ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES
DEPARTMENT OF ZOOLOGICAL SCIENCES

Exploring the potentials of Native *Bacillus thuringiensis* (Berliner) isolates in the management of chickpea pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Arsi and East Shoa Zones of Oromia Regional State, Ethiopia

By

LEMMESSA GEMMEDA ABDI

A PhD Dissertation submitted to the School of Graduate Studies of the Addis Ababa University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Zoological Sciences (Insect Sciences)

October 2024
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October 2024
Addis Ababa, Ethiopia

BIOGRAPHY

Dr. Lemmessa Gemmeda Abdi born in 1976 from his father Gemmeda Abdi and his mother Hatatu Aregu, in West Shoa, Gindeberet Woreda. After his age reached for School, he joined his primery School at Gute Andode primery School, he took the ESSLC in 1997 at Gerbeguracha Primery and Secondary School, North Shoa. He joined Hawassa College of Agriculture, the then called Hawassa University and graduated BSc. Degree in Plant Production and Dry Land Farming (PPDF) in 2002. Dr. Lemmessa Gemmeda, employed by Ministry of Agriculture as Lecturer at Kombolcha TVET College, Eastern Hararghe and he has served as Head Department of Plant Sciences for four years. Next appointed as acting Vice Dean for Administrative and transferred to Chiro TVET College, West Hararghe and served for two years fully as Administrative Vice Dean. Finally, Dr. Lemmessa Gemmeda moved to Asella TVET College, the then called Arsi University, he has given an opportunity for his MSc. Degree and joined Haromaya University in 2008 and graduated in 2011 in the field of Integrated Pest Management (IPM). Dr. Lemmessa Gemmeda additinally ingaged him self working on community service and research activites like as team member of Integrated Seed Sector Development (ISSD) program for four years giving advise, training for five seed producer co-operative farmers in Arsi Zone, suppervised five Woreds in Arsi Zone when the first maize fall army worm inveds Ethiopia as well as worked incollaboration with different NGO's like GIZ by giving training on pesticides application and pesticide safety for pesticide aplicators, LVIA LOT 3-Oromia on reducing pesticide application and introducing Integrated Pest Management for vegetable producer co-operative farmers in Shirka and Tiyo District. After serving for four years as senior Lecturer, he has given an opportunity to pursue his PhD at Addis Ababa University in the Department of Zoological Sciences, Insect Sciences. My future aspiration is strengthen biological pest control strategies in Ethiopia through formulating and developing biological pesticides to minimize the impacts of chemical pesticides on environment and human health.

Sincerely

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DEDICATION

I dedicated this PhD dissertation to my late Father Gemmeda Abdi and my late brothers Bekele Gemmeda and Iticha Obsu. I also dedicated to all my family members, especially to my beloved wife Aselefech Kebede; my Kids, Abdissa Lemmessa, Moti Lemmessa and Lemmi Lemmessa for their dedicated partnership in the success of my education.

DECLARATION

I, **Lemmessa Gemmeda Abdi**, declare that the research reported in this dissertation, except where otherwise indicated, and is my own original research. This dissertation has not been submitted for any degree or examination purpose at any other university and all sources of materials used for this dissertation has been duly acknowledged.

Lemmessa Gemmeda Abdi

Signature: ----------

Date:----------

As advisors, we approved and signed this Ph.D Dissertation for submission.

1. Emana Getu (Ph.D), Professor of Entomology

Signature:----------Date:----------

2. Diriba Muleta (Ph.D), Associate Professor of Microbiology

Signature:----------Date:----------

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
APPROVAL SHEET

This is to certify that the Dissertation prepared by Lemmessa Gemmeda Abdi, entitled '**Exploring the potentials of Native *Bacillus thuringiensis* (Berliner) isolates in the management of chickpea pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Arsi and East Shoa Zones of Oromia Regional State, Ethiopia**' and submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy in Zoological Sciences (Insect Sciences) complies with the regulations of the University and meets the accepted standards with respect to originality and quality. We read and approved its submission.

Signed by Board of the Examiners:

Advisor: Prof. Emana Getu Signature_____Date_____

Advisor: Dr. Diriba Muleta Signature_____Date_____

Examiner: Prof. Mulugeta Negari Signature_____Date_____

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Chair of the Department Signature_____Date_____

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ABBREVIATIONS

ALP	Alkaline phosphatase
APN	Amino peptidase-N
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
CADR	Cadherin
cfu	colony forming unit
cm	centimeter
CRD	Complete Randomized Design
Cry	Cry toxin or protein
cry	*cry* gene
CSA	Centeral Statistical Agency
Cyt	cytolytic toxin
EABC	Ethiopian Agricultural Busines Cooporation
EIAR	Ethiopian Institute of Agricultural Research
EPPO	European Plant Protection Organization
EtBr	Ethidium bromide
FL	Fiducial limit
gm	gram
GPS	Global positioning system
GPI	Glycosylphosphatidyl-inositol
GY	Grain yield
h	hour
ha	hectare
HSD	Highest significant diffirence
ICARDA	International Center for Agricultural Research in the Dry Areas
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
LD_{90}	Lethal Dose ninty
LD_{50}	Lethal Dose fifty
LT_{50}	Lethal time fifty
m.a.s.l	meter above sea level

mg	milligram
min	minute
mL	milliliter
mm	milimeter
µL	micro liter
nm	nanometer
NPV	Nuclear Polyhedrosis Virus
OD	Optical Density
°C	degree centigrade
PCR	Polymrase Chain Reaction
PD	Pod damage
RCBD	Randomized Complete Block Design
RH	Relative humidity
rpm	Revolution per minute
SAS	Statistical Analysis Soft ware
t	ton/tons
TNPD	Total number of pods
T_a	Annealing temprature
TBE	Tris borate-EDTA (Ethylene diamine tetra acetic acid)
T_m	Melting temprature
TSI	Tripile sugar iron
UV	Ultera Violete
WP	Wetable Powder

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ABSTRACT

Chickpea is an important staple food legume that provides proteins and health beneficial phytochemicals across the globe. It also improves soil fertility by hosting some nitrogen fixing soil bacteria. However, its production and productivity are highly affected by different factors including insect pests. Chickpea pod borer, *Helicoverpa armigera* is the most economically significant insect pest of chickpea globally. An over-reliance on insecticides leads to chemical residue in food, harmful to humans health, and disastrous to the natural ecosystem, which in turn prompts the biological pest management alternatives. *Bacillus thuringiensis (Bt)* gains popularity as bioinsecticide to control insect pests and vectors of human diseases through its insecticidal crystal toxic proteins encoded as *cry* and *cyt* genes. Thus, the objective of this study was to search for native *Bacillus thuringiensis* isoates that possess lepidopteran active *cry* genes chiefly *cry*1 and *cry*2. Out of the 121 soil samples collected, 23 *Bacillus* species were isolated and purified on suitable medium. Screening biotoxicity assay was conducted under laboratory along with a reference *Bt* .var. *thuringiensis*. Experiments were replicated three times. Ten 3rd instar larvae were used per replicate. Likewise, the ten *Bt* isolates were morphologically, and biochemically characterised. Further their *cry* genes profile was analysed using PCR. Five universal primers cry1, *cry*2, *cry*3, *cry*4, *cry*7, 8 and one specific primer *cry*9 and 16S rRNA universal primer were used for the analysis of *cry* genes. Biotoxicity assay was conducted at five concentrations ranging from 0.25-2 mL. $LD_{50, 90}$ values were 0.84-1.24 mL and 1.54-1.98 mL, respectively. Furthermore, pathogenicity was evaluated in the greenhouse and further verified under field conditions at Kulumsa Agricultural Research Center and Gonde Basic Seed Farm Center in 2023 main crop growing season. Treatments used were *Bt* concentrations at LD_{90} in the greenhouse and double of LD_{90} under field conditions. *Bt* concentrations first adjusted to 0.5 McFarland standard ($1.5x10^8$ cfu/mL) and mixed with 0.5 mL Triton x-100 (0.1%), 1 mL Tween 80 (2%) and one gram milk powder. Control plots sprayed with distilled water. Consequently, ten native *Bt* isolates were screened based on their larval mortality efficacy that ranged between 27.78% and 75.83% and LT_{50} values of 45.71-143.9 h. Statistically no significant ($p>0.05$) difference was observed among the tested native bacterial isolates and the reference *Bt*.var. *thuringiensis*. Some of the *Bt* isolates share the same morphological and biochemicals characteristics, but there are also some variations. All the *Bt* isolates possessed endospores, insecticidal crystal proteins, rod shaped cells as well as positive to catalase and motility tests.

Similarly, they all were positive to glucose fermentation and citrate utilisation, but negative to urease and indole tests. Two or more *cry* genes were detected in all *Bt* isolates, where *cry*4 (100%) detected followed by *cry*2 (90%), *cry*1 (50%), *cry*9 (40%) and *cry*7, 8 (10%). All the *Bt* isolates in the current study amplified 16S rRNA genes. As of the combinations of *cry* genes, *Bt* isolates KDL, AUPOS, GHTSW and GHTSW-1 carried (*cry*2+*cry*4), AUGHS-1 (*cry*1+*cry*4), ZDS (*cry*2+*cry*4+*cry*9), AUGHS-3 (*cry*1+*cry*2+*cry*4), ZDS-3 and AUASG-2 (*cry*1+*cry*2+*cry*4+*cry*9) and AUSD-1 (*cry*1+*cry*2+*cry*4+*cry*7,8+*cry*9). *Bt* grew under varied values of temperature that ranged from 15 °C-40 °C. All the *Bt* isolates showed growth at all the values of the tested temperatures. After 35 °C, the growth of all the *Bt* isolates declined indicating that 30 °C to 35 °C is the optimum growth temperature range. There was statistically significant ($p<0.05$) difference between *Bt* isolates tested and the control plots under the greenhouse conditions. Larval mortality (%), pod damage (%) and grain yield (t/ha) found to be in the range of 66.67±8.70% to 90.48±16.49%, 8.09±2.49% to 36.59±9.71% and 0.91±0.06 t/ha to 2.31±0.54 t/ha, respectively. Whereas, the control plots resulted in 36.59±9.71% pod damage and 0.91±0.06 (t/ha) grain yield. Under field trials, post 1st, pre 2nd and post 2nd *Bt* sprayed larval population counted per plant both at Gonde and Kulumsa showed statistically ($p<0.05$) significant variations between *Bt* isolates and the control plots that ranged from 0.97±0.17 to 3.50±0.23, 0.87±0.25 to 3.91±0.31 and 0.84±0.29 to 2.82±0.14 at Gonde. At Kulumsa, it was found in between 0.99±0.16 and 2.66±0.12, 1.00±0.21 and 2.69±0.20, 0.73±0.17 and 2.70±0.40, separately. Pod damage (%) and grain yield (t/ha) at Gonde ranged from 10.42±6.63 to 49.35±15.02 and 0.75±0.16 to 2.05±0.71, while, at Kulumsa ranged from 8.63±2.57 to 52.25±9.72 and 1.07±0.19 to 2.42±0.02 (t/ha) that was statistically ($p<0.05$) different from the control plots with the highest 47.53±9.51% pod damage and the lowest 0.97±0.03 (t/ha) grain yield. We concluded that all the *Bt* isolates tested harboured either *cry1* or *cry2* or both *cry* genes in combinations with other *cry* genes and efficiently reduced larval population, percentage pod damage with higher grain yield (t/ha). Hence, the *Bt* isolates were identified as potential candidate for *H. armigera* management and other related insect pests. Therefore, continuous exploration of *Bt* specific *cry* gene families is prompted and testing under multiple field trials for commercialisation and development of *Bt* bioinsecticides.

Keywords: Chickpea pod borer; *cry* genes*;* Crystal proteins; mortality; Native *Bt* isolates

CHAPTER ONE

1. GENERAL INTRODUCTION

1.1. Background and Justifications

Chickpea (*Cicer arietinum* L.) family Leguminosae is a staple food legume cultivated in many countries worldwide. The crop plays an enhanced role in the diets since it is a good source of energy, protein, minerals, vitamins, fiber, and health beneficial phytochemicals. Generally, chickpea provides 357- 446 Kcal/100g energy,12.6-29.0% protein, 3.4-8.8% fatty acid, 54-71% carbohydrate, 4.95% crude fiber and also a good source of minerals like Ca, Mg, K, P, and vitamins particularly, Vitamin Bs (Redden *et al.,* 2007). Chickpea plays an important role in improving soil fertility by hosting some N-fixing bacteria that fix up to 140 kg N per ha to meet most of its atmospheric nitrogen requirements (Saxena and Singh, 1987). Worldwide, the production of chickpea reaches 18.09 million tons from 14.81 million hectares (FAOSTAT, 2024). In Africa, chickpea is cultivated in the North and Eastern parts, Morocco, Algeria, Sudan, Ethiopia, Tanzania and Malawi are the largest producers with a total production of 0.78 million tons from 0.476 million hectares of land. Ethiopia shares 0.49 million tons of chick production from 0.227 million hectares of land from Africa (FAOSTAT, 2024).

Chickpea is the second most important pulse crop after faba beans, accounting for 15.18% of Ethiopia's total production (CSA, 2022). Ethiopia produces more chickpeas than any other country in the world, ranking first in Africa and fifth globally in terms of productivity (2.17 t/ha). However, it is the sixth-largest exporter of chickpeas (Charlie, 2016; FAOSTAT, 2024). But under favorable conditions, production can reach 5 t/ha (ICARDA, 2019). Ethiopia accounts for more than 60% of the African chickpea market, which are essential to the country’s economy. The average yearly export of chickpeas is 48,739 tons, with projected foreign exchange profits of $22.56 million (Ojiewo, 2016). However, there is still a wide gap between the growing demand and the production of chickpea due to insect pests and diseases that severely affect the production and productivity of chickpea in Ethiopia.

Among economically important fungal diseases of chickpea are *Ascochyta rabiei* (Pass) and *Fusarium oxysporum* f. sp. *ciceris* (Foc) among others, while, *Helicoverpa armigera* (Hübner), *Agrotis segtum* (Hfn.), *Aphis craccivora* (Koch) and, *Callosobruchus chinensis* L. are the important insect pests. Chickpea pod borer, *H. armigera* remains the most important insect pest of chickpea. It is among the world most economically important insect pest due to its wider geographical locations. In the current scenario, this pest is invading Africa, Asia, the Caribbean, Europe, Oceania, and South America. According to Gilligan and Passoa, (2014); EPPO, (2023) the pest has been spreading to North and Central America. The insect has voracious nature, multivoltin, and great dispersal ability that allows it to travel up to 200 km with the wind aid (EPPO, 2023). The phenology of the species is poorly understood and causes a several of problems, which made it difficult to identify the pest in the past. (Hardwick, 1965). However, the author has attempted to resolve the species complex in his review as the New and Old World bollworms, the majority of which were formerly referred to as either *H. armigera* or *H. obsoleta* under the genus *Heliothis*. Hardwick tried to refine his hypothesis by arguing that *H. zea* (New World) and *H. armigera* (New World)) that distinguished them as separate species based on differences in their genitalia and both belonged to the genus *Helicoverpa* (Hardwick, 1965; Pogue, 2004; Ueiroz-SAntos *et al.*, 2018).

Pogue, (2004) has found that there are certain problems with using the genitalia to identify the species due to some overlapping dimensions in the male vesica of *H. zea* and *H. armigera*. It was elucidated that the number of diverticula at the base, the length and number of coils, the number of cornuti visible on the vesicula inside the aedeagus, and the valve length are the best characteristics on the male genitalia for the identification of species complex (Pogue, 2004; Smith-Pardo, 2014; Ueiroz-Antos *et al*., 2018). Furthermore, the most reliable methods for correctly identifying the species in the *Helicoverpa* genus to date include genitalia dissection and molecular markers (Smith-Pardo, 2014). It was reported that 96 cultivated and 61 uncultivated plant species were identified as host plants for *H. armigera* (Pawar *et al*., 1986). The larvae attack 135 plant species in 35 families where the top six families are Asteraceae, Brassicaceae, Fabaceae, Malvaceae, Poaceae, and Solanaceae (Cunningham and Zalucki, 2014). Reed and Pawar, (1982) have reported 60 cultivated and 67 other plant species in 39 families as hosts of the insect pest.

Fitt, (1989) has found that 182 plant species were *H. armigera* hosts, 56 of which had showed significant damage and 126 of which less damaged. Tomato, green gram, and Egyptian clover were the most preferable hosts with a larval survival rate of over 80%. Likewise, the most significant host crops for *H. armigera* that have been documented include maize, sorghum, cotton, peas, pigeon pea, chickpeas, tomatoes, and cowpea (Gilligan and Passoa, 2014; Wasihun, 2016). Preferably the larvae feed on nitrogen rich, and economically important plant parts such as foliage, flowers, and developing seeds and cause substantial yield losses.

All over the world, several scholars have reported on the yield losses caused by *H. armigera* on chickpea. For instance, a single larva can damage up to 40 chickpea pods in its larval stage (El Fakhouri *et al.*, 2022; Hossain *et al*., 1970). It is one of the disastrous pest to the chickpea industry in Bangladesh causing up to 90% yield loss (Verma *et al.*, 2018). Zahid and his colleagues found that 5 larvae per meter row resulted in at least 47.31% of crop damage that caused a yield loss of 43.46% in chickpea (Zahid *et al.*, 2008). Moreover, a density of one larva per meter row averagely caused up to 155 to 157 kg yield loss per hectare (Zahid *et al.*, 2008). Whilst in India 20-30% and 10-90% yield loss was reported (Verma *et al.*, 2018). It is estimated that the losses resulting from *H. armigera* alone to chickpea in the semiarid tropical regions amount to around 328 million dollars (ICRISAT, 1992). In Ethiopia, 80 percent of pod damage was reported in early sown chickpea (ICRISAT, 1992). Gelatu and Million, (1996) have reported that in the central highlands of Ethiopia pod damage can reach 21-36 percent and a yield loss of 20-30% per annum.

Farmers use insecticides widely at higher dosages to combat the insect pest problem. As a result, the application of synthetic chemicals is hazardous to natural enemies, non-target organisms, and human beings. Even with the repeated application of insecticides, the pests are not amenable with the pesticides which in turn poses resistance to many groups of chemical insecticides (Gupta, 2009). There has been growing concern all over the world about the adverse effects of pesticides in agriculture, human health, and the environment including soil health, and water pollution (Al-Zaidi *et al*., 2011). The adverse effect of these chemicals needs urgent management options such as the use of microbial agents against insect pests of chickpea. The potential benefits of microbial control of insects to agriculture and public health through the use of microbial insecticides are considerable in light of the drawbacks associated with chemical pesticides (Raza *et al.*, 2015).

As a result, there is a growing need to augment microbial control agents through the use of Entomopathogenic bacteria, *B. thuringiensis*, Entomopathogenic nematodes, *Nuclear Polyhedrosis Virus*, *Baculovirus*, *Cytoplasmic polyhedrosis virus* and fungi particularly *Beauveria basiana* and *Metarhizium anisopliae* (Gupta *et al.*, 2010; Prasad, 2010; Ahmad, 2016). The entomopathogenic bacteria, *Bacillus thuringiensis* (*Bt*) (Berliner) is the most widely used bioinsecticide against a broad range of insect pests in the order Lepidoptera, Diptera, Coleoptera, Orthoptera, Hemiptera, and the rest (Lacey and Brooks, 1997; Rubio-Infante and Moreno-Fierros, 2016). Thus, the bacterium naturally occurs in several environments where it produces parasporal inclusion during sporulation that contains toxic crystal protein and delta-endotoxin encoded as *cry* genes with thermostable beta-exotoxin that distinguish *Bt* isolates from the other members of the *Bacillus* species (Palma *et al*., 2014; Das *et al*., 2021). It was first recognized and isolated by Ishiwata in 1901 in Japan from diseased silkworms, *Bombyx mori*. At the time, the author thought that diseases in silkworms was caused by an unidentified bacterium (Ishiwata, 1901). The same year, Ishiwata discovered that a rod-shaped, spore-forming bacterium he named "sotto bacillus" was the cause of a particularly troublesome disease known as "sotto" or "sudden collapse." He then postulated that the symptoms were brought on by a toxin that might be found either inside the spore or close to the spore (Dimock, 2002; Sansinenea, 2012).

The second discovery was made by Ernst Berliner, in Germany in 1911 who rediscovered *Bt* from diseased Mediterranean flour moths caterpillars, *Anagasta kuhniella* Zeller in the province of Thuringia from the grain store in silos (Berliner, 1915). Then the investigator named as *Bacillus thuringiensis* (*Bt*) which is used as the formal scientific name of the bacterium to date (Berliner, 1915; Dimock, 2002). *Bacillus thuringiensis* occurs in the environment naturally and is also artificially added to an ecosystem to attain insect pest control. Thus, its occurrence can be defined as both natural and artificial. It is native to many environments including soils, insect cadavers, stored product dust, leaves of plants, and aquatic environments (Lambert and Peferoen, 1992). Moreover, *Bt* has been detected recently in Antarctic soils and marine sediments (Forsyth and Logan, 2000). *Bacillus thuringiensis* grows naturally as a saprophyte, feeding on dead organic matter. The spores of *Bt* persist in soil and vegetatively proliferate in the presence of nutrients and conducive weather conditions (Lambert and Peferoen, 1992). Three prevailing latent niches for *Bt* was proposed that including an entomopathogen, a phylloplane inhabitant, and a soil microbe (Osman *et al.*, 2015 ; Anil *et al*., 2017).

Thus, it was discovered that between 30 and 100% of the phyllosphere's spore formers were *B. thuringiensis* (Martin and Travers, 1989). Spores are heat resistant and pre-treating samples with heat makes it easier to isolate *B. thuringiensis* strains since heat treatment usually eliminates other undesirable, non-spore-forming, and vegetative bacteria (Travers *et al.*, 1987). Hence, heat treatment in combination with an acetate medium is normally used to establish a selective isolation process and remains a golden standard during the screening activity of *Bacillus* species from the environments (Travers *et al.*, 1987). *Bacillus thuringiensis* accounts for about 5-8% of *Bacillus* spp. population in the environment (Martin and Travers, 1989; Chatterjee *et al*. 2007). Approximately 27,000 isolates gathered from 100 soil samples worldwide were used to show that *B. thuringiensis* could be isolated from every type of habitat, including those with an abundance of insects or diseased insects (Martin and Travers, 1989).

Among few of them, *Bacillus thuringiensis* subspp. *kurstaki*, Strain HD-1 was isolated from a pink bollworm, *Pectinophora gossypiella* Saunders, in a mass rearing facility (Dulmage, 1970), *Bacillus thuringiensis.* var. *thuringiensis* isolated from the larch fly, *Pristiphora erichsonii* (Hartig) in Canada (Health Canada, 2018), *Bacillus thuringiensis* subsp. *aizawai* was isolated from dust in a silkworm rearing facility during a second outbreak of the disease in silkworms in Japan. *Bacillus thuringiensis* subspp. *kenyae* was isolated from grain dust from a storage facility in Kenya (Norris and Burges, 1963). *Bacillus thuringiensis* subspp. *israelensis* isolated from mosquito larvae from mosquito rearing pond in the Negev desert in Israel (Goldberg and Margalit, 1977). Krieg and his colleagues isolated *Bacillus thuringiensis* subsp. *tenebrionis* from a dead meal worm larva, *Tenebrio molitor* L (Krieg *et al*., 1983). Unlike the *Bt* products developed for insect control in agriculture and against mosquito species are *B. thuringiensis* var. *kurstaki* HD1 expresses Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa proteins or HD73 that produces Cry1Ac; *B. thuringiensis* var. *aizawai* HD137 produces a little diverse Cry toxins such as Cry1Aa, Cry1Ba, Cry1Ca and Cry1Da; *B. thuringiensis* var. *sandiego* and *B. thuringiensis* var. *tenebrionis* produce Cry3Aa toxin and *B. thuringiensis* .var. *israelensis* contains Cry4A, Cry4B, Cry11A and Cyt1Aa toxins (Flexner and Belnavis, 2000; Kaur, 2001; Sanahuja *et al.*, 2011; Bravoa *et al*., 2013)**.**

To date the lepidopteran active cry toxic proteins identified were, Cry1 (A-K), Cry2 (A-B), Cry7 (B), Cry8 (D), Cry9 (A-C, E), Cry14 (A), Cry18 (A-C), Cry20 (A-B), Cry22 (A), Cry32 (A), Cry51 (A), Cry54 (A-B), Cry59 (A-B) and Cyt2 (B) (Palma *et al*., 2014; David *et al.*, 2019; Domínguez-Arrizabalaga *et al*., 2020; Das *et al.*, 2021).

About 20–30% of the dry weight of the sporulated cells that are ultimately prone to crystallization is made up of the *Bt* toxic crystalline inclusion. Therefore, it was primarily achieved by controlling the transcription of toxin genes to stabilize toxin transcript (Lambert and Peferoen, 1992). The transcriptional regulatory factors known as sigma factors (σ) are shared among *Bt* and are utilized to control the expression of important genes at various phases of sporulation. σ^{K} and σ^{E}, which are active during sporulation, control the majority of genes encoding Cry and Cyt toxins. The 130-140 kDa *Bt* toxins, which are part of the Cry1 classes of toxins that are anti-lepidopteran active toxin, depend on an additional crystallization mechanism. These toxins have a C-terminal region known as the crystallization domain, which is highly conserved among 130–140 kDa toxins and accounts for around half of the protoxin size and it's obligatory is for their crystallization (Tetreau *et al*., 2021).

Hannay first identified the cry toxin of *Bacillus thuringiensis* in 1953, and then he thought that the toxin was found to be sensitized within the bacterial cell (Hannay, 1953). The first *Bacillus thuringiensis* crystal preparations was utilized in Europe in order to suppress *Ostrinia mubitalis*, Hübner, the European corn ear borer in 1928. The initial *B. thuringiensis* commercial product under the trade name "Sporein," was produced in France in 1938 (Lambert and Peferoen, 1992). Thus,1981 was the first insecticidal crystal proteins cloned by Schnepf and Whiteley that led to the revolutionary development of *Bt* transgenic crops that exhibit insect pest resistance (Schnepf and Whiteley, 1981). Subsequently, reduced chemical insecticides were used on cotton, tomatoes, maize, and potatoes, among other crops. Thereafter, a number of *B. thuringiensis* products were produced in France, Germany, Soviet Union and the United States during the green revolution in the 1960s (Fitt, 2008; Naranjo, 2011). The growth of *Bt* transgenic crops impacted the insecticides used resulting in a 94.5 million kilogram reduction in the amount of the insecticide active ingredients used. The economic benefit of utilizing *Bt* at the farm level was US$1.73 billion, while the total agricultural revenue benefit from 1996 to 2005 was approximately $7.51 billion, even though solely 50% cotton was produced during this time. The use of *Bt* transgenic crops with insect resistance, especially transgenic chickpea, has resulted in a significant decrease in the use of pesticide sprays, which has decreased exposure to pesticides, increased the activity of natural enemies, decreased levels of pesticide residues in food, and made a safe environment for life (Sharma and Dhillon, 2018).

Currently, data bridge market research analysis (DATA BRIDGE, 2021) has forecasted that the *B. thuringiensis* bio-pesticides market will project to a compound annual growth rate of 16.10% during the period of 2021-2028 from the current 15.2% growth rate. Similarly, the market values will be expected to increases from $1.80 billion in 2020 to $ 5.94 billion by the year 2028. One of the most interesting features of cry toxins is their insect specificity, which is mostly brought about by the specific binding of the protein's receptor sites found in the larvae micro villi mid gut cells. Numerous toxin receptor sites have been discovered in insects. These toxins have distinct receptor sites on the brush boundary membrane for different insect species (Lambert and Peferoen, 1992). According to Pigott and Ellar, (2007) review, Cry toxin binding proteins receptors sites identified in lepidopteran insects include cadherin (CADR) like proteins, glycosylphophatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP), a 270 kDa glycoconjugate, and a 250 kDa protein known as P252.

Regarding *Bt* toxin activity in the gut of insects, two theoretical frameworks were suggested where the toxin was initially ingested by the susceptible insects. The combination of the mid gut proteinase enzyme and the alkaline pH causes the crystal proteins to become soluble and activated. On the apical micro villi of mid gut cells, active toxins bind. A 60 kDa α-glucosidase anchored in the apical mid gut membrane via protein receptor site for *Bt* toxins is to be bound. Following toxin binding to the receptor site, a portion of the toxin inserts into the lipid bilayer of the membrane to form an ionic-selective channel. This allows water to enter the cell and ions and other larger components to exit, causing the cell to swell and lyse via a colloid-osmotic lysis mechanism.The second theoretical framework postulates that oligomers, which are essential for pore formation, are formed by the crystal toxins that *Bt* produces. The most severe cytological alterations brought on by *Bt* toxin have been shown to include lysis and swelling of mid gut epithelial cells, as well as late damage to skeletal muscles and neural structures (El-Bendary, 2006; Bravoa *et al.*, 2013; Adang *et al*., 2014; David *et al.* 2019).

Compared to Cry toxins, First, unsaturated phospholipids on the midgut epithelial cells of the insect bind to cytotoxins as monomers. Over time, the epithelial cells develop aggregates of the Cyt toxins (Gill et al., 1992). The aggregates have the potential to reach a size of between 300 and 400 kDa According to Gill *et al*. (1992), these toxin clumps on the epithelial cell membrane cause pores to open up, which then cause cytolysis.

Thus, a number of variables impacted *Bt*'s development, sporulation, and toxic production. Temperature, pH levels, and nutrients like sources of carbon and nitrogen are some of the most important variables. Higher crystalline inclusions with higher protein contents and insecticidal action were seen in response to higher glucose concentrations. Maximum yields of endotoxin and protein were obtained in a semi-synthetic medium containing 6–8 g/l of glucose (Peter and Peter, 1973). *Bacillus thuringiensis* grows over a wide range of temperature values.

For *Bt* growth, the minimum and maximum temperatures are 10-15 °C and 40-45 °C, respectively (De Vos *et al.* 2009). Heimpel and Angus, (1958) have observed that *Bt* grows well at temperatures between 28 and 35 °C. According to data generated by Health Canada *B. thuringiensis* strain ATCC 13367 grew well in trypticase soy broth and in fetal bovine serum at 27 °C, 32 °C, 37 °C and 42 °C (Health Canada, 2018). During the fermentation process, *Bt* uses sugars typically glucose, fructose, maltose, ribose, molasses and starch to produce acid. As a carbon source, *B. thuringiensis* uses citrate slowly and has the ability to convert nitrates to nitrites. The growth and germination of *B. thuringiensis* spores have been restricted by pH, with *B. thuringiensis* ssp. *kurstaki* and *B. thuringiensis* ssp. *israelensis* exhibiting optimal growth at a pH of 7.5 ± 1.0 (Seligy *et al*. 1997). El-Bendary, (2006) have reported that *B. thuringiensis* ssp. *thuringiensis* grows best on nutrient agar at pH levels of 6.7 and 6.4; at pH 6.0 and 5.6, growth was reduced ten times; at pH 5.1, growth was further reduced by 10,000 times; and at pH 4.4, growth was absent. Therefore, using an entomopathogenic bacterium, *B. thuringiensis* against *H. armigera* in chickpeas has been documented by several researchers (Nguyen *et al*., 2007; Nandish, 2016; Estela *et al.*, 2004; Qayyum *et al.*, 2015).

A study evaluated the toxicity of *Bacillus thuringiensis* Cry proteins to *Helicoverpa armigera*, a significant pest in southern Africa (Li and Bouwer, 2012). The toxicity was determined through diet contamination and droplet feeding methods. Second instar larvae were less susceptible, and Cry1Ab, Cry1Ac, and Cry2Aa were the most toxic proteins. This study provides an initial benchmark for assessing individual Cry protein toxicity in South Africa. *Helicoverpa punctigera* and *H. armigera* were tested for susceptibility to insecticidal proteins from *B. thuringiensis* using bioassay where Cry1Ab, Cry1Ac, Cry2Aa, and Cry2Ab were found to be effective in killing *H. armigera* (Liao *et al*., 2002). Another previous study (Chandrasekaran *et al*., 2015) has evaluated the biological effects of *Bacillus thuringiensis* and *B. subtilis* on *Helicoverpa armigera* in an artificial diet.

The results showed that *Bt/Bs* food consumption, digestion, and growth rate declined significantly compared to different concentrations of *Bt* and *Bs* but approximate digestibility increased. The consumption-dependent growth efficiency was higher in bacteria free diets. The lethal concentration values were lower in the *Bt/Bs* mixture. The authors further demonstrated that the susceptibility of American boll worm larvae to *Bacillus thuringiensis*. var. *kurstaki* using a diet incorporation method was enhanced. Varied lethal concentrations and temporal variations in insect populations were reported across nine locations in India. Temperature also influenced susceptibility with an increase in ambient temperature increased susceptibility by 7.5-fold. The study highlights the role of selection pressure, host plant, xenobiotics, and agroecological conditions in *H. armigera* susceptibility (Chandrasekaran *et al*., 2015). The study scrutinised that the susceptibility of American bollworm larvae to *Bacillus thuringiensis*. var. *kurstaki* using a diet incorporation method. Results showed varied lethal concentrations and temporal variations in insect populations across nine locations in India. Temperature also influenced susceptibility, with an increase in ambient temperature increasing it by 7.5-fold. The study highlights the role of selection pressure, host plant, xenobiotics, and agroecological conditions in *H. armigera* susceptibility (Chandrashekar *et al*., 2015).

Transgenic chickpea plants expressing Cry1Ac protein showed high insect toxicity and plant protection against pod borer larvae, *H. armigera* with plants expressing Cry1Ac protein above10 ng/mg that showed 80-85% protection and high insect mortality, while plants expressing between 5 and 10 ng/mg showed early pupation and moderate insect mortality (Sanyal *et al.*, 2005). The majority of studies on *B. thuringiensis* have focused on the strains that are effective against Lepidoptera and Diptera such as *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *israelensis*, and *B. thuringiensis* subsp. *aizawai*. Lone *et al*., (2017) have discovered that of the four putative toxic isolates investigated, JK12 was more toxic against *H. armigera* than *B. thuringiensis* HD1. Then, the authors further highlighted the significance of continuing research into novel *Bt* stains from a variety of ecological locations around the world. In Ethiopia, attempts have not been made to utilize native isolates of *B. thuringiensis* against pod borer, *H. armigera* in chickpea. However, the existence of native *Bt* isolates and their potential against insect pest control have been evaluated and reported previously (Tesfaye and Emiru, 2010; Aynalem *et al*., 2021; Gebremariam *et al*., 2021). Therefore, it can be hypothesized that there are virulent and effective native *Bt* isolates that may serve as an alternative to chemical insecticides to control noxious insect pest such as *H.*

armigera in chickpea production. Thus, this study proposed with the following general and specific objectives:

1.2. Objectives

1.2.1. General objective

The general objective of this study was to investigate and isolate virulent native *Bacillus thuringiensis* isolates that could be used for the control of *H. armigera* to boost the production and productivity of chickpea crop

1.2.2. Specific objectives:

1. To isolate, screen and test the pathogenicity of native isolates of *Bacillus thuringiensis* against chickpea pod borer, *H. armigera* (Hübner)
2. To identify and characterize native isolates of *Bacillus thuringiensis* crying *cry1* and *cry2* gens for the management of *H. armigera* (Hübner)
3. To evaluate the pathogenicity of native isolates of *Bacillus thuringiensis* against *H. armigera* (Hübner) under the greenhouse conditions
4. To test field pathogenicity of some selected native isolates of *Bacillus thuringiensis* against *H. armigera* (Hübner)

1.3. Significance of the study

The findings of this study evidenced that native *Bt* isolates can effectively suppress the chick pod borer, *H. armigera*, used as alternative to chemical insecticides. Furthermore, it benefited the global scientific community by publishing findings on native *Bt* isolates from Ethiopian sources, where few investigations have been under taken. It also opens up new research avenues for the investigation of more potent native *Bt* isolates in the management of the target insect pest, chick pod borer, *H. armigera* and other economically significant agricultural crop pests. Additionally, it helps public health sectors control human diseases causing vectors to reduce the use of insecticides, which in turn reduces risks to human and environmental health.

1.4. Scope of the study

Our study was targeting specific agro-ecosystems particularly chickpea and tomato farms as well as few insect cadavers for *Bt* sources to be studied to control the target insect pest, *H. armigera*. So that, targeting only a single economically important insect pest of chickpea, *H. armigera* with few native *Bt* isolates collected.

Further the collected *Bt* isolates were morphologically and biochemically characterized, the toxic *cry* genes they harbouring were molecularly identified.

1.5. Limitations of the study

The current study covered very limited agro-ecosystems (chickpea and tomato farm) and few insect cadavers for *Bt* collection compared to the vast biodiversity exisisted within different agro-ecosystems with an immense resources which were intact. Those *Bt* isolates currently studied were not sequenced and identified to the strain level as a result not registered. Those identified *Bt* isolates were also not verified under multiple field trials on the target insect pest, *H. armigera* and its associated host crops.

1.6. Structure of the dissertation

This Dissertation comprises six chapters that encompass experimental chapters (chapters 2-5), each was prepared in an article form adhering to the specific objectives listed in subsection 1.2.2 in a chronological order.

Chapter two: The first experimental chapter refers to **Paper I** which is adhered to the first specific objective that addressed the laboratory screening of native *Bt* isolates through pathogenicity testing. **Paper I** is entitled ‘’Pathogenicity testing of native *Bacillus thuringiensis* isolates against chickpea pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Ethiopia’’ and published in Elseveir, Crop Protection (2023), Vol. 174, pp. 1-9.

Chapter three (**Paper II**) is in line with the second specific objective that delt with morphological and biochemical identification of the *Bt* isolates with molecular characterization of their toxic cry genes responsible for the toxicity against *H. armigera*. **Paper II** is entitled as ‘’*Bacillus thuringiensis* isolates and their *cry* gens toxic to chickpea pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) from Ethiopia’’ that has been published in Indian Journal of Entomology, Ref. No. e24107, DoI. No.: 10.55446/IJE.2024.2107.

