EVALUATION OF *Bacillus thuringiensis* ISOLATES' POTENTIAL IN THE MANAGEMENT OF POD BORERS *(Lepidoptera*: *Noctuidae*) INFESTING PIGEON PEAS

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DECLARATION

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

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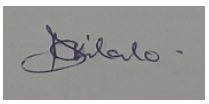
Signature:  Date: 06/08/2023

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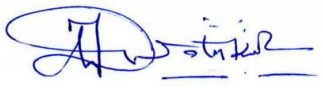
Signature:  Date: 28/07/2023

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DEDICATION

This Thesis is dedicated to my beloved husband Cornelius Tanui for his continuous support, understanding and encouragement throughout my study and project work, to my parents Barnabas Arusei and Teresiah Arusei for supporting my education. To my daughters Faith Jepkosgei, Joy Jebet, son Ryan Kiplagat and baby Blessing Jepchirchir for their love, patience, endurance and encouragement during my entire study and project work.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMNS

ASAL	Arid and semi-arid land
Bt	*Bacillus thuringiensis*
ANOVA	Analysis of Variance
FAOSTAT	Food and Agricultural Organization of the United Nations
Ha	Hectare
Kg	Kilo gram
LT	Lethal time
LC	Lethal concentration
HaNPV	Nuclear Polyhedrosis Virus
FAO	Food and Agricultural Organization
MoA	Ministry of Agriculture
IPM	Integrated Pest Management
OD	Optical Density
PCR	Polymerase Chain Reaction
KALRO	Kenya Agricultural Research Organization
IPM	Integrated pest management
Mg/Ml	Micrograms per millilitres
BioRi	Biotechnology Research Institute
FCRI	Food Crop Research Institute
KSCAP	Kenya Climate Smart Agriculture Project
ITOC	ICIPE, Thomas Odhiambo Campus

ABSTRACT

Pod borer infestation, especially *Helicoverpa armigera*, is a key constraint affecting pigeon pea (*Cajanus cajan*) production. Farmers find it difficult to adopt the use of synthetic pesticide because pigeon pea is a subsistence crop pesticides are expensive and there is danger of pesticide resistance development. The objectives of this study were to i) evaluate the response of pod borer against *Bacillus thuringiensis (Bt)* isolates in the laboratory and ii) determine the efficacy of *Bacillus thuringiensis cry gene* against the pigeon pea pod borer. Soil samples from pigeon pea growing farms within Machakos and Makueni Counties were isolated with a selective technique using nutrient broth mixed with 0.25M Sodium Acetate ($C_2H_3NaO_2$). The *Bacillus thuringiensis* isolates were grown on nutrient agar plates to establish colony features and were subjected to gram reaction to ascertain the *Bacillus thuringiensis* morphological characteristics. Molecular detection of ten (10) *Bacillus thuringiensis* isolates was done using PCR with specific primers for *Cry*1 and *Cry*2 genes. The isolates were tested at different dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) for bioassay and bio efficacy experiment in the field to determine the virulence against pigeon pea pod borer. Second and third instar larvae used for bioassay were reared in ITOC- ICIPE. Out of the 10 characterized isolates, two isolates, J7 and J10, with strong bands of *Cry1* and *Cry2* genes and were selected for the bioassay. The same isolates at the effective dose rates determined in the laboratory were used to test their bio efficacy potential in controlling pod borers in the field. In the laboratory, *Bacillus thuringiensis Cry*1 and *Cry*2 significantly ($P<0.05$) caused higher mortality of 2nd and 3rd instar larvae of pod borer compared to control. *Bacillus thuringiensis Cry*1 significantly ($p<0.05$) caused higher mortality to 2nd and 3rd instar larvae of pod borers compared to *Bt Cry*2. In the field, *Bt Cry*1, *Bt Cry*2 and the mixture of *Bt Cry1* and *Bt Cry2* at concentrations 10^{-3} and $^{10-4}$ significantly ($p<0.05$) reduced the number of damaged pods in the field compared to the control. Among the treatments, *Bacillus thuringiensis Cry*1 recorded the least number of damaged pods in the field and was significantly ($p<0.05$) different from the mixture of *Cry*1 & *Cry*2 and *Cry*2 alone. These results demonstrate the potential of locally isolated *Bacillus thuringiensis Cry*1 and *Cry*2 to manage pod borer infestation of pigeon peas in the field. Further evaluation and development can be done to provide an alternative method of managing the pod borers for sustainable production of pigeon peas.

CHAPTER ONE
INTRODUCTION

1.1 Background

Pigeon pea (*Cajanus cajan*) is an important cultivated legume crop, especially in the Eastern part of Kenya (Snapp *et al*., 2003). The crop adapts well to drought and low soil fertility. It is grown as a monocrop or intercropped with other crops such as sorghum, maize, beans, cow peas, millet and cassava. The crop increases soil coverage, reduces soil erosion, improves soil structure and enhances yields by increasing productivity (Recha *et al*., 2013). Pigeon pea has an excellent nutritional value as a major protein source especially for vegetarian diet, high fiber and low fat content, vitamin B complex for body metabolism, Vitamin C and K and body essential minerals like phosphorus and magnesium. These act as supplements to consumption of animal proteins which are expensive and not readily available to the poor farmer (Saxena *et al*., 2010). Proteins are very important because they reduce malnutrition in children which is common in developing countries as a result of eating unbalanced diet or diet rich in carbohydrates only occasioned by poverty levels.

Production of pigeon peas in Kenya faces major challenges caused by pests and diseases such as pod boring insects especially the pod borer, *Helicoverpa armigera Hübner* (*Lepidoptera: Noctuidae*). The pod boring insects majorly reduce pigeon pea yield estimated to be up to 80% (Baker *et al*., 2002). The pod borer larvae feed on the reproductive structures that are irreplaceable thus lowering grain yield. The pesticides are expensive, making it difficult for smallholder subsistence farmers to adopt their use (Jadhav *et al.*, 2012; Pandey, 2017). In addition , there are risks for the pest developing resistance to synthetic pesticides.Therefore, there is need to develop alternative, environmentally safe and efficient pest management methods which include the use of biological control agents such as *Bacillus thuringiensis* (*Bt*) (Kalra & Khanuja, 2007).

1.2 Statement of problem

Pests and diseases limit pigeon pea production. *Helicoverpa armigera* a lepidopteran insect pest, is particularly, a menace to pigeon pea farmers in arid and semi-arid lands (ASAL) of Kenya. It attacks the crop during the vegetative and reproductive stages, thereby lowering the yields drastically. It causes crop damage which results in more than 80% yield loss (Cheboi *et al*., 2016;

Kooner & Cheema, 2006). The young larvae feed excessively on young buds and flowers while mature larvae penetrate inside the pods ingesting the grains completely.

Helicoverpa armigera is a polyphagous pest that utilizes a wide host range of crops such as cotton (*Gossypiu m spp*), cowpeas (*Vigna unguiculata*), pigeon pea (*Cajanus cajan*), sorghum (*Sorghum bicolor*), tomato (*Solanum lycopersicum*), beans (*Phaseolus vulgaris*), sun flower (*Helianthus annuus*), chickpea (*Cicer arietinum*) and maize (*Zea mays*) (Cunningham & Zalucki, 2014). The females have high fecundity resulting in increase in population growth. The survival mechanisms of *H. armigera* are a characteristic diapause and high mobility to facilitate the spread, distribution and its establishment worldwide. Lack of resistant pigeon pea varieties, use of synthetic pesticides by farmers, and expensive registration of new and safe pesticides have made management of the pest difficult. Pesticide use is also a threat to human health, environment and non-target organism in the agricultural ecosystems (Thacker, 2002). There are also risks for pest resistance development. Therefore, there is need for an alternative and environmentally safe method for the management of the pest.

1.3 Justification of study

Pigeon pea is a major pulse crop grown in ASAL regions. It is a high value crop and suitable for climate smart interventions in terms of nutritional value (Thacker, 2002), improving soil fertility, soil structure (Kwena, 2018) and its medicinal importance (Odeny, 2007). In Africa, studies have revealed that pigeon pea seeds are used to treat hepatitis and measles, avert malnutrition in children and are utilized in vegetarian meals (Saxena *et al*., 2010; Sharma *et al*., 2011). Pigeon pea pods, leaves, seeds, and seed byproducts are used to make livestock feeds due to richness on protein content with high palatability value (Odeny, 2007). They are also grown for environmental management purposes, as wind breaks in agro forestry and as a source of fire wood for households.

Despite all these benefits, the crop is affected by the insect pests which lower its quality and production. Not much attention has been accorded to the crop to reduce the losses. The crop is attacked by pod boring insects, pod sucking bugs and pod fly causing severe damage. Pod boring insect pest, *Helicoverpa armigera Hübner (Lepidoptera: Noctuidae)* is the most destructive pest

worldwide causing considerable losses (Srinivas *et al.*, 2019) that are estimated to 80% due to young larval invasion of the crop during the vegetative and reproductive stages. *Helicoverpa armigera* have a facultative diapause, high mobility, and high fecundity in females allowing it to adapt to different ecological environments, subsequently leading to the spread, distribution and establishment of this pest species worldwide (Cunningham & Zalucki, 2014). Farmers depend majorly on synthetic insecticides to control and manage the pest promoting extensive and indiscriminate use. Indiscriminate use of the pesticides has become a threat to human health, the environment, and is destroying non-target and beneficial organisms in the agricultural ecosystem (Kranthi *et al*., 2002). Pesticides are expensive to the low earning smallholder farmer because the crop needs to be sprayed 3 to 6 times in a season with no much results, hence poor crop yield. Use of cultural practices is not effective because they are time consuming and are limited in application. For example, there is limited land for crop rotation, it is cumbersome and most farmers do not adhere to the stipulated practices.

Biological control agent, *Bacillus thuringiensis (Bt),* a gram positive bacteria, occurs naturally in the soil, gut of moths and butterflies, aquatic environment, under the dark surfaces of the leaves, in dust and marine sediments (Maeda *et al*., 2000). The bacteria has been identified as a potential biocontrol which has been used successfully and efficiently in controlling pod borers in chick pea, cotton and tomatoes (Kalra & Khanuja, 2007). During sporulation, *Bt* produces crystalline inclusions known as *cry* proteins (δ-endotoxin) with insecticidal properties used against lepidopterans (El-Menofy *et al*., 2014). It's specific in its mode of action, thus making it effective on the target pests. *Bacillus thuringiensis* as a useful bacterium is still underutilized. It can be used as a biopesticide to reduce the use of synthetic insecticides which are not eco-friendly. This study was conducted to control *H. armigera* using locally isolated *B. thuringiensis* from soil to enhance the productivity of pigeon pea crop.

1.4 Objectives of the study

1.4.1 General objectives

The general objective of the study was to evaluate the potential of using *Bacillus thuringiensis* as a biopesticide to manage pod borers infesting pigeon peas for improved pigeon pea productivity.

1.4.2 Specific objectives

1. To evaluate the in vitro response of pod borer larvae to infection by *Bacillus thuringiensis* isolates.
2. To determine the effect of local *Bacillus thuringiensis* isolates for the management of pod borers infesting pigeon peas in the field.

1.5 Hypothesis

1. Application of *Bacillus thuringiensis* has no effect against the pod borers infesting pigeon peas in the laboratory and in the field.

2. Both 1^{st} and 2^{nd} instar larvae of pigeon pea pod borer are not susceptible to *Cry1* or *Cry2* genes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and taxonomy of pigeon peas

Pigeon peas (*Cajanus cajan*) is an excellent pulse crop which is believed to have its native origin from India, Eastern Ghats due to its hereditary diversity in the germplasm and where its wild relatives *(Cajanus cajanifolia*) are found, in East Africa and West Africa. Pigeon pea is widely grown in India. During 17th century it gained entry into American continent through slave trade carried out by Europeans (Van der Maesen, 1990). It arrived to East Africa in the 19th century through the people who came to work as railway employees (Hillocks *et al*., 2000). In East Africa, mainly in Kenya it's grown in Eastern region in the following districts Machakos, Makueni, Kitui, Mwingi and Mbeere (Shanower *et al*., 1999). Pigeon pea is classified into Kingdom: *Plantae*, Order*: Fabales*, Family: *Leguminoceae/Fabaceae*, Genus: *Cajanus*, Species: *Cajanus cajan*, sub tribe *Cajaninae*, tribe *Phaseoleae* and its binomial name is *Cajanus cajan (L.) Millspaugh* (Kumar *et al*., 2017). From the *Cajaninae* sub tribe pigeon pea is the lone crop cultivated comprising 11 correlated genera and 32 species (Mallikarjuna *et al*., 2011). Apparently *Cajanus cajan* originated from *Cajanus cajanifolius* but the difference arises from the morphology of the floral parts and pod, pod color, seed color and weight.

Pigeon pea is a perennial leguminous plant which can be grown yearly and attain a height of 0.5–4.0 m (1.6–13.1 ft) (Kumar *et al*., 2017). It's a long taproot rooted plant with extensive roots and secondary branches, the roots have nodules which result to the mutual relationship leading nitrogen fixation in the soil. Leaves are simple and form a compound trifoliate, hairy, and pubescent and the leaflets are elliptical. Branches can be bushy or upright depending the spacing of the crop in the field. Flowers are zygomorphic, hermaphrodite at the terminal or auxiliary and are brightly yellow with streaks of purple or red color. Pigeon pea fruit is the pod, pods are linear while some are sickle shape and varies in length and color from green to dark brown depending on the variety. Seed quantity in each pod depends with variety, shorter duration variety have two to three seeds and long duration variety have four to five seeds. Pigeon pea seeds vary in size, shape and size. They are round and lens shaped, the color varies from white to dark mottled brown with yellow cotyledons (Varshney *et al*., 2017).

Pigeon peas are classified according to the flowering arrangement and maturity period, determinate and non-determinate. Determinate complete their reproductive phase and the vegetative phase which enables easy management. These varieties are adversely invaded by pod borer, *Helicoverpa armigera (Hubner)* and *Maruca testulalis (Geyer)* culminating in reduced yield production (Reddy *et al.*, 2005). Non determinate have continuous vegetative and reproductive phases (Singh *et al*., 1995). Following the length of maturity there are three types; short, medium and long maturing variety. Short duration variety takes 3-4 months to reach maturity, and perform well in medium altitude (600-1500m) in warmer temperatures (Kimani *et al*., 1994). Medium duration variety takes 5-6 months to mature and the long duration variety takes 8-9 nine months.

