BIOCONTROL AGAINST INSECT PESTS BY *Bacillus thuringiensis* ISOLATED FROM ORGANIC AGRICULTURAL FARM

A

Thesis Dissertation Submitted to

Department of Microbiology

Central Campus of Technology, Tribhuvan University, Dharan, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Masters of Science in **(Agriculture)**

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ABSTRACT

Insects are a significant factor in the destruction of agricultural crops. The management must be done in such a way that is free of potentially dangerous chemicals and that remains environmentally benign. *Bacillus thuringiensis* is a soil bacterium whose main characteristic is the development of parasporal crystalline inclusions that contain the crystal protein which possesses insecticidal effects. The study's primary objective is to evaluate the insecticidal activity of Bt as a biocontrol agent against insects pests. A total of 60 soil samples were randomly taken from four organic farms in Dharan, Tarahara, Itahari and Jhumka, Nepal. The bacteria were isolated using sodium acetate selection method and were conventionally identified to determine whether the isolates were Bt. Four isolates namely SR1, SI7, ST9 and SJ11 displayed the characteristics of Bt which were bioassayed against two insect pests, *Spodoptera frugiperda* and *Myzus persicae*. Bioassay was carried out by preparing crystal/spore mixture (1mg/ml) and feeding it to the insects which were grown in the laboratory by placing them in the disposable plastic glass and directly dipping maize and bean leaves before being given to the appropriate insect. All the four isolates showed insecticidal activity against the insect pest and gave positive result with great potentiality to infect and manage *S. frugiperda* and *M. persicae*. The mean difference between treatment and control was statistically significant ($p>0.05$). The findings showed that the use of living organisms to control pests by implementing bioactive agents or microorganisms that are entomopathogenic to insect pests is an appealing course of action and a sustainable approach for crop protection because they degrade quickly, resulting in fewer exposures and primarily preventing pollution issues brought on by synthetic pesticides.

Keywords:*Bacillus thuringiensis*, entomopathogen, bioassay, insecticidal activity, crystal/spore, efficacy

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LIST OF ABBREVIATIONS

B. thuringiensis: *Bacillus thuringiensis*

S. frugiperda: *Spodoptera frugiperda*

M. persicae: *Myzus persicae*

BD: Bacterial death

OD: Other reason death

S: Survival

IPM: Integrated Pest Management

EPM: Entomopathogenic microorganism

EPB: Entomopathogenic bacteria

RARS: Regional Agricultural Research Station

SR1: Isolated *Bacillus thuringiensis* SR1

SI7: Isolated *Bacillus thuringiensis* SI7

ST9: Isolated *Bacillus thuringiensis* ST9

SJ11: Isolated *Bacillus thuringiensis* SJ11

LB: Luria-Bertani

NB: Nutrient Broth

FAW: Fall armyworm

MR-VP: Methyl Red and Voges-Prauskauer

SIM: Sulphide-indole-motility

HSD: Honest Significant Difference

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CHAPTER I

INTRODUCTION

1.1 Background

Agriculture pests include weeds, arthropods (mostly insects and mites), molluscs (slugs and snails), and a few vertebrates in addition to plant pathogens (fungi, oomycetes, bacteria, viruses and nematodes). They lower a product's output and quality by eating crops. Millions of pest species are thought to exist worldwide. They significantly affect an agriculture output restriction that has caused a 40% decrease in the possible world crop yields as a result of these setbacks (Mantzoukas& Eliopoulos, 2020). Numerous naturally occurring bacteria, fungi, nematodes, and viruses that infect a variety of organisms are hosted by mites and other insect pests. Insect pests are regarded as significant deterrents because they are thought to be responsible for 10.80% of global agriculture losses in the post-green revolution era (Dhaliwal *et al*., 2015). Because of their ease of use, great utility, and timeliness, insecticides are employed to minimize these losses and have subsequently established themselves as a crucial technique for managing insect pest infestation (Sharma, 2019). However, in up to 80% of instances, the intensive application of pesticides has led to the development of resistance to one or more kind of insecticides.

Microorganisms that are pathogenic to insect pest and mites are known as entomopathogens. Many types of naturally occurring bacteria, fungi, nematodes, and viruses infect a range of insect pests and are crucial to managing them. Some entomopathogens are manufactured in large quantities in vivo (nematodes and viruses) or in vitro(bacteria, fungi, and nematodes) and sold commercially. Therefore, entomopathogens, which include bacteria, viruses, protozoa, and fungi, are viewed as controlling pest infestations. Natural entomopathogens play a significant role in controlling insect populations (Roy & Cottrell, 2008). The families Bacillaceae.Pseudomonadaceae, Enterobacteraceae, Streptococcaceae, and

Micrococcaceae contain the vast majority of bacterial infections in insects. However, the Bacillaceae have received the majority of attention (Bahadur, 2018).

In general, biological control techniques including the use of bacterial entomopathogens are regarded to be safer than using chemical pesticides and offer a number of advantages. Because it doesn't leave behind any chemical residues that could harm people or other organism, bio-control is frequently seen as a progressive and environmentally friendly method of eradicating pest organism. When it works, it can also provide broadly applicable, essentially permanent control with an excellent cost-benefit ratio. For instance, their mode of action is often more complicated than that of conventional pesticides, targeting a variety of locations where resistantbugs are more likely to evolve (Ruiu, 2015). Since entomopathogenic bacteria can be employed as an independent pest management technique, for optimal effectiveness and environmental sustainability, they are best used in rotation or in conjunction with insecticides. Numerous investigations have shown that chemical substances and entomopathogenic microbes can coexist as well as function cooperatively (Musser *et al*., 2006). The further benefits of using bio-pesticides in pest management programs includes crop residues reduction, and harvest flexibility due to short or nonexistent pre-harvest time.

Bacillus thuringiensis is a Gram-positive, rod-shaped, motile, facultatively anaerobic, and spore-forming bacterium that is proven to be a bio-control agent against pests (Fernando *et al*., 2010). *B. thuringiensis* is an omnipresent bacterium that originates in numerous ecological habitats essentially soil, sediment, stored products, dust, dead insects, phylloplane and aquatic environment (Iriarte *et al*., 2000). *B. thuringiensis* is usually referred to as soil-dwelling bacterium that can act as opportunistic pathogen under suitable circumstances, because of the abundance of its spores in the environment. The entomocidal bacterium *B. thuringiensis* has been the topic of most of the investigation and implementation in terms of biological control of phyto-pathogenic insects, mostly because of its entomotoxic properties. *B. thuringiensis* is familiar for its capacity to form crystalline inclusions that contain insecticidal protein that is toxic to numerous insects pests including

Lepidoptera, Coleoptera, Diptera, Hemiptera and it also possesses several biological activities functioning as a molluscicidal, nematicide and acaricide (Abd El-GhanyA & Abd El-Ghany N, 2017). Hence, it has been commercially taken as the best-selling biopesticide (Jenkins & Dean, 2001).

B. thuringiensis toxins have a high molecular potency compared to chemical pesticides: they are 80,000 times more potent than organophosphates and 300 times more potent than synthetic pyrethroids (Feitelson *et al*., 1992). Bt strains are capable of synthesizing Crystal 'cry' and cytolytic 'Cyt' toxins, named as δ-endotoxins, in the time of sporulation and on the onset of stationary growth phase as parasporal crystalline inclusions. These crystals are made by proteins called insecticidal crystal proteins (ICPs). The prime action of Cry protein is to lyse the midgut epithelium cells and produce pores (Bravo *et al*., 2007). These crystals are dissolved in the midgut after it gets ingested by the insects, the midgut proteases proteolytically activate the toxins and bind to specific receptors located in the insect plasma membrane resulting in cell disruption and insect dying (Bravo *et al*., 2007). Understanding crystal composition provides a forecast of its insecticidal activity (Biag & Mehnaz, 2010). Contrastingly, the involvement of Bt proteases has also been reported initiating lethargic protoxins (Brar *et al*., 2007). Unlike the stationary phase, *B. thuringiensis* isolates are also capable of synthesizing additional insecticidal proteins in the course of the vegetative growth phase, these proteins are eventually secreted into the culture medium and turn out to beidentified as vegetative insecticidal protein 'Vip' and secreted insecticidal protein 'Sip' (Donovan *et al*., 2006).Additionally, the insecticidal crystal protein (ICP) genes have been effectively inserted into cotton, corn, soybeans and rice, which have had a huge positive impact on the economy (Crickmore, 2006). Thusly the preference for synthetic pesticides, Bt endotoxins have come to be the most determined bio-pesticides in commercial agriculture (Capalo *et al*., 2001).