Chapter four (**Paper III**) is dedicated to the greenhouse experiment and adhered to the third specific objective that specifically addressed the pathogenicity of the screened *Bt* isolates for further evaluation under the greenhouse conditions. **Paper III** is entitled ‘’Native *Bacillus thuringiensis* isolates against the old-World bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Ethiopia’’ and published in Tayler and Francis, Biocontrol Science and Technology (2024), Vol.34, Issue. 7.pp. 616-629.

Chapter five (Paper IV) is adhered to the forth specific objective entitled as 'Evaluation of Native *Bacillus thuringiensis* (Berliner) isolates against chickpea pod borer, *Helicoverpa armigera* (Hübner) under field conditions'' Paper IV has been submitted and revised for publication in an International Journal.

Chapter six: Deal with general descussion, general conclusion and recommendations separatly.

CHAPTER TWO

Laboratory set up and procedures for Bt isolation and H. armigera larval rearing, Arsi University

CHAPTER TWO

Paper I: Pathogenicity testing of Native *Bacillus thuringiensis* isolates against chickpea pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Ethiopia

ABSTRACT

Helicoverpa armigera (Hübner) is the most significant insect pest of chickpea in Ethiopia. Sole reliance on insecticide causes both human and environmental health problems. A common soil bacterium, *Bacillus thuringiensis* used as a bioinsecticide to control numerous Lepidopteran larvae. Therefore, native isolates were collected, screened, tested for pathogenicity against *H. armigera* with a reference *Bacillus*. var. *thuringiensis*. From 121 soil samples collected, 23 *Bacillus* species were isolated and purified. *Bt* index ranged from 0.33 to 1.00 and the mean was 0.73 average *Bt* index. Absorbance values ranged from 0.46 to 0.98. Screening biotoxicity assay was conducted at the rate of 1 mL against 3rd instar larvae of *H. armigera*. Experiments were replicated three times; ten larvae were used per replicate and reputed thrice. Ten native *Bt* isolates were screened based on larval mortality which ranged between 27.78% and 75.83% and LT_{50} values of 45.71–143.9 h. The highest mortality of 75.83% and LT_{50} of 45.71 h was recorded from reference *Bt* followed by native *Bt* isolate KDL with mortality percentage of 69.16% and LT_{50} of 48.49 h. Statistically no significant difference was observed among the tested bacterial isolates. Biotoxicity assay was conducted at five concentrations ranging from 0.25 to 2 mL. The LD_{50} and LD_{90} values were 0.84–1.24 mL and 1.54–1.98 mL, respectively. The lowest $LD_{50, 90}$ of 0.84 mL and 1.54 mL were recorded from KDL *Bt* isolate compared to reference *Bt* whose LD_{50} and LD_{90} were 0.84 mL and 1.66 mL. Results of *Bt* growth under varied values of temperature ranged from 15 °C to 40 °C. All the *Bt* isolates showed growth at all the values of the tested temperatures with steady growth from 15 °C to 20 °C and uniform growth observed from 20 °C to 30 °C. After 35 °C, the growth of all the *Bt* isolates declined indicating that 30 °C– 35 °C is the optimum growth temperature range. Protein content ranged from 0.43 to 0.97 (mg/mL). However, protein content and larval mortality was not linearly correlated. It was concluded that all the tested native *Bacillus* isolates were virulent enough and identified as a promising candidate bioinsecticide against *H. armigera*. Hence, recommended for further intensive evaluations under greenhouse and field trails.

Keywords: Africa Bollworm Natural product Polyphagous Soil bacterium

1.1. INTRODUCTION

Chickpea pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is the most devastating and economically important insect pest of chickpea all over the world including Ethiopia (Ndemah, *et al.*, 2001; Sharma *et al.*, 2005; Mazzi and Dorn, 2012). It is widely distributed throughout the world, currently spreading to South and Central America (EPPO, 2023b). In Ethiopia, it exists in all chickpea-growing agro-ecologies, including the Tigray region, East and West Gojam of the Amhara region, East, West, and South-West Shewa of the Oromia region, as well as Bale and Guji Zones. However, the development time of *H. armigera* is influenced by factors such as degree days and temperature; it undergoes 6^{th} larval instar stage and spans six generations time with a total average developmental period of 27.5 to 45 days to complete egg to adult stage (Geremew and Surachate , 2003).

It is a polyphagous pest attacking a wide range of agricultural crops including cotton, tomato, maize, flax, bean, peas and many other agricultural crops (Armes *et al.*, 1992 ; Sori, 1996 ; Damte *et al*., 2002; Huang *et al.*, 2007). To overcome the pest problem, producers relay on intensive insecticide use. Consequently, excessive use of chemical insecticides leads to human and environmental health problems. As a result, there is a growing need to shift to bioinsecticide based pest management strategy. Therefore, the potential benefits of *Bacillus thuringiensis (Bt)* to agriculture and public health have received due attentions because of the drawbacks associated with chemical insecticide (Kaur *et al.*, 2006; Sarwar, 2015; Pérez *et al*., 2017; Saiyad, 2017; Bahri *et al*., 2021). Hence, bioinsecticide improves performance, has low resistance with host specific and environmentally friendly (Lacey, 1997). *Bt* is a gram positive spore forming soil bacterium and encountered in all ecosystems including cadavers (Martin and Travers, 1989; Swiecicka *et al.*, 2008; Nair *et al.*, 2018). It is characterized by the production of toxic crystal proteins against a wide range of Lepidopteran larvae (Lacey, 1997; WHO, 1999; Fernández-chapa *et al*., 2019). Among several microbial control agents, *B. thuringiensis* occupied 98% formulations and has more than 50% market share (Lacey, 2015; Osman *et al*., 2015). Several researchers have reported its pathogenicity against *H. armigera* on chickpea (Baig *et al*., 2010; Li and Bouwer, 2012; Qayyum *et al.*, 2015; Majeed *et al*., 2018). Likewise, in Ethiopia, the existence of native *Bacillus* species with entomopathogenic activity against some insect pests has been evaluated and reported (Tenssay *et al*., 2009; Gebremariam *et al*., 2021;Aynalem *et al.*, 2021).

Nevertheless, evaluations against *H. armigera* management have not been documented regardless of the severity of the problem. Yet, there are still unexplored habitats that contain enormous resources. Hence, there is ample opportunity to obtain competent native *Bt* isolates. Therefore, the hypothesis of this study outlined that there are virulent and proficient native isolates of *Bt*, alternative to insecticides used to control *H. armigera* on chickpea. Thus, this study was aimed at evaluating the suitability of native *Bacillus* isolates against *H. armigera* management under *in vitro* conditions.

1.2. MATERIALS AND METHODS

1.2.1. Descriptions of the study area

Helicoverpa armigera larval culture was maintained at Kulumsa Agricultural Research Center, EIAR and Gonde Basic Seed Farm Centre, Ethiopian Agricultural Business Cooperation, EABC. Experiments on bioassay were conducted at Gonde Basic Seed Farm Centre. Both located in Arsi Administrative Zone of Oromia Regional State. Kulumsa Agricultural Research Center situated at $8^0$01'88''N and $39^0$07'$39^0$10'E and altitude of 2210 m.a.s.l. The area is wet with 809 mm mean annual rain fall and maximum and minimum temperatures of 23.08 and 9.9 ^{0}C, respectively (Abayneh *et al.*, 2003). Gonde Basic Seed Farm Centre is located at $8^0$06'25''N and $39^0$07'38''E with an altitude of 2268 m.a.s.l. The area is wet with 800 mm mean annual rain fall and maximum and minimum temperatures of 23 °C and 10 °C, respectively.

1.2.2. Soil sample collection

Sample sites were purposively selected targeting chickpea and tomato farms. Three to four farms per kebele were selected based on farm size, accessibility and habitats where *Bt* exists using knowledge from literature (Martin and Travers, 1989; Doolotkeldieva *et al.*, 2018). Soil samples of 200 g were collected in sterile plastic bags from five points within the farm by scraping 5-10 cm top soil surface using sterile spatula to make soil composite. The study site comprise chickpea growing districts of Arsi Zone (Gobesa, Tiyo, and Digaluna and Tijo), tomato producing areas in the Rift valley Ziway, Dugda (Meki), Bora (AlemTena), Lome (Koka) and Adama (Wonji). Moreover, samples from cadavers, forest soil, water, animal sewage, animal feed store dust and waste paper were included. The collected soil samples were dried in the laboratory at room temperature by spreading on paper. Finally, the dried soil samples were crushed using sterile mortar and pestle. Final samples were sieved and kept in plastic vials and stored at 4 °C in the refrigerator until use. Sampling locations were recorded using global positioning system (GPS) (Table 1, Figure 1).

Table 1 Sampling sites, sampling types and altitudes recorded for each sampling point

No.	*Bt.* Code	Districts	Kebeles	Sample Types	No. of Samples	Altitude (m.a.s.l)
1	TALS	Tiyo	Asella lake	Soil	1	2494
2	TABFS	Tiyo	Asela Biofarm	Soil	1	2504
3	TTCS	Tiyo	Tulu Chabi	Soil	3	2371
4	TTCS	Tiyo	Tulu Chabi	Soil	3	2255
5	TTCS	Tiyo	Tulu Chabi	Soil	3	2255
6	TTCS	Tiyo	Tulu Chabi	Soil	3	2371
7	TShS	Tiyo	Shala	Soil	3	2557
8	TTKDL	Tiyo	Tulu kuche	Dead larvae	1	2890
9	GBCS	Gobesa	Birbo fi Chole	Soil	3	2325
10	GBCS	Gobesa	Birbo fi Chole	Soil	3	2325
11	GBCS	Gobesa	Birbo fi Chole	Soil	3	2325
11	GHZS	Gobesa	Hela Zenbaba	Soil	3	2329
12	GHZS	Gobesa	Hela Zenbaba	Soil	3	2350
13	GHZS	Gobesa	Hela Zanbaba	Soil	3	2337
15	GHTS	Gobesa	Hela Tareta	Soil	3	2322
16	GHTS	Gobesa	Hela Tareta	Soil	3	2297
17	GHTSW	Gobesa	Hela Tareta	Spider web	1	2451
18	DTBAS	Digaluna Tiyo	Burkitu Alkasa	Soil	3	2540
19	DTBAS	Digaluna Tiyo	Burkitu Alkasa	Soil	3	2540
20	DTBAS	Digaluna Tiyo	Burkitu Alkasa	Soil	3	2541
24	DTBAS	Digaluna Tiyo	Burkitu Alkasa	Soil	3	2540
21	DTKMS	Digaluna Tiyo	Kacama Murkicha	Soil	3	2490
22	DTKMS	Digaluna Tiyo	Kacama Murkicha	Soil	3	2472
23	DTKMS	Digaluna Tiyo	Kacama Murkicha	Soil	3	2490
25	LKS	Lome	koka	Soil	3	1607
26	LKS	Lome	koka	Soil	3	1593
27	LKS	Lome	koka	Soil	3	1606
28	AWS	Adama	Wonji	Soil	3	1543
29	AWS	Adama	Wonji	Soil	3	1542
30	AWS	Adama	Wonji	Soil	3	1543
31	BBS	Bora	Bote (Alemtena)	Soil	3	1649
32	BBS	Bora	Bote (Alemtena)	Soil	3	1649
33	BBS	Bora	Bote (Alemtena)	Soil	3	1649
34	DMS	Dugda	Maki	Soil	3	1662
35	DMS	Dugda	Maki	Soil	3	1662
36	DMS	Dugda	Maki	Soil	3	1662
37	ZDS	Ziway Dugda	-	Soil	3	1724
38	ZDS	Ziway Dugda	-	Soil	3	1724
39	ZDS	Ziway Dugda	-	Soil	3	1724
40	AUSD	Arsi Un	Erbeta	store dust	1	2374
41	AUES	Arsi Un	Erbeta	Soil	1	2354
42	AUPOS	Arsi Un	Sewage Pond	Soil	1	2370
43	AUDSP	Arsi Un	Erbeta	Dead spider	1	2354
44	AUCS	Arsi Un	Campus	Soil	1	2410
45	AUGHS	Arsi Un	Green house	Slug	1	2354
46	AUCHS	Arsi Un	Club house	Soil	1	2461
47	AUWP	Arsi Un	Erbeta	Waste paper	1	2386
48	AUASG	Arsi Un	Erbeta	Animal sewage	1	2370
49	AUGHS	Arsi Un	Green house	Soil	1	2386
50	KDL	Kuyu	-	Dead larvae	1	2198
51	KSS	Kuyu	North Shoa	soil	1	2198
Total Samples					**121**	

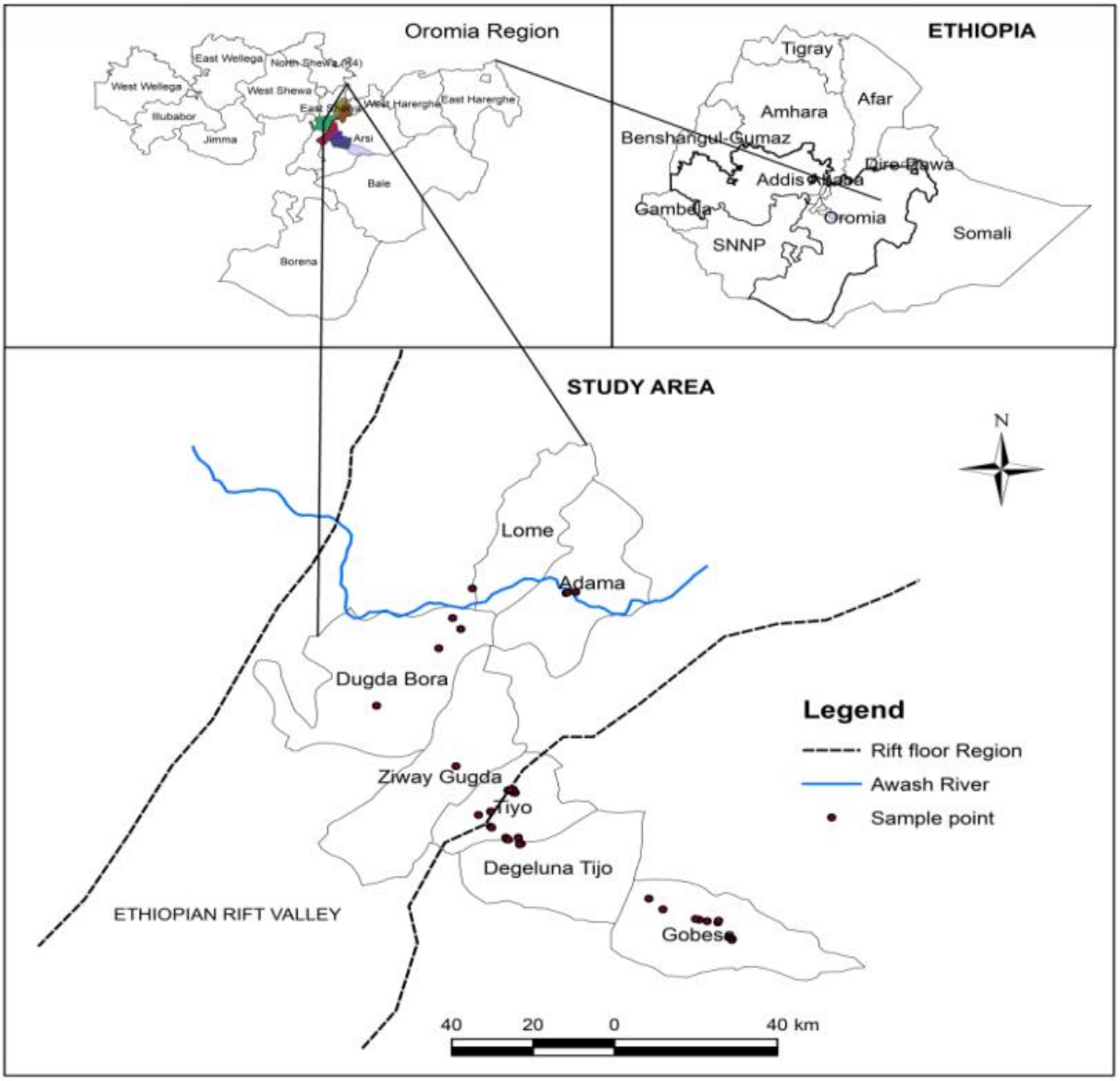


Figure 1 Map of the study areas

1.2.3. Isolation of *B. thuringiensis* from soil

The collected soil samples were processed at Holeta microbial biotechnology laboratory following standard procedures for efficient isolation of *B. thuringiensis* from environmental samples (Travers *et al.*, 1987; Ammouneh *et al.*, 2011; Palma, 2015). One gram of each soil sample was suspended in 9 mL of sterile distilled water in a test tube and mixed vigorously by vortexing for 1 min. Thereafter, the solid matter was allowed to settle out for 2 min, and 5 mL of the supernatant was transferred to another sterile test tube with the capacity of 10 mL. The supernatant was then heat shocked at 80 °C for 3 min in hot shaking water bath to kill the non-spore forming organisms (Travers *et al.*, 1987).

Hundred microliter of the suspension was taken and cultured on nutrient agar plates using micro pipette and incubated for 48 h at 30 °C. *Bt* like single colony was selected at random using colony counter and further cultured on nutrient agar medium for purification. The cultures were checked for spore production and the presence of parasporal inclusions under phase contrast microscope. Thus, *Bt* like colonies showed the presence of spores and parasporal inclusion were washed using orbital shaker at 120 rpm with 10 mL sterile distilled water and stored at -20 °C in a freezer until used (Thiery and Frachon, 1997; Travers *et al*., 1987).

1.2.4. Isolation of *B. thuringiensis* from cadavers

Each dead larva was collected using sterile forceps and placed in sterile plastic screw-top bottles. The surface of the insect body was sterilized in 70% alcohol for 3 min to avoid contact with foreign microflora and washed repeatedly by distilled water and then flame dried in a spirit lamp (Lacey and Brooks, 1997). Thereafter, each dead larva was crushed in sterile crucible and added to a tube containing 10 mL of distilled water in a test tube. The homogenate was thoroughly mixed by vortexing and incubated for 4 h at 30 °C. Then, 1mL of the homogenate was taken and heat shocked at 80 ^{o}C for 3 min in water bath shaker and finally 100 µL of this was plated on the nutrient agar plates and incubated for 24 h at 37 °C (Travers *et al*., 1987; Bhalla *et al.*, 2005).

1.2.5. Measuring Absorbance and cell density of *Bt* isolates

Absorbance of each *Bacillus* species was measured using Micro plate Readers RE, ver.5.0.0.42 loaded with SkanIt Software 5.0 at Holeta Microbial Biotechnology.100 µL of the *Bacillus* suspension from each isolate was added to microplate well, three wells each filled with nutrient broth medium. The blank filled with pure nutrient broth medium only used as a negative control and incubated at 30 ^{o}C for 24 h for sporulation. The Micro plate contained the sporulated *Bacillus* culture was inserted to Micro plate reader and run the software and absorbance was read at 600 nm weave length. The summary results with blank subtractions were displayed and the average absorbance was calculated for each isolate. Cell density for each isolate was determined from absorbance measured using McFarland turbidity standard (Mcfarland, 1907; Leboffe and Pierce, 2016; Stevenson *et al*., 2016; Souza and Bitencourt, 2020).

1.2.6. Preparation of spore-crystal mixture

Laboratory scale production of spore-crystal mixtures was carried out following standard procedures (Dulmage, 1971). From the preserved culture, a loopful of the culture was taken and inoculated onto Modified Broth Solution (MBS) containing 6.8 g KH_2PO_4, 0.3 g $MgSO_4.7H_2O$, 0.2 g $CaCl_2.2H_2O$, 10 g Bactotryptose, 2 g yeast extract, 10 mL of stock solutions prepared from (2 g $MnSO_4.H_2O$, 2 g Fe_2 $(SO_4)3$, 2 g $ZnSO_4.7H_2O$ and 1litre of distilled water), 17 g of nutrient agar and 1liter of distilled water. The inoculated plates were incubated at 30 °C for 24 h to induce sporulation. A single colony was taken and inoculated into 100 µL liquid nutrient broth medium in Eppendorf tube and then incubated at 30 ^oC for 24 h. From the sporulated *Bacillus* culture, 100 µL was taken and transferred to Erlenmeyer flasks having a capacity of 250 mL with 150 mL nutrient broth medium and allowed to ferment for 72 h at room temperature using orbital shaker at 180 rpm (Ahmed *et al*., 2015). The suspensions were centrifuged at 10,000 rpm for 3 min at 4 ^oC in a sterile test tube. Pallets were washed twice in 20 mL of sterile distilled water and centrifuged as above. The supernatant was discarded and the spore-crystal mixtures were collected and re-suspended in 20 mL of sterile distilled water and stored in a deep freezer at -20 °C until use in a 20% glycerol (Andrews, 2001).

1.2.7. Standardization of *Bacillus* species

McFarland standard was used as a reference to adjust the *Bacillus* species cell density in a suspension so that the number of bacteria was within a given range, where a 0.5 McFarland standard corresponds to 1.5 x 10^8 cfu/mL. *Bacillus* species suspension was adjusted to achieve a turbidity that is equivalent to a 0.5 McFarland standard. Hence, the inoculated tube was visually compared with the 0.5 McFarland standard against a card with white background and contrasting black lines (Mcfarland, 1907). *Helicoverpa armigera* field culture was established on small plots of chickpea planted at Kulumsa Agricultural Research Center, EIAR and Gonde Basic seed farm, EABC. Fifth instar larvae were collected from field culture and kept in insect rearing box feed with chickpea diet for pupation. Pupae were collected and transferred to plastic pots embedded with saw dust. The emerged adults were supplied with artificial diet prepared from 5 g honey and 200 mL water. Male and female in pairs were placed in insect cages and reared separately in the laboratory at 25± 2 ^oC temperature, 65±5% RH and photoperiod of 12L:12D (Armes *et al.*, 1992).

The adults were allowed to lay eggs on the cheese cloth and the hatched larvae were collected using camel brush and fed on artificial diet in Petri plates that were sealed well. Starting from second instar, individually reared larvae in small plastic cups (1 cm height and 1.5 cm radius) were supplied with artificial diet to avoid cannibalism.

1.2.8. Reference strain

The reference *Bt* named as *Bacillus thuringiensis*. var. *thuringiensis* (Trade name, Bitoxybacillin^{TM}, producer Company, Sibbiopharm ltd., Russian Federation) was commercially available in the form of wettable powder (WP). It was obtained from Gallica flower farm, Menegasha, Ethiopia. Its preparation contains bacterial spores of (10^6 spores/mL) and proteinaceous crystals (delta-endotoxin) and thermo-stable b-exotoxin of *Bt* culture used to control many Lepidopteran larvae and red spider mite. Bitoxybacillin^{TM} is pale-beige to brown color powder with 1500 UA/mg biological activity and 0.6–0.8% exotoxin content (Teferra and Dubale, 2020; Adilkhankyzy *et al*., 2022).

1.2.9. Entomopathogenicity screening bioassay

The Experiments were laid out in a Completely Randomized Design (CRD) with three replications. A bioassay was conducted to determine LT_{50} values for each of the *Bacillus* species based on percentage larval mortality assessed for seven days. The treatments used were 23 native *Bacillus* isolates along with the reference *Bt*. var. *thuringiensis.* Ten 3rd instar larvae per replicate were subjected to a treatment of single concentration of one milliliter (1 mL) of the standardized *Bacillus* isolates approximately containing ($1.5x10^8$ cfu/mL) (Navon, 2000). Chickpea diet for larvae was prepared according to McGuire *et al.*, (1997). A 10 g diet was dispensed to feeding cup and dilution was made using 1ml of *Bacillus* species cells and stirred well for four minutes. Thereafter, one larva was released into feeding cup to avoid cannibalism by ensuring lids fastening securely to prevent the escape of the larvae (McGuire *et al.*, 1997). Then the larvae were kept at temperature of 25±2 °C, 65 ±5% RH and 12L: 12D photoperiod. Native *Bacillus* isolates that caused larval mortality of 50% and above were considered as virulent and further evaluated for dose response biotoxicity assay.

1.2.10. Response biotoxicity assay

The biotoxicity of each native *Bacillus* isolate with the reference *Bacillus thuringiensis* var. *thuringiensis* was assessed using diet incorporated dose response method. The experiment was replicated three times and repeated three times in CRD design. Diet for larvae was prepared according to (McGuire *et al*, 1997) after it was cooled to 55 °C.

Serial dilution was made using 0.25 mL, 0.50 mL, 1 mL, 1.50 mL and 2 mL of *Bacillus* isolate concentrations that approximately contained ($1.5x10^8$ cfu/mL) to 10 g chickpea diet and mixed thoroughly for four minutes. Controls larvae were supplied with the same amount of diet without *Bt* concentration and kept at temperature of 25 ± 2 °C, 65 ± 5% relative humidity and 12L:12D photoperiod (Baig *et al*., 2010).

1.2.11. Temperature tolerance of *Bacillus* isolates

Each *Bacillus* culture of 100 µL was inoculated to microplate wells that contained nutrient broth with three wells for each isolate. The blank filled with pure nutrient broth medium only as a negative control. Before incubation, absorbance was measured for each temperature levels (0 ^oC, 15 ^oC, 20 ^oC, 25 ^oC, 30 ^oC, 35 ^oC and 40 ^oC). Absorbance of each *Bacillus* culture was measured after 24 h for each temperature levels using micro-plate reader and blank subtracted values were taken as *Bacillus* absorbance values at the selected temperatures and the optimum growth temperature was determined for each of the isolates (Matter and Gesraha, 2012).

1.2.12. Determination of protein content of *Bacillus* species

Protein concentration for the selected *Bacillus isolate* was measured spectrophotometrically at wavelength of 280 nm following the methods commonly used to measure the concentration of *Bacillus* species in the suspension (Gill and Hippel, 1989). Bovine serum albumin (BSA) was used as standard for protein quantification of the *Bacillus* species (Layne, 1957).

1.2.13. Data analysis

Before the data were subjected to analysis, the cumulative mortality data over the given period was corrected using Abbott's formula, when mortality in the control greater than 5% and less than 10%, above 10% mortality was rejected (Abbott, 1925).

$$\text{Corrected mortality (P)} = \frac{Pc-Pt}{Pc} \text{ x } 100$$, Where:

- (P) is the estimated percentage of insects killed by the entomopathogens alone

- (Pc) is the percentage of the control insects that are living, and

- (Pt) is the percentage of the treated insects that are living after the experimental period.

Then after, mean larval mortality data was checked for normality using chi-square test as well as homogeneity among the treatments at $\alpha = 0.05$.

Biotoxicity of *Bacillus* species were determined by working out median lethal time (LT_{50}) values after seven days post exposure using probit analysis in SPSS software vr. 23 at 95% fiducial limit (Finney, 1971).

$$\text{Mean lethal time (LT50) (hrs)} = \frac{\text{x1y1} + \text{x2y2} + \cdots \text{xnyn}}{\text{Total x mortality}}$$

Where: X1 indicated the number of larvae died in the 1st day (y1) and continuous until day (Yn).

Thereafter, the corrected data for percent mortality for lethal dose (LD_{50} and LD_{90}) was subjected to analysis of variance using SPSS statistical software var.23. Probit analysis was used for analysis of mean lethal dose. Percentage larval mortality was assessed and it was calculated using the formula described by Senthil- Nathan *et al*. (2008).

$$\text{Mean larval mortality (\%)} = \frac{\text{Number of dead larvae in treatments}}{\text{Total larvae tested}} \text{ x } 100$$

Significant differences between treatments were set at alpha 0.05 and means were compared using Tukey's highest significant difference (HSD) test. Microsoft office excel 2010 was used for all graphs and regression analysis made between absorbance and cell density of *Bt* species.

1.3. RESULTS

1.3.1. Isolation of *Bacillus* species and index

From a total of 121 samples collected, 48 *Bt* like colonies were observed. A total of 23 *Bacillus* isolates presumptively designated as *B. thuringiensis*. In general, the number of *Bt* colonies showed variation with indices that ranged from 0.33 to 1.00 with an average *Bt* index of 0.73. Each of the sample categories like animal sewage (0.33), animal feed store dust (0.67), dead insect larvae (0.50). Nevertheless, spider web, dead spider, slug, waste paper, sewage pond and lake resulted in the highest *Bt* index of 1.00.

Table 2 Sample categories and number of samples with its respective presumptive *Bt* and index

Sample categories	No. of samples	*Bt* like colonies Observed	Presumptive *Bt*	*Bt* index
Soil from tomato farm	45	4	2	0.50
Soil from chickpea farm	60	24	8	0.33
Animal sewage	1	3	1	0.33
Animal feed store dust	1	3	2	0.67
Dead insect larvae	2	3	1	0.50
Spider web	1	2	2	1.00
Dead spider	1	1	1	1.00
Slug	1	1	1	1.00
Waste paper	1	1	1	1.00
Waste sewage pound	1	1	1	1.00
Lake	1	1	1	1.00
Other natural habitats	6	4	2	0.50
Total	**121**	**48**	**23**	**0.73**

1.3.2. Growth and Cell density of presumptive *Bt* Isolates

Absorbance of each of the 23 presumptive *Bt*. Isolates and the reference *Bt*. var. *thuringiensis* was measured at 600 nm wave length after growing in micro-plate reader. Results of absorbance measured and their respective cell density (cfu/mL) values are presented. The maximum (0.98) absorbance value was recorded from native presumptive *Bt* isolate of AUWP followed by AUASG-2, AUGHS-1 and AUDSP with their respective absorbance values of 0.97, 0.95 and 0.85, respectively. Nevertheless, the minimum (0.46) was recorded from AUSD-3.

Absorbance values of the rest of presumptive *Bt* isolates including the reference *Bt*. var. *thuringiensis* fell between the maximum and minimum absorbance values indicated. Direct correlation was observed between absorbance and cell density ($R^2=1$, $P<.0001$) (Fig.2).

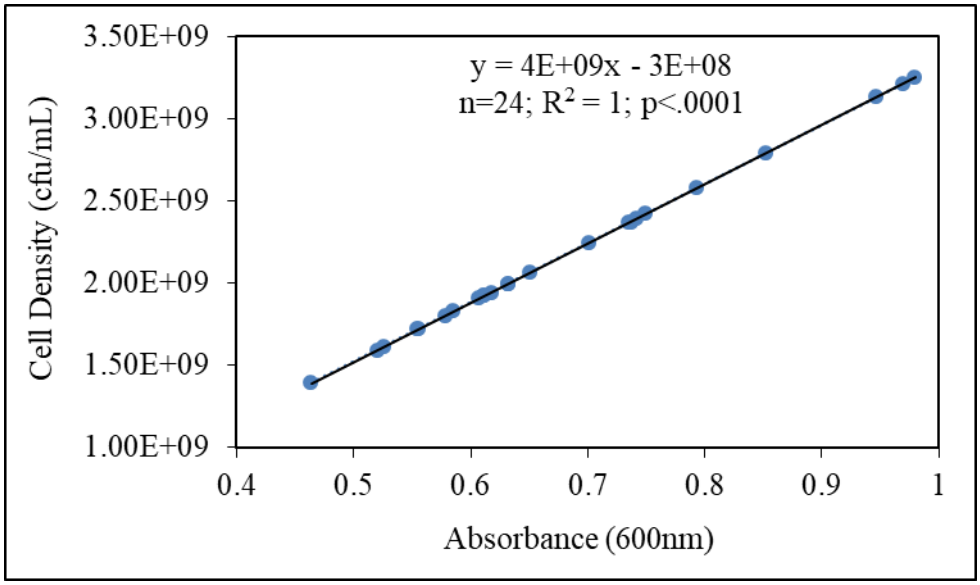


Figure 2 Correlation between absorbance and cell density of *Bt* isolate

McFarland Standard	$BaCl_2$ 1%V(ml)	H_2So_4 1%V(ml)	Bacterial Cell (cfu/ml) ($x10^8$)	Absorbency Range	
0.5	0.05	9.95	1.5	0.08	0.13
1	0.1	9.9	3	0.14	0.17
2	0.2	9.8	6	0.27	0.31
3	0.3	9.7	9	0.38	0.42
4	0.4	9.6	12	0.51	0.55
5	0.5	9.5	15	0.67	0.70
6	0.6	9.4	18	0.74	0.77
7	0.7	9.3	21	0.83	0.88
8	0.8	9.2	24	0.94	0.98

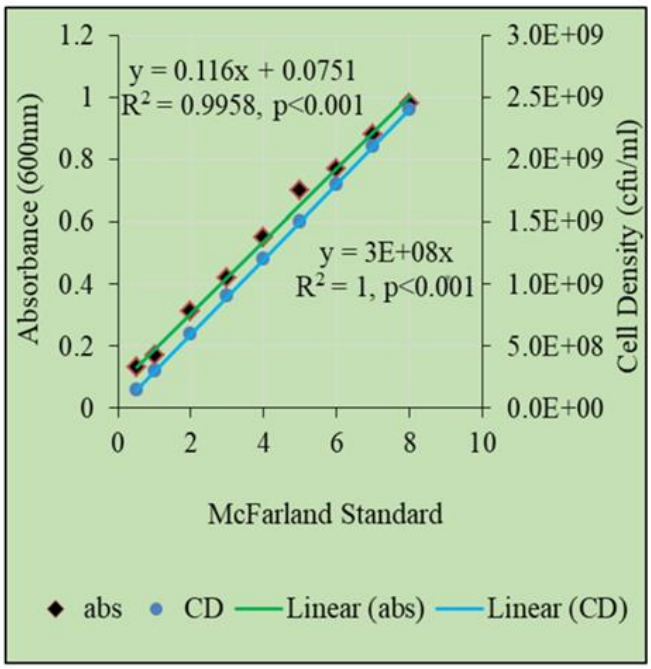


Figure 3 McFarland standard (a) McFarland standard, rates of chemical reaction and bacterial cell density its respective absorbance range (b) correlation graph between McFarland standard, absorbance and cell density of *Bt* isolates

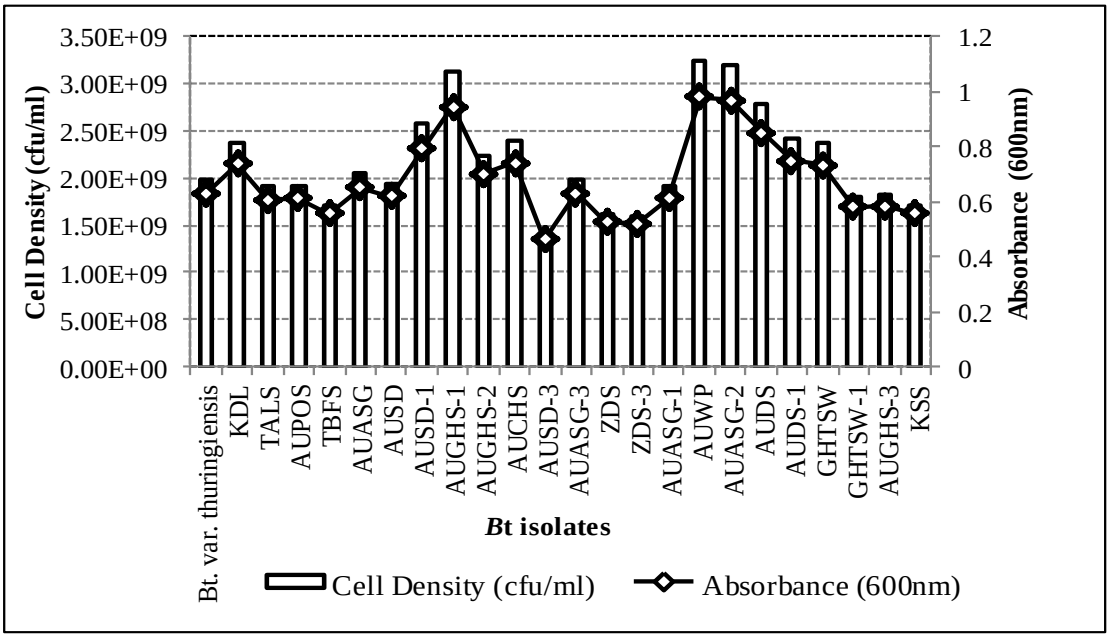


Figure 4 Absorbance (600 nm) and Cell Density (cfu/mL) of presumptive *Bt* isolates and the reference *Bt*. var. *thuringiensis*

1.3.3. Entomopathogenicity screening bioassay of presumptive *Bt* isolates against *H. armigera*

Results of entomopathogenicity screening bioassay revealed that significant ($p<0.05$) variations observed among the presumptive *Bt* isolates tested against 3rd instar larvae of *H. armigera* (Table 3). Of the 23 presumptive *Bt* isolates subjected to screening bioassay, only ten presumptive *Bt* isolates achieved larval mortality of 50% and beyond. The highest percentage mortality was recorded from the reference *Bt*. var. *thuringiensis* that achieved 75.83% mortality followed by native presumptive *Bt* isolates of KDL, AUPOS, AUGHS-1 and AUSD-1 that resulted in (69.16%, LT_{50}, 48.49 h), (68.05%, LT_{50}, 50.05 h), (66.94%, LT_{50}, 50.55 h) and (64.72%, LT_{50}, 55.10 h) larval mortality, respectively. There was no significant ($p>0.05$) difference between the reference *Bt*. var. *thuringiensis* and native presumptive *Bt* isolates above (Table 3). However, there was a significant ($p>0.05$) difference between the reference *Bt*. var. *thuringiensis* and the rest presumptive native *Bt* isolates (Table 3). Therefore, based on the larval mortality the isolates achieved and their LT_{50} values, 10 of the 23 native presumptive *Bt* isolates were selected and further evaluated for biotoxicity assay under laboratory conditions to determine their respective LD_{50} and LD_{90} values.