2.2 Production of pigeon peas

The legume crop is majorly grown in India, leading by a total production of 73% and the other major pigeon pea producing countries are Myanmar (12%), Malawi, 7%, Tanzania (5%), and Kenya (1%) (FAOSTAT, 2018) (Figure 2.2). According to FAO statistics of 2018, in terms of area coverage, Kenya with 2% is ranked fifth after India with 82%, Myanmar 8%, Tanzania 4%, and Malawi 4% (Figure 2.1). In Kenya, pigeon pea crop is ranked third, after common beans (*Phaseolus vulguris*) and cow peas (*Vigna aunguiculata L*.). Smallholder farmers cultivate pigeon peas both for consumption and for export (Mergeai *et al*., 2001). In the last five years the yield production has ranged between 89,000 and 196, 0000 tonnes, with average area coverage of 240, 000 hectares per year and the yield average 0.65 tonnes per hectare (Table 2.1). On average the country population consume 106,280 tonnes per year and the excess being exported to other countries (Ministry of Agriculture (MoA), Economic Review of Agriculture, 2015).

In Kenya pigeon pea farming is majorly carried out by small scale growers who cultivate the crop in acreage of 0.2 ha and 1.4 ha. Pigeon pea is normally cultivated during the short rains season between September and October. Short and medium duration pigeon pea varieties are grown by farmers as their preferred option due there early maturity.

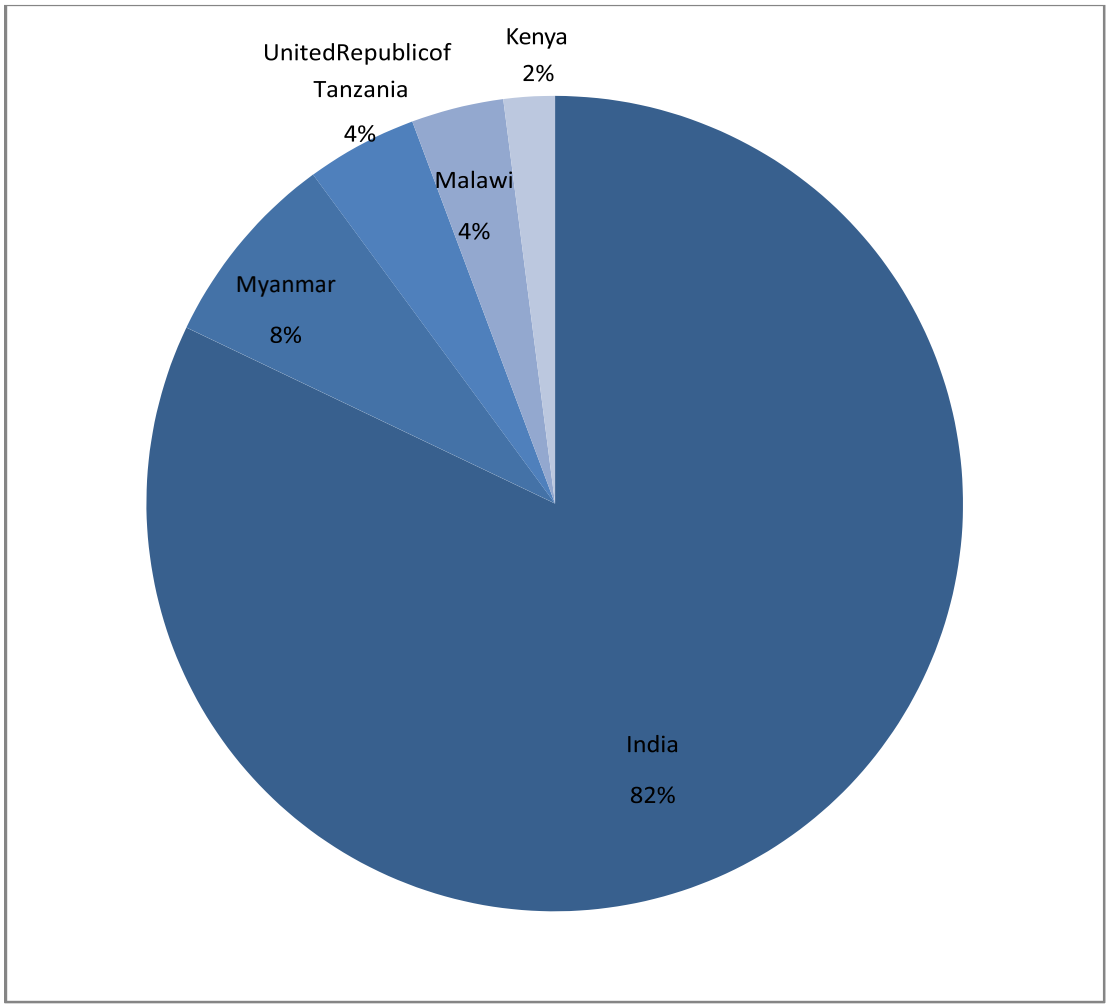

Figure 2.1: Global Pigeon pea production (FAOSTAT, 2014**)**

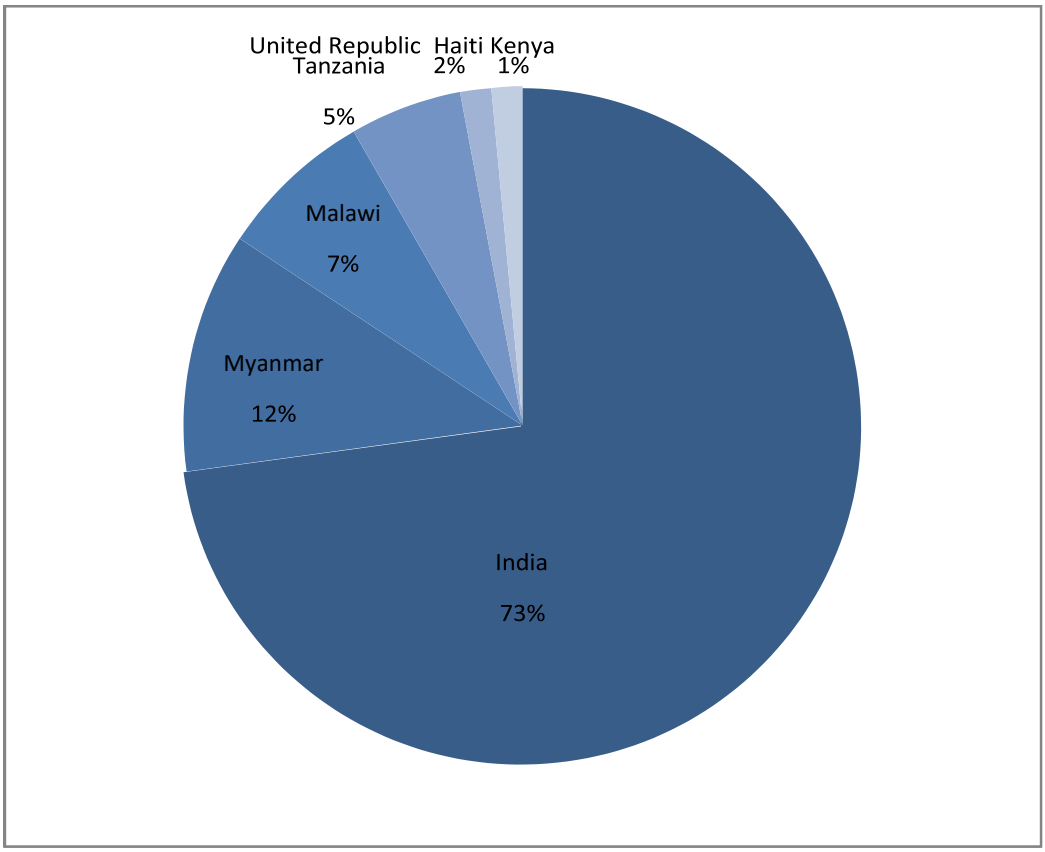

Figure 2.2: Pigeon pea area coverage (FAOSTAT 2018)

Table 2.1: Pigeon pea production tendencies in Kenya (2010-2014)

	Area(Ha)	Production(t)	Yield(Ha)	Exports(t)	Imports(Mt)
2010	158 746	103 324	0.65		2 956
2011	138 708	88 813	0.64		17 467
2012	271 136	167 623	0.62	61 343	
2013	256 396	165 636	0.65	59 356	
2014	276 134	196 324	0.71	90 044	

2.3 Economic importance of pigeon peas

Pigeon pea is an important crop in arid and semi-arid areas as a major protein source amounting to 20% to 22% more so in vegetarian meal. It also contributes other sources of nutrients like vitamins, and essential elements e.g. Folate, Calcium (Ca), Iron (Fe), Magnesium (Mg), Phosphorous (P) and Potassium (K) (Odeny, 2007; Saxena *et al*., 2010). In the traditional African society it replaces the consumption of animal products as the only source of protein and other common cereals like beans which are deficient of recommended protein calories. Proteins are body building macronutrients, required by the body well-being to facilitate cell and muscle development especially in children below 5 years, expectant women and lactating mothers (Kinyua *et al*., 2016). Proteins are important because they reduce malnutrition in children which is common in developing countries as a result of eating unbalanced diet or diet rich in carbohydrates only. Pigeon pea can be used when raw in its green stage as a vegetable which is very nutritious and easily digestible. When mature it is dried and used to make food stews to be served with other foods like rice (Snapp *et al*., 2003).

The crop adapts well in ASAL regions because it has long deep tap root system that break the soil pan, improving the soil structure and the leaves have osmotic adjustment which help maintain cell turgidity through the building up of very tiny insoluble substances (Subbarao *et al*., 2000). Pigeon pea grows well in low soil fertility, and best grown in arid and semiarid areas. It can be grown as a monocrop or intercropped with other crops like sorghum, millet, maize or cassava which makes it very suitable for climate smart intervention and improves soil fertility through nitrogen fixation facilitated by the presence of rhizobacterium association. It also increase soil organic matter through falling leaves and old roots thus increasing crop yields in sustainable cropping system (Kwena, 2018).It's used as animal feed. Pods and leaves are rich in protein and have high palatability value when fed to livestock. Seeds and seed byproducts are used as livestock and poultry feeds for the high protein content contributed. It can also be mixed with other grain cereals like maize and used to make animal feeds (Saxena *et al*., 2010).

Pigeon peas are grown for environmental management purposes, as wind breaks in agro forestry, roofing, weaving baskets in Africa and their dry stems used as a major source of fire wood after charcoal in Northern India. In Kenya, especially the Eastern part, where the crop is grown,

smallholder farmers use them as firewood and as fencing materials. Pigeon pea is an important crop to bees, the flowers provide nectar for the production of quality and nutritious honey (Orwa *et al*., 2009). Pigeon pea has medicinal properties where the polyphenols and flavonoids are used to treat some ailments in humans. The leaves, flowers, roots and roots are used to treat and cure illnesses related to respiratory, digestive and reproductive system. In Africa studies have shown that pigeon pea seeds can be used to treat hepatitis and measles (Sharma *et al.*, 2011).

Due to their determinate flowering characteristic, they allow continuous podding, enabling farmers to have grain harvest continuously without replanting again. This production can achieved by pruning the crop to facilitate the growth of new shoots and flowering. Despite all these excellent qualities and benefits, pigeon pea encounters a lot of limiting constraints from insect pests which affects its quality and production of the crop yields.

2.4 Biotic constraints of pigeon pea

Pigeon pea production, during cultivation of both local and improved varieties globally, is limited by various biotic challenges. They are affected by both living and nonliving components which constrain the yield of the crop. The main biotic stresses are the insect pests and diseases which affect the crop hence reducing production. Major insect pests attack pigeon pea and damage the flowers, leaves, pods and the seeds. These insect pests include Pod borers one of them being, *Helicoverpa armigera Hubner*, pod sucking bugs and pod fly which cause up to 50% of the field losses of the crop. The crop is mainly invaded by pod borers threatening a yield loss of 100% (Cheboi *et al*., 2016).

Helicoverpa armigera attacks pigeon pea and other crops in all countries that cultivate the crop. Diseases are also important factors affecting yield production on pigeon pea production. The major pigeon pea diseases causing substantial yield of over 50% are Cercospora leaf spot (*Cercospora cajani Hennings*), powdery mildew, nematode, rust (*Uredo cajani*), Phytophthora blight (*Phytophthora drechsleri Tucker f.sp.cajani*) and Fusaruim wilt (*Fusarium udum Butler*). There are also storage pests like weevils and grain borers that invade the pigeon pea grains hence lowering its quality. Abiotic stress include drought, poor soil fertility and soil salinity, seed

variety, temperature, humidity and photoperiod affects the pigeon pea productivity (Sultana *et al.*, 2014).

2.5 Pests and diseases of pigeon peas

Pigeon pea as an outstanding legume crop worldwide has a great potential to produce more yield given the necessary research attention. Pigeon pea production is seriously threatened by pest and disease constraints affecting the crop from the vegetative stage to maturity stage of the plant. The most common and threatening diseases affecting the plant in the farm is fusarium wilt (*Fusarium udum*) which is a nuisance in areas growing pigeon peas thus causing a lot of damage. It is reported to cause a yield loss amounting to US $5 million in East Africa. Nematodes also as important pests pose a challenge to the production of pigeon peas especially in the regions affected by fusarium wilt. The main nematode species causing damage to the crop include root knot nematode (*Meloidogyne javanicum*) and reniform nematode (*Rotylenchus parvus*) (Hillocks *et al*., 2000).

Accordingly, pigeon pea crop is infested by insect pests on the reproductive structures such as buds, flowers and pods hence lowering the crop yield. The main insect pests attacking pigeon pea plant are classified based on their mode of invasion. Those affecting the flowers and the pods are Lepidopterans. Hemipterans suck the pods while dipterans and hymenopterans feed on seeds. Insect pests cause a yield loss ranging from 16% to 69% annually.

