Round	White-pink	Clay buff, buff edges
PPA 2	3 grains	Convex	Wooly	Round	White	Buff
PPA 4	0 grain	Convex	Wooly	Round	White	Vinacious and buff
PPA 5	2 grains	Convex	Wooly	Round	White-pink	Clay buff, straw edges
PPA 6	2 grains	Convex	Wooly	Round	White-pink	Vinacious, purple edge
PPA 7	3 grains	Convex	Wooly	Round	White-pink	Vinacious, purple edge
PPA 9	2 grains	Convex	Wooly	Round	White	Vinacious , purple edge
PPA 10	0 grain	Convex	Wooly	Round	White-pink	Clay pink , buff edge
PPA 11	4 grains	Convex	Wooly	Round	White-pink	Purple
PPA 12	0 grains	Convex	Wooly	Round	White-pink	Vinacious and clay pink
PPA 12	0 grains	Convex	Wooly	Round	White-pink	Clay buff, buff edge

3.4 Recovery of *Fusarium* isolates on PDA media

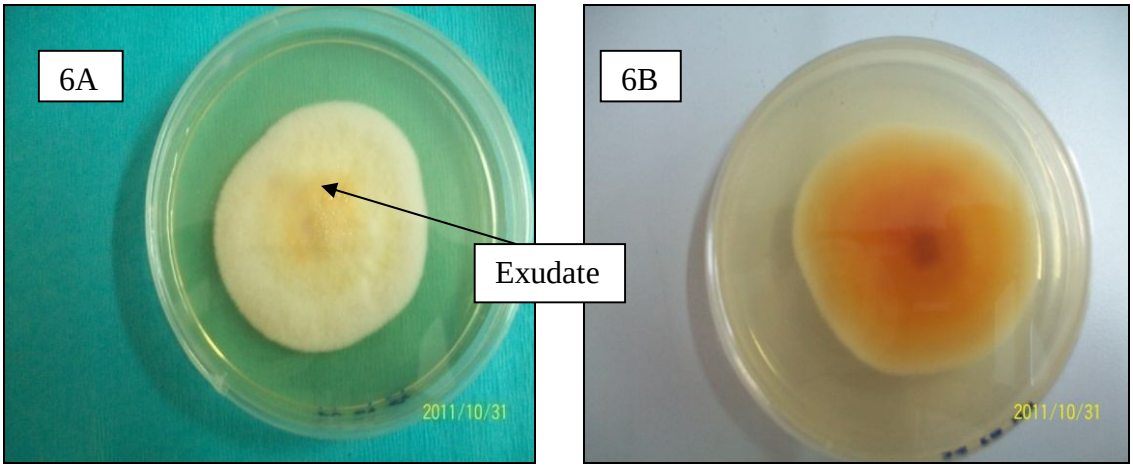
Fusarium cultures obtained from PPA media were recovered on PDA slants (Plate 5).

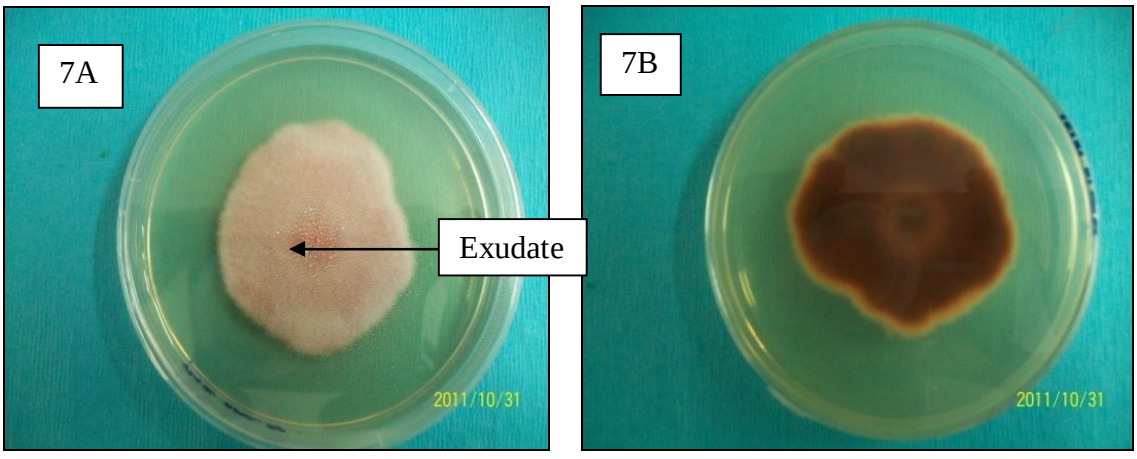
Plate 5: Seven days old pure *Fusarium* isolates on PDA media slants. The arrows point towards the middle part, used during inoculation, the white area around is the aerial mycelia.

3.5 Characterization of *Fusarium* cultures obtained

3.5.1 *Fusarium* cultures on PDA media

The plates show morphological characteristics of *Fusarium* isolates on PDA plates, observed after 7 days of being kept in the dark. Aerial mycelium of the isolates FV 012 and FV 003 formed a convex shape that was cottony in texture (Plates 6 and 7). The top of the *Fusarium* Isolates appeared pink (Plates 6, 8, 9, 10) to dark purple (Plate 7), while the bottom appeared orange to dark purple. The mycelial color appeared different for the isolates. The textures of the isolates differ. The top (A) and the bottom (B) color of the *Fusarium* culture media on plates was also observed.





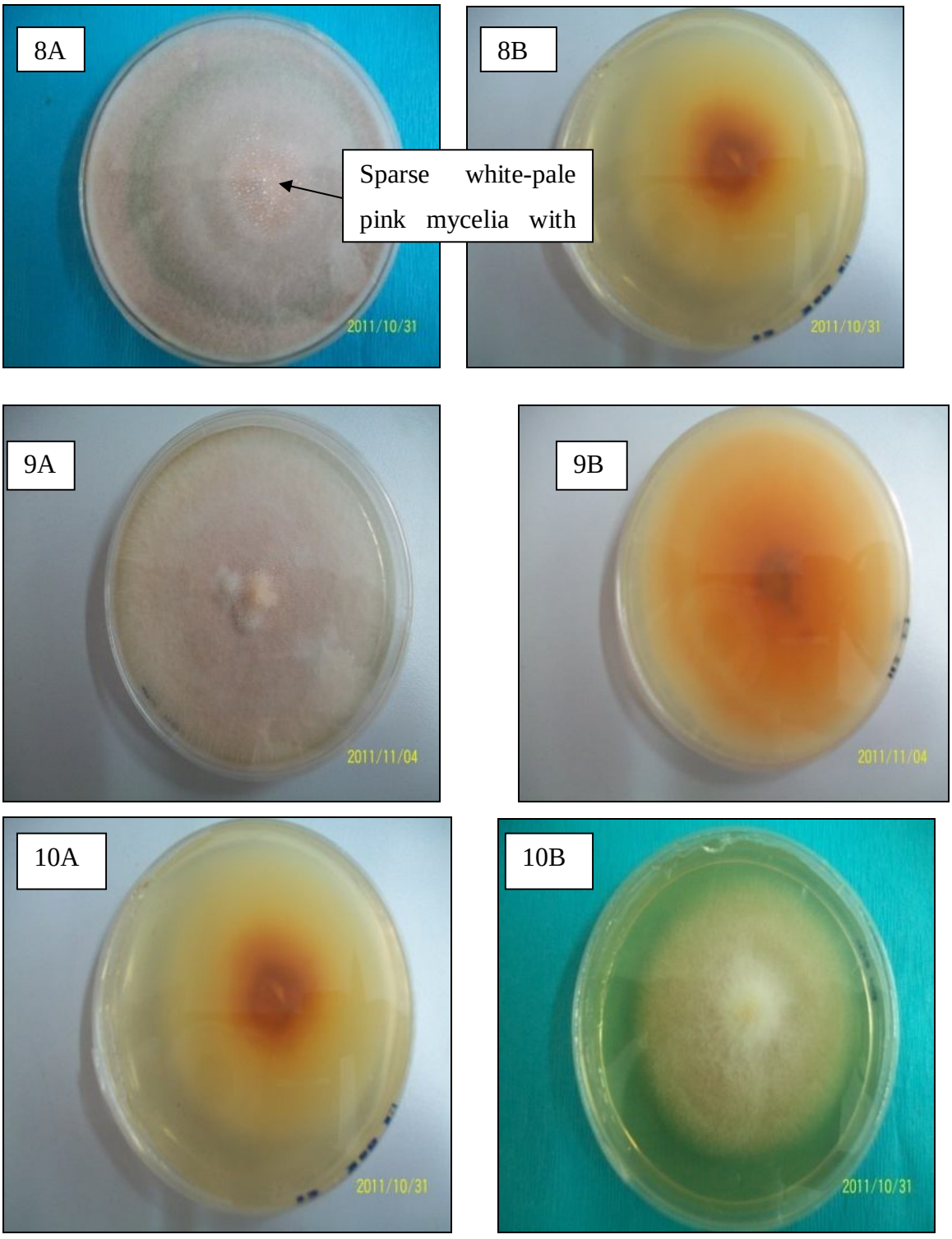


Plate 6-10: *Fusarium* Isolates on PDA. **A** (top) and **B** (bottom) of the different isolates; FV 003; FV 012; FV 010, FV 006 and FV 008 respectively. (Magnification X 1000)

3.5.2 Rate of growth of *Fusarium* isolates cultured on PDA media

Growth characteristics of the *Fusarium* isolates were observed, for 10 days while isolates were kept in the dark at 26°C (Fig. 5). Moderate/intermediate rate of growth for all cultures except for FV 011, FV 003 and FV 001, whose growth was rapid. FV 011 and FV 003 growth covered the petri plate fully at the end of the seven days (Fig. 5). Growth diameters on PDA were used to determine if the *Fusarium* species was *F. verticillioides*. The growth of the culture during the 10 day period showed an average rate of growth (Fig. 6).

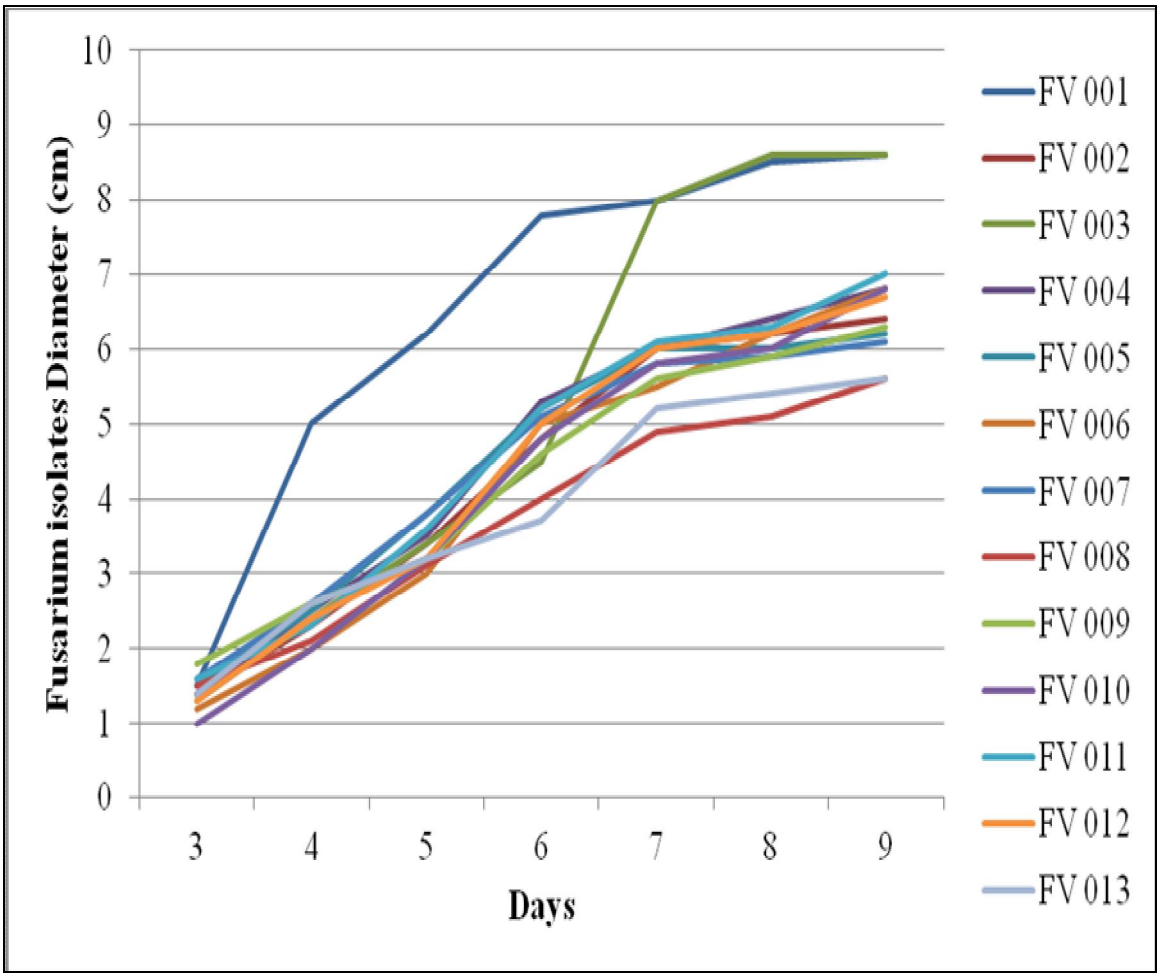


Fig 5: Radial growth of *Fusarium* isolates

3.5.3 Morphological characteristics of *Fusarium* isolates cultured on PDA media

Table 4: Morphological characteristics of *Fusarium* isolates on 7 day old PDA cultures.

KEY: (+) Present; (++) average/moderate growth; (+++) High growth

White aerial mycelia were observable on all the cultures, typical of *Fusarium* species.

Isolate	Fv 1	Fv2	Fv3	Fv4	Fv5	Fv6	Fv7	Fv8	Fv 9	Fv 10	Fv TO	Fv 11	Fv 12	Fv 13
Top														
Color	coral	Pink	White	salmon	salmon	Pink	pink	pink	Pink	rose	rose	Pink	Pink	Vinaceous
Mycelia length	++	++	+++	++	++	++	++	++	++	++	++	++	++	++
Exudates	_	+	+	_	_	+	+	_	+	_	_	+	+	_
Bottom														
Color	Rust	Saffron, orange and apricot	Apricot orange, saffron	Saffron and orange	Saffron and orange	Radial rust and salmon edge	Orange rust/ tawny Saffron	Orange and rust straw	Purple	Orange and rust, with straw	apricot and rust	Siena	purple	Purple
Diffusate	-	-	Orange	Apricot and pale cream	Peach	Vinaceous	Yellow-cream	Pink-brown	Peach	Peach-brown	Brown-tan to dark purple	Tan	Tan	Vinaceous

3.5.4 Microscopic characterization of *Fusarium* isolates

Polyphialides were not observed in all the cultures, monophialides were short for FV 010 and the branched monophialides had a rabbit eared appearance in FV 012. The length of the chains was measured relative to the number of spores that were observed to be present on the monophialides (Table 5). Shapes for all of the isolates macroconidia appeared thin walled and more dorsal curved than ventral, except FV010 which appeared thin walled, falcate to straight. The macroconidia were present sparsely for all except FV 010, FV 011 and FV 05. The microconidia were all oval shaped, 0 septa and abundant in culture. The apical cells were all conical shaped, except FV 008 which appeared hooked, and all basal cells appeared notched/ foot shaped (Table 6).

Microconidial shape was oval, short and unicellular and 0 septa for all the isolates. They were all very abundant in media, and produced in chains in the aerial mycelium. Macroconidia for most of the isolates were sparsely produced, and were narrow and straight. The apical cells were hooked and short and the basal cells not notched/blunt. No distinct form. The others were all notched, not distinct and their basal cells appeared. All macroconidia were 3-5 septate. FV 010 had 3 septa, and appeared falcate to straight (Table 7). The macroconidia appear long and slightly falcate (Plate 11). They were thin-walled with apical conical cells and notched basal cells in both plates. Macroconidia appeared long and slender (11 A). Plate (11 B) macroconidia ranged from short to medium length. They were thin walled, slender and falcate to straight.

All macroconidia in plate B, belonging to the *Fusarium* isolate FV 010 had 3 septa. The blue arrows show notched / foot shaped basal cell morphology (Plate 11A), while the apical cell morphology (Plate 11 B) is indicated by the blue arrow in FV 010.The macroconidia in FV 012 (Plate 11A) have 3-5 septa. The microconidia on all plates appear oval and unicellular, for most of the isolates. Unicellular microconidia on CLA were observed (Plate 12), both in long and short chains and some chlamydospores on the aerial hyphae (Plate 12C). The microconidia were abundant in some cultures (Plate 12A and B), and rare or fewer in some cultures (Plate 12 C). Microconidia and false head appear in abundance in culture, and branched monophialides were present.

Most cultures had monophialides with false heads (Plate 13). Some appeared branched and there was abundant production of microconidia observed (Plate 13A and 13B). Very long hyphae was present (Plate 13 B), and there was absence of polyphialides. Monophialides were however abundant (Plate 13C and D). Some monophialides possessed branches (13C) conidia at the top and false heads (13D).

Table 5: Microscopic characteristics of *Fusarium* cultures obtained X1000 Mg

Fusarium spp	Monophialide /Polyphialide presence	Length (No. of spores)	False heads presence	Hyphae Breadth (µm)	Chlamydo-spores
FV 001	Monophialides	8 to10	Present	4.8	None
FV 002	Branched monophialides	6 to12	Long& slender	7.2	None
FV 004	Branched monophialide chains	7 to 10	Present	3.92	None
FV 005	Branched monophialides	8 to 11	in chains	1.44	None
FV 006	Monophialide chains.	7 to 9	Present	1.48	None
FV 007	Branched monophialide chains	6 to 12	Present	1.28	None
FV 008	Branched monophialide chains	7 to 10	Abundant	1.76	None
FV 009	Branched monophialide chains	9	Present	1.68	None
FV 010	Monophialide chains	4 to 6	Present	1.6	Present (rough walled)
FV 011	Branched monophialide chains	12	Present	5.08	None
FV 012	Branched monophialide chains (Formed rabbit ears)	3 to 18	Present	4.48	None
FV TO	monophialide chains	12	Present	1.4	None

Table 6: Microscopic characters of the *Fusarium* cultures on 7 day old Carnation Leaf Agar and Synthetic Nutrient Agar cultures. Spores on Sporodochia X1000Mg

KEY: (+) Present; (++) average/moderate growth; (+++) High growth

MACROCONIDIA					MICROCONIDIA	
Fusarium spp	abundance	Length (µm)	width (µm)	Septate	Length (µm)	width (µm)
FV 001	+	39.11	4.41	3-4	7.25	2.0
FV 002	+	39.71	5.29	3	8.53	2.5
FV 004	+	52.5	3.48	3	10.73	2.92
FV 005	++	56.7	2.94	3	10.0	1.97
FV 006	+	33.97	4.85	3	10.29	2.20
FV 007	+	57.8	3.38	3-5	10.44	2.17
FV 008	+	58.3	1.96	3	8.82	2.40
FV 009	+	27.9	2.35	3	7.35	2.22
FV 010	+++	58.8	3.68	3	7.2	2.40
FV 011	++	48.09	2.94	3-4	2.94	3.24
FV 012	+	72.79	5.15	3-5	3.41	2.80
FV TO	+	58.2	1.94	4	8.82	2.94

Table 7: Microscopic characters of the *Fusarium* cultures, Spores on aerial mycelia X1000Mg

	MACROCONIDIA		MICROCONIDIA	
	Length(µm)	width (µm)	length (µm)	width (µm)
FV 001	35.6	4.11	10.29	2.94
FV 002	47.06	3.53	9.70	2.64
FV 004	58.82	2.79	11.17	2.0
FV 005	17.88	2.94	9.11	2.65
FV 006	25.29	2.65	8.09	2.8
FV 007	44.12	2.94	9.7	2.88
FV 008	27.06	2.65	8.96	3.24
FV 009	25.74	4.26	10.29	2.52
FV 010	48.09	3.94	7.35	2.05
FV 011	52.94	4.12	8.00	2.5
FV 012	52.5	4.26	7.4	2.65
FV TO	42.6	4.41	8.8	2.72

3.5.4.1 Macroconidia on Sporodochia on Carnation Leaf Agar media

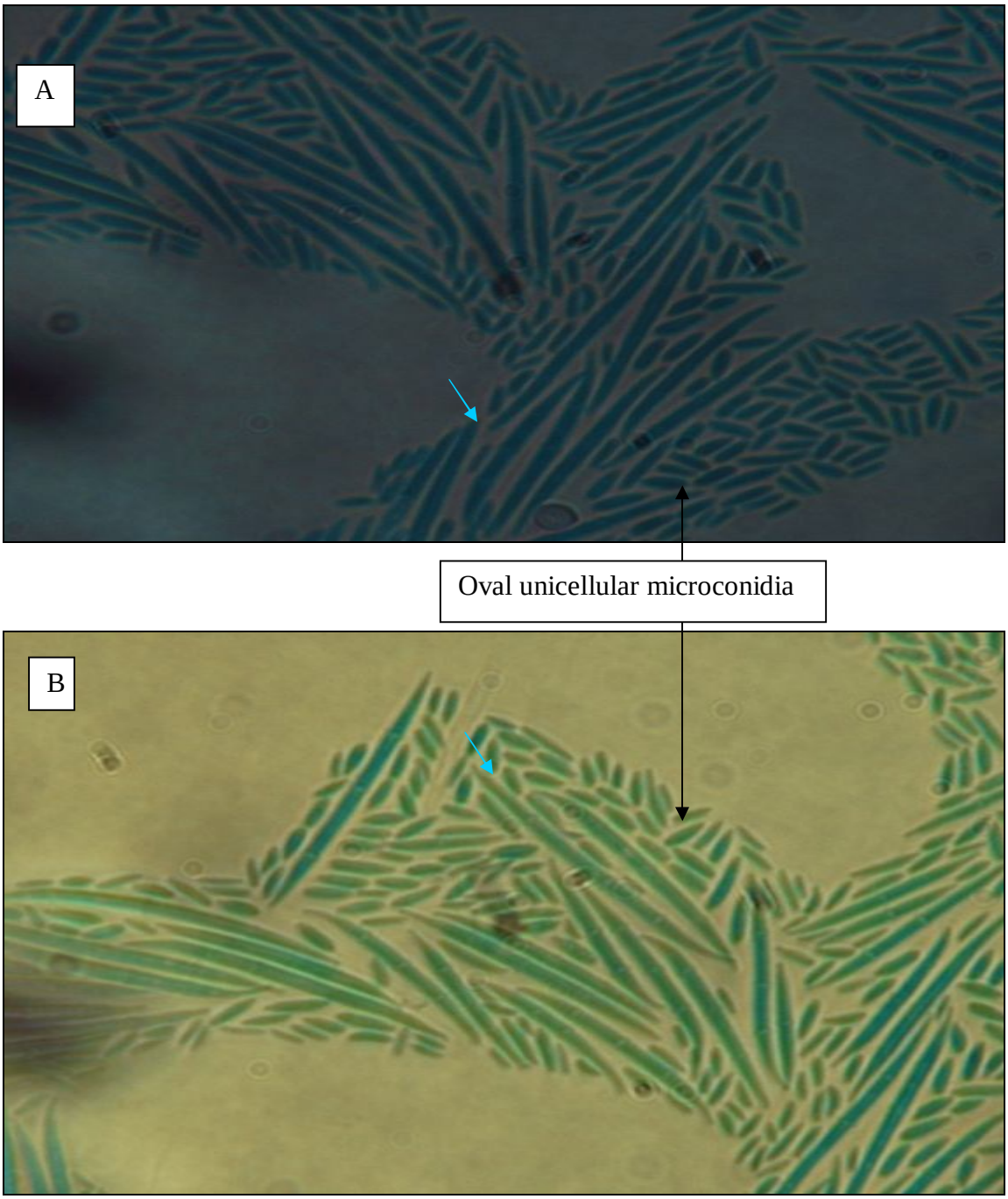


Plate 11: Abundant Macroconidia; **A:** FV 012 on aerial mycelium; **B:** FV 010 on Sporodochia. (Magnification X 1000).