Table 3 Mean larval mortality (%) and lethal time (LT_{50}) (h) of the tested native presumptive *Bt* isolates and the reference strain *Bt*. var. *thuringiensis* with p-values at (5%) significance level

Bt isolates	Mortality (%)	**LT_{50}** (hrs.)	95% FL (lower, upper)	χ^2	Regression Equations	r^2	*P*-value
Bt. vr. *thuringiensis*	75.83a	45.71	(38.95-54.44)	24.28	y=-3.318+0.186x	0.81	*P<.027*
KDL	69.16ab	48.49	(40.83-59.95)	18.83	y=-2.862+0.168x	0.84	*P<.017*
TALS	47.11d	73.03	(58.58-87.81)	24.70	y=0.73+0.058x	0.89	*P<.001*
AUPOS	68.05abc	50.05	(43.94-58.68)	28.77	y=-3.928+0.183x	0.95	*P<.001*
TABFS	34.44efg	96.88	(83.44-118.1)	25.39	y=0.36+0.55x	0.89	*P<.001*
AUASG	27.78g	143.9	(117.4-170.4)	17.55	y=-1.151+0.0486 x	0.86	*P<.001*
AUSD	35.78efg	92.29	(80.04-109.7)	29.24	y=-0.552+0.0604x	0.94	*P<.001*
AUSD-1	64.72abc	55.10	(48.49-63.79)	31.04	y=-3.224+0.152x	0.86	*P<.001*
AUGHS-1	66.94abc	50.55	(42.62-63.61)	17.32	y=-2.000+0.139x	0.80	*P<.030*
AUGHS-2	38.44def	87.16	(75.25-102.7)	30.98	y=-0.162+0.0585x	0.84	*P<.001*
AUCHS	39.77def	84.75	(73.34-98.83)	33.77	y=-0.600+0.0667x	0.97	*P<.001*
AUSD-3	31.11fg	117.8	(93.65-199.9)	9.660	y=0.746+0.0360x	0.83	*P<.001*
AUASG-3	34.44efg	96.26	(82.59-118.0)	24.13	y=-0.333+0.0556x	0.93	*P<.001*
ZDS	63.60bc	62.64	(54.81-74.78)	23.76	y=-2.188+0.115x	0.91	*P<.001*
ZDS-3	62.49bc	66.49	(59.23-78.22)	27.16	y=-2.472+0.109x	0.92	*P<.001*
AUASG-1	33.78efg	105.3	(88.59-139.2)	17.73	y=0.0638+0.0463x	0.90	*P<.001*
AUWP	31.11fg	115.9	(93.11-185.9)	10.57	y=0.282+0.0429x	0.84	*P<.001*
AUASG-2	60.27bc	61.09	(55.36-68.02)	43.54	y=-4.111+0.153x	0.93	*P<.001*
AUDS	33.78efg	108.0	(86.15-171.7)	10.08	y=1.067+0.0361x	0.72	*P<.001*
AUDS-1	31.78efg	109.7	(75.96-143.4)	10.19	y=1.000+0.0361x	0.60	*P<.001*
GHTSW	57.42c	63.29	(57.13-71.56)	36.80	y=-3.366+0.133x	0.92	*P<.001*
GHTSW-1	61.39bc	65.89	(58.30-78.33)	25.78	y=-2.833+0.119x	0.92	*P<.001*
AUGHS-3	63.61bc	60.39	(54.09-69.31)	32.97	y=-3.482+0.141x	0.89	*P<.001*
KSS	40.44de	83.70	(72.23-97.63)	33.60	y=-0.533+0.0667x	0.96	*P<.001*
Control	-	-	-	-	-	-	-

Means with the same letters were not significantly different. LT_{50}, lethal time (50%), mortality (%) (*df= 24, 50, f-value = 10.37, pr<.0001*) according to Tukey's Highest Significant Difference (HSD) test at 5% probability level.

1.3.4. Dose response biotoxicity assay of *Bt* isolates against *H. armigera*

Biotoxicity of the virulent native presumptive *Bt* isolates with the reference *Bt*. var. *thuringiensis* was tested against 3rd instar larvae of *H. armigera* under laboratory conditions by using diet incorporated dose response test method (Table 5 & Figure 4). Variations were observed among all the tested presumptive *Bt* isolates (*p<0.05*) (Table 4 & Figure 5).

All the tested native *Bacillus* isolates along the reference *Bt*. var. *thuringiensis* showed mortality against *H. armigera* to some extent at all concentrations tested. However, percentage mortality increases as both the concentrations of *Bacillus* species and exposure time increased (Table 4 & Figure 5). The highest percentage larval mortality was recorded at concentrations of 1.50 mL and 2 mL. Among the native *Bacillus* isolates tested, KDL achieved 100% larval mortality followed by AUASG-2 and GHTSW, where both achieved 96.66% larval mortality at 2 mL that was comparable to the reference strain *Bt.* var. *thuringiensis* (96.66%) larval mortality at the same concentration (Table 4). The highest LD_{50} was recorded from native *Bacillus* isolate ZDS that was 1.24 mL and the lowest 0.84 mL was recorded from *Bt*. var. *thuringiensis* and native *Bacillus* isolate KDL, where both showed the same LD_{50} followed by native *Bacillus* isolate GHTSW (Table 5).The corresponding LD_{90} varied among the test isolates . The lowest LD_{90} was recorded from KDL that was1.54 mL followed by AUASG-2 with 1.63 mL. Whereas, the reference *Bt*. var. *thuringiensis* and native *Bacillus* isolate GHTSW, both resulted in LD_{90} value of 1.66 mL (Table 5).

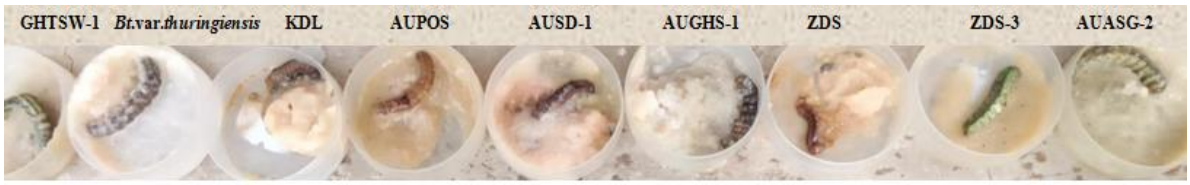


Figure 5 *Helicoverpa armigera* Larval Killed by some of the native *Bacillus* isolates and reference *Bt*. var. *thuringiensis* during biotoxicity assay

Table 4 Mean larval mortality (%) by native potent *Bacillus* isolates and reference *Bt.* var. *thuringiensis* tested at different concentrations

	Concentrations (ml)				
Bt isolates	0.25	0.5	1.0	1.5	2.0
Bt.var. *thuringiensis*	16.66a	36.66a	46.667ab	90.00a	96.66a
KDL	13.33ab	33.33ab	50.00a	90.00a	100.0a
AUPOS	13.33ab	26.66abc	36.66abc	83.33ab	93.33a
AUSD-1	6.66c	23.33bcd	40.00abc	86.66ab	93.33a
AUGHS-1	13.33c	33.33abc	40.00abc	83.33ab	93.33a
ZDS	3.33c	13.33d	30.00c	70.00b	90.00a
ZDS-3	3.33c	13.33d	36.66abc	83.33ab	93.33a
AUASG-2	3.33c	20.00cd	40.00abc	90.00a	96.66a
GHTSW	13.33ab	30.00abc	43.33abc	90.00a	96.66a
GHTSW-1	3.33c	13.33d	40.00abc	83.33ab	93.33a
AUGHS-3	6.66bc	20.00cd	33.33bc	76.66ab	93.33a
Control	0.00d	0.00c	0.00d	0.00b	0.00b

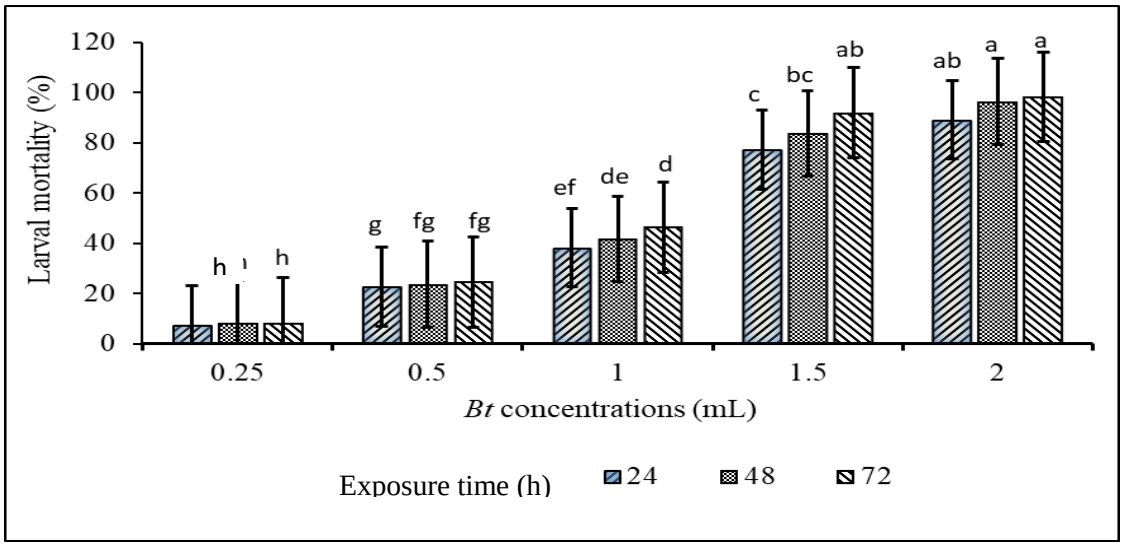


Figure 6 Mean larval mortality (%) by native *Bacillus* isolates and reference *Bt.* var. *thuringiensis* tested at different concentrations levels and time intervals (h). Means with the same letters were not significantly different. Means with the same letters were not significantly different. Effects *of Bt* concentrations and exposure time (h) on *H. armigera* larval mortality (*df=8,100; f=value=2.48; pr<.0170*) according to Tukey's HSD test at 5% probability level.

Table 5 LD_{50} and LD_{90} values of the selected virulent native *Bacillus* isolates tested and their respective fiducial limit (95%) and p-values at 5% significance level

Bt. strains	**LD_{50}** (ml/10g)	95% FL (lower, upper)	**LD_{90}** (ml/10g)	95% FL (lower, upper)	χ^2	Regration Equations	r^2	*P*-value
Bt.var. *thuringiensis*	0.84	(0.68-1.00)	1.66	(1.44-2.01)	65.03	y=0.791+4.707x	0.89	*P<.001*
KDL	0.84	(0.70-0.98)	1.54	(1.35-1.83)	78.49	y=3.546+3.357x	0.89	*P<.001*
AUPOS	1.01	(0.85-1.17)	1.85	(1.62-2.22)	65.34	y=-0.004+4.829x	0.96	*P<.001*
AUSD-1	1.02	(0.88-1.17)	1.76	(1.56-2.08)	79.15	y=-0.506+5.244x	0.96	*P<.001*
AUGHS-1	1.03	(0.88-1.18)	1.79	(1.58-2.12)	75.10	y=-0.378+5.122x	0.96	*P<.001*
ZDS	1.24	(1.09-1.39)	1.98	(1.76-2.32)	77.55	y=-1.270+5.146x	0.94	*P<.001*
ZDS-3	1.12	(0.98-1.26)	1.77	(1.59-2.06)	92.00	y=-1.256+5.577x	0.95	*P<.001*
AUASG-2	1.02	(0.89-1.15)	1.63	(1.46-1.89)	97.45	y=-0.976+5.691x	0.96	*P<.001*
GHTSW	0.91	(0.76-1.06)	1.66	(1.46-1.98)	73.77	y=0.191+5.024x	0.96	*P<.001*
GHTSW-1	1.10	(0.97-1.24)	1.76	(1.57-2.04)	91.59	y=-1.181+5.569x	0.94	*P<.001*
AUGHS-3	1.12	(0.97-1.27)	1.89	(1.67-2.23)	75.12	y=-0.787+5.130x	0.93	*P<.001*
Control	-	-	-	-	-	-	-	-

1.3.5. Effect of temperature on the growth of *Bacillus isolates*

The growths of all the *Bacillus* isolates were tested under various temperature intervals (Figure 7). Results of absorbance values measured indicated that all the tested *Bacillus* isolates showed growth at the tested temperature values, but variation in growth was observed between the isolates at all the tested temperature values. At 15 ^{o}C temperature, the minimum 0.12 was obtained from *Bacillus* isolate AUASG-2 and maximum growth of 0.26 was obtained from AUGHS-3 compared to the reference strain *Bt.* var. *thuringiensis* that resulted in 0.21 growths.

Similarly, at 20 ºC temperature, the minimum and maximum growth was obtained from *Bacillus* isolates AUASG-2 and AUGHS-3 with 0.18 and 0.42, respectively. Moreover, at 25 °C, isolate AUGHS-3 showed the highest growth (0.73) compared to the reference *Bt* resulted in 0.68, whereas the lowest 0.39 was recorded from KDL. At 30 °C the highest growth (0.91) was recorded from reference strain *Bt.* var. *thuringiensis*, followed by 0.87, 0.83 and 0.80 growths from native *Bacillus* isolates of AUSD-1, AUGHS-3 and AUGHS-1, respectively. All the *Bacillus* isolates showed the highest growth at 35 °C, whereas some native *Bacillus* isolates AUASG-2, AUGHS-1 and AUSD-1 showed significant growth increment to 1.30, 1.09 and 1.04, respectively compared to the reference *Bt*. var. *thuringiensis* that showed 0.98 growth and the lowest 0.67 and 0.75 recorded from the *Bacillus* isolates APOS and ZDS, respectively. Generally, similar trends were observed under all the tested temperature values. There was a steady growth from 15 °C to 20 °C except for isolates GHTSW and AUGHS-3 and uniform growth was observed from 20 °C to 30 °C. The growth of all the *Bacillus* isolates declined beyond 35 °C.

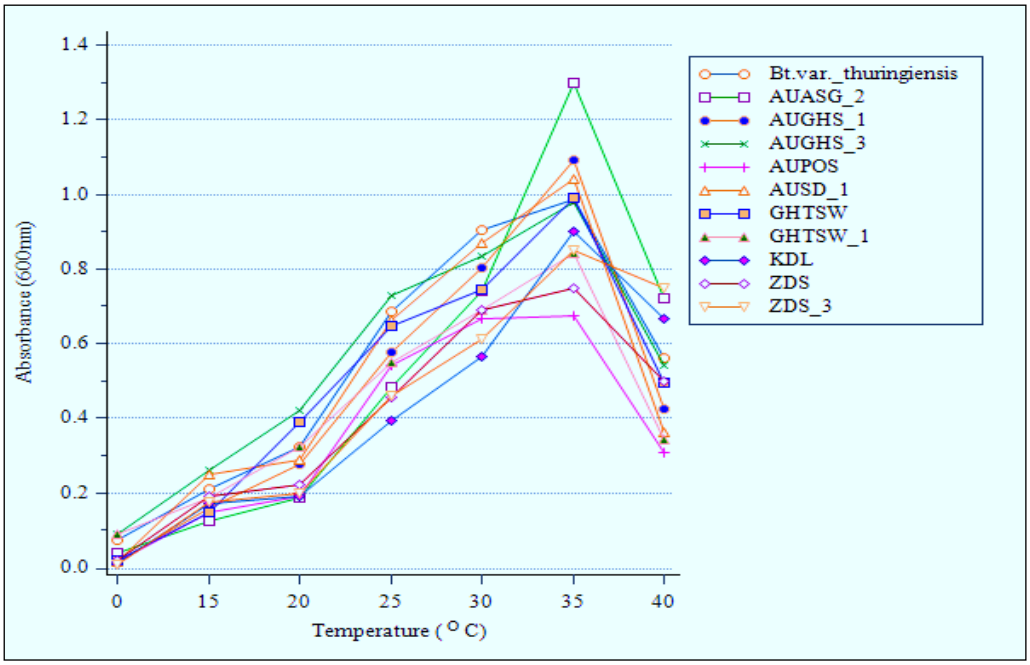


Figure 7 Effect of temperature levels on the growth of native *Bacillus* and reference *Bt*. var. *thuringiensis* as measured by Absorbance at (600 nm).

1.3.6. Protein content of *Bacillus thuringiensis* isolates

Results from protein concentration showed that the protein content of all the selected native *Bacillus* isolates including the reference *Bt*. var. *thuringiensis* was varied (Table 6). The protein content ranged from 0.43 to 0.97mg/mL.

The highest (0.97) and the lowest (0.43) protein concentration was obtained from native *Bacillus* isolates of AUASG-2 and ZDS compared to the reference *Bt*. var. *thuringiensis* resulted in 0.54 protein content (Table 6).

Table 6 Absorbance at (280 nm) and protein content of the selected native *Bacillus* isolates and reference *Bt*. var. *thuringiensis.*

Bt. isolates	Absorbance (280 nm)	Protein content (mg/ml)
Bt. var. *thuringiensis*	0.36	0.54
KDL	0.57	0.85
AUPOS	0.55	0.82
AUSD-1	0.55	0.82
AUGHS-1	0.31	0.46
ZDS	0.29	0.43
ZDS-3	0.37	0.55
AUASG-2	0.65	0.97
GHTSW	0.53	0.79
GHTSW-1	0.47	0.70
AUGHS-3	0.34	0.51

1.4. DISCUSSION

Results revealed that 48 *Bt* like colonies isolated and characterized. Among 48 isolates, 23 *Bacillus* isolates were further tested. *Bacillus* isolates index ranged from 0.33 to 1.00 with an average index of 0.73. Compared to other sample categories, *Bacillus* species isolated from soil samples collected from chickpea and tomato farms were less that could be due to intensive pesticides used by the farmers year round. Similarly Lone, (2017) who isolated 56 crystalliferous colonies from 207 samples with 0.62 average *Bt* index, where 0.33-0.84 and 0.50-1.00 *Bt* index was from Agricultural land and forest in northwestern Himalayas. Moreover, (Doolotkeldieva *et al*., (2018) reported 35% *Bt* isolates from 240 soil samples, 14.5% from litter and from dead bodies of insect larvae, 18.67%, 17.95%, 13.77%, 9.61% that were recorded from Lepidoptera, Coleoptera, Orthoptera and Diptera orders, respectively in Kyrgyzstan. Similarly, Meadows *et al*., (1992) have reported the highest number of *Bt* from insect webbing (3), guano (3) and mammalian fecal pellets (2) out of 3, 3 and 2 samples collected, respectively.

The highest ($3.25x10^9 \pm 4.0x10^8$ cfu/mL) and the lowest ($1.39x10^9 \pm 3.7x10^7$cfu/mL) cell density were recorded from native *Bacillus* isolates of AUSD-3 and AUWP, respectively. Direct correlation was observed between absorbance and cell density. The current result on cell density of native *Bacillus* isolate, agrees with that of (Peñuelas-Urquides *et al.*, (2013) who demonstrated a correlation between OD 600 nm, cell density (cfu/mL) and McFarland standard. Results on entomopathogenicity screening revealed that there were statistically significant ($p<0.05$) differences among *Bacillus* isolates tested. Percentage larval mortality ranges from 27.78% to 75.83%. Among the native *Bacillus* isolates subjected to screening bioassay, ten of them were further screened based on larval mortality (%) and LT_{50} values they achieved. From the ten native *Bacillus* isolates screened, four isolates such as KDL, AUPOS, AUGHS-1 and AUSD-1 showed better percentage larval mortality and LT_{50} values and comparable with the reference *Bt.* var. *thuringiensis.* Biotoxicity assay tested at five doses of the selected effective *Bacillus* isolates and the results revealed that larval mortality increases as dose of the test isolates increases (Table 4). The highest larval mortality was recorded at higher doses (1.5 mL and 2mL). Similarly, larval mortality increases as doses and exposure time increased. The selected *Bacillus* isolates showed better growth between 30-35 °C.

Likewise, Tenssay *et al.*, (2009) have reported that 30 ºC is the optimum growth for *B. thuringiensis* isolated from soil in Ethiopia. Similarly, Matter and Gesraha, (2012) have demonstrated the insecticidal crystal protein to be 30-35 ºC with optimum value of 30 ºC . In another study, Kwalimwa, (2012) in his study indicated that 37 °C is the optimum temperature for delta-endotoxin production of *B. thuringiensis*. The protein content ranges from 0.43-0.97 (mg/mL). The highest (0.97) protein content recorded from native *Bacillus* isolate AUASG-2 (Table 6). Similarly, as absorbance of *Bt* strains increased, protein content was also increased as noted in earlier study (Haggag and Yousef, 2010) where the authors showed direct correlation of absorbance and protein concentration. However, protein concentration and larval mortality was not linearly correlated. This is possibly due to the types and compositions of toxic crystal protein complex present in each *Bt* isolates (Hofte and Whiteley, 1989; Liao *et al*., 2002; Lone *et al.*, 2017).

1.5. CONCLUSION

Results obtained from pathogenicity screening and biotoxicity assay of native *Bt* isolates against 3rd instar larvae of *H. armigera* suggested that percentage larval mortality, LT_{50}, LD_{50} and LD_{90} values of the potent native *Bacillus* isolates were comparable to that of the reference *Bt*.var. *thuringiensis* indicating their virulence against *H. armigera* management of chickpea and other related host crops. Hence, further intensive evaluation under greenhouse and field trails could promote the incorporation of these bioinsecticide in to integrated *H. armigera* management systems.

CHAPTER THREE

Molecular and biochemical characterization of Bt species, suspension preparation and bioassay

CHAPTER THREE

Paper II: *Bacillus thuringiensis* isolates and their *cry* genes toxic to chickpea pod borer, *Helicoverpa armigera* (Hübner) from Ethiopia

ABSTRACT

Helicoverpa armigera (Hubn) is one of the most destructive insect pests of chickpea in Ethiopia. For sustainable management of insect pests of food crops, *Bacillus thuringiensis* (*Bt*) is a widely used bioinsecticide. This study was aimed at exploring native *Bt* isolates that harbour *cry* genes to control *H. armigera*. Ten native *Bt* isolates were analyzed for their *cry* genes. Accordingly, all the native *Bt* isolates were observed to harbour two or more *cry* genes. Statistically significant ($p<0.05$) variations were observed among *Bt* species in influencing larval incidence, pod damage (%) and grain yield (t/ha).Three potential native *Bt* isolates were identified with their respective *cry* genes that included KDL (*cry*2 + *cry*4), AUGHS-1 (*cry*1 + *cry*4), and AUSD-1 (*cry*1 + *cry*2 + *cry*4 + *cry*7, 8 + *cry*9). Native *Bt* isolates exhibited a strong potential in the management of chickpea pod borer. Development of commercial bioinsecticide and other *Bt* technologies using *B. thuringiensis* from Ethiopian sources will be a new avenue to be addressed.

Key words: Bioinsecticide, *Bt*, *cry* genes, crystal protein, *Helicoverpa armigera*, larval mortality, pathogenicity, pod damage, grain yield, toxicity

3.1. INTRODUCTION

Soil microbes have been identified as potential candidates for use in biocontrol against insect pests (Riba and Silvy, 1989). For instance, *Bacillus thuringiensis* (*Bt*) is the most important entomopathogenic microbe used to date as an alternative to chemical insecticides against insect pests of agriculture such as chickpea pod borer. *Bt* is a gram-positive, rod shaped, and spore-forming bacterium that produces proteinaceous parasporal crystal proteins encoded as *cry* genes (Bravo *et al*., 2013; Das *et al.*, 2021). These crystals are composed of *cry* genes that are toxic against a range of insect pests from the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Hemiptera as well as against mites and nematodes (Palma *et al*., 2014 ; Das *et al.*, 2021). These toxins are uniquely specific, safe, and completely biodegradable (Rubio-Infante and Moreno-Fierros, 2016).

Most of the commercial *B. thuringiensis* formulations used for the control of lepidopteran larvae (e.g., *Helicoverpa armigera*) mainly contain toxins of the *cry*1A and *cry*2A gene families, especially *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*2Aa, and *cry*2Ac genes (Rubio-Infante and Moreno-Fierros, 2016). *H. armigera* was found susceptible to various *cry* genes (*cry*1Ab, *cry*1Ac, *cry*2Aa, and *cry*2Ab) with complete eradication of the target pest (Liao *et al*., 2002). The discovery of novel *cry* genes with broad spectra of toxicity is important for the development of new products for the management of insect pests of crops. As a result, the search for new *cry* genes is an on-going effort worldwide with more than 800 *cry* genes discovered so far (Rabha *et al*., 2023). Aynalem *et al.*, (2021) have made a study on *Tuta absoluta* using native *Bt* isolates and found that lepidopteran active *cry* genes were found in Ethiopian isolates. Therefore, this study was designed to characterize native *Bt* isolates by testing their pathogenicity against chickpea pod borer, *H. armigera* 3rd instar larvae.

3.2. MATERIALS AND METHODS

3.2.1. Source of *Bt* isolates

Ten native *Bt* isolates previously collected, isolated and their bio-toxicity assay was conducted under laboratory at a single concentration and proofed to be proficient were used (Gemmeda *et al.,* 2023). A single pure colony was taken and inoculated in to 100 µL liquid nutrient broth medium in Eppendorf tube and then incubated at 30 °C for 24 h for sporulation. The sporulated culture was stored at -20 °C in lab freezer for further use.

3.2.2. Reference *Bt* strain

The commercially accessible form of *Bt* also known as *Bacillus thuringiensis* was produced by Sibbiopharm ltd. in the Russian Federation under the trade name BitoxybacillinTM and the Gallica Flower Farm in Menegasha, Ethiopia, provided it. The preparation of commercial *Bt* included proteinaceous crystals (delta-endotoxin), thermo-stable b-exotoxin of *Bt* culture, and bacterial spores of (10^6 spores/mL), which are employed to control numerous Lepidopteran larvae and red spider mite. BitoxybacillinTM is pale beige to brown powder with a biological activity of 1500 UA/mg and an exotoxin content of 0.6-0.8% (Teferra and Dubale, 2020; Adilkhankyzy *et al*., 2022).

3.2.3. Morphological characterisation

Prior to each experiment, single colonies of each *Bt* isolates were routinely cultured on nutrient agar medium incubated for 24 h at 37 °C temperature. *Bt* colonies were observed under colony counter and morphologically characterized using the criteria selected such as texture, surface, colour, form, elevation and margin. Whereas, cell morphology likes cell shape and cell arrangement were included.The use of a reference strain, *Bt. v*ar. *thuringiensis* ensured that they have different genetic background (Leboffe *et al.,* 2016; Maza *et al*., 2020).

3.2.4. Biochemical characterization of *Bt* isolates

3.2.4.1. Gram staining

To determine whether bacteria were gram positive and to isolate just the rod-shaped ones, gram staining was used. Samples were smeared on clean glass slides, followed by heat fixing and air drying. The smears were then stained with the primary stain, crystal violet, followed by treatment with iodine solution, which functions as a mordant.

Finally, the smears were counter stained with the basic dye, safranin, and air dried. The smeared slides were then observed under 100x oil immersion microscope. The differential aspect of the Gram stain can be shown as Gram positive bacteria retained crystal violet and Gram negative bacteria lose their crystal violet and stain pink color (Smith and Hussey, 2005; Doolotkeldieva *et al.*, 2018).

3.2.4.2. Endospore stain

The bacterial culture grown on nutrient agar medium for 48-72 h were smeared on a clean glass slide and allowed to air dry. Smear was heat fixed by passing the slide over flame followed by heating over steam for 15 minutes with continuous addition of five percent aqueous solution of malachite green. After steam fixation, excess stain was washed under running tap water. The slide was counter stained with 0.5 per cent aqueous solution of safranin for 30 seconds and washed thoroughly under tap water. The slide was blot dried and observed under oil immersion microscope (Hussey and Zayaitz, 2016).

3.2.4.3. Insecticidal crystal protein staining

Depending on the insecticidal crystal protein composition, the crystal adjacent to the endospore during sporulation stages of three to four distinguishes *Bacillus thuringiensis* from other *Bacillus* species. It was performed according to the procedures developed by (Sharif and Alaeddinoĝlu, 1988). The stained bacterial cell was observed under Euromex iScope Series (Holland) to visualise the crystal proteins present.

3.2.4.4. Catalase test

An inoculum from the pure culture was streaked on a sterile nutrient agar plate and it was incubated at 37 ˚C for 24 hours. Growth from the plate is smeared on a clean slide and a drop of hydrogen peroxide (3% H_2O_2) is placed on the bacterial smear. The hydrogen peroxide is neutralized by the catalase enzyme generated by these bacteria, and copious bubbles liberated indicating that the bacteria able to break down hydrogen peroxide into water and oxygen that showed a positive test (Al-joda, B-M. and Jasim, 2021).

3.2.4.5. Motility testing

B. thuringiensis isolates were aseptically streaked by stabbing. After incubating the stabbed culture, motile bacteria typically give diffused, hazy growth that spreads throughout the medium rendering it slightly opaque. Non motile bacteria generally give growth that are confined to stab line and have sharply defined margins leaving the surrounding medium clearly transparent (Peter *et al.*, 1973).

3.2.4.6. Citrate reduction

Citrate agar was used which contains citrate and inorganic ammonium as carbon and nitrogen sources. The test *Bt* was aseptically inoculated in to the slant by stabbing in to the butt and streaking on the surface of the slants. The inoculated slants are incubated at 37 °C for 24 to 48 h in an incubator. If the bacteria capable of utilizing citrate as a carbon source, the enzyme citrase hydrolysis citrate in to oxaoloacetic acid and acetic acid and colour changes on the slant from green to blue indicated citrate utilisation positive (MacWilliams, 2009).

3.2.4.7. Terhalose fermentation

The *Bt* inoculum from a pure culture is aseptically transferred to a sterile tube of phenol red trehalose broth. The inoculated tube is incubated at 37 ^{o}C for 18 to 24 h and the results are determined. A positive test consists of a colour change from red to yellow, indicating a pH change to acidic (Reiner, 2012).

3.2.4.8. Maltose fermentation

Aseptically inoculate each test tube with 1 to 2 drops of *Bt* culture incubated for 24 h using an inoculating loop. Incubate tubes at 37 °C for 18 to 24 h. By employing phenol red as a pH indicator, fermentation outcomes can be determined. A yellow colour suggests that the sugar has fermented sufficiently to produce an acidic product that brings the pH lower to 6.8 or less. Results of gas production indicated that the presence of trapped bubbles in the Durham tube signifies the generation of gas. No bubbles within the Durham tube indicate a anaerobic organism (Reiner, 2012).

3.2.4.9. Urease utilisation

The urea broth was prepared contains urea and phenol red and cultures were inoculated aseptically using pure *Bt* inoculum. It was incubated for 24-48 h at 37 °C and checked for the change in colour from phenol red to deep pink. An enzyme urease catalysis urea to CO_2 and NH_3, ammonia combines with water to produce ammonium hydroxide, a strong base which increases pH of the medium. Increase in the pH causes phenol red to turn to deep pink that indicative of positive for urease utilisation (Brink, 2010).

3.2.4.10. Triple Sugar Iron (TSI)

The TSI agar slants containing 1% each of lactose and sucrose, and 0.1% glucose with phenol red indicator. Butt and slant are used to prepare the media. The culture inoculated by stab and streak method was incubated for 24 h at 37 ^{o}C and observed for the change in colour from red to yellow.

Within 6-8 h of inoculation, both the slant and butt colour shift to yellow due to acid generation, if the bacteria can use glucose in both aerobic and anaerobic circumstances. If the bacteria are able to use sucrose and lactose, acid production continue and the colour of the media remains yellow. If the bacteria are a strict aerobe, the colour of the butt not changed and the response only take place in a slant. If it can't use sucrose or lactose, the bacteria turn to amino acid, which makes the medium alkaline and turns the colour of the medium red owing to phenol red (Lehman, 2005).

3.2.4.11. Indole production

Inoculate the test tube containing tryptone broth with a small amount of *Bt* pure culture and incubate it at 37 °C for 24 to 48 hours. Add 5 drops of Kovács reagent directly to the tube for indole production,. An amino acid tryptophan goes under deamination and hydrolysis by bacteria that express tryptophanase enzyme. The formation of a pink to red colour in the reagent layer on top of the medium forming red ring indicated positive response to indole production (Maza *et al*., 2020).

3.2.5. Molecular characterization

3.2.5.1. Bacterial Genomic DNA preparation and Extraction

Molecular work was carried out at Holeta Molecular Biotechnology. Pure culture of each the native *Bt* isolates was prepared from a single colony. The obtained pure culture was streaked on nutrient broth medium and incubated at -20 ^{o}C for DNA extraction. Total genomic DNA was extracted from the pure culture using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. To produce the PCR products, the universal and specific primer pairs (Table 7) were employed. The extracted DAN was stored at -20 ^{o}C for further study.

3.2.5.2. Oligonucleotide primer pairs

Five universal primer pairs and one specific primer pairs (forward and reverse) sintesized by SANGON biotech manufacture (China) were used to explore encoding *cry* genes (Table 7).

Table 7 properties of primer pairs used for detection of different *cry* genes in the native *Bt* isolates

Oligonucleotide primers pairs	Sequence (5' to 3')	Band size (bp)	T_m (^oC)	T_a (^oC)
***cry*1 (universal)**	CAT GAT TCA TGC GGC AGA TAA AC	274	54.4	
***cry*1 (un) (R)**	TTG TGA CAC TTC TGC TTC CCA TT	277	56.6	56.1
***cry*2 (Un) (F)**	GTT ATT CTT AAT GCA GAT GAA TGGG	689	51.4	
***cry*2 (Un) (R)**	CGG ATA AAA TAA TCT GGG AAA TAGT	701	49.5	50.0
***cry*7,8 (un) (F)**	AAG CAG TGA ATG CCT TGT TTAC	420	53.3	
***cry*7,8 (un) (R)**	CTT CTA AAC CTT GAC TAC TT	423	45.4	49.5
***cry*9 (F)**	CAC ATG AGT TTT CTT CCT AT	440	46.1	
***cry*9 (R)**	AGA TAC GAT GCT TGT TGT AA	440	47.8	49.5
***cry*3 (un) (F)**	CGT TAT CGC AGA GAG AGA TGA CAT	589	56.1	
***cry*3 (un) (R)**	CAT CTG TTG TTT CTG GAG GCA AT	595	55.3	58.0
***cry*4 (un) (F)**	GCA TAT GAT GTA GCG AAA CAA GCC	439	56.5	
***cry*4 (un) (R)**	GCG TGA CAT ACC CAT TTC CAG GTCC	439	52.6	58.0

Ben-Dov *et al.*, 1997, T_a, annealing temperature, T_m, melting temperature, F, forward primers, R, reverse primers

3.2.5.3. Optimization of annealing temperature (T_a)

One of the important variables influencing the purity yield of the reaction products is the annealing temperature (Ta). Reaching the optimum T_a is critical for reaction specificity, as none specific products may be formed as a result of none optimal T_a. Optimization was done by applying temperature gradient PCR, where PCR carried with different T_a. Based on the primer pairs five temperature levels were chosen. For instance, if the calculated melting temprature (T_m) of one primer was 51 °C it started 3 °C below the lowest calculated T_m of the primer pair and then it sated at 48 ^{o}C to 60 ^{o}C and increased the temperature level by 3 ^{o}C intervals, so that the T_a was fall in between 48 ^{o}C and 60 °C (Rychlik *et al*., 1990; Ammouneh *et al.*, 2011; Jasmina *et al*., 2013).

3.2.5.4. DNA amplification

Twenty microliter of total genomic DNA extracted from *Bt* isolates was used as template for PCR amplification mixed with TaqDNA polymerase enzyme (1 unit), 10X buffer (2 µl), 2 µl $MgCl_2$ (2500 µM), 2 µl deoxynucleotide triphosphate (dNTPs) (2500 µM), 1 µl of each forward and reverse primer (10 pmol), and 14.8 µl sterile distilled water. The PCR condition was a 95 degree initial denaturation for 5 minutes followed by 35 cycles of denaturation at 95 degrees for 30 seconds, annealing at temperatures indicated (Table 7) 55 degrees for 1 minute and extension at 72 degrees for 30 seconds with a final extension at 72 degrees for 5 minutes (da Silva and Valicente, 2013).

3.2.5.5. Analysis for *cry* genes by gel electrophoresis

The presence of specific *cry* genes of the amplicons of all *Bt* isolates were analysed by PCR method using gene specific primers according to (Ben-Dov *et al*., 1997; da Silva and Valicente, 2013). Agarose gel was used to run gel electrophoresis where nucleic acid molecules are size separated by the aid of an electric field. Five µl of the PCR amplified product was stained with bromophenol blue. Nucleic acid fractionation was made using agarose gel electrophoresis for further purification of a band of interest. Further, excising the desired band from a stained gel was made and analyzed by electrophoresis (100 V/45 min/1×TBE) using 3% agarose gel mixed with 2 µL loading dye (gel-red) for gel documentation. After electrophoresis the gels were visualized and the photographs were captured by UV light. The size of the amplicons was estimated based on 100 bp ladder loaded as size markers.

3.2.5.6. 16S rRNA gen analysis

The primer pair RD1 AAGGAGGTGATCCAGCC, and FD1AGAGTTTGATCCTGGCTCAG was used to generate a 1500 bp (Weisburg *et al*. 1991). The PCR condition was a 95 degree initial denaturation for 5 minutes followed by 35 cycles of denaturation at 95 degrees for 30 seconds, annealing at 55 degrees for 1 minute and extension at 72 degrees for 30 seconds with a final extension at 72 degrees for 5 minutes. in 25µl reaction mixture (forward and reverse primers 0.5µM each, 50–100 ng of template, 0.5 U of Taq DNA polymerase (Promega, USA), 200µM dNTPs, 10 mM Tris, 50 mM KCl and 1.5 mM $MgCl_2$). PCR product (5µl) was analyzed in 3% (w/v) Agarose (Promega, USA) gel electrophoresis at 100 V for 45 min in 1×TBE buffer and visualized under UV transillumination in a gel documentation system (Alpha imager mini, USA) following staining in EtBr (Sigma, USA) solution (0.5µg/ ml).

3.3. RESULTS

3.3.1. Morphological characteristics of *Bt* isolates

Results of colony morphology showed that two colony textures (Brittle and Viscous), five colony surfaces (Dull, smooth, rough, Veined and Glistening), three colors (creamy white, white to off white), three forms (circular, wavy and oval), two types of elevations (flat and raised) and two types of margin (curled and entire) were identified (Table 8, Fig. 8a). All *Bt* isolates had rod shaped cell from Gram stained, whereas, five cell arrangements were observed (short chain, short chain with v-arrangement, short chain with curved, Palisade and long chain) (Table 8, Fig. 8b).