The vast majority of animal species on the globe are insects, which outnumber all other animal species combined. Every year, one-fifth of the world's total crop production is lost due to herbivorous insects. Numerous invasive insect pests have emerged in the last ten years as a result of global exportation and

the climate emergency, posing a threat to agriculture and ecosystem health (Liebhold *et al*., 2016). Fall armyworm or 'FAW' are destructive species of insects belonging to the Lepidoptera order and family Noctuidae (FAO, 2017; CABI, 2017). The FAWis regarded as the most devastating pest species in the world, seriously harming important cereal crops like sorghum, rice and maize. 186 plant species from 42 families are consumed by FAW (Early *et a*l., 2018) out of which corn and rice is the key host (Hoy, 2013). The earliest FAW observation in Nepal, also known as Phaujikira, has been reported by the Nepal Agriculture Research Council (NARC)(Pokharel, 2019). The presence of this pest in Nepal was proven by the Plant Quarantine and Pesticide Management Center on August 12, 2019, following confirmation in the lab test report with morphological and molecular diagnosis (NPPO, 2019). Because they consume their host plants, fall armyworm larvae are incredibly damaging. New leaves can be discovered with larvae of fall armyworm, leaf whorls, tassels or cobs depending on the stage of maize growth (Goergen *et al*., 2016). It has recently been in the Nawalparasi district in maize, and if the pest is not carefully handled, crop losses of up to 100% are anticipated (Bhusal & Bhattarai, 2019).

Aphids, another common pest of vegetables and fruits, feed on plant sap, which causes sooty mould to appear on damaged plants (Tang *et al*., 2017). Among them, *Myzus persicae* (green peach aphid), is a pest that has a large economic impact on both agriculture and horticulture because it directly harms plants by consuming nutrients and indirectly spreads a variety of viral infections (Diaz *et al*., 2009). It is the most harmful aphid pest, inhibiting growth, shriveling leaves andkilling diverse tissues. They can cause harm such as chlorosis, necrosis, wilting, stunting, abortion of flowers and fruits, distortion of leaves and defoliation (Sayed *et al*., 2019).

1.2 Objectives

1.2.1 General objective

To study the bio-control against insect pest by *Bacillus thuringiensis* isolated from organic agricultural farm.

1.2.2 Specific objectives

i. To isolate *Bacillus thuringiensis* from soil samples collected from organic agricultural farm.

ii. To identify *B. thuringiensis* isolate using conventional and biochemical techniques.

iii. To rear insect pests of order Lepidoptera and Hemiptera under laboratory condition.

iv. To detect the insecticidal activity of the bacterium against insect pests.

CHAPTER II

LITERATURE REVIEW

2.1 Biological management of insect pests

Humans beings consuming crop plants as food are damaged by 10,000 species of insects, 30,000 species of weeds, 10,000 diseases (resulted by bacteria, fungi, viruses and other organisms) however, 10 percent of the entirely associated pest species are investigated as the vital pests of crop plants (Dhaliwal *et al*., 2007). The animal groups as agricultural pests of greatest economic influence are insects, mites, nematodes and gastropod molluscs (Speiser, 2002). Insects are in charge of straight injury they cause to the plants as well as unintended damage in which the insect either transfer or permit entrance of fungal, bacteria or viral infections (Campbell *et al*., 2002). Major crops that are affected by Coleopteran pest include cereals, oilseeds, vegetables, fruits, plantation crops and stored grains (Patole, 2017). Similarly, Lepidopteran pests are responsible for notable depletion in numerous cropping systems in the temperate, subtropical and tropical regions of the world (Vreysen *et al*., 2007).

The term “entomopathogens” was introduced by Tanzini *et al* (2001) to characterize microorganisms that reduce insect pest population to levels that do not hurt crop plants economically. Delgado and Murcia (2011) defined the term referring to the microbial population that can attack insect pests by integrating them into their life cycles and using them as hosts, as well as classifying these microorganisms as either facultative or obligate parasites attacking insect pests with high survival potential. Entomopathogenic microorganisms (EM) are effective against insect pests and environmentally safe for both people and non-target animals (reduced pesticides residues) when used as pest control agents. EPMs are crucial in the environmentally friendly control of this pest because of the preferred habitat of this insect. The presence of natural enemies in an agro-ecosystem is crucial for preventing insect populations from rising to harmful levels. According to the type of pest and the biological characteristics of the control agent, biological control agents

(BCAs) are used in various ways. These agents have a number of appealing qualities, including host specificity, absence of toxic residues, no phytotoxic effects, human safety, and the potential for self-sustaining pest management, to name just a few. However, effective application demands a detailed understanding of both the ecology of the insects and its natural antagonist. As an effective biological control agent, entomopathogens (fungi, bacteria, viruses, and nematodes) must be developed. This requires a complete understanding of bioassay processes as well as manufacturing, formulation, and application strategies.

2.2 *Bacillus thuringiensis* as a bio-control agent

The usage of entomopathogenic microorganisms as bio-control agents set off the most successful substitutes to synthetical pest control. Entomopathogenic bacteria (EPB) have gained interest due to their small host range, high virulence, safety for non-target species, compatibility with many other types of insecticides, ease of application and simplicity. An exceptional opportunity to conduct prospective and predictive research in the pesticide industry exists in the field of microbial pesticides (Bahadur, 2018). Collectively, *Bacillus thuringiensis* is the prime entomopathogenic microorganism employed for the protection of crops. Bt also naturally occurs in the rhizosphere, phylloplane, freshwater, deciduous and coniferous leaves, grain dust, and also from insects, crustaceans, annelids, insectivorous mammals, the gut of caterpillars of different types of moths and butterflies, besides on the animal excreta, insect rich environment, flour mills, and grain storage areas (Madigan & Martinko, 2005; du Rand & Nicolette, 2009). It is frequently utilized in agricultural (Deist *et al*., 2014), forestry (Zhang *et al*., 2006) and public health programmes (Ibrahim *et al*., 2010) as bio-control agent. This bacterium's parasporal inclusion body (crystal), which forms during sporulation, sets it apart from other species of this genus (Hofte & Whiteley, 1989). The Cry proteins that make up this crystal are produced by Cry genes (Crickmore *et al*., 1998).

Additionally, recent research has confirmed additional new potentials of certain *B. thuringiensis* strains. The bioremediation of heavy metals and other

chemicals (Aceves-Diez *et al*., 2015; Melo *et al*., 2016), anticancer activities (Periyasamy *et al*., 2016), the production of polymers (Singh *et al*., 2013) and antagonistic effects against plant and animal pathogenic microorganisms are some of these new features (Roy *et al*., 2013).

2.2.1 Ecology and Prevalence

Bacillus thuringiensis is primarily a soil bacterium that lives in both a saprophytic and parasitic state. Many habitats appear to be home to *Bacillus thuringiensis*. Numerous habitats including soil, insects, dead insects, sericulture environments, forest and cultivated soils, stored dust products, coniferous and deciduous leaves, and soil, have all been used to isolate strains. Many studies investigated into the distribution of Bt in various geographic areas and diverse sources due to the economic significance of Bt (Schnepf *et al*., 1998). Meadows (1993) stated that *B. thuringiensis* may occupy three different environmental niches, including those of an entomopathogen, phylloplane occupant, and soil microbe. Furthermore, a lot of *B. thuringiensis* strains collected from various environments exhibit no insecticidal activity. A high level of genetic flexibility, at least in part, is responsible for the astounding diversity of Bt strains and toxins. One sign of the vast genetic diversity against *B. thuringiensis* isolates is the variation in flagellar H-antigen agglutination reactions (Schnepf *et al*., 1998).

B. thuringiensis's survivality is directly influenced by the local microbiota as well as soil characteristics including pH, humidity, mineral and organic matter concentrations, which can either positively or negatively affect germination, growth, sporulation, and protein production (Polanczyk *et al*., 2009). It has been isolated artificially or naturally from the phylloplane (Maduell et al 2008) and demonstrated that *B. thuringiensis* can enter this niche by rain splashing from the soil to lower leaves because it is transported by seeds (Prabhakar *et al*., 2009) that germinate from animal excrement, such as those from insects or birds and from dead insects.

2.2.2 Morphology and Biology

Bacillus thuringiensis creates white colonies that spread across the plate. *B. thuringiensis*'s length and width range from 3-5m and 1-1.2m, respectively. The organism's spores are ellipsoidal, uninflated, and located in the sub terminal region of the cell. The presence of parasporal crystal inclusionsis the most reliable indicator for differentiating *B. thuringiensis* from other bacillus species. *B. thuringiensis* strains can create two different kinds of δ-endotoxins. The proteins are referred to as Cry and Cyt. A single gene results in the production of each insecticidal endotoxin. Crystal proteins include those that are part of the pore-forming Cry toxins, whether or not cytolytic Cyt toxins are present. While Cyt toxins work in a way similar to a detergent on the membrane phospholipids of the insect midgut, Cry toxins specifically interact with a receptor in this membrance (Brave *et al*., 2013).