3.5.4.2 Microconidia on aerial mycelium on Carnation Leaf Agar media

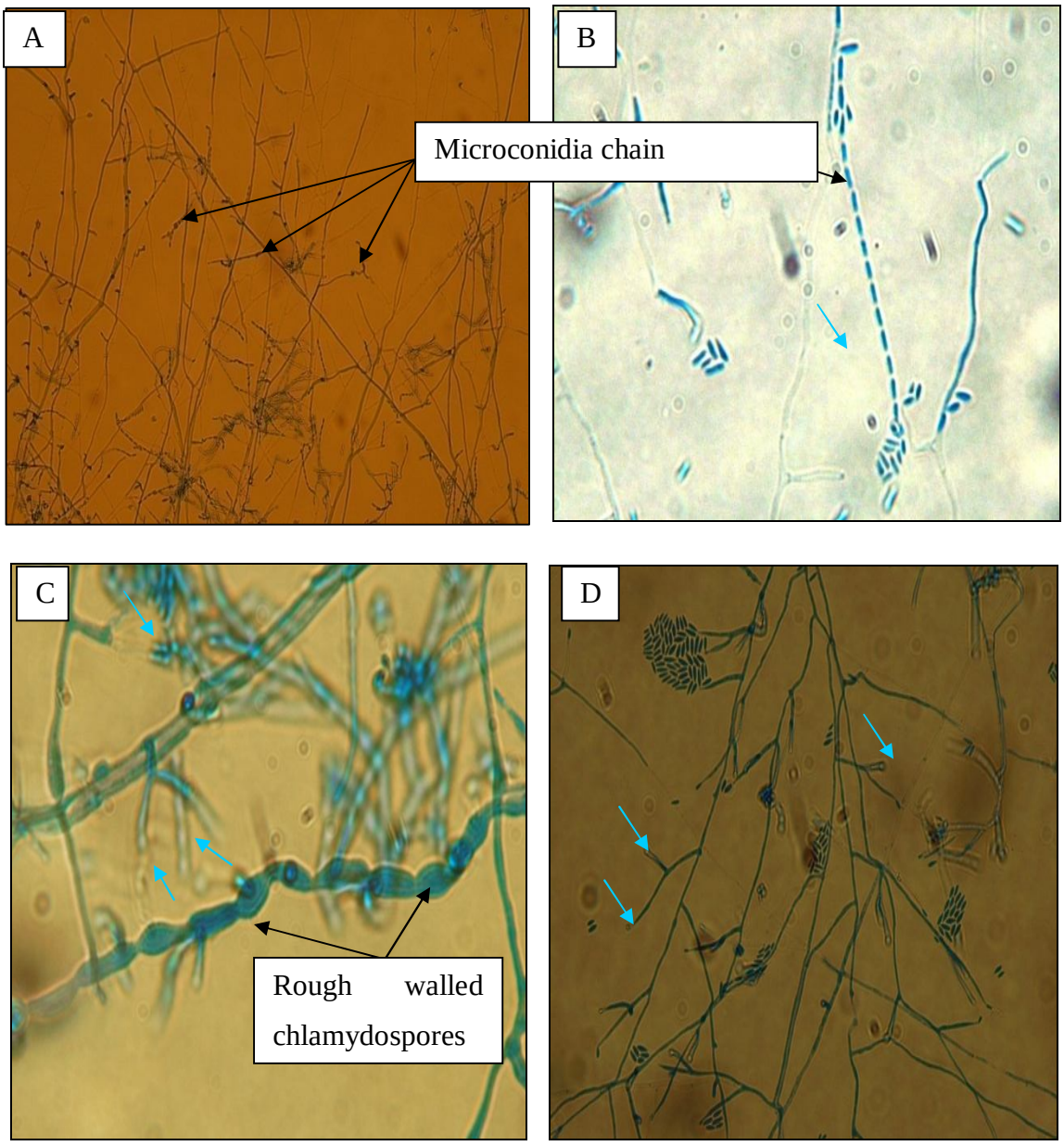


Plate 12: Microconidia growth on culture; **A:** FV 001 with monophialides and microconidia on short chains branched and unbranched monophialides with false heads on hyphae, are shown using blue arrow in the plates in 10 above; **B:** FV 012 long chains of microconidia; **C:** FV 010 chlamydospores (rough walled) formed in chains on surface hyphae; **D:** FV 002. (Magnification X 1000).

3.5.4.3 Microconidia on Sporodochia and aerial mycelium in Synthetic Nutrient Agar medium

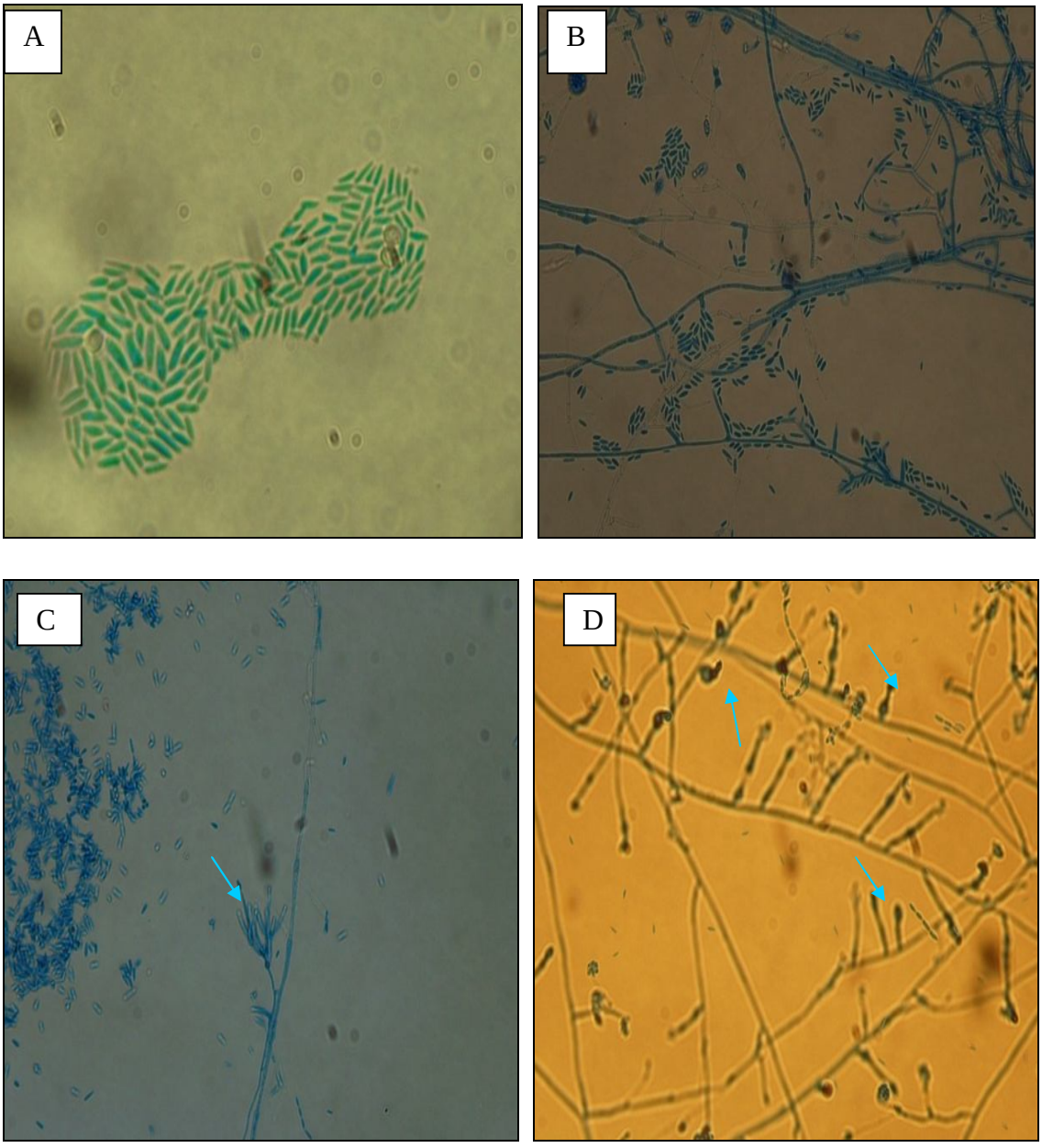


Plate 13: Microconidia were observed from culture, they appeared as oval uniseptate spores as observed in the FV 010 isolate; **A:** observed on aerial mycelium, monophialides on hyphae are shown using arrows; **B:** FV 005 microconidia on hyphae and Sporodochia; **C:** FV 012 Branched monophialides; **D:** FV 007 monophialides with, false heads. (Magnification X1000).

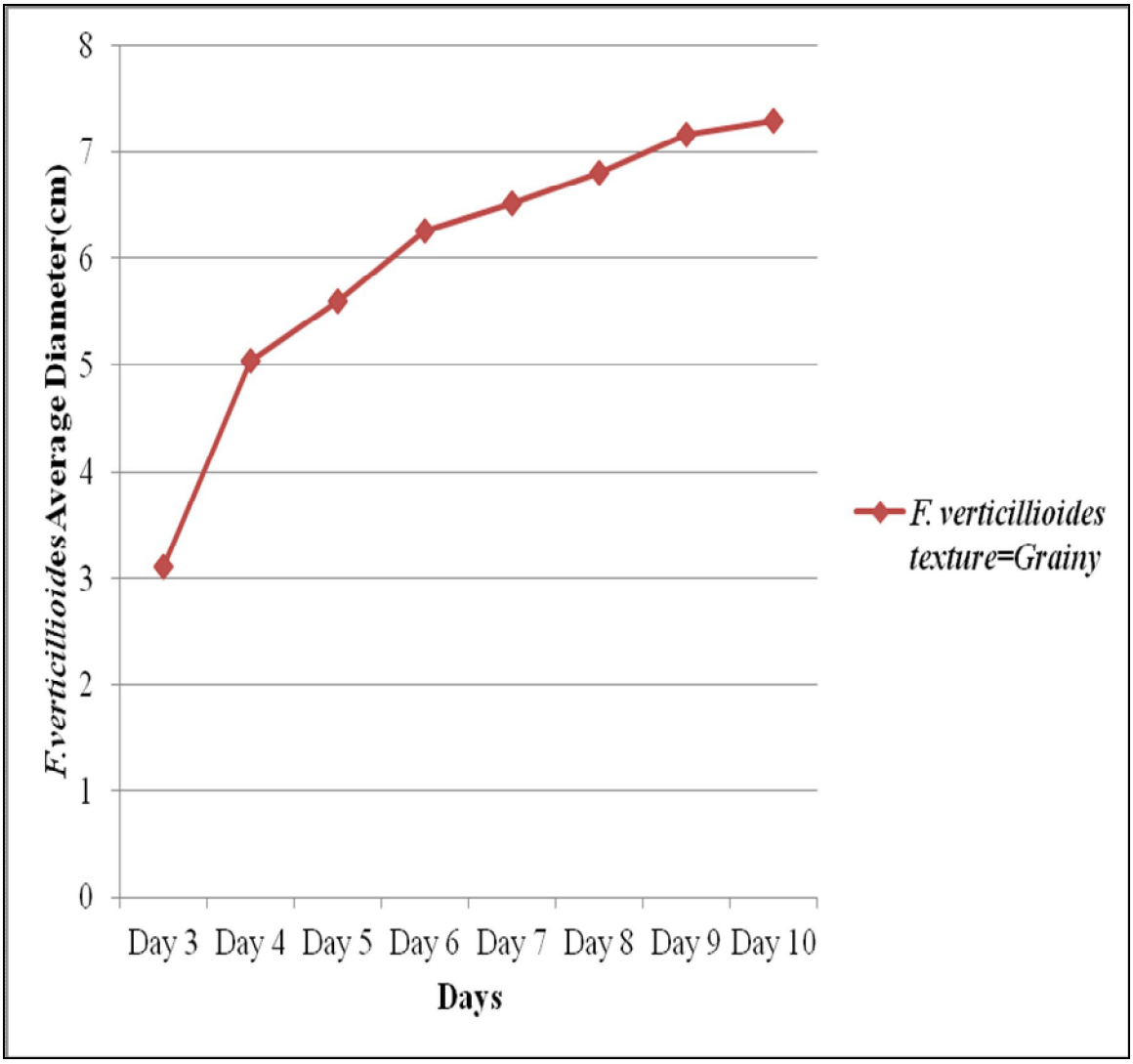


Fig. 6: *Fusarium verticillioides* culture growth rate over seven days (cm)

3.6 PCR bands from gel electrophoresis of the *Fusarium* isolates

VERT-1(5'-GCGGGAATTCCAAAGTGGCC-3') and reverse VERT-R (5'-CGACTCACGGCCAGGAAACC-3') which are species specific PCR primers for the identification of *F. verticillioides* were used for further identification. PCR bands approximately 1kb in size were obtained. This identified isolates with the VERT gene, thus positively identified as *Fusarium verticillioides* (Plate 14).

Specific primers, VERTF-1, VERTF-2 and Fro-fum8-rev were used to analyze the isolated *F. verticillioides* for fumonisin production. Isolates positive for the FUM gene and thus identified as fumonisin producers showed positive bands (Plate 15).

The presence of fumonisin toxins from the isolates obtained from the maize grains were recorded, after observation of the gels obtained (Table 8). When PCR results were obtained on the isolated *Fusarium*, both *F. verticillioides and G. moniliformis* tested positive for presence of the vert gene (Fig. 7).

The frequency shows that a large number of the *F. verticillioides* isolates in both sexual and asexual stages produce fumonisins (Fig. 8). Seventy two percent of the isolates obtained produced fumonisins, twenty eight percent of the isolates however did not test positive for the Fum gene when PCR was carried out (Fig. 9). A larger frequency of toxigenic isolates was observed than that of the non toxic isolates (Fig. 10).

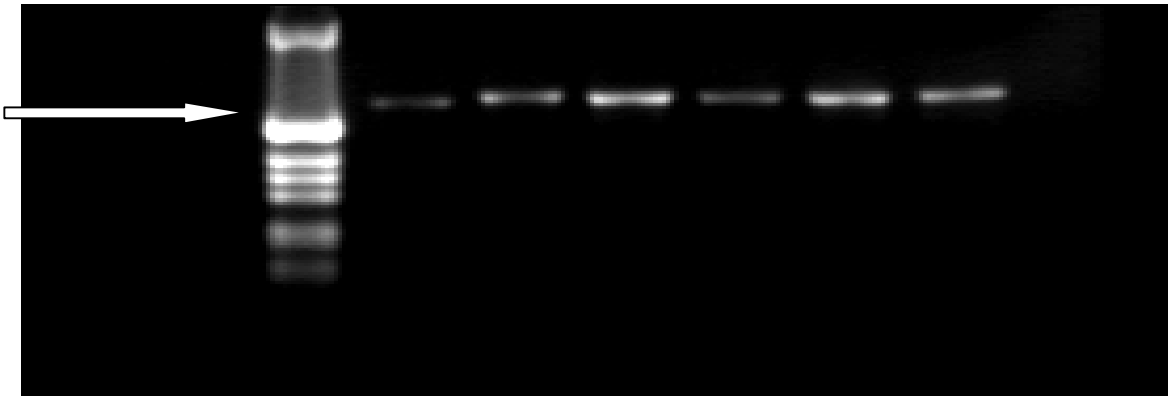


Plate 14: Agarose gel showing amplified products of VERTF-1 and VERT-R set of primers. The first lane is the marker lane. The arrow shows the size of the bands are 1000bp. All bands were positive for *verticillioides* gene, except lane 7, where *Fusarium oxysporum* PCR product was used for the negative control lane.

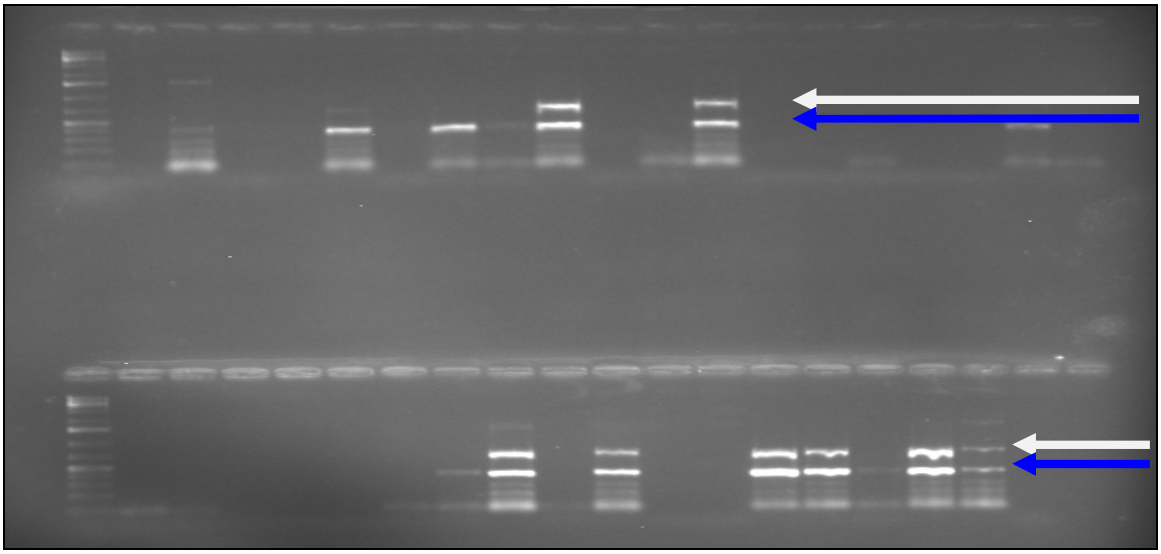


Plate 15: PCR amplification of *Fusarium* isolates. *Fusarium* isolates confirmed to be *Fusarum verticillioides* were indicated by 1000bp size bands, indicated by the white arrow, they were also shown to produce fumonisins as they had the FUM gene present. Fumonisin producing *F. verticillioides* strains were shown using the primers for FUM gene VERTF-1 and VERTF-2, and they showed bands whose size was 500kb as shown by the blue arrow. *F. oxysporum* strain was used as negative control, and they neither formed bands positive for the VERT gene, nor those for the FUM gene.

Table 8: Toxigenic *Fusarium* species from isolates

Fusarium obtained from initial isolation on Peptone PCNB Agar (PPA) media						
Plate No.	*Fusarium* species isolated per plate (5 grains each) for six replicates					
	Replicates used for the isolation					
	I	II	III	IV	V	VI
PPA 1	*F.vert* (+Fum)	*G.mon* (+Fum)	*F.oxysporum*	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)
PPA 2	*F.vert* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon*
PPA 4	*F.vert*	*G.mon* (+Fum)	*F.oxysporum*	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon*
PPA 5	*G.mon*	*G.mon* (+Fum)	*G.mon*	*G.mon*	*G.mon* (+Fum)	*G.mon* (+Fum)
PPA 6	*G.mon*	*G.mon* (+Fum)	*F.oxysporum*	*G.mon* (+Fum)	*G.mon*	*G.mon* (+Fum)
PPA 7	*G.mon*	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)
PPA 9	*G.mon*	*G.mon* (+Fum)	*G.mon*	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)
PPA 10	*F.vert* (+Fum)	*G.mon* (+Fum)	*F.oxysporum*	*G.mon*	*G.mon*	*G.mon* (+Fum)
PPA 11	*F.vert* (+Fum)	*G.mon* (+Fum)	*F.oxysporum*	*G.mon* (+Fum)	*G.mon* (+Fum)	*G.mon* (+Fum)
PPA 12	*F.vert* (+Fum)	*G.mon* (+Fum)	*F.oxysporum*	*G.mon* (+Fum)	*G.mon*	*G.mon*