The greatest limiting factor affecting the production of pigeon pea are the insect pests that attack reproductive structures. These are the pod suckers and pod borers. *Helicoverpa armigera* and *Maruca vitrata* remain key insect pests threatening the production of pigeon peas among other pests worldwide. According to Fitt (1989) *H. armigera* is characterized with the following features that makes the insect pest a serious and most wide spread worldwide hindering its control and management. The insect has high prolificacy, can feed on a variety of crops, robust mobility, and then experience a period of dormancy during harsh conditions. *Helicoverpa armigera* attacks mainly the reproductive parts of the plant because it has nutrients like nitrogen necessary for the growth of the larvae and when the flowering period is over, they nourish on plant leaflets. The adult moth oviposits on the flowers because they are attractive, the adults feed on the nectar for growth and development and also they are able to hide the eggs from invasion by the predators. *Helicoverpa armgera* has a wide host range which enables its population to multiply incessantly

during the cropping season. It has continuous successions with different hosts such as cotton, tomato, okra and chick pea, thus making it a challenge to control (Hillocks *et al*., 2000).

2.6 Impact of pigeon pea pod borer, *Helicoverpa armigera* on pigeon pea crop

Helicoverpa armigera is a significant agricultural pest insect which destroys pigeon pea globally. It is commonly known as pod borer, African cotton bollworm, corn earworm, tomato fruit worm and tobacco budworm. *Helicoverpa armigera* causes economic yield loss of pigeon peas of up to 80% total yield. The larvae feed on reproductive plant parts such as young buds, flowers and later the mature larvae make holes on the pods thus facilitating the entry of disease pathogens escalating the destruction. The damage caused by the pod borers varies depending on regions and locations where the crop is grown, crop variety and the season cultivated hereafter causing enormous economic losses and socioeconomic costs (Cunningham & Zalucki, 2014). The estimated yield loss in Kenya, of legumes and cotton is up to 50% while that of other crops like sorghum and millet is 20% (Maulana, 2018).

Pigeon pea pod borer is polyphagous. It attacks more than 180 hosts belonging to 45 plant families which include both wild and cultivated crops. Therefore, it is difficult to eradicate because of its survival, spread and distribution mechanisms in the natural environment, (Mathukumalli *et al*., 2016). Farmers rely mainly on synthetic insecticides to control and manage the insect pest which has proved to be in vain. The extensive and indiscriminate use of synthetic insecticides has become a threat to human health and the environment. There are risks of insect pests developing resistance and destruction of non-target organisms and beneficial organisms in the agricultural ecosystem. Most of the pesticides are expensive for the smallholder farmers. They, therefore, use substandard and low dosages which are not useful for the control of insect pests. Due to lack of skilled labor and training, farmers do not use the correct method of application of the pesticides on the crop, leading to pesticide resistance.

2.7 Biology and ecology of pigeon pea pod borer

Eggs of *H. armigera* are laid individually on the reproductive parts of the host plant. They are small with pomegranate shape with a diameter of 0.4 to 0.6nm and yellowish to white during the early stages, but change to brownish color before emerging to the next stage. The females oviposit

over 3000 eggs which is completed between 5-24 days during flowering stage (Maulana, 2018). During cold weather the eggs take longer period than warm season (Shanower *et al*., 1997). When eggs are hatched, young whitish yellow larvae emerge with a darkish brown head having spiracles that give them the dark spotted appearance. Mature larvae are 35-40nm long. The instars change from one development stage to another depending on the environmental factors and the quality of the diet. Fully mature larvae fall off from the host and hide under the soil to complete the pupation process. Pupae are normally sparkly brown with smooth surface, 14 to 18nm long, with two analogous spines that are posteriorly located on the physique (Maulana, 2018). Adult moths are nocturnal, light brownish in color and on the surface of the wings they have a black spot, gray asymmetrical streaks and an obscure reniform mark on the front wings. Hind wings are pale white in color covered by a dark spot laterally on the edge. The surface area of the moth wingspan is approximately 35 to 40mm to facilitate robust mobility when flying to establish the host.

2.8 Lifecycle of pigeon pea pod borer

The lifecycle of *H. armigera* which takes 25 to 60 days relies on the temperature prevailing in the surrounding environment. After the adult moth hatches the eggs, takes 3 to 5 days to hatch, larva to pupa stage 17 to 35 days and pupa to adult stage is completed after 17 to 20 days (Fig 3).

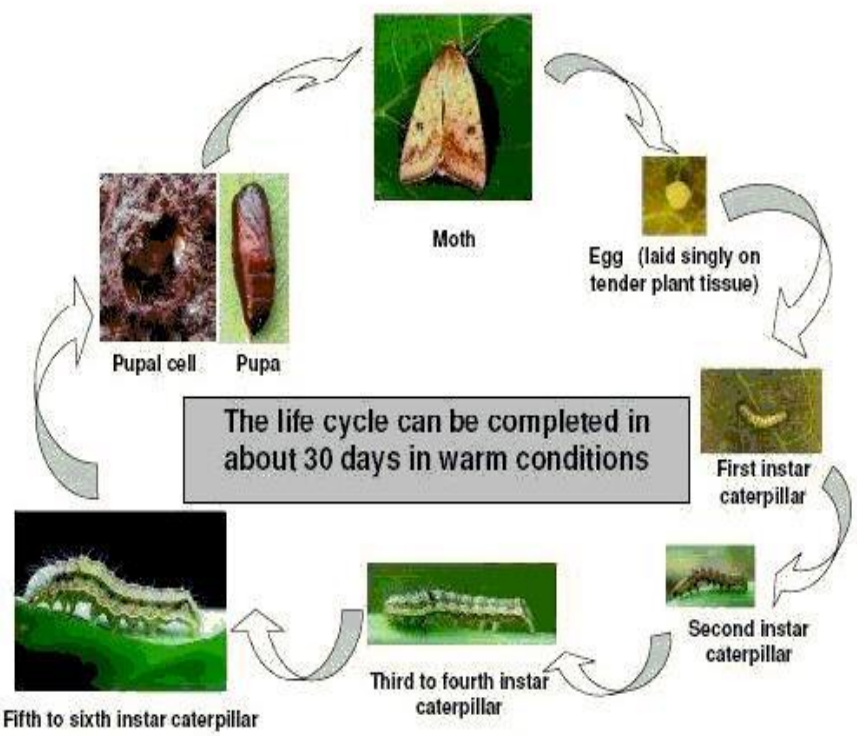

Figure 2.3: Life cycle of African bollworm (Helicoverpa armigera) (Seif *et al*., 2002)

2.9 Insect pest management of pigeon peas

Pigeon pea pest management has encountered a lot of challenges due to the different biologies of the three major insect pests attacking the crop. The insect pest exhibit different host range, varying mouthparts (chewing, sucking and piercing), and the mode of feeding. These pests also have unpredictable population changing aspects depending on seasons, sites and pesticide resistance that have been reported. All-important pests affecting pigeon pea attack the main parts of the plant that are beneficial to the farmer and they are proficient of damaging the whole plant. Pest infestation on the crop is affected by temperature and presence of moisture in the soil thus making problematic to manage this occurs due to global warming leading to climate change. Interferes with the whole process of insect pest management due changing seasons of pigeon pea planting and its development. Also socioeconomic constraints such as low income, lack of improved varieties, and lack of quality seeds affect the production of pigeon peas. Farmers also are not properly enlightened on agronomic skills on the overall management of the insect pests in their farms hence posing a lot of risks and loses of the crop yields. Most marginalized crops are not

attended to for so many years hence leading to greater losses. Pigeon pea has not been given much attention by farmers, policy makers and researchers as it's a marginalized crop, thus limiting its management of pest (Hillocks *et al*., 2000).

2.9.1 Use of synthetic pesticides in the management of pod borers

Chemical control as mode of controlling pests in pigeon pea farms have become unsuccessful due to various challenges related to its mode of application, handling, persistence on the ground and disposal on the environment. Most of the synthetic pesticides used have posed unfavorable hazards to human, environment and to non-target organisms. The over usage and persistent use of this chemicals have led to development of resistance to the pest and resurgence of secondary insect pests. It has been reported that insect pest of pigeon pea, *H. armigera* and *Melangromyza* species have developed resistance to organophosphates and pyrethroids posing threats to management of pests using pesticides thus affecting pigeon pea production. Synthetic pesticides are becoming a menace to human health by causing chronic illness like cancer, neurological disorders, and respiratory effects, metabolic and thyroid effects and to the environment by killing beneficial microorganisms which makes it not the better option for controlling pests and diseases. It causes environmental pollution as a result of water runoff in the existing water bodies both surface and ground water. The chemical residues are ingested by the animals like fish, water used for household activities are contaminated hence causing diseases. Also the harmful residues persist for a long time in the soil thus affecting the soil structure and exhausting soil fertility. They cause air pollution especially during aerial spraying (Aktar *et al.*, 2009). Due to the economic constraints faced by smallholder farmers cultivating the crop in the marginal areas they are not able to purchase the chemicals to control the pests hence opting to utilize other management practices which are not sustainable. Therefore due to all these challenges posed by the use of pesticides, there is need for establishment of effective and safe management strategies (Hillocks *et al*., 2000).

2.9.2 Cultural management practices

Cultural management practices are wide range of management techniques or skills which may be incorporated together by farmers to reduce disease and insect pest invasion leading to increase in crop production. Pigeon pea is affected by over 200 species of insect pests, other management practices have proved inadequacy in the control of these pests. Practices like intercropping in

pigeon pea have been ascertained to give noble results. Pigeon pea intercropped with maize or sorghum reduce the effect of pests like *H. armigera* and nematodes. This is because the maturity of the crops differs and they facilitate to break the lifecycle of the pests. Using pest tolerant varieties is a priority to manage these pest but the few short and medium duration varieties that have been developed are not embraced well by farmers because of the taste, seed color, size and are susceptible to diseases. This new pest tolerant varieties are also susceptible to pest infestation making farmers to opt for their old varieties as they are tolerant and can survive despite the attack of the pests and diseases. They are not readily available in the market stores for farmers to access and purchase limiting them to their old varieties. Lack of training to the farmers on the importance of using resistant varieties is a challenge hence making them not to embrace the varieties.

Management using insect resistant cultivar is also affected by the insect pest population dynamics and ecology. The crop can be planted early to overcome the challenges posed by these pests but due to global warming that has led to climate change, it affects the different biology of the diverse insect pests invading the crop and it cannot aid the management of the pests effectively. Ratoon plants and volunteer plants are discouraged because they are a major source of remnant pests carried over to the next season leading to increased insect pest build up interfering with pest management.

Field sanitation is recommended to reduce host plants that can harbor the pests because of its extensive variety of hosts and uncover the pupa towards natural enemies or destroyed by high temperatures. Scouting and rogueing is done regularly by removing of the mature larvae or complete removal of the infested plant to reduce the multiplication of pests as way of management, though it is time consuming. All the collected debris should be assembled in a pit hole and burnt to ensure complete destruction of the pest in the farm. Proper spacing should be adhered to prevent overcrowding and allow breeding of the pests. Conserving of the natural enemies is an important factor in the management of the pest. Crop rotation is an indigenous method of alternating different crops during the growing season to break the lifecycle of the pest and reduce insect build up in soil, but the method has yielded little success because of limited farming land for rotating the crops (Minja, 2001).Consequently, use of cultural methods to gap the damage caused by the insect pests

is cumbersome, expensive, and knowledge based which most pigeon pea farmers are cannot adopt and accomplish giving poor results in the long run.

2. 9.3 Biological control of pigeon pea pod bores

Biocontrol is a mode of controlling pests using other living organisms to manage pest multiplication below detrimental levels. They are classified into three; predators, parasitoids, and pathogens. Predators trap and feed on their prey for example beetles and red ants. Parasitoids don't feed on the host directly for example egg parasitoids and larvae parasitoids parasitize on maggots. It's reported that *Trichogramma spp* has worked effectively in controlling *H. armigera* in pigeon pea, cotton and tomato but due to changes in environmental condition, use of conventional insecticides and the ecology of parasitoids is not sustainable (Romeis & Shanower, 1996).

Predators and parasitoids utilization in the management of *H. armigera* in pigeon peas is comparatively low as a result of the presence of trichomes and trichome exudates from pigeon pea buds which impede their mode of action (Shanower *et al*., 1997). Insect disease pathogens have been identified as useful biocontrol agents in controlling harmful insects attacking legumes. They are gaining popularity in the agricultural sector due to the beneficial components; they are environmental friendly; specific in there mode of action; regularly efficient in insignificant amounts and decay rapidly leaving no detrimental effects on the environment. It involves the use of fungi, virus and bacteria to control *Helicoverpa armigera*. For example insect pathogens like Nuclear Polyhedrosis Virus (HaNPV) is reported to function effectively and its safe in controlling *H. armigera* but its usage is limited due to the effect of ultraviolet light, lack of virulence and substantial amount of titers that can eradicate larvae (Sharma *et al*., 2011).

Entomopathogenic fungi (EPFs)e.g. *B. bassiana* and *M. anisopliae* have been evaluated against *H. armigera* exhibiting successful results in managing *H. armigera* in chickpea, under laboratory and field conditions. The EPFs were effective against 3rd instar larvae though when the residues remain on the grains it releases some secondary metabolites which are harmful to human health (Bayissa *et al.*, 2017). Similarly, botanical extracts like neem extract have proven to be efficacious in decreasing the population of larvae damaging the pods (Bhushan *et al*., 2011).

2.9.4 Integrated pest management

The nature and major pest status of the pod borer (h. armigera) affecting pigeon pea necessitates a combination of different techniques that are safe, cost-effective and applicable as management approaches (Minja, 2001). IPM can be implemented because of its variability of techniques. Cultural methods have been used for a long time because they are affordable and practical but not successful in managing the pod borers. Use of chemical control is effective for management of pod borer although injudicious utilization of synthetic insecticides by farmers has led to pest resistant making it a challenge to the farmers to control the pest. Furthermore, synthetic insecticides have become a threat to the well-being of mankind, animals and its environs due to accumulation of harmful residues in the ecosystem (Kranthi *et al*., 2002). Therefore, there is need for a safe method of managing pod borers to enhance yield. Using natural enemies and parasitoids is the key approach of controlling this destructive pigeon pea pest. High populations of predators have been analyzed and have shown the potential in controlling *H. armigera*. Consequently more information is needed on the relationship between natural enemies and pest of pigeon pea.