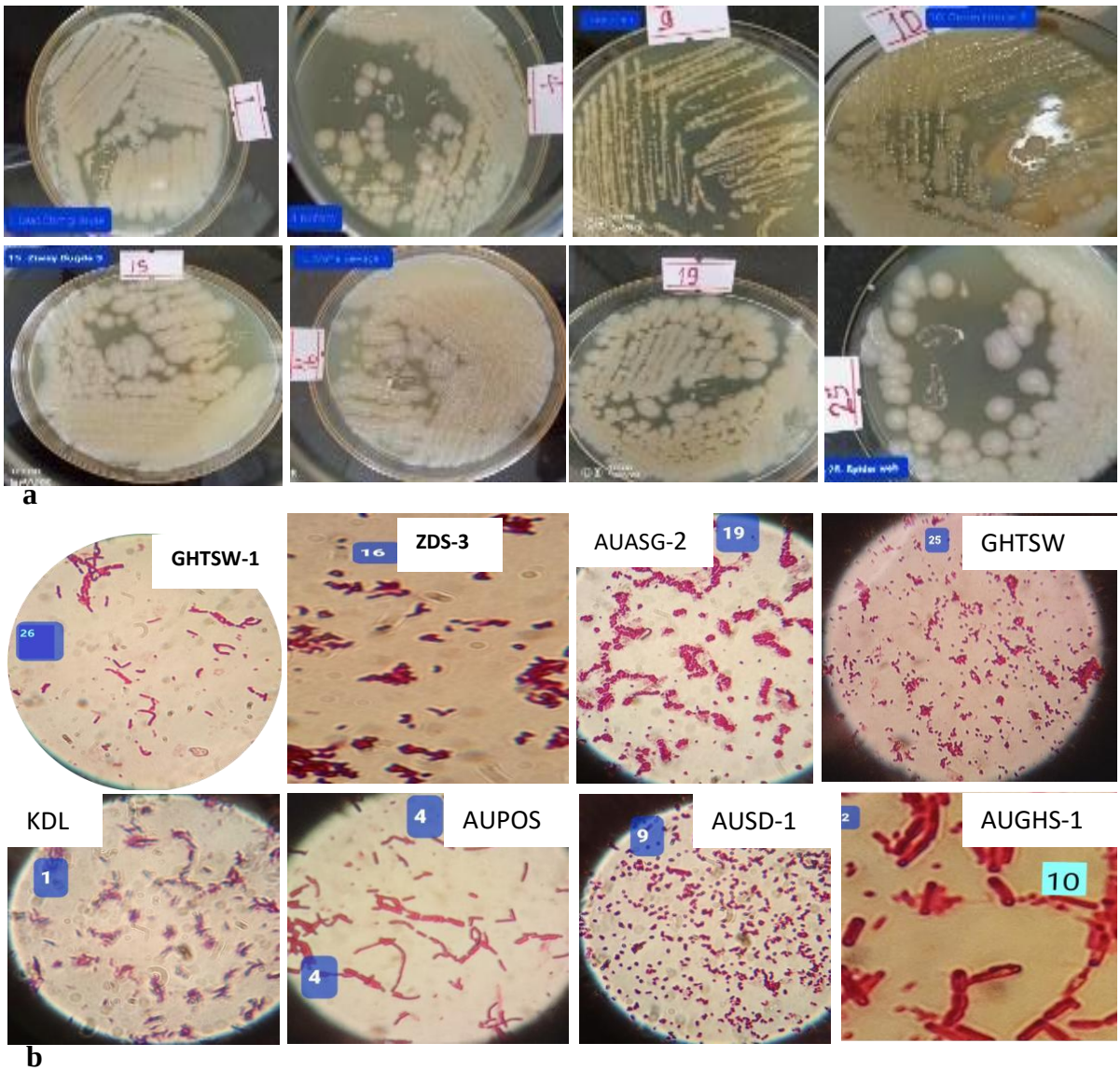


Figure 8 Morphological characterisation (**a**) colony morphology (**b**) cell shape and arrangement.

Table 8 Detailed descriptions of morphological characteristics of native *Bt* isolates

***Bt* isolates**	**Morphological Characteristics**							
	Texture	**Surface**	**Colour**	**Form**	**Elevation**	**Margin**	**Cell shape**	**Cell Arrangement**
Bt. var. *thuringiensis*	Brittle	Dull	White	Circular	Raised	Entire	Rod	Palisade
KDL	Brittle	Dull	Creamy white	Circular	Flat	Entire	Rod	Short chain
AUPOS	Brittle	Dull	White	Circular	Raised	Entire	Rod	Short chain (V)
AUSD-1	Viscous	Smooth	Off white	Oval	Flat	Entire	Rod	Long chain
AUGHS-1	Viscous	Smooth	Off white	Circular	Raised	Entire	Rod	Short chain (C)
ZDS	Brittle	Veined	Creamy white	Wavy	Flat	Curled	Rod	Palisade (V)
ZDS-3	Viscous	Glistening	White	Circular	Flat	Entire	Rod	Palisade
AUASG-2	Brittle	Rough	White	Circular	Flat	Entire	Rod	Long chain
GHTSW	Brittle	Rough	Creamy white	Circular	Raised	Entire	Rod	Palisade
GHTSW-1	Brittle	Dull	White	Circular	Raised	Entire	Rod	Short chain
AUGHS-3	Brittle	Dull	White	Circular	Raised	Entire	Rod	Short chain (V)

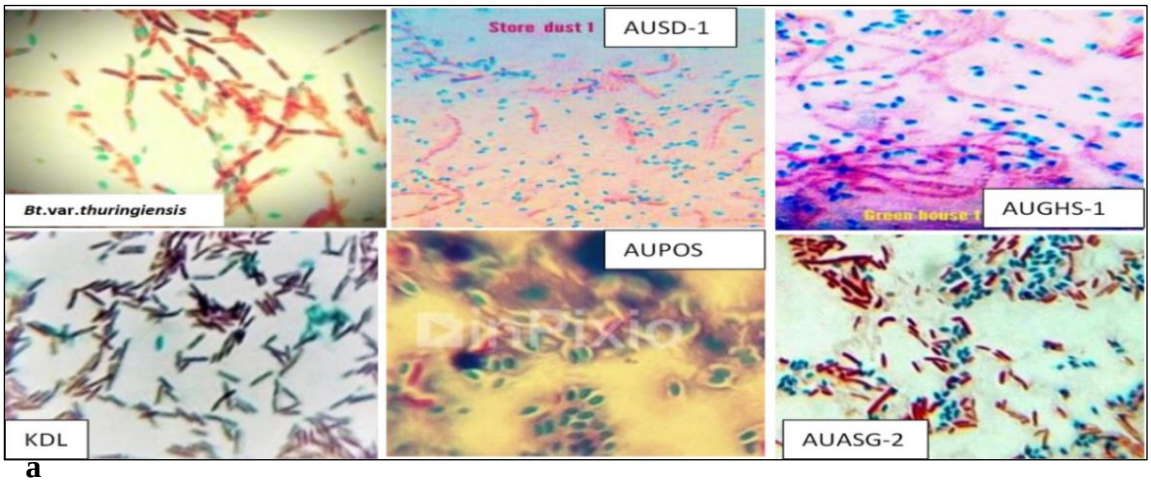
3.3.2. Biochemical characteristics of *Bt* isolates

It was shown that from gram stain and motility test result all the tested *Bt* isolates were gram positive and motile (Table 10). Results of endospore and crystal protein stain revealed that all *Bt* isolates were positive for both endospore and crystal proteins (Table 9 & Fig. 9b), where, small brightly stained green spores were clearly visible within pink vegetative cells that contain green elliptical structures (Fig. 9a). The catalase test was performed for all *Bt* isolates and they were positive for catalase degradation. Upon introduction of hydrogen peroxide to the bacterial smears, samples underwent a violent reaction with bubbles forming at rapid rates and similar results were also seen in the reference *Bt* strain (Table 10, Fig. 10b). All *Bt* isolates were showed positive reaction to glucose, maltose and citrate utilisation test, whereas, they all showed negative to urease and indole test (Table 10, Fig. 10a). Variations were observed between *Bt* isolates in response to triple sugar iron and trehalose test. Five *Bt* isolates showed positive and three showed negative result to trehalose test, whereas, three isolates were not identified (Table 10, Fig. 10a). Five *Bt* isolates showed negative to triple sugar iron test and five indicated as R/Y implied that the *Bt* isolates were only utilised glucose, whereas, one *Bt* isolate not identified (Table 10).

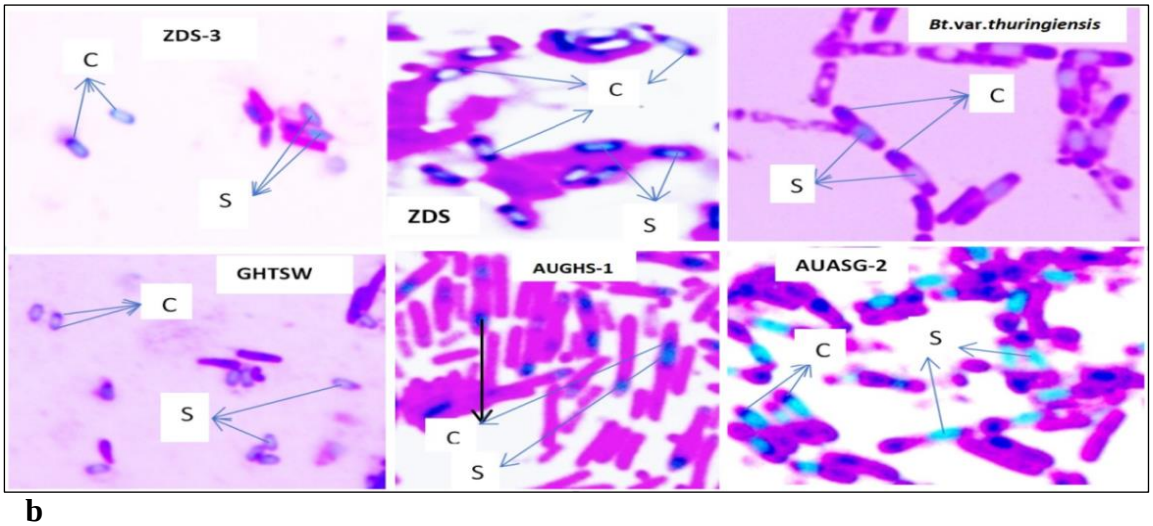
3.3.3. Insecticidal crystal proteins

Table 9 Native *Bt* isolates and their insecticidal crystal proteins

***Bt* isolates**	**Insecticidal crystal proteins**
KDL	present
AUPOS	present
AUSD-1	present
AUGHS-1	present
ZDS	present
ZDS-3	present
AUASG-2	present
GHTSW	present
GHTSW-1	present
AUGHS-3	present
Bt. var. *thuringiensis*	present

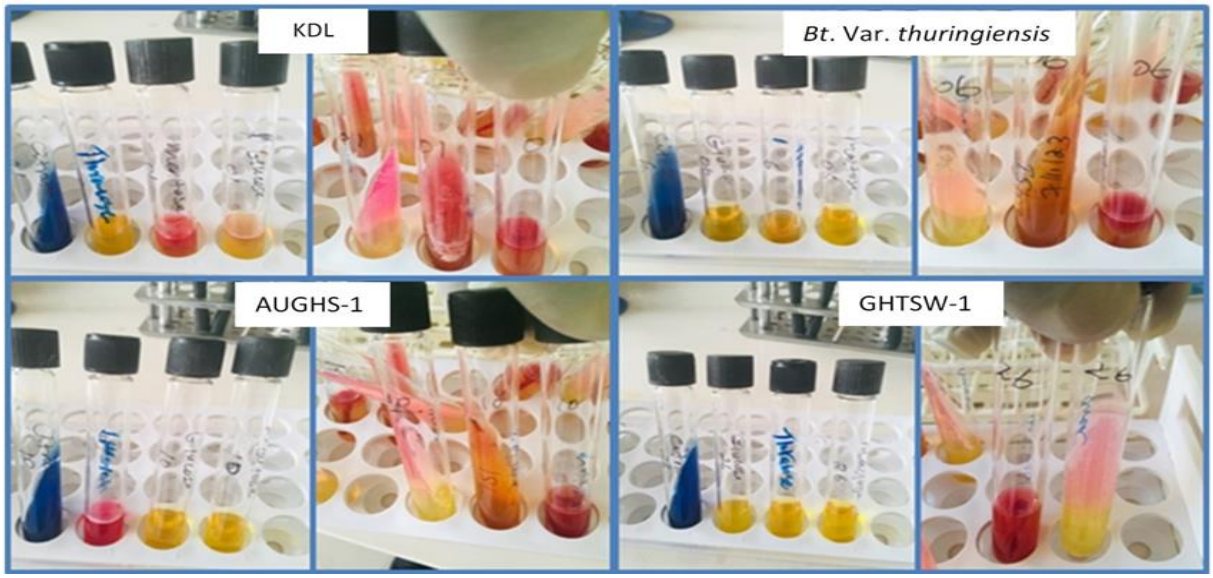


a

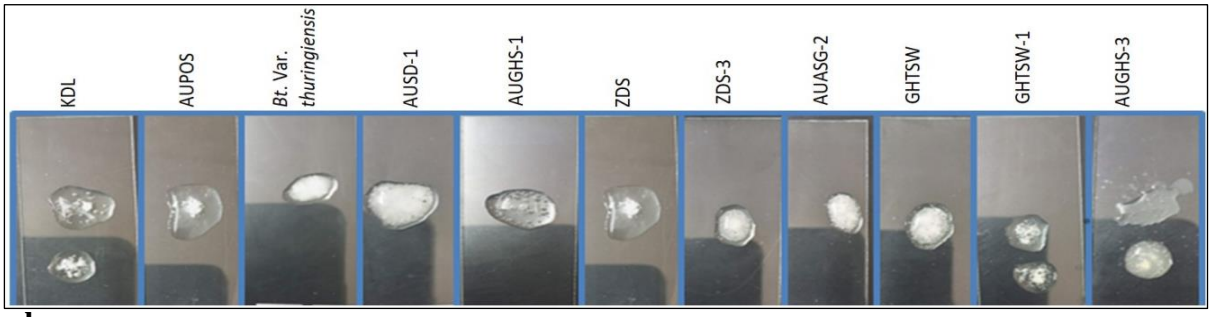


b

Figure 9 Biochemical characteristics (**a**) Gram and endospore stain (**b**) Insecticidal Crystal protein stain



a



b

Figure 10 Biochemical test (**a**) Citrate, indole, glucose and traholese test (**b**) Catalase test

Table 10 Detailed descriptions of biochemical test of native *Bt* isolates and the reference *Bt*. Var. *thuringiensis*

Biochemical characteristics	*Bt* isolates										
	KDL	AUPOS	AUSD-1	AUGHS-1	ZDS	ZDS-3	AUASG-2	GHTSW	GHTSW-1	AUGHS-3	*Bt*.var. *thuringien*
Gram Stain	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Endospore stain	+	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	-	+	+	+	+	+	+	+	+
Terhalose	-	N	+	+	+	-	-	N	+	N	+
Maltose	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-
TSI	R/Y	-	R/Y	-	-	R/Y	R/Y	-	-	N	R/Y
Indole	-	-	-	-	-	-	-	-	-	-	-

(+) positive response (-) negative response (N) no response (R/Y) red or yellow

3.3.4. *cry* genes detected in Bt isolates

The typing of primary rank of *cry* genes *cry*1, *cry*2, *cry*3, *cry*4, *cry*7,8 and *cry*9 was performed for ten native *Bt* isolates by PCR amplification using universal and specific primers given in (Table 11). All *Bt* isolates were found to be positive for the presence of *cry* genes. No isolates harbouring a single gene, rather, combinations of multiple *cry* genes were detected. Off which native *Bt* isolate AUSD-1 carried *cry*1,*cry*2, *cry*4, *cry*7,8 *and cry*9 genes. Whereas 5 (50%) isolates had combinations of two *cry* genes, 2 (20%) isolates had combination of three *cry* genes and 2 (20%) isolates had combinations of 4 *cry* genes and 1(10%) isolates had 5 *cry* genes combinations (Table 11, Fig. 11). Any of the isolates not amplified *cry*3 genes. All the native isolates were harbouring (100%) *cry*4 genes (Table 11, Fig. 11).

Table 11 Native *Bt* isolates showed positive results to different *cry* genes

Bt isolates	*cry* gens					
	cry1	*cry2*	*cry3*	*cry4*	*cry7, 8*	*cry9*
KDL	-	+	NA	+	-	-
AUPOS	-	+	NA	+	-	-
AUSD-1	+	+	NA	+	+	+
AUGHS-1	+	-	NA	+	-	-
ZDS	-	+	NA	+	-	+
ZDS-3	+	+	NA	+	-	+
AUASG-2	+	+	NA	+	-	+
GHTSW	-	+	NA	+	-	-
GHTSW-1	-	+	NA	+	-	-
AUGHS-3	+	+	NA	+	-	-
Bt. var. *thuringiensis*	+	+	NA	+	+	-

(+) genes detected (-) no genes detected (NA) not amplified

3.3.5. 16S rRNA gen profile of *Bt* isolates

Amplicons of about 1500 bp were obtained from PCR amplification of 16S rRNA gene of native *Bt* isolates (Fig. 12). All native *Bt* isolates except AUASG-2 and AUGHS-3 augmented 16S rRNA gene that means it confirmed that the isolates were grouped to *Bacillus thuringiensis*. The two above isolates were not tested due to insufficient DNA extracted and the original source samples were far to replace it.

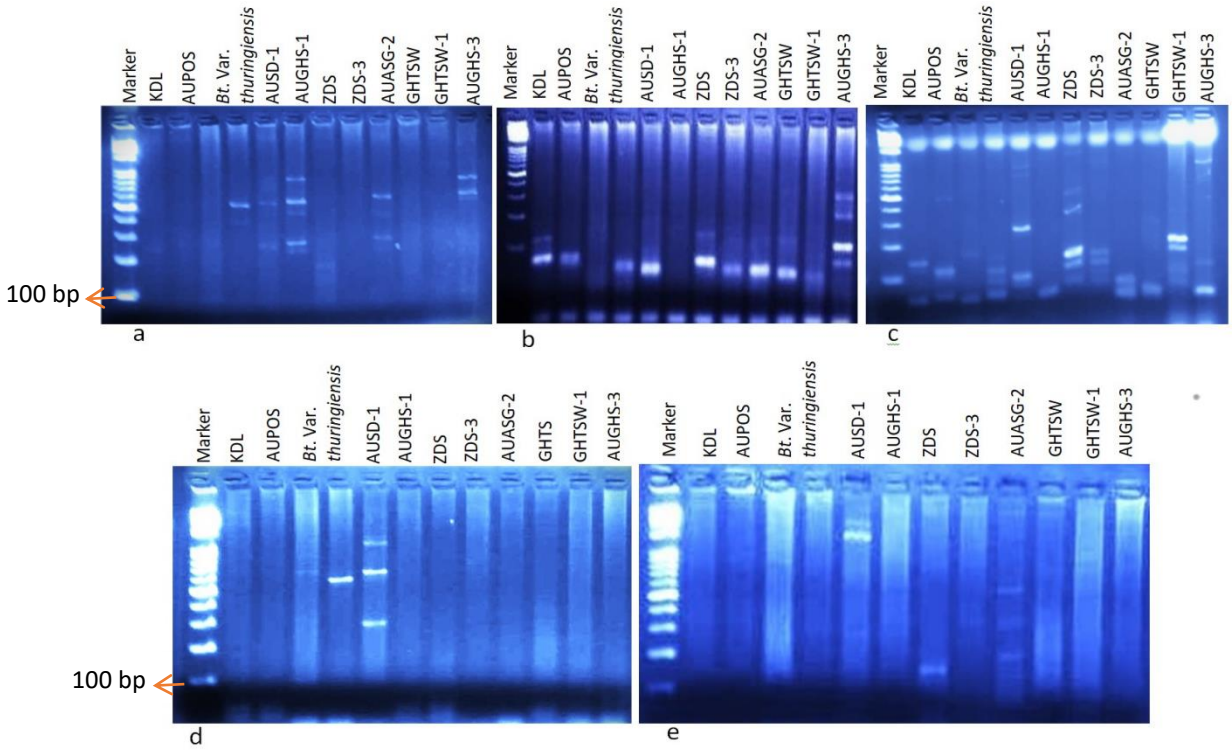


Figure 11 PCR analysis of *cry* gene profile of native *Bt* isolates and their respective band size reading (bp) (**a**) *cry*1, (**b**) *cry*2, (**c**) *cry*4, (**d**) *cry*7, 8, (**e**) *cry*9

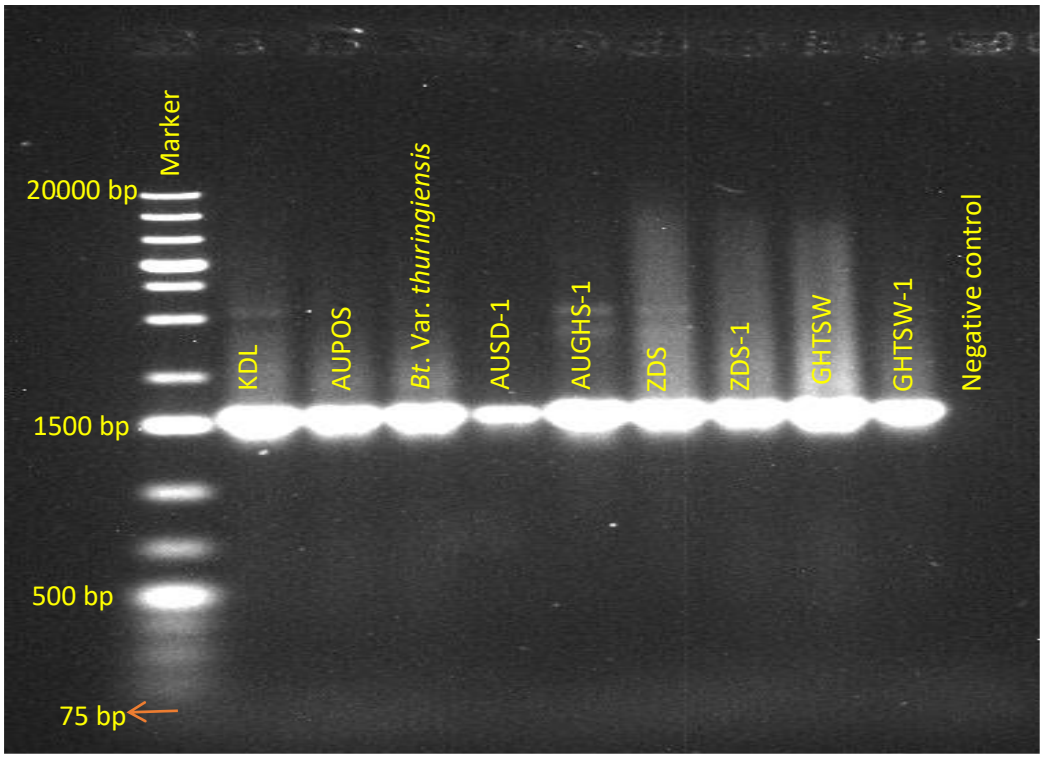


Figure 12 PCR result for 16S rRNA gen amplification visualized under UV light after 3% agarose gel electrophoresis labelled with the names of *Bt* isolates studied, standard *Bt*. Var. *thuringiensis* and negative control

3.4. DISCUSSION

The purpose of this study was to search for native *Bacillus thuringiensis* (*Bt)* isolates harbouring *cry*1, *cry*2, cry7, 8 and cry9 genes which could be highly potent to lepidopteran larvae. Therefore, in the present study 10 native *Bt* isolates were morphologically, biochemically and molecularly characterised. Results of morphological characterization are variable, but, biochemical test is more specific and consistent, mainly gram and endospore stain, motility and catalase test. Results of these biochemical test showed that all *Bt* isolates possessed endospores, road shape cells and motile as well as produce catalase enzyme (Karen, 2010; Smith and Hussey, 2005, 2013 ; Ghosh *et al.*, 2017). These were in agreement with almost all *Bt* studies and strongly supported. All native *Bt* isolates were positive to glucose fermentation and citrate utilisation and negative to urease and indole test (Abirami *et al*., 2016; Jyothi and Priya, 2018; Gholamveisi *et al.*, 2018).

Most *Bt* studies were not consistent; this is possibly due to different *Bt* species respond differently to the test. PCR analysis was done for their *cry* genes profile to detect the insecticidal crystal proteins that highly toxic to *H. armigera* larvae. Results obtained confirmed that all native *Bt* isolates were belongs to *Bacillus thuringiensis* group. The *cry* gene profile of the 10 native *Bt* isolates showed that the presence of *cry*1, *cry*2, *cry*4, *cry*7, 8 and *cry*9 genes. None of the native *Bt* isolates harboured single gene and any of the *Bt* isolates were not amplified *cry*3 gene. However, when looking at lepidopteran active *cry* genes separately like *cry*1, *cry*2, *cry*7,8 and *cry*9 genes, native *Bt* isolates KDL, AUPOS, GHTSW and GHTSW-1 harboured only *cry*2 gene and AUGHS-1 possessed *cry*1 gene alone. The results obtained above were not agreed with most literatures because of single *cry* gene rarely frequent (Khojand *et al.*, 2013).

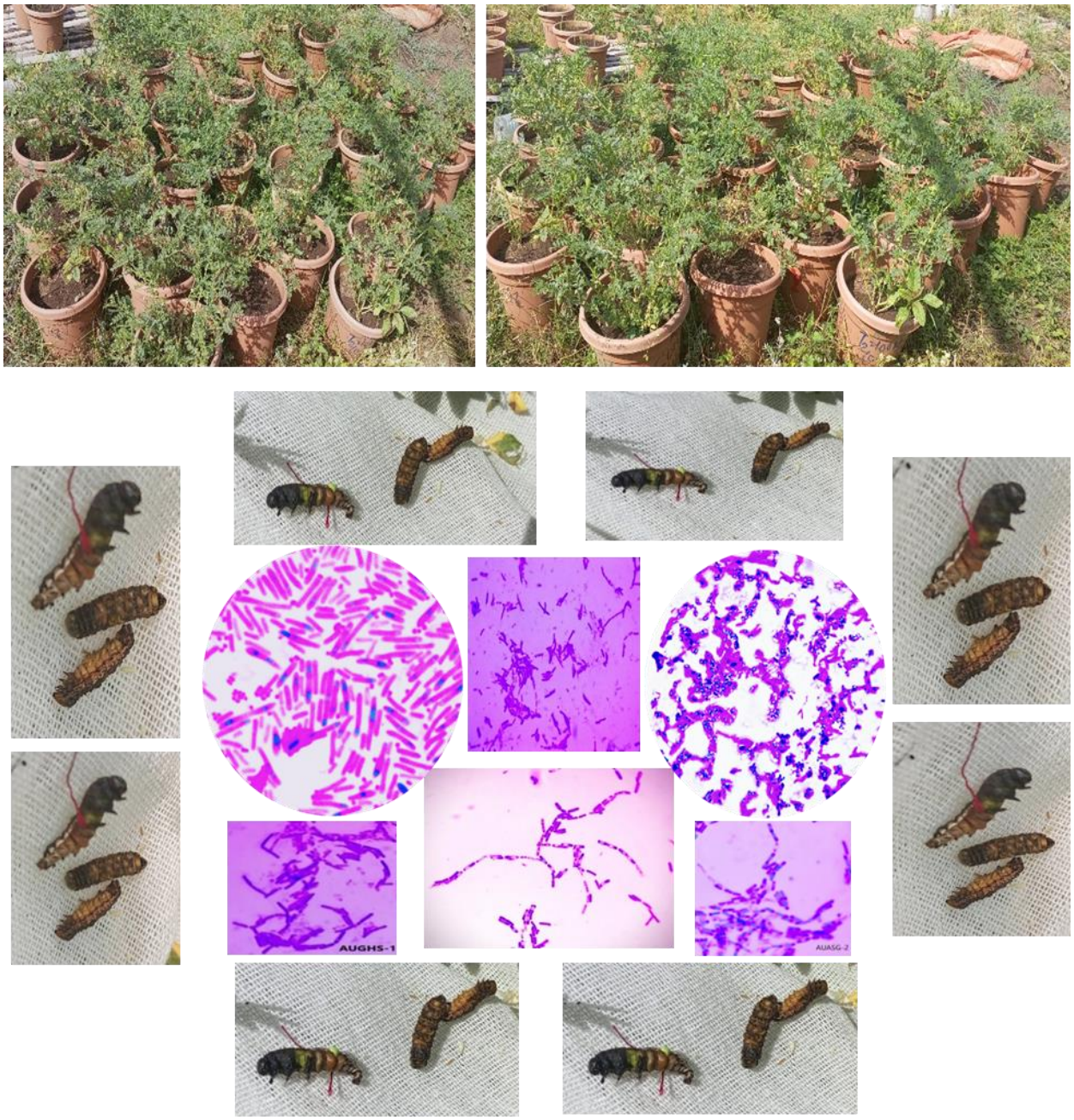
Considering the frequency of incidence of single *cry* gene, *cry*4 gene was predominantly detected in all *Bt* isolates (100%) followed by *cry*2 gene (90%), *cry*1 gene (50%), *cry*9 gene (40%) and *cry*7,8 gene (10%). Aynalem *et al*. (2021) have reported that in Ethiopian *Bt* isolates against tomato whit fly, *Tuta absoluta*, found that *cry*2 and *cry*9 genes were more frequently detected genes than *cry*1gene. However, Hassan *et al*. , (2021) detected *cry*1 gene (100%) compared to *cry*2 gene, however, similar result was obtained in respective of *cry*4 gene (84.61%) detected. Baig *et al*. (2010) made study on *cry* Genes profiling and the toxicity of *Bacillus thuringiensis* against *H. armigera.*

He found that the percentage incidence of *cry* gens, out of 50 *cry* gene positive *Bt* isolates 6 isolates were positive to *cry*2 gene and 22 were positive to *cry*4 gene, moreover, out of 17 single *cry* gene positive *Bt* isolates 18 and 65 of the isolates were positive to *cry*2 and *cry*4 genes respectively. Two native *Bt* isolates, ZDS harboured *cry*2+*cry*4+*cry*9 genes and AUGHS-3 harboured *cry*1+*cry*4+*cry*2 genes. Three *cry* genes *cry*1+*cry*2+*cry*4+*cry*9 combination were detected in native *Bt* isolates of ZDS-3 and AUASG-2, *Bt* isolate AUSD-1 harbouring 5 *cry* genes *cry*1+*cry2*+*cry*4+*cry*7,8+*cry*9, this result agreed with most *Bt* studies as of *cry* genes found in combinations (Khojand *et al*., 2013; Hassan *et al*., 2021).

3.5. CONCLUSION

The morphology of the native bacterial isolates along with the results obtained from the biochemical tests and molecular characterization was confirming that the 10 isolated bacterial species from Ethiopia were belongs to *Bt.* Their *cry* genes were analysed, thus, indicated all native *Bt* isolates are harbouring two or more *cry* genes. Typically *cry*2 gene is the most frequently detected in all *Bt* isolates except AUGHS-1 native *Bt* isolate which lack *cry*2 but, it possessed *cry*1 gene. Off all *cry* genes detected *cry*7, 8 and *cry*9 are less frequently identified and *cry*4 gene was detected in all *Bt* species. Hence, *cry*1 and *cry*2 genes that express insecticidal crystal proteins to lepidopteran larvae were isolated and identified from Ethiopian *Bt* isolates. This implied that the presence of potent *Bt* isolates against lepidopteran larvae that necessitate continuous experimental work to verify under greenhouse and field conditions against *H. armigera* in chickpea.

CHAPTER FOUR



Dead H. armigera larvae killed by Bt species under the Greenhouse experiment

CHAPTER FOUR

Paper III: Native *Bacillus thuringiensis* isolates against the Old-World bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera:Noctuidae) in Ethiopia

ABSTRACT

Chickpea **(***Cicer arietinum* L.) production in Ethiopia has been seriously constrained by the insect pest, *Helicoverpa armigera*. Insecticides have been used for decades to control this noxious pest. However, the restriction on chemical insecticides necessitates biological pest control alternatives. Therefore, the present study investigated the effectiveness of native *Bacillus thuringiensis (Bt)* isolates against *Helicoverpa armigera* 3rd instar larvae. The treatments consisted of *Bt* concentrations at the LD_{90} values, whereas the control plots were sprayed with distilled water and a commercial *Bt*. var. *thuringiensis* product. The results revealed statistically ($p<0.05$) significant differences between the native *Bt* isolates and the control plots. Post 1st, pre 2nd and post 2nd *Bt* sprayed larval populations counted per plant ranged from 0.8±0.3 to 3.1±0.1, 0.6±0.1 to 3.6±0.0 and 0.5±0.1 to 3.6±0.4, respectively. Pod damage (%) and grain yield (t/ha) ranged between 9.7±3.1 and 47.5±9.5, 1.0 ±0.0 and 2.6±0.5, respectively compared to 47.5±9.5% pod damage and 1.0±0.0 (t/ha) grain yield from the control plots. These results indicate the potential of these bio-insecticides in the management of chickpea pod borer. Future research in Ethiopia should focus on additional collection, screening, characterisation, and testing in field trials.

Keywords: Biological control; crystal proteins; LD_{50}; larval mortality; pod damage

4.1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) plays a significant role in the human diet as a staple food legume (Charlie, 2016; Redden *et al*., 2007). Nevertheless, chickpea production is highly impacted by insect pests such as *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *H. armigera* is among the most economically important insect pest with annual yield losses that exceed $2 billion worldwide (Pedgley, 1985; Sharma *et al*., 2005; Sharma *et al*., 2006). In the semiarid tropics, yield losses were estimated to be 328 million USD (ICRISAT, 1992). A study made by El Fakhouri *et al*. (2022) in Morocco indicated yield loss inflicted by *H. armigera* to be between 14.3% and 31.2%. In early sown chickpea, 80% of pod damage was reported in Ethiopia (ICRISAT, 1991). In the central highlands of Ethiopia, pod damage can range between 21% and 36% with 20% and 30% annual yield loss (Gelatu and Million, 1996). In Ethiopia, chemical insecticides are widely used to control *H. armigera.* Subsequently, insect pests have developed resistance to the insecticides that prompt frequent applications. Intensification of chemical insecticides may also have harmful effects on human and environmental health (Nicolopoulou-Stamati *et al*., 2016; Pathak *et al*., 2022; Rani *et al*., 2020). There is also a growing interest in residue free produce by consumers in the developed countries. Thus, there is a need for alternative management options (Patil *et al*., 2017). Hence, the potential of biological-based insecticides such as *Bacillus thuringiensis (Bt)* to control *H. armigera* needs to be explored (Mohammad *et al*., 2010; Patil *et al*., 2017).

Bacillus thuringiensis is a gram-positive, soil born bacterium that produces parasporal crystalline inclusions during sporulation that harbours insecticidal crystal proteins and *ß*-endotoxin with toxicity against many Lepidopteran larvae (Kumar *et al*., 2016; Li and Bouwer, 2012; Majeed *et al*., 2018). Thus, it has been used to control *H. armigera* in chickpea worldwide (Estela *et al*., 2004; Qayyum *et al*., 2015). Few studies in Ethiopia reported the existence and potential of native *Bt* isolates against insect pest control (Aynalem *et al*., 2021; Tesfaye and Emiru, 2010; Tesfaye *et al*., 2012). In this study, the effectiveness of ten native *B. thuringiensis* isolates was tested against *H. armigera* 3rd instar larvae in the greenhouse and under field conditions.

4.2. MATERIALS AND METHODS

4.2.1. Descriptions of the study area

Greenhouse and field experiments were conducted in 2023 at the Kulumsa Agricultural Research Centre, Ethiopian Institute of Agricultural Research (EIAR) and Gonde Basic Seed Farm Center, Ethiopian Agricultural Business Corporation (EABC) both located at 8˚01'88"N and 39˚07'10"E, 8˚06'25"N and 39˚07'38"E; at an elevation of 2210 and 2268 metres above sea level, respectively. Both locations receive 800 mm and 809 mm mean annual rain fall with 23 °C and 10 °C 23.08 °C and 9.9 °C maximum and minimum temperatures, respectively (Abayneh *et al*., 2003).

4.2.2. Sources of *Bt* isolates and production of spore-crystal mixtures

Ten native *Bt* isolates were retrieved earlier from soil samples and cadavers (Gemmeda *et al*., 2023). The virulence of the *Bt* isolates was tested against *H. armigera* under laboratory conditions (Gemmeda *et al*., 2023). Laboratory scale production of spore-crystal mixtures was performed at Holeta Microbial Biotechnology laboratory following fermentation procedures (Dulmage, 1971). From the stored culture, a loopful of culture was grown on Modified Broth Solution (MBS) containing 6.8 g KH_2PO_4, 0.3 g $MgSO_4$. $7H_2O$, 0.2 g $CaCl_2$. $2H_2O$, 10 g Bactotryptose, 2 g yeast extract, 10 mL of stock solutions prepared from (2 g $MnSO_4$. H_2O, 2 g $Fe_2(SO_4)_3$, 2 g $ZnSO_4$. $7H_2O$ and 1liter of distilled water), 17 g of nutrient agar and one liter of distilled water. The solution was sterilised and the pH was adjusted to 7.2. The inoculated plates incubated at 30 °C for 24 h to induce sporulation.

A single pure colony was taken and inoculated into 100 µL nutrient broth medium in an Eppendorf tube and then incubated at 30 °C for 24 h. From the sporulated *Bt* culture, 100 µL was transferred to 250 mL Erlenmeyer flasks filled with 150 mL nutrient broth medium and fermented for 72 h at room temperature using an orbital shaker at 180 rpm (Ahmed *et al*., 2015). The suspensions were centrifuged at 10,000 rpm for 3 min at 4 °C in a sterile test tube. The supernatant was discarded but the pellets were washed twice in 20 mL of sterile distilled water and centrifuged. The spore-crystal mixtures were collected and re-suspended in 20 mL of sterile distilled water and stored at −20 °C until use (Andrews, 2001).