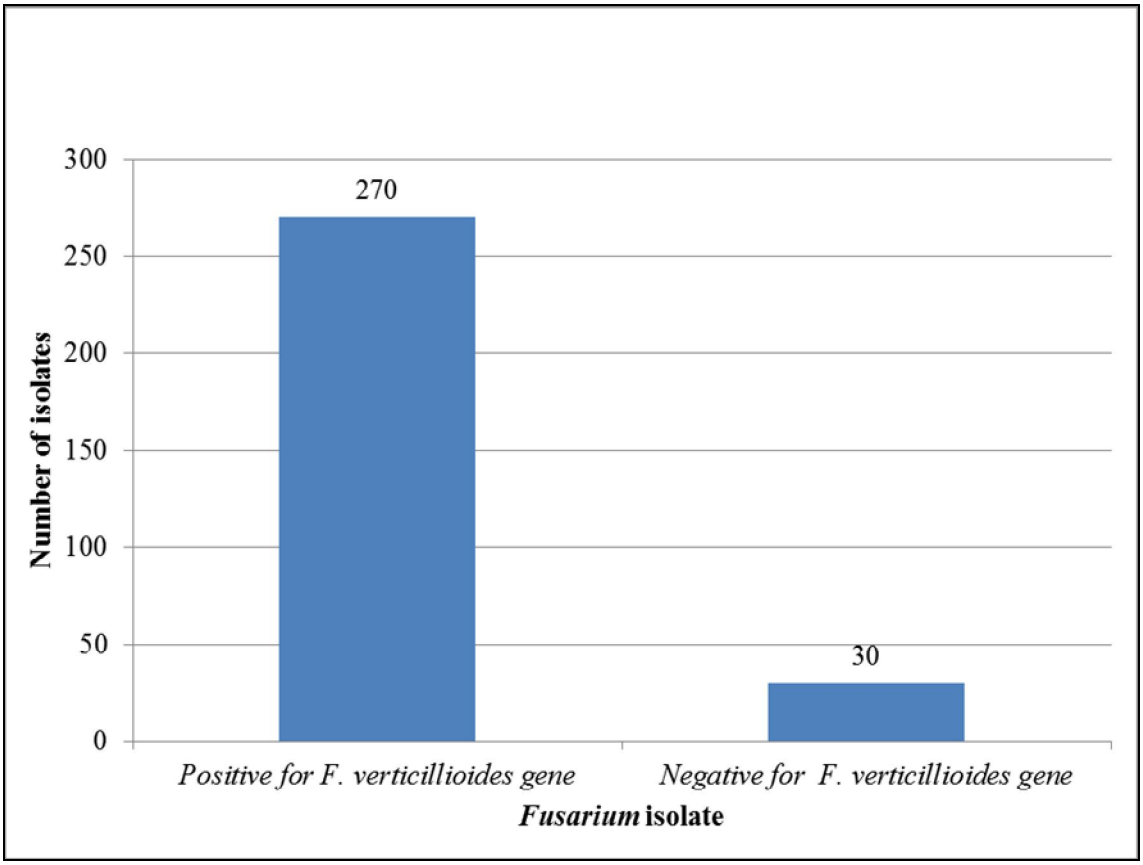


Fig. 7: *Fusarium* isolates testing positive for *vert.* gene from isolates

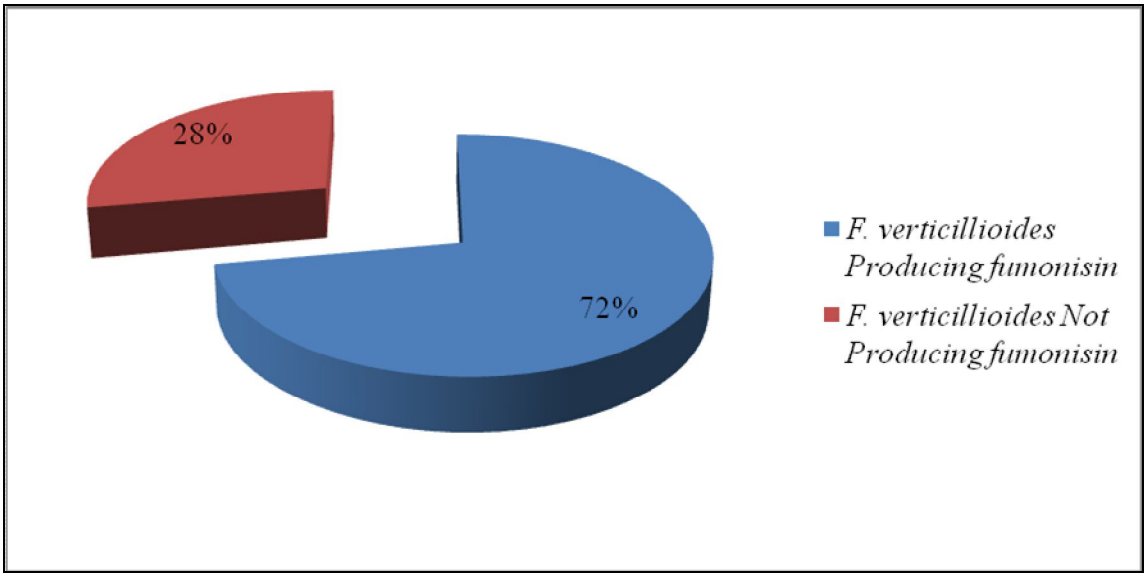


Fig. 8: Frequency of *Fusarium verticillioides* isolates producing fumonisin

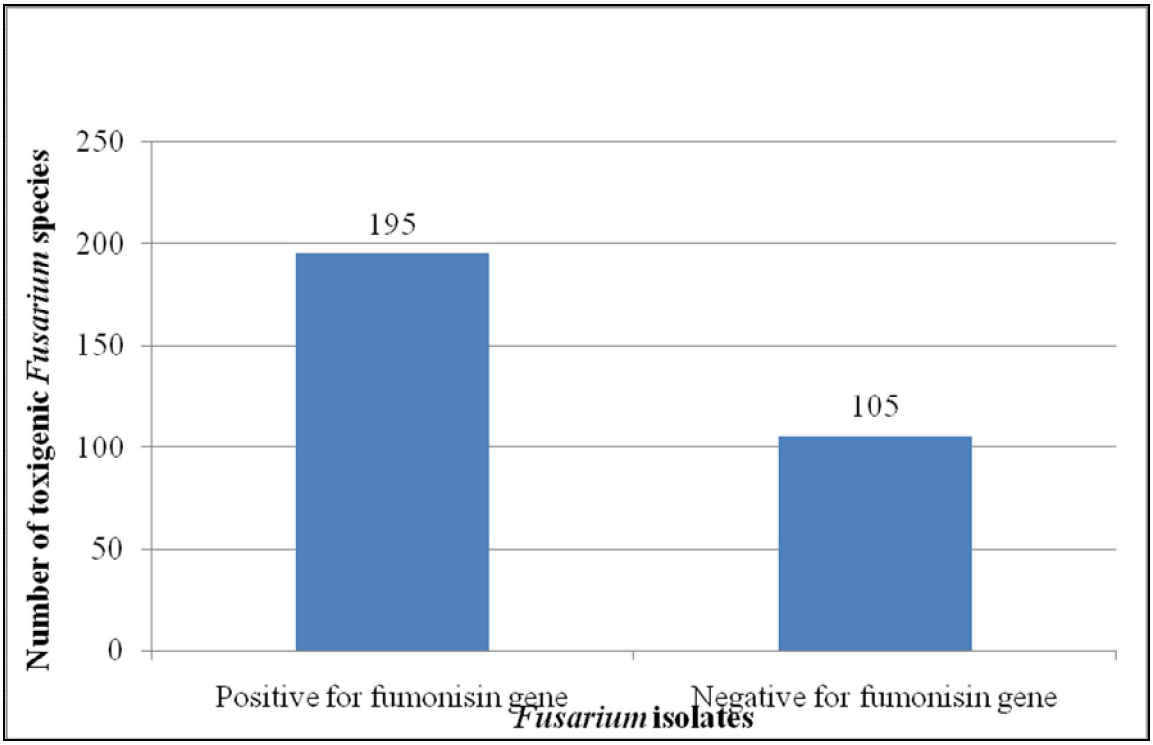


Fig. 9: Fumonisin production by *Fusarium* isolates

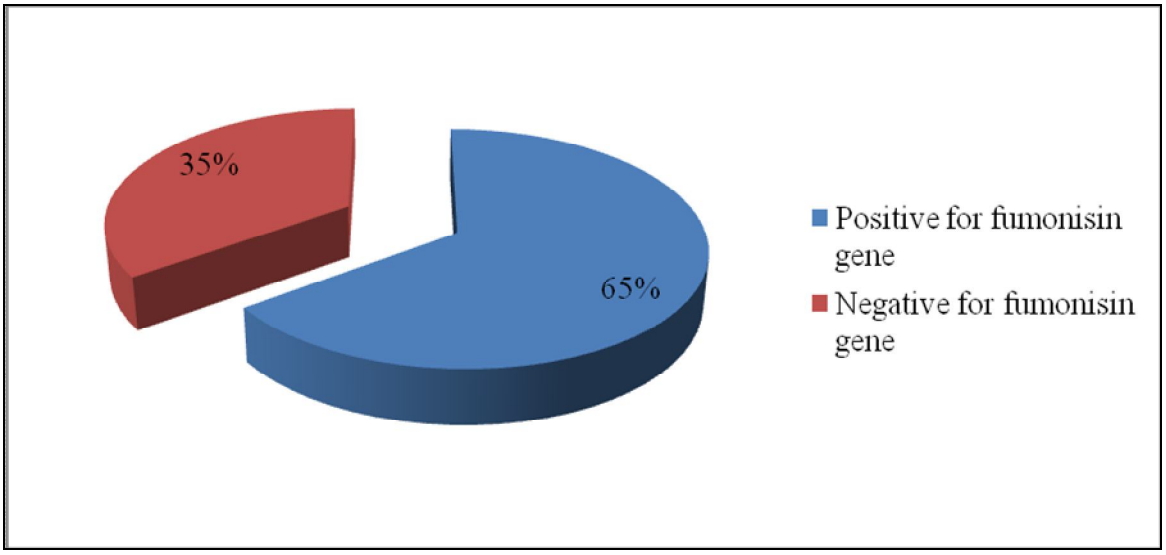


Fig. 10: Frequency of fumonisin producing *Fusarium* isolates

3.7 *Fusarium* isolates Sequences

The 14 isolates used for comparison, further sequenced gave the resulting sequences shown in APPENDIX X. These results were summarized in the table below. The results confirmed the presence of *F. verticillioides* in both its sexual and asexual forms *Giberella moniliformis* and *F. verticillioides* respectively. *F. oxysporum* isolates were also present (Table 9).

Table 9: *Fusarium* cultures sequenced using geneious program

ISOLATE	SPECIES NAME
FV001	*Giberella moniliformis*
FV002	*Giberella moniliformis*
FV004	*Giberella moniliformis*
FV005	*Giberella moniliformis*
FV006	*Giberella moniliformis*
FV007	*Giberella moniliformis*
FV010	*Fusarium oxysporum*
FVT0	*Giberella moniliformis*
FV011	*Giberella moniliformis*
FV012	*Fusarium verticillioides*

3.8 Laboratory maintenance and growth of *Bacillus thuringiensis*

Bacillus thuringiensis cultures were maintained in nutrient broth and nutrient agar cultures at 30°C. Colonies appeared turbid in nutrient broth (Plate 16 A) and smooth and, pale cream on nutrient agar (Plate 16 B) after 48hours incubation.

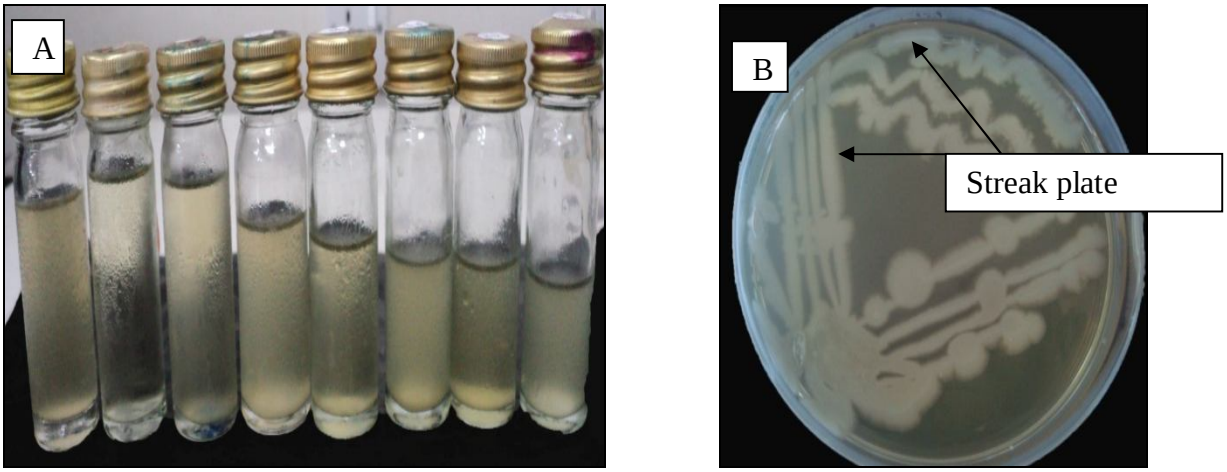


Plate 16: *Bacillus thuringiensis* cultures in; **A:** turbidity indicates growth resulting from microbial multiplication; **B:** streaks show clear pale –cream colonies.

3.9 Determination of Purity of *Bacillus thuringiensis* crystals using microscopy

The presence of endospores, vegetative cells and sporulating *Bt*. cells was confirmed using microscopy. Intracellular formed endospores appeared green (Plate 17A and C) while vegetative cells were stained red (Plate 17 A and B). The vegetative cells (Plate 18) all have endospores within them that are devoid of stain. The vegetative cells are rod shaped, with some occuring in chains(18 A) and (18 B) and some as single cells (18 B). Blue arrows show refractive spores independent of the *Bt*. vegetative cells.

3.9.1 Schaeffer-Fulton Stain

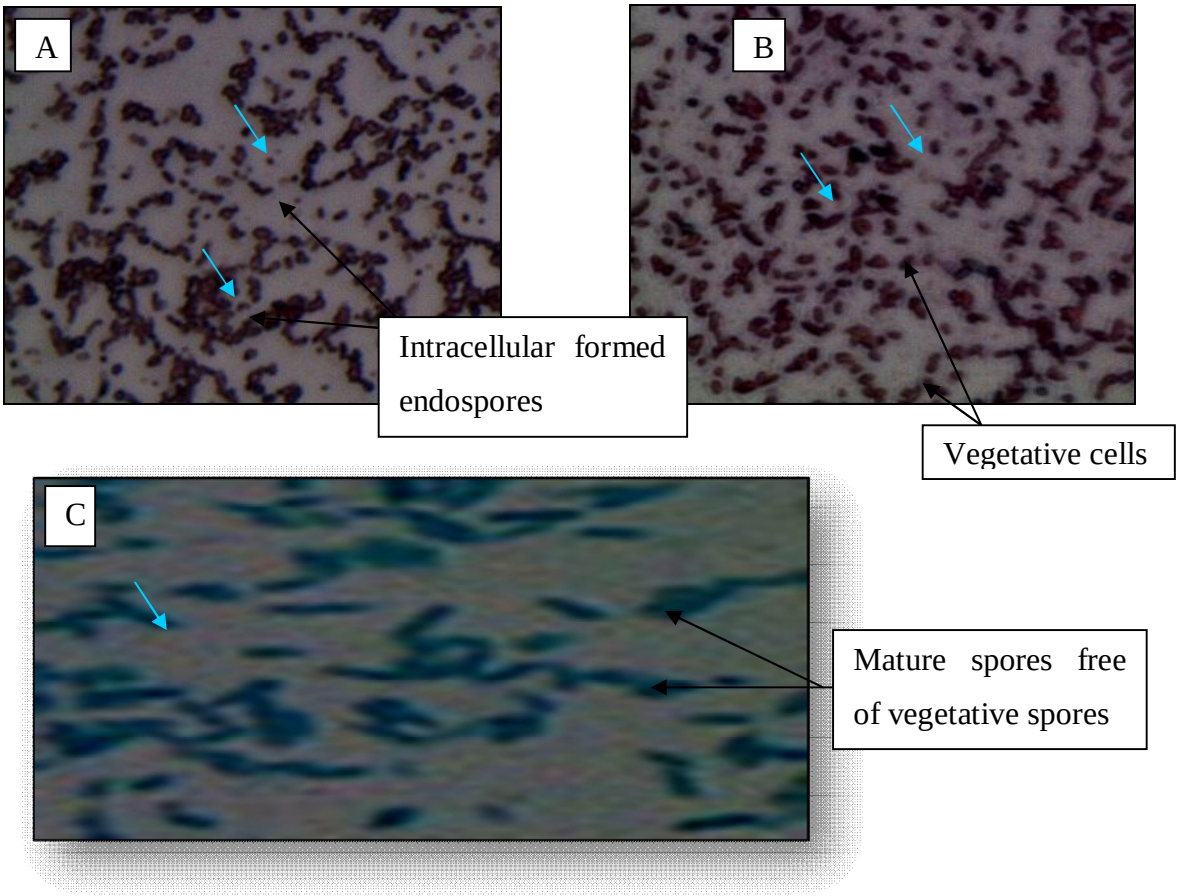


Plate 17: *Bacillus thuringiensis* isolates vegetative cells; **A:** KM 31; **B:** Tenebrionis (TEN); **C:** KG 12; intercellular spores indicated above using blue arrows. (MgX1000)

3.9.2 Gram Stain

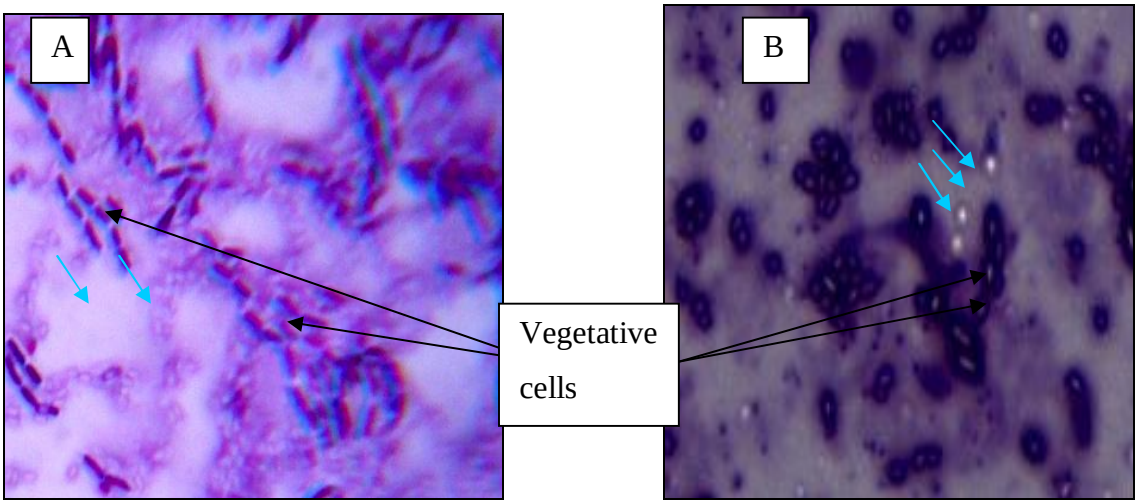


Plate 18: Gram stain reaction for *Bacillus thuringiensis* isolates; **A:** KG 20; **B:** TEN when Sporulating. Black arrows indicate cells with unstained intracellular refractile clear spores, while blue arrows show the spores independent of the cells. Mg X1000

3.9.3 Smirnoff stain

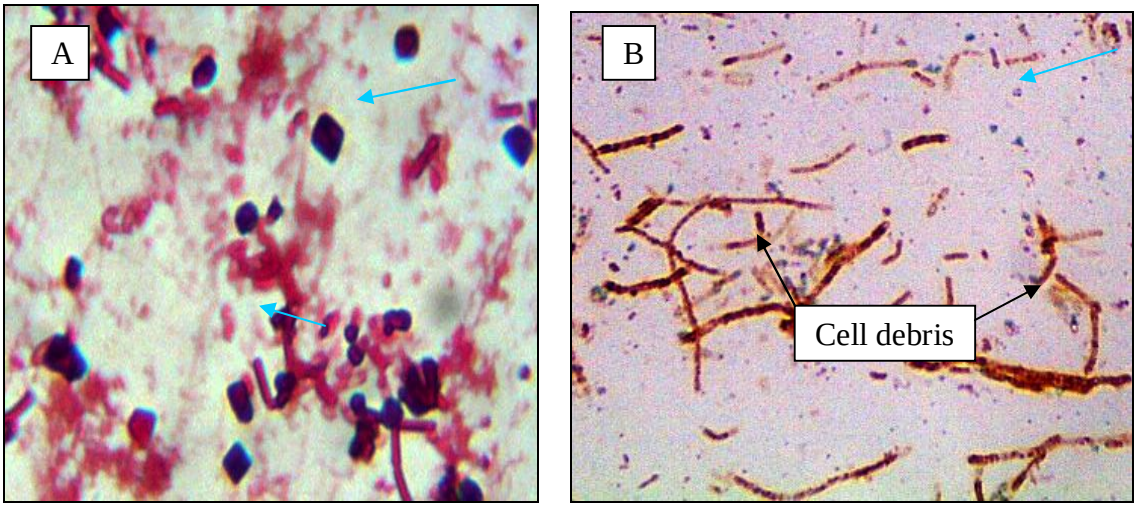


Plate 19: A Parasporal Crystal protein in *Bacillus thuringiensis* isolates; **A:** *Bt.* isolates KG 20; **B:** KM 24, with blue arrows pointing at parasporal crystals outside the cells and cell debris. Mg X1000

3.10 Protein estimation of solubilized crystals

Protein estimation was carried out on both the solubilized protoxin and insoluble protoxin fractions. After isolation from *Bacillus thuringiensis*, crystals from KG 20 showed the highest absorbance rates at 1.62, followed by ISR, KG 411 at 0.78, TEN, KM 31, KM 24 at 0.62, KUR at 0.61, BSA, and finally KG 12-0 at 0.52 (Fig. 11). The absorbance rates of the *Bt*. toxins were similar in rates of absorbance, apart from the toxins from isolates ISR and KG 411 which had higher absorbance readings than the others. The readings were similar to BSA readings, with the exception of KG 20, ISR and KG 411 which had much higher readings than the standard BSA readings.

The highest range of absorption of the soluble solubilized *Bt*. protein was that of KG 20 at 1.1 after obtaining a line of best fit, then ISR, KM 24, KG 411 after TEN, KM 31, BSA, KG 12-0 and KUR (Fig. 12). All the *Bt*. toxins, except KG 20 had similar rates of absorption to that of BSA. The absorption rates were similar to that of the proteins originally isolated from the *Bt*., except for KM 24, whose absorption rate increased greatly from 0.62 to 0.0.85 and KUR, whose absorption rate decreased slightly, from 0.61 to 0.39.When compared to the standard BSA readings, the BSA readings were mostly similar to those of KM31, KG 12-0 and KUR, and similar to all the rest except KG20, which still showed readings as high as those in the original readings after isolation. The readings obtained were lower than those obtained after direct isolation of the toxins from *Bt*., before solubilization.

The highest absorption rate was exhibited by ISR with 0.802 followed by KG 20 readings at 0.79, TEN, KUR, BSA, KM 24, KM 31, at 0.398 and KG 12-0 at 0.37 (Fig. 13). The order of the toxins readings was similar, with slight changes. BSA and KUR had increased intensity in relation to the others. The highest readings changed from the original readings after isolation. The solubilized insoluble protoxin had lower readings. All readings were in line with the standard BSA readings.

Highest absorption rate for soluble *Bt*. Toxins after protease treatment was obtained from ISR with readings at 1.2; this was similar to the readings obtained after Solubilization. KG 20 with readings up to 1.00, KG 12-0, KM31, KM 24, KG411, TEN, KUR, and BSA had the lowest readings at 0.5(Fig. 14).

All readings appeared in line with the BSA standard readings. Especially readings obtained from KG 411, TEN and KUR. There was a slight decrease in readings of KG 20 from 1.1 during the Solubilization treatment to 1.0. ISR readings increased from 0.9 during Solubilization to 1.2. KM24 had similar readings from 0.95 during Solubilization to 0.94 after undergoing protease treatment. The rest had similar readings, thus not much change was observed.

After protease treatment, the insoluble protoxin readings (Fig. 15) were similar to the protein toxin readings obtained right after isolation. KG 20 readings increased from 0.802 after solubilisation treatment to 1.2, ISR, KM 24, BSA, TEN, KG 411, KM31, KUR, KG 12-0, showed the lowest readings at 0.56,which was an increase from previous readings obtained after solubilisation at 0.0.37. The insoluble protease readings were similar to soluble protease readings in that the ISR toxin readings in the insoluble protoxin protease treatment are similar to those obtained by the highest readings by soluble toxin from KG20 after protease treatment, at 1.2. The lowest too, in the soluble protease treatment, KG 411, TEN, KUR and BSA showing similar readings to those shown by the lowest readings in the insoluble protease treatment readings at 0.56 for KG 12-0.