These endotoxin-producing genes are frequently found on sizable, contagious plasmids. Proteins Cry and Cyt are structurally different. The pathogenicity of these proteins to insects and each crystal protein's specific host range are their key characteristics. The growth conditions and the presence of extra chaperone proteins affect the size and morphology of *B.thuringiensis* crystals and spores. Variations in culture conditions (culture media composition, pH, oxygen availability, and the culture's initial inoculumvolume) have been shown to have a major impact on the sporulation phase's spore count as well as the generation and appearance of crystal inclusions in various investigations (Yezza *et al*., 2006). There are five different types of crystal morphologies: bipyramidal crystals related to Cry 1 protein, cuboidal crystals connected to Cry 2 proteins, flat-square crystals, typical of Cry 3 proteins, and bar-shaped inclusion connected to Cry 4D proteins (Subedi and Bhattarai, 2002).

It has an easy life cycle. The spore germinates resulting in the formation of vegetative cell that develops and uses binary fission for reproduction when nutrition and environmental factors are favorable for growth. Until one or more substances, such as carbohydrates, amino acids, or oxygen, are no longer sufficient for further vegetative development, cells will continue to divide. The bacteria sporulates in these circumstances, resulting in a spore and parasporal body, the latter of which, as mentioned above, is mostly made up of one or

more insecticidal proteins in the form of crystalline inclusions. These are selectively poisonous to diverse species of several invertebrate phyla and are known in the literature as insecticidal crystal proteins (ICP) or δ-endotoxins. ICPs are made up of both the more common Cry (crystal) proteins and the Cyt (cytolytic) proteins that some Bt strains generate. Upon intake, cry proteins become solubilized and are typically broken down into active toxins by proteolytic enzymes. Crystal proteins are beneficial alternative to chemical pesticides for the control of insect pests in agriculture, forestry, and the home due to their great specificity and environmental safety.

B. thuringiensis is a species that is split into more than 70 subspecies based on the antigen characteristics of the flagellar (H) antigen rather than insecticidal protein complements or target spectra (Lecadet *et al*., 1999). A new H antigen serovariety number and subspecific name are given to each new isolate that carries a flagellar antigen type that differs noticeably from the others in immunological testing. There are four main subspecies *B. thuringiensis* subsp. *kurstaki* (H 3a3b3c) and *B. thuringiensis* subsp. aizawai (H 7), which are used to combat lepidopteran pests; *B. thuringiensis* subsp. israelensis (H 14), which is used to combat mosquitoes and blackfly larvae.

2.2.3 History of *Bacillus thuringiensis*

Shigetane Ishiwata made the initial discovery of *B. thuringiensis* in Japan in 1901 as the cause of the sotto disease in silkworm (*Bomyxmori*) larvae. A few years later, in 1911, a German researcher named Ernst Berliner discovered dead Mediterranean flour moth larvae in a flour mill in the German state of Thuringia. From these larvae, Berliner was able to identify a similar strain. He gave the organism the appropriate name *Bacillus thuringiensis*. Inclusion bodies or "Restkorper," were discovered by Berliner when studying the bacterium in addition to the endospore (Berliner, 1911; Kleter, 2007).

2.2.4 Taxonomy of *Bacillus thuringiensis*

Alternative strategies for classifying and dividing *B. thuringiensis* strains and isolate requires further research considering their economic significance. In order to discover novel Bt strains with exceptional insecticidal abilities,

numerous screening programs have been established. Many Bt strains that are active against lepidopteran, dipteran, and coleopteran insects have been isolated as a result. Additionally, *B. thuringiensis* strains that are effective against worms, mites, protozoa, and insects from the orders Hymenoptera, Homoptera, Orthoptera, and Mallophaga have been isolated.

Although the genus Bacillus and Bergey's Manual of Determinative Bacteriology were published in 1973 and 1974, respectively, the designation of *B. thuringiensis* as a distinct species within the genus Bacillus has been the subject of debate (Gordan *et al*., 1973; Buchanan & Gibbons, 1974). One of the most diverse genera in the class Bacilli is the genus Bacillus, which contains rod-shaped, Gram-positive, facultatively anaerobic bacteria with G+C levels between 32 and 69% (Garbeva *et al*., 2003). The fact that many species in these genera produce antibiotics and peptides with anti-microbial, anti-viral and anti-tumor properties makes them important in practical terms. Additionally, they produce compounds and thermostable enzymes that can inhibit phytopathogenic organisms that are found in soil (Giacomodonato, 2001).

The vegetative cells of *B. thuringiensis* are characterized by their size and sturdiness. They are either straight or slightly curved, with rounded ends. Usually, they appear in pairs or brief chains. Gram-positive *B. thuringiensis* is non-capsulated, motile, and has peritrichous flagella.

Table 1: General Taxonomy of *Bacillus thuringiensis* (Source: Integraded Taxonomic Information System-Report)

Kingdom:	Bacteria
Subkingdom:	Posibacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Bacillales
Family:	Bacillaceae
Genus:	*Bacillus*
Species	*Bacillus thuringiensis* (Berliner, 1915)

2.2.5 *Bacillus thuringiensis* genome

The genome size of *B. thuringiensis* strains ranges from 2.4 to 5.7 million base pair. The majority of *B. thuringiensis* isolates contains a number of extra-chromosomal elements, some of which are circular and others of which are linear. Large plasmids encode the proteins that make up the parasporal crystal. *B. thuringiensis* chromosomes also contain a region that hybridizes to cry gene probes, though it is unknown how much this chromosomal homolog contributes to crystal generation (Schnepf *et al*., 1998). Additionally, it is a significant source of gene for transgenic expression and offers plants with insect resistance (Li *et al*., 2008).

B. thuringiensis's insecticidal toxins, also known as "Cry toxins" or "δ-endotoxins" have several insect-specific properties (Hemipel, 1967). The cry gene family contains the genes responsible for producing these toxins (Schnepf *et al*., 1998). They are expressed during the stationary period of growth, which is a common trait of cry genes. The final product of cry gene expression, cry proteins make about 20-30% of the dry weight of cells and typically build up in the mother cell starting in stage III of sporulation and continuing until stage VII (Bulla *et al*., 1980).

Cry genes have a high degree of flexibility, which is an intriguing characteristic. This particular trait might help Cry poisons to be more

adaptable in terms of the range of insects they can infect. The multiple transposons and insertion elements that surround the cry genes are the most likely cause of this genetic flexibility. Indeed, these transposable elements might promote the spread of genes and emergence of novel poisons (de Maagd *et al*., 2001). Additionally, the presence of cry genes on transmissible plasmids increase the possibility of horizontal gene transfer between various *B. thuringiensis* strains, which results in the emergence of novel strains with various cry toxin combinations (Thomas *et al*., 2001).

According to a prior study, *B. thuringiensis* can also manufacture certain antibiotics, such Zwittermycin A, to boost its insecticidal toxins and prevent pathogenic bacteria. (Broderick *et al*., 2000). The strain *B.cereus* UW85 was the first to contain the entire gene cluster for antibiotic production (Kevany et al 2009). When insects are exposed to *B.thuringiensis*, it can also create virulence factors including collagenases and enhancers (Peng *et al*., 2016). Numerous virulence genes that play important roles in these bacteria's pathogenicity are frequently encoded on plasmids. Plasmids include the virulence genes, which typically results in a variety of phenotypes and diseases. (Zwick *et al*., 2012).

2.2.6 Toxin structure

Parasporal crystalline inclusions of crystal proteins are produced during the stationary phase of growth. The δ-endotoxins, such as the Cry and Cyt toxins, are the most widely recognized. Depending on their homology and molecular structure, cry proteins can be categorized into different classes (Okumura *et al*., 2014). A Cry toxin is a "simple" toxin, which is described as a monomer or oligomer of a toxic simple protein, based on how it works (Lamanna & Sakaguchi, 1971). These proteins have high and specific cytocidal activity against human-cancer cell lines (following protease activation), and they either belong to the three-domain (Cry31 or Ps1, Cry41 or Ps3, Cry63 or Ps6) or the ETX_MTX2 family proteins (Cry45 or Ps4, Cry 46 or Ps2, Cry64 or Ps5) (Ohba, 2009). Another set of unique hazardous qualities may be caused by some Cry proteins. The Cyt proteins develop into a smaller, more specific category of crystal proteins that are insecticidal to several dipteran larvae,

including mosquitoes and blackflies (Ben-Dov, 2014). In addition, some Cyt toxins can enhance the insecticidal effects of other Bt proteins (Soberon, 2013).