Considering all these, it is necessary to integrate various alternative strategies for management of pigeon pea pest. Biocontrol is a valuable method that involves the use of naturally occurring microorganisms which are beneficial in the ecosystem and has captured attention in the agricultural sector when incorporated in IPM as a management strategy to control *H. armigera* hence increasing crop yield. This is due to the negative impact to both human and the environment that has been portrayed by the use of chemical control, time consuming, cumbersome and expensive cultural methods and therefore it's essential to find a safe, affordable and ecofriendly strategy to control the pod borer, *H. armigera* in pigeon pea. Consequently, significant efforts globally are made to produce the best auspicious biological control organism from naturally occurring bacteria, *Bacillus thuringiensis* which has proven successful against lepidopterans i.e. pod borers.

2.9.5 *Bacillus thuringiensis*

Bacillus thuringiensis is a gram positive soil bacterium with an aerobic nature, identified by the capability to sporulate hence producing *cry* and *cyt* toxins which consist of insecticidal proteins known as delta endotoxin (El-Menofy *et al*., 2014). *Bacillus thuringiensis* produces crystal proteins during sporulation. *Bt* bacterium is found in various places such as soil, insects and their

environment, forests, grain products and aquatic environment (Martin & Travers, 1989). The bacterium can survive longer even in harsh environmental conditions. *Bacillus thuringiensis* contain crystalline proteins that are toxic and specific to Lepidoptera, Coleoptera, Hymenoptera, Diptera (Bravo *et al*., 2017) and nematodes. *Bacillus thuringiensis* works efficiently when the target insect ingests the bacteria into the midgut. The *Cry* toxins get attached to the wall of the gut leading to the establishment of apertures that permit the toxins to penetrate into the blood system therefore resulting to blood poisoning. The insect larvae means of feeding will be halted causing septicaemia and death due to starvation.

2.9.6 *Bacillus thuringiensis* as a biopesticide

Bacillus thuringiensis has captured the world's attention because of its potential use as mode of biological control agent that can be used in the management of various insect pests of in the agricultural and medical sector for more than 60 years and contributes up to 80% to 90% of the microbial agents utilized (Argôlo-Filho & Loguercio, 2013). There are *Bt* isolates that have proved to be novel and have been used in the control of insect pests. These isolates belong to the genus *B. thuringiensis Subsp. Kurstaki* and *B. thuringiensis var. aziwai* used against various lepidopterans, and *B. thuringiensis Subsp. israelensis* is active on mosquitoes and black flies.

Bacillus thuringiensis exists as specific in its mode of action in that it doesn't affect non-target organism and it's safe for use in controlling insect pests of crops, forest plants and vectors of human diseases in public health sector. Moreover, it degrades completely on the environment leaving no harmful residues to accumulate on environment making it safe, efficient, ecofriendly and excellent method of managing insect pests when incorporated in integrated pest management program. It is also a key source of genes for transgenic expression to provide pest resistance in plants. Bt formulations that are in the market included Thuricide®, Javeline®, Dipel®, worm killer® and Bactospine® which were formulated from *B. thuringiensis Kurstaki* and *B. thuringiensis aizawai* (Wabule *et al.*, 2003). The commercial products are in powder form which are diluted during spraying and applied on the crop and they have proved to control caterpillars attacking crops hence improved productivity. Considering the safety depicted by *B. thuringiensis*, it gives a potential to be used in making biopesticide an option for synthetic pesticide.

In Kenya, research has been done on *B. thuringiensis* isolated from different habitats like soil, insect cadavers, and grain dusts and have showed great significance of microbial agents in the agricultural sector. *Bacillus thuringiensis* biopesticide screening efficacy has been carried out on the following lepidopteran insects: *Prostephanus truncatus*, *Busseola fusca*, *Chillo partellus*, *Sesamia calamistis,* and *Maruca testulalis* and they have revealed that *Bt* is a useful product to be used although resistance of *Helicoverpa armigera* to *Bt* has been reported from studies on transgenic crops like cotton (Alvi *et al*., 2012). This challenge can be fixed by conducting more screening of *B. thuringiensis* cry toxins more often to obtain novel genes for the continuous control of pigeon pea pod borer hence increasing crop productivity to enhance smallholder farmers' livelihood.

CHAPTER THREE

EVALUATION OF THE RESPONSE OF *HELICOVERPA ARMIGERA* LARVAE TO INFECTION BY *BACILLUS THURINGIENSIS* ISOLATES

ABSTRACT

Bacillus thuringiensis (*Bt*) is a bacteria that is found in diverse agro ecological zones. It contains crystalline insecticidal proteins that are beneficial in the management of destructive agricultural pests. The present study addresses the use of *Bt* isolated from soil and screened using molecular techniques as a biocontrol in managing pod borers, *Helicoverpa armigera* in pigeon pea. Ten soil samples (J1, J2, J3, J4, J5, J6, J7, J8, J9 and J10) from pigeon pea growing farms within Machakos and Makueni Counties were collected and *Bt* isolated using a selective method comprising nutrient broth and 0.25M Sodium Acetate. Isolates were grown on nutrient agar plates to establish colony features and were subjected to gram reaction technique to establish the *Bt* morphological characteristics. Molecular detection of the *Bt* isolates was done using PCR with specific primers to differentiate *Cry*1 and *Cry*2 genes. The results revealed that all the samples grown on nutrient agar exhibited *Bacillus thuringiensis* colony morphological features. Further analysis using gram staining technique indicated that eight soil samples (J1, J2, J3, J4, J6, J7, J9, and J10) were gram positive under the phase contrast microscope. Molecular analysis showed that *Bt* isolates J4, J6, J7 and J10 had gel bands indicating the presence of *Bt* genes. Two *Bt* isolates J7 and J10 had clear bands with the expected sizes; (J7) *Cry*2 and (J10) *Cry*1 expressed 600 bp and 1500 bp, respectively. These isolates were preserved and multiplied for use in bioassay and field experiments. Bioassay results showed that *Bt Cry*1 and *Bt Cry*2 significantly ($p<0.05$) caused mortality to *H. armigera* larva compared to control and that mortality was significantly ($p<0.05$) higher for *Cry*1 and *Cry*2 at 24 hours for 2nd and 3rd instar larvae. Mortality decreased as concentration of the *Bacillus thuringiensis* inoculum decreased as portrayed by 2nd instar larvae compared to 3rd instar larvae, which was attributed to the effect of larval stage and *Bt* concentration. The number of missing larvae was realized in both second and third larval instars due to cannibalism observed in *Helicoverpa armigera* larvae. The study concludes that *H. armigera* larvae especially 2nd and 3rd instars succumb to *Bt* infection and that soils in pigeon pea growing areas are a resource for obtaining *Bacillus thuringiensis* Cry genes which can be used as biopesticide.

3.1 INTRODUCTION

Bacillus thuringiensis (*Bt*) is an entomopathogenic microorganism that is gram positive, aerobic, spore forming bacterium. It is an indigenous microorganism that is found in diverse ecological environment and can adapt well to high temperatures (Martin & Travers, 1989). Various *Bt* strains can be isolated from soil (Ammouneh *et al.*, 2011; Martin & Travers, 1989; Mwathi, 2006), carcasses of dead insects (Asokan, 2007; Rajashekhar *et al*., 2017), forests (Lee *et al*., 2012; Lone *et al*., 2017a), stored grain products (Mwathi, 2006) and aquatic environment (Iriarte *et al*., 2000). It has the capability to sporulate hence producing *Cry* and *Cyt* toxins which consist of insecticidal proteins known as delta endotoxin (El-Menofy *et al*., 2014). During sporulation, this bacterium produces crystal proteins that can survive longer even in harsh environmental conditions. *Bacillus thuringiensis* contain crystalline proteins that are toxic and specific in their mode of action to the following insect orders: Lepidoptera (Caterpillars), Coleoptera (beetles), Hymenoptera (black flies), Diptera (Mosquitoes) (Bravo *et al*., 2017) and nematodes (Hui *et al*., 2012; Ravari & Moghaddam, 2015) These factors enable the bacterium not to affect non-target organism and is safe for use in controlling insect pests of crops, forest plants and vectors of human diseases in public health sector like mosquitoes.

Bacillus thuringiensis acts efficiently when used to control target organisms. However, when the target insect ingests the bacteria in to the midgut, the *Cry* toxins get attached to the wall of the gut leading to the establishment of openings that allow the toxins to penetrate into the blood system therefore resulting to blood poisoning. The insect larvae mode of feeding is paused causing septicaemia and death due to starvation (Bravo *et al*., 2007). Consequently, the *Bt* toxins are degrades completely on the environment leaving no harmful residues to accumulate on environment making it safe, efficient, ecofriendly and excellent method of managing detrimental agricultural insect pests when incorporated in integrated pest management system. Considering the safety demonstrated by *Bt*, it gives a potential to be used as a biocontrol agent to produce biopesticide which can be used to minimize the use of synthetic pesticide which are harmful to human health, the environment and beneficial non-target organism (Crickmore, 2006; Raymond *et al*., 2010). *Bt Cry* gene has been used to manage pod borers invading pigeon pea crop.

3.2 MATERIALS AND METHODS

3.2.1 Collection of soil samples for isolation of *Bacillus thuringiensis*

Ten soil samples were collected within Machakos County in the following agro ecological zones, upper midland zones (UM4) and Lower midland zone (LM5) where pigeon pea is grown. Soil sampling was done randomly at a depth of approximately 15cm. At least 100g of soil was divided and put into khaki bags that were labelled and transported to the laboratory inside a cool box and stored at -20°C awaiting isolation of *Bacillus thuringiensis* bacteria.

3.2.2 Media preparation for culturing of *Bacillus thuringiensis*

Nutrient agar plates were prepared using commercial nutrient agar. Nutrient agar was weighed based on the required amount to be used. Weighed 28 grams of nutrient agar and placed in a 1 litre reagent bottle and then added 1 litre of sterile distilled H_2O and mixed evenly for 2 minutes. The mixture was heated on a magnetic hot plate until it started boiling to allow the mixture to dissolve completely. The media was sterilized in the autoclave at 121°C, pressure 15pa for 20 minutes. To cool media, a water bath was used to lower the temperature of the media to 45°C before dispensing into the petri dishes following aseptic techniques inside a laminar flow hood and allowed to set before storage in a sterile environment. The agar plates were stored in the refrigerator at 4°C to be used later in the experiment.

3.2.2.1 Nutrient broth preparation for *Bacillus thuringiensis* preservation

Weighed 13g of commercial nutrient broth and placed in a 2 Litre conical flask and added 1 Litre of sterile distilled H_2O. The mixture was mixed thoroughly by stirring using magnetic stirrer, then dispensed into 250ml conical flasks and covered with cotton wool wrapped with aluminum foil. The mixture was autoclaved for 20 minutes at 121°C, 15 pascal pressure. The nutrient broth was kept for preservation of *Bacillus thuringiensis* bacteria.

3.2.3 Isolation of *Bacillus thuringiensis* from soil samples

Isolation of *Bacillus thuringiensis* was carried out by weighing 0.5g of soil sample and suspending it in to a sterilized 250ml conical flask containing nutrient broth medium and 0.25M sodium acetate (Astuti *et al*., 2018; Martin & Travers, 1989). The combination was mixed using a shaker incubator (Lab-line) at 200 revolution per minute for 72 hours at 30°C before heat treating in a hybridization

oven (hybaid) for 3 minutes at 80°C in order to kill non-spore-forming bacteria and other vegetative cells. The isolates were then grown on nutrient agar (Toxoid) and incubated inside a CO_2 incubator at 30°C for 24 hours to establish colony features and were later subjected to gram staining reaction to ascertain the *Bacillus thuringiensis* morphological characteristics (Martin & Travers, 1989). Colonies that exhibited *Bt* colony characteristics were further sub cultured to obtain pure colonies. The pure cultures were stored at 4°C in sterilized glycerol and nutrient broth in the ratio 1:1 to be processed later (Ammouneh *et al*., 2011).

3.2.4 Detection of *Bacillus thuringiensis cry* genes using molecular methods

Ten purified *Bacillus thuringiensis* isolates were screened for presence of cry genes using Polymerase chain reaction (PCR) technique. DNA extraction and purification was done using DNA genomic kit from Zymo Research Company, South Africa following the manufacturer's instructions and DNA were amplified by preparing 25µL master mix which was composed of 50ng sample DNA, 1U Taq polymerase (Hot start), 1.0 µM of each primer, 0.2 mM dNTPs, 2mM $MgCl_2$, and 1 x buffer. *Bacillus thuringiensis var. aizawai* was used as positive control while DNA and PCR grade water was used as a negative control. The cycling conditions were as follows; initial denaturation at 94ºC for 15 minutes, denaturation at 94ºC for 1 minute, annealing between 47ºC to 54ºC for 1 minute, polymerization at 72ºC for 1 minute involving 40 cycles and a final extension at 72ºC between 5 to 10 minutes. PCR amplicons were analyzed using 2% agarose gel (Top vision) stained with ethidium bromide (Sigma) to facilitate proper visualization. The amplified products were visualized and documented using a UV imager (UVITEC) (Astuti *et al*., 2018; Lone *et al*., 2017a).

3.2.5 Assessment of the response of pod borer larvae to *Bacillus thuringiensis* containing *Cry*1 and *Cry*2 gene in the laboratory

The experiment was conducted at KALRO, Food Crop Research Institute, Muguga Crop Protection Unit in a control temperature room. Pod borers (*H. armigera*) larvae were sourced from Thomas Odhiambo Campus ICIPE, Mbita. The colony was multiplied and maintained in the controlled temperature (CT) laboratory room and was used during the experiment. The insect bioassay was performed in the laboratory, using petri dishes that are covered with whatman filter paper at the bottom in order to retain the moisture inside. The larvae were fed using pigeon pea

flowers and pods which have been sprayed with six different concentrations of *B. thuringiensis* formulation constituted in the laboratory from the two identified bacteria containing *Cry1*(J10) and *Cry2*(J7) genes, *B. thuringiensis aiziwai* and control. Ten Instar 2^{nd} and 3^{rd} larvae were introduced into each petri dish that were covered with a net to allow proper air circulation. The whole experiment was replicated four times with nine treatments. The petri dishes were incubated in the controlled temperature room at 27 ± 1°C, and relative humidity of 70 ± 10%. Larval response and mortality rate was recorded at an interval of 24, 48 and 72 hours and at each evaluation the larvae were categorized depending on the movement response to slight touch that is alive (sluggish movement) or dead (no response to touch). Mortality rate was recorded and evaluated by MS excel to establish lethal concentration and lethal time.