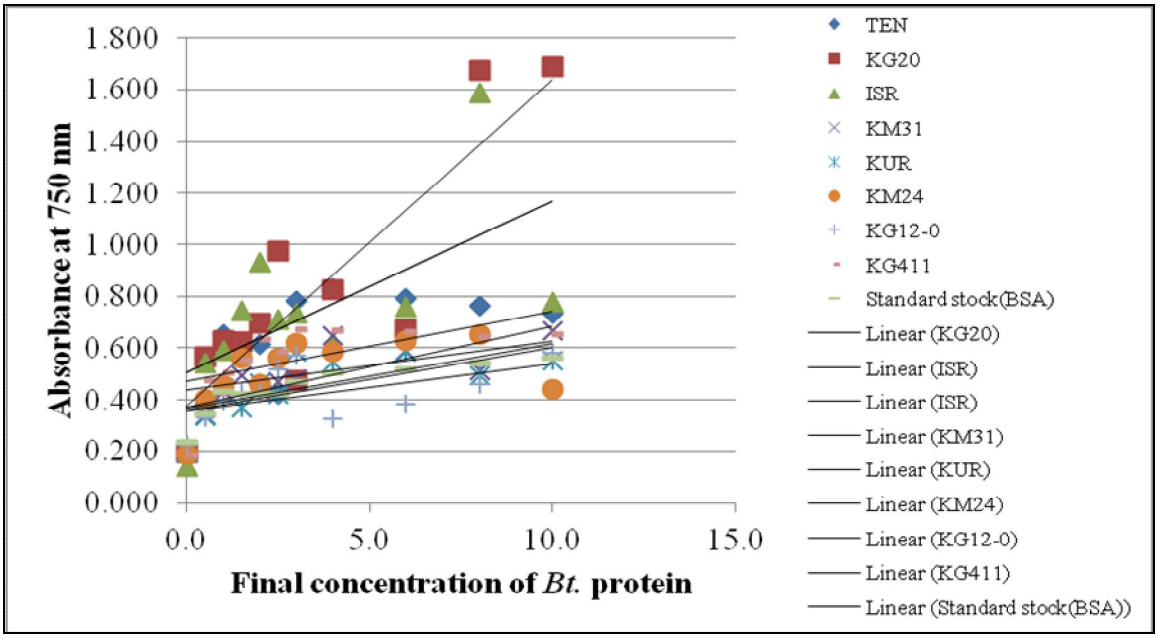


Fig.11: Protein estimation of *Bacillus thuringiensis* toxins after isolation from bacteria

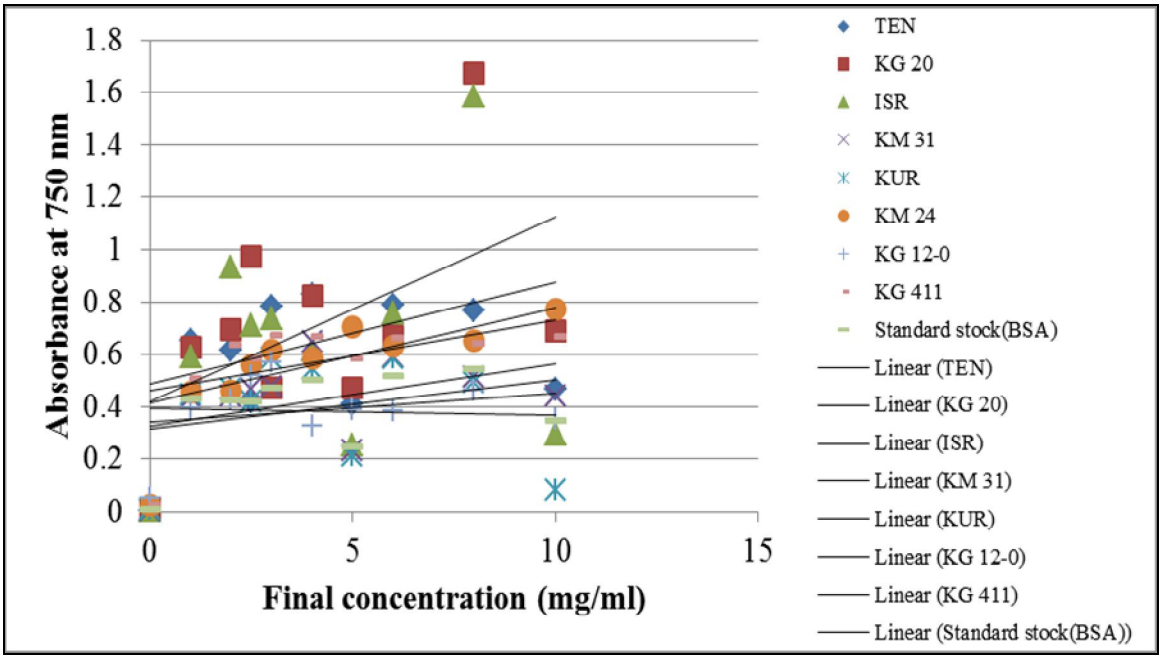


Fig. 12: Protein estimation of soluble *Bacillus thuringiensis* toxins after Solubilization

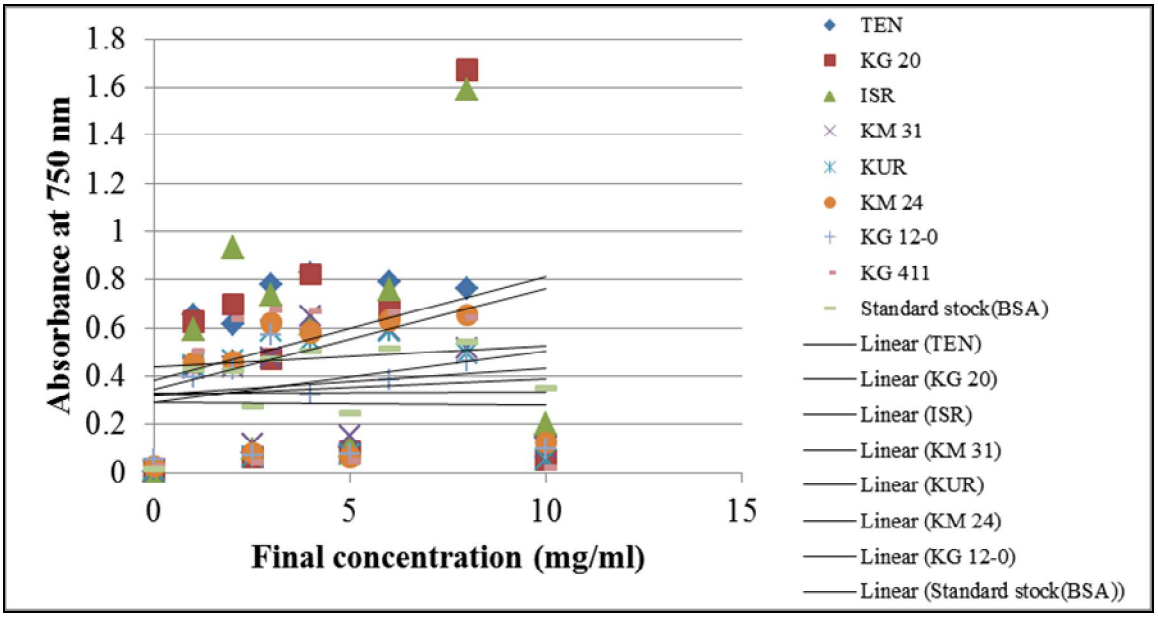


Fig. 13: Lowry assay for insoluble *Bacillus thuringiensis* toxins after Solubilization treatment

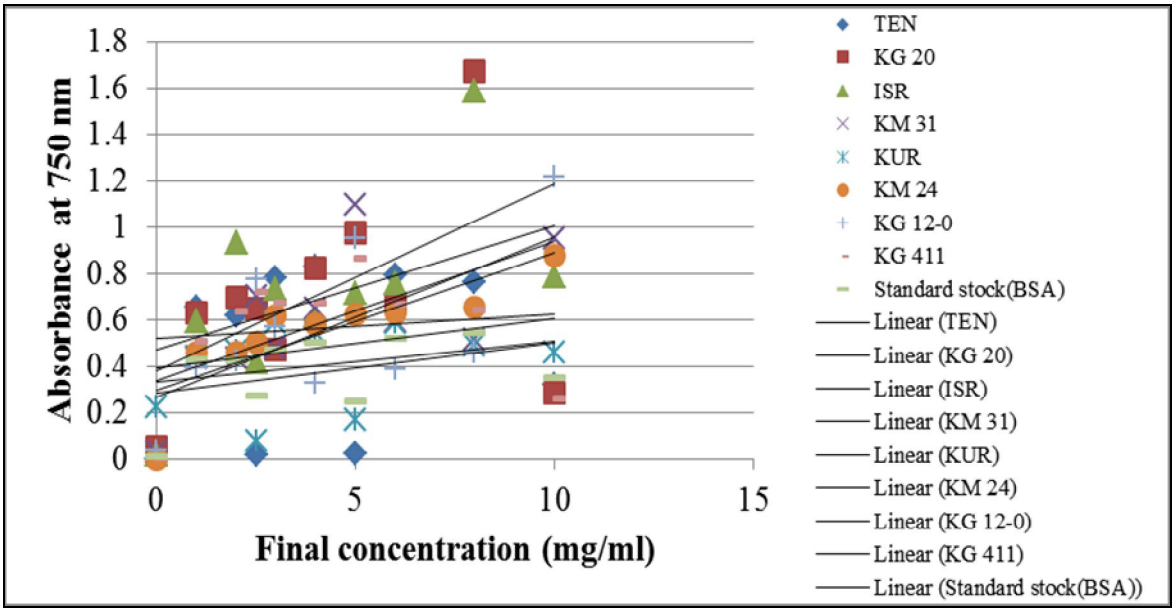


Fig. 14: Lowry assay for soluble *Bacillus thuringiensis* toxins after protease treatment

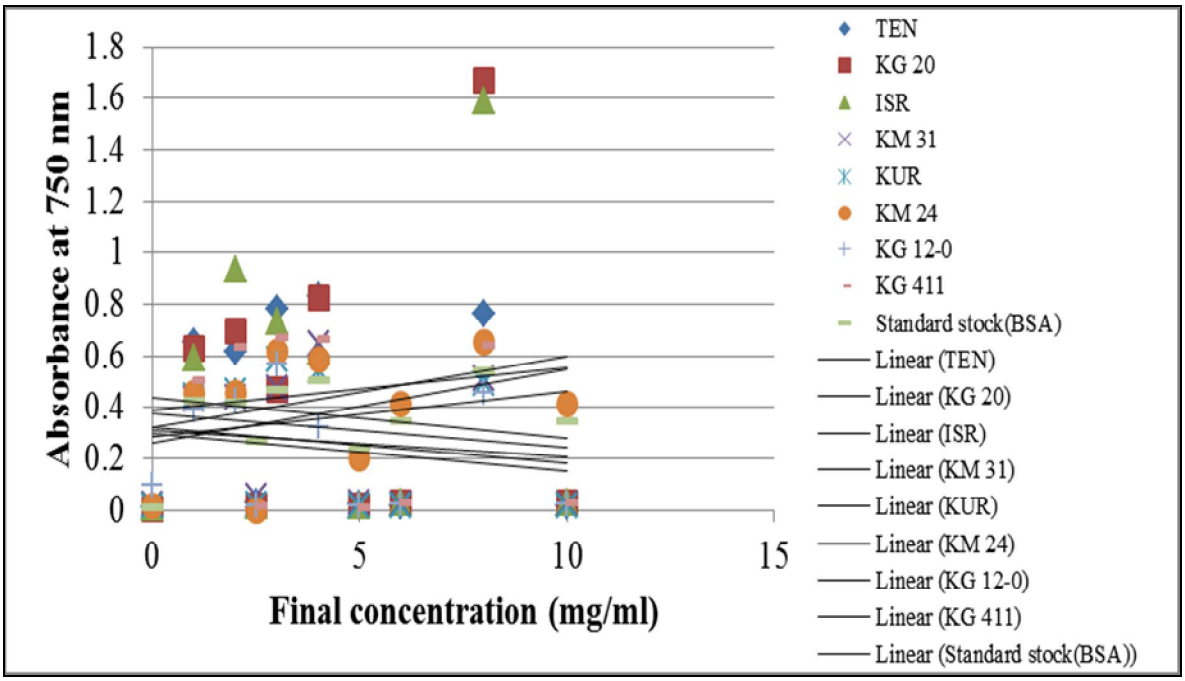


Fig. 15: Lowry assay for insoluble *Bacillus thuringiensis* toxins after protease treatment

3.11 Screening Assays

The *Bt.* toxins in the well-diffusion assays appeared to have different effects on the fumonisin producing *F.verticillioides*. The inhibition zones, devoid of fungal growth around the wells with the toxin, appeared to be short in length (Plate 20).

3.11.1 *Bacillus thuringiensis* toxicity bioassay using *Fusarium verticillioides*

There was no significant difference at p value of 0.05, in the length of inhibition zones among the *F. verticillioides* that had the different *Bt*. toxins (Plate 20). The most effective toxin was by KUR which created small zones of inhibition of 4.1mm, followed by KG 411with a mean length of 3.3mm ISR, TEN, KG 12, KM 24 and KG 20 respectively (Fig. 16). The least effective was KM 31 where the wells with the toxin appeared to have overgrowth of the fungi and no zone of inhibition, same as the control. Compared to the control, significant differences of the toxins were observed at p value of 0.05, and the significant levels were less than this for all cultures except those treated using KM 31, whose significance level was greater than the p value (Appendix VI).

The mean growth inhibitions of *F.verticillioides* under various treatments of *Bt* toxins were compared with the growth inhibition of *F.verticillioides* in the control group. This indicated clear levels of control. Growth of the *F.verticillioides* was either completely inhibited or reduced (Plate 21). This indicated the effectiveness of *Bt*. toxins, against fumonisin producing *F.verticillioides.* Where the toxin was mixed with growth media, significant differences were observed when fungal diameters were compared implying that the various toxins from different strains of *B. thuringiensis* affected growth of *F. verticillioides* differently. The most effective *Bt.* toxins against *F. verticillioides* were TEN at 2.1 cm, KG 411 at 2.2 cm, followed by ISR at 2.3 cm, KG 20at 2.9cm , KG 12 at 3.4, KM 31 at 3.75cm and finally KM 24 at 3.95 cm respectively (Fig. 17).

The mean fungal diameters of KM 24 at 3.95cm and KUR at 4cm were least effective and their toxicity was not significantly different to that of the control where distilled water was used in place of the toxins.

3.11.1.1 *In vitro* plate assay for evaluation of antagonistic activity using well-diffusion assay:

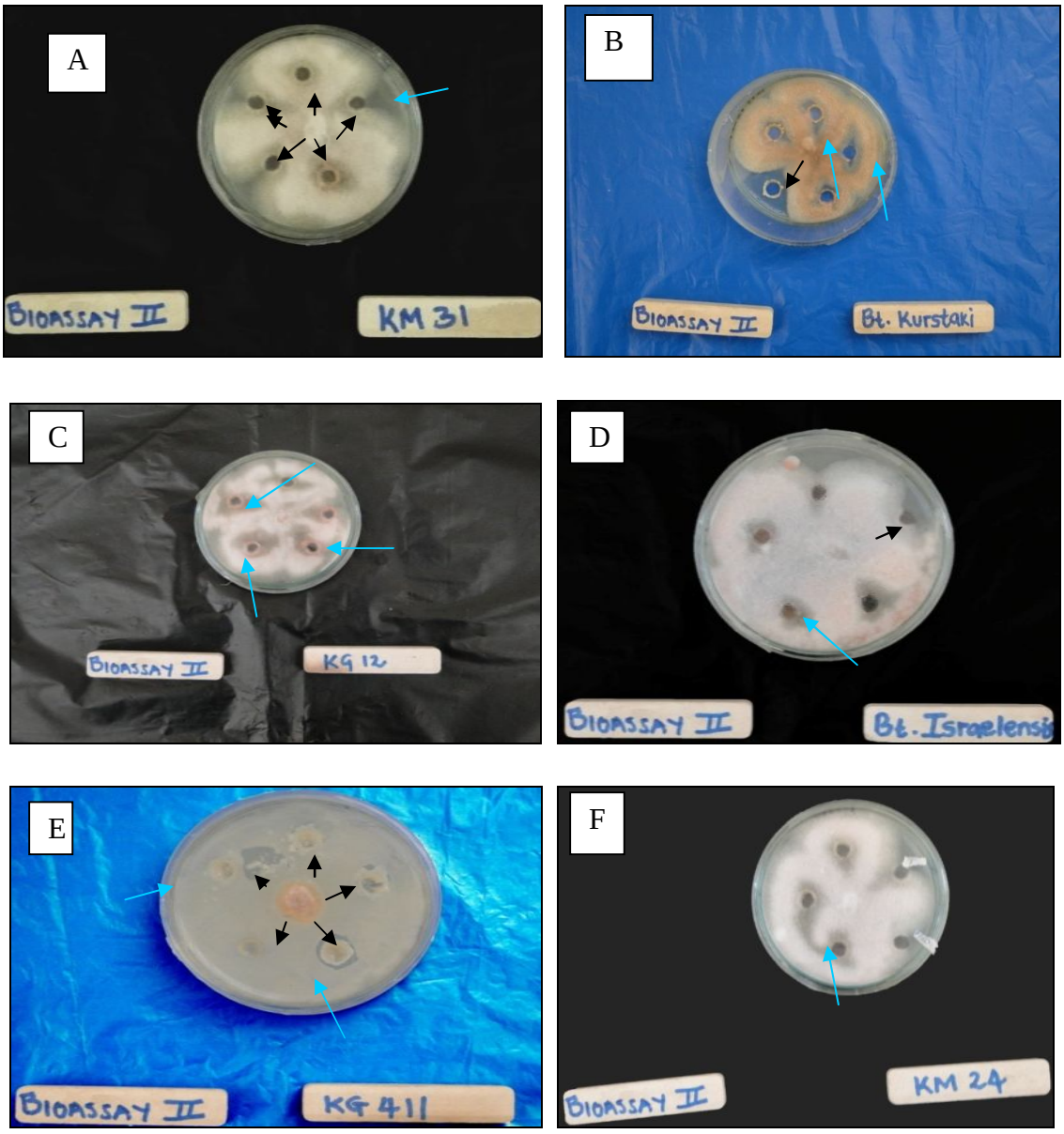


Plate 20: Well-diffusion assays showing *Bacillus thuringiensis* toxins in the wells, creating zones of inhibition of *Fusarium verticillioides* around them. Inhibition is shown using blue arrows. Black arrows show the wells on the plates' periphery ; **A:** KM 31 toxin; **B:** Kurstaki toxin (KUR); **C:** KG 12 toxin; **D:** Israelensis toxin (ISR); **E:** KG 411 *Bacillus thuringiensis* toxins completely inhibits growth of the fungi, and; **F:** Diffusion-wells containing KM 24 *Bacillus thuringiensis* toxins with small areas of inhibition.

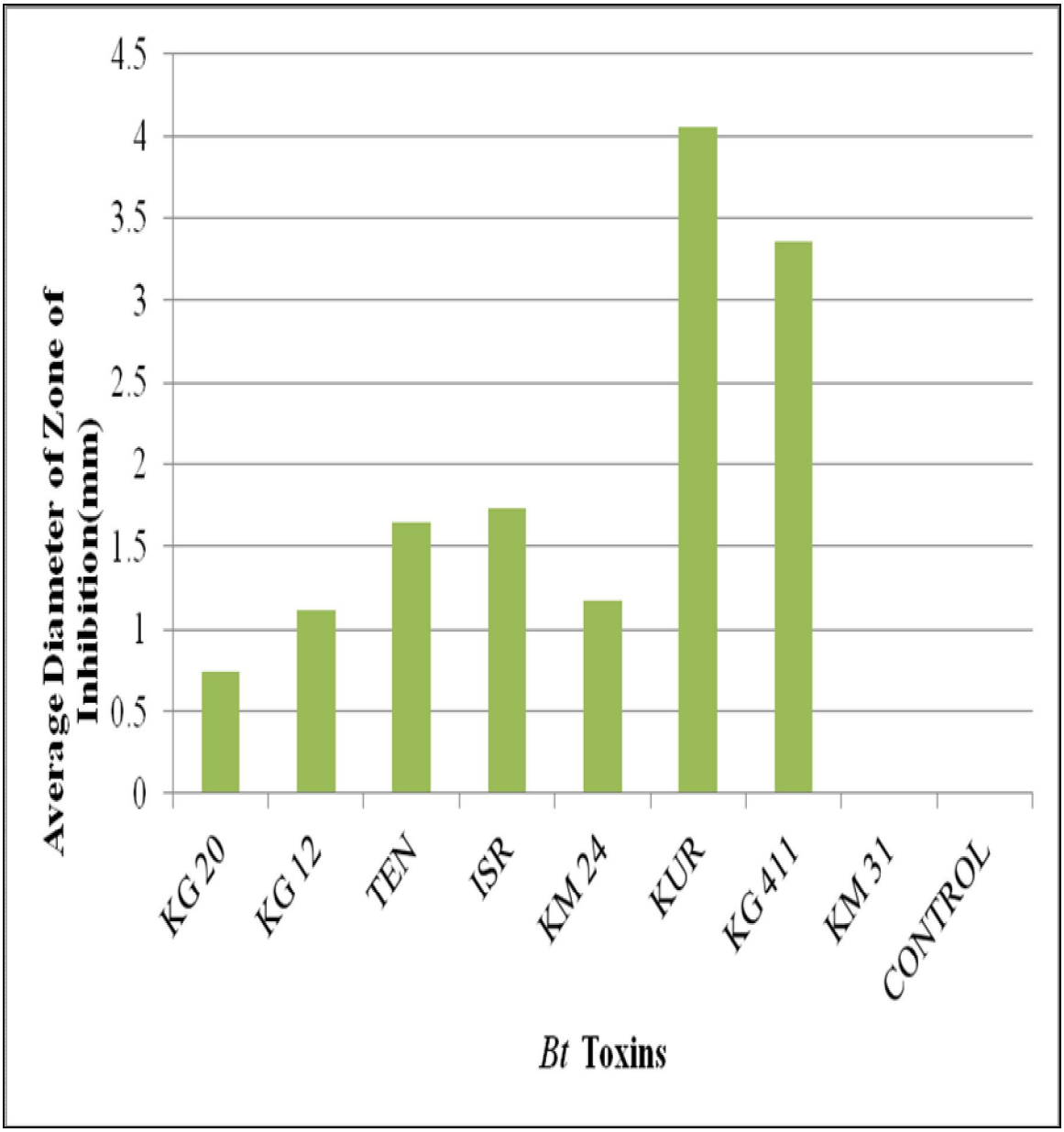


Fig. 16: Zones of inhibition of *Fusarium verticillioides* cultures subjected to different *Bacillus thuringiensis* toxins after 7days (mm)