The fact that a protein crystallizes into a parasporal structure gives it the name "Cry toxin". As a result, Cry toxins comprise a variety of unconnected lineages rather than being members of a single, homologous protein family. Other Crytoxins, such as binary Bin- and ETX_MTX2-like toxins generated by *Lysinibacillussphaericus* (Ls, formerly known as *Bacillus sphaericus),* belong to different protein families, but the well-known three-domain Cry proteins make up the largest category. (Berry, 2012).

There are more than 200 members in at least 50 subgroups of the Cry proteins. The wider family of Cry proteins known as the three-domain family consists of globular molecules with three structural domains joined by a single linker. The inclusion of protoxins with two different lengths is one distinctive trait of the members of this family. About twice as many protoxins are present in one large group as there are poisons. According to de Maagd *et al* (2001), the C-terminal extension present in lengthy protoxins is not necessary for toxicity and is thought to contribute to the development of crystal inclusion bodies inside the bacteria. Two closely related gene families, Cyt1 and Cyt2, make up cytotoxins(Crickmore et al 1998). Six separate three- domain Cry proteins, Cry1Aa, Cry2Aa, Cry3Aa,Cry3Bb, Cry4Aa, Cry4Bb, have each had their tertiary structures identified by X-ray crystallography to date (Morse *et al*., 2001).

Upon cleavage in the insect, the 130KDa component of the crystal protein that is toxic to Lepidoptera larva produces the functional (insecticidal) proteins of lower molecular weight; quite frequently, the crystal that is created is an assembly of several proteins (Crickmore *et al*., 1998). In contrast to Cyt2A, Cry3A and Cry1Aa both have three domains. Helix 5 is surrounded by the other helices in domain I, which is made up of a bundle of seven antiparallel-helices in a 10 helix structure. Three antiparallel –sheets that are connected in a classic "Greek Key" topology, or "prism fold" (330,343), make up Domain II. According to Schnepf *et al*., (1998), Domain III is made up of two twisted,

antiparallel-sheets that come together to form a –sandwich with a "jelly roll" topology. On the other hand, Cyt proteins feature a single α-β domain that is made up of two outer layers of –helix hairpins encircling a α-sheet. Volvatoxin A2, a PFT cardiotoxin made by the straw mushroom *Volvariella volvacea*, is structurally linked to cytotoxin (Lin *et al*., 2004).

2.2.8 Mode of action of *Bacillus thuringiensis*

Research to reduce agricultural pests has primarily focused on histological investigations using the stomach of insects. This is because modifications in gut can have an impact on both their growth and key physiological occurrence including altered absorption of nutrient, degenerative transformation, hunger loss and food abandonment, intestinal paralysis, physiological ilness, and complete paralysis. The most common symptoms as sson as the vulnerable insect swallow the Bt spores and crysals are blackened color, a defining indication of infections induced by this microbe, resulting in insect mortality (Monnerat & Bravo 2000). Lepidopteran mouthparts that are used for chewing encourage the consumption of *B. thuringiensis* toxins both as products and as toxin-containing GMP. One of the most crucial points of contact between the insect and its surrounding is the preintestine (front), midgut, and hindgut, which make up its digestive tract (Levy *et al*., 2004).

The midgut's alkaline (pH 9 to 12) environment helps the crystals dissolve after intake (Knowles, 1994). Lepidoptera-specific proteins are soluble at pH values higher than 9.5 (Knowles, 1993). The specific activity of Cry toxins is greatly influenced by the pH of an insect's midgut. Some toxins (Cry111A) are activated in alkaline environments, whereas others (Cry1b) are active in neutral to acidic environments (Bravo *et al*., 2007). The activation of Cry toxins and the specificity of the toxin in certain insects both depend on the cleavage of the toxin. Proteases generate protoxins when toxins are solubilized, creating active proteins that are 60-70 kDa (Bravo *et al*., 2005). The protoxins in the midgut of lepidopterans are activated by digestive enzymes and bind to certain receptors in the microvilli of the apical membrane of the columnar cells (Höfte & Whiteley 1998). The specificity of Cry toxins is determined by their affinity for the apical microvilli of the insect's

membrane vesicles (Bravo *et al*., 2007). By attaching to specific receptors on the apical membranes of intestinal cells, the Cry toxins can pass through the peritrophicmembrane , creating or forming pores that are then followed by cell breakdown due to an osmotic imbalance between the intracellular and external environments. As a result, the microvilli are destroyed, the insect stops feeding, and it eventually dies (Bravo *et al*., 2007; Sousa *et al*., 2010).

By referencing signal transduction, Zhang *et al*., (2006) have developed a second model to explain the method of action of Cry toxins. Altough it has only been investigated in insect cells, the process of binding the protein created by the proton's cleavage in intestinal microvilli is the same as in the first model. Stink bugs have small channels that can inject saliva into the salivary plant tissue and needle-like sucking mouthparts made of two mandibular and two maxillary stylets. These insects digest their food without using their mouths, secreting saliva into the food, which uses salivary proteases to break down the sap proteins (Zhu *et al*., 2003). Nutrient absorption occurs after predigested nutrients are thoroughly digested by proteases in the gut.

Due to these insects' differing feeding preferences, Hemiptera have a variety of midgut morphologies and functional activities. There are three distinct areas:a posterior dilated region, a medium tubular region, and an anterior dilated region (Guedes *et al*., 2007). In addition to transporting ions and water, breaking down carbs, and storing lipids, the anterior area is also in charge of maintaining electrolyte balance. In the intermediate and posterior regions, the process of digestive enzyme secretion is typically visible. Histologically, the pentatomids' midgut is composed of regenerative and digesting cells that are scattered throughout the midgut and has a basic epithelial layer. Since hemipterans lack peritrophic membrane, they are not shielded by a chitin and protein membrane that keeps infections and other harmful organisms out (Hegedus *et al*., 2009).

2.3 Fall armyworm (*Spodoptera frugiperda*)

The majority of people in Sub-Saharan Africa rely on maize as a major grain crop for their diet and way of life. In addition to being a significant staple,

maize is a crucial raw material for the manufacture of confections, formulas for animal feed, and syrup for pharmaceuticals. Due to a significant shift toward higher protein foods caused by rising personal income and urbanization, maize has seen greater demand than other grains globally (FAO, 2015).

The primary insect pest of maize is the fall armyworm (*Spodoptera frugiperda*). Although it is the major pest of maize, it also affects more than 80 other crop species including rice, millet, sorghum and cotton (FAO, 2020). It has more than 85 host species and is polyphagous in nature. It was discovered in Nepal's Nawalparasi district in maize crop for the first time in Nepal. The Nepal Agriculture Research Council (NARC) has reported the first encounter of FAW, also known as Phaujikira locally (Pokharel, 2019). The fall armyworm has already made several losses by attacking several states of India, particularly in the tropical areas. There is a high risk of this insect spreading to Nepal because of open border with India (Bhusal & Chapagain, 2020). Free open borders and improper quarantine can cause the pest to spread in Nepal. This insect thrives in Nepal's terai region because of the regions climate (Bhusal & Chapagain, 2020).

2.3.1 Classification

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Superfamily: Noctuoidea

Family: Noctuidae

Genus: *Spodoptera*

Species: *Spodoptera frugiperda*

2.3.2 Morphology

The morphology of FAW is similar to other members of the Order and Family such as Spotted stem borer (*Chilopartellus*), African maize stalk borer (*Busseolafusca*), African cotton leafworm (*Spodoptera littoralis*), beet armyworm (*Spodoptera exigua*) and African armyworm (*Spodoptera exempta*). FAW has distinctive features at adult caterpillar phase to separate it from other species. The FAW has four pimples-like dark spots with hair on it, making it looks rough to the sight. However, it has a smooth to tough body. The smooth-skinned larvae of the fall armyworm range in color from a light tan or green to practically black. Their backs have three yellow-white hairlines. There is a larger dark stripe next to the yellow lines on each side of their bodies. Next to it is a similarly broad, wavy, yellow stripe that has red spots on it.

The body is divided into segments, and each segment consists of four dark dots that may or may not form squares as they progress from the first to the last. While the head area is marked with a chewing mouth section and a white and dark Y-shaped pattern (Benson, 2017). At the pupa stage, the genital aperture and the anal slot can be used to identify between the female and male FAW. Compared to males, the distance between the female genitalia and the anal slot is greater (Sharanabasappa *et al*., 2018). Females' forewings are uniformly grayish brown to finely mottled with grey and brown. Female forewings, in contrast, have a brown and grey coat, a triangular white patch at the apex, and a circular spot in the middle of the wing. Sharanabasappa *et al*., (2018) found that the average wing length for males and females, respectively, varies from 3.00 to 3.4 and 3.00 to 3.50. The hind wing of FAW is silver-white with a little dark margin on both the male and female (Sharanabasappa *et al*., 2018).