3.3 RESULTS

3.3.1 Isolation and identification of *Bacillus thuringiensis* based on colony morphological characteristics

Ten *Bacillus thuringiensis* isolates obtained from the soil samples collected from various agro ecological zones were identified using morphological features (Table 3.1). However, purification and molecular detection of the ten *Bacillus thuringiensis* isolate was conducted and two samples J7 and J10 contained *Cry*1 and *Cry*2 genes respectively of the ten isolates (Figure 3.3).

3.3.2 Identification of colony morphological characteristics

The results showed that the bacteria isolates exhibited *Bacillus thuringiensis* morphological features. This characteristics were cream white in color, distinct, separated, slightly elevated with pinhead and a wracked edge shape as described by Glare & O'callaghan (2000). The characteristics named are shown in the image (Figure 3.1).

Table 3.1 Morphological characteristics of *Bacillus thuringiensis* isolates and there locality**.**

Isolate No.	Color	Cell morphology	Gram staining	Shape	Locality
J1	Cream white	Distinct, elevated, wracked edge	Positive	Purple Rod	Machakos
J2	Cream white	Distinct, slightly elevated, wracked edge	Positive	Purple Rod	Machakos
J3	Cream white	Distinct, slightly elevated, wracked edge	Positive	Purple Rod	Machakos
J4	Cream white	Distinct, slightly elevated, wracked edge	Positive	Purple Rod	Machakos
J5	Cream white	Distinct, slightly elevated, wracked edge	Negative	Pink Rod	Machakos
J6	White	Distinct, slightly elevated, wracked edge	Positive	Purple Rod	Kiboko
J7	Cream White	Distinct, elevated with pin head in the middle, wracked edge	Positive	Purple Rod	Kiboko
J8	Cream white	Distinct, slightly elevated, wracked edge	Negative	Pink Rod	Kiboko
J9	Cream white	Distinct, slightly elevated, wracked edge	Positive	Purple Rod	Kiboko
J10	Cream white	Distinct, slightly elevated, wracked edge	Positive	Purple Rod	Kiambu

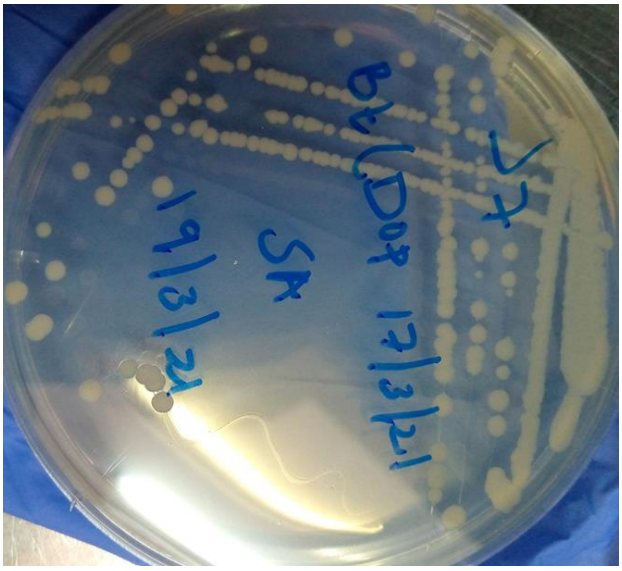

Figure 3.1: Pure culture of *Bacillus thuringiensis* after incubation

3.3.3 Gram staining of pure isolated *Bacillus thuringiensis* colonies

Analysis using gram reaction technique (Bagari *et al*., 2013) revealed that 8 isolates had purple rods with bipolar ends, some were single while others were paired in chains (Figure 3.2). Two isolates had pink rods instead of purple rods (Table 3.1).

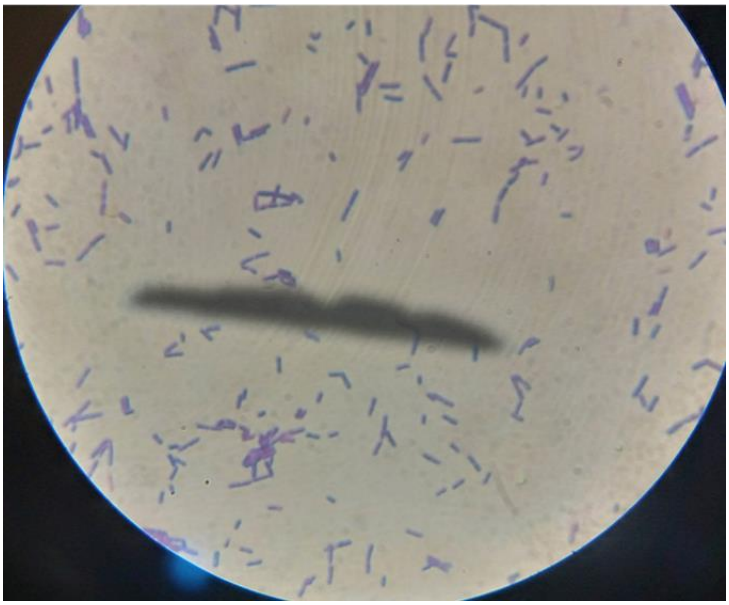

Fig 3.2: *Bacillus thuringiensis* gram positive purple rods

3.3.4 Molecular detection of *Bacillus thuringiensis* isolates

Out of the eight (8) *Bacillus thuringiensis* isolates only four (4) isolates expressed the *Bt Cry*1 and *Cry*2 using specific primers. *Bt Cry*1 had the band size of 1500bp as observed in J10 while the *Bt Cry*2 had band size 600bp as observed in J4, J6 and J7 (Figure 3.3), 8 isolates had *Cry*1 and *Cry*2 genes.

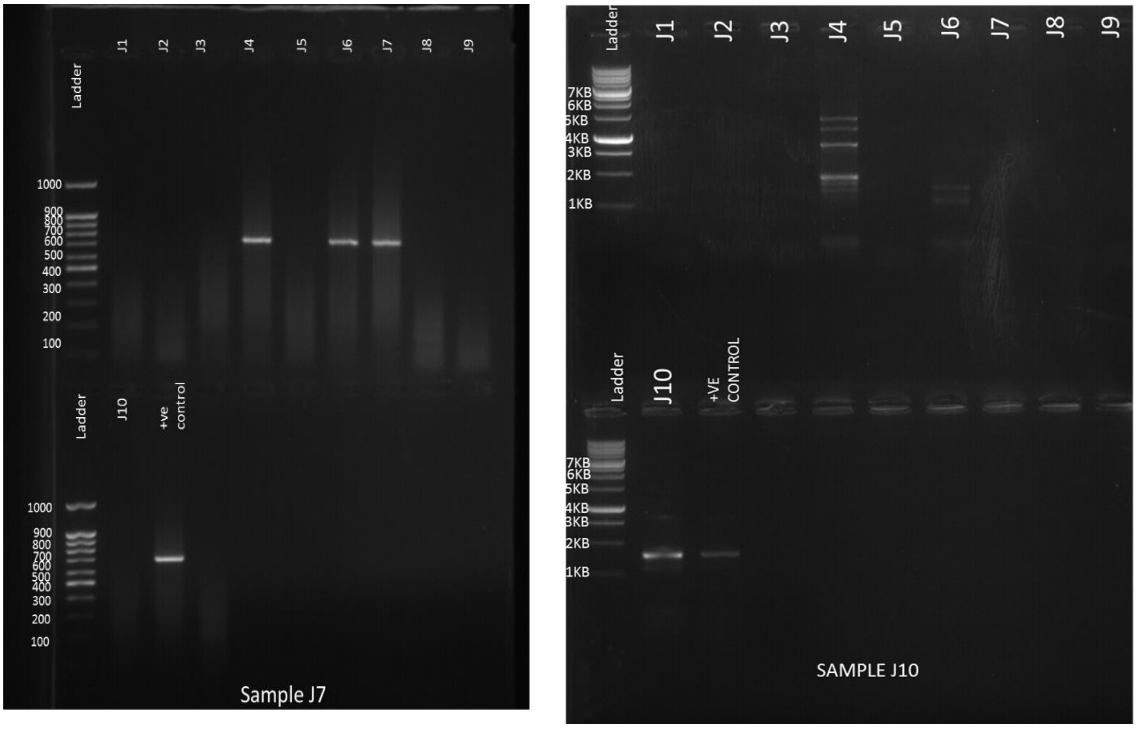

Fig 3.3 a& b. Agarose gel electrophoresis of PCR amplicons of *Cry*1 (J10) and *Cry*2 (J7) from locally isolated *Bacillus thuringiensis* strains using 1KB and 100bp molecular weight ladder respectively.

3.3.5 Effect of *Bacillus thuringiensis* isolates on pod borers in a controlled environment

3.3.5.1 Effect of concentration of *Bacillus thuringiensis* isolates containing Cry1 gene on the second instar larvae of pigeon pea pod borers

Exposure of pigeon pea pod borer larvae to various concentrations of *Bacillus thuringiensis* showed adverse effect on the second instar larvae of pigeon pea pod borers (Table 3.2). No differences were observed between experiment one and experiment two. In both experiments, *Bt cry1* caused significant ($p<0.05$) mortality of pod borer larvae compared to control with low dilutions causing higher mortality compared to higher dilutions. Exposure of pod borer larvae to various *Bt* concentrations resulted in mortality which was concentration dependent. In the first experiment, the highest mortality was recorded in *Bt* (10^{-2}) which was significantly different from all the other concentrations. It was followed in the second place by *Bt* (10^{-1} and (10^{-3}) while the lowest mortality was recorded in the control where no *Bt* was applied. Baciguard, *Bt* (10^{-1}) and *Bt* (10^{-3}) had a similar effect in causing mortality on the pod borer larvae. In the second experiment, the highest mortality was recorded in *Bt* (10^{-1}) and Baciguard which was significantly ($p<0.05$)

different from all other concentrations tested while the lowest mortality was recorded in the control with no *Bt*. All the other concentrations had no differences but the mortality caused was significantly ($p<0.05$) different from that of control. Cannibalism was observed among the larvae.

Table 3.2: Effect of various concentrations of *Bacillus thuringiensis* containing *Cry*1 gene on second instar of pigeon pea pod borer larvae

Treatments	Experiment One			Experiment Two		
	Alive	Level of Cannibalism	Dead	Alive	Level of Cannibalism	Dead
Treatment 10^{-1}	5.4a	3.1ab	0.73b	5.3ab	2.1a	1.13a
Treatment 10^{-2}	5.1a	2.8ab	1.33a	4.6b	3.8a	0.60b
Treatment 10^{-3}	6.5a	1.9b	0.73b	5.1ab	3.3a	0.67b
Treatment 10^{-4}	6.4a	2.7ab	0.46bc	6.1ab	2.7a	0.47b
Treatment 10^{-5}	5.2a	4.3a	0.33cd	4.9b	3.6a	0.53b
Treatment 10^{-6}	6.4a	3.1ab	0.26cd	6.5ab	1.9a	0.67b
Baciguard(*Bt* std)	6.1a	2.9ab	0.60b	5.7ab	2.1a	1.13a
Control	6.1a	3.6ab	0.07d	6.8a	3.0a	0.00c
Mean	5.9	3.1	0.56	5.6	2.8	0.65
LSD ($P\leq 0.05$)	2.2	2.3	0.32	1.8	2.1	0.31
P value	0.926	0.001	0.013	0.001	0.191	0.006
CV (%)	29.5	60.2	24.8	26.2	31.7	24.7

LSD – Least significant differences, CV- Coefficient of Variation. Values followed by a different letters within a column are significantly different at 5% probability

3.3.5.2 Effect of various concentrations of *Bacillus thuringiensis* isolates containing *Cry*1 gene on the third instars of pigeon pea pod borer larvae

Exposure of pigeon pea pod borers to various concentrations of *Bt* isolates containing *Cry*1 genes revealed different effects on the third instar larvae of pigeon pea pod borers (Table 3.3). There were no significant difference between experiment one and experiment two. In both experiments, no significant differences were observed between the treatments on the number of live and missing third instar larvae of pigeon pea pod borers. In both experiments, *Bt* containing *Cry* l genes significantly ($p<0.05$) caused mortality of the third instar larvae compared to control. Exposure of pod borers to various concentrations of *Bt* containing *Cry* l genes resulted in mortality which was concentration dependent. In the first experiment, the highest mortality was recorded in

concentration *Bt* (10^{-2}) while the lowest mortality was recorded in the control treatments where no *Bt* was applied. In the second experiment, the highest mortality was recorded in the standard (Baciguard) followed by *Bt* (10^{-1}) concentration while the lowest mortality was recorded in the control where no *Bt* was applied (Table 3.3).

Table 3.3: Effect of various concentrations of *Bacillus thuringiensis* isolates containing *Cry*1gene on third instar of pigeon pea pod borers

Treatments	Experiment One			Experiment Two		
	Alive	Level of Cannibalism	Dead	Alive	Level of Cannibalism	Dead
Treatment 10^{-1}	7.93a	1.27a	0.26bc	7.60a	1.00a	0.47ab
Treatment 10^{-2}	7.60a	1.27a	0.60a	7.53a	1.67a	0.40ab
Treatment 10^{-3}	7.33a	1.93a	0.40ab	8.00a	1.33a	0.33ab
Treatment 10^{-4}	7.33a	2.00a	0.33ab	8.26a	1.40a	0.20ab
Treatment 10^{-5}	7.87a	1.67a	0.20ab	8.53a	1.33a	0.13ab
Treatment 10^{-6}	8.06a	1.60a	0.13bc	8.00a	1.06a	0.40ab
Baciguard (*Bt* std)	7.73a	1.40a	0.47ab	7.60a	1.13a	0.73a
CONTROL	8.00a	1.87a	0.06c	8.60a	1.40a	0.00b
Mean	7.73	1.62	0.31	8.02	1.30	0.33
LSD ($P \leq 0.05$)	1.13	1.16	0.20	0.90	1.00	0.42
P value	0.642	0.664	0.003	0.526	0.894	0.024
CV (%)	20.3	30.7	18.9	16.1	29.9	18.8

LSD – Least significant differences, CV- Coefficient of Variation. Values followed by a different letter within a column are significantly different at 5% probability.