KEY: TEN-*Bt. Tenebrionis*

KUR- *Bt. Kurstaki*

ISR- *Bt. israelensis*

3.12.1.2 *In vitro* plate assay using *Bacillus thuringiensis* toxin in culture

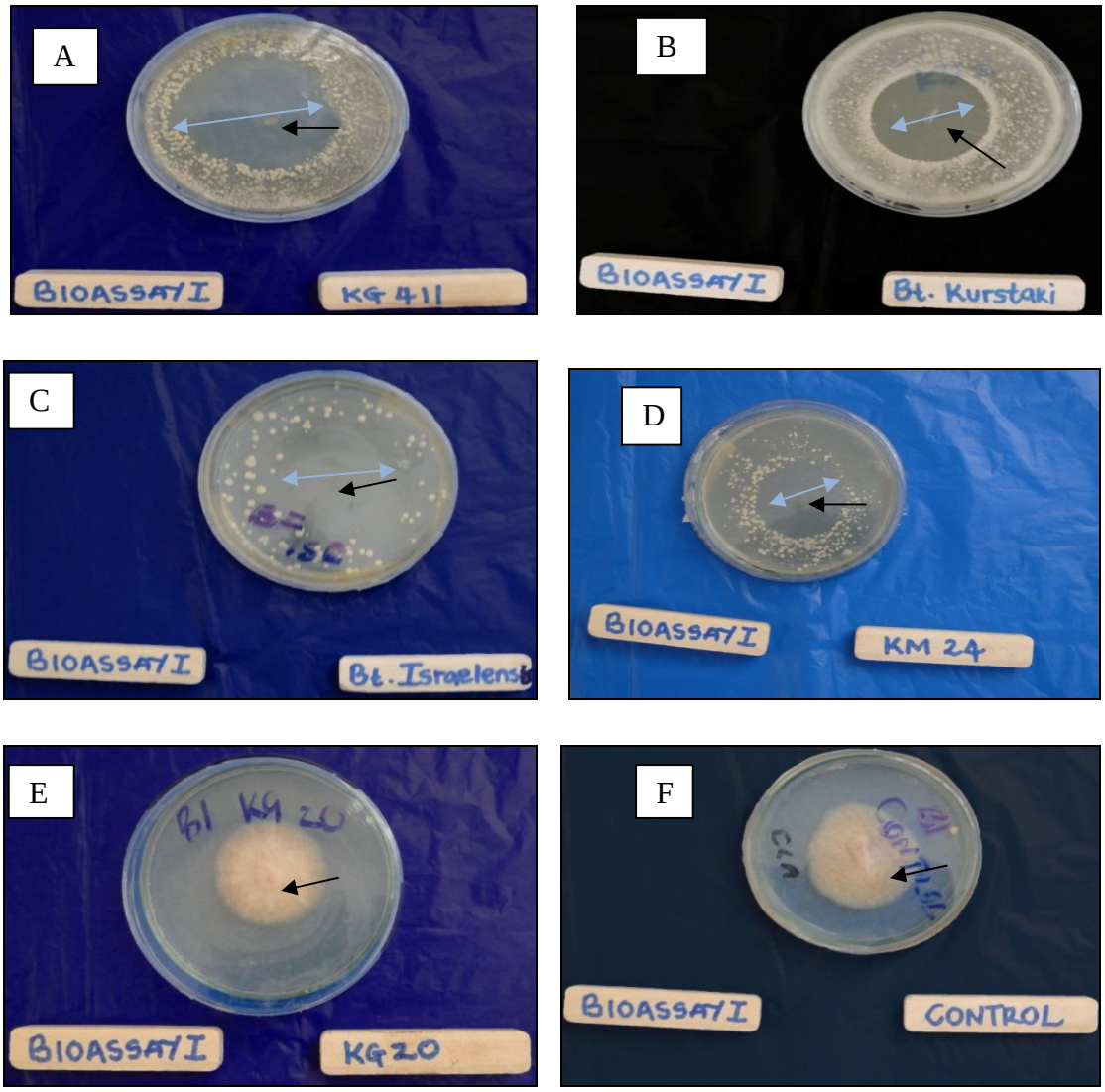


Plate 21: Inhibition of *F. verticillioides* growth in 7 day culture by *Bacillus thuringiensis* toxins mixed in the media; Black arrow shows the fungi plugged in the middle of the plate, while blue arrows show the inhibition zone created as a result of the *Bt*. toxins mixed in the media after 7 days. **A:** definate zone on media with KG 411 toxin; **B:** *Bacillus thuringiensis Kurstaki* toxin; **C:** *Bacillus thuringiensis Israelensis* toxin;**D:** KM 24 toxins. Some reduction of growth as the *Fusarium verticillioides* diameter has been shortened, but no zone of inhibition is created ; **E:** by KG 20 toxin in media, compared to the contol as shown in ;**F:** where no growth inhibition is observed and no zone of inhibition.

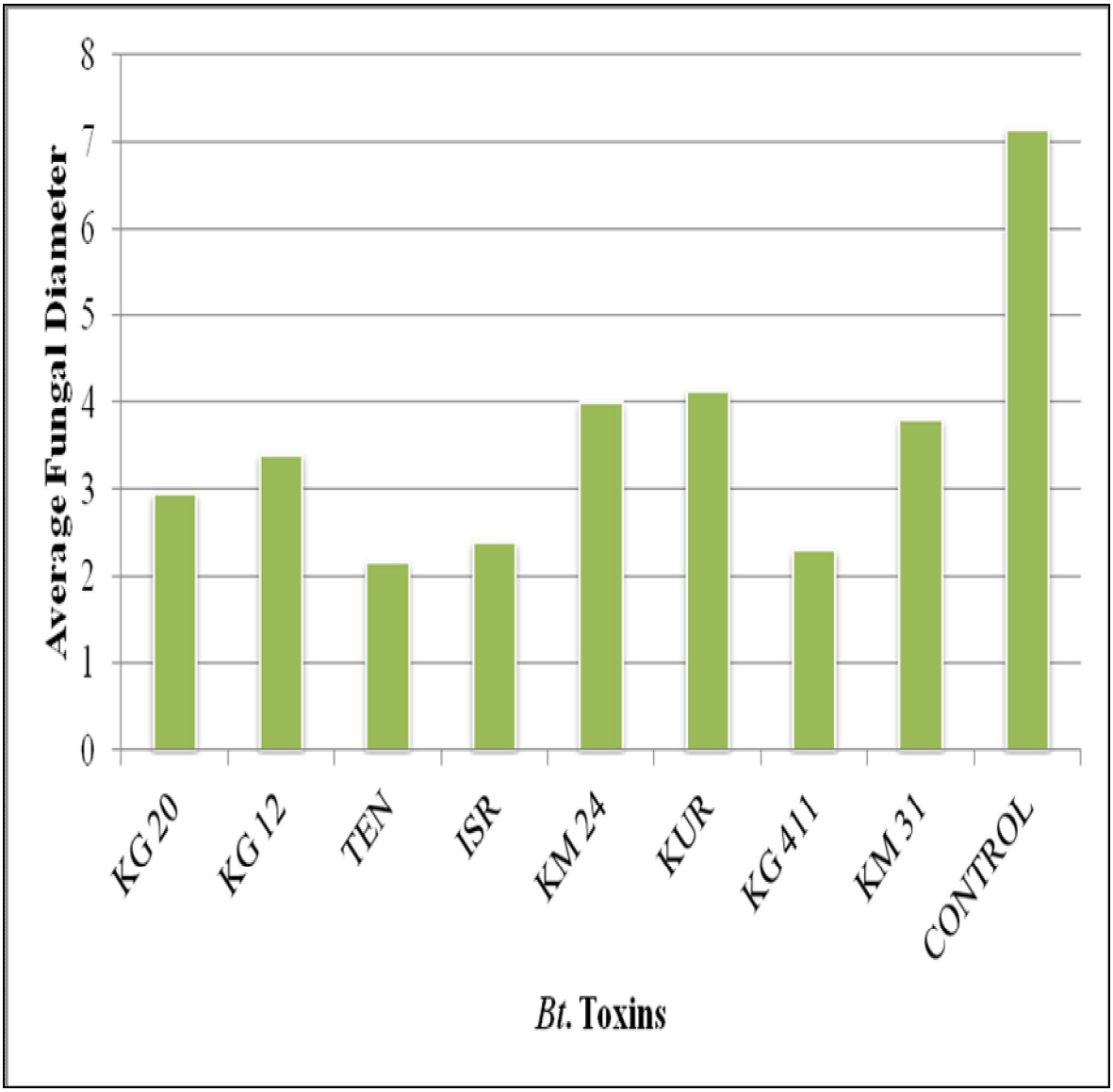


Fig. 17: Fungal diameters of *Fusarium verticillioides* in PDA cultures with different *Bacillus thuringiensis* toxins after 7days (cm)

KEY: TEN-*Bt. Tenebrionis*

KUR- *Bt. Kurstaki*

ISR- *Bt. israelensis*

3.12 *Bacillus thuringiensis* toxicity bioassay using *Prostephanus truncatus*

3.12.1. Toxicity of *Bacillus thuringiensis* Isolates Based on Three Forms of Toxin Delivery; Dry Maize Grains, Dry Maize Particles and Maize Flour Pellets.

Three different modes were used to deliver the *Bt*. toxin to *P.truncatus*, mixing the toxins with maize grains, maize particles or maize flour and as a result, significant differences at p value of 0.05 were observed in mortality. The most active *Bt*. toxin against *P.truncatus* was KG 20; delivered through mixing with maize particles. This gave the highest average mortality of 5.51 (Appendix II). This was followed by maize grains and least in maize flour (Fig. 18).

3.12.2 Toxicity of *Bacillus thuringiensis* Isolates based on solubility of the toxins;

Soluble toxins produced higher mortality rates than those of the insoluble toxins. In soluble form, the maize particles induced the highest rate of mortality. The least was that of ISR, which gave mortality rates that were not significantly different to that of the control, where distilled water was used in place of the toxins. KG 20 showed the highest mortality rate in this form, producing an average mortality of 5.51.The insoluble toxins produced much lower rates of mortality, with KG 12 showing the highest rates at 4.8 (Appendix I). The toxins each appeared to act differently on *P. truncatus*. The highest rates in soluble form, appeared to be highest in KG20, followed by KG 12, KG411, KM31, KM24, KUR, ISR, respectively, all which showed significant effect against *P. truncatus*, TEN, however appeared to be least effective, with mortality rates that were negligible and similar to those exhibited by the control, where distilled water was used in place of the *Bt*. toxin (Table 10; Appendix III). Mortality of *P.truncatus* due to *Bt* toxins in maize particles was highest unlike in maize flour where least mortality of *P.truncatus* was observed (Fig 18). Insoluble form of the toxins were less active than the soluble form against *P.truncatus*. The insoluble *Bt* toxin with the highest activity against *P.truncatus* was KG 12 on maize grains(Average mortality=4.80 Appendix II) . The effect of the insoluble toxins on *P.truncatus* was high in maize grains, followed by maize particles while the effect was least on maize flour (Fig.19).

Table 10: Average Mortality of *Prostephanus truncatus* when subjected to different *Bacillus thuringiensis* toxins in using 3 different modes of delivery Maize Grains (MG), Maize Particles (MP) and Maize Flour (MF)

	KM24						KG12					
	M G		M P		M F		M G		M P		M F	
	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	sol	Insol
MM	3.47	2.20	2.98	2.17	2.16	2.90	4.36	4.80	3.30	2.85	2.85	2.85
	KG20						KG411					
	M G		M P		M F		M G		M P		M F	
	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	sol	Insol
MM	2.88	4.11	5.51	3.28	3.01	1.88	2.95	4.15	3.20	2.90	2.50	2.70
	ISR						TEN					
	M G		M P		M F		MG		MP		M F	
	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	sol	Insol
MM	2.80	1.94	2.81	2.30	2.67	2.30	3.80	3.59	2.35	2.90	2.40	1.85
	KUR						KM31					
	M G		M P		M F		M G		M P		M F	
	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	Sol	Insol	sol	Insol
MM	2.84	2.50	2.95	2.80	2.63	3.13	2.15	3.00	3.17	3.15	2.42	2.96
	CONTROL											
	M G		M P		M F							
	Sol	Insol	Sol	Insol	Sol	Insol						
MM	1.1	1.1	1.1	2.5	1.2	1.2						

Key:

Sol = Soluble form of *Bt.* toxin, Insol = Insoluble form of *Bt.* toxin, MM=Mean Mortality

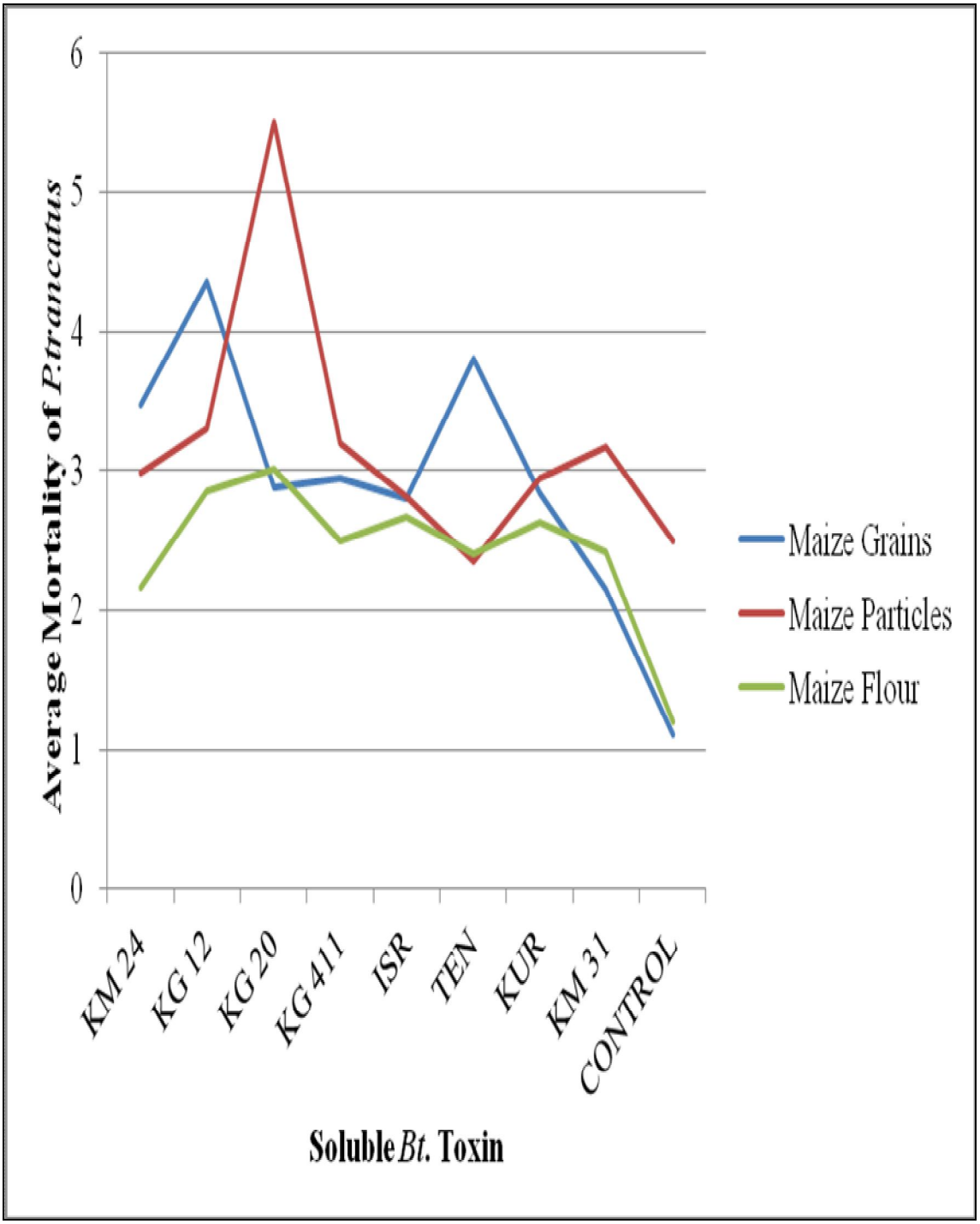


Fig. 18: Soluble *Bacillus thuringiensis* toxins in different delivery forms against *Prostephanus truncatus*

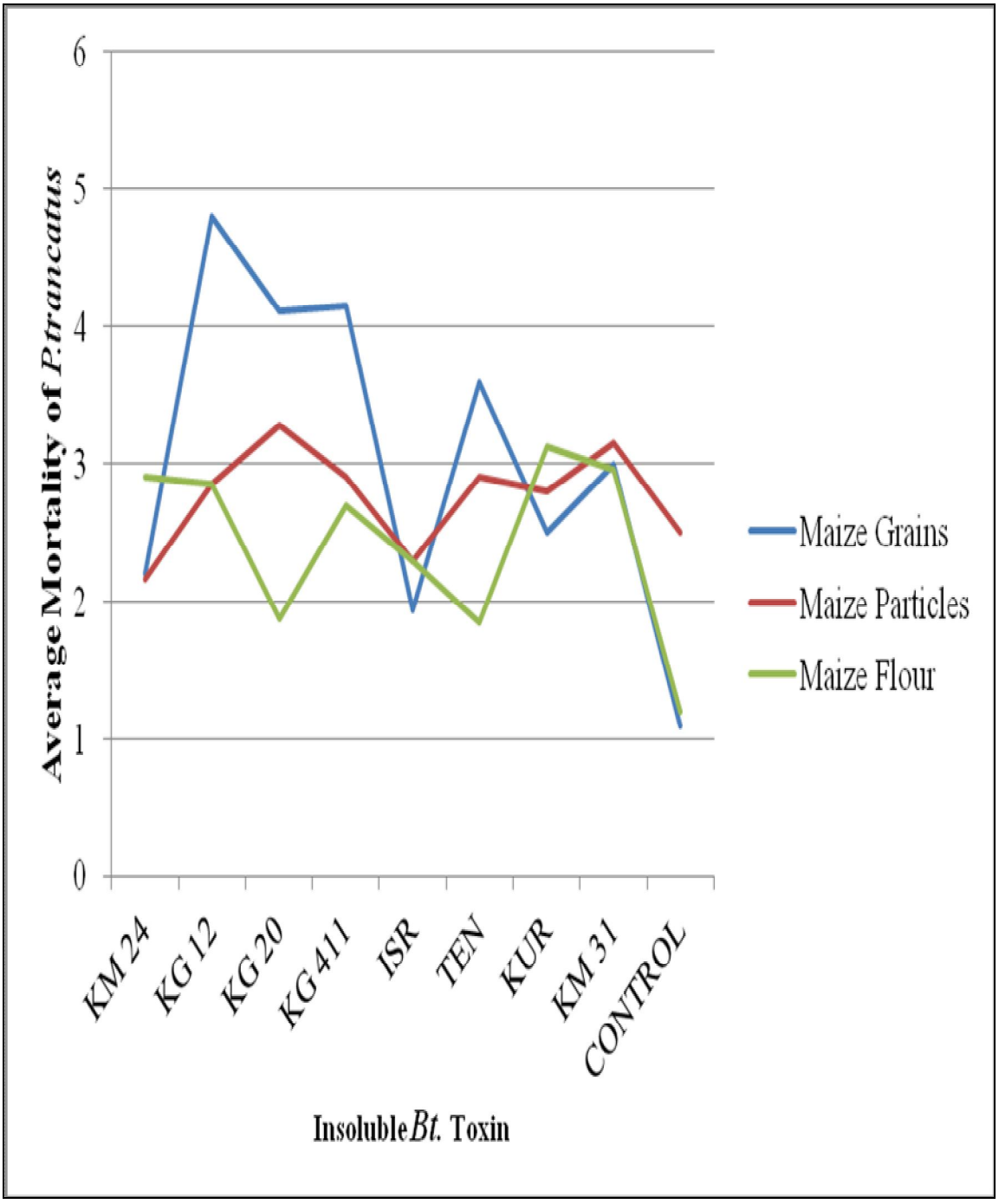


Fig. 19: Insoluble *Bacillus thuringiensis* toxins in different delivery forms against *Prostephanus truncatus*

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Discussion

4.1 Isolation and Characterization of *Fusarium verticillioides*

Fusarium isolates were successfully obtained, from both maize grains appearing clean and those that appeared contaminated.One hundred and eighty three clean maize grains were used during isolation, in addition to 117 that appeared contaminated on the surface, and there was isolation of *Fusarium* from all grains used. This indicates that *Fusarium* prevalence was high despite a higher frequency of maize grains that appeared clean being used for isolation at 61% as observed from the results, than the infected grains at 39%.

A large number of *Fusarium* isolates were obtained after isolation from maize grains. *Fusarium* isolation from 100% of the grains used for isolation as observed confirms other sources of literature which state that *Fusarium* is widely spread in Makueni district, in Kenya's Eastern province and occurs naturally within the region (Muthomi *et al*., 2002; Okoth and Siamento, 2011; Kedera *et al*., 1999; Kheseli *et al*., 2011).

Purification of the *Fusarium* cultures and further characterization showed that most isolates produced convex, wooly, and round configured cultures, and most had white aerial mycelia (Gerlach and Nirenberg, 1982), they were also easily distinguished by the presence of other morphological characters such as exudate production, diffusate presence, and the color at the bottom of the cultures (Wollenweber, 1913). Characterization using microscopy was similar to that of Burgess *et al*., (1988), whereby, the shape of the microconidia, the number of septa and the presence of monophialides and abundance of false heads verified the type of *Fusarium* in each case. This consequently led to identification of the *Fusarium* isolates, however the isolates obtained each appeared to have some differences in terms of their morphological characteristics, this in turn brought about a problem in that it made the identification process more complex, since isolates of the same species sometimes appeared to differ in features, this brought about irregularities as observed in the results obtained.

The observation made enabled the comparability of features between the isolates. Characterization and identification of *F. verticillioides* was carried out as their rate of growth is moderate to fast and should be approximately 7-8cm in seven days, compared to other species in sections that are known to be slow growers thus confirming the work by Gerlach and Nirenberg in 1982. The sparse amount of macroconidia and large production of microconidia in aerial mycelia was similar to results documented by Messiaen and Cassini in 1968 that indicates these conditions in *F. verticillioides* cultures. Cultures in CLA media, indicated spore shapes as illustrated by Gerlach and Nirenberg (1982). The absence of chlamydospores in most of the isolated cultures typically indicated the presence of *F.verticillioides*, as did, the presence of both branched and unbranched monophialides and abundant false heads. The absence of polyphialides was also a main indicator of *F. verticillioides*, as documented by Gerlach and Nirenberg in 1982. The frequency of the isolated species after comparison showed a high level of isolation of *F. verticillioides* from the maize grains. The use of molecular methods in identification of the species was however more precise as it was able to identify species specifically, according to their DNA. VERT-1(5'-GCGGGAATTCCAAAGTGGCC-3') and reverse VERT-R (5'-CGACTCACGGCCAGGAAACC-3') which are species specific PCR primers for the identification of *F. verticillioides* were used after morphological methods of identification were carried out. Specific primers, VERTF-1, VERTF-2 and Fro-fum8-rev were used to analyze the isolated *F. verticillioides* for fumonisin production. Translation Elongation Factor TEF-1a gene was amplified using primers EF-1 and EF-2 and the resulting PCR product was used to carry out sequencing for confirmation of identity of the isolated species. This methods used together consequently led to the positive identification of *F. verticillioides* at 80%, *G. moniliformis* at 10% and *F. oxysporum* at 10% of the isolates obtained.