4.2.3. Rearing of *H. armigera* larvae

At Kulumsa Agricultural Research Center and Gonde Basic Seed Farm Center, *H. armigera* field culture was established on small chickpea plots. From the unsprayed plot in the field, *H. armigera* larvae were collected at their fifth instar and allowed to pupate in rearing boxes supplied with a larval diet prepared from chickpea (McGuire *et al*., 1997). Pupae were transferred to plastic pots embedded with sawdust. Adults were fed with a diet prepared from honey and sugar (2.5% v/v) using cotton swab dipped into the diet by suspended it in a cage. Male and female moths were reared separately in insect rearing cages in pairs for mating under a temperature of 25 °C, a relative humidity of 65%, and a photoperiod of 12L:12D (Armes *et al*. 1992). Adult females oviposited on the cheese cloth, and the newly hatched larvae were removed using a camel brush and transferred to tightly sealed Petri plates. Starting from the second instar, larvae were individually reared to avoid cannibalism in small plastic cups (1cm height and 1.5 cm radius) supplied with one gram of larval diet which was changed every two days.

4.2.4. Reference *Bt* strain

The commercially accessible form of *Bacillus thuringiensis* under the trade name of Bitoxybacillin$^{TM.}$ was used. BitoxybacillinTM is a pale beige to brown powder with a biological activity of 1500 UA/mg and an exotoxin content of 0.6–0.8% (Teferra and Dubale, 2020; Adilkhankyzy *et al*., 2022).

4.2.5. Greenhouse experiment

4.2.5.1. Experimental procedures and treatments

The experiment was laid out in a Complete Randomised Design (CRD) with three replications. Plastic pots having 18 cm top diameter and 12 cm bottom diameter with 15 cm height were prepared and filled with topsoil, sand, and forest soil. Three chickpea seeds were planted in each pot. After germination, the seedlings were thinned to one plant/pot. Pots were watered every two days. The spray volume was calibrated on the pot's surface area before use. During the experimental period, greenhouse temperatures ranged from 27 to 35 ^{o}C with 45±5% relative humidity and 12L:12D photoperiod. Seven *H. armigera* third instars were released at the pod setting stage on different leaves. Thereafter, the pots were covered with muslin material tied to the pots. A waiting period of 24 h was given for the larvae to settle on their host (Lawo *et al*., 2008).

Ten promising native *Bacillus thuringiensis* (10^6 spores/mL) isolates KDL, AUPOS, AUSD-1, AUGHS-1, ZDS, ZDS-3, AUASG-2, GHTS, GHTSW-1 and AUGHS-3 at concentrations of their LD_{90} values 1.54 mL,1.85 mL,1.76 mL,1.79 mL,1.98 mL,1.77 mL,1.63 mL,1.66 mL,1.76 mL and 1.89 mL, respectively with the reference *Bt*. var. *thuringiensis* at LD_{90} value of 1.66 mL were tested (Chandrashekar *et al*., 2015; WHO, 2005). *Bt* concentrations were first adjusted to 0.5 McFarland turbidity units (Mcfarland, 1907). Before spraying, the *Bt* suspension was mixed with 0.5 mL Triton x-100 (0.1%), 1 mL Tween 80 (2%), and one gram of milk powder. Similarly, an equal amount of distilled water was sprayed onto the control pots. The crop canopy in pots was completely covered with water (9 mL). A hand-held sprayer with a 500 mL capacity fitted with a cone nozzle was used for the application.

4.2.6. Data collection

4.2.6.1. Larval mortality

Larval mortality under the greenhouse experiment was assessed seven days after treatment applications. Percentage larval mortality per plant per pot was calculated for the three replicates and used for analysis of variance.

4.2.6.2. Total number of pods and percentage of pod damage

At maturity, all the pods were collected from the chickpea plant in each pot. The damaged and total numbers of pods were counted. Percentage pod damage per plant was calculated for the three replicates and used for analysis of variance.

4.2.6.3. Grain yield

Grain yield was determined at maturity from the collected and threshed chickpea pods per plant in each pot. The threshed pods were cleaned and dried to the recommended moisture content. The grain yield obtained from each pot was converted to tons per hectare using the area of the pot which was 0.07 m^2.

4.2.7. Field experiment

4.2.7.1. Experimental design and treatments

A randomised complete block design (RCBD) with three replications was used. The plot size was 2 m × 1.5, 1.50 m between plots, and 2 m between blocks. Chickpea seeds, Arerti (Kabuli type) were sown at the recommended seed rate and depth (Dadi *et al*., 2005). Each plot received a random set of treatments. Treatments of ten promising native isolates of *B. thuringiensis* that were screened and tested under *in vitro* conditions (Gemmeda *et al*., 2023) and the reference *Bt*. var. *thuringiensis* as standard check were administered at the LD_{90} values as previously verified under the greenhouse experiment. Treatment application coincided with the insect generation time.

The laid eggs were carefully examined and on average 0.40 eggs per plant were counted. The average number of *H. armigera* larvae per plant was recorded to be 1.02. Applications were made by comparing these values with *H. armigera* economic threshold level determined by Zahid *et al*.(2008). The sprayed volume per plot was 50 mL which was sufficient to cover the plot. The application was made using a hand-held sprayer of 500 mL capacity with a cone nozzle.

4.2.8. Data collection

4.2.8.1. Larval mortality

Larval mortality assessment was done pre and 24 h after treatment applications for 14 consecutive days in two day intervals from ten chickpea plants selected and tagged per plot. Data on each of the three replicates were averaged and the percentage of larval mortality was calculated. Thereafter, the means of the two locations were pooled for analysis.

4.2.8.2. Total number of pods and percentage of pod damage

All the pods were collected at maturity from ten randomly selected and tagged chickpea plants per plot. The damaged and total number of pods were counted and averaged. The Percentage of pod damage per plant was calculated as described in the greenhouse experiment. Thereafter, means from the two locations were pooled and used for analysis of variance.

4.2.8.3. Grain yield

Data on grain yield from field experiments were obtained by harvesting the whole chickpea plants per plot and dried under the sun. The dried pods were then threshed, cleaned and separately weighed for each replicate. Data from the two locations were pooled and averaged. Afterwards, the means were used to calculate grain yield in tons per hectare for analysis of variance.

4.2.9. Statistical analysis

Data for all the collected parameters were analysed using SAS statistical software packages, version 9.4 (SAS, 2013). Means were compared and separated using Tukey's Highest Significant Difference (HSD) test at alpha 0.05. All the data were checked for normality using a chi-square test at a 5% probability level. For the graphs on the percentage of live larvae that pupated, the percentage of adults that emerged and successful adults after emerged as well as the correlation analysis made between the mean percentage of pods damaged and larval mortality per plant and grain yield in tons per hectare were computed using Minitab Statistical Software Package, vr.17 (Minitab, 2000). Data on the percentage the pod damage were calculated (Lateef and Reed, 1983).

4.3. RESULTS

4.3.1. Effect of *Bt* applications on *H. armigera* larval mortality, percentage pod damage and grain yield under the greenhouse conditions

Significant ($p<0.05$) variation was observed between native *Bt* isolates and the reference *Bt*. var. *thuringiensis* BitoxybacillinTM treatments in terms of *H. armigera* larval mortality, chickpea pod damage and grain yield (Table 12). Larval mortality, chickpea pod damage and grain yield ranged from 66.7±8.7% to 90.5±16.5%, 8.1±2.5% to 36.6±9.7% and 1.0±0.1 t/ha to 2.3±0.5 t/ha, respectively. The highest larval mortality percentage 90.48±16.49%, 90.48±16.25%, 85.7±1.7%, and 90.5±8.2% were recorded from reference *Bt*. var. *thuringiensis,* native *Bt* isolates of KDL, AUPOS and AUSD-1, respectively followed by AUGHS-1, ZDS-3, GHTSW, GHTSW-1and AUASG-2. However, no significant ($p>0.05$) difference occurred between the tested *Bt* isolates. The lowest larval mortality was recorded from AUGHS-3.

There were statistically significant ($p<0.05$) differences among the *Bt* treatments in percentage pod damage (Table 12). The lowest percentage pod damage was recorded from KDL and ZDS (8.4±0.8%) and the reference *Bt*. var. *thuringiensis* (8.3±2.0%) compared to the control plots with pod damage of 36.6±9.7%. Whereas values of the rest native *Bt* isolates were intermediate and no significant difference was observed between them except the native *Bt* isolate of AUGHS-3 that showed 20.2±2.4% pod damage and it was significantly ($p<0.05$) different from the rest *Bt* isolates tested (Table 12). Grain yield in tons per hectare did not show significant ($p>0.05$) difference between the reference *Bt* and native *Bt* isolates tested (Table 12), but significantly ($p<0.05$) different from the control. Values (2.3±0.5 t/ha) obtained from reference *Bt*. var. *thuringiensis*, native *Bt* isolates of KDL (2.1±0.2 t/ha), AUPOS (1.9±0.5 t/ha) and AUSD-1 (2.0±0.2 t/ha) were relatively superior than the rest *Bt* isolates and they were significantly ($p<0.05$) different from the control plots that gave 0.9±0.1 t/ha grain yield (Table 12).

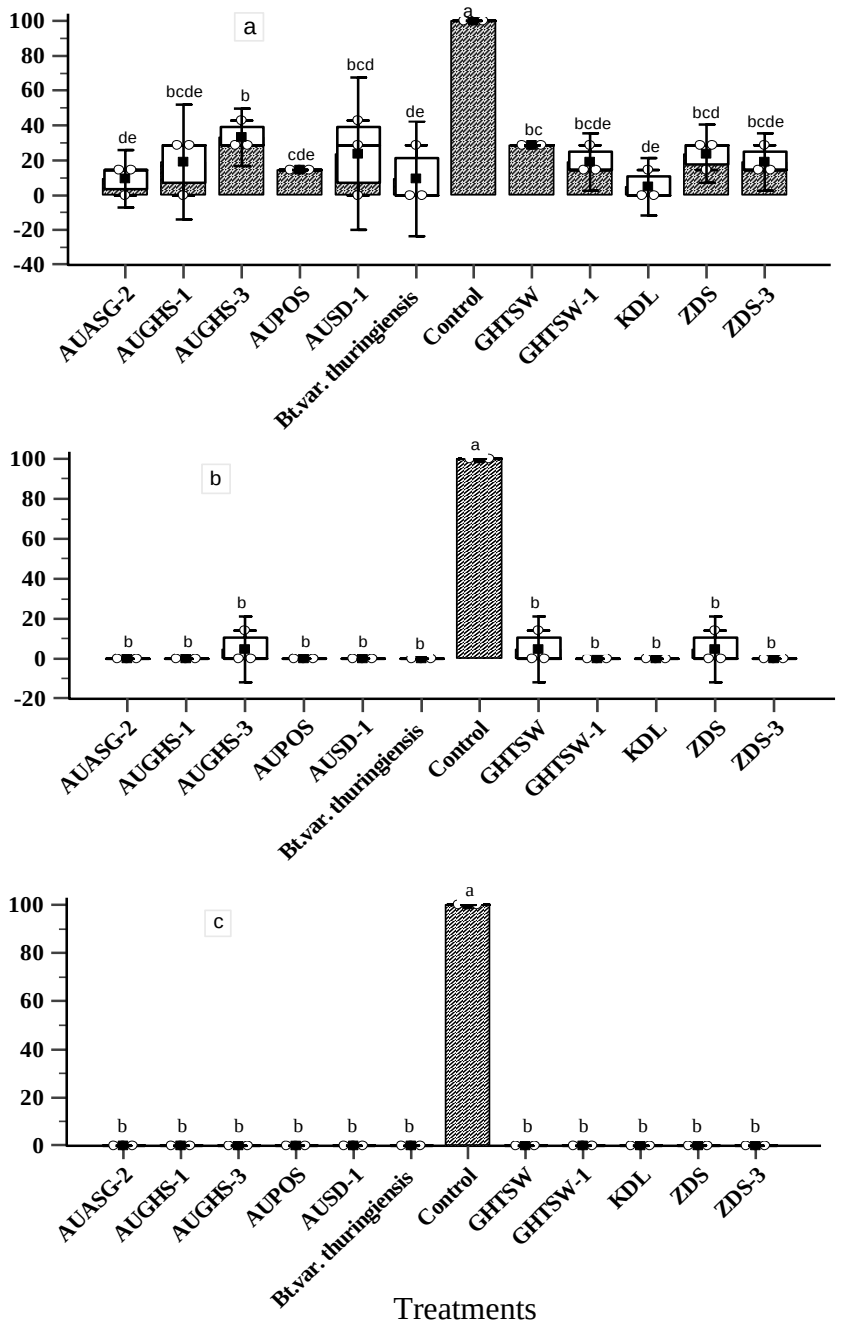
A significant ($p<0.05$) difference was observed between *Bt* isolates in the percentage live larvae pupated and adult emerged as well as successful adults after emergence (Fig. 13). All the live larvae (100%) in the control pots were successfully pupated. However, in the pots treated with *Bt*, the highest (33.3%) live pupae were recorded from the native *Bt* isolate of AUGHS-3 (Fig. 13a). On the other hand, the lowest pupae were recorded from native *Bt* isolates of ZDS-3 (9.5%), KDL (9.8%) and the reference *Bt*. var. *thuringiensis* (9.5%) (Fig.13a).

Percent (4.8%) adult emergence was recorded from native isolates of ZDS, GHTSW and AUGHS-3 (Fig.13b). All the emerged adults were desiccated compared to the successful control adults (Fig. 13c).

Table 12 Mean larval mortality percentage and mean percentage pod damage per plant per pot and grain yield (t/ha) in *Bt* sprayed under greenhouse at Kulumsa Agricultural Research Center in 2023

Treatments	Concentrations LD_{90} (mL/10g)	Parameters		
		[a]LM (%) plant^{-1}	[b]PD (%) plant^{-1}	[c]GY (t/ha)
KDL	1.54	90.48±16.3^{ab}	8.09±2.5^{c}	2.13±0.2^{a}
AUPOS	1.85	85.71±1.7^{abc}	9.74±2.8^{c}	1.92±0.5^{a}
AUSD-1	1.76	90.48±8.2^{ab}	8.42±0.8^{c}	1.97±0.2^{a}
AUGHS-1	1.79	80.95±16.5^{abc}	11.01±4.3^{c}	1.70±0.2^{ab}
ZDS	1.98	71.43±21.8^{cd}	12.28±4.5^{c}	1.56±0.4^{ab}
ZDS-3	1.77	80.95±8.2^{abc}	9.78±2.6^{c}	1.65±0.3^{ab}
AUASG-2	1.63	76.19±1.7^{bcd}	11.94±3.2^{c}	1.62±0.2^{ab}
GHTSW	1.66	80.95±0.0^{abc}	9.91±4.2^{c}	1.75±0.3^{ab}
GHTSW-1	1.76	76.19±8.2^{bcd}	11.01±5.8^{c}	1.65±0.2^{ab}
AUGHS-3	1.89	66.67±8.7^{d}	20.23±2.4^{b}	1.45±0.3^{ab}
Bt.var. *thuringiensis*	1.66	90.48±16.5^{ab}	8.29±2.0^{c}	2.31±0.5^{a}
control	-	0.00±0.0^{e}	36.59±9.7^{a}	0.91±0.1^{b}

Means with the same letters within the same columns were not significantly different. Data were average of three replicates ± SD, ten sampled plants per plot. Means were separated using Tuke's HSD test at Probability of 5%. [a]LM (%), Larval mortality (****Pr<0.0001*); [b]PD (%), percentage pod damage (****Pr<0.0001*); [c]GY (t/ha), Grain yield tons per hectare (****Pr<0.0001*).



Means with the same letters were not significantly different. Data were average of three replicates ± SD. Means were separated using Tuke's HSD test at 5% probability level. [a]Live larvae pupated (%) (****pr<0.0001*), [b]adult emerged (%) (****pr<0.0001*), [c]successful adult after emerged (%) (****pr<0.0001*).

Figure 13 (**a)** Mean percentage live larvae pupated, (**b)** Percentage adult emergence and, (**c)** Successful adult after emerged, counted in *Bt* sprayed under greenhouse at Kulumsa Agricultural Research Center in 2023.

4.3.2. Effect of *Bt* applications on *H. armigera* larval population under field conditions

The results of the pooled data analyzed showed that mean *H. armigera* larval populations counted per plant at pre and post 1st and 2nd as well as the control ranged from 1.6±0.5-2.3±0.7, 0.8±0.3-3.1±0.1, 0.6±0.1-3.6±0.0 and 0.5±0.1-3.6±0.4, respectively. Pre 1st *Bt* sprayed did not show a significant ($p>0.05$) difference between *Bt* isolates and the control (Table 13). However, at post 1st and pre 2nd *Bt* sprayed larval populations counted per plant did not show a statistically significant ($p>0.05$) difference among the tested *Bt* isolates, but there was significant ($p<0.05$) difference from the control plots. Moreover, at post 2nd *Bt* sprayed there was only a significant difference between native *Bt* isolates of KDL and AUGHS-3. Nevertheless, all the *Bt* isolates tested showed significant ($p<0.05$) difference compared to the control plots (Table 13). Comparatively, *Bt* treated plots with native *Bt* isolate KDL showed lower larval populations of 0.8±0.3, 0.6±0.1 and 0.5±0.1 at post 1st, pre 2nd and post 2nd , respectively. Whereas, the highest larval populations of 3.1±0.1, 3.6±0.0 and 3.6±0.4 were recorded from the control plots at post 1st, pre 2nd and post 2nd *Bt* sprayed, respectively (Table 13).

Table 13 Pooled data on mean total number *of H. armigera* larval population per plant counted pre and post 1st and 2nd *Bt* sprayed during main crop growing season in 2023

Treatments	concentrations $2*LD_{90}$(mL/10g)	1st sprayed		2nd sprayed	
		[a]Pre plant^{-1}	[b]Post plant^{-1}	[c]Pre plant^{-1}	[d]Post plant^{-1}
KDL	3.08	1.82±0.4^{a}	0.76±0.3^{b}	0.60±0.1^{b}	0.52±0.1^{c}
AUPOS	3.70	1.97±0.3^{a}	0.83±0.1^{b}	0.69±0.1^{b}	0.65±0.1^{bc}
AUSD-1	3.52	1.96±0.6^{a}	0.90±0.2^{b}	0.72±0.1^{b}	0.68±0.2^{bc}
AUGHS-1	3.58	1.63±0.2^{a}	1.05±0.2^{b}	0.71±0.1^{b}	0.74±0.0^{bc}
ZDS	3.96	1.57±0.5^{a}	0.90±0.2^{b}	0.77±0.1^{b}	0.77±0.1^{bc}
ZDS-3	3.54	2.02±0.6^{a}	1.15±0.1^{b}	0.71±0.2^{b}	0.76±0.1^{bc}
AUASG-2	3.26	2.02±0.5^{a}	1.10±0.1^{b}	0.71±0.1^{b}	0.71±0.2^{bc}
GHTSW	3.32	2.26±0.7^{a}	1.27±0.1^{b}	0.88±0.2^{b}	0.88±0.1^{bc}
GHTSW-1	3.52	1.87±0.5^{a}	1.08±0.3^{b}	0.74±0.2^{b}	0.78±0.2^{bc}
AUGHS-3	3.78	1.66±0.4^{a}	1.15±0.1^{b}	0.83±0.1^{b}	0.91±0.1^{b}
Bt. vr. *thuringiensis*	3.32	2.17±0.4^{a}	0.82±0.1^{b}	0.62±0.1^{b}	0.59±0.1^{bc}
control	-	1.96±0.1^{a}	3.10±0.1^{a}	3.62±0.0^{a}	3.61±0.4^{a}

Means with the same letters within the same columns were not significantly different. Data were average of three replicates ± SD, ten sampled plants per plot. Means were separated using Tuke's HSD test at Probability of 5%. [a]Pre, Pre 1st sprayed larval counted per plant (*Pr<0.529*); [b]Post, Post 1st sprayed larval counted per plant (****Pr<0.0001*); [c]Pre, pre 2nd sprayed larval counted per plant (****Pr<0.0001*); [d]Post, post 2nd sprayed larval counted per plant (****Pr<0.0001*).

4.3.3. Effect of *Bt* applications on percentage chickpea pod damage and grain yield under field conditions

The results of the pooled data analyzed showed that the total number of pods, percentage pod damage per plant and grain yield (t/ha) were found to be in the range of 81.3±17.2 to 111.6±10.1, 9.7±3.1% to 47.5±9.5% and 1.0±0.0 t/ha to 2.6±0.5 t/ha, respectively (Table 14). The total number of pods per plant did not show a significant ($p>0.05$) difference. Similarly, there was no significant ($p>0.05$) difference between *Bt* species in percentage pod damage. However, all the tested *Bt* isolates showed significant ($p<0.05$) differences in the percentage of pod damage compared to the control plots.

Grain yield in tons per hectare showed a significant ($p<0.05$) difference among *Bt* isolates and control plots. Thus, grain yield due to native *Bt* isolate AUGHS-3 was significantly ($p<0.05$) different from the reference *Bt. var. thuringiensis* and the native *Bt* isolate KDL, but there was no significant ($p>0.05$) difference from the control plots and the rest *Bt* isolates (Table 14). Both the reference *Bt*. var. *thuringiensis* (2.6±0.5 t/ha) and native *Bt* isolate KDL (2.5±0.3 t/ha) relatively showed higher grain yield followed by native *Bt* isolates of AUPOS, AUSD-1, AUGHS-1, ZDS ZDS-3, AUASG-2, GHTSW,GHTSW-1 and AUGHS-3 with their respective grain yield (t/ha) of 2.3±0.2, 2.0±0.2, 2.1±0.1, 1.9±0.3, 2.1±0.1, 2.1±0.3, 1.8±0.2 and 1.7±0.2, respectively compared to the control plots that gave 47.5±9.5% pod damage and 1.0±0.0 t/ha grain yield (Table 14).

Table 14 Effect of *Bt* applications on the mean total number of pods per plant, mean percentage pod damage per plant and grain yield (t/ha) during main crop growing season in 2023

Treatments	Concentrations $2*LD_{90}$ (mL/10g)	Parameters		
		[a]TNPD plant^{-1}	[b]PD (%) plant^{-1}	[c]GY (t/ha)
KDL	3.08	111.61±10.1^{a}	10.01±3.0^{b}	2.52±0.3^{a}
AUPOS	3.70	93.00±23.1^{a}	11.07±5.7^{b}	2.24±0.2^{ab}
AUSD-1	3.52	87.00±6.2^{a}	10.60±4.0^{b}	2.27±0.2^{ab}
AUGHS-1	3.58	81.92±6.5^{a}	13.77±6.9^{b}	2.00±0.2^{ab}
ZDS	3.96	83.50±8.3^{a}	12.83±5.0^{b}	2.11±0.2^{ab}
ZDS-3	3.54	87.37±13.1^{a}	14.11±6.8^{b}	1.88±0.3^{ab}
AUASG-2	3.26	104.83±6.8^{a}	11.76±4.1^{b}	2.11±0.1^{ab}
GHTSW	3.32	81.29±17.2^{a}	11.45±1.0^{b}	2.06±0.3^{ab}
GHTSW-1	3.52	81.29±17.2^{a}	13.06±3.0^{b}	1.82±0.2^{ab}
AUGHS-3	3.78	83.87±5.2^{a}	13.58±5.5^{b}	1.75±0.2^{bc}
Bt. var. *thuringiensis*	3.32	91.71±18.8^{a}	9.67±3.1^{b}	2.61±0.5^{a}
Control	-	99.04±22.1^{a}	47.53±9.5^{a}	0.97±0.0^{c}

Means with the same letters within the same columns were not significantly different. Data were average of three replicates ± SD, ten sampled plants per plot. Means were separated using Tuke's HSD test at Probability of 5%. [a]TNPD, Total number of pods per plant (*Pr<0.1720*); [b]PD (%), percentage pod damage per plant (****Pr<0.0001*); [c]GY (t/ha) Grain yield (****Pr<0.0001*).

4.3.4. Relationship among *H. armigera* larval mortality, percentage pod damage and grain yield

The results of correlation analysis in the greenhouse showed that percentage *H. armigera* larval mortality per plant had strong negative relationship (r= -0.92; P<0.001) with percentage chickpea pod damage per plant (Fig. 14a). Similarly, grain yield (t/ha) was inversely related (r= -0.86; P<0.001) to percentage chickpea pod damage per plant (Fig. 14b). Under field conditions, the pooled data from the two locations showed strong negative relationship (r= -0.76; P<0.001) between percentage pod damage per plant and grain yield (t/ha; Fig. 15).

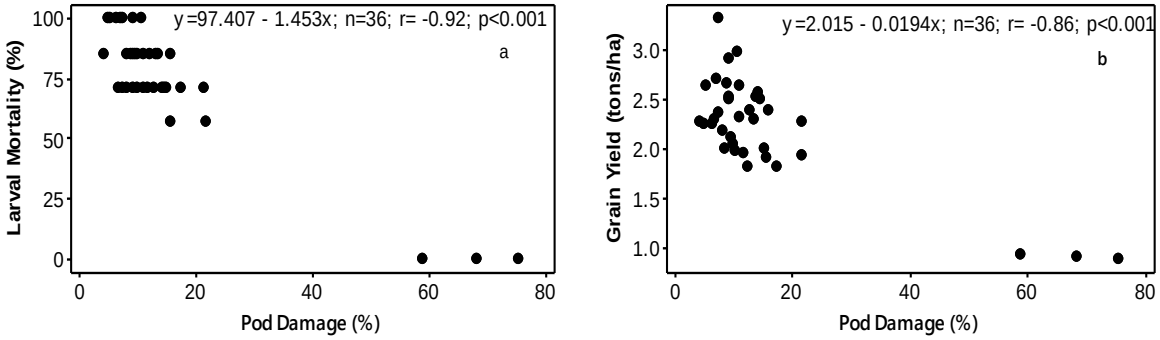


Figure 14 (**a**) The relationship of larval mortality (%) with pod damage (%), (**b**) Pod damage (%) with grain yield (t/ha), as affected by *Bt* applications under greenhouse at Kulumsa Agricultural Research Center in 2023

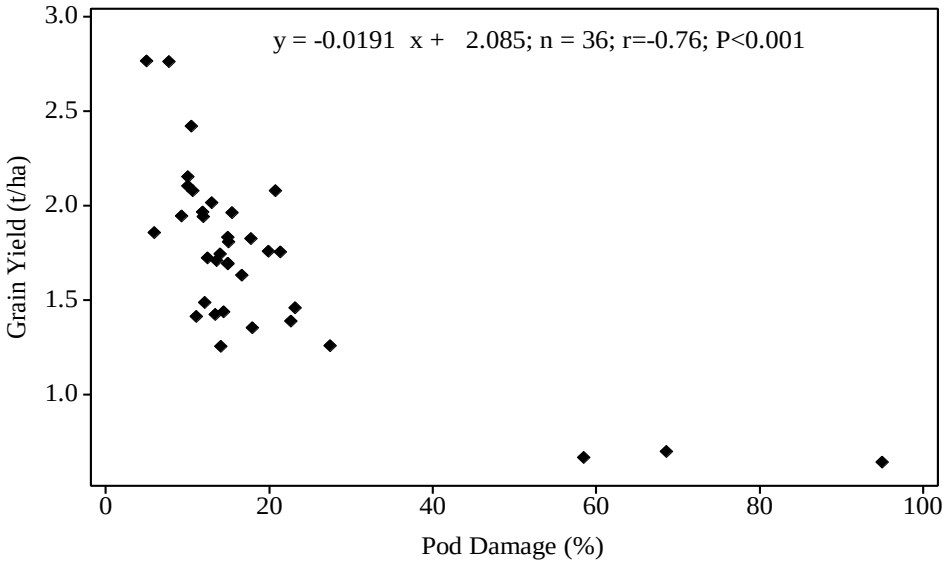


Figure 15 Correlation analysis of means of pooled data of pod damage (%) and chickpea grain yield (t/ha) from two locations as affected by *Bt* sprayed.

4.4. DISCUSSION

All the native *Bt* isolates achieved more than 50% larval mortality under the greenhouse. Values obtained from the native *Bt* isolates KDL, AUPOS and AUSD-1 on percentage larval mortality were moderately higher than the rest of native *Bt* isolates but comparable to the reference *Bt*. var. *thuringiensis*, followed by native *Bt* isolates of AUGHS-1, ZDS-3 and GHTSW, GHTSW-1 and AUASG-2. Of all the native *Bt* isolates tested, ZDS and AUGHS-3 demonstrated lower larval mortality. However, Lawo *et al*. (2008) have demonstrated 86.4% larval mortality when susceptible *H. armigera* was exposed to *cry*2A under a greenhouse. Similarly, native *Bt* isolates KDL, AUPOS and AUSD-1 relatively showed a lower percentage of pod damage with higher grain yield (t/ha) compared to the control plot that resulted in the highest percentage of pod damage and the lowest grain yield (t/ha).

On the other hand, the values of the rest native *Bt* isolates were intermediate. Only native *Bt* isolate AUGHS-3 exhibited a higher percentage of pod damage but lower grain yield (t/ha). The highest pupated live larvae were counted from the control, but the lowest were from the native *Bt* isolates of KDL, AUASG-2 and the reference *Bt*. From native *Bt* isolates of AUGHS-3, GHTSW and ZDS, adult insects emerged but they were unable to survive after emergence compared to the control plots. In contrast to this study, Tarekegn *et al*. (2019) have conducted a study using a commercial *Bt* product, *Bt*. var. *kurstaki* (WP) at two concentrations (0.025, 0.5 g/200 mL) against *H. armigera* under greenhouse and found that the percentage of healthy adults emerged was higher than the deformed ones at both concentrations. The overall implication is that the native *Bt* isolates have exhibited a promising potential in the management of this deleterious insect pest of chickpea.

Under field trial, application of all the native *Bt* isolates and the reference *Bt*. var. *thuringiensis* reduced larval population per plant at pre, post 1st and 2nd per plots. Among, the native *Bt* isolates KDL, AUPOS and AUSD-1 and the reference *Bt* relatively showed a lower larval population per plant. Similarly, their values on percentage pod damage were comparatively lower than the rest of the *Bt* isolates and resulted in higher grain yield (t/ha) as previously reported by Kumar *et al*. (2016) using the liquid formulation of *Bt* strain PDBC *Bt*1 and commercial *Bt* k (Halt 5% WP) against *H. armigera* of pigeon pea. The authors have demonstrated that a lower larval population per plant on the 3rd and 7th days after 1st and 2nd rounds of spray gave a lower percentage pod damage per plant and higher grain yield (t/ha).

Ahmed *et al*. (2015) studied bio-pesticide DiPel 2x alone and with mixtures of milk powder, molasses and K_2CO_3 to control chickpea pod borer that showed significantly lower pod damage and higher grain yield with and without the mixtures. Unlike the current study, Singh and Dhkal (2019) have investigated *H. armigera* of chickpea using the commercial *Bt* product *Bt*. var. *kurstaki* (WP) (DOR *Bt*-1) that reduced percentage pod damage with increased chickpea grain yield. Larval mortality percentage had an inverse relationship with the percentage of pod damage per plant. Correspondingly, the percentage of pod damage was inversely related to grain yield (t/ha) in the greenhouse. Furthermore, at Kulumsa field conditions, the percentage of pod damage per plant and grain yield (t/ha) had strong negative relationship. Nevertheless, the relationship was intermediate at Gonde. This result is in agreement with the findings of Aderajew *et al*. (2020) who reported the percentage of pod damage of 63.6% with a significant reduction in grain yield of chickpea due to *H. armigera*. Generally, our findings indicate the possibility of using bioinsecticides in the management of chickpea pod borer with extensive exploration of Phyto-beneficial soil bacteria in different agro-ecologies of Ethiopia and beyond.

4.5. CONCLUSION

Although the values among native *Bt* isolates varied, all of the tested isolates effectively reduced the number of larvae per plant, and the percentage of pod damage, but increased the grain yield compared to the control, and the tested isolates exhibited similar results with the reference *Bt*. var. *thuringiensis*. Therefore, this study strongly confirms the existence of potent native *Bt* isolates that are effective against *H. armigera*. Thus, the continuation of *Bt* research in Ethiopia with a focus on widespread collection, screening and testing under multiple field trials should be emphasised.

CHAPTER FIVE

Field evaluation of Bt species on H. armigera at Kulumssa Agricultural Research Center and Gonde Basic Seed Farm in 2023

CHAPTER FIVE

Paper IV: Evaluation of Native *Bacillus thuringiensis* (Berliner) isolates against chickpea pod borer, *Helicoverpa armigera* (Hübner) under field conditions

ABSTRACT

Chickpea pod borer, *Helicoverpa armigera* remains disastrous to chickpea production. *Bacillus thuringiensis (Bt)* is a widely used bioinsecticide to control insect pests of food crops. This study aimed at to explore native *Bt* isolates harbouring *cry* genes pathogenic to *H. armigera*. Ten native *Bt* isolates were analyzed for their *cry* genes. Therefore, all the native *Bt* isolates were harbouring two or more *cry* genes. Statistically significant ($p<0.05$) variations were observed among *Bt* species in larval population and pod damage (%) reduction and higher grain yield (t/ha). Three potential native *Bt* isolates were identified with their respective *cry* genes they harboured, namely KDL (*cry*2 + *cry*4), AUGHS-1 (*cry*1 + *cry*4), and AUSD-1 (*cry*1 + *cry*2 + *cry*4 + *cry*7, 8 + *cry*9). Hence, we recommend that the development of commercial bioinsecticide and other *Bt* technologies using *B. thuringiensis* from Ethiopian sources will be a future new research avenue to be addressed.

Keywords: Bioinsecticide; *Bt*; chickpea pod borer**;** *cry* gens; crystal proteins; larval mortality; pod damage

5.1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is rich in protein, dietary fibre and plays a significant role in human diet as a staple food legume (Charlie, 2016; Redden *et al*., 2007). It also improves soil fertility by hosting some nitrogen fixing soil bacteria (Saxena and Singh, 1987). Nevertheless, its production is highly deteriorated due to insect pests. Chickpea pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is among the most economically important insect pest attacking chickpea worldwide with significant yield losses that exceed $2 billion (Pedgley, 1985; Sharma *et al*., 2005; Sharma *et al*., 2006). In the semiarid tropics, the extent of losses has been estimated to be 328 million dollars (ICRISAT, 1992). A study made by El Fakhouri *et al*. (2022) in Morocco indicated that yield loss inflicted by *H. armigera* ranged between 14.3% and 31.2%. As evidenced somewhere else, a single larva can damage up to 40 chickpea pods in its larval period. In early sown chickpea, 80% of pod damage was reported in Ethiopia (ICRISAT, 1991). In the central highlands of Ethiopia, pod damage can range between 21%-36% with 20%-30% annual yield loss (Gelatu and Million, 1996).

The economic injury level and economic threshold level of 2.35 and 1.76 larvae per meter row for *H. armigera* in chickpea were found by Singh *et al*., (2021). Zahid *et al*., (2008) calculating the economic threshold level from the economic injury level and discovered that there were 0.90 and 0.73 larvae per meter row for two consecutive years. According to Hadiya *et al*., (2023) the economic threshold level for *H. armigera* was 0.60 larvae per plant, while the economic injury level was 0.67 larvae per plant. Chemical insecticides alone were used for decades for the control of *H. armigera*. However, human and environmental health associated with chemical insecticides becomes disastrous added to other problems like cost and appearance of chemical resistant insect pests (Nicolopoulou-Stamati *et al*., 2016; Pathak *et al*., 2022; Rani *et al*., 2020).

There is also a growing interest for residues free produce by consumers in the developed countries. Consequently, searching alternatives to chemical insecticides such as Lambda-cyhalothrin, Karate, confidence and Golden used for *H. armigera* management was elucidated (Patil *et al*., 2017). Hence, the potential benefits of the soil bacterium *Bacillus thuringiensis (Bt)* to control insect pests of agricultural crops and public health should be harnessed (Mohammad *et al*., 2010; Patil *et al*., 2017).

Bacillus thuringiensis is a gram-positive, soil born bacterium that produces parasporal crystalline inclusions during sporulation that harbours insecticidal crystal proteins and *ß*-endotoxin with toxicity to many Lepidopteran larvae (Kumar *et al*., 2016; Li and Bouwer, 2012; Majeed *et al*., 2018). Thus, it has been used to control *H. armigera* in chickpea worldwide (Estela *et al*., 2004; Qayyum *et al*., 2015).

Few studies in Ethiopia reported the existence and potential of native *Bt* isolates against insect pests control (Aynalem *et al*., 2021; Tesfaye and Emiru, 2010; Tesfaye *et al*., 2012). Despite the substantial resources available, the study conducted became very rare and not well established, particularly with regards to *H. armigera*. Because of the abundance of resources and biodiversity, there have been many opportunities that have not yet been fully explored and utilized. Therefore, the hypothesis of this study outlined that there are virulent and effective native *Bt* isolates that may serve as alternative to chemical insecticides to control this noxious pest of chickpea pod borer, *H. armigera* on chickpea. Thus, in this study the effectiveness of ten native *Bacillus thuringiensis* isolates were tested against *H. armigera* 3rd instar larvae under field conditions.

5.2. MATERIALS AND METHODS

5.2.1. Descriptions of the study area

Experiments were conducted under field conditions at two locations, Kulumsa Agricultural Research Centre, Ethiopian Institute of Agricultural Research Center (EIAR) and the Gonde Basic Seed Farm Center, Ethiopian Agricultural Business Cooperation (EABC). Both are situated in Arsi Administrative Zone of Oromia Regional State. The location of Gonde Basic Seed Farm Center is at $8^0$06'25"N and $39^0$07'38"E with an altitude of 2268 meter above sea level (m.a.s.l). The area receives 800 mm mean annual rainfall with maximum and minimum temperatures of 23 ˚C and 10 ˚C, respectively. Kulumsa Agricultural Research Center is located at $8^0$01'88"N and $39^0$07'10"E and elevation of 2210 m.a.s.l. The area is wet with an average annual rain fall of 809 mm and maximum and minimum temperatures 23.08 ˚C and 9.9 ˚C, respectively (Abayneh *et al*., 2003).