3.3.5.3 Effect of various concentrations of *Bacillus thuringiensis* isolates containing *Cry2* gene on the second instars of pigeon pea pod borers

Application of various concentrations of *Bacillus thuringiensis* isolates containing *cry2* gene had adverse effect on the second instar larvae of pigeon pea pod borers (Table 3.4). There were no significant difference between the two experiments and among the treatments except for the number of dead pod borers in the second experiment. There were no significant difference among the treatments on the number of dead pod borers in the first experiment. However, in the second experiment, Bt cry 2 caused significant ($p<0.05$) mortality of pod borer larvae compared to control. Exposure of pod borers to various *Bt* concentrations resulted in mortality and the highest mortality rate was recorded in plates that received *Bt* standard (Baciguard) followed in the second place by

mortality recorded in *Bt* (10^{-1}) while the lowest mortality was recorded in the plates with no *Bt* applied.

Table 3.4: Effect of various concentrations of *Bacillus thuringiensis* isolates containing *Cry*2 gene on second instar of pigeon pea pod borers

Treatments	Experiment One			Experiment Two		
	Alive	Level of Cannibalism	Dead	Alive	Level of Cannibalism	Dead
Treatment 10^{-1}	8.33a	1.40a	0.13a	7.46a	1.46a	0.46a
Treatment 10^{-2}	8.20a	1.53a	0.13a	7.86a	1.53a	0.27ab
Treatment 10^{-3}	8.07a	1.80a	0.06a	8.20a	1.06a	0.33ab
Treatment 10^{-4}	7.40a	2.06a	0.20a	7.46a	1.86a	0.33ab
Treatment 10^{-5}	7.67a	2.20a	0.06a	7.06a	2.00a	0.40a
Treatment 10^{-6}	7.67a	2.06a	0.13a	7.13a	2.33a	0.27ab
Baciguard (Bt std)	7.46a	2.20a	0.33a	7.33a	1.53a	0.60a
CONTROL	8.20a	1.73	0.06a	8.06a	1.87a	0.00b
Mean	7.88	1.88	0.14	7.58	1.70	0.33
LSD ($P \leq 0.05$)	1.05	1.08	0.26	1.26	1.20	0.38
P value	0.467	0.811	0.564	0.526	0.673	0.127
CV (%)	18.4	27.5	14.3	23.1	32.0	18.7

LSD – Least significant differences, CV- Coefficient of Variation. Values followed by a different letters within a column are significantly different at 5% probability

3.3.5.4 Effect of various concentrations of *Bacillus thuringiensis* isolates containing *Cry2* genes on third instar larvae of pigeon pea pod borers

Results showed that *Bt* isolates containing *Cry*2 gene affected the pigeon pod borers at several concentrations (Table 3.5). There were no significant differences between the two experiments, and between treatments on the number of live pod borers recorded in the laboratory after treatments applications. However, in both experiments the numbers of live pod borers were more in concentration *Bt* (10^{-6}) treated plots and control. The effect of *Bt* treatment on the number of missing pod borers was not significant in both experiments. *Bacillus thuringiensis* isolates containing *Cry2* caused significant ($p<0.05$) mortality on pod borer larvae compared to control in both experiments.. Exposure of pod borers to various *Bt* concentrations resulted in mortality which was dependent on the concentration and the stage of the larvae. In the first experiment, the highest mortality was recorded in *Bt* standard (Baciguard) and at *Bt* (10^{-2}) concentration which were

significantly ($p<0.05$) different from the rest of the test concentrations while the lowest mortality was recorded in the control. The rest of the concentrations recorded significant ($p<0.05$) mortality of the pod borer larvae compared to control. In the second experiment, the highest mortality was recorded in *Bt* standard (Baciguard) followed by *Bt* (10^{-1}) in the second position and were significantly ($p<0.05$) different from the rest of the test concentrations while the lowest mortality was recorded in the control. The rest of the test concentrations recorded significant ($p<0.05$) mortality of pod borer larvae compared to control

Table 3.5: Effect of various concentrations of *Bacillus thuringiensis* isolates containing *Cry*2 genes on third instar of pigeon pea pod borers

Treatments	Experiment One			Experiment Two		
	Alive	Level of Cannibalism	Dead	Alive	Level of Cannibalism	Dead
Treatment 10^{-1}	8.10a	1.53a	0.20b	8.3ab	1.10a	0.27ab
Treatment 10^{-2}	8.27a	1.27a	0.33a	8.7a	1.00a	0.20bc
Treatment 10^{-3}	8.53a	1.20a	0.13b	8.5ab	1.30a	0.20bc
Treatment 10^{-4}	8.00a	1.40a	0.20b	7.4ab	2.10a	0.13c
Treatment 10^{-5}	7.67a	2.00a	0.13b	7.6ab	2.00a	0.20bc
Treatment 10^{-6}	8.47a	1.27a	0.20b	8.6ab	1.00a	0.13c
Baciguard (*Bt* std)	7.53a	2.12a	0.33a	6.9b	2.50a	0.33a
CONTROL	8.40a	1.33a	0.07c	9.1a	0.90a	0.00d
Mean	8.12	1.52	0.200	8.13	1.50	0.18
LSD ($P\leq 0.05$)	1.59	1.55	0.132	1.70	1.70	0.137
P Value	0.425	0.725	0.784	0.001	0.067	0.687
CV (%)	15.60	29.8	16.9	17.1	32.5	17.7

LSD – Least significant differences, CV- Coefficient of Variation. Values followed by a different letters within a column are significantly different at 5% probability

3.3.5.5 Lethal effects of various *Bacillus thuringiensis* concentrations on mortality of pigeon pea pod borers

Effects of several concentrations of *Bacillus thuringiensis* on instars of pigeon pea pod borer were determined to get LD_{50} concentration. The results show that after 72 hours of exposure of pod borer instars to various concentration of *Bacillus thuringiensis* the LD_{50} and LT_{50} was not attained. *Bacillus thuringiensis* with *Cry*1 gene caused significant ($P\leq 0.05$) deaths on second instar pod borer larvae. There were significant differences among the treatments with *Bt* (10^{-2}) concentration

recording the greatest mortality (30%) on the second instar pod borers larvae in the first experiment while the *Bt* standard (Baciguard) exerted the greatest mortality (25%) in the second experiment. *Bacillus thuringiensis* with *Cry*1 gene caused significant (P≤0.05) deaths on third instar pod borer larvae. Treatments with *Bt* (10^{-2}) concentration recorded the greatest mortality (25%) on the third instar pod bore larvae in the first experiment while the *Bt* standard exerted the greatest mortality (30%) in the second experiment. *Bacillus thuringiensis* with *Cry*2 gene caused significant (P≤0.05) deaths on pod borers larvae. In both experiments and among the treatments *Bt* standard caused significantly (p<0.05) higher mortality on second and third pigeon pea pod borers instars (Fig. 3.4 a & b and Fig. 3.5 a & b)

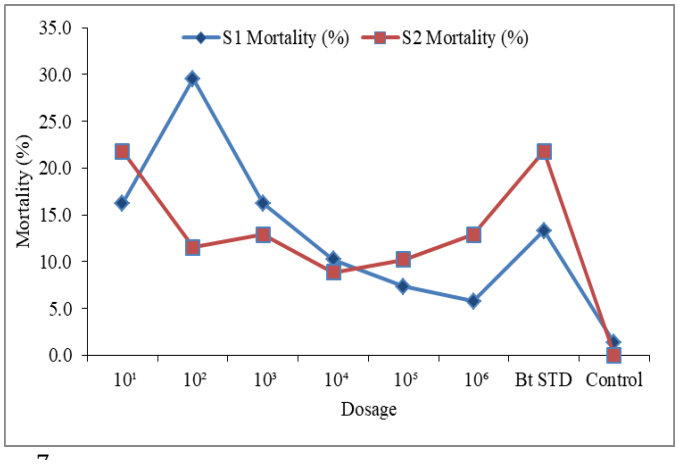

Fig 3.4a: Effect of various concentrations of *Cry*1 *Bt* on the second instars of pigeon pea pod

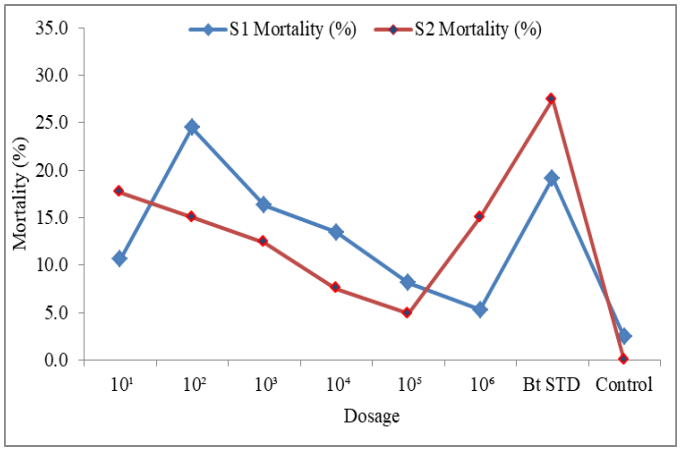

Fig 3.4b: Effect of various concentrations of *Cry*1 *Bt* on the third instars of pigeon pea pod

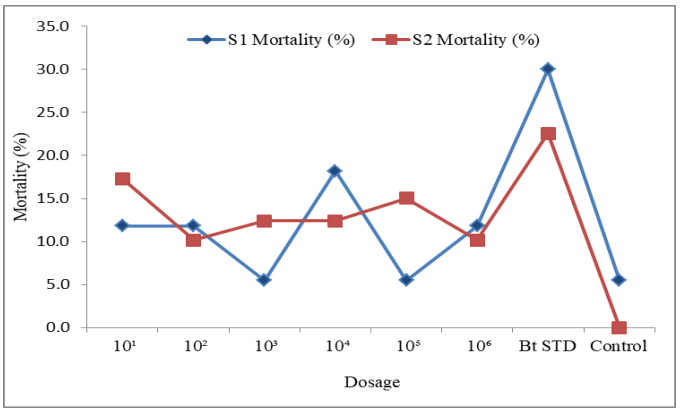

Fig 3.5a: Effect of various concentrations of *Cry*2 *Bt* on the second instars of pigeon pea pod

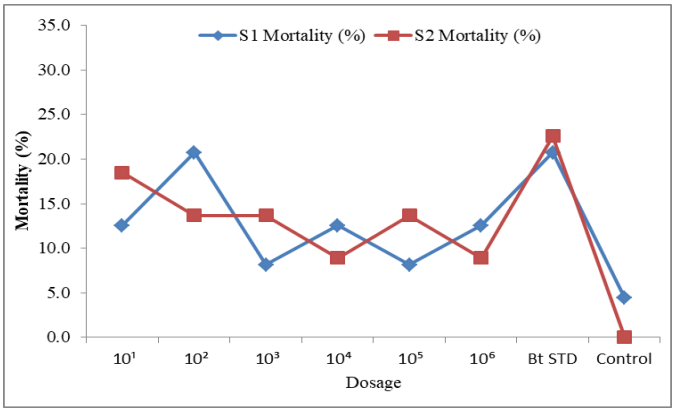

Fig 3.5b: Effect of various concentrations of *Cry*2 *Bt* on the third instars of pigeon pea pod

3.4 DISCUSSION

This study has shown that soil samples collected from diverse agro ecological zones had *Bacillus thuringiensis* bacteria. It agrees with results of Chatterjee *et al.* (2007) and Martin & Travers (1989) who reported ecology, diversity and abundance of *B. thuringiensis* isolate. Morphological characteristics revealed that 8 isolates were bacilli. However molecular detection using PCR carried out using specific primers revealed that 4 out of the 8 isolates had *Cry*1 and *Cry*2 genes. These were *Bt* isolate J4, J6, J7 and J10. Isolate J4 had multiple bands sizes on the gel showing that this isolate is harboring other Cry genes. Two isolates did not show the presence of either *Cry*1 or *Cry*2 indicating that they may contain other Cry genes not tested in the study. This was done on all the *B. thuringiensis* isolates to be able to establish the target cry gene, which is *Cry*1 and *Cry*2. The results agreed with those of Lone *et al*. (2017) who isolated *B. thuringiensis* bacteria from diverse environments harboring *Cry*1 and *Cry*2 genes that have specific toxicity against *Helicoverpa armigera*. PCR screening using specific primers for the detection of *Cry*1 and *Cry*2 genes displayed successful amplification. *Bacillus thuringiensis* isolate J10 was obtained from natural forest soil, which demonstrates that undisturbed forest soils are also a significant source of isolates of *Bt* with *cry* gene. Similarly isolates J4, J6 and J7 were obtained from soils with human activities (farming). Different *B. thuringiensis* strains can be obtained from diverse environment experiencing different climatic conditions and human activities as reported by Asokan *et al*. (2014).

The two potential isolates J7 and J10 were subjected to virulence screening in the laboratory against second and third instar larvae of *Helicoverpa armigera* using various dilutions with different *Bt* insecticidal crystals concentrations from the highest *Bt* (10^{-1}) to the lowest *Bt* (10^{-6}). From the study, mortality of the larvae was recorded after 24, 48 and 72 hours. Larval mortality was noted to be higher after 24 hours which showed that the larvae stopped feeding on the treated flowers and leaves due to the cry toxin effect in the midgut. This concurs with the study carried out by Tende *et al*. (2010) on two lepidopteran insects, *Chilo partellus* and *Busseola fusca* that stopped feeding after 2 hours. Mortality was not reported on all control samples (untreated sample) especially after 24 hours compared to the treated samples which agrees with Ammouneh *et al*. (2011) findings.

From this study, *Bt* with *Cry*1 (J10) and *Cry*2 (J7) genes applied at a concentration of *Bt* (10^{-1}) and *Bt* (10^{-2}) recorded the highest larval mortality on both second and third instar larvae of *H. armigera*. The mortalities are attributed to the stage of the insect, whereby 2nd instars feed more aggressively compared to 3rd instar who slow down their feeding during this stage. These findings are consistent with those of Lone *et al*. (2017b). *Bacillus thuringiensis* insecticidal *Cry* toxins also affect the mortality of the *H. armigera* larvae, whereby *Cry*1 proteins affects lepidopterans only while *Cry*2 has a specific action on lepidopteran and dipteran (Adang *et al.*, 2014; Schnepf *et al*., 1998).