Gel electrophoresis bands were analyzed and the results were similar to those obtained by O'Donnell *et al.*, in 1998. The bands indicated the presence of *F. verticillioides* in both its sexual and asexual Stages. Isolates from 270 of the maize grains used were positive for the vert gene, and were thus identified as *F. verticillioides*. The results similarly, indicated the presence of the Fum gene, which was indicated by the bands in the gel, and the size of the DNA, by confirming its molecular weight in the gel, as compared with the

DNA ladder. The frequency of *F. verticillioides* isolates producing fumonisins was observed to be at 72%, while those of *F.verticillioides* isolates not producing fumonisins was 28%, this indicates high levels of production of fumonisins by *F. verticillioides* species found in Kenya. Molecular analysis of the *Fusarium* isolates indicated that 195 of the isolates were positive for the Fum gene, while 105 of the isolates were not. This thus indicates that at 65% a higher frequency of the isolates, are fumonisin producers, while, a lower frequency of the isolates at 35% are non fumonisin producers. Sequencing described as, "the gold standard method of confirmation of a species true identity" (Balajee *et al*., 2005), was finally done, and confirmed findings obtained through morphological and microscopic evaluation. The findings corroborated those found by Balajee *et al*. in 2005. This showed that *F. verticillioides* is dominantly present in maize grains in Kenya and that most of them are fumonisin producers and thus presents high risk because fumonisins cause disease in man and animals.

4.2 Confirmation of *Bacillus thuringiensis* Parasporal Crystals presence for Bioassay and Protein Estimation of their toxins

Bacillus thuringiensis cultures revealed smooth pale cream colonies similar to those shown by Wang'ondu in 2001. The Schauffer-Fulton stain, revealed red vegetative cells with green endospores as inclusions within the cells, similar to the findings by Sneath in 1986. Endospores were found present both within and independent of the vegetative cells. The vegetative cells appeared to have taken the red safranine counter stain, while the green endospores, appeared highly resistant to this dye. Mature spores stained green whether free or still in the vegetative sporangia. The gram stain, revealed cells stained a deep purple color (Wang'ondu, 2001), and appearing rod shaped as documented by Aizawa (1901). The endospores were readily recognized microscopically, by their intracellular site of formation as shown by Rasko (2005). Some were also seen outside the cells as were also shown in the results. Smirnoff stain helped in the observation of cell debris, and parasporal bodies. This confirmed that the microbes used were *B. thuringiensis*, their purity and their ability to produce spores and subsequently toxins for the control of *F.verticillioides* and *P.truncatus* when carrying out the bioassays was also confirmed.

Protein Estimation of *Bt.* toxins was carried out as directed by Lowry (1951). This revealed that the readings obtained were high after direct isolation from the *B. thuringiensis* microbes, and then lowered considerably after solubilization treatment, Protease treatment then increased the readings obtained slightly. The toxins used for the bioassays involving *F. verticillioides* were not activated using the proteases or subjected to solubilization treatments. Solubilization provides alkaline conditions and Proteases activate the protoxin to toxin, thus enhances the action of the *Bt*. toxins in insect pests.

Protease treatment appeared most effective in the soluble protoxins, showing more mortality, than in the case of the insoluble protoxins used, when subjected to *P. truncatus*. This was similar to findings shown by Likhayo *et al*., (2002). There was no significant difference in terms of Protein activity, between the insoluble and soluble protoxins when protein content was measured after solubilization and protease treatments.

4.3 *Bacillus thuringiensis* toxicity bioassay against *Fusarium verticillioides*

Disk diffusion wells were used, as described with slight modification by Iqbal *et al.* (1999) to contain the *Bt.* toxins and the presence of inhibition zones were observed and their lengths recorded. These showed some rate of inhibition, and no significant difference between the reactions of the different toxins. This inhibition rates were similar findings to those by Kheseli *et al.,* (2011).

When *In vitro* plate assays using *B. thuringiensis* toxin in culture were used, the toxins acted as inhibiters of growth against the fungi. No significant difference was exhibited when the toxins effects were compared amongst themselves. When compared against the control, there appeared to be a large amount of significance in the controlling effects of *F. verticillioides* growth using the *Bt*. toxins. The *F. verticillioides* cultures' growth appeared to be successfully inhibited by the *Bt*. toxins, to a great extent. This was done in triplicates and the mean results, showed high rates of inhibition *F. verticillioides* cultures treated using the *Bt*. toxins. These results appeared similar to those presented by Kheseli *et al.,* (2011), showing complete inhibition of growth and formation of inhibition zones.

4.4 *Bacillus thuringiensis* toxicity bioassay using *Prostephanus truncatus*

The form of delivery with the highest mortality rate was maize particles, followed by maize grains, and finally maize flour, this is in line with findings by Kheseli *et al*., (2011). This was as a result of polling together mortality rates observed using all 3 modes of delivery, in both the soluble and insoluble toxin treated maize cultures.

The soluble form of the toxins produced higher mortality rates than that of the toxins that were insoluble. In soluble form, the maize particles induced the highest rate of mortality; here the best toxins appeared to be that of KG 20, followed by 12-0, KG 411, KM 24, KM 31, KUR, ISR, TEN appeared to have the lowest mortality rate, just as low as that exhibited by the control in this bioassay. Lower mortality rates were exhibited when insoluble toxins were used, with highest mortality being observed in maize grains. Highest mortality on *P.truncatus* in insoluble form was by KG 12-0 whose average mortality was lower than that of the soluble maize grains. These findings are similar to those shown by Likhayo *et al*., (2002). It also confirms that solubilized crystals exhibit higher activity, when used against insect pests. The results obtained, show that native species of *B. thuringiensis*, can be used effectively as biocontrol, against both *F. verticillioides* and *P. truncatus*. This is similar to findings and conclusions by Kheseli *et al*., (2011) showing the effectiveness of *Bt*. to against both fungi and insect pests, and by Likhayo *et al*., (2002) documenting effective strains of *Bt*. against *P. truncatus* in Kenya.

4.5 Conclusions

In conclusion, this study shows that *F. verticillioides* has a high rate of incidence in maize grains in Kenya. This brings to attention a worrying issue, since *F. verticillioides* was isolated even from maize grains that did not show any signs of infection, or disease. This study shows varieties of native *B. thuringiensis* able to counter the threat brought about by *P. truncatus* and *F. verticillioides*, it also shows that native varieties of *B. thuringiensis* could be used to lessen the effects of pests in an effective, affordable and safe way.

4.6 Recommendations

Potency and effectiveness of *Bt.* as a biopesticide could be brought about by carrying out additional research through more isolation of novel *Bt.* locally, for use against agricultural pests, and subsequent introduction of more methods that are easier and cost effective, for characterization of *Bt.* and their toxins.

Methods should be devised and used to further enhance potency of the above *Bt.* varieties that have been shown to be effective against both *P.truncatus* and *F.verticillioides* so as to avail *Bt.* toxins that are safe and more potent than currently available, unsafe cocktails of chemical pesticides.

More fungal varieties and insect pests of maize grains should undergo similar treatments to find out if there is a larger scope of pests that can be effectively eradicated using this, and other locally isolated *Bt.* toxins. More tests can be conducted using Vegetative insecticidal proteins (Vips), from the varieties used in this study and other novel varieties.

The varieties used in this study and confirmed to be effective, may be used to create transgenic plants, with resistance, or reduced susceptibility to the above plant pests. This will do away with problems brought about as a result of topical application of these toxins, which has short lived results, due to Ultra violet rays exposure from the sun.

REFERENCE

Abeysinghe S. (2006) Biological control of *Fusarium oxysporum* f.sp. *Radicis-cucumerinum*, the casual agent of root and stem rot of *Cucumis sativus* by non-pathogenic *Fusarium oxysporum*. *Ruhuna Journal of Sci*ence, 1, 24-31.

Agarry, O.O., Akinyosoye F.A. and Adetuyi F.C. (2005) Antagonistic properties of microorganisms associated with cassava (*Manihot esculenta Crantz*) products. *African Journal of Biotechnol*ogy, 4, 627-632.

Aizawa K. (2001) Shigetane Ishiwata: His discovery of sottokin (*Bacillus thuringiensis*) in 1901 and subsequent investigations in Japan. *Proceedings of a Centennial Symposium Commemorating Ishiwata's Discovery of Bacillus thuringiensis*.

Allah-Fadl M.E. (1998) Occurrence and toxigenicity of *Fusarium moniliforme* from freshly harvested maize ears with special references to fumonisin production in Egypt. *Mycopathologia,* 140, 99–103.

Alastruey-Izquierdo .A.; Estrella M.C.; Monzo´ n A.; Mellado E.; Tudela J.L.R. (2008) Antifungal susceptibility profile of clinical *Fusarium* spp. isolates identified by molecular methods *Journal of Antimicrobial Chemotherapy,* 61, 805–809.

Altschul S.F.; Gish W.; Miller W.; Myers E.W; Lipman D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biol*ogy,215 , 403-410.

Anon. (1969) Royal Botanic Garden Edinburgh. Flora of the British Fungi. Colour Identification Chart. *Edinburgh Her Majesty´s Stationery Office*.

Aronson A.I.; Beckman W.; Dunn P. (1986) *B. thuringiensis* and related insect pathogens. *Microbiol*ogy, 50, 1-24.

Baba-Moussa A.A.M.T. (1998) La microflore associée aux dégâts des lépidoptères foreurs de tiges et mineurs d'épis de maïs (*Zea mays*) dans la région Sud du Bénin avec référence spéciale à *Fusarium moniliforme* Sheld. Mémoire d'Ingénieur Agronome. Université du Bénin, Lomé, Togo.

Bacon CW.; Hinton D.M. (2000) Biological control of *Fusarium moniliforme* in corn by competitive exclusion using *Bacillus mojavensis*. Aflatoxin/Fumonisin Workshop. October 25–27, 2000.

Balajee S. A.; Borman A. M.; Brandt M. E.; Cano J.; Cuenca-Estrella M.; Dannaoui E.; Guarro J.; Haase G.; Kibbler C.C.; Meyer W.; O'Donnell K.; Petti C. A.; Rodriguez-Tudela J. L.; Sutton D.; Velegraki A.; Wickes B. L. (2009) Sequence-Based Identification of *Aspergillus, Fusarium*, and *Mucorales* Species in the Clinical Mycology Laboratory: Where Are We and Where Should We Go from Here? *Journal of Clinical Microbiology*, 47(4), 877–884.

Balajee, S. A.; Gribskov J.; Brandt M.; Ito J.; Fothergill A.; Marr K. A. (2005) Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. *Journal of Clinical Microbiol*ogy, 43, 5996–5999.

Balzer, A.; Tardieu D.; Bailly J.D.; Guerre P. (2004) The *trichothecenes*: Toxins nature, natural occurrence in food and feeds and ways of struggle. *Journal of Veterinary Medicine*., 155, 299-314.

Bedford, O. (1980) Biology, ecology and control of palm rhinoceros beetles. *Annual Review of Entomology,* 25, 309-339.

Beiriger, R.L., Sites, R.W. (1996). The *Bostrichidae* (*Coleoptera*) of Missouri. *Journal of the Kansas Entomological Society*, 69(1), 45-68.

Bezuidenhout, S.C.; Gelderblom W.C.A; Gorst-Allaman C.P.; Horak R. M.; Marasas W.F.O.; Spiteller G.; Vleggaar. R. (1988) Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *Journal of the Chemical Society, Chemical Communications*., 743-745.

Biliwa, A.; Böye J.; Fischer H. U.; Helbig J.; (1992) Stratégie de lâchers et études de suivi de *Teretriosoma nigrescens* au Togo, pp. 138-142. *In* Böye, J., M. Wright, and G.-A. Laborius (eds.). *Implementation of and further Research on Biological Control of the Larger Grain Borer*. *Proceedings of an FAO/GTZ Co-ordination Meeting*. FAO, Rome, Italy, and GTZ, Eschborn, Germany. FAO, Rome, Italy.

Booth C. (1971) 'The Genus *Fusarium*' (Commonwealth Mycological Institute: Kew, Surray, UK).

Borgemeister, C.; Djossou F.; Adda C.; Schneider H.; Djomamou B.; Azoma K.; Markham R. H..(1997a) Establishment spread and impact of *Teretriosoma nigrescens* Lewis (*Coleoptera: Histeridae*), an exotic predator of the larger grain borer *Prostephanus truncatus* (Horn) (*Coleoptera: Bostrichidae*), in south-western Bénin. *Environmental Entomology*, 26, 1405-1415.

Borgemeister, C., Holst, N. & Hodges, R.J. (2003) Biological control and other pest management options for larger grain borer *Prostephanus truncatus*. pp. 311–328 *in* Neuenschwander, P., Borgemeister, C., & Langewald, J. (*Eds*) *Biological control in IPM systems in Africa*. Wallingford, Oxon, CAB International.

Brownbridge M. (1989) Isolation of new entomopathogenic strains of *B. thuringiensis and Bacillus sphaericus*. Israel *Journal of Entomology.*, 23, 109- 113.

Burgess, L.W., Liddell, C. M., and Summerell, B. A. (1988) Laboratory manual for *Fusarium* research. 2nd ed. University of Sydney, Sydney, Australia. Pp. 156.

Burgess L.W, Summerell B.A, Bullock S, Gott K.P, Blackhose D (1994) 'Laboratory Manual for *Fusarium* Research.'(University of Sydney:Sydney, Australia)

Burgess L.W, Summerell B.A.,Nelson P.E.(1991) An evaluation of several media for use in the identification of some *Fusarium* species. *Australian Plant Pathology,* 20, 86-88.

Cahagnier, B., Melcion D., and Richard-Molard D. (1995) Growth of *Fusarium moniliforme* and its biosynthesis of fumonisin B1 on maize grain as a function of different water activities. *Letters in Applied Microbiology*., 20, 247-251.

Cavaglieri L., Orlandoa J., Rodríguez M.I., Chulze S., Etcheverry M. (2005) Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro and at the maize root level . *Research in Microbiology,* 156, 748–754.

Charles W. Bacon, Ida E. Yates, Dorothy M. Hinton, Filmore Meridith. (2001) Biological Control of *F. moniliforme* in maize, *Environmental Health Perspectives,* 109(2), 325-332.

Chelkowski, J. (1998) Distribution of *Fusarium* Species and Their Mycotoxins in Cereal Grains. In: *Mycotoxins in Agriculture and Food Safety*, Sinhaand, K.K. and D. Bhatnagar (Eds.) pp: 520.

De Barjac, H. and Frachon, E. (1990) Classification of *Bacillus thuringiensis* strains. *Entemophaga,* 35, 233-240.

De Maagd, R. A., Bravo A., and Crickmore N. (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics*., 17, 193–199.

Demianyk, C.J., Sinha, R.N. (1988) Bioenergetics of the larger grain borer, *Prostephanus truncatus* (Horn) (*Coleoptera: Bostrichidae*), feeding on corn. *Annals of the Entomological Society of America*, 81 (3), 449-459.

Desjardins, A. E.; Plattner, R. D. and Gordon, T. R. (2000) *Gibberella fujikuroi* mating population A and *Fusarium subglutinans* from *teosinte* species and maize from Mexico and Central America. *Mycological Research.*, 104, 865-872.

Dulmage, H. T.; Boeing, O. P.; Rehnborg, C. S.; Hansen, G.D. (1971) A proposed standard bioassay for formulations of *B. thuringiensis* based on the international unit. *Journal of Invertebrate Pathology*, 18, 240–245.

Dunstan, W.R. and Magazini, I.A. (1981) Outbreaks and new records. Tanzania. The larger grain borer on stored products. *FAO Plant Protection Bulletin* 29, 80–81.

Dutton, M.F. (1966) Fumonisins,Mycotoxins of increasing importance:their nature andtheir effects. *Pharmacological therapy* 70, 137-161.

Dutton,M.F. and Westlake, K.(1985) Occurrence of mycotoxinsin cereals and animal foodstuffs in Natal, South Africa. *Journal of the Association of official Analytical Chemists* 68, 839-842.

Duvik, J., (2001) Prospects for reducing fumonisin contamination of maize through genetic modification. *Environtal Health Perspectives*., 109, 337-342.

Dunstan, W.R. and Magazini, I.A. (1981) Outbreaks and new records. Tanzania. The larger grain borer on stored products. *FAO Plant Protection Bulletin* 29, 80–81.

Dutton, M.F. (1966) Fumonisins,Mycotoxins of increasing importance:their nature andtheir effects. *Pharmacological therapy,* 70,137-161

Dutton,M.F. and Westlake, K.(1985) Occurrence of mycotoxinsin cereals and animal foodstuffs in Natal, South Africa. *Journal of the Association of official Analytical Chemists,* 68, 839-842

Duvik, J., (2001) Prospects for reducing fumonisin contamination of maize through genetic modification. *Environtal Health Perspectives*., 109, 337-342.

Edgar, Robert C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Research*, 32(5), 1792-97.

El-Hamshary, O.I.M. and Khattab A.A. (2008) Evaluation of antimicrobial activity of *Bacillus subtilis* and *Bacillus cereus* and their fusants against *Fusarium solani*. *Research Journal of Cell and Molecular Biology*., 2, 24-29.

El-Shabrawy, E. M. (2001) Studies on ear and kernel rot of maize caused by *Aspergillus* and *Fusarium* spp. M.Sc. Thesis, Fac. Agric., Tanta Univ. 80.

Environmental Health Criteria (EHC). (2000) Environmental Health Criteria 219: fumonisin B1, International Programme on Chemical Safety (IPCS; UNEP, ILO and WHO). Eds. W.H.O.

Estruch, J. J., Warren G. W., Mullins M. A., Nye G. J., Craig J. A., and Koziel M. G. (1996) Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proceedings of the National Academy of Sciences USA,* 93, 5389–5394.

Farrell, G., Hill, M.G., Nang‘ayo, F.L.O. and Stabrawa, A. (1996b) A review of investigations to improve pest management of stored maize in smallholder farm in Kenya. *Integrated Pest Management Reviews,* 1, 251–263.

Fandohan P., Hell K., Marasas W.F.O., Wingfield M.J.(2003) Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African Journal of Biotechnology*, Vol. 2 (12), 570-579.

Farrell, G. and Schulten, G.G.M. (2002) Larger grain borer in Africa, a history of efforts to limit its impact. *Integrated Pest Management Review*, 7, 67 – 84.

Federici, B. A. (2005) Insecticidal bacteria: an overwhelming success for invertebrate pathology. *Journal of Invertebrate Pathology*, 89, 30–38.

Fisher, W.S. (1950). A revision of the North American species of beetles belonging to the family *Bostrichidae*. US Department of Agriculture, Miscellaneous Publication 698, 36-42.

Food and Agriculture Organization (FAO). (2002) FAOSTAT Database, Food and Agriculture Organization, Roma, Italy Flamboyant, Benin. 125 pp.

Galán-Wong L.J., Pereyra-Alférez B., Luna-Olvera H.A. (2006) Bacterias entomopatógenas. Biotecnología financiera aplicada a bioplaguicidas. In: García-Gutiérrez C, Medrano-Roldán H, Eds. México pp. 43-63.

Gelderblom, W.C.A., Kriek, N.P.J, Marasas, W.F.O. and Farber (1992) The cancer initiating potential of Fumonisin B mycotoxins. *Carcinogenesis*, 13, 433-437.

Gerlach, W. and Nirenberg H. (1982) The genus *Fusarium* a pictorial adas. Mitt. Biol. Bundesanst. Land-Forst-wirtsch., *Berlin-Dahlem*, 209, 1-406.

Gile P.H. and Kibata G.N. (1992) Recent developments in Kenya on the ecology and control of *Prostephanus truncatus*. In; *Implementation of the further research on Biology and control of the Larger Grain Borer*, Proceedings of the FAO/GTZ Coordination Meeting , Lome, Togo, November, 1990, Boeye, J.MM, Wright and G.A. Larius (Eds,),pp 14-16.

Golob, P. (1988) Chemical control of the larger grain borer pp. 53–69 *in* Schulten, G.G.M. and Toet, and A.J. (*Eds*) *Report II Technical papers presented at the workshop. Workshop on the containment and control of the larger grain borer, Arusha, Tanzania, (16–21 May 1988)*. Ministry of Agriculture and Livestock Development, Tanzania, and Food and Agriculture Organization of the United Nations, Rome, Italy.

Groves F.D., Zhang L., Chang Y.S., Ross P.F., Casper H., Norred W.P., You W.C., Fraumeni J.F. (1999) Jr: *Fusarium* mycotoxins in corn and corn products in a high-risk area for gastric cancer in Shandong Province, China. *Journal of the Association of Official Analytical Chemists International.*, 82, 657-62.

Guarro, J., and Gene J. (1992) *Fusarium* infections. Criteria for the identification of the responsible species. *Mycoses,* 35, 109–114.

Gueye, M.T., Goergen, G., Badiane, D., Hell, K. and Lamboni, L. (2008) First report on occurrence of the larger grain borer *Prostephanus truncatus* (Horn) (*Coleoptera: Bostrichidae*) in Senegal. *African Entomology*, 16 (2), 309 – 311.

Gwinner, J., Harnisch, R. and Müch, O. (1996) Manuel sur la manutention et la conservation des grains après-récolte. GTZ, Eschborn, Germany. 368 pp.

Haider, M. Z., Knowles, B. H. and Ellar, D. J. (1986) *European Journal of Biochemistry*., 156, 531-540.

Harman G.E. (2006) Overview of Mechanisms and uses of *Trichoderma* spp. *Phytopathology*, 96, 190-194.

Harnisch, R. & Krall, S. (1984) Togo: further distribution of the larger grain borer in Africa. *FAO Plant Protection Bulletin,* 32, 113–114.

Haubruge .E, Gaspar .C (1990) Détermination en laboratoire des zones favorables au dévelopement des populations du grand capucin du grain, *Prostephanus truncatus* (Horn), en Afrique. *L'Agronomie Tropicale*, 45, 251-258.

Hendricks K. (1999) Fumonisins and neural tube defects in south Texas. *Epidemiology*, 10, 198-200.

Hennequin, C.; Lavarde V.; Poirot J. L.; Rabodonirina M.; Datry A.; Aractingi S.; Dupouy C. J.; Caillot D.; Grange F.; Kures L.; Morin O.; Lebeau B.; Bretagne S.; Guigen C.; Basset D. and Grillot R. (1997) Invasive *Fusarium* infections: a retrospective survey of 31 cases. The French Groupe d'Etudes des Mycoses Opportunistes. *Journal of Medical and Veterinary Mycology*., 35, 107–114.