5.2.2. Sources of *Bt* isolates and production of spore-crystal mixtures

Native *Bt* isolates were previously isolated from soil samples and cadavers. The isolated and purified *Bt* isolates were maintained at appropriate chilling temperature in 20% glycerol for subsequent activities. The virulence of the *Bt* isolates was tested against *H. armigera* under laboratory conditions. Consequently, ten potent native *Bt* isolates were selected and screened based on larval mortality they achieved (Gemmeda *et al*. 2023). To further confirm their efficacy under field conditions laboratory scale production of spore-crystal mixtures were carried out at Holeta microbial biotechnology laboratory following liquid fermentation procedures (Dulmage, 1971). From the stored culture, a loopful of the culture was taken and grew on Modified Broth Solution (MBS) containing 6.8 g KH_2PO_4, 0.3 g $MgSO_4.7H_2O$, 0.2 g $CaCl_2.2H_2O$, 10 g Bactotryptose, 2 g yeast extract, 10 mL of stock solutions prepared from (2 g $MnSO_4.H_2O$, 2 g $Fe_2(SO_4)_3$, 2 g $ZnSO_4.7H_2O$ and 1liter of distilled water), 17 g of nutrient agar and 1liter of distilled water. The solution was sterilized at 120 ºC for 15 min and the PH was adjusted to 7.2 and incubated at 30 °C for 24 h to induce sporulation. A single pure colony was taken and inoculated in to 100 µL liquid nutrient broth medium in Eppendorf tube and then incubated at 30 °C for 24h. From the sporulated *Bt* culture 100 µL was taken and transferred to Erlenmeyer flasks having a capacity of 250 mL filled with 150mL nutrient broth medium and fermentation was carried out for 72 h at room temperature using orbital shaker at 180 rpm (Ahmed *et al*., 2015).

The suspensions were centrifuged at 10,000 rpm for 3 min at 4 °C in a sterile test tube. Pallets were washed twice in 20 mL of sterile distilled water and centrifuged as above. The supernatant was discarded and the spore-crystal mixtures were collected and re-suspended in 20 mL of sterile distilled water and stored in freeze at -20 °C until use in a glycerol (Andrews, 2001).

5.2.3. Standardization of *Bacillus* species

McFarland standard was used as a reference to adjust the *Bacillus* species cell density in a suspension so that the number of bacteria was within a given range, where a 0.5 McFarland standard corresponds to 1.5 x 10^8 cfu/ml. *Bacillus* species suspension was adjusted to achieve a turbidity that is equivalent to a 0.5 McFarland standard. Hence, the inoculated tube was visually compared with the 0.5 McFarland standard against a card with white background and contrasting black lines (Mcfarland, 1907).

5.2.4. Reference *B*t strain

The commercially accessible form of *Bt* also known as *Bacillus thuringiensis* was produced by Sibbiopharm ltd. in the Russian Federation under the trade name BitoxybacillinTM and the Gallica Flower Farm in Menegasha, Ethiopia, provided it. The preparation of commercial *Bt* included proteinaceous crystals (delta-endotoxin), thermo-stable b-exotoxin of *Bt* culture, and bacterial spores of (10^6 spores/mL), which are employed to control numerous Lepidopteran larvae and red spider mite. BitoxybacillinTM is pale beige to brown powder with a biological activity of 1500 UA/mg and an exotoxin content of 0.6-0.8% (Teferra and Dubale, 2020; Adilkhankyzy *et al*., 2022).

5.2.5. Chickpea variety

The cultivar utilized was Arerti (kabuli type), with creamy seed color, released by Debre Zeit Agricultural Research Center in 1999/2000 and received from Gonde Basic Seed Farm Centre. The variety was released for an altitude range of 1800-2600 m.a.s.l and initial planting date is mid-August at 30 cm and 20 cm between rows and plants, respectively. The optimum seed rate per hectare was between 100 and 115 k. The grain yield per hectare in the research field and the farmer's field was between 1.6 and 5.2 tons and 1.6 and 3.2 tons, respectively (Dadi *et al*., 2005).

5.2.6. Experimental Design and procedures

Field experiments were carried out between July 2022 and January 2023 at Kulumsa Agricultural Research Center and Gonde Basic Seed Farm Center. The studies were set up using a randomized complete block design (RCBD) with three replications. The plot size used was 2 m x 1.5 m, 1.50 m between plots, and 2 m between blocks. Chickpea seeds were sown in rows at a depth of 5-10 cm with the spacing of 20 cm between plants and 30cm between rows. Each plot received a random set of treatments. All the plots received the same for weeds and other management practices.

5.2.7. Detailed Treatments

Table 15 Treatments of ten promising native *Bt* isolates with reference *Bt*. var. t*huringiensis*, their respective LD_{90} values and total sprayed volume tested under field trails, in 2023

Treatments		Total sprayed volume and mixtures		
Bt isolates	Concentrations (LD_{90} ml/10g)	2*LD_{90} (ml/ 10g) (*Bt*)	*Bt*+0.5ml 0.1%Triton x-100 +1ml 2% Tween 80	Total sprayed volume (ml)
Bt.var. *thuringiensis*	1.66	3.32	4.82	50
KDL	1.54	3.08	4.58	50
AUPOS	1.85	3.70	5.20	50
AUSD-1	1.76	3.52	5.02	50
AUGHS-1	1.79	3.58	5.08	50
ZDS	1.98	3.96	5.46	50
ZDS-3	1.77	3.54	5.04	50
AUASG-2	1.63	3.26	4.76	50
GHTSW	1.66	3.32	4.82	50
GHTSW-1	1.76	3.52	5.02	50
AUGHS-3	1.89	3.78	5.28	50
Control	-	-	1.50	50

LD_{90}, lethal dose millilitre per 10 gram diet, *Bt*, represented 2*LD_{90} (mL/10g) tested

5.2.8. Treatment applications

Treatments (Table 15) of ten promising native isolates of *B. thuringiensis* that were collected, isolated, characterized and screened under *in vitro* were used as treatments (Gemmeda *et al*., 2023). The reference *Bt*. var. *thuringiensis* was used as standard check. Control plots were sprayed with distilled water.

Treatments were applied with the model of concentrations of twice of the LD_{90} values (WHO, 2005; Chandrashekar *et al*., 2015). Before being applied, the *Bt* formulations were adjusted to 0.5 McFarland turbidity units, approximately contained $1.5x10^8$ cfu/mL (Mcfarland, 1907). Before application *Bt* formulations were combined with 0.5mL Triton x-100 (0.1%), 1 mL Tween 80 (2%) and one gram of milk powder.Treatment application was coincided with the insect generation time. The laid eggs were carefully examined and recorded 0.40 eggs per plant and the number of *H. armigera* larvae per plant recorded was reached 1.02. Applications were made by comparing the current values with *H. armigera* economic threshold level determined by Hadiya *et al*., (2023); Singh *et al*., (2021); Zahid *et al*., (2008). The sprayed volume per plot was determined by calibrating in situ, and thus 50 mL sprayed volume was determined and it was sufficient to fully cover the plant canopy. Application was made using a hand-held sprayer of 500mL capacity with cone nozzle.

5.2.9. Data collected

5.2.9.1. Larval mortality counted

Larval mortality assessment was done pre and post after 24 h of 1st and 2nd *Bt* sprayed consequently in two days interval from ten randomly selected and tagged chickpea plants. Data of the ten plants per replicates were averaged and means for percentage larval mortality were calculated per plant and used for analysis of variance.

5.2.9.2. Total number of pods and percentage pod damage

All the pods were collected at maturity from ten randomly selected and tagged chickpea plants per plot. The damaged and total number of pods were counted and averaged. Percentage pod damage per plant was calculated using the formula described by (Lateef and Reed, (1983) . Hereafter, means were used for analysis of variance.

$$\text{Percentage pod damage/plant} = \frac{\text{Average number of damaged pods}}{\text{Average total number of pods}} \text{ X } 100$$

5.2.9.3. Grain yield:

Data on grain yield from field experiments were obtained by harvesting the whole chickpea plant per plot and dried under sun. The dried pods were then threshed, cleaned and weighed separately for each replicate. Afterwards, the means were used to calculate grain yield tons per hectare and used for analysis of variance.

5.2.10. Data analysis

Analysis of the data on mean percentage larval mortality, average percentage pod damage per plant and grain yield tons per hectare were performed using SAS statistical software packages, version 9.4 (SAS, 2013). Means were compared and separated using Tukey's Highest Significant Difference (HSD) test at alpha 0.05. All the data were checked for normality using chi-square test at 5% probability level. For the graphs on correlation analysis made between the mean percentage pods damaged and larval mortality per plant, grain yield in tons per hectare and percentage pod damage per plant were performed using Minitab Statistical Software Package, vr.17 (Minitab, 2000).

5.3. RESULTS

5.3.1. Effect of *Bt* applications on *H. armigera* larval population

Results of field experiment revealed that larval population counted per plant pre 1st *Bt* sprayed both at Gonde and Kulumsa not significantly ($p>0.05$) (Table 16, 17) different. However, post 1st, pre 2nd and post 2nd *Bt* sprayed larval population counted statistically showed significant ($p<0.05$) difference among the tested *Bt* isolates and the control plots at both locations (Table 16, 17). At Gonde significant ($p<0.05$) variations were observed in larval population counted per plant between the reference *Bt*.var. *thuringiensis*, native *Bt* isolates tested and the control as well as within native *Bt* isolates themselves during post 1st *Bt* sprayed (Table 16). The lowest number of larvae per plant 0.97±0.17 and 0.97±0.20 were recorded from native *Bt* isolates of AUSD-1 and AUGHS-1 and the highest 3.50±0.23 was obtained from control plot. Similarly, they significantly ($p<0.05$) differed from the reference *Bt* .var. *thuringiensis* which resulted in 1.70±0.60 larval per plant.

Within the native *Bt* isolates, AUSD-1 and AUGHS-1 also significantly ($p<0.05$) differed from AUPOS, GHTSW and GHTSW-1 where their corresponding larval population of 1.50±0.25, 1.75±0.10 and 1.60±0.00 per plant, respectively. Pre and post 2nd *Bt* sprayed larval population counted per plant showed significant ($p<0.05$) difference only between *Bt* species tested and control plots. The highest larval population of 3.91±0.31 and 2.82±0.14 per plant were recorded from control plots both pre and post 2nd *Bt* sprayed, separately. The lowest 0.87±0.25 and 0.84±0.32 larval population per plant obtained from native *Bt* isolate of AUGHS-1 pre and post *Bt* sprayed (Table 16, 17). Larval population counted per plant post 1st, pre 2nd and post 2nd *Bt* sprayed at Kulumsa indicated that significant ($p<0.05$) difference was observed between *Bt* species and control plots, where the highest 3.50±0.23, 3.91±0.31 and 2.82±0.14 recorded from control and the lowest 0.99±0.17 and 1.00±0.21 both post 1st and pre 2nd *Bt* sprayed obtained from native *Bt* isolate of AUGHS-1, whereas, the lowest 0.73±0.17 post 2nd *Bt* sprayed was from native *Bt* isolate of AUSD-1.

Table 16 Effect of *Bt* applications on *H. armigera* larval population per plant at Gonde Basic Seed Farm Center, EABC during main crop growing season in 2023

Treatments	concentrations 2*LD_{90}(mL/10g)	1st spray		2nd spray	
		[a]PrSC plant^{-1}	[b]PoSC plant^{-1}	[c]PrSC plant^{-1}	[d]PoSC plant^{-1}
Bt. vr. *thuringiensis*	3.32	2.40±1.00^{a}	1.70±0.60^{bc}	0.97±0.76^{b}	0.95±0.19^{b}
KDL	3.08	1.43±0.75^{a}	1.40±0.26^{bcd}	0.95±0.24^{b}	0.95±0.15^{b}
AUPOS	3.70	2.05±0.65^{a}	1.50±0.25^{bc}	1.13±0.05^{b}	0.92±0.27^{b}
AUSD-1	3.52	1.86±1.38^{a}	0.97±0.17^{d}	0.95±0.20^{b}	0.89±0.09^{b}
AUGHS-1	3.58	1.03±0.58^{a}	0.97±0.20^{d}	0.87±0.25^{b}	0.84±0.32^{b}
ZDS	3.96	1.40±0.98^{a}	1.20±0.06^{bcd}	0.90±0.32^{b}	0.85±0.03^{b}
ZDS-3	3.54	2.10±0.80^{a}	1.10±0.15^{bcd}	1.05±0.17^{b}	0.87±0.29^{b}
AUASG-2	3.26	1.93±1.24^{a}	1.03±0.30^{bcd}	0.99±0.28^{b}	0.86±0.25^{b}
GHTSW	3.32	1.95±0.65^{a}	1.75±0.10^{b}	1.17±0.18^{b}	1.17±0.09^{b}
GHTSW-1	3.52	1.75±0.65^{a}	1.60±0.00^{bc}	1.08±0.16^{b}	0.91±0.01^{b}
AUGHS-3	3.78	1.95±1.00^{a}	1.40±0.10^{bcd}	1.04±0.24^{b}	1.00±0.03^{b}
control	-	1.50±1.38^{a}	3.50±0.23^{a}	3.91±0.31^{a}	2.82±0.14^{a}

Means with the same letters within the same columns were not significantly different. Data were means ± SD, means were separated using Tukey's highest Significant Difference (HSD) test. [a]PrSC, pre first sprayed larval counted (*df=11,22, f-value=1.82, pr<0.1107*); [b]PoSC, post first sprayed counted, (*df=11,22, f-value=14.33, pr<0.0001*); [c]pre second sprayed larval counted (*df=11, 22, f-value=21.90, Pr<0.0001*); [d]PoSc, post second sprayed larval counted (*df=11, 22, f-value=35.31, Pr<0.0001*).

Table 17 Effect of *Bt* applications on *H. armigera* larval population per plant at Kulumsa Agricultural Research Center, EIAR, during main crop growing season, in 2023

Treatments	concentrations 2*LD_{90} (mL/10g)	1st spray		2nd spray	
		[a]PrSC plant^{-1}	[b]PoSC plant^{-1}	[c]PrSC plant^{-1}	[d]PoSC plant^{-1}
Bt. var. *thuringiensis*	3.32	1.75±0.06^{a}	1.32±0.14^{b}	1.48±0.70^{b}	0.73±0.50^{b}
KDL	3.08	1.20±0.10^{a}	1.16±0.21^{b}	1.28±0.07^{b}	0.76±0.20^{b}
AUPOS	3.70	1.63±0.40^{a}	1.21±0.08^{b}	1.35±0.22^{b}	0.80±0.55^{b}
AUSD-1	3.52	1.37±0.15^{a}	1.05±0.17^{b}	1.08±0.08^{b}	0.73±0.17^{b}
AUGHS-1	3.58	1.83±0.46^{a}	0.99±0.17^{b}	1.00±0.21^{b}	0.76±0.40^{b}
ZDS	3.96	1.83±0.20^{a}	1.07±0.16^{b}	1.21±0.08^{b}	0.80±0.15^{b}
ZDS-3	3.54	1.47±0.80^{a}	1.13±0.36^{b}	1.22±0.21^{b}	0.80±0.10^{b}
AUASG-2	3.26	1.73±0.26^{a}	1.15±0.17^{b}	1.22±0.11^{b}	0.83±0.23^{b}
GHTSW	3.32	1.77±0.15^{a}	1.45±0.36^{b}	1.59±0.02^{b}	0.80±0.15^{b}
GHTSW-1	3.52	1.87±0.10^{a}	1.19±0.31^{b}	1.33±0.05^{b}	0.80±0.10^{b}
AUGHS-3	3.78	1.77±0.15^{a}	1.14±0.16^{b}	1.25±0.04^{b}	0.83±0.00^{b}
control	-	1.62±0.42^{a}	2.66±0.12^{a}	2.69±0.20^{a}	2.70±0.40^{a}

Means with the same letters within the same columns were not significantly different. Data were means ± SD, means were separated using Tukey's highest Significant Difference (HSD) test. [a]PrSC, pre first sprayed larval counted (*df=11,22, f-value=1.56, pr<0.1794*); [b]PoSC, post first sprayed counted, (*df=11,22, f-value=15.46, pr<0.0001*); [c]PrSC, pre second sprayed larval counted (*df=11,22, f-value=11.49, pr<0.0001*); [d]PoSC, post second sprayed larval counted (*df=11, 22, f-value=14.33 Pr<0.0001*).

5.3.2. Effect of *Bt* applications on percentage pod damage and grain yield

Total number of pods per plant did not show significant ($p>0.05$) difference among *Bt* isolates tested and the control at both locations (Table 18, 19). However, there was significant ($p<0.05$) variations in percentage pod damage and grain yield in tons per hectare between *Bt* tested and control plots. Percentage pod damage ranged from 10.42±6.63 to 49.35±15.02 and 8.63±2.57 to 52.25±9.72, similarly, grain yield tons per hectare found in between 0.75±0.16 and 1.95±0.48, 1.07±0.19 and 2.42±0.23 both at Gonde and Kulumsa, respectively. The lowest 10.42±6.63 and 8.63±2.57 percentage pod damage at both locations and the highest grain yield tons per hectare 1.95±0.48 at Gonde obtained from native *Bt* isolate AUGHS-1, but the highest grain yield was recorded from KDL at Kulumsa compared to control plots resulted in the highest 49.35±15.02 and 52.25±9.72 percentage pod damage and the lowest 0.75±0.16 and 1.07±0.19 grain yield in tons per hectare (Table 18, 19). But no significant ($p>0.05$) difference was observed among native *Bt* isolates and the reference *Bt*. Var. *thuringiensis* in percentage pod damage at both locations (Table 18, 19).

Table 18 Effect of *Bt* applications on total number of pods and percentage pod damage per plant and grain yield (t/ha) at Gonde Basic Seed Farm Center, during main crop growing season in 2023

	Concentrations	Parameters		
Treatments	2*LD_{90}(mL/10g)	[a]TPDS plant^{-1}	[b]PD (%) plant^{-1}	[c]GY (t/ha)
Bt. var. *thuringiensis*	3.32	96.50±12.58^{a}	10.58±3.48^{b}	1.89±0.05^{ab}
KDL	3.08	111.0±27.60^{a}	10.99±5.09^{b}	1.95±0.27^{ab}
AUPOS	3.70	73.33±37.20^{a}	11.24±3.90^{b}	1.75±0.23^{ab}
AUSD-1	3.52	94.67±33.30^{a}	10.57±1.89^{b}	1.89±0.52^{ab}
AUGHS-1	3.58	76.83±17.60^{a}	10.42±6.63^{b}	1.95±0.48^{ab}
ZDS	3.96	76.00±25.00^{a}	11.52±3.26^{b}	1.58±0.31^{ab}
ZDS-3	3.54	67.83±32.90^{a}	11.35±3.36^{b}	1.73±0.15^{ab}
AUASG-2	3.26	85.33±21.10^{a}	14.70±4.09^{b}	1.78±0.16^{ab}
GHTSW	3.32	103.67±24.0^{a}	13.91±4.63^{b}	1.50±0.15^{ab}
GHTSW-1	3.52	77.17±28.50^{a}	13.32±3.35^{b}	1.68±0.16^{ab}
AUGHS-3	3.78	77.83±56.70^{a}	17.81±9.38^{b}	1.33±0.77^{b}
Control	-	104.25±15.75^{a}	49.35±15.02^{a}	0.75±0.16^{c}

Means with the same letters within the same columns were not significantly different. Data were means ± SD, means were separated using Tukey's highest Significant Difference (HSD) test. [a]TPDS, total number of pods per plant (*df=11,22, f-value=0.62, pr<0.7898*); [b]PD, percentage pod damage per plant, (*df=11,22, f-value=3.30, pr<0.0082*); [c]GY, grain yield tons per hectare, (*df=11,22, f-value=2.13, pr<0.0063*).

Table 19 Effect of *Bt* applications on total number of pods and percentage pod damage and grain yield (t/ha) at Kulumsa Agricultural Research Center during main crop growing season in 2023

Treatments	Concentrations 2*LD_{90} (mL/10g)	Parameters [a]TPDS plant^{-1}	[b]PD (%) plant^{-1}	[c]GY (t/ha)
Bt. vr. *thuringiensis*	3.32	96.92±13.07[a]	8.65±2.49[b]	2.34±0.02[ab]
KDL	3.08	112.25±10.5[a]	8.72±4.34[b]	2.42±0.23[ab]
AUPOS	3.70	90.42±15.09[a]	10.31±0.82[b]	1.80±0.71[ab]
AUSD-1	3.52	91.33±15.87[a]	8.63±2.57[b]	2.36±0.41[ab]
AUGHS-1	3.58	97.17±27.71[a]	9.13±3.18[b]	2.19±0.19[ab]
ZDS	3.96	87.83±9.64[a]	17.13±4.24[b]	1.55±0.57[ab]
ZDS-3	3.54	99.17±15.39[a]	15.87±5.76[b]	1.57±0.73[ab]
AUASG-2	3.26	89.42±6.24[a]	11.42±2.40[b]	1.87±0.23[ab]
GHTSW	3.32	106.0±12.49[a]	8.99±2.75[b]	2.01±0.36[ab]
GHTSW-1	3.52	85.42±24.98[a]	12.81±2.02[b]	1.62±0.19[ab]
AUGHS-3	3.78	89.92±15.09[a]	12.34±4.74[b]	1.58±0.98[ab]
Control	-	93.83±6.00[a]	52.25±9.72[a]	1.07±0.19[b]

Means with the same letters within the same columns were not significantly different. Data were means ± SD, means were separated using Tukey's highest Significant Difference (HSD) test. [a]TPDS, total number of pods per plant (*df=11,22, f-value=0.44, pr<0.9208*); [b]PD, percentage pod damage per plant, (*df=11,22, f-value=15.59, pr<0.0001*); [c]GY, grain yield tons per hectare, (*df=11,22, f-value=2.52, pr<0.0314*).

5.3.3. Relationship of percentage pod damage and grain yield

The relationship between chickpea pod damage (%) and grain (t/ha) was studied under field conditions at two locations. Results obtained showed that they have an inverse relationship, where at Gonde Basic Seed Farm Center (*r= -0.68; P<0.001*) (Fig. 16a) and at Kulumsa Agricultural Research Center (*r= -0.83; P<0.001*) (Fig. 16b) .

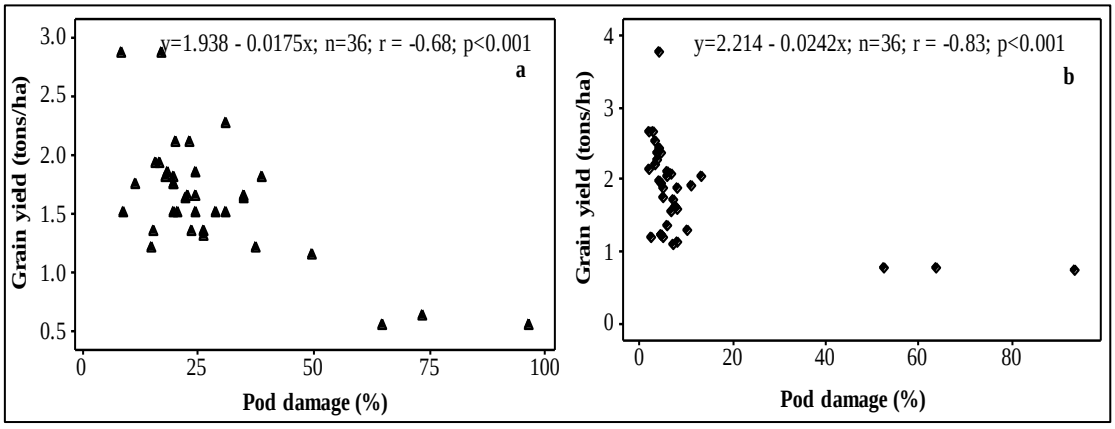


Figure 16 Correlation graph of pod damage (%) with grain yield under field conditions (**a**) Gonde Basic Seed Farm Center, (**b**) Kulumsa Agricultural Research Center.

5.4. DISCUSSION

Results of this study revealed that all the native *Bt* isolates achieved more than 60% larval mortality under field conditions. Values obtained from the native *Bt* isolates KDL, AUPOS AUGHS-1 and AUSD-1 on percentage larval mortality were moderately higher than the rest native *Bt* isolates and comparable to the reference *Bt*. var. *thuringiensis*, followed by native *Bt* isolates of ZDS-3 and GHTSW, GHTSW-1 and AUASG-2,. Off all native *Bt* isolates tested, ZDS and AUGHS-3 demonstrated lower larval mortality. Lawo *et al*. (2008), made study on *cry*2A to susceptible *H. armigera*, revealed that 86.4% larval mortality under the greenhouse. Similarly, native *Bt* isolates KDL, AUPOS, AUGHS-1 and AUSD-1 relatively showed lower percentage pod damage and resulted in higher grain yield (t/ha), compared to the reference *Bt*. var. *thuringiensis*. The control plot gave highest percentage pod damage and lowest grain yield (t/ha). However, the values of the rest of native *Bt* isolates were intermediate. Only native *Bt* isolate AUGHS-3 showed higher percentage pod damage and lower grain yield (t/ha).

Under field, all the native *Bt* isolates and the reference *Bt*. var. *thuringiensis* reduced larval population per plant at pre and post 1st and 2nd *Bt* spayed. Among, the results obtained from native *Bt* isolates of KDL, AUPOS, AUGHS-1and AUSD-1and the reference *Bt* relatively showed lower larval population per plant and values were consistent. Therefore, they are comparatively superior to the rest native *Bt* isolates. Similarly, values of the above listed native *Bt* isolates and reference *Bt* on percentage pod damage comparatively lower than the rest *Bt* isolates and resulted in higher grain yield (t/ha), similarly Kumar *et al*. (2016) have used liquid formulation of *Bt* strain PDBC *Bt*1and commercial *Bt* k (Halt 5% WP) on *H. armigera* in pigeon pea indicated that lower larval population per plant on the 3rd and 7th days after 1st and 2nd sprayed and gave lower percentage pod damage per plant and higher grain yield (t/ha). Ahmed *et al*. (2015) have studied for two years on bio-pesticide DiPel 2x alone and with mixtures of milk powder, molasses and K_2CO_3 to control chickpea pod borer that showed significantly lower pod damage and higher grain yield with and without the mixtures. Alike the current study, Singh and Dhkal (2019) have made two years study on *H. armigera* in chickpea using commercial *Bt* product *Bt*. var. *kurstaki* (WP) (DOR *Bt*-1) that reduced percentage pod damage and increased chickpea grain yield.

Larval mortality percentage had inverse relationship with percentage pod damage per plant, as well as percentage pod damage was inversely related to grain yield (t/ha) in the greenhouse. Similarly, under field at Kulumsa, percentage pod damage per plant and grain yield (t/ha) had strong negative relationship, whereas, at Gonde the relationship was intermediate. Similarly, Aderajew *et al*. (2020) have regretted percentage pod damage with chickpea grain yield indicated that 63.6% grain yield was tune due to *H. armigera* damage on chickpea.

5.5. CONCLUSION

Though the values among native *Bt* isolates varied, all of the examined isolates effectively reduced the number of larvae per plant, percentage of pod damage, and gaves higher grain yield in tons per hectare compared to the control, and they all substantiated comparable results with the reference *Bt*. var. *thuringiensis*. Therefore, this confirmed to us the existence of Ethiopian *Bt* isolates harbouring *cry*1 and *cry*2 genes that are potent to *H. armigera* larvae. Our study leads us to the conclusion that they were recognized as potential candidates for *H. armigera* management in chickpea and other related host crops. Due to *Bt* poor field persistence, it must be used considering *H. armigera* economic threshold level, or else the formulation must be improved to boost its persistence. Experimental work to explor their specific *cry* gene families and that will be verified under a range of locations for further commercialization and development of *Bt* technologies.

CHAPTER SIX

6.1. General Discussion

Chickpea is a protein rich legume crop plays a significant role in the human diet (Redden *et al., 2007).* Pod borer, *H. armigera* is the world most significant insect pest of chickpea that cause a substantial amount of yield loss to the crop (Reed and Pawar, 1982; Sehgal and Ujagir, 1990; Cherry *et al*., 2003; Singh *et al*., 2021). The pest developed resistance againest many chemical insecticides, due to this overdose and frequent applications of the insecticides leads to human safety and environmental problems, chemical residue in food and catastrophic to the natural eco-system (Saleem, and Yunus, 1982;Vitale *et al.*, 2007). A comprehensive research is currently under way to find alternatives to chemical insecticides to control the insect pest (Fitt, 2008; Naranjo, 2011). Thus, the discovery of some strains of *B. thuringiensis* (*Bt*) had insecticidal activities against insect pest in a divers procured over a hundred years ago, but rigorous research into their development was initiated more recently (Fitt, 2008; Naranjo, 2011). Consequently, one could argue that the foundation of commercial biopesticides with *B. thuringiensis* strains is still a very recent field of study (Sharma, and Dhillon, 2018).

The main objective of this study was to explore native *Bt* isolates pathogenic to chickpea pod borer, *H. armigera*. For *Bt* sources to be studied, soil samples were collected purposefully targeting chickpea and tomato farm (Table 1 and Fig. 1). Moreover, samples from few insect cadavers, store dust, animal sewage and ponds were also included. Thus, the collected soil samples were processed under the laboratory and isolation was made followed the procedures of (Travers *et al*., 1987). Intensive screening was carried out against *H. armigera* 3rd instar larvae under laboratory conditions using biotoxicity assay at a single concentration (1 mL). Therefore, based on the results of larval mortality they achieved ten potent native *Bt* isolates namely KDL, AUPOS, AUSD-1, AUGHS-1, ZDS, ZDS-3, AUASG-2, GHTSW, GHTSW-1 were screened. Then after, morphologically and biochemically characterized and their pathogenicity was tested to *H. armigera* 3rd instar larvae at a *Bt* concentrations of 0.25 mL. 0.5 mL, 1.5 mL, 2 mL and 2.5 mL to determine the $LD_{50, 90}$ values. Results showed that there are similarities and differences in some of the morphological characters between the native *Bt* isolates tested. As to the biochemical test results the *Bt* isolates are positive to gram stain, endospore stain, crystal stain, motility and catalase test similar to that of the reference *Bt*. var. *thuringiensis*.

Furthermore, molecular characterisation was done, to identify the *cry* genes they harbouring mostly *cry*1 and *cry*2 genes using five universal primers *cry*1, *cry*2, *cry*3, *cry*4 and *cry*7,8 and specific primer *cry*9. All the *Bt* isolates harbour one or more *cry* gen/genes, among *cry*4 gene was detected 100%, *cry*2 90% and *cry*1 50%. In line with this Gebremariam *et al*., (2021); Aynalem *et al*., (2021) reported that *cry*2 and *cry*9 genes were more prevalent than the rest of *cry* genes identified in *Bt* isolates from Ethiopian sources. With the exception of *cry*3 gene, which was not amplified in any of the isolates examined, all the *cry* genes, peculiarly found in native *Bt* isolate AUSD-1, which was isolated from store dust. Finally, those *Bt* isolates were analysed for 16S rRNA gene, all native *Bt* isolates except AUASG-2 and AUGHS-3 amplified the gen (Janda and Abbott, 2007; Baig *et al*., 2010).

6.2. General conclusion

Results of the current study revealed that morphological and biochemical characterizations of the native *Bt* isolates along with the results of molecular characterization confirmed us the ten native bacterial isolates were belongs to *Bt.* The persistence of *Bt* under field conditions is less due to environmental factors. Hence, the growth conditions of the *Bt* isolates were tested under various temperature ranges which indicated that the optimum growth temperature requirement for the *Bt* to efficiently control the target insect pest. Nutrient utilization and enzyme production ability of the native *Bt* isolates indicated that *Bt* sprayed can survived in the natural environment by enzymatically converting naturally available nutrients and it helps the *Bt* to get sufficient nutrient for sporulation and production of toxic proteins. Their *cry* gene profile were analysed, thus, indicated the presence of multiple genes active to diverse insect orders, so that *Bt* isolates harbouring *cry*1 and *cry*2 genes that express insecticidal crystal proteins to lepidopteran larvae were isolated and identified from Ethiopia. Similarly all the native *Bt* isolates tested for 16S rRNA universal primer were amplified the gen at a band size of 1500 bp which indicated the native *Bt* isolates have similar homology analogous to reference *Bt*. var. *thuringiensis* except native *Bt* isolates of AUASG-2 and AUGHS-3 due to insufficient DNA extracted and so far we are unable to replace from the original source sample. It was perceived that from the pathogenicity test result in the greenhouse and under field conditions.

All the native *Bt* isolates were demonstrated similar results compared to the reference *Bt*. var. *thuringiensis* in suppressing *H. armigera* larval population, lowering percentage chickpea pod damage and substantiated higher chickpea grain yield compared to the control. Therefore, this study strongly suggested the existence of potent native *Bt* isolates that are effective against *H. armigera* 3rd instar larvae.

6.3. General recommendation

Since little is known about native *Bt* isolates, there are many opportunities for further investigation. Given that there are a lot more economically significant insect pests of agricultural crops that needs to be studied, including vectors of human diseases. Compared to the huge biodiversity that existed under various agro-ecologies that were not efficiently explored. It was expected that many *B. thuringiensis* isolates that are potent to the Lepidoptera order are still undiscovered. So that a methodical quest for novel isolates with a broad range of activities against a variety of insect groups. To do this extensive collection on the larger scale should be required. It will also be crucial to identify, characterize, and evaluate their pathogenicity under several field trials.The range of toxicity of the currently collected isolates should also be examined. Since the *Bt* isolates showed several *cry* genes, including the 100% detected *cry*4 gene and the 90% detected *cry*2 gene, which are thought to be active in other insect order like Diptera. Sequencing and identification of the insecticidal crystal toxic protein subfamilies are necessary. In a method that will allow for verification through numerous field trials on the target insect pest, *H. armigera* and other related host crops. As such, we recommend that the development of commercial bioinsecticide and other *Bt* technologies using *B. thuringiensis* isolates from Ethiopian sources will be a future new research avenue to be addressed.