2.3.3 Life cycle

The pest's life cycle is divided into four stages: Egg, Larvae, Pupa and Adult. The length of the lifecycle varies depending on season; in summer it takes 30 days while in autumn and spring, it takes 60 days. During winter, however,

this period is extended to 60 days (Luginbil, 1928). The different stages of fall armyworm are described briefly below.

Egg: The female produces 50 to 150 white dome-shaped eggs in clusters on the underside of the leaves, towards the base where the stem and leaves meet. (Hardke *et al*., 2015). The eggs are 0.8mm in diameter and 0.3mm in height. They are initially creamy and pale yellow in appearance, but before hatching, they turn light brown. Eggs can mature in 2-3 days at a temperature of 20 to 30 ^{o}c.

Larvae: Six larval instars are required for the fall armyworm to complete the larval stage. Larvae are greenish in color in their first instar but turn orange in their second instar. Larvae have a first instar length of about 1mm and a sixth instar length of 45mm. (Prasanna *et al*., 2018). The head is reddish brown during the fourth and sixth instars, with white spots and lateral lines. (Igyuve *et al*., 2018).

Pupae: The caterpillar pupates in soil between 2 and 8 cm deep after 14 days. (Prasanna *et al*., 2018). During pupation, a loose oval silk cocoon that is 20 to 30 mm long is secreted. (CABI, 2017b). A pupa of reddish brown color measuring 14 to 18 mm in length and 4.5 mm in width can be found inside this cocoon. The length of the pupal stage varies with the climate; typically, it lasts 8-9 days in the summer and 20-30 days in the winter (CABI, 2019).

Adult: Adults are naturally nocturnal, and they are most active in the hot, muggy evenings. In general, female moths are larger than male moths. The wingspan of an adult moth is 32 to 40 mm. Male moths can be identified by their speckled forewing and triangular white markings on the tip and middle of their wings. The adult phase lasts between 7 to 21 days.

2.4 Green peach aphid(*Myzus persicae*)

Aphids are widespread vegetables and fruit pests that feed on plant sap and cause sooty mould on damaged plants (Tang *et al*., 2017) and they cause a variety of plant diseases including wilting, shrinkage, floral and fruit abortion, leaf distortion, yellowing of leaves, and death of various plant tissues (Sayed *et al*., 2019). Diaz *et al*., (2009) suggested that among them, *Myzus persicae* is

an economically significant plague for agricultural and horticultural crops because it creates direct and indirect damage by consuming plant nutrients and transferring a variety of viral vectors. *M. persicae* also has an impact on the plant's photosynthetic ability by producing sugary honeydew on the surface of the leaves and causing water stress (Frantz *et al*., 2004).

The peach potato or green peach aphid, *Myzus persicae* is the most major aphid crop pest worldwide (Sulzer, 1976) (Hemiptera: Aphididae) (van Emden and Harrington, 2007). This species' reputation as a pest has been exacerbated by several variables, including its distribution, host range, causes of plant damage, life cycle, ability to spread, and potential to develop pesticide resistance. *M. persicae* is a highly polyphagous species with over 400 host species in 40 distinct plant families, including several commercially significant crop plants. It prefers peaches but also affects fruits, vegetables, sunflowers, tobacco, and suger beet. Its color is typically green, though it can also be pinkish or pale-brown. Adults can be both apterous and alate at different times of the year, depending on the surrounding environment and climatic conditions. The best cultural techniques are removing crop leftovers and weed hosts. Aphids feed on sap from developing leaves and florets. Infected leaves may curl downward, get brown, and eventually die. Aphids damage can kill plants when infestation are severe and can decrease plant vigor, growth, and yield.

2.4.1 Classification

Kingdom: Metazoa

Phylum: Arthropoda

Subphylum: Uniramia

Class: Insecta

Order: Hemiptera

Suborder: Sternorrhyncha

Superfamily: Aphidoidea

Family: Aphididae

Genus: *Myzus*

Species: *Myzus persicae*

2.4.2 Morphology of *Myzus persicae*

The elliptical-shaped eggs of this species are around 0.6 millimeters (0.024 in) length and 0.3 millimeters (0.012 in) broad. The eggs start of yellow or green before turning black. At first, green nymphs quickly turn golden and resemble viviparous adults. Pinkish nymphs can develop into winged females (Capinera, 2005). Adult winged aphids have a dark dorsal patch, a yellowish green abdomen and a black head and thorax. They are between 0.071 and 0.083 inches (1.8 to 2.1 millimeters) long. The adult aphids lack wings and are yellow or green in color, with possible medial and lateral green stripes. Their cornicles are relatively lengthy, the same color as their skin, and unevenly inflated all the way down. The extremities are pallid. Due to morphological variations, the mature green peach aphid's colour canrange from yellowish-green to red or brown. These factors are mostly determined by the host plants,diet, and temperature (Singh, 2021).

2.4.3 Life cycle of *Myzus persicae*

M. persicae alternates between peach and other summer host plants, and it is a heteroecious holocyclic (host alternating, sexual reproduction occurring during a portion of the life cycle) species. In the absence of enemies or predators, the average life span is about 23 days. The temperature affects the life cycle of *Myzus persicae*. In mild regions, the life cycle has been found to vary often between 10 to 12 days for an entire generation and over 20 generations reported annually (Gahatraj, 2019).

Myzus persicae spend the winter on Prunus (Peach) trees and when the trees come out of dormancy, the eggs hatch, and the nymphs begin to feed on the blossoms, young leaves, and stems of the plant (Capinera, 2005). Winged female Prunus spp. aphids that overwinter lay eggs lay nymphs on summer hosts after some generations. In cold climates, adults return to Prunus species

in the autumn for mating and egg-laying. The aphid undergoes cyclical parthenogenesis, in which several generations of apomictic parthenogenesis are followed by a single sexual generation, to reproduce regularly (Vorburger *et al.*, 2003). Females give birth to their offspring 6-17 days following conception, at an average initial birth age of 10.8 days. Generally, it takes 14.8 days to complete reproduction.

The peach is the aphid's major host, but also attacks other herbaceous plants on occasion (secondary hosts). In the spring and early summer, *Myzus persicea* reproduces parthenogenetically on peaches, and in the autumn, it reproduces sexually (Karagounis *et al.*, 2006). It includes the following phases.

i) Eggs: The eggs are elliptical-shaped and are roughly 0.6 mm long and 0.3 mm broad. Eggs start yellow or green before quickly turning black.

ii) Nymphs: Nymphs are the name of immature *Myzus persicae*. They have three dark lines on the back of the abdomen, which are absent in the adult, and are a pale yellowish-green color. There are four nymphal stages in Hawaii. The nymphal development cycle lasts between 6 and 11 days.

iii) Adult: The adult aphid lacks wings and ranges in color from light green to pale yellow. Adults with wings are green with dark brown or black patterns on their abdomens. Adult aphids are small to medium-sized, measuring between 1/25 and 1/12 of an inch, and their antennae are 2/3 as long as the body. Each adult female gives birth to about 50 nymphs.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials required

The materials equipment, media and reagent used in this study are systematically listed in appendix-I.

3.2 Methods

3.2.1 Study design

The study was conductedfrom December 2021 to June 2022. This study was laboratory based cross-sectional study. All the work concerning to this research was carried out in the Microbiology laboratory at the Central Campus of Technology, Hattisar, Dharan and Entomological Laboratory of the Regional Agricultural Research Station, Nepal Agricultural Research Center (NARC), Tarahara, Nepal.

3.2.2 Laboratory set up

The microbiology laboratory of the Central campus of technology in Dharan was used for the isolation of *Bacillus thuringiensis* and rearing of *Spodoptera frugiperda* and *Myzus persicae* as well as the bioactivity of isolates against insect pests was performed in the Entomological Laboratory of the Regional Agricultural Research Station, Nepal Agricultural Research Center (NARC), Tarahara, Nepal.

3.2.3 Site justification and planning

A Simple Random Sampling Technique was used to select the sampling site randomly from the four different organic farms (Dharan, Tarahara, Itahari and Jhumka) in Sunsari District, Nepal. Furthermore, each organic farm was divided into separate sections according to the extent of their land holdings. For this, a map of the entire sampling site was divided in to 100 blocks and 10 blocks with a range of 10 numbers were picked from each site

3.2.4 Sample collection site

The soil samples were collected from the agricultural field of the Organic farms of Dharan, Tarahara, Itahari and Jhumka, Sunsari, Nepal whereas the insect pests were collected from the various infected agriculture fields of Tarahara, Nepal.