Hodges, R.J. (1983) A review of the biology and control of the greater grain borer *Prostephanus truncatus* (Horn). *Tropical Stored Products Information*, 43, 3-9.

Hodges, R.J. (1986) The biology and control of *Prostephanus truncatus* (Horn) (*Coleoptera: Bostrichidae*) - A destructive storage pest with an increasing range. *Journal of Stored Products Research*, 22(1), 1-14.

Hodges, R. J.(1994) Recent advances in the biology and control of *Prostephanus truncatus* (*Coleptera: Bostrichidae*). In: Stored Product Protection. Proceedings of the 6th International Working Conference on Stored Product Protection. 17-23 April 1994, Canberra, Australia. Ed. by Highleye, Wright, E. J., Banks, H. J., Champ, B. R. Wallingford, UK: CAB International, 929-934.

Hodges, R.J., Farrell, G. & Golob, P. (1996) Review of the larger grain borer outbreak in East Africa – rate of spread and pest status. pp. 29–41 *in* Farrell, G., Greathead, A.H., Hill, M.G. & Kibata, G.N. (*Eds*) *Management of farm storage pests in East and Central Africa. Proceedings of the East and Central African Storage Pest Management*

Workshop, 14–19 April 1996, Naivasha, Kenya. International Institute of Biological Control (IIBC), Ascot, Berks.

Höfte H, Whiteley H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev.*, 53, 242-255.

Iqbal A.; Ahmed S.; Abid Ali S. and Rasool S.A. (1999). Isolation and partial purification of Bac201: A plasmid associated bacteriocin-like inhibitory substance from *Staphylococcus aureus* AB201. *Journal of Basic Microbiology*, 39, 325-336.

Iriarte J, Caballero P. (2001) Biología y ecología de *Bacillus thuringiensis*. Bioinsecticidas: Fundamentos y Aplicaciones de *Bacillus thuringiensis* en el Control Integrado de Plagas. Caballero P, Ferré J, Eds. *Phytoma-España*, 15-44.

Julian, A.M.; Wareing P.W; Phillips S.I.; Medlock V.F.P.; Macdonald M.V. and Del Rio L.E., (1995) Fungal contamination and selected mycotoxins in pre-and post-harvest maize in Honduras. *Mycopathologia*, 129, 5-16

Kassou D.K, Aho N. (1993) Stockage et conservation des grains alimentaires tropicaux: principes et pratiques. *Les Editions du Flamboyant*, Benin. 125.

Kedera C.J., Plattner R.D. and Desjardins A.E. (1999) Incidence of *Fusarium* spp. and levels of fumonisin B1 in maize in Western Kenya. *Appl. Environ. Microbiol*., 65, 41-44.
Keil, H. (1988) Losses caused by the Larger Grain Borer in farm stored maize. In: *Technical papers from the workshop on the Containment and Control of the Larger Grain Borer*, Arusha, and Tanzania16-21 May 1988. Schulten, G.G.M. and A.J. Toet (Eds.), pp.28-52.FAO Rome

Kheseli P.O., Ochanda J.O., Okoth S.A. (2011) Evaluation of potency of native *Bacillus thuringiensis* against maize insect pests and aflatoxin producing fungi in Kenya *Journal of Tropical Microbiology and Biotechnology*,7, 1.

Kingsolver, J.M. (1971) Key to the genera and species of Bostrichidae commonly intercepted in USDA plant quarantine inspections. USDA Agriculture Quarantine Inspection Memorandum 697, 1-11.

Knowles, B.H., (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. *Advances in Insect Physiology*, 24, 275-308.

Larkin, R.P., Hopkins, D.L., and Martin, F.N. (1993) Ecology of *Fusarium oxysporum* f.sp. *niveum* in soils suppressive and conducive to *Fusarium* wilt of water melon. *Phytopathology* 83, 105-1116.

Lee M.K., You T.H., Young B.A., Cotrill J.A., Valaitis A.P., Dean D.H. (1996) Aminopeptidase N purified from gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* Cry1Ac toxin. *Journal of Applied Environmental Microbiology* 62(8), 2845-2849.

Leslie J.F and Summerell B.A. (2006) The *Fusarium* Laboratory Manual. First Edition. Blackwell Publishing Ltd.

Levinson B.L.; Kasyan K. J.; Chiu T.; Currier C.T.S; Gonzales J.M.; J.R. (1990). Ecogen Inc., Langhorne, Pennsylvania 19047

Likhayo, P., Machuka J., Nang'ayo F. and Maniania N. (2002) Prospecting for *Bacillus thuringiensis* for the control of *Prostephanus truncatus* (Horn) and *Sitophilus zeamais* Motschulsky. Kenya Agricultural Research Institute, Biotechnology Centre. Department of Entomopathology, International Centre for Insect Physiology and Ecology.

López-Meza, J.E., Ibarra J.E. (1996). Characterization of a novel strain of *Bacillus thuringiensis. Journal of Applied Environmental Microbiology*, 62, 1306-1310.

Lord J.C. (2005) From Metchnikoff to Monsanto and beyond: The path of microbial control. *Journal of Invertebrate Pathology.*, 89 (1), 19-29.

Lorence A., Darszon A., Díaz C., Liévano A., Quintero R., Bravo A. (1995) Endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers. *FEBS Lett.*, 360 (3), 217-222.

Lowry, O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*., 193, 265-275.

MacDonald, M.V. and Chapman R. (1997) The incidence of *Fusaium moniliforme* on maize from Central America, Africa and Asia during 1992-1995. *Plant Pathology*., 46, 112-126.

Mannon J and Johnson, E. (1985) Fungi down on the farm. *New Scientist*., 12–16.

Marasas W.F.O, Jaskiewicz K, Venter F.S., Van Schalkwyk D.J. (1988) *Fusarium moniliforme* contamination of maize in oesophageal cancer areas in Transkei. *South African Medical Journal.*, 74, 110-114.

Marasas, W.F.O., (1966) Fumonisins: history, worldwide occurrence and impact, p.1-17. In L.S. Jackson, J.W. De Vries, and L.B. Bullerman(ed.), *Fumonisins in food*. Plennum Press, New York, N.Y.

Marasas W.F.O. (1995) Fumonisins: their implications for human and animal health. *Natural Toxins,* 3, 193-8.

Marasas, W. F. O. (2001). Discovery and occurrence of the fumonisins. *Environmental Health Perspectives*., 109(2), 239–243.

Marasas W.F.O., Miller J.D., Riley, R.T. and Visconti A. (2001) Infection of Maize by *Fusarium* species and contamination with -AJOL. *African Journal of Biotechnology*, 2, 12.

Marroquin L. D., Elyassnia D., Griffitts J.S., FeitelsonJ.S., and Aroian R.V. (2000) *Bacillus thuringiensis* (*Bt*) Toxin Susceptibility and Isolation of Resistance Mutants in the Nematode *Caenorhabditis elegans.Genetics,* 155 (4), 1693-1699.

Martin, R.A., Macleod J.A. and Caldwell C. (1991) Influences of production imputs on incidence of infection by *Fusarium* species on cereal seed. *Plant Disease*., 75, 784-788.

Meikle, W.G., Gutierrez A.P. and Herren H.R. (1996) Decision making by smallholder farmers in an oil palm based economy in southern Benin, West Africa. *Trop. Agric.* (Trinidad), 73, 217-225. 13 (2011), 73-8073.

Mirocha, C.J., Mackintosh C.G., Mirza U.A., Xie W., Xu Y. and Chen J. 1992 Occurrence of fumonisin in forage grass in New Zealand. *Journal of Applied Environmental. Microbiology*., 58, 3196-3198.

Mohamed A. F. (2011) Biocontrol of *Fusarium* Moulds and Fumonisin B1 Production. *World Rural Observations*, 3(3), 53-61

Morgan, G.A., Leech, N.L., Gloeckner, G.W., Barrett, K.C. (2004) *SPSS for Introductory Statistics, Use and Interpretation*. Second Edition. Laurence Erlbaum Associates Publishers, London

Morrison, V. A., Haake R. J., and Weisdorf D. J. (1993) The spectrum of non-*Candida* fungal infections following bone marrow transplantation. *Medicine*, 72, 78–89.

Mugo S., Gichuki S. (2009) Taming Major Maize Field Pests in Kenya: The Role of Biotechnology *KARI, CIMMYT*

Mugo S., Songa J., DeGroote H., Hoisington D. (2002) Insect resistant maize for Africa (IRMA) project: an overview. Proceeding of a Symposium on "Perspectives on the Evolving Role of Private/Public Collaborations in Agricultural Research." Syngenta Foundation for Sustainable Agriculture, Washington, D.C

Muthomi J.W.; Riungu G.M.; Ndung'u J.K.; Narla R.D.; Gathumbi J.K. and Wagacha J.M. (2002) Identity and Pathogenicity of *Fusarium* spp. isolated from wheat Department of Plant Science and Crop Protection, University of Nairobi, P.O. Box 30197, 00100 GPO, Nairobi, Kenya

Munkvold, G.P. and Desjardins, A.E. (1997) Fumonisins in maize can we reduce their occurrence. *Plant Disease.*, 81, 556-565.

Munkvold, G.P., Hellmich, R.L., and Rice, L.G. (1999) Comparison of fumonisin concentrations in kernels of transgenic *Bt* maize hybrids and non-transgenic hybrids. *Plant Disease*., 83, 130-138.

Nang'ayo, F.L.O. (1996) Ecological studies on Larger Grain Borer in savanna woodlands of Kenya. Ph.D. dissertation. Imperial College, London.

Nang'ayo F.L.O, Hill, M.G. and Wright, D.J. (2002). Potential hosts of *Prostephanus truncatus* (Coleoptera: Bostrichidae) among native and agroforestry trees in Kenya. *Bulletin of Entomological Research*, 92(6), 499-506.

Narasimha R. K., Vijaypal R. B., Girisham S. and Madhusudhan R.S. (2009) Influence of volatile compounds and food preservatives in the management of fumonisins (B1) production by *Fusarium moniliforme. African Journal of Biotechnology,* 8 (17), 4207-4210.

Narva, K.E., Payne, J.M., Schwab, G.E., Hickle, L.A., Galasan, and WHO (2007) Novel *Bacillus thuringiensis* microbes active against nematodes, and genes encoding novel-nematode –active toxins cloned from *Bacillus thuringiensis* isolates © WHO.

National Toxicology Program (NTP). (1999) NTP technical report on the Toxicology and Carcinogenesis studies of fumonisin B1 (CAS NO. 116355-83-0) in F344/N rats and B6C3F1 mice (Feed studies). NTP TR 496, NIH Publication No. 99-3955, US Department of Health and Human Services, Public Health Service, National Institute of Health. 1 – 46.

Nelson, P. E., Toussoun T. A., and Marasas W. F. O. (1983) *Fusarium* species: An illustrated manual for identification. Pennsylvania State University Press, University Park. 193.

Ninfa M. Rosas-Garcia (2009) Biopesticide Production from *Bacillus thuringiensis*: An Environmentally Friendly Alternative *Recent Patents on Biotechnology*, *3,* 28-36

Nour E. K., Sarmia G., Mebrouk K. and Jamal E. H. (2010) Biological control of *Fusarium oxysporum* f.sp. *lycopersici* Isolated from Algerian Tomato by *Pseudomonas fluorescens, Bacillus cereus, Serratia marcescens and Trichoderma harzianum*. *Research Journal of Agronomy,* 4(2), 31-34.

O'Donnell K., Kistler H.C., Cigelnik E., Ploetz R.C. (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences, USA 95, 2044 9.

Okoth S.A. and Siamento E. (2011) Evaluation of selected soil fertility management interventions for suppression of *Fusarium* spp. in a maize and beans intercrop, *Journal of Yeast and Fungal Research*, 1(2), 035-043.

Ominski K.H., Marquardt R.R., Sinha R.N., Abramson D. (1994) Ecological aspects of growth and mycotoxin production by storage fungi. In: Miller JD, Trenholm HL. eds. Mycotoxins in Grains. Compounds other than Aflatoxin. Eagen Press, USA. 287–305.

Orsi R. B., Corrêa, B., Possi, C. R., Schammass, E. A., Nogueira, J. R., Dias, S.M.C., Malozzi, M. A. B. (2000) Mycoflora and occurrence of fumonisins in freshly harvested and stored hybrid maize. *Journal of Stored Products Research*., 36, 75-87.

Pantenius, C.U. (1988) Stored losses in traditional maize granaries in Togo, *Insect science and its Application,* 9,725-735.

Pelizza S.A., Stenglein S.A., Cabello M.N., Dinolfo M.I., C.E. (2010) First record of *Fusarium verticillioides* as an entomopathogenic fungus of grasshoppers *Lange Journal of Insect Science,* 11(70), 1536-2442.

Perez, C., Pauli, M. and Bazerque, P. (1990) Antibiotic assay by agar-well diffusion method. *Acta Biologiae et Medecine Experimentaalis*, 15, 113-115.

Pierce, S. and Schmidt, G.H. (1992) On the knowledge of *Prostephanus truncatus* (Horn) (*Coleopters: Bostrichidae*), a dangerous pest of maize in Africa. Anzeiger fur Schadlingskunde, *Pflanzen Schutz, Umweltschutz*, 65, 25-32.

Pigott C.R, Ellar D.J. (2007) Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews.*, 71(2), 255–281

Pittet, A., Parisod V. and Schellenberg M., (1992) Occurrence of fumonisins B I and B 2 in cornbased products from the Swiss market. *Journal of Agricultural and Food Chemistry*., 40, 1352-1354.

Plattner R., Proctor R.H., Brown D.W., Desjardins A.E. (2000) Potential for minimization of fumonisin levels in corn using fumonisin nonproducing strains. Aflatoxin/Fumonisin Workshop. Tenaya Lodge, Fish Camp, Yosemite, CA, USA. pp 35-37.

Poinars G.O. Jr. and Thomas G.M. (1978) Diagnostic manual for the identification of insect pathogens. Plenum Press, New York, pp. 218.

Pung, S.H., Sivasithamparam, K., and Barbetti, M.J. (1992) Interactions between *Fusarium oxysporum* and *meloidogyne arenaria* as root pathogens of subterranean clover affected by soil sterilization and inoculum substrate. *NewZealand Journal of Agriculture Research.*, 35, 83-91.

Rasko, D. A., Altherr M. R., Han C. S., and Ravel J. (2005) Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiology Reviews*., 29, 303–329.

Rheeder, J.P. (1992) *Fusarium moniliforme* and fumonisins in corn in relation to human oesphageal cancer in Transkei. *Phytopathology*, 82, 535-537.

Rheeder, J.P., Marasas, W.F.O. and Vismer, H.F. (2002) Production of fumonisins analogs by *Fusarium* species. *Applied Environmental Journal of Plant Sciences,* 3(1),52-60.

Romies, J. R. Hellmich, M. Candolfi, K. Carstens, A. de Schrijer, A. Gatehouse, R. Herman, J Huesing, M. McLean, A. Raybould, A. Shelton and A. Waggoner. (2011) Recommendations for the design of laboratory studies on non-target arthropods for risk assessment of genetically engineered plants. *Transgenic Research* 20, 1-22.

Ross, P.F., Rice L.G., Plattner R.D., Wilson T.M. and Owens D.L. (1991) Concentrations of fumonisin B1 in feeds associated with animal health problems. *Mycopathologia*, 114, 129-135.

Samson, R. A. (1991) Identification of food-borne *Penicillium*, *Aspergillus*, and *Fusarium* species. In: Champ BR, Highley E, Hocking AD, Pitt JI. eds. Fungi and mycotoxins in stored products. Proceedings of an international conference, Bangkok, Thailand, 23–26 April 1991.

Sanahuj G., Banakar R., Twyman R.M, Capell T and Christou P. (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications *Plant Biotechnology Journal*, 9, 283–300.

Schnepf, E., Crickmore N., Van Rie J., Lereclus D., Baum J., Feitelson J., Ziegler D. R., and Dean D H.(1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews.*, 62, 775–806.

Shires S.W. (1979) Influence of temperature and humidity on survival development period and adult sex ratio in *Prostephanus truncatus* (Horn) (*Coleoptera: Bostrichidae*). *Journal of Stored Products Research*., 15, 5-10.

Sick, T., A.J.: JP2007006895 (2007) In: Ninfa M. Rosas-Garcia. (2009) Biopesticide Production from *Bacillus thuringiensis*: An Environmentally Friendly Alternative *Recent Patents on Biotechnology.*, *3*, 28-36.

Sneath, P.H.A. (1986) Endospore- forming Gram-positive rods and cocci. In Bergey's Manual of Systematic Bacteriology ed. Sneath P.H.A., Mair, N.S., Sharpe, M.E., and Holt, J.G. pp. 1104-1140, Baltimore :Williams and Wilkins.

Snyder W.C, Hansen H.N. (1940) The species concept in *Fusarium*. *American Journal of Botany*, 27, 64-67

Snyder W.C, Toussoun T.A. (1965) Current status status of taxonomy in *Fuasarium* spp.and their perfect stages. *Phytopathology*, 55, 833-837.

Sreenivasa M.Y., Gonzalez J.M.T., Dass R.S., Charith R.A.P., Janardhana G.R. (2009) A PCR-based assay for the detection and differentiation of potential fumonisin producing *Fusarium verticillioides* isolated from Indian maize kernels. *Food Biotechnology*., 22(2), 160-170.

Sutton, J.C. (1982). Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology*., 4, 195-209.

Sydenham, E.W., Marasas, W.F.O., Shephard, G.S., Thiel P.G. and Hirooka, E.Y. (1992) Fumonisin concentrations in Brazilian feeds associated with field outbreaks of confirmed and suspected animal mycotoxicoses. *Journal of Agricaltural and Food Chemistry*., 40, 994-997.

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Marasas, W.F.O. and Stockenström, S. (1991) Fumonisin contamination of commercial cornbased human foodstuffs. *Journal of Agricultural and Food Chemistry*., 39, 2014-2018.

Szczech, M.M. (1999) Suppressiveness of vermicompost against *Fusarium* wilts of tomatoto. *Journal of Phytopathology*., 147, 155-161.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*., 28(10), 2731–2739.

Tatiana F.P, Laira P. F., Gabriela A.M. (2008) Inoculum Padronization For The Production Of Cutinase By *Fusarium oxysporum* , *Brazilian Journal of Microbiology* 39, 74-77.

Thompson, M., Gaertner, F.H.: EP0461799 (1991) Biopesticide Production from *Bacillus thuringiensis*: In: Ninfa M. Rosas-Garcia , (2009), Biopesticide Production from *Bacillus thuringiensis*: An Environmentally Friendly Alternative *Recent Patents on Biotechnology*, 3, 28-36.

Thompson, M., Knuth, M., Cardineau, G.: (2004) In: Ninfa M. Rosas-Garcia , (2009), Biopesticide Production from *Bacillus thuringiensis*: An Environmentally Friendly Alternative *Recent Patents on Biotechnology*, 3, 28-36.

Tian, J., H. L. Collins, J. Romeis, S. E. Naranjo, R. L. Hellmich and A. M. Shelton. (2012) Using field-evolved resistance to Cry1F maize in a lepidopteran pest to demonstrate no adverse effects of Cry1F on one of its major predators. *Transgenic Research* 21, 1303-1310.

Tyrell, D.J., Bulla, L.A. JR., Andrews, R.E. JR., Kramer, K.J., Davidson, L.I. & Nordin, P. (1981) Comparative Biochemistry of entomocidal parasporal crystals of selected *Bacillus thuringiensis* strains *Journal of Bacteriology*, 145, 1052-1062

Ueno Y., Iijima K., Wang S.D., Sugiura Y., Sekijima M., Tanaka T., Chen C., Yu S.Z.: (1997). Fumonisins as a possible contributory risk factor for primary liver cancer: a 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. *Food and Chemical Toxicology.*, 35, 1143-50.

Van Frankenhuyzen K, Gringorten J.L., Gauthier D., Milne R.E., Mason L., Peferoen M. (1993) Toxicity of activated CryI proteins from *Bacillus thuringiensis* to six forest lepidoptera and *Bombyx mori. Journal of Invertebrate Pathology*., 62, 295-301.

Wambugu F.M. (2001) Modifying Africa: how biotechnology can benefit the poor and hungry, a case study from Kenya. Nairobi, Kenya, 76.

Wamunyokoli F.A**.** (1992) Isolation, purification and characterization of *Bacillus thuriengiensis* endotoxin active against mosquito larvae. University of Nairobi Biochemistry Department Thesis.

Wang J, *et al*., (2003) Food contamination of fumonsisn B_1 in high-risk area of esophageal and liver cancer. *The Journal of Toxicological Sciences.* 72, 188–196.

Wang'ondu, V. W. (2001) Isolation and partial characterization of *Bacillus thuringiensis* from Kakamega and Machakos districts in Kenya. University of Nairobi M.Sc. Thesis 96 – 105.

WHO and FAO, Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, WHO Headquarters Geneva, Switzerland 24 – 28 September 2001 © WHO and FAO.