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Appendix 8.1 Microplate reader instrument information

Instrument information			
Name			Multiskan FC
ESW version			1.01.16
Serial number			357-914734
Instrument modules			
Filter			
	Name		
	Position		1
	Wavelength		340
	Bandwidth		
	Date and time of definition		6/12/2022 2:13:00 PM
Filter			
	Name		
	Position		2
	Wavelength		600
	Bandwidth		
	Date and time of definition		6/12/2022 2:12:00 PM
Filter			
	Name		
	Position		3
	Wavelength		630
	Bandwidth		
	Date and time of definition		8/10/2020 11:11:00 PM
Filter			
	Name		
	Position		4
	Wavelength		470
	Bandwidth		
	Date and time of definition		10/26/2020 1:54:00 AM
Filter			
	Name		
	Position		5
	Wavelength		517
	Bandwidth		
	Date and time of definition		12/3/2020 11:09:00 PM
Filter			
	Name		
	Position		6
	Wavelength		660
	Bandwidth		
	Date and time of definition		8/10/2020 11:12:00 PM
Filter			
	Name		
	Position		7
	Wavelength		720
	Bandwidth		
	Date and time of definition		8/10/2020 11:13:00 PM
Filter			
	Name		
	Position		8
	Wavelength		520
	Bandwidth		
	Date and time of definition		10/21/2020 3:59:00 AM
	Incubator		No

Appendix 8.2 Micro-plate reader computer set up for absorbance measure

Name	Plate 1
Plate template	ANSI/SBS Standard, 96-well

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1	Blank1 Group 1
B	1 Group 1 1:1	1 Group 1 1:1	1 Group 1 1:1	2 Group 1 1:1	2 Group 1 1:1	2 Group 1 1:1	3 Group 1 1:1	3 Group 1 1:1	3 Group 1 1:1	4 Group 1 1:1	4 Group 1 1:1	4 Group 1 1:1
C	5 Group 1 1:1	5 Group 1 1:1	5 Group 1 1:1	6 Group 1 1:1	6 Group 1 1:1	6 Group 1 1:1	7 Group 1 1:1	7 Group 1 1:1	7 Group 1 1:1	8 Group 1 1:1	8 Group 1 1:1	8 Group 1 1:1
D	9 Group 1 1:1	9 Group 1 1:1	9 Group 1 1:1	10 Group 1 1:1	10 Group 1 1:1	10 Group 1 1:1	11 Group 1 1:1	11 Group 1 1:1	11 Group 1 1:1	12 Group 1 1:1	12 Group 1 1:1	12 Group 1 1:1
E	13 Group 1 1:1	13 Group 1 1:1	13 Group 1 1:1	14 Group 1 1:1	14 Group 1 1:1	14 Group 1 1:1	15 Group 1 1:1	15 Group 1 1:1	15 Group 1 1:1	16 Group 1 1:1	16 Group 1 1:1	16 Group 1 1:1
F	17 Group 1 1:1	17 Group 1 1:1	17 Group 1 1:1	18 Group 1 1:1	18 Group 1 1:1	18 Group 1 1:1	19 Group 1 1:1	19 Group 1 1:1	19 Group 1 1:1	20 Group 1 1:1	20 Group 1 1:1	20 Group 1 1:1
G	21 Group 1 1:1	21 Group 1 1:1	21 Group 1 1:1	22 Group 1 1:1	22 Group 1 1:1	22 Group 1 1:1	23 Group 1 1:1	23 Group 1 1:1	23 Group 1 1:1	24 Group 1 1:1	24 Group 1 1:1	24 Group 1 1:1
H	25 Group 1 1:1	25 Group 1 1:1	25 Group 1 1:1									

General information	
Software version	SkanIt Software 5.0 for Microplate Readers RE, ver. 5.0.0.42
Optical response compensation	Yes

Appendix 8.3 *Bt* absorbance values at temperature of 15 °C incubated for 24 hrs

Results summary							Measurement results temp 15 °C @24hrs					
General												
Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction1 (600nm)		Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction1 (600nm)
Plate 1	A01	Group 1	Blank1	0.1575	-0.01586		Plate 1	A07	Group 1	Blank1	0.1688	-0.004558
Plate 1	B01	Group 1	1	0.2992	0.1258		Plate 1	B07	Group 1	3	0.3825	0.2091
Plate 1	C01	Group 1	5	0.3418	0.1684		Plate 1	C07	Group 1	7	0.4181	0.2447
Plate 1	D01	Group 1	9	0.4517	0.2783		Plate 1	D07	Group 1	11	0.3431	0.1697
Plate 1	E01	Group 1	13	0.2796	0.1062		Plate 1	E07	Group 1	15	0.3226	0.1492
Plate 1	F01	Group 1	17	0.411	0.2376		Plate 1	F07	Group 1	19	0.1744	0.001042
Plate 1	G01	Group 1	21	0.3127	0.1393		Plate 1	G07	Group 1	23	0.2995	0.1261
Plate 1	H01	Group 1	25	0.4109	0.2375		Plate 1	A08	Group 1	Blank1	0.1694	-0.003958
Plate 1	A02	Group 1	Blank1	0.1564	-0.01696		Plate 1	B08	Group 1	3	0.373	0.1996
Plate 1	B02	Group 1	1	0.3106	0.1372		Plate 1	C08	Group 1	7	0.3434	0.17
Plate 1	C02	Group 1	5	0.38	0.2066		Plate 1	D08	Group 1	11	0.4046	0.2312
Plate 1	D02	Group 1	9	0.6105	0.4371		Plate 1	E08	Group 1	15	0.3565	0.1831
Plate 1	E02	Group 1	13	0.3234	0.15		Plate 1	F08	Group 1	19	0.2193	0.04594
Plate 1	F02	Group 1	17	0.4825	0.3091		Plate 1	G08	Group 1	23	0.3844	0.211
Plate 1	G02	Group 1	21	0.3728	0.1994		Plate 1	A09	Group 1	Blank1	0.1812	0.007842
Plate 1	H02	Group 1	25	0.4843	0.3109		Plate 1	B09	Group 1	3	0.385	0.2116
Plate 1	A03	Group 1	Blank1	0.1635	-0.009858		Plate 1	C09	Group 1	7	0.4236	0.2502
Plate 1	B03	Group 1	1	0.3352	0.1618		Plate 1	D09	Group 1	11	0.4318	0.2584
Plate 1	C03	Group 1	5	0.3883	0.2149		Plate 1	E09	Group 1	15	0.3271	0.1537
Plate 1	D03	Group 1	9	0.6261	0.4527		Plate 1	F09	Group 1	19	0.3481	0.1747
Plate 1	E03	Group 1	13	0.3115	0.1381		Plate 1	G09	Group 1	23	0.4097	0.2363
Plate 1	F03	Group 1	17	0.5743	0.4009		Plate 1	A10	Group 1	Blank1	0.1715	-0.001858
Plate 1	G03	Group 1	21	0.3784	0.205		Plate 1	B10	Group 1	4	0.0453	-0.1281
Plate 1	H03	Group 1	25	0.3963	0.2229		Plate 1	C10	Group 1	8	0.3545	0.1811
Plate 1	A04	Group 1	Blank1	0.1649	-0.008458		Plate 1	D10	Group 1	12	0.4119	0.2385
Plate 1	B04	Group 1	2	0.3872	0.2138		Plate 1	E10	Group 1	16	0.2794	0.106
Plate 1	C04	Group 1	6	0.3705	0.1971		Plate 1	F10	Group 1	20	0.3986	0.2252
Plate 1	D04	Group 1	10	0.3601	0.1867		Plate 1	G10	Group 1	24	0.3393	0.1659
Plate 1	E04	Group 1	14	0.3067	0.1333		Plate 1	A11	Group 1	Blank1	0.1636	-0.009758

Appendix 8.3 (*Continued)*

Plate 1	F04	Group 1	18	0.9698	0.7964		Plate 1	B11	Group 1	4	0.3428	0.1694
Plate 1	G04	Group 1	22	0.3308	0.1574		Plate 1	C11	Group 1	8	0.3603	0.1869
Plate 1	A05	Group 1	Blank1	0.1769	0.003542		Plate 1	D11	Group 1	12	0.0425	-0.1309
Plate 1	B05	Group 1	2	0.3646	0.1912		Plate 1	E11	Group 1	16	0.3243	0.1509
Plate 1	C05	Group 1	6	0.3883	0.2149		Plate 1	F11	Group 1	20	0.3988	0.2254
Plate 1	D05	Group 1	10	0.2514	0.07804		Plate 1	G11	Group 1	24	0.3511	0.1777
Plate 1	E05	Group 1	14	0.3355	0.1621		Plate 1	A12	Group 1	Blank1	0.2256	0.05224
Plate 1	F05	Group 1	18	0.1839	0.01054		Plate 1	B12	Group 1	4	0.3172	0.1438
Plate 1	G05	Group 1	22	0.4555	0.2821		Plate 1	C12	Group 1	8	0.3421	0.1687
Plate 1	A06	Group 1	Blank1	0.181	0.007642		Plate 1	D12	Group 1	12	0.3347	0.1613
Plate 1	B06	Group 1	2	0.3615	0.1881		Plate 1	E12	Group 1	16	0.2935	0.1201
Plate 1	C06	Group 1	6	0.3913	0.2179		Plate 1	F12	Group 1	20	0.3515	0.1781
Plate 1	D06	Group 1	10	0.2343	0.06094		Plate 1	G12	Group 1	24	0.309	0.1356
Plate 1	E06	Group 1	14	0.3289	0.1555							
Plate 1	F06	Group 1	18	0.3504	0.177							
Plate 1	G06	Group 1	22	0.3357	0.1623							

Appendix 8.4 *Bt* absorbance values at temperature of 20 °C incubated for 24 hrs

Results summary					Measurement results temp 20 °C @N 24hrs							
General												
Plate	Well	Group	Sample	Absorbance 1 (630nm)	Blank Subtraction 1 (600nm)		Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction 1 (600nm)
Plate 1	A01	Group 1	Blank1	0.1976	0.01505		Plate 1	A07	Group 1	Blank1	0.1685	-0.01405
Plate 1	B01	Group 1	1	0.2573	0.07475		Plate 1	B07	Group 1	3	0.2426	0.06005
Plate 1	C01	Group 1	5	0.2467	0.06415		Plate 1	C07	Group 1	7	0.2414	0.05885
Plate 1	D01	Group 1	9	0.2934	0.1109		Plate 1	D07	Group 1	11	0.3107	0.1281
Plate 1	E01	Group 1	13	0.2443	0.06175		Plate 1	E07	Group 1	15	0.2689	0.08635
Plate 1	F01	Group 1	17	0.3586	0.1761		Plate 1	F07	Group 1	19	0.3461	0.1636
Plate 1	G01	Group 1	21	0.2738	0.09125		Plate 1	G07	Group 1	23	0.2216	0.03905
Plate 1	H01	Group 1	25	0.4149	0.2324		Plate 1	A08	Group 1	Blank1	0.1777	-0.00485
Plate 1	A02	Group 1	Blank1	0.2421	0.05955		Plate 1	B08	Group 1	3	0.2525	0.06995
Plate 1	B02	Group 1	1	0.2292	0.04665		Plate 1	C08	Group 1	7	0.3032	0.1207
Plate 1	C02	Group 1	5	0.2415	0.05895		Plate 1	D08	Group 1	11	0.2987	0.1162
Plate 1	D02	Group 1	9	0.2577	0.07515		Plate 1	E08	Group 1	15	0.2117	0.02915
Plate 1	E02	Group 1	13	0.2271	0.04455		Plate 1	F08	Group 1	19	0.2923	0.1098
Plate 1	F02	Group 1	17	0.3222	0.1397		Plate 1	G08	Group 1	23	0.2399	0.05735
Plate 1	G02	Group 1	21	0.231	0.04845		Plate 1	A09	Group 1	Blank1	0.1839	0.00135
Plate 1	H02	Group 1	25	0.3965	0.214		Plate 1	B09	Group 1	3	0.2539	0.07135
Plate 1	A03	Group 1	Blank1	0.1657	-0.01685		Plate 1	C09	Group 1	7	0.2708	0.08825
Plate 1	B03	Group 1	1	0.2469	0.06435		Plate 1	D09	Group 1	11	0.4326	0.2501
Plate 1	C03	Group 1	5	0.2405	0.05795		Plate 1	E09	Group 1	15	0.2206	0.03805
Plate 1	D03	Group 1	9	0.2596	0.07705		Plate 1	F09	Group 1	19	0.325	0.1425
Plate 1	E03	Group 1	13	0.253	0.07045		Plate 1	G09	Group 1	23	0.3494	0.1669
Plate 1	F03	Group 1	17	0.4324	0.2499		Plate 1	A10	Group 1	Blank1	0.1677	-0.01485
Plate 1	G03	Group 1	21	0.2777	0.09515		Plate 1	B10	Group 1	4	0.2689	0.08635
Plate 1	H03	Group 1	25	0.3539	0.1714		Plate 1	C10	Group 1	8	0.279	0.09645
Plate 1	A04	Group 1	Blank1	0.177	-0.00555		Plate 1	D10	Group 1	12	0.2808	0.09825

Appendix 8.4 (*Continued)*

Plate 1	B04	Group 1	2	0.2591	0.07655		Plate 1	E10	Group 1	16	0.2321	0.04955
Plate 1	C04	Group 1	6	0.2439	0.06135		Plate 1	F10	Group 1	20	0.2882	0.1057
Plate 1	D04	Group 1	10	0.2473	0.06475		Plate 1	G10	Group 1	24	0.3167	0.1342
Plate 1	E04	Group 1	14	0.0904	-0.09215		Plate 1	A11	Group 1	Blank1	0.17	-0.01255
Plate 1	F04	Group 1	18	0.1856	0.00305		Plate 1	B11	Group 1	4	0.2743	0.09175
Plate 1	G04	Group 1	22	0.2709	0.08835		Plate 1	C11	Group 1	8	0.2503	0.06775
Plate 1	A05	Group 1	Blank1	0.177	-0.00555		Plate 1	D11	Group 1	12	0.2776	0.09505
Plate 1	B05	Group 1	2	0.2472	0.06465		Plate 1	E11	Group 1	16	0.2424	0.05985
Plate 1	C05	Group 1	6	0.231	0.04845		Plate 1	F11	Group 1	20	0.2815	0.09895
Plate 1	D05	Group 1	10	0.1958	0.01325		Plate 1	G11	Group 1	24	0.2743	0.09175
Plate 1	E05	Group 1	14	0.2166	0.03405		Plate 1	A12	Group 1	Blank1	0.1992	0.01665
Plate 1	F05	Group 1	18	0.3659	0.1834		Plate 1	B12	Group 1	4	0.2916	0.1091
Plate 1	G05	Group 1	22	0.4257	0.2432		Plate 1	C12	Group 1	8	0.2704	0.08785
Plate 1	A06	Group 1	Blank1	0.1642	-0.01835		Plate 1	D12	Group 1	12	0.2753	0.09275
Plate 1	B06	Group 1	2	0.2732	0.09065		Plate 1	E12	Group 1	16	0.2765	0.09395
Plate 1	C06	Group 1	6	0.291	0.1085		Plate 1	F12	Group 1	20	0.298	0.1155
Plate 1	D06	Group 1	10	0.2421	0.05955		Plate 1	G12	Group 1	24	0.2902	0.1077
Plate 1	E06	Group 1	14	0.2376	0.05505							
Plate 1	F06	Group 1	18	0.3754	0.1929							
Plate 1	G06	Group 1	22	0.2939	0.1114							

Appendix 8. 5 *Bt* absorbance values at temperature of 25 °C incubated for 24 hrs

Results summary					Measurement results temp 25 °C @ 24hrs							
General												
Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction 1 (600nm)		Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction 1 (600nm)
Plate 1	A01	Group 1	Blank1	0.1645	-0.007592		Plate 1	A07	Group 1	Blank1	0.1738	0.001708
Plate 1	B01	Group 1	1	0.2575	0.08541		Plate 1	B07	Group 1	3	0.2669	0.09481
Plate 1	C01	Group 1	5	0.2429	0.07081		Plate 1	C07	Group 1	7	0.2903	0.1182
Plate 1	D01	Group 1	9	0.2096	0.03751		Plate 1	D07	Group 1	11	0.2674	0.09531
Plate 1	E01	Group 1	13	0.2759	0.1038		Plate 1	E07	Group 1	15	0.2494	0.07731
Plate 1	F01	Group 1	17	0.266	0.09391		Plate 1	F07	Group 1	19	0.3191	0.147
Plate 1	G01	Group 1	21	0.2449	0.07281		Plate 1	G07	Group 1	23	0.3062	0.1341
Plate 1	H01	Group 1	25	0.2759	0.1038		Plate 1	A08	Group 1	Blank1	0.1724	0.0003083
Plate 1	A02	Group 1	Blank1	0.1683	-0.003792		Plate 1	B08	Group 1	3	0.2701	0.09801
Plate 1	B02	Group 1	1	0.2624	0.09031		Plate 1	C08	Group 1	7	0.2984	0.1263
Plate 1	C02	Group 1	5	0.2676	0.09551		Plate 1	D08	Group 1	11	0.2694	0.09731
Plate 1	D02	Group 1	9	0.2369	0.06481		Plate 1	E08	Group 1	15	0.1743	0.002208
Plate 1	E02	Group 1	13	0.2517	0.07961		Plate 1	F08	Group 1	19	0.3003	0.1282
Plate 1	F02	Group 1	17	0.3013	0.1292		Plate 1	G08	Group 1	23	0.2769	0.1048
Plate 1	G02	Group 1	21	0.2244	0.05231		Plate 1	A09	Group 1	Blank1	0.1689	-0.003192
Plate 1	H02	Group 1	25	0.3532	0.1811		Plate 1	B09	Group 1	3	0.2922	0.1201
Plate 1	A03	Group 1	Blank1	0.1679	-0.004192		Plate 1	C09	Group 1	7	0.2492	0.07711
Plate 1	B03	Group 1	1	0.2748	0.1027		Plate 1	D09	Group 1	11	0.2821	0.11
Plate 1	C03	Group 1	5	0.2623	0.09021		Plate 1	E09	Group 1	15	0.2596	0.08751
Plate 1	D03	Group 1	9	0.259	0.08691		Plate 1	F09	Group 1	19	0.2978	0.1257
Plate 1	E03	Group 1	13	0.3138	0.1417		Plate 1	G09	Group 1	23	0.066	-0.1061
Plate 1	F03	Group 1	17	0.3092	0.1371		Plate 1	A10	Group 1	Blank1	0.1742	0.002108
Plate 1	G03	Group 1	21	0.2671	0.09501		Plate 1	B10	Group 1	4	0.5629	0.3908
Plate 1	H03	Group 1	25	0.3487	0.1766		Plate 1	C10	Group 1	8	0.274	0.1019
Plate 1	A04	Group 1	Blank1	0.1725	0.0004083		Plate 1	D10	Group 1	12	0.2939	0.1218
Plate 1	B04	Group 1	2	0.3037	0.1316		Plate 1	E10	Group 1	16	0.301	0.1289
Plate 1	C04	Group 1	6	0.2538	0.08171		Plate 1	F10	Group 1	20	0.2484	0.07631
Plate 1	D04	Group 1	10	0.2449	0.07281		Plate 1	G10	Group 1	24	0.2811	0.109

Appendix 8.5 (*Continued)*

Plate 1	E04	Group 1	14	0.2526	0.08051		Plate 1	A11	Group 1	Blank1	0.1775	0.005408
Plate 1	F04	Group 1	18	0.284	0.1119		Plate 1	B11	Group 1	4	0.3185	0.1464
Plate 1	G04	Group 1	22	0.255	0.08291		Plate 1	C11	Group 1	8	0.2714	0.09931
Plate 1	A05	Group 1	Blank1	0.1715	-0.0005917		Plate 1	D11	Group 1	12	0.2971	0.125
Plate 1	B05	Group 1	2	0.0894	-0.08269		Plate 1	E11	Group 1	16	0.2638	0.09171
Plate 1	C05	Group 1	6	0.3415	0.1694		Plate 1	F11	Group 1	20	0.2631	0.09101
Plate 1	D05	Group 1	10	0.2473	0.07521		Plate 1	G11	Group 1	24	0.2945	0.1224
Plate 1	E05	Group 1	14	0.2438	0.07171		Plate 1	A12	Group 1	Blank1	0.1903	0.01821
Plate 1	F05	Group 1	18	0.3205	0.1484		Plate 1	B12	Group 1	4	0.3584	0.1863
Plate 1	G05	Group 1	22	0.2712	0.09911		Plate 1	C12	Group 1	8	0.2961	0.124
Plate 1	A06	Group 1	Blank1	0.1633	-0.008792		Plate 1	D12	Group 1	12	0.292	0.1199
Plate 1	B06	Group 1	2	0.2951	0.123		Plate 1	E12	Group 1	16	0.2857	0.1136
Plate 1	C06	Group 1	6	0.2913	0.1192		Plate 1	F12	Group 1	20	0.2488	0.07671
Plate 1	D06	Group 1	10	0.2534	0.08131		Plate 1	G12	Group 1	24	0.3097	0.1376
Plate 1	E06	Group 1	14	0.2676	0.09551							
Plate 1	F06	Group 1	18	0.2954	0.1233							
Plate 1	G06	Group 1	22	0.2559	0.08381							

Appendix 8.6 *Bt* absorbance values at temperature of 30 °C incubated for 24 hrs

Results summary					Measurement results temp 30 °C@ 24hrs							
General												
Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction 1 (600nm)		Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction 1 (600nm)
Plate 1	A01	Group 1	Blank1	0.1976	0.00405		Plate 1	A07	Group 1	Blank1	0.2006	0.00705
Plate 1	B01	Group 1	1	0.2522	0.05865		Plate 1	B07	Group 1	3	0.3522	0.1587
Plate 1	C01	Group 1	5	0.2423	0.04875		Plate 1	C07	Group 1	7	0.2901	0.09655
Plate 1	D01	Group 1	9	0.6496	0.4561		Plate 1	D07	Group 1	11	0.4081	0.2146
Plate 1	E01	Group 1	13	0.381	0.1875		Plate 1	E07	Group 1	15	0.2698	0.07625
Plate 1	F01	Group 1	17	0.4788	0.2853		Plate 1	F07	Group 1	19	0.2811	0.08755
Plate 1	G01	Group 1	21	0.3457	0.1522		Plate 1	G07	Group 1	23	0.435	0.2415
Plate 1	H01	Group 1	25	0.2893	0.09575		Plate 1	A08	Group 1	Blank1	0.2035	0.00995
Plate 1	A02	Group 1	Blank1	0.1803	-0.01325		Plate 1	B08	Group 1	3	0.3257	0.1322
Plate 1	B02	Group 1	1	0.2465	0.05295		Plate 1	C08	Group 1	7	0.2583	0.06475
Plate 1	C02	Group 1	5	0.308	0.1145		Plate 1	D08	Group 1	11	0.3582	0.1647
Plate 1	D02	Group 1	9	0.6029	0.4094		Plate 1	E08	Group 1	15	0.2489	0.05535
Plate 1	E02	Group 1	13	0.4447	0.2512		Plate 1	F08	Group 1	19	0.3615	0.1679
Plate 1	F02	Group 1	17	0.4617	0.2682		Plate 1	G08	Group 1	23	0.3732	0.1797
Plate 1	G02	Group 1	21	0.3527	0.1592		Plate 1	A09	Group 1	Blank1	0.2123	0.01875
Plate 1	H02	Group 1	25	0.3078	0.1143		Plate 1	B09	Group 1	3	0.3364	0.1429
Plate 1	A03	Group 1	Blank1	0.1864	-0.00715		Plate 1	C09	Group 1	7	0.255	0.06145
Plate 1	B03	Group 1	1	0.273	0.07945		Plate 1	D09	Group 1	11	0.3915	0.1979
Plate 1	C03	Group 1	5	0.293	0.09945		Plate 1	E09	Group 1	15	0.3283	0.1348
Plate 1	D03	Group 1	9	0.7396	0.5461		Plate 1	F09	Group 1	19	0.3601	0.1666
Plate 1	E03	Group 1	13	0.3815	0.188		Plate 1	G09	Group 1	23	0.399	0.2055
Plate 1	F03	Group 1	17	0.4747	0.2812		Plate 1	A10	Group 1	Blank1	0.1822	-0.01135
Plate 1	G03	Group 1	21	0.3312	0.1377		Plate 1	B10	Group 1	4	0.3956	0.2021
Plate 1	H03	Group 1	25	0.3303	0.1368		Plate 1	C10	Group 1	8	0.3039	0.1104
Plate 1	A04	Group 1	Blank1	0.1585	-0.03505		Plate 1	D10	Group 1	12	0.4217	0.2282
Plate 1	B04	Group 1	2	0.2882	0.09465		Plate 1	E10	Group 1	16	0.2271	0.03355
Plate 1	C04	Group 1	6	0.1223	-0.07125		Plate 1	F10	Group 1	20	0.3434	0.1499
Plate 1	D04	Group 1	10	0.311	0.1175		Plate 1	G10	Group 1	24	0.2819	0.08835

Appendix 8.6 (*Continued)*

Plate 1	E04	Group 1	14	0.2626	0.06905		Plate 1	A11	Group 1	Blank1	0.2022	0.00865
Plate 1	F04	Group 1	18	0.3102	0.1167		Plate 1	B11	Group 1	4	0.3894	0.1959
Plate 1	G04	Group 1	22	0.361	0.1674		Plate 1	C11	Group 1	8	0.2846	0.09105
Plate 1	A05	Group 1	Blank1	0.1899	-0.00365		Plate 1	D11	Group 1	12	0.3878	0.1943
Plate 1	B05	Group 1	2	0.2687	0.07515		Plate 1	E11	Group 1	16	0.3498	0.1563
Plate 1	C05	Group 1	6	0.2901	0.09655		Plate 1	F11	Group 1	20	0.3114	0.1179
Plate 1	D05	Group 1	10	0.2989	0.1054		Plate 1	G11	Group 1	24	0.309	0.1155
Plate 1	E05	Group 1	14	0.5077	0.3142		Plate 1	A12	Group 1	Blank1	0.2344	0.04085
Plate 1	F05	Group 1	18	0.32	0.1265		Plate 1	B12	Group 1	4	0.2942	0.1007
Plate 1	G05	Group 1	22	0.3555	0.162		Plate 1	C12	Group 1	8	0.2902	0.09665
Plate 1	A06	Group 1	Blank1	0.1747	-0.01885		Plate 1	D12	Group 1	12	0.3332	0.1397
Plate 1	B06	Group 1	2	0.3388	0.1453		Plate 1	E12	Group 1	16	0.346	0.1525
Plate 1	C06	Group 1	6	0.2836	0.09005		Plate 1	F12	Group 1	20	0.3185	0.125
Plate 1	D06	Group 1	10	0.2822	0.08865		Plate 1	G12	Group 1	24	0.2918	0.09825
Plate 1	E06	Group 1	14	0.3597	0.1662							
Plate 1	F06	Group 1	18	0.3197	0.1262							
Plate 1	G06	Group 1	22	0.3078	0.1143							

Appendix 8.7 *Bt* absorbance values at temperature of 35 °C incubated for 24 hrs

Results summary					Measurement results temp 35 °C@ 24hrs							
General												
Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction 1 (600nm)		Plate	Well	Group	Sample	Absorbance1 (600nm)	Blank Subtraction1 (600nm)
Plate 1	A01	Group 1	Blank1	0.1703	-0.05875		Plate 1	A07	Group 1	Blank1	0.253	0.02395
Plate 1	B01	Group 1	1	1.0833	0.8543		Plate 1	B07	Group 1	3	1.0636	0.8346
Plate 1	C01	Group 1	5	0.8841	0.6551		Plate 1	C07	Group 1	7	1.1474	0.9184
Plate 1	D01	Group 1	9	1.1426	0.9136		Plate 1	D07	Group 1	11	1.4243	1.195
Plate 1	E01	Group 1	13	0.8932	0.6642		Plate 1	E07	Group 1	15	0.7331	0.5041
Plate 1	F01	Group 1	17	1.3418	1.113		Plate 1	F07	Group 1	19	1.541	1.312
Plate 1	G01	Group 1	21	1.1122	0.8832		Plate 1	G07	Group 1	23	0.9225	0.6935
Plate 1	H01	Group 1	25	0.8855	0.6565		Plate 1	A08	Group 1	Blank1	0.2064	-0.02265
Plate 1	A02	Group 1	Blank1	0.2268	-0.00225		Plate 1	B08	Group 1	3	0.9954	0.7664
Plate 1	B02	Group 1	1	1.1774	0.9484		Plate 1	C08	Group 1	7	1.208	0.979
Plate 1	C02	Group 1	5	0.8389	0.6099		Plate 1	D08	Group 1	11	1.3973	1.168
Plate 1	D02	Group 1	9	1.4237	1.195		Plate 1	E08	Group 1	15	0.948	0.719
Plate 1	E02	Group 1	13	0.8766	0.6476		Plate 1	F08	Group 1	19	1.4046	1.176
Plate 1	F02	Group 1	17	1.3271	1.098		Plate 1	G08	Group 1	23	0.9048	0.6758
Plate 1	G02	Group 1	21	1.2125	0.9835		Plate 1	A09	Group 1	Blank1	0.2466	0.01755
Plate 1	H02	Group 1	25	0.9636	0.7346		Plate 1	B09	Group 1	3	0.8688	0.6398
Plate 1	A03	Group 1	Blank1	0.2306	0.00155		Plate 1	C09	Group 1	7	0.8663	0.6373
Plate 1	B03	Group 1	1	1.1304	0.9014		Plate 1	D09	Group 1	11	1.4622	1.233
Plate 1	C03	Group 1	5	0.9565	0.7275		Plate 1	E09	Group 1	15	1.2564	1.027
Plate 1	D03	Group 1	9	1.2433	1.014		Plate 1	F09	Group 1	19	1.6336	1.405
Plate 1	E03	Group 1	13	0.928	0.699		Plate 1	G09	Group 1	23	0.9248	0.6958
Plate 1	F03	Group 1	17	1.3527	1.124		Plate 1	A10	Group 1	Blank1	0.2389	0.00985
Plate 1	G03	Group 1	21	1.4724	1.243		Plate 1	B10	Group 1	4	0.9992	0.7702
Plate 1	H03	Group 1	25	0.9809	0.7519		Plate 1	C10	Group 1	8	1.0942	0.8652
Plate 1	A04	Group 1	Blank1	0.2366	0.00755		Plate 1	D10	Group 1	12	1.0778	0.8488
Plate 1	B04	Group 1	2	1.0966	0.8676		Plate 1	E10	Group 1	16	1.0441	0.8151
Plate 1	C04	Group 1	6	1.1141	0.8851		Plate 1	F10	Group 1	20	1.1385	0.9095
Plate 1	D04	Group 1	10	1.3399	1.111		Plate 1	G10	Group 1	24	1.05	0.821

Appendix 8.7 (*Continued)*

Plate 1	E04	Group 1	14	0.7699	0.5409		Plate 1	A11	Group 1	Blank1	0.2441	0.01505
Plate 1	F04	Group 1	18	0.6065	0.3775		Plate 1	B11	Group 1	4	0.9489	0.7199
Plate 1	G04	Group 1	22	1.229	1		Plate 1	C11	Group 1	8	0.9086	0.6796
Plate 1	A05	Group 1	Blank1	0.2456	0.01655		Plate 1	D11	Group 1	12	1.0891	0.8601
Plate 1	B05	Group 1	2	1.0681	0.8391		Plate 1	E11	Group 1	16	1.2217	0.9927
Plate 1	C05	Group 1	6	1.1792	0.9502		Plate 1	F11	Group 1	20	1.5276	1.299
Plate 1	D05	Group 1	10	1.2188	0.9898		Plate 1	G11	Group 1	24	1.1307	0.9017
Plate 1	E05	Group 1	14	0.9875	0.7585		Plate 1	A12	Group 1	Blank1	0.2082	-0.02085
Plate 1	F05	Group 1	18	0.5818	0.3528		Plate 1	B12	Group 1	4	0.7567	0.5276
Plate 1	G05	Group 1	22	1.2004	0.9714		Plate 1	C12	Group 1	8	1.0454	0.8164
Plate 1	A06	Group 1	Blank1	0.2415	0.01245		Plate 1	D12	Group 1	12	0.9071	0.6781
Plate 1	B06	Group 1	2	1.0049	0.7759		Plate 1	E12	Group 1	16	0.9728	0.7438
Plate 1	C06	Group 1	6	1.109	0.88		Plate 1	F12	Group 1	20	1.3115	1.082
Plate 1	D06	Group 1	10	1.4025	1.173		Plate 1	G12	Group 1	24	1.0049	0.7759
Plate 1	E06	Group 1	14	0.6779	0.4489							
Plate 1	F06	Group 1	18	0.4955	0.2665							
Plate 1	G06	Group 1	22	1.0911	0.8621							

Appendix 8.8 *Bt* absorbance values at temperature of 40 °C incubated for 24 hrs

Results summary					Measurement results temp 40 °C @24hrs							
General												
Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction 1 (600nm)		Plate	Well	Group	Sample	Absorbance 1 (600nm)	Blank Subtraction1 (600nm)
Plate 1	A01	Group 1	Blank1	0.1383	-0.04881		Plate 1	A07	Group 1	Blank1	0.1716	-0.01551
Plate 1	B01	Group 1	1	0.939	0.7519		Plate 1	B07	Group 1	3	0.5621	0.375
Plate 1	C01	Group 1	5	1.208	1.021		Plate 1	C07	Group 1	7	0.7702	0.5831
Plate 1	D01	Group 1	9	0.4228	0.2357		Plate 1	D07	Group 1	11	0.3793	0.1922
Plate 1	E01	Group 1	13	1.2821	1.095		Plate 1	E07	Group 1	15	1.0682	0.8811
Plate 1	F01	Group 1	17	1.2031	1.016		Plate 1	F07	Group 1	19	0.8336	0.6465
Plate 1	G01	Group 1	21	1.3517	1.165		Plate 1	G07	Group 1	23	1.2063	1.019
Plate 1	H01	Group 1	25	0.7787	0.5916		Plate 1	A08	Group 1	Blank1	0.159	-0.02811
Plate 1	A02	Group 1	Blank1	0.118	-0.06911		Plate 1	B08	Group 1	3	0.5378	0.3507
Plate 1	B02	Group 1	1	0.5433	0.3562		Plate 1	C08	Group 1	7	0.9439	0.7568
Plate 1	C02	Group 1	5	1.0992	0.9121		Plate 1	D08	Group 1	11	0.3689	0.1818
Plate 1	D02	Group 1	9	0.4005	0.2134		Plate 1	E08	Group 1	15	0.3361	0.149
Plate 1	E02	Group 1	13	1.1795	0.9924		Plate 1	F08	Group 1	19	0.937	0.7499
Plate 1	F02	Group 1	17	1.2504	1.063		Plate 1	G08	Group 1	23	0.6762	0.4891
Plate 1	G02	Group 1	21	1.3651	1.178		Plate 1	A09	Group 1	Blank1	0.1855	-0.001608
Plate 1	H02	Group 1	25	0.7181	0.531		Plate 1	B09	Group 1	3	0.3997	0.2126
Plate 1	A03	Group 1	Blank1	0.1881	0.0009917		Plate 1	C09	Group 1	7	0.6038	0.4167
Plate 1	B03	Group 1	1	1.0785	0.8914		Plate 1	D09	Group 1	11	0.4825	0.2954
Plate 1	C03	Group 1	5	1.0111	0.824		Plate 1	E09	Group 1	15	0.6612	0.4741
Plate 1	D03	Group 1	9	0.8258	0.6387		Plate 1	F09	Group 1	19	0.9603	0.7732
Plate 1	E03	Group 1	13	1.3105	1.123		Plate 1	G09	Group 1	23	1.2008	1.014
Plate 1	F03	Group 1	17	1.1101	0.923		Plate 1	A10	Group 1	Blank1	0.2469	0.05979
Plate 1	G03	Group 1	21	0.0668	-0.1203		Plate 1	B10	Group 1	4	0.2901	0.103
Plate 1	H03	Group 1	25	0.7692	0.5821		Plate 1	C10	Group 1	8	1.2376	1.05
Plate 1	A04	Group 1	Blank1	0.2116	0.02449		Plate 1	D10	Group 1	12	0.4517	0.2646
Plate 1	B04	Group 1	2	0.6884	0.5013		Plate 1	E10	Group 1	16	1.2168	1.03
Plate 1	C04	Group 1	6	1.3443	1.157		Plate 1	F10	Group 1	20	1.1848	0.9977
Plate 1	D04	Group 1	10	0.5365	0.3494		Plate 1	G10	Group 1	24	1.3036	1.116
Plate 1	E04	Group 1	14	1.5079	1.321		Plate 1	A11	Group 1	Blank1	0.2281	0.04099

Appendix 8.8 (*Continued)*

Plate 1	F04	Group 1	18	0.9138	0.7267		Plate 1	B11	Group 1	4	0.7011	0.514
Plate 1	G04	Group 1	22	1.6386	1.451		Plate 1	C11	Group 1	8	1.0752	0.8881
Plate 1	A05	Group 1	Blank1	0.2133	0.02619		Plate 1	D11	Group 1	12	0.5323	0.3452
Plate 1	B05	Group 1	2	0.5058	0.3187		Plate 1	E11	Group 1	16	1.1853	0.9982
Plate 1	C05	Group 1	6	1.2231	1.036		Plate 1	F11	Group 1	20	1.1379	0.9508
Plate 1	D05	Group 1	10	0.5689	0.3818		Plate 1	G11	Group 1	24	1.0932	0.9061
Plate 1	E05	Group 1	14	1.0909	0.9038		Plate 1	A12	Group 1	Blank1	0.2351	0.04799
Plate 1	F05	Group 1	18	0.7286	0.5415		Plate 1	B12	Group 1	4	0.4993	0.3122
Plate 1	G05	Group 1	22	1.3322	1.145		Plate 1	C12	Group 1	8	1.3516	1.164
Plate 1	A06	Group 1	Blank1	0.1498	-0.03731		Plate 1	D12	Group 1	12	0.581	0.3939
Plate 1	B06	Group 1	2	0.5683	0.3812		Plate 1	E12	Group 1	16	0.4079	0.2208
Plate 1	C06	Group 1	6	1.3779	1.191		Plate 1	F12	Group 1	20	0.5348	0.3477
Plate 1	D06	Group 1	10	0.7293	0.5422		Plate 1	G12	Group 1	24	1.5026	1.315
Plate 1	E06	Group 1	14	1.2384	1.051							
Plate 1	F06	Group 1	18	1.0924	0.9053							
Plate 1	G06	Group 1	22	1.6922	1.505							

Appendix 8. 9 Abotte's table

s.no.	*Bt* isolates		x (% living in control)	Y (% living in treated)					Difference	Significance of diff.	% control
			x	1	2	3	sum of Y	mean of y	x-y		x-y/x
1	KDL		30	2.3	3.9	3.0	9.2	3.067	20.8		69.3
2	TALS		30	4.0	5.8	6.0	15.8	5.267	14.2		47.3
3	KSS		30	5.0	6.0	7.0	18.0	6.000	12.0		40.0
4	AUPOS		30	2.5	4.0	3.0	9.5	3.167	20.5		68.3
5	TABFS		30	6.6	7.3	5.8	19.7	6.567	10.3		34.3
6	*Bt*. var. *thuringiensis*		30	2.4	3.0	2.0	7.4	2.467	22.6		75.3
7	AUASG		30	6.8	7.0	8.0	21.8	7.267	8.2		27.3
8	AUSD		30	7.8	6.5	5.0	19.3	6.433	10.7		35.7
9	AUSD-1		30	2.7	3.3	4.7	10.7	3.567	19.3		64.3
10	AUGHS-1		30	3.3	4.0	2.7	10.0	3.333	20.0		66.7
11	AUGHS-2		30	5.6	7.0	6.0	18.6	6.200	11.4		38.0
12	AUCHS		30	6.7	5.4	6.0	18.1	6.033	11.9		39.7
13	AUSD-3		30	5.8	6.9	7.9	20.6	6.867	9.4		31.3
14	AUASG-3		30	7.3	6.6	5.8	19.7	6.567	10.3		34.3
15	ZDS		30	3.4	3.6	4.0	11.0	3.667	19.0		63.3
16	ZDS-3		30	3.7	3.7	3.8	11.2	3.733	18.8		62.7
17	AUASG-1		30	7.0	5.0	8.0	20.0	6.667	10.0		33.3
18	AUWP		30	6.9	7.0	6.8	20.7	6.893	9.3		31.1
19	AUASG-2		30	2.8	3.5	5.6	11.9	3.967	18.1		60.3
20	AUDS		30	7.2	6.9	5.8	19.9	6.633	10.1		33.7
21	AUDS-1		30	6.8	5.9	7.8	20.5	6.833	9.5		31.7
22	GHTSW		30	4.2	5.3	3.2	12.7	4.233	17.3		57.7
23	GHTSW-1		30	4.7	3.9	2.9	11.5	3.833	18.5		61.7
24	AUGHS-3		30	5.3	3.0	2.7	11.0	3.667	19.0		63.3

Appendix 8.9 (*Continueded*)