3.2.5 Sampling

15 samples from each place were collected using adequate sampling procedure. About 60 soil samples were collected from the four different organic fields of the Sunsari district. Sterile gloves were used for sample collection and proper tags were given to each sample. About 10 gm of soil samples were collected from 2-5 cm depth and were aseptically placed in the soil sample bags/plastic bags and the bags were zip-locked. All soil samples were transferred to the laboratory and preserved at 4ºC until processed. Similarly, insect pests were collected from the affected area in the field which were placed in clean, sterile plastic containers and transported to the Entomology laboratory of the Regional Agricultural Research Station under a controlled situation for further rearing process.

3.2.6 Biosafety

The standard biosafety protocol is not required for the study. But the isolated micro-organisms were assayed against insect pests as a pathogen during the laboratory work.

3.3 Laboratory work

The study was carried out at the Central Campus of Technology, Hattisar, Dharan and Regional Agricultural Research Station (RARS) Tarahara Nepal.

3.3.1 Isolation of *Bacillus thuringiensis*

Bacillus thuringiensis were isolated by using the sodium acetate selection method. 2gm of soil were placed in a 10ml saline solution and heat-shocked at 80ºC for 15 minutes to eliminate all bacteria that are not capable of producing spores. It was appropriate to infer that *Bacillus thuringiensis* was present in the heat-treated sample because it produces spores.

The samples were diluted five times to reduce the total number of CFUs in each sample and to remove the humic material they contained. The resulting spore mixture was cultivated in Luria Bertani media containing sodium acetate 0.25 M concentration and incubated for 24 hours at 37ºC to promote spore germination. On Luria Bertani agar plates, Bt-like colonies with rounded, smooth forms and an earthy color were streaked to obtain a single colony. Strong proof that the isolate was Bt was supplied by this test, which was then used for additional identification.

3.3.2 Identification of *Bacillus thuringiensis*

The isolated organism was identified according to the Bergey's Manual of Systematic Bacteriology, Volume 2; 1986 using standard microbiological techniques including morphological, physiological and biochemical characteristics as listed below:

3.3.2.1 Microscopic Examination

The isolates were inspected using light and compound microscopes to evaluate the morphology of the cell, size, spore, and crystal, which was accomplished using a variety of staining techniques including Gram staining, Spore staining and Crystal staining

a. Gram staining

A thin smear of fresh culture was prepared in clean grease free slide and heat fixed. Smear was flooded with crystal violet for one minute and washed with distilled water. The slide was flooded with Grams iodine for 45 seconds and washed with distilled water. Then it was flooded with 95% ethyl alcohol (decolorize) for 10 seconds. The smear was flooded with Safranin (counter stain) for one minute and washed with distilled water. Excess water was removed by blotting paper, air dried and observed under microscope (Provine& Gardner, 1974; Bergey's Manual of Systematic Bacteriology, 1986).

b. Crystal staining

A thin smear of fresh culture was prepared in clean glass slide. The smear was air dried and heat fixed at 110ºC for 10 minutes. The smear was stained with 0.25% Coomassie Brilliant blue for 3 minutes and washed with distilled water and cbserved under microscope.

c. Spore staining

A thin film of five days old culture is made on clean grease free slide. The smear was flooded with Malachite green and steamed over boiling water bath for ten minutes, keeping the smear moist by adding dye as required. Slide was washed with distilled water and counterstained with Safranin for 30 seconds. It was washed with tap water, air dried and observed under microscope.

3.3.2.2 Biochemical Characteristics

For the study of biochemical characteristics, the pure colonies were transferred to NB and incubated at 37ºC for 24 hours.

a. Catalase test

A small amount of bacteria colony was extracted from NA and placed on a glass slide that had been cleaned and sanitized. A sterilized glass rod was used to place a drop of 3% H_2O_2 into the slide. The presence of gas effervescence signified a successful test.

b. Citrate utilization test

Simmons's Citrate agar slant was prepared. Organism was streaked on the slant and incubated at 37ºC. Positive results were verified by the emergence of a vivid blue color and the slanting growth of the organisms.

c. MR-VP test

The medium used was Glucose phosphate broth which was inoculated with the organisms taken from 18-24 hour pure culture. The broth was transferred into a clean test tube and the broth was incubated for 24 hours. The remaining broth was re-incubated for a further 24 hours. 2 to 3 drops of methyl red indicator and α-napthol was added to differentiate the bacteria based on acid production (Methyl Red test) and acetone production (Voges-Proskauer reaction). It was found that the organism showed MR negative and VP positive results.

d. Sulfide-indole-motility (SIM) test

A bacterial colony with a needle was stabbed into SIM medium placed in the test tubes. About two-third of the way into the deep, the inoculating needles were stabbed, and then were taken out by going back in the same direction. The tubes were incubated at 37ºC for 24 to 48 hours, or until growth is visible. A diffuse cloud of development away from the site of injection was a sign of a successful motility test. Further, five drops of Kovác's reagent were added to the top of the well to check for the presence of indole (a by-product of tryptophan metabolism). The layers remained yellow or appear slightly hazy after introducing the reagent, which indicated the organism was indole negative.

e. Sugar utilization test

For this test, 1% of carbohydrate was added to a base medium that included peptone, sodium chloride, and phenol red. Durham's tube were inserted inside the test tubes and turned upside down. Solution was initially placed into the tube. The solution was then autoclaved. After being inserted into tubes, these isolates underwent a 24-hour incubation period at 37ºC. The pink color of broth turning yellow and the appearance of gas effervescence are signs of a successful test. Sugar substituted with glucose.

3.3.2.3 Hydrolysis Test

a. Starch hydrolysis test

The isolates were inoculated onto solidified starch agar and cultured for 24 hours at 37ºC. Positive tests revealed a clean zone surrounding the colonies after flooded with Gram's iodine solution.

b. Gelatin hydrolysis test

Two test tubes were used for each isolate for greater precision of outcome. Test organisms were stabbed onto nutritive gelatin medium in a tube and cultured at 37ºC for a week with un-inoculated medium as control. To check for gelatin liquefaction, tubes were taken out of the incubator each day and placed in the refrigerator for 15 to 20 minutes. Positive test results indicated partial or total liquefaction despite being exposed to freezing temperature.

c. Lecithinase activity

Organisms were streaked on egg yolk emulsion medium and incubated at 37ºC for 24 hours. Opalescence around the colony indicated a successful test.

3.4 Rearing of insect pests

Insect pest larvae were raised in the entomological laboratory of the Regional Agriculture Research Station (RARS), Nepal, by providing the required nutrients and recreating the proper habitat for each individual insect pest in the quarantine that was previously randomly collected from the affected zone. To shield it from direct sunshine and UV rays, the quarantine room was darkened.

3.4.1 Rearing of *S.frugiperda* and *Myzus persicae*

First, sufficient organic manure was used to sow the seeds of maize, red beans, and black beans into flower pots (maize in 12 pots and beans in 12 pots). The pots were than kept in the glass house on the Tarahara farm. Watering was done every two to three days. After the plant started to grow, aphids and fall armyworm larvae were gathered from the affected field. They were retained on the plants until the pure bacterial culture was ready. The collected larvae were then brought inside RARS entomological laboratory to be checked for

mechanical damage. The gathered larvae were then placed into a container with 1000 ml capacity while being covered with new maize leaves. To keep the larvae from escaping, a muslin cloth was used to seal the container. *Myzus persicae* received fresh bean leaves in a similar manner. Both leaves were replaced daily. At room temperature and 70% relative humidity, these task were carried out in the laboratory (Ramanujam *et al*., 2020)

3.5 Preparation of mass culture of *Bacillus thuringiensis*

Luria Bertani broth was used to prepare mass culture of *Bacillus thuringiensis*. Bt pure culture was inoculated in sterile nutrient broth and incubated for 4 days in water bath shaker at 25ºC. Centrifugation at 10000 rpm for 15 minutes was used to extract vegetative cells. Pellets were rinsed with distilled water twice. The spore mixture was added in a sterile microfuge tube with 10µl of the solution to 10µl of sterile water. It was stored at -20ºC until use.

3.6 Bioassay

During bioassay, *S. frugiperda* and *Myzus persicae* of the orders Lepidoptera and Hemiptera were exposed to bacterial suspension and their reactions were recorded. Three replications of the experiment were carried out for treatment with bacteria and one for the control. In the case of *S. frugiperda*, 5 larvae were taken per replication, but for *Myzus persicae*, 25 aphids were taken per replication in a disposable plastic glass with a porous lid of cotton cloth that was tightened with rubber. The spore suspension of about 10ml was taken in a small beaker with a sporeconcentration of 1mg/ml. Then, the fresh leaves of maize and beans were immersed into the suspension for 15 seconds and fed to the insect pests. Controls were fed with the leaves drowned only with sterile water. Then, the glasses housed by *S.frugiperda* and *Myzus persicae* were incubated under controlled conditions (65±5% RH and 25±2 ºC). Insect numbers affected by the bacteria were observed and compared to a negative control.