Wie, S.I., Andrews, R.E., Hammock, B.D, Faust R.M. & Bulla, Land .A. JR. (1982) Enzyme –linked immunosorbent assays for the detection and quantification of the entomocidal parasporal crystalline protein of *Bacillus thuringiensis* subsp. *Kurstaki* and *israelensis. Journal of Applied and Environmental Microbiology*, 43, 891-894

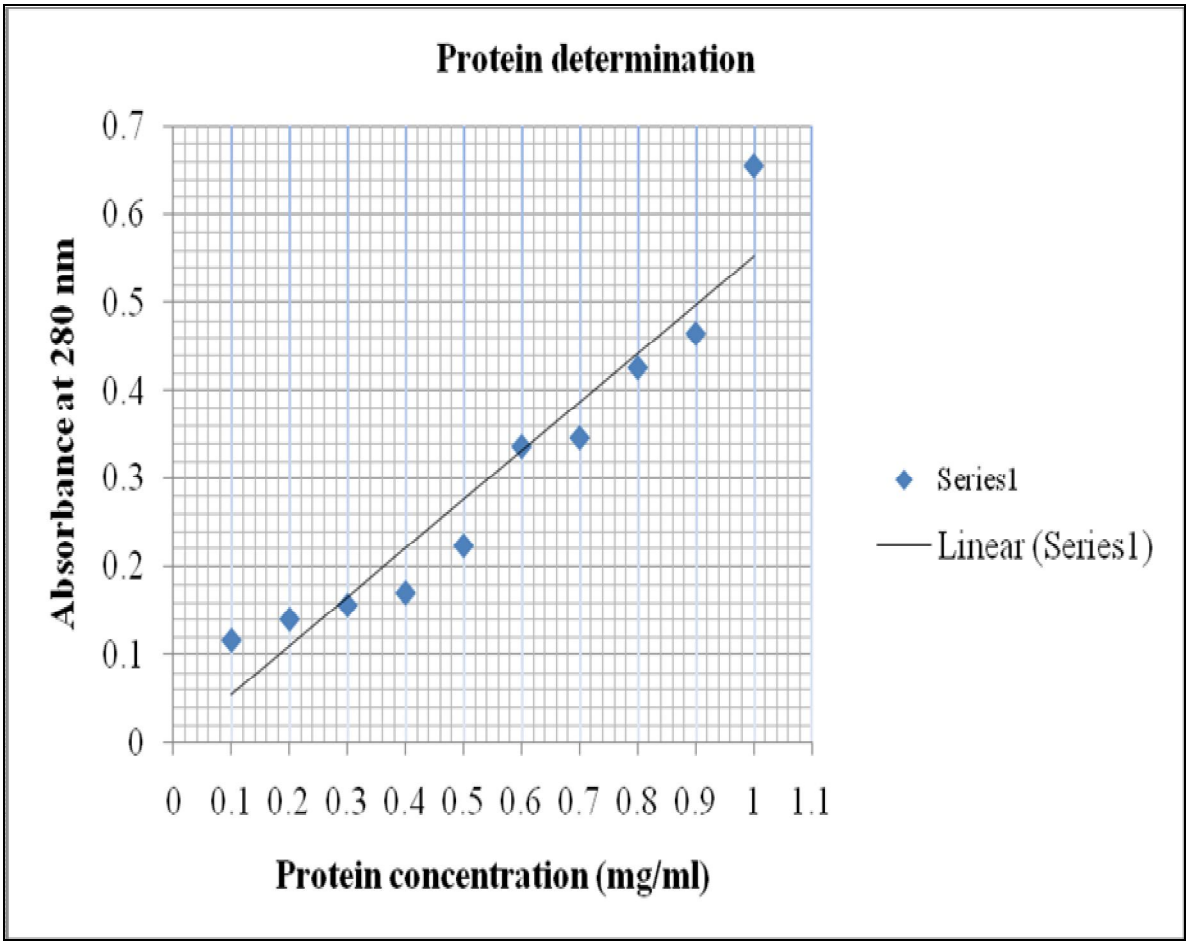
Wollenweber H.W. (1913) Studies on the *Fusarium* problem. *Phytopathology*, 3, 24-39

Yates I., Arnold J., Hinton D.M., Basinger W., Walcott R. (2000). The use of an iodine-based product as a biocompatible control of *Fusarium moniliforme*. Aflatoxin/Fumonisin Workshop, October 25–27, 2000.

Yoshizawa T., Yamashita A., Luo Y.: (1994), Fumonisin occurrence in corn from high- and low-risk areas for human esophageal cancer in China. *Journal of Applied and Environmental Microbiology.*, 60, 16269.

5.0 APPENDICES

APPENDIX I



A standard curve for determination of protein concentration.

OD280nm=0.4195=0.4754*Protein conc. (mg/ml)

Correlation(r) = 0.99426

Alpha (p) = 0.05 (95% confidence)

APPENDIX II

Descriptive of the mortalities arising from the different *Bt* toxins

Descriptive Statistics					
Dependent Variable: Mortality of *Prostephanus truncatus*					
Bt Toxins	Maize Form	Solubility	Mean	Std. Deviation	Count (N)
KM 24	Maize Grains	Soluble	3.4700	1.82395	10
		Insoluble	2.2000	1.64046	10
	Maize Particles	Soluble	2.9800	1.66320	10
		Insoluble	2.1700	1.60142	10
	Maize Flour	Soluble	2.1600	1.91381	10
		Insoluble	2.9000	2.45674	10
KG 12	Maize Grains	Soluble	4.3600	3.28234	10
		Insoluble	4.8000	2.71006	10
	Maize Particles	Soluble	3.3000	2.21359	10
		Insoluble	2.8500	2.26139	10
	Maize Flour	Soluble	2.8500	1.76462	10
		Insoluble	2.8500	2.32200	10
KG 20	Maize Grains	Soluble	2.8800	2.95138	10
		Insoluble	4.1100	2.49241	10
	Maize Particles	Soluble	5.5100	2.78865	10
		Insoluble	3.2800	2.33657	10
	Maize Flour	Soluble	3.0100	2.81522	10
		Insoluble	1.8800	1.52738	10
KG 411	Maize Grains	Soluble	2.9500	2.14022	10
		Insoluble	4.1500	1.97273	10
	Maize	Soluble	3.2000	2.04396	10

	Particles	Insoluble	2.9000	1.82270	10
	Maize Flour	Soluble	2.5000	1.90029	10
		Insoluble	2.7000	2.12394	10
ISR	Maize Grains	Soluble	2.8000	2.23855	10
		Insoluble	1.9400	1.33600	10
	Maize Particles	Soluble	2.8100	1.46170	10
		Insoluble	2.3000	1.71917	10
	Maize Flour	Soluble	2.6700	1.90499	10
		Insoluble	2.3000	1.94651	10
TEN	Maize Grains	Soluble	3.8000	2.38281	10
		Insoluble	3.5900	3.38081	10
	Maize Particles	Soluble	2.3500	1.94437	10
		Insoluble	2.9000	2.10555	10
	Maize Flour	Soluble	2.4000	2.19596	10
		Insoluble	1.8500	1.47290	10
KUR	Maize Grains	Soluble	2.8400	2.19048	10
		Insoluble	2.5000	2.02649	10
	Maize Particles	Soluble	2.9500	2.01674	10
		Insoluble	2.8000	2.25142	10
	Maize Flour	Soluble	2.6300	1.76134	10
		Insoluble	3.1300	2.18380	10
KM 31	Maize Grains	Soluble	2.1500	1.63384	10
		Insoluble	3.0000	1.81046	10
	Maize Particles	Soluble	3.1700	1.90499	10
		Insoluble	3.1500	1.97273	10
	Maize Flour	Soluble	2.4200	1.66052	10

		Insoluble	2.9600	1.73666	10
CONTROL	Maize Grains	Soluble	1.1000	.87560	10
		Insoluble	1.1000	.87560	10
	Maize Particles	Soluble	2.5000	1.71594	10
		Insoluble	2.5000	1.71594	10
	Maize Flour	Soluble	1.2000	1.22927	10
		Insoluble	1.2000	1.22927	10

APPENDIX III

Three way Anova

Tests of Between-Subjects Effects

Dependent Variable: Mortality of *Prostephanus truncatus*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	370.299[a]	53	6.987	1.659	.003
Intercept	4220.730	1	4220.730	1002.225	.000
Toxins	153.125	8	19.141	4.545	.000
Maize Form	37.561	2	18.781	4.460	.012
Solubility	1.612	1	1.612	.383	.536
Toxins * Maize Form	100.944	16	6.309	1.498	.096
Toxins * Solubility	19.207	8	2.401	.570	.803
Maize Form * Solubility	7.529	2	3.764	.894	.410
Toxins * Maize Form * Solubility	50.321	16	3.145	.747	.746
Error	2046.721	486	4.211		
Total	6637.750	540			
Corrected Total	2417.020	539			

a. R Squared = .153 (Adjusted R Squared = .061)

b. Computed using alpha = .05

APPENDIX IV

Post hoc ANOVA; LSD Test and Dunnet Test

Multiple Comparisons							
Dependent Variable: Mortality of *P.truncatus* *Prostephanus*							
						95% Confidence Interval	
	(I) *Bt* Toxins	(J) *Bt* Toxins	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
LSD	KM 24	KG 12	-.8550*	.37467	.023	-1.5912	-.1188
		KG 20	-.7983*	.37467	.034	-1.5345	-.0622
		KG 411	-.4200	.37467	.263	-1.1562	.3162
		ISR	.1767	.37467	.637	-.5595	.9128
		TEN	-.1683	.37467	.653	-.9045	.5678
		KUR	-.1617	.37467	.666	-.8978	.5745
		KM 31	-.1617	.37467	.666	-.8978	.5745
		CONTROL	1.0467*	.37467	.005	.3105	1.7828
	KG 12	KM 24	.8550*	.37467	.023	.1188	1.5912
		KG 20	.0567	.37467	.880	-.6795	.7928
		KG 411	.4350	.37467	.246	-.3012	1.1712
		ISR	1.0317*	.37467	.006	.2955	1.7678
		TEN	.6867	.37467	.067	-.0495	1.4228
		KUR	.6933	.37467	.065	-.0428	1.4295
		KM 31	.6933	.37467	.065	-.0428	1.4295
		CONTROL	1.9017*	.37467	.000	1.1655	2.6378
	KG 20	KM 24	.7983*	.37467	.034	.0622	1.5345
		KG 12	-.0567	.37467	.880	-.7928	.6795
		KG 411	.3783	.37467	.313	-.3578	1.1145
		ISR	.9750*	.37467	.010	.2388	1.7112
		TEN	.6300	.37467	.093	-.1062	1.3662
		KUR	.6367	.37467	.090	-.0995	1.3728
		KM 31	.6367	.37467	.090	-.0995	1.3728
		CONTROL	1.8450*	.37467	.000	1.1088	2.5812
	KG 411	KM 24	.4200	.37467	.263	-.3162	1.1562
		KG 12	-.4350	.37467	.246	-1.1712	.3012
		KG 20	-.3783	.37467	.313	-1.1145	.3578

		ISR	.5967	.37467	.112	-.1395	1.3328
		TEN	.2517	.37467	.502	-.4845	.9878
		KUR	.2583	.37467	.491	-.4778	.9945
		KM 31	.2583	.37467	.491	-.4778	.9945
		CONTROL	1.4667*	.37467	.000	.7305	2.2028
	ISR	KM 24	-.1767	.37467	.637	-.9128	.5595
		KG 12	-1.0317*	.37467	.006	-1.7678	-.2955
		KG 20	-.9750*	.37467	.010	-1.7112	-.2388
		KG 411	-.5967	.37467	.112	-1.3328	.1395
		TEN	-.3450	.37467	.358	-1.0812	.3912
		KUR	-.3383	.37467	.367	-1.0745	.3978
		KM 31	-.3383	.37467	.367	-1.0745	.3978
		CONTROL	.8700*	.37467	.021	.1338	1.6062
	TEN	KM 24	.1683	.37467	.653	-.5678	.9045
		KG 12	-.6867	.37467	.067	-1.4228	.0495
		KG 20	-.6300	.37467	.093	-1.3662	.1062
		KG 411	-.2517	.37467	.502	-.9878	.4845
		ISR	.3450	.37467	.358	-.3912	1.0812
		KUR	.0067	.37467	.986	-.7295	.7428
		KM 31	.0067	.37467	.986	-.7295	.7428
		CONTROL	1.2150*	.37467	.001	.4788	1.9512
	KUR	KM 24	.1617	.37467	.666	-.5745	.8978
		KG 12	-.6933	.37467	.065	-1.4295	.0428
		KG 20	-.6367	.37467	.090	-1.3728	.0995
		KG 411	-.2583	.37467	.491	-.9945	.4778
		ISR	.3383	.37467	.367	-.3978	1.0745
		TEN	-.0067	.37467	.986	-.7428	.7295
		KM 31	.0000	.37467	1.000	-.7362	.7362
		CONTROL	1.2083*	.37467	.001	.4722	1.9445
	KM 31	KM 24	.1617	.37467	.666	-.5745	.8978
		KG 12	-.6933	.37467	.065	-1.4295	.0428
		KG 20	-.6367	.37467	.090	-1.3728	.0995
		KG 411	-.2583	.37467	.491	-.9945	.4778
		ISR	.3383	.37467	.367	-.3978	1.0745
		TEN	-.0067	.37467	.986	-.7428	.7295
		KUR	.0000	.37467	1.000	-.7362	.7362
		CONTROL	1.2083*	.37467	.001	.4722	1.9445

Dunnett t (2-sided)[a]	KM 24	CONTROL	1.0467*	.37467	.035	.0493	2.0440
	KG 12	CONTROL	1.9017*	.37467	.000	.9043	2.8990
	KG 20	CONTROL	1.8450*	.37467	.000	.8477	2.8423
	KG 411	CONTROL	1.4667*	.37467	.001	.4693	2.4640
	ISR	CONTROL	.8700	.37467	.117	-.1273	1.8673
	TEN	CONTROL	1.2150*	.37467	.009	.2177	2.2123
	KUR	CONTROL	1.2083*	.37467	.009	.2110	2.2057
	KM 31	CONTROL	1.2083*	.37467	.009	.2110	2.2057
Based on observed means. The error term is Mean Square (Error) = 4.211.							
*. The mean difference is significant at the .05 level.							
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.							

APPENDIX V

Analysis of Inhibition zones

Descriptive

Zone of Inhibition

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
KG20	3	2.1433	.12503	.07219	1.8327	2.4539	2.00	2.23
KG12	3	1.7167	.38188	.22048	.7680	2.6653	1.30	2.05
TEN	3	4.5667	.30551	.17638	3.8078	5.3256	4.30	4.90
ISR	3	4.9333	.40415	.23333	3.9294	5.9373	4.50	5.30
KM24	3	3.2667	.25166	.14530	2.6415	3.8918	3.00	3.50
KUR	3	4.0667	.10408	.06009	3.8081	4.3252	3.95	4.15
KG411	3	4.9000	.85440	.49329	2.7776	7.0224	4.00	5.70
KM31	3	.0000	.00000	.00000	.0000	.0000	.00	.00
Control	3	.0000	.00000	.00000	.0000	.0000	.00	.00
Total	27	2.8437	1.92115	.36972	2.0837	3.6037	.00	5.70

ANOVA

Zone of Inhibition

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	93.516	8	11.690	86.072	.000
Within Groups	2.445	18	.136		
Total	95.961	26			

Alpha (p) = 0.05.

The level of significance obtained after one way ANOVA is 0.000 (i.e. <0.001). This level is less than the 0.05 significance level required implying that growth inhibitions by toxins from various strains of *B.thuringiensis* were significantly different.

APPENDIX VI

Dunnet Test

Multiple Comparisons

Zone of Inhibition
Dunnett t (2-sided)

(I) *Bt* Toxin	(J) *Bt* Toxin	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
KG20	Control	2.14333*	.30090	.000	1.2601	3.0265
KG12	Control	1.71667*	.30090	.000	.8335	2.5999
TEN	Control	4.56667*	.30090	.000	3.6835	5.4499
ISR	Control	4.93333*	.30090	.000	4.0501	5.8165
KM24	Control	3.26667*	.30090	.000	2.3835	4.1499
KUR	Control	4.06667*	.30090	.000	3.1835	4.9499
KG411	Control	4.90000*	.30090	.000	4.0168	5.7832
KM31	Control	.00000	.30090	1.000	-.8832	.8832

*. The mean difference is significant at the 0.05 level.
i.e. p=0.05

In Post hoc ANOVA, the mean growth inhibitions of *F.verticillioides* under various treatments of *Bt* toxins have been compared with the growth inhibition of *F.verticillioides* in the control group using Dunnet Test, a post hoc ANOVA test. The level of significance obtained in each comparison is 0.000 which is < 0.05 except in the comparison between KM31 & Control group which gave as sig. level of 1.00 which is > 0.05. This shows that the growth inhibition by the various *Bt* toxins were different from the growth inhibition in the control group except growth inhibition of the KM31 which was not significantly different from the inhibition of the control group.

APPENDIX VII

Analysis of Fungal Diameter

Descriptives

Fungal Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
KG 20	3	2.9667	2.23681	1.29142	-2.5899	8.5232	.40	4.50
KG 12	3	3.4000	1.00000	.57735	.9159	5.8841	2.40	4.40
TEN	3	2.1667	1.66233	.95975	-1.9628	6.2961	.40	3.70
ISR	3	2.4000	1.47309	.85049	-1.2594	6.0594	1.50	4.10
KM 24	3	4.0000	.62450	.36056	2.4487	5.5513	3.30	4.50
KUR	3	4.1333	.64291	.37118	2.5363	5.7304	3.40	4.60
411	3	2.0333	1.76163	1.01708	-2.3428	6.4095	.40	3.90
KM 31	3	3.8000	.90000	.51962	1.5643	6.0357	2.90	4.70
CONTROL(H2O)	3	7.1333	.63509	.36667	5.5557	8.7110	6.40	7.50
Total	27	3.5593	1.86202	.35835	2.8227	4.2959	.40	7.50

APPENDIX VIII

One Way ANOVA

fungal diameter

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	58.032	8	7.254	4.066	.006
Within Groups	32.113	18	1.784		
Total	90.145	26			

When the fungal diameter means were compared using one way ANOVA, the significance level obtained was 0.006. When the significance level is less than 0.05(alpha<0.05) which is the required Level of Significance it denotes that the means being compared are significantly different (Morgan *et al*., 2004).

APPENDIX IX

Post Hoc Tests

Multiple Comparisons

fungal diameter

Dunnett t (2-sided)

(I) *Bt* Toxin	(J) *Bt* Toxin	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
KG 20	CONTROL(H2O)	-4.16667*	1.09059	.008	-7.3677	-.9656
KG 12	CONTROL(H2O)	-3.73333*	1.09059	.018	-6.9344	-.5323
TEN	CONTROL(H2O)	-4.96667*	1.09059	.002	-8.1677	-1.7656
ISR	CONTROL(H2O)	-4.73333*	1.09059	.003	-7.9344	-1.5323
KM 24	CONTROL(H2O)	-3.13333	1.09059	.057	-6.3344	.0677
KUR	CONTROL(H2O)	-3.00000	1.09059	.072	-6.2010	.2010
411	CONTROL(H2O)	-5.10000*	1.09059	.001	-8.3010	-1.8990
KM 31	CONTROL(H2O)	-3.33333*	1.09059	.039	-6.5344	-.1323

*. The mean difference is significant at the 0.05 level.

APPENDIX X

The PCR products were sequenced using geneious program. Molecular analyses of the generated sequences were conducted using *MEGA* version 5 (Tamura, Peterson, Stecher, Nei, and Kumar 2011) and MUSCLE was used to generate multiple alignments of the nucleotide sequences (Edgar and Robert, 2004).

FV001_EF1_EF_2

CTTTGATGAGACTGTAACGACACCGGTAAAGCTACCCGCACCGGACACGACG
GGTCTCCGGTCAGACCCCGGCATCCAATTGGATCCGGGGTCTGACCGGAGAC
CCGTCGTCCGGTGACGGGTAGCTGTTCACCGGTGTCTTACAGTCTCATTACCA
TGGTATTA

FV002_EF1__EF2

CCTGGCGTCAGTAGTACAACCAAACGGCTAGAACTTCACGTTGGCAATCGCT
TCGTTGCCTCACATTAGTTGTAATCGATTACAACTAATGTGAGGCAACGAAGC
GATTGCCAACGTGAAGTTCTAGCCGTTTGGTTGTACTACCAATCGCGACMCC
CCAAA

FV004_EF1 and EF2

TTCGAGATAGAATCAGGATTCAAATCGTCGAAGAAACAGAGTCTTCTGGTAC
GGAGCACCTGGGCGCCCAGGTGCTCCGTGACCAGAAGACTCTGTTTCTTCGA
CGATTTGAATCCTGATTCTATCTCAAGCGCCGTTAGCTCCAA

FV005_EF1__EF2

GGTGAATAACAGACCATCAGGGATCAAGACTCACCGTCGTGGAAGTCTTTCT
CGCATGTGGACTGACGTCAGTCCACATGCGAGAAAGACTTCCACGACGGTGA
GTCTTGACCCTGATGGTCTGTTATTCACCTTACTGCTCA

FV006_EF1__EF2

GGGGTGAGGAACACGCAGGGGTCAATGTACTCAGGACAGATAGTAGCTTTTT
GTTCTCCTCGGAGAAGCGTCGACGCTTCTCCGAGGAGAACAAAAAGCTACTA
TCTGTCCTGAGTACATTGACCCCTGCGTGTCCTCACTCAAGCGGAGACTAAGA
TCYAAA

FV007_EF1__EF2

TTCGGCGTCATTGAGTGACGTAGCTACCTGACGTGACATGTAGCGGGTTCTAG
AACATTGATACCTCACGCGGCGTCAGCGGTAGAACCTAGTCTTCCTTTCGGTT
GACCTGAACGTCGAGCCAATTGGAGGGGGGACCGCCCTCGAGGGCGGTTGCC
CCCTCCAATTGGCTCGACGTTCAGGTCAACCGAAAGGAAGACTAGGTTCTAC
CGCTGACGCCGCGTGAGGTATCAATGTTCTAGAACCCGCTACATGTCACGTC
AGGTAGCTACGTCATCATGACGCCGTACGATAGATTCTCAAA

FV008_ EF1 _EF2

AGTCATGGGAACGGGACCAAGCGGGTCAAATAGATGTCGCCCTACCGCCCGA
GGCTTTATGGTTATCCGAGCCTGGCGAATATTCGCCAGGCTCGGATAACCATA
AAGCTCGGGCGGTAGGGCGACATCTATTTGACCCGCTGGTCCGTTCCCATGA
CGCCGTACGATAGATTCTCCAAA

FV009_EF 1_EF2

GTCGTCATGATGACGTAGCTACCTGACGTGACTAGCATCAACCGGGGATTCA
TTGATACCTCACGCGGCGTCAGCGGTAGAACCTAGTCTTCCTTTCGGTTGACC
TGAACGTCGAGCCAATTGGAGGGGGGACCGCCCTCGAGGGCGGTTGCCCCCT
CCAATTGGCTCGACGTTCAGGTCAACCGAAAGGAAGACTAGGTTCTACCGCT
GACGCCGCGTGAGATTACCGCCCTTACTGCAAA

FV10_EF1__EF2

TGGTAGTACAAAACCACGGGTCAAAGAGTCGCCTACCGCCCGAGGCTTATGG
TTATCACATGTAGATATGTAATGATCCTGAGGTGCTCGGGTGACCAAAGACT
CTGTTTCTTCGAGATTTTGAATCCTGATTCCTAAGCGCCGTTAGCTCTCA

FV TO _EF1__EF2

GGGTCATGTTGAGTGATGCGACGGAGCGTTCAAGAATGTCCCACCTCGAACA
AGCTTTTTTTGCGACCCTGGGTAGACTTGTAATCATTCCAGTGCTCGCGAGTA
AGACTTCCACGACGGTGAAGCTTGATCCTGAGCTGATCCTCGTCCCATGACG
CCGTACGATGATTCCCCAAA

FV011_EF1 _EF2

CGGTTTGAGTTATGCCGACGGAGCGTTCAAGATGTCCCACCTCGAACAAGCT
TTCTTTTGCGACTGGGAGACGGTACCGCTCCCAGTCGCAAAAGAAAGCTTGTT
CGAGGTGGGACATCTTGAACGCTCCGTCGGCATAACTCCGGCGACGTTATCT
CA

FV012_EF1__EF2

TGGCGTCTTGGTAGTACAACCAGCGGGTCAAATAGATGTCGCCCTACCGCCC
GAGGCTTTATGGTTATCCGAGCCTGGCGAATATTCGCCAGGCTCGGATAACC
ATAAACTCGGGCGGTAGGGCGACATCTATTTGACCCGCTGGTCCCGTTCCCAT
GACGCCGTACGATAGATTCTTCAAA