Larval Mortality (hrs)																											
24hrs (R1)	24hrs (R2)	24hrs (R3)	sum	100%	48hrs(R1)	48hrs(R2)	48hrs(R3)	sum		100%	72hrs(R1)	72hrs(R2)	72hrs(R3)	sum		100%	96hrs(R1)	96hrs(R2)	96hrs(R3)	sum		100%	120hrs1	120hrs2	120hrs3	sum	Total sum	100%
3	2	4	9	30.00	4	4	3	11	20	66.67	2	3	2	7	27	90.00	1	0	2	3	30	100.00	0	0	0	0	30	100.00
0	1	1	2	6.67	1	1	2	4	6	20.00	1	2	1	4	10	33.33	1	2	2	5	15	50.00	0	0	0	0	15	50.00
2	3	2	7	23.33	2	4	5	11	18	60.00	3	1	3	7	25	83.33	1	2	1	4	29	96.67	0	1	0	1	30	100.00
3	3	2	8	26.67	4	4	3	11	19	63.33	2	2	4	8	27	90.00	0	0	0	0	27	90.00	0	1	0	1	28	93.33
0	1	0	1	3.33	1	2	2	5	6	20.00	4	0	0	4	10	33.33	0	0	1	1	11	36.67	1	2	2	5	16	53.33
3	2	2	7	23.33	3	3	5	11	18	60.00	2	0	3	5	23	76.67	0	1	1	2	25	83.33				0	25	83.33
1	0	0	1	3.33	1	1	0	2	3	10.00	1	0	1	2	5	16.67	2	1	3	6	11	36.67	2	0	1	3	14	46.67
1	0	1	2	6.67	0	1	0	1	3	10.00	0	1	1	2	5	16.67	3	1	2	6	11	36.67	0	1	1	2	13	43.33
1	3	2	6	20.00	3	3	4	10	16	53.33	1	3	0	4	20	66.67	0	1	0	1	21	70.00	0	0	0	0	21	70.00
4	2	3	9	30.00	3	4	5	12	21	70.00	2	3	1	6	27	90.00	0	1	0	1	28	93.33				0	28	93.33
1	1	2	4	13.33	1	1	2	4	8	26.67	0	1	2	3	11	36.67	0	2	1	3	14	46.67	1			1	15	50.00
1	1	0	2	6.67	2	2	1	5	7	23.33	1	2	1	4	11	36.67	1	1	0	2	13	43.33	1	1	0	2	15	50.00
1	0	3	4	13.33	0	2	1	3	7	23.33	2	2	3	7	14	46.67	1	0	0	1	15	50.00	0			0	15	50.00
0	1	0	1	3.33	2	3	4	9	10	33.33	0	2	2	4	14	46.67	0	1	1	2	16	53.33	2			2	18	60.00
3	2	3	8	26.67	1	3	4	8	16	53.33	2	3	1	6	22	73.33	0	0	0	0	22	73.33	0			0	22	73.33
4	3	2	9	30.00	3	4	2	9	18	60.00	0	1	2	3	21	70.00	1	0	1	2	23	76.67	1			1	24	80.00
2	1	2	5	16.67	1	3	1	5	10	33.33	0	0	1	1	11	36.67	0	1	1	2	13	43.33	1			1	14	46.67
2	3	1	6	20.00	2	3	4	9	15	50.00	1	3	2	6	21	70.00	0	0	0	0	21	70.00	0			0	21	70.00
3	4	2	9	30.00	3	4	3	10	19	63.33	2	2	1	5	24	80.00	0	2	0	2	26	86.67	0			0	26	86.67
2	2	1	5	16.67	0	2	3	5	10	33.33	0	1	1	2	12	40.00	0	0	1	1	13	43.33	0			0	13	43.33
1	2	3	6	20.00	1	2	2	5	11	36.67	4	2	3	9	20	66.67	1	2	1	4	24	80.00	0			0	24	80.00
3	2	4	9	30.00	3	4	2	9	18	60.00	1	3	2	6	24	80.00	0	1	1	2	26	86.67	0			0	26	86.67
2	3	2	7	23.33	5	3	2	10	17	56.67	1	2	3	6	23	76.67	0	0	1	1	24	80.00	0			0	24	80.00
4	1	2	7	23.33	3	2	3	8	15	50.00	0	2	1	3	18	60.00	3	1	2	6	24	80.00	1			1	25	83.33

Appendix 8. 10 Analysis of variance table for determination of LT_{50} values of *Bt* isolates

Bt.* Var. *thuringiensis

Variable	Coefficient	Std. Error	Wald	P
hrs	0.18621	0.011208	20.0714	<0.0001
Constant	-3.31848	0.50643	20.5451	<0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.10	20.19161	0.61114	28.96549
0.20	28.95250	15.41529	36.06855
0.25	32.28081	20.77169	39.03480
0.50	45.71279	38.95195	54.44210
0.75	59.14476	51.26371	75.71791
0.80	62.47307	53.89935	81.40492
0.90	71.23396	60.53322	96.67827
0.95	78.46886	65.82572	109.47712
0.975	84.74406	70.34223	120.65216
0.980	86.61172	71.67732	123.98728
0.990	92.04033	75.54006	133.69915

KDL

Variable	Coefficient	Std. Error	Wald	P
hrs	0.16818	0.010536	16.5154	<0.0001
Constant	-2.86233	0.48580	18.2672	<0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.10	18.56173	-6.76431	28.73412
0.20	28.83617	12.10175	36.92521
0.25	32.73948	18.88404	40.42204
0.50	48.49198	40.83337	59.95604
0.75	64.24449	54.58845	87.68428
0.80	68.14780	57.54014	95.01172
0.90	78.42224	65.00576	114.60325
0.95	86.90705	70.98782	130.96546
0.975	94.26636	76.10397	145.22961
0.980	96.45667	77.61772	149.48394
0.990	102.82314	82.00016	161.86723

Appendix 8.10 (*Continued*)

AUPOS

Variable	Coefficient	Std. Error	Wald	P
hrs	0.18331	0.012619	21.3581	<0.0001
Constant	-3.92803	0.60056	23.6245	<0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.05	21.84912	3.07003	30.42598
0.10	28.07888	13.43160	35.33343
0.20	35.62264	25.42341	41.83122
0.25	38.48854	29.68573	44.59318
0.50	50.05443	43.94586	58.68080
0.75	61.62032	54.22080	76.75362
0.80	64.48623	56.49480	81.50390
0.90	72.02998	62.25868	94.22962
0.95	78.25975	66.87487	104.88245
0.975	83.66314	70.81835	114.18257
0.980	85.27132	71.98434	116.95821
0.990	89.94575	75.35817	125.04129

AUSD-1

Variable	Coefficient	Std. Error	Wald	P
hrs	0.15275	0.010109	24.2256	<0.0001
Constant	-3.22480	0.53294	26.4679	<0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.010	8.34971	-19.77074	21.28533
0.020	13.82838	-10.90225	25.39722
0.025	15.71326	-7.86325	26.82399
0.05	22.04632	2.28403	31.68137
0.10	29.34793	13.79543	37.46938
0.20	38.18961	27.19705	45.01598
0.25	41.54860	32.00679	48.16456
0.50	55.10444	48.49743	63.79120
0.75	68.66027	60.71102	83.69489
0.80	72.01926	63.42570	88.93853
0.90	80.86094	70.31521	102.99724
0.95	88.16256	75.83944	114.77242
0.975	94.49562	80.56190	125.05462
0.980	96.38050	81.95869	128.12360
0.990	101.85917	86.00129	137.06138

Appendix 8.10 (*Continued*)

AUGHS-1

Variable	Coefficient	Std. Error	Wald	P
hrs	0.13919	0.010595	15.1213	0.0001
Constant	-2.00067	0.48615	18.3525	<0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.10	19.44489	-7.03635	29.81005
0.20	30.12298	13.03121	38.39203
0.25	34.17964	20.18469	42.12265
0.50	50.55100	42.61865	63.61343
0.75	66.92237	56.48660	93.67021
0.80	70.97903	59.49747	101.54343
0.90	81.65711	67.14033	122.55011
0.95	90.47526	73.28015	140.06958
0.975	98.12369	78.53691	155.33373
0.980	100.40006	80.09292	159.88527
0.990	107.01665	84.59890	173.13172

ZDS

Variable	Coefficient	Std. Error	Wald	P
hrs	0.11598	0.0084525	20.1976	<0.0001
Constant	-2.28847	0.47659	24.9268	<0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.10	28.90240	7.41597	38.93491
0.20	40.48346	26.35482	48.56997
0.25	44.88315	33.08379	52.69635
0.50	62.63892	54.80838	74.78055
0.75	80.39470	69.81613	103.58158
0.80	84.79439	73.17818	111.07488
0.90	96.37544	81.77192	131.05506
0.95	105.93928	88.70873	147.71509
0.975	114.23448	94.65989	162.23065
0.980	116.70335	96.42286	166.55910
0.990	123.87944	101.53089	179.15666

Appendix 8.10 (*Continued*)

ZDS-3

Variable	Coefficient	Std. Error	Wald	P
hrs	0.10908	0.0096540	20.8489	<0.0001
Constant	-2.47297	0.56981	26.4581	<0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.05	29.17639	6.38666	39.47395
0.10	37.41811	20.15079	45.94086
0.20	47.39819	35.96957	54.62030
0.25	51.18967	41.49865	58.39821
0.50	66.49087	59.23582	78.22106
0.75	81.79207	72.03315	102.98376
0.80	85.58355	74.93700	109.38689
0.90	95.56363	82.37356	126.44855
0.95	103.80536	88.38087	140.67229
0.975	110.95383	93.53475	153.06583
0.980	113.08139	95.06144	156.76170
0.990	119.26546	99.48448	167.51876

AUASG-2

Variable	Coefficient	Std. Error	Wald	P
hrs	0.15304	0.0046403	20.4892	<0.0001
Constant	-4.11197	0.30197	14.9859	0.0001

Hours-Response table

Probability	hrs	95% Confidence interval	
0.10	-5.35999	-31.29600	20.57603
0.20	15.58483	-9.75190	40.92157
0.25	23.54186	-1.62567	48.70939
0.50	61.09391	55.36619	68.02164
0.75	87.76597	62.71650	112.81543
0.80	95.72300	70.53267	120.91332
0.90	116.66782	90.94987	142.38576
0.95	133.96441	107.64731	160.28151
0.975	148.96662	122.01813	175.91511
0.980	153.43167	126.27632	180.58701
0.990	166.40994	138.60667	194.21321

Appendix 8.11 *H. armigera* larval mortality at different *Bt* concentration and time in hours

Bt isolates	Conc	larvae	24hrs(R1)	24hrs(R2)	24hrs(R3)	sum	48hrs(R1)	48hrs(R2)	48hrs(R3)	sum	sum48	72hrs(R1)	72hrs(R2)	72hrs(R3)	sum	sum72	96hrs(R1)	96hrs(R2)	96hrs(R3)	sum	sum96	120hrs(R1)	120hrs(R2)	120hrs(R3)	sum	sum120	lcon	ltrt	%cont
KDL	0.25	30	2	1	1	4	1	2	2	5	9	0	1	2	3	12	1	0	0	1	13	0	0	0	0	13	30	17	43.33
	0.50	30	1	2	2	5	2	1	2	5	10	1	0	2	3	13	1	0	0	1	14	0	0	0	0	14	30	16	46.67
	1.00	30	2	3	2	7	2	4	3	9	16	2	0	2	4	20	2	1	0	3	23	0	0	0	0	23	30	7	76.67
	1.50	30	3	4	2	9	3	5	4	12	21	2	1	3	6	27	0	0	0	0	27	0	0	1	1	28	30	2	93.33
	2.00	30	3	4	3	10	4	5	4	13	23	1	1	3	5	28	0	0	0	0	28	0	0	0	0	28	30	2	93.33
AUPOS	0.25	30	2	1	1	4	1	3	2	6	10	0	1	2	3	13	1	0	0	1	14	0	0	0	0	14	30	16	46.67
	0.50	30	1	2	2	5	2	1	3	6	11	1	0	2	3	14	1	0	0	1	15	0	0	0	0	15	30	15	50.00
	1.00	30	2	3	2	7	2	4	3	9	16	2	0	2	4	20	2	1	0	3	23	0	0	0	0	23	30	7	76.67
	1.50	30	3	3	2	8	3	5	4	12	20	2	1	3	6	26	0	0	0	0	26	0	0	1	1	27	30	3	90.00
	2.00	30	2	3	3	8	4	6	5	15	23	1	1	2	4	27	0	0	0	0	27	0	0	0	0	27	30	3	90.00
Bt.var.thuringiensis	0.25	30	2	2	1	5	1	2	2	5	10	0	1	2	2	12	1	0	0	1	13	0	1	0	1	14	30	16	46.67
	0.50	30	1	2	3	6	2	1	2	5	11	1	0	2	3	14	1	0	0	1	15	0	0	0	0	15	30	15	50.00
	1.00	30	2	2	4	8	2	4	3	9	17	2	0	2	4	21	2	1	0	3	24	0	0	0	0	24	30	6	80.00
	1.50	30	2	3	2	7	4	5	4	13	20	2	1	3	6	26	0	0	0	0	26	0	0	1	0	26	30	4	86.67
	2.00	30	3	4	3	10	4	5	4	13	23	1	1	3	5	28	0	0	0	0	28	0	0	0	0	28	30	2	93.33
AUSD-1	0.25	30	2	1	1	4	1	2	2	5	9	0	1	2	3	12	1	0	0	1	13	0	1	0	1	14	30	16	46.67
	0.50	30	1	2	2	5	2	1	2	5	10	1	0	2	3	13	1	0	0	1	14	0	0	0	0	14	30	16	46.67
	1.00	30	2	3	2	7	2	4	3	9	16	2	0	2	4	20	2	1	0	3	23	0	0	0	0	23	30	7	76.67
	1.50	30	3	2	2	7	3	5	4	12	19	2	1	3	6	25	0	0	0	0	25	0	0	1	1	26	30	4	86.67
	2.00	30	3	4	3	10	4	5	5	14	24	1	1	2	4	28	0	0	0	0	28	0	0	0	0	28	30	2	93.33
AUGHS-1	0.25	30	2	1	1	4	1	2	2	5	9	0	1	2	3	12	1	0	0	1	13	0	1	0	1	14	30	16	46.67
	0.50	30	1	2	2	5	2	1	2	5	10	1	0	2	3	13	1	0	0	1	14	0	0	0	0	14	30	16	46.67
	1.00	30	2	3	2	7	2	4	3	9	16	2	0	2	4	20	2	1	0	3	23	0	0	0	0	23	30	7	76.67
	1.50	30	3	5	2	10	3	3	4	10	20	2	2	3	7	27	0	0	0	0	27	0	0	1	1	28	30	2	93.33
	2.00	30	3	4	3	10	4	5	4	13	23	1	1	3	5	28	0	0	0	0	28	0	0	0	0	28	30	2	93.33

Appendix 8.11 (*Continued*)

ZDS-3	0.25	30	2	1	1	4	1	2	2	5	9	0	1	2	2	11	1	0	0	1	12	0	1	0	1	13	30	17	43.33
	0.50	30	1	2	2	5	2	1	2	5	10	1	2	2	5	15	1	0	0	1	16	0	0	0	0	16	30	14	53.33
	1.00	30	2	3	2	7	2	4	3	9	16	2	1	2	5	21	2	1	0	3	24	0	0	0	0	24	30	6	80.00
	1.50	30	2	3	2	7	3	5	4	12	19	2	1	2	5	24	0	0	0	0	24	0	0	1	1	25	30	5	83.33
	2.00	30	3	4	3	10	4	5	4	13	23	1	1	3	5	28	0	0	0	0	28	0	0	0	0	28	30	2	93.33
AUASG-2	0.25	30	2	1	1	4	1	2	2	5	9	0	1	2	3	12	1	0	0	1	13	0	1	0	1	14	30	16	46.67
	0.50	30	1	2	3	6	2	1	3	6	12	1	0	2	3	15	1	0	0	1	16	0	0	0	0	16	30	14	53.33
	1.00	30	4	3	2	9	2	4	3	9	18	2	0	2	4	22	2	1	0	3	25	0	0	0	0	25	30	5	83.33
	1.50	30	3	4	2	9	3	5	4	12	21	2	1	2	5	26	0	0	0	0	26	0	0	1	1	27	30	3	90.00
	2.00	30	3	4	3	10	4	3	4	11	21	1	1	3	5	26	0	0	0	0	26	0	0	0	0	26	30	4	86.67
GHTSW	0.25	30	1	1	3	5	1	2	2	5	10	0	1	2	3	13	1	0	0	1	14	0	1	0	1	15	30	15	50.00
	0.50	30	1	3	2	6	2	1	3	6	12	1	0	2	3	15	1	0	0	1	16	0	0	0	0	16	30	14	53.33
	1.00	30	2	3	2	7	2	3	3	8	15	2	3	2	7	22	2	1	0	3	25	0	0	0	0	25	30	5	83.33
	1.50	30	3	2	4	9	3	5	3	11	20	2	1	2	5	25	0	0	0	0	25	0	0	1	1	26	30	4	86.67
	2.00	30	3	4	3	10	4	3	4	11	21	1	1	3	5	26	0	0	0	0	26	0	0	0	0	26	30	4	86.67
GHTSW-1	0.25	30	2	1	1	4	1	2	2	5	9	0	1	2	3	12	1	0	0	1	13	0	1	0	1	14	30	16	46.67
	0.50	30	1	2	2	5	2	1	2	5	10	1	0	2	3	13	1	0	0	1	14	0	0	0	0	14	30	16	46.67
	1.00	30	2	3	2	7	2	4	3	9	16	2	0	2	4	20	2	1	0	3	23	0	0	0	0	23	30	7	76.67
	1.50	30	3	3	2	8	3	5	4	12	20	2	1	3	6	26	0	0	0	0	26	0	0	1	1	27	30	3	90.00
	2.00	30	3	3	3	9	4	6	4	14	23	1	1	3	5	28	0	0	0	0	28	0	0	0	0	28	30	2	93.33
AUGHS-3	0.25	30	0	1	1	2	1	2	2	5	7	0	1	2	3	10	1	0	0	1	11	0	1	0	1	12	30	18	40.00
	0.50	30	1	2	0	3	2	1	2	5	8	1	0	2	3	11	1	0	0	1	12	0	0	0	0	12	30	18	40.00
	1.00	30	2	1	1	4	2	4	3	9	13	2	0	2	4	17	2	1	0	3	20	0	0	0	0	20	30	10	66.67
	1.50	30	2	2	1	5	3	5	4	12	17	2	1	3	6	23	0	0	0	0	23	0	0	1	1	24	30	6	80.00
	2.00	30	2	3	3	8	4	5	4	13	21	1	1	3	5	26	0	0	0	0	26	0	0	0	0	26	30	4	86.67

Appendix 8.12 Analysis of variance tables for dose-reponse variables

Bacillus thuringiensis.* var. *thuringiensis

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	killed

Sample size	150
Positive cases	86 (57.33%)
Negative cases	64 (42.67%)

Overall Model Fit

Null model -2 Log Likelihood	204.706
Full model -2 Log Likelihood	139.681
Chi-squared	65.025
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.3518
Nagalkerke R^2	0.4725

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.57220	0.22627	48.2808	<0.0001
Constant	-1.33060	0.23434	32.2414	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.20	0.31101	0.023915	0.49868
0.25	0.41732	0.16236	0.59096
0.50	0.84633	0.68383	1.00067
0.75	1.27534	1.11206	1.50361
0.80	1.38164	1.20671	1.63970
0.90	1.66146	1.44518	2.00858
0.95	1.89254	1.63545	2.31986

Dependent Y	killed
Independent X	con

Least squares regression

Sample size	15
Coefficient of determination R^2	0.8911
Residual standard deviation	1.1319

Regression Equation

y = 0.7907 + 4.7073 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	0.7907	0.5613	-0.4221 to 2.0034	1.4085	0.1825
Slope	4.7073	0.4564	3.7212 to 5.6934	10.3131	<0.0001

Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	136.2768	136.2768
Residual	13	16.6565	1.2813

F-ratio	106.3608
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.8121)

KDL

Dose variable	con
Variable for number of cases	larvae
Variable for number of positive cases	Dead

Sample size	150
Positive cases	86 (57.33%)
Negative cases	64 (42.67%)

Overall Model Fit

Null model -2 Log Likelihood	204.706
Full model -2 Log Likelihood	126.209
Chi-squared	78.497
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.4074
Nagalkerke R^2	0.5472

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.84269	0.25748	51.2165	<0.0001
Constant	-1.55684	0.25240	38.0458	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.14940	-0.15510	0.34333
0.20	0.38814	0.15540	0.54756
0.25	0.47884	0.27046	0.62805
0.50	0.84487	0.70281	0.98487
0.75	1.21091	1.06424	1.41263
0.80	1.30161	1.14537	1.52704
0.90	1.54035	1.35063	1.83652
0.95	1.73751	1.51471	2.09752

Dependent Y	Dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9378
Residual standard deviation	1.3427

Regression Equation

y = 0.3374 + 5.1415 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	0.3374	0.3977	-0.6936 to 1.3683	0.8483	0.4116
Slope	5.1415	0.3672	4.3484 to 5.9347	14.0039	<0.0001

Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	353.5668	353.5668
Residual	13	23.4378	1.8029

F-ratio	196.1091
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.5970)

AUPOS

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	killed

Sample size	150
Positive cases	76 (50.67%)
Negative cases	74 (49.33%)

Overall Model Fit

Null model -2 Log Likelihood	207.917
Full model -2 Log Likelihood	142.579
Chi-squared	65.339
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.3531
Nagalkerke R^2	0.4709

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.51676	0.21163	51.3652	<0.0001
Constant	-1.53150	0.24017	40.6621	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.16479	-0.19523	0.39081
0.20	0.45483	0.18502	0.63761
0.25	0.56502	0.32571	0.73513
0.50	1.00971	0.85004	1.17217
0.75	1.45440	1.28065	1.70293
0.80	1.56459	1.37734	1.84446
0.90	1.85464	1.62285	2.22599
0.95	2.09416	1.81988	2.54679

Dependent Y	killed
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9237
Residual standard deviation	1.2402

Regression Equation

y = 0.03467 + 4.7904 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	0.03467	0.4059	-0.9635 to 1.0329	0.08543	0.9332
Slope	4.7904	0.3819	3.9654 to 5.6154	12.5444	<0.0001

Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	242.0195	242.0195
Residual	13	19.9937	1.5380

F-ratio	157.3624
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.3476)

AUSD-1

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	75 (50.00%)
Negative cases	75 (50.00%)

Overall Model Fit

Null model -2 Log Likelihood	207.944
Full model -2 Log Likelihood	128.799
Chi-squared	79.145
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.4100
Nagalkerke R^2	0.5467

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.73959	0.22896	57.7286	<0.0001
Constant	-1.78602	0.25969	47.2985	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.28999	-0.0047575	0.48334
0.20	0.54289	0.31804	0.70281
0.25	0.63896	0.43725	0.78960
0.50	1.02669	0.88274	1.17548
0.75	1.41442	1.25757	1.63202
0.80	1.51050	1.34280	1.75279
0.90	1.76339	1.55971	2.07814
0.95	1.97223	1.73390	2.35175

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9326
Residual standard deviation	1.3084

Regression Equation

y = -0.5830 + 5.3221 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	-0.5830	0.3607	-1.5394 to 0.3734	-1.6163	0.1300
Slope	5.3221	0.3969	4.4648 to 6.1795	13.4104	<0.0001

Analysis of Variance

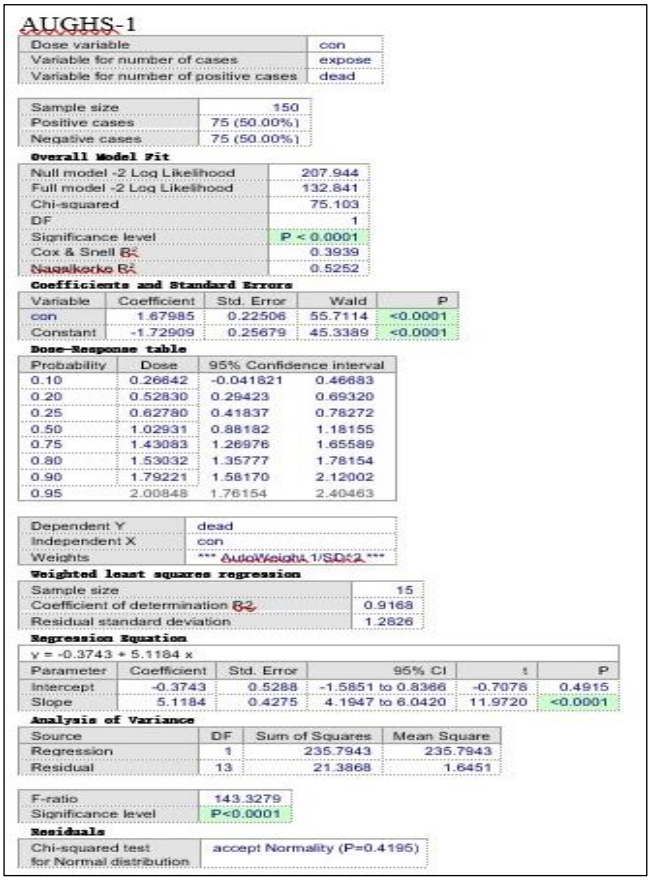
Source	DF	Sum of Squares	Mean Square
Regression	1	307.8615	307.8615
Residual	13	22.2544	1.7119

F-ratio	179.8384
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.3976)

Appendix 8.12 (*Continued*)



AUGHS-1

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	75 (50.00%)
Negative cases	75 (50.00%)

Overall Model Fit

Null model -2 Log Likelihood	207.944
Full model -2 Log Likelihood	132.841
Chi-squared	75.103
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.3939
Nagalkerke R^2	0.5252

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.67985	0.22506	55.7114	<0.0001
Constant	-1.72909	0.25679	45.3389	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.26642	-0.041821	0.46683
0.20	0.52830	0.29423	0.69320
0.25	0.62780	0.41837	0.78272
0.50	1.02931	0.88182	1.18155
0.75	1.43083	1.26976	1.65589
0.80	1.53032	1.35777	1.78154
0.90	1.79221	1.58170	2.12002
0.95	2.00848	1.76154	2.40463

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9168
Residual standard deviation	1.2826

Regression Equation

y = -0.3743 + 5.1184 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	-0.3743	0.5288	-1.5851 to 0.8366	-0.7078	0.4915
Slope	5.1184	0.4275	4.1947 to 6.0420	11.9720	<0.0001

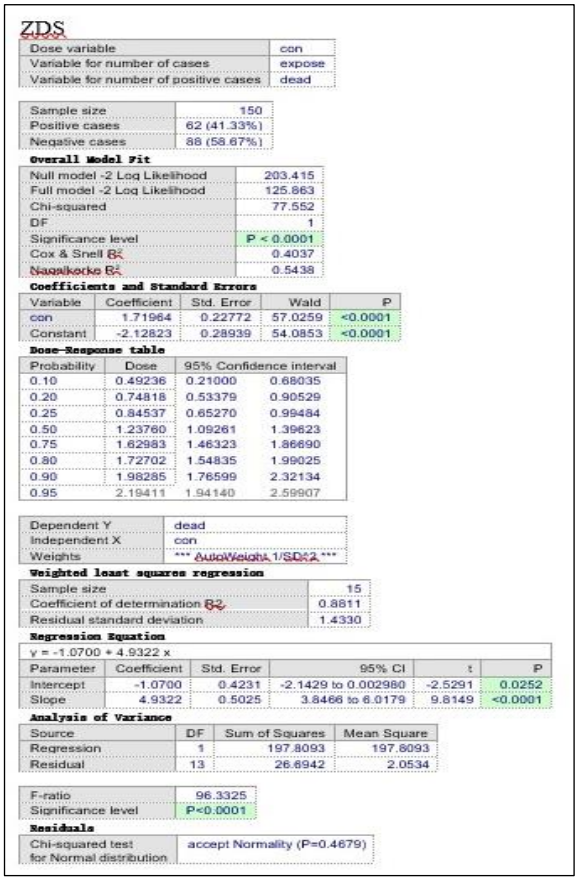
Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	235.7943	235.7943
Residual	13	21.3868	1.6451

F-ratio	143.3279
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.4195)



ZDS

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	62 (41.33%)
Negative cases	88 (58.67%)

Overall Model Fit

Null model -2 Log Likelihood	203.415
Full model -2 Log Likelihood	125.863
Chi-squared	77.552
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.4037
Nagalkerke R^2	0.5438

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.71964	0.22772	57.0259	<0.0001
Constant	-2.12823	0.28939	54.0853	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.49236	0.21000	0.68035
0.20	0.74818	0.53379	0.90529
0.25	0.84537	0.65270	0.99484
0.50	1.23760	1.09261	1.39623
0.75	1.62983	1.46323	1.86690
0.80	1.72702	1.54835	1.99025
0.90	1.98285	1.76599	2.32134
0.95	2.19411	1.94140	2.59907

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.8811
Residual standard deviation	1.4330

Regression Equation

y = -1.0700 + 4.9322 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	-1.0700	0.4231	-2.1429 to 0.002980	-2.5291	0.0252
Slope	4.9322	0.5025	3.8466 to 6.0179	9.8149	<0.0001

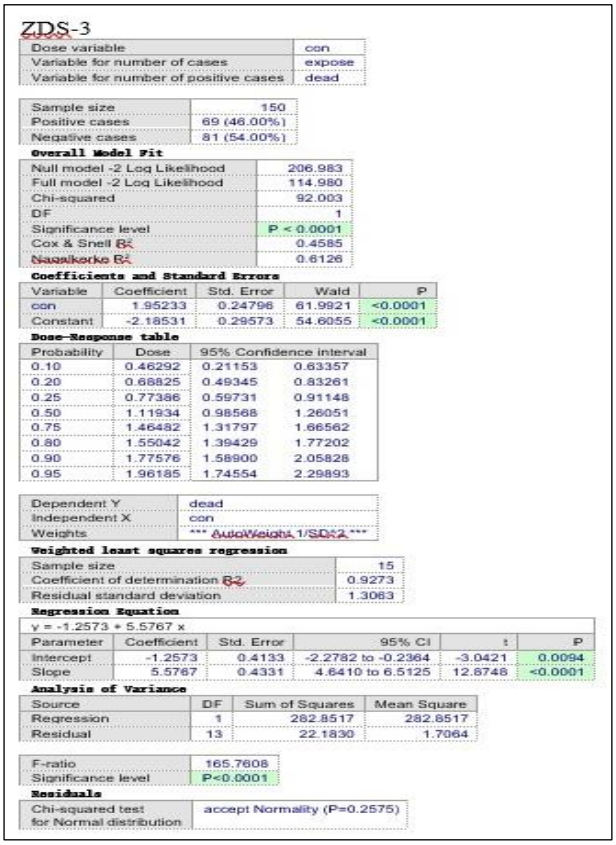
Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	197.8093	197.8093
Residual	13	26.6942	2.0534

F-ratio	96.3325
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.4679)



ZDS-3

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	69 (46.00%)
Negative cases	81 (54.00%)

Overall Model Fit

Null model -2 Log Likelihood	206.983
Full model -2 Log Likelihood	114.980
Chi-squared	92.003
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.4585
Nagalkerke R^2	0.6126

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.95233	0.24796	61.9921	<0.0001
Constant	-2.18531	0.29573	54.6055	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.46292	0.21153	0.63357
0.20	0.68825	0.49345	0.83261
0.25	0.77386	0.59731	0.91148
0.50	1.11934	0.98568	1.26051
0.75	1.46482	1.31797	1.66562
0.80	1.55042	1.39429	1.77202
0.90	1.77576	1.58900	2.05828
0.95	1.96185	1.74554	2.29893

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9273
Residual standard deviation	1.3083

Regression Equation

y = -1.2573 + 5.5767 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	-1.2573	0.4133	-2.2782 to -0.2364	-3.0421	0.0094
Slope	5.5767	0.4331	4.6410 to 6.5125	12.8748	<0.0001

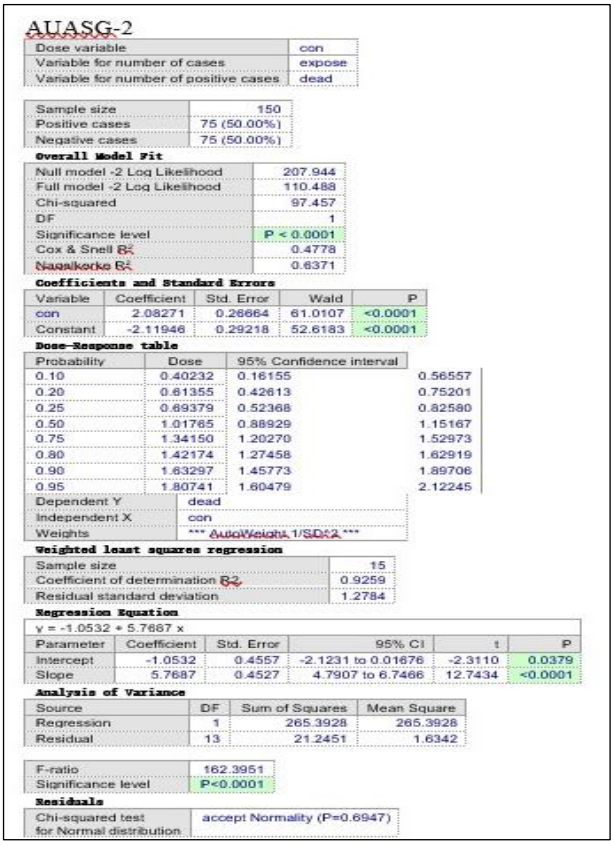
Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	282.8517	282.8517
Residual	13	22.1830	1.7064

F-ratio	165.7608
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.2575)



AUASG-2

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	75 (50.00%)
Negative cases	75 (50.00%)

Overall Model Fit

Null model -2 Log Likelihood	207.944
Full model -2 Log Likelihood	110.488
Chi-squared	97.457
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.4778
Nagalkerke R^2	0.6371

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	2.08271	0.28664	61.0107	<0.0001
Constant	-2.11946	0.29218	52.6183	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.40232	0.16155	0.56557
0.20	0.61355	0.42613	0.75201
0.25	0.69379	0.52368	0.82580
0.50	1.01765	0.88929	1.15167
0.75	1.34150	1.20270	1.52973
0.80	1.42174	1.27458	1.62919
0.90	1.63297	1.45773	1.89706
0.95	1.80741	1.60479	2.12245

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9259
Residual standard deviation	1.2784

Regression Equation

y = -1.0532 + 5.7687 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	-1.0532	0.4557	-2.1231 to 0.01676	-2.3110	0.0379
Slope	5.7687	0.4527	4.7907 to 6.7466	12.7434	<0.0001

Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	265.3928	265.3928
Residual	13	21.2451	1.6342

F-ratio	162.3951
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.6947)

Appendix 8.12 (*Continued*)

GHTSW

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	82 (54.67%)
Negative cases	68 (45.33%)

Overall Model Fit

Null model -2 Log Likelihood	206.636
Full model -2 Log Likelihood	132.860
Chi-squared	73.775
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.3885
Nagelkerke R^2	0.5195

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.69564	0.23280	53.0523	<0.0001
Constant	-1.54330	0.24506	39.6605	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.20	0.41381	0.16663	0.58293
0.25	0.51238	0.29165	0.67031
0.50	0.91016	0.76078	1.05835
0.75	1.30794	1.15151	1.52481
0.80	1.40650	1.23925	1.64946
0.90	1.66595	1.46151	1.98630
0.95	1.88020	1.63941	2.27010

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9160
Residual standard deviation	1.3005

Regression Equation

y = 0.1626 + 5.0523 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	0.1626	0.4706	-0.9500 to 1.2752	0.3455	0.7353
Slope	5.0523	0.4243	4.1355 to 5.9690	11.9061	<0.0001

Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	239.7608	239.7608
Residual	13	21.9877	1.6914

F-ratio	141.7563
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.2967)

AHTSW-1

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	70 (46.67%)
Negative cases	80 (53.33%)

Overall Model Fit

Null model -2 Log Likelihood	207.277
Full model -2 Log Likelihood	115.680
Chi-squared	91.597
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.4570
Nagelkerke R^2	0.6102

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.94613	0.24745	61.8530	<0.0001
Constant	-2.15086	0.29307	53.8606	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.44669	0.19393	0.61802
0.20	0.67274	0.47698	0.81751
0.25	0.75862	0.58127	0.89654
0.50	1.10520	0.97136	1.24627
0.75	1.45178	1.30495	1.65250
0.80	1.53766	1.38152	1.75925
0.90	1.76371	1.57685	2.04646
0.95	1.95039	1.73388	2.28792

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9367
Residual standard deviation	1.3222

Regression Equation

y = -1.2311 + 5.6189 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	-1.2311	0.3863	-2.2264 to -0.2358	-3.1867	0.0071
Slope	5.6189	0.4051	4.7437 to 6.4941	13.8695	<0.0001

Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	336.2970	336.2970
Residual	13	22.7270	1.7482

F-ratio	192.3641
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.6259)

AUGHS-3

Dose variable	con
Variable for number of cases	expose
Variable for number of positive cases	dead

Sample size	150
Positive cases	69 (46.00%)
Negative cases	81 (54.00%)

Overall Model Fit

Null model -2 Log Likelihood	206.983
Full model -2 Log Likelihood	131.865
Chi-squared	75.118
DF	1
Significance level	P < 0.0001
Cox & Snell R^2	0.3939
Nagelkerke R^2	0.5264

Coefficients and Standard Errors

Variable	Coefficient	Std. Error	Wald	P
con	1.66714	0.22189	56.4506	<0.0001
Constant	-1.86924	0.26438	49.9897	<0.0001

Dose-Response table

Probability	Dose	95% Confidence interval	
0.10	0.35251	0.052031	0.54977
0.20	0.61639	0.38869	0.77941
0.25	0.71664	0.51275	0.87049
0.50	1.12122	0.97377	1.27771
0.75	1.52580	1.36025	1.75947
0.80	1.62605	1.44844	1.88643
0.90	1.88993	1.67337	2.22780
0.95	2.10785	1.85436	2.51447

Dependent Y	dead
Independent X	con
Weights	*** AutoWeight 1/SD^2 ***

Weighted least squares regression

Sample size	15
Coefficient of determination R^2	0.9302
Residual standard deviation	1.2203

Regression Equation

y = -0.7424 + 5.0864 x

Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	-0.7424	0.4240	-1.7612 to 0.2763	-1.7509	0.1035
Slope	5.0864	0.3863	4.2519 to 5.9210	13.1668	<0.0001

Analysis of Variance

Source	DF	Sum of Squares	Mean Square
Regression	1	258.1662	258.1662
Residual	13	19.3589	1.4891

F-ratio	173.3651
Significance level	P<0.0001

Residuals

Chi-squared test for Normal distribution	accept Normality (P=0.6367)