FAWs' death that occurred due to bacteria was identified by starvation of the larvae that were lacking the ability or strength to move in which the larvae eventually turned black after its demise while other reason death included

active feeding of the larvae but sudden death in which its body was filled instead of starving. Death occurred due to bacteria and other reason in case of green peach aphid were distinguished by the indication of causal death (BD) in which no body parts were harmed or brokenwhereas removal of wings and missing body parts that might have been occurred by physical injury lead to OD.

3.7 Statistical analysis

The data recorded from the bioassay was noted and tabulated. The data (BD, OD and S) within five treatments (Control, SR1, SI7,ST9 and SJ11), each having three replicas were statistically analyzed by using IBM SPSS Statistic 20 Statistical software. For this, a two-way ANOVA was performed on survival and mortality data for insects (bacterial death and other reason death). Tukey's honestly significant difference (HSD) post hoc test was used to further compare treatment averages at the conventional level of probability (0.05).

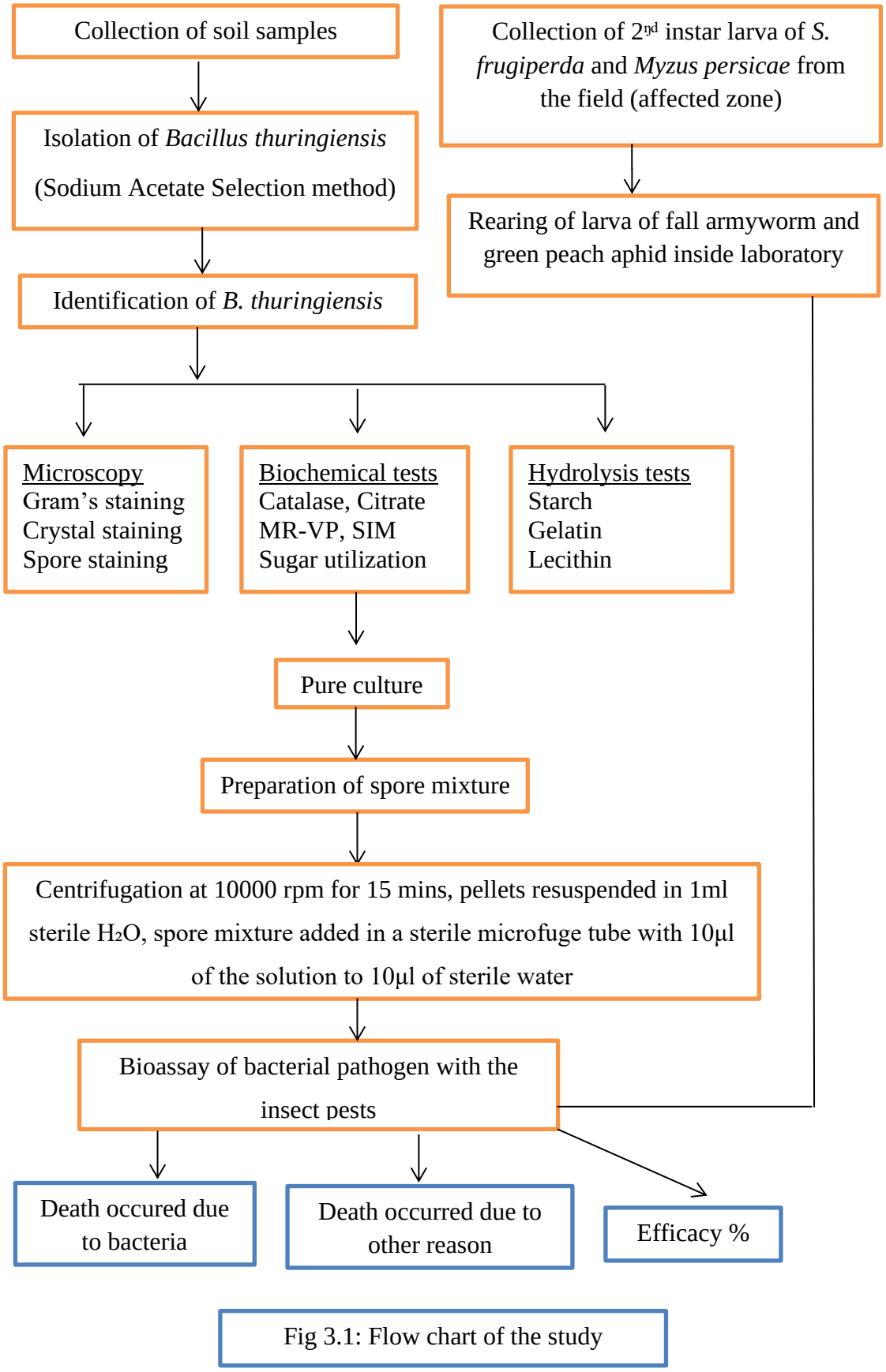


Fig 3.1: Flow chart of the study

CHAPTER IV

RESULTS

The study was conducted in Central Campus of Technology, Hattisar Dharan and RARS Tarahara. In total 60 samples were collected and isolated using sodium acetate selection method. Identified *B. thuringiensis* isolates were used to study their insecticidal properties against the insect pests.

4.1 Sampling of soil

The soil sample was taken from various organic farms of Dharan, Tarahara, Itahari, and Jhumka of Sunsari district, Nepal. 15 samples from each place were collected.

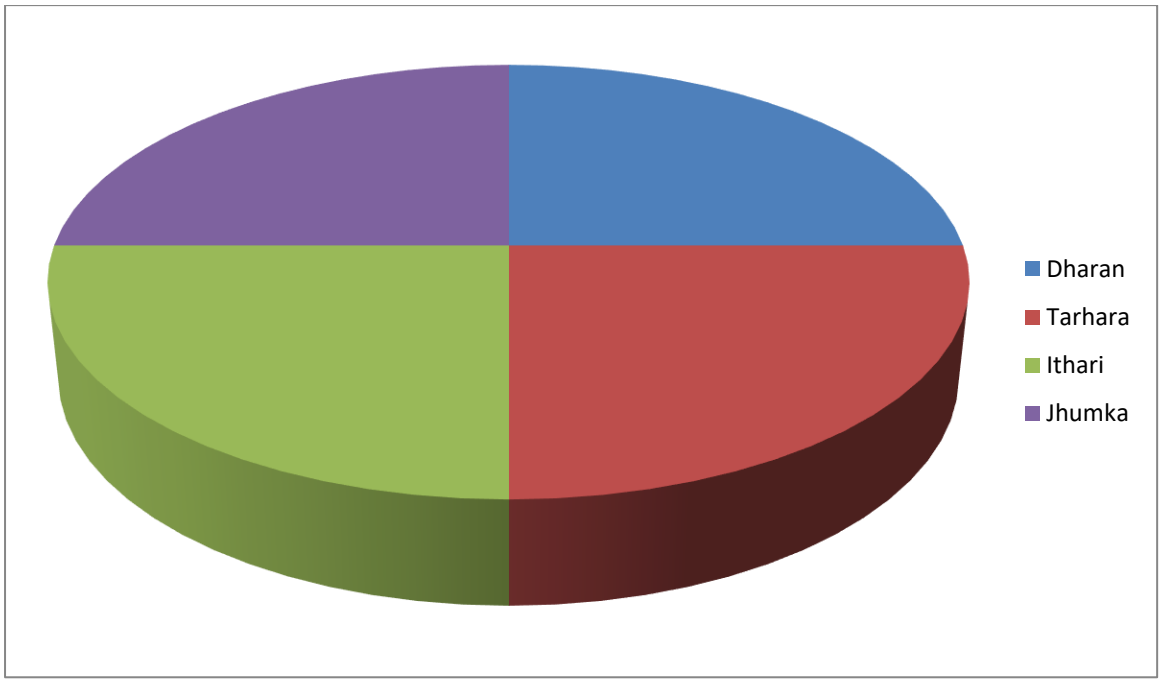


Figure **4.1** Soil Sampling Site

4.2 Identification of *B. thuringiensis*

All 4 isolates were purified and characterized microscopically as well as using set of biochemical tests.