The study revealed that treatments had an effect on larval mortality depending on concentration and time. *Bacillus thuringiensis* with *Cry*1 (J10) and *Cry*2 (J7) genes caused higher mortality at concentration *Bt* (10^{-1}) and *Bt* (10^{-2}), respectively. This indicates that the isolated *B. thuringiensis* is effective at higher concentrations compared to lower concentration on both 2nd and 3rd instar larvae of pigeon pea pod borer as reported by Da Silva *et al*. (2018) and Lone *et al*. (2017). Therefore, the higher the concentration of *B. thuringiensis* inoculum the more effective it is against the pod borer larvae, demonstrating its potential to manage insect pest for increased yield productivity.

Mortality also decreased as the concentration of the *B. thuringiensis* decreased, particularly for 2nd instar larvae compared to 3rd instar larvae. This finding agrees with the studies done by Muigano (2014) who reported the lowest mortality rate of both 2nd and 3rd pigeon pea pod borer instar larvae was in control samples (untreated) compared to the treated samples. The results are in agreement with the work done by Ammouneh *et al*. (2011) and Wang'ondu (2001) who tested the insecticidal activity of *B. thuringiensis* against various insect pests. The differences in the performance of *B. thuringiensi*s was also recorded between *Cry*1 and *Cry*2 proteins. Their different mode of action is linked to the origin of sample and its insecticidal activity. Isolate with *Cry*1 genes was collected from forest soil while isolate with *Cry*2 gene was obtained from pigeon pea farms, hence they performed differently in there potency. These occurs as a result of excessive farming activities which exposes *B. thuringiensis* to extreme sunlight hence reducing its toxicity. In addition, host specificity contributes to the performance of the different cry genes (Martin & Travers, 1989; Muigano, 2014). Larval mortality from the various *B. thuringiensis* concentrations was recorded nevertheless lethal effects, LD_{50} and LT_{50} were not attained after 72 hours. This was attributed to

the toxicity levels and pathogenic action of bacteria. Similarly, *B. thuringiensis* as a biocontrol agent requires longer exposure time to kill the target organism and different strains have diverse host specificity against insect pests of the same order hence affecting its lethal effects. This study agrees with the work done on the evaluation of *B. thuringiensis* against *H. armigera*, both in the laboratory and field where low mortalities were recorded and the lethal effects were not achieved (Abedi *et al*., 2014; Fite *et al*., 2020; Lone *et al*., 2017).

Cannibalism is a common behavior in insect species and especially at larval stage. Most lepidopterans engage in this kind of behavioral activity (Kakimoto *et al.*, 2003; Tang *et al*., 2016). From the current study, larval cannibalism was observed from both second and third instars of *H. armigera*. This was attributed to the type of diet fed on the larvae, environmental conditions i.e. temperature and the stage of the larvae. Cannibalism in *H. armigera* have only been demonstrated in work done in the laboratory and this is confirmed by work done on the factors affecting cannibalism in *H. armigera* (Kakimoto *et al*., 2003; Tang *et al*., 2016).

The study concludes that The study concludes that *B. thuringiensis* bacteria has potential in the management of pigeon pea pod borers and that soil is a great resource it can be a good source of important micro-organisms such as *B. thuringiensis* that can be used for different purposes, including as bio-control agents. Consequently, more research should be done on the *Bt* isolated from other habitats to ascertain their effectiveness and develop them for controlling agricultural pests.

CHAPTER FOUR

BIOEFFICACY OF *BACILLUS THURINGIENSIS* ISOLATES CONTAINING *CRY1* AND *CRY2* GENES ON POD BORERS, *HELICOVERPA ARMIGERA* INFESTING PIGEON PEA IN THE FIELD

ABSTRACT

Pigeon pea is an important legume crop that is grown by smallholder farmers in the Eastern region of Kenya. The crop is attacked by different insect pests, among them being pigeon pea pod borer (*Helicoverpa armigera*), resulting in reduction of crop yields. Most farmers depend on synthetic insecticides to control *H. armigera*, which later on causes detrimental effects to the environment, human health, non-target organisms and pest resistance. The objective of the study was to evaluate the bio efficacy of different isolates of *Bacillus thuringiensis* bacteria containing *Cry*1 and *Cry*2 genes on pod borers infesting pigeon peas in the field. Two *B. thuringiensis* isolates, containing *Cry*2 gene (isolate J7) and *Cry*1 gene (isolate J10), were evaluated at different concentrations both in the field. Field experiments were set up in KALRO - Kiboko for two seasons, the short and long rain season of 2020 and 2021, respectively. *Bacillus thuringiensis* at selected concentrations 10-3 and 10-4 was applied on pigeon peas to control pod borer larvae. Damaged pods were assessed and data analyzed. *Bacillus thuringiensis* treatments did not significantly ($P>0.05$) affect the larvae and hence the number of damaged pods compared to control in the first season. However, in the second season *Bacillus thuringiensis* treatments significantly ($P\leq0.05$) reduced the number of damaged pods compared to control and that the highest number of damaged pods was observed in control plots. The yields from both seasons were significantly ($P\leq0.05$) different, short rain season of 2020 had higher yield production compared to long rain season of 2021. In the short rain season of 2020, there was no significant effect of the various treatments on pigeon pea yield compared to the long rain season, where the treatments significantly ($P\leq0.05$) decreased pigeon pea yield compared to control. In the long rain season of 2021, there was a decrease in yield production compared to the short rain season of 2020. From the study findings, it is demonstrated that the locally isolated *B. thuringiensis* containing *Cry*1 and *Cry*2 genes have the potential of managing pod borers, *H. armigera* infesting pigeon peas in the field thus resulting to increased productivity.

4.1 INTRODUCTION

In East Africa, food production has become a challenge to the smallholder farmers due to the effect of both biotic and abiotic stresses that have proved to be detrimental to crop productivity. Climate change has tremendously contributed to most pest invasion on different agricultural crops, increasing pre and post-harvest losses, hence affecting crop yield and becoming a threat to countries food security. Insect pests can cause up to 100% losses especially during the early stages of crop development and when weather is favourable for the pest to multiply. Pigeon peas are attacked by various insects of the order Lepidoptera, especially pod borer (*Helicoverpa armigera*). The pest invades the crop during its vegetative and reproductive stages, thus disturbing the development of flower buds and pods. Reduction of the pod borer damage on pigeon pea as a climate smart crop is of significance among smallholder farmers. Therefore, efficient control and management of pigeon pea pod borer will contribute to increase in yield production of pigeon pea, farmer income and food security in the arid and semi-arid areas.

Farmers use conventional chemical pesticides to control *H. armigera.* However, the use of chemicals has been a challenge, resulting in pest resistance due to the indiscriminate use, pest resurgence, while chemical residues in the environment cause pollution in the soil, air and water bodies hence affecting human health, and outbreak of secondary pests. Regulations governing pesticides registration of new products are also not cost effective. Moreover, there is need for a safe, effective and ecofriendly pesticide to control pod borer on pigeon pea. Microbial agents incorporated with integrated pest management have the potential of managing insect pests infesting various crops amongst pigeon pea. Bacteria, fungi and viruses are among the microbial agents used for the control of agricultural pests. Bacterium, specifically *Bacillus thuringiensis* (*Bt*) consist of insecticidal crystal proteins (*Cry1* and *Cry2* genes) that are specific in their mode of action and are effective against different insect orders attacking crops like tomatoes, cotton, beans, chickpeas and maize. *Bacillus thuringiensis* biopesticide is ecofriendly, target specific, easily degradable and suitable when used as a component of an IPM program. Consequently, more research is needed to be carried out to evaluate *Bt* toxins' efficacy and virulence used in controlling pigeon pea pod borers.

4.2 MATERIALS AND METHODS

4.2.1 Reactivation and multiplication of *Bacillus thuringiensis* isolate in the laboratory

The *B. thuringiensis* bacteria isolated and kept in the laboratory fridge at 4°C (see Section 3.2.2) was used in this study. Preparation and multiplication of the *B. thuringiensis* isolate was done by culturing it in nutrient agar plates prepared under sterile condition inside a laminar flow hood using spread plate technique. The inoculated sample was incubated inside a CO_2 incubator for 48 hours and at the temperature of 30°C. Heat fixed smears were made and gram staining experiment was done to ascertain the morphological characteristics of *Bt* isolate by observing under oil immersion at the magnification X 1000 in a phase contrast microscope.

4.2.2 Preparation of *B. thuringiensis* inoculum as a biopesticide for use in experiments

The already constituted and sterilized nutrient broth was inoculated with the *B. thuringiensis* bacteria from the isolated samples. The bacteria was picked from the Petri dishes in a zigzag manner using a sterilized micropipette with a tip and dropped inside a flask containing the broth. Every sample was replicated and the control included and labelled clearly. The flask containing the sample broth was sealed using an aluminum foil and tied tightly with a parafilm to prevent spillage of the sample and mixed by swirling gently. The entire sub culturing work was done under sterile conditions inside the laminar flow hood. The broth was incubated in a water bath shaker incubator for 72 hours at a speed of 200 rpm at 37°C. After 48 hours the color of the broth was checked if it has changed to cloudy appearance, as compared to the control sample (distilled water) and the speed was reduced to 150 rpm. The samples were removed after 72 hours and the optical density (OD) checked using spectrophotometer machine and those with 600nm wavelengths were selected and used in the experiment. Serial dilution was conducted on the selected bacterial cultures from 10^{-1} to 10^{-6} from which 10^{-3} and 10^{-4} dilution factors were used in field experiments alongside Baciguard (bio-pesticide) and Actara®25WG 8g/20L (synthetic pesticide).

4.2.3 Efficacy of *B. thuringiensis* on pod borers affecting pigeon pea in the field

4.2.3.1 Establishing the crop

The field experiment was established in pigeon pea agro ecological zone specifically in Machakos County situated in Eastern province of Kenya. The recommended pigeon pea variety, KARI Mbaazi 1, a short duration variety which matures within 3-4 months was grown by following the

recommended agronomic practices. The crop was grown in plots measuring 4.0 × 5.0 m each and the space between plants and rows was 50 cm ×150cm, respectively consisting of twenty plants per plot. All other agronomic practices were followed; land preparation, sowing, irrigation, weeding, roughing and disease control as recommend except application of insecticides.

4.2.3.2 Experiment layout

The experiment was laid out using a randomized complete block design (RCBD) consisting of nine treatments and four replications. The *B. thuringiensis* isolate that was locally obtained and reactivated in the first experiment was used. There were 6 different *B. thuringiensis* concentrations at optimal concentration, commercial pesticide Actara®25WG 8g/20L, *B. thuringiensis* formulation in the market and untreated control. The experiment was repeated twice in different seasons. For identification, pod borers in the field were collected and taken to the laboratory to be sorted out based on their morphological characteristics using stereomicroscope and hand lens and recorded prior to the spraying of the treatment plots. The spraying was done at the beginning of flowering stage and monitoring was carried out regularly up to maturity. Spraying was done at an interval of two weeks with fresh inoculum. The number of healthy and unhealthy pods was counted in every five plants that were selected randomly and tagged per plot and recorded before every spraying session to depict the damaged caused by the pod borers. The total yield production was evaluated based on the dry pods harvested and threshed.

4.2.4 Data analysis of field and bioassay data

Data was collected, organized and analyzed using Microsoft office excel 2013 and Genstat Fifteenth edition (Genstat Model Release 15.3 (PC/Windows 8) (Lawes Agricultural trust, Rothamsted Experimental station, UK). Least Significant Difference Turkey at 95% was used to compare the means.

4.3 RESULTS

4.3.1 Plant stand

Pigeon pea Plant stand was significant ($p \leq 0.05$) between the two different seasons (Figure 4.1). Generally there were more pigeon pea plants in the short season 2020 averaging 87.5% than the long rain season 2021 at (73.1%).

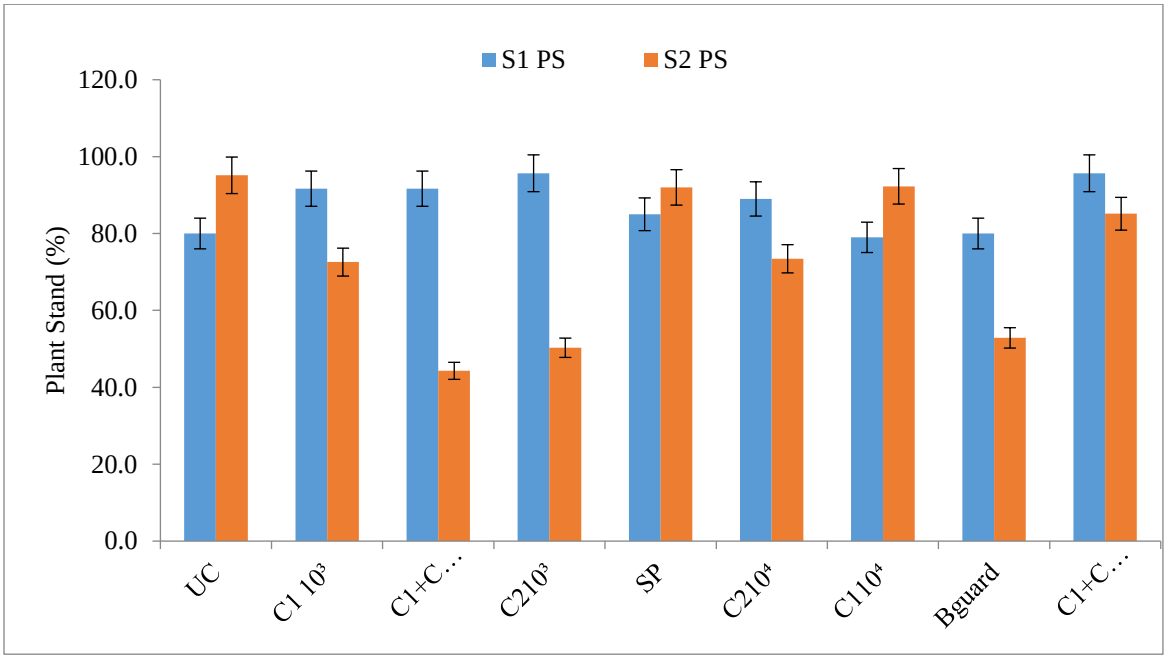

Figure 4.1: Effect of different *Bacillus thuringiensis* treatments on the plant stand of pigeon pea. UC- Untreated control, *Cry*1 (10^{-3})- *Cry*1 (10^{-3}), *Cry*1 + *Cry*2 (10^{-4}) - *Cry*1 + *Cry*2 (10^{-4}), *Cry*2 (10^{-3}) - *Cry*2 (10^{-3}), SP- synthetic pesticide, *Cry*2 (10^{-4}) - *Cry*2 (10^{-4}), Bguard – Baciguard, *Cry*1 + *Cry*2 (10^{-3}) - *Cry*1 + *Cry*2 (10^{-3}).

4.3.2 Effect of *Bacillus thuringiensis* isolate on the number of pods of pigeon peas in the short and long rains

Application of various *Bacillus thuringiensis* treatments had no significant effect on the number of damaged pods per plot compared to untreated plot. There were also no differences on the number of pods between the treatments in the short rain season 2020. However, in the long rain season of 2021, application of *Bt* treatment concentrations significantly ($P \leq 0.05$) decreased the number of pods compared to untreated plots. However, the number of pods per treatment did not vary (Table 4.1). Untreated plots had the highest number of damaged pigeon pea pods while plots that received Baciguard recorded the least number of damaged pods. No difference were observed among the treatments on the number of pods recorded per plot in the two seasons of experimentation. Untreated control had the least number of pods in the first season where there was a lot of wetness but had the most number of pods in the second season with less rain. Actara the standard pesticide had the second most number of pods in the first and second seasons.

Table 4.1: Effect of various *Bacillus thuringiensis* treatments on pigeon pea damaged pods and number of pods

Treatments	Season One		Season Two	
	Damaged pods	No. of Pods	Damaged pods	No. of Pods
Untreated control	2.9a	124.1a	1.3a	33.3a
*Cry*1 10^{-3}	3.1a	156.3a	0.56b	25.4a
Mixture *Cry*1 + *Cry*2 (10^{-4})	3.2a	148.8a	0.6ab	15.5a
*Cry*2 10^{-3}	3.1a	153.2a	0.48b	17.6a
Actara®25WG(Synthetic pesticide)	2.7a	155.1a	0.61ab	32.2a
*Cry*2 10^{-4}	2.0a	127.3a	0.78ab	25.7a
*Cry*1 10^{-4}	2.3a	126.3a	0.61ab	32.3a
Baciguard(Bt std)	3.2a	142.7a	0.31b	18.5a
Mixture *Cry*1 + *Cry*2 (10^{-3})	2.6a	146.4a	0.63ab	29.8a
Mean	2.8	142.4	0.7	25.6
LSD (P≤0.05)	1.8	36.8	0.7	18.4

LSD – Least significant differences, CV- Coefficient of Variation. Values followed by a different letters within a column are significantly different at 5% probability

4.3.2 Effect of *Bacillus thuringiensis* isolate on pigeon pea yield in the short and long rains

In the first and second season, there were no significant (P>0.05) differences of pigeon pea moisture content compared to control and among treatments (Table 4.2). Generally, higher yield were recorded in the first season than in the second season. There were no significant (P>0.05) effects on pigeon pea yield caused by various treatments on pigeon pea in the first season. In the second season, there were significant (P≤0.05) varied difference among treatments on yield compared to control. Plots treated with synthetic pesticides (Actara®25WG) had significantly (p<0.05) higher yields compared to the rest of the treatments while plots that received *Cry*1 (10^{-4}) and untreated control recorded the lowest yield. *Cry*1 (10^{-3}) followed Baciguard (*Bt* std) in the second place and *Cry*2 (10^{-4}) in the third position in order of decreasing yield realized.

Table 4.2: Effect of various *Bacillus thuringiensis* treatments on moisture content and pigeon pea yield

Treatments	Season One		Season Two	
	Moisture Content	Yield (Kg/ha)	Moisture Content	Yield (Kg/ha
Untreated control	12.6a	300.0a	10.5a	11.3b
*Cry*1 10^{-3}	12.4a	250.0a	9.7a	20.0ab
Mixture *Cry*1 + *Cry*2 (10^{-4})	12.3a	262.5a	11.0a	10.0b
*Cry*2 10^{-3}	12.9a	275.0a	9.7a	15.0b
Synthetic Pesticide	13.1a	300.0a	11.5a	30.0a
*Cry*2 10^{-4}	12.1a	300.0a	10.5a	16.3ab
*Cry*1 10^{-4}	12.9a	237.5a	10.3a	13.8b
Baciguard (*Bt* std)	12.8a	300.0a	13.0a	15.0b
Mixture *Cry*1 + *Cry*2 (10^{-3})	12.3a	250.0a	11.0a	15.0b
Mean	12.6	275.0	11.03	16.3
LSD ($P \leq 0.05$)	1.0	144.7	7.6	8.51
P Value	0.278	0.970	0.874	0.003
CV (%)	5.6	36.1	24.7	35.9

LSD – Least significant differences, CV- Coefficient of Variation. Values followed by a different letters within a column are significantly different at 5% probability

4.4 Discussion

This study involved carrying out the evaluation of identified native *Bacillus thuringiensis* isolates carrying insecticidal *Cry* protein activities against pigeon pea pod borer, *Helicoverpa armigera*. Isolates of *Bacillus thuringiensis* with *Cry*1 and *Cry*2 genes evaluated in this study have shown insecticidal properties against the pigeon pea pod borer, *H. armigera*. The current study shows that the plant population was influenced by the prevailing weather conditions during the seasons. During the short rain season of 2020, the rainfall was sufficient and temperatures conducive for crop growth and development hence reducing pest infestation. It also resulted in increased flowering and podding. These results agrees with the work done previously by Cheboi, *et al*., (2016), Esilaba (2021) and Wambua *et al*. (2017). Although there were no differences, the untreated contrl recorded most damage and least number of pods.

During the long rain season of 2021, weather conditions were not favorable. It was cloudy and low temperature were realized thereby affecting crop growth and development. It's been reported that

low temperatures and cloudy weather affect flowering and fruiting og pigeon pea leading to poor pod development, pod filling and may lead to increased pest infestation by pigeon pea pod borers, *H. armigera* (Esilaba, 2021)

From the current study there treatment effects varied as it depended on the *B. thuringiensis* inoculum concentration. The effect of *Cry gene* was observed in the current study where the plots sprayed with bacteria containing *Cry*2 genes at a concentration of *Bt* (10^{-3}) and a mixture of bacteria with *Cry*1 + *Cry*2 genes at the same concentration *Bt* (10^{-3}) had the highest plant population resulting in increased number of pods and reduced pod borer infestation. These led to increased yield production of pigeon pea due to the effectiveness of *Bt Cry* gene in managing pod borers. Lone *et al*. (2017) reported that *Bt* Cry gene work more efficiently when both *Cry*1 and *Cry*2 are combined in managing *H. armigera* compared to individual *Cry*1 and *Cry*2 isolates. It was also observed that prevailing weather conditions affected the performance of *Bt* bacteria, whereby during the short rain season of 2020, plots treated with *Bt* inoculum had higher plant stand and number of pods compared to the long rain season of 2021. Moreover, due to proper crop growth and development, conducive weather conditions contributed to low pod borer infestation causing increased number of pods per pigeon pea crop during the short rain season of 2020. During the long rain season of 2021 the weather was chilly with low temperatures hence affecting the action of *Bt* in controlling the pod borers. *Bacillus thuringiensis* has been reported to work effectively in controlling *H. armigera* and other pests of agricultural importance without affecting non target organism, human and environment health (Pandey, 2017).

The effect of the seasons and the treatment application was reflected on the pigeon pea crop yield during the two seasons. The yields were higher during the 2020 short rain season compared to the long rain season 2021. During the 2021 long rain season pest infestation was high as a result of the weather conditions since planting time up to plant maturity hence affecting the development of the pigeon pea crop (Mathukumalli *et al*., 2016). Temperatures make the conditions favourable and enables the pod borer larvae to attack the crop during flowering, fruiting and pod development hence lowering the crop yield and affecting the quality of seeds (Esilaba, 2021; Muli *et al*., 1997; Sharma *et al*., 2015).

CHAPTER FIVE:

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The study evaluated the potential efficacy and toxicity of *Bacillus thuringiensis* as a biocontrol agent targeting pigeon pea pod borer, *Helicoverpa armigera* of the insect order Lepidoptera at its larval most destructive stage. The findings showed that *B. thuringiensis* bacteria have great advantages as a source of biocontrol agent. It can be obtained easily from diverse agro ecological zones of the world (Chatterjee *et al*., 2007; Martin & Travers, 1989; Raymond *et al*., 2010). *Bacillus thuringiensis* is an ecofriendly biocontrol in comparison to the synthetic pesticides which are detrimental to non-target organism, human, the environment and cause pesticide resistance (Crickmore, 2006; Raymond *et al.,* 2010).

The Cry genes obtained from *B. thuringiensis* have demonstrated their effectiveness with high specificity to the target host especially those insect pests of agricultural importance. *Bacillus thuringiensis* based biopesticide have contributed majorly in the field of biological control. Up to over 90% of products consist of *B. thuringiensis* genes. But there are various challenges surrounding the production and formulation of the *Bacillus thuringiensis* biopesticide products. The challenges include high cost of production, insect larval specificity, sensitivity of the product to the prevailing climatic conditions i.e. solar radiations and limited activity to pod borer insects. These challenges constraint and limit the developing countries like Kenya from importing such useful products for use by their smallholder farmers hence leaving the option of using conventional chemicals for the management of pod borers.

From the study, it was revealed that prevailing climatic conditions from different seasons affects infestation of the crop by the pigeon pea pod borer, *H. armigera*. Insect pests thrive well at different temperatures orchestrated by climate change hence causing severe damage. The rate of application and concentration of *B. thuringiensis* inoculum caused mortality of *H. armigera* larval stages. When the rate of application increased, mortality rate of the 2nd and 3rd instar increased. Also the higher the concentration of the *B. thuringiensis* inoculum, the higher the mortality rate of the instars. Mortality occurred after 72 hours and mostly on the 2nd instar stage of the pod borer. This occurrence is attributed to larval ingestion of the cry toxins inoculated on the flowers and pods of pigeon peas. *Bacillus thuringiensis* toxins concentration effect decreases (degrades) with time

when exposed to the environment consequently affecting its efficacy on the mortality of targeted insect pest (Avilla *et al*., 2005). Cannibalism effect demonstrated by *H. armigera* at different developmental stages can be utilized as a way of reducing insect population amongst lepidopterans of agricultural importance

The rate of *B. thuringiensis* toxicity was measured using LD_{50} and LT_{50}. LD_{50} is the dose of the *B. thuringiensis* inoculum at which 50% kill of the *H. armigera* is achieved and LT_{50} is the time at which 50% mortality was attained. Both depend on the dosage, and mode of action causing biochemical changes in the body of an organism hence interfering with the normal cell functions. It's noted that the smaller the LD_{50}, the more toxic the biopesticide. From the findings, the two isolates *B. thuringiensis*, one containing *Cry*1 gene (isolate J10) and the one with *Cry*2 gene (isolate J7) were potent against the pigeon pea pod borer larvae, *H. armigera*. Isolate J10 was obtained from the forest soil and it was more effective than Isolate J7 which was sampled from pigeon pea farm in Machakos County. The difference in its potency is due to the origin (diversity) of the sample type and its insecticidal activity as reported by Martin & Travers (1989).

Controlling pigeon pea pod borers in Kenya has been a challenge. Production and formulation process of this *Bt* biopesticide is not cost effective. The process of registration is expensive, strains of *B. thuringiensis* are numerous and therefore require funding to facilitate proper characterization of specific genes to the target pest i.e. employing gene stacking approach. For this reason more studies should be carried out to formulate the two isolates that have been determined to be effective against pigeon pea pod borer infesting pigeon peas.

From the study, it was observed that timely planting, application of the *B. thuringiensis* inoculum regularly as required and with the recommended concentration resulted to increased crop production. Failure to observe the required management practices, then the pod borer, *H. armigera* attacks the crop at the reproductive stage hence affecting the podding process of pigeon pea. The study concludes that *B. thuringiensis* as a biological control agent can be used efficiently in managing insect pests of agricultural importance when properly formulated and repackaged as a biopesticide for use by farmers. In addition, climate change and pesticide resistance have caused resurgence of secondary pests thereby affecting crop production and income for small scale farmers to sustain their livelihoods. Therefore, this ecofriendly biopesticide for controlling the pod borer infesting pigeon peas can be formulated and upscaled for use by farmers. These isolates can

be further screened for their effectiveness against other insect pest like mosquito larvae due to the diversity, host specificity and toxicity. The information generated will provide an appropriate and safe technique of controlling pod borers infesting pigeon pea.

5.2 Conclusion

The study shows that *B. thuringiensis Cry*1 and *Cry*2 has insecticidal properties and causes mortality of the pigeon pea pod borer larvae both in the laboratory and field. Higher concentration of *B. thuringiensis* caused increased larval mortality of second instar larvae of *H. armigera* compared to lower concentrations and third instar larvae. Therefore, timely application of *B. thuringiensis* isolate in controlling pigeon pea pod borer will facilitate reduction in infestation hence increasing pigeon pea productivity.

In the field, *B. thuringiensis* was efficacious at higher concentration and in a mixture against pigeon pea pod borers. There was increased plant growth and development resulting to increased number of pods hence leading to increased yields. In addition, the study shows that the insecticidal activity of both, *Cry*1 and *Cry*2 improved pigeon pea yield production by controlling *H. armigera.* Therefore, *B. thuringiensis* as an ecofriendly biocontrol agent, can be used in controlling pigeon pea pod borers in the field to control pigeon pea pod borer to improve pigeon pea crop productivity.

5.3 Recommendation

Based on the results of this research study, the following recommendation can be made;

i. Additional analysis of all the *Bacillus thuringiensis* isolates to purify, identify and establish their efficacy against other organisms of agricultural importance needs to be done.

ii. Timely application of *Bacillus thuringiensis Cry*1 should be done immediately after flowering stage of the crop and at higher concentration in order to reduce the pod borer's larvae infestation.

iii. Formulate and upscale the production of the Bt isolates evaluated for use by farmers to sustainably produce pigeon pea crop

iv. Farmers are encouraged to do early planting of the crop in every season in combination with *B. thuringiensis* inoculum and other agronomic management practices in order to reduce pod borer infestation and achieve increased yield productivity (IPM).

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