Table 4.1: Morphological and biochemical characteristics of Bt isolates

Test performed	SR1	SI7	ST9	SJ11
Pigmentation	Creamy	Creamy	Creamy	Creamy
Grams staining	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod
Endospore	+ve	+ve	+ve	+ve
Crystal	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve
Citrate	+ve	+ve	+ve	+ve
Motility	+ve	+ve	+ve	+ve
Indole	-ve	-ve	-ve	-ve
MR	-ve	-ve	-ve	-ve
VP	+ve	+ve	+ve	+ve
Sugar utilization	+ve	+ve	+ve	+ve
Starch hydrolysis	+ve	+ve	+ve	+ve
Gelatin	+ve	+ve	+ve	+ve
Lecithin	+ve	+ve	+ve	+ve

Among the tested isolates (+ve denotes for positive results whereas –ve denotes for negative results), all isolates showed exact morphological and biochemical properties to the *B. thuringiensis*.

4.3 Death record of *S.frugiperda* during Bioassay

Three replications of the *S. frugiperda* bioassay using five treatments (Control, SR1, SI7, ST9, and SJ11) were used for recording the daily death record of the larvae. Five *S. frugiperda* replicas/larvae were used in each treatment during the test. Following that, larvae were given maize leaves that had already been prepared by soaking them in spore suspension (10 ml) taken in a beaker. Daily bioassay for dead and live record of larvae was recorded until pupation. During the bioassay, dead larvae were found after daily observation for a variety of reasons, including bacterial infection, physical injury, and other pathogens like fungus and nematodes. Larval death due to bacteria was identified as inert, wrinkled, curved, dry and black in color. In this study, no death due to bacteria was seen in the control treatment; however it was reported that the isolated Bacillus strains SR1, SI7, ST9, and SJ11 resulted in the death of 14, 13, 13 and 13 larvae respectively.

Table 4.2: Death record of larvae of *S. frugiperda*

No. of larvae taken: 5 per treatment plate

Days	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**	**TD**	**BD**	**OD**
Treatment Replica	**TD BD OD**	**TD BD OD**	**TD BD OD**	**TD BD OD**	**TD BD OD**	**TD BD OD**	**TD BD OD**			
Control										
1	0	0	1 0 1	0 0 0	0	0 0 0	0	1	0	1
2	0	0	0	1 0 1	1 0 1	1 0 1	0	3	0	3
3	0	0	0	1 0 1	0	1 0 1	0	2	0	2
							Total	6	0	6
SR1										
1	0	1 1 0	3 3 0	1 1 0	0	0	0	5	5	0
2	1 1 0	2 2 0	2 2 0	0	0	0	0	5	5	0
3	0	2 1 1	1 1 0	2 2 0	0	0	0	5	4	1
							Total	15	14	1
SI7										
1	1 0 1	0	3 3 0	0	1 1 0	0	0	5	4	1
2	0	0	0	2 2 0	2 2 0	1 1 0	0	5	5	0
3	0	0	1 1 0	1 1 0	2 1 1	1 1 0	0	5	4	1
							Total	15	13	2
ST9										
1	1 0 1	1 1 0	2 2 0	1 1 0	0	0	0	5	4	1
2	0	1 1 0	3 2 1	1 1 0	0	0	0	5	4	1
3	0	2 2 0	1 1 0	1 1 0	1 1 0	0	0	5	5	0
							Total	15	13	2
SJ11										
1	0	3 3 0	1 1 0	1 1 0	0	0	0	5	5	0
2	1 0 1	1 0 0	2 2 0	1 1 0	1 1 0	0	0	5	4	1
3	0	2 2 0	1 1 0	0	2 2 0	0	0	5	5	0
							Total	15	14	1

4.4 Death record of *Myzus persicae* during Bioassay

The death records of *Myzus persicae* was recorded weekly among three replicates utilizing five treatments (Control, SR1, SI7, ST9, and SJ11). In the bioassay, 25 aphids/replicas were taken. After that, leaves of beans that were immersed into the spore suspension were fed to the aphids. During the bioassay, several insect were discovered dead after daily monitoring for a variety of reasons, including bacterial infection, physical harm and other pathogens like fungus and nematodes. In this study, no death due to bacteria was observed in the control treatment: nevertheless, it was reported that the isolated Bacillus strains SR1, SI7, ST9, and SJ11 infected 57, 60, 57, and 56 aphids, respectively, and caused their death as well.

Table 4.3: Death record of *Myzus persicae*

No. of aphids taken: 25 per treatment plate

Days	**1st week**			**2nd week**			**TD**	**BD**	**OD**
Treatments Replica	**TD**	**BD**	**OD**	**TD**	**BD**	**OD**			
Control									
1	3	0	3		0		3	0	3
2	7	0	7		0		7	0	7
3	5	0	5	1	0	1	6	0	6
				Total			16	0	16
SR1									
1	17	15	2	7	5	2	24	20	4
2	20	14	6	5	5	0	25	19	6
3	19	15	4	3	3	0	22	18	4
				Total			71	57	14
SI7									
1	18	15	3	4	4	0	22	19	3
2	21	16	5	3	3	0	24	19	5
3	24	22	2		0		24	22	2
				Total			70	60	10
ST9									
1	20	18	2	3	1	2	23	19	4
2	21	19	2	3	0	3	24	19	5
3	22	19	3		0		22	19	3
				Total			69	57	12
SJ11									
1	18	15	3	4	4	0	22	19	3
2	19	16	3	5	5	0	24	21	3
3	15	12	3	6	4	2	21	16	5
				Total			67	56	11

4.5 Mortality of *S. Frugiperda* within three treatments

The mortality of *S. frugiperda* larvae was computed using the bioassay data after the experiment, and it was discovered that the control had a mortality rate of 40%, while the *B. thuringiensis* isolates SR1, SI7, ST9, and SJ11 had the same mortality rate (100%) within three treatments.

Table 4.4: Mortality% of *S. Frugiperda* within three treatments

Treatments	Total no. of larvae tested	No. of dead larvae of *S. frugiperda*	Mortality (%)	Survival (%)
Control	15	6	40	60
SR1	15	15	100	0
SI7	15	15	100	0
ST9	15	15	100	0
SJ11	15	15	100	0

4.6 Mortality of *Myzus persicae* within three treatments

The mortality of *Myzus persicae*was computed using the bioassay data after the experiment, and it was found that the control showed a death rate of 21.33%, whereas the isolates (SR1, SI7, ST9, and SJ11) had mortality rates of 94.67%, 93.33%, 92% and 89.33%, respectively.

Table 4.5: Mortality of *Myzus persicae* within three treatments

Treatments	Total no. of larvae tested	No. of dead aphids	Mortality (%)	Survival (%)
Control	75	16	21.33	78.67
SR1	75	71	94.67	5.33
SI7	75	70	93.33	6.67
ST9	75	69	92	8
SJ11	75	67	89.33	10.67

4.7 Death records of *S. frugiperda* due to bacteria during the bioassay

Bt isolates SR1 and SJ11 have been identified to be more virulent than the other treatments during the bioassay. Table 4.6 shows the specific relation between treatment and mortality of *S. frugiperda* caused by the bacteria calculated by two-way ANOVA.

Table 4.6: Death records of *S. frugiperda* due to bacteria during the bioassay calculated by two-way ANOVA

Treatment	Replication	BD	P value
Control	1	0	
	2	0	
	3	0	
Total		0	
SR1	1	5	
	2	5	
	3	4	
Total		14	
SI7	1	4	
	2	5	
	3	4	
Total		13	0.000
ST9	1	4	
	2	4	
	3	5	
Total		13	
SJ11	1	5	
	2	4	
	3	5	
Total		14	

4.8: Death records of *Myzus persicae* due to bacteria during the bioassay

Bt isolates SR1 and ST9 were found to be more virulent than the other treatment during the bioassay. Table 4.7 shows the specific relationship between treatment and mortality of *Myzus persicae* caused by the bacteria

Table 4.7: Death records of *Myzus persicae* due to bacteria during the bioassay calculated by two-way ANOVA

Treatment	Replication	BD	P value
Control	1	0	
	2	0	
	3	0	
Total		0	
SR1	1	20	
	2	19	
	3	18	
Total		57	
SI7	1	19	
	2	19	
	3	22	
Total		60	0.000
ST9	1	19	
	2	19	
	3	19	
Total		57	
SJ11	1	19	
	2	21	
	3	16	
Total		56	

4.9 Efficacy of *B. thuringiensis* isolates on *S. frugiperda*

A modified version of Abbotto's technique was used to calculate the efficiency or efficacy% of the isolates from the bioassay data record (Abbott, 1925). In this investigation, a 100% success rate was achieved by all Bt isolates (SR1, SI7, ST9, and SJ11) inducing the death of*S. frugiperda* larvae.

Table 4.8: Efficacy % of *B. thuringiensis* isolates on *S. frugiperda*

Treatment	Death % by Bt	Other reason death %	Survival %	Efficacy%
Control	0	40	60	-
SR1	93.33	6.67	0	100
SI7	86.67	13.33	0	100
ST9	86.67	13.33	0	100
SJ11	93.33	6.67	0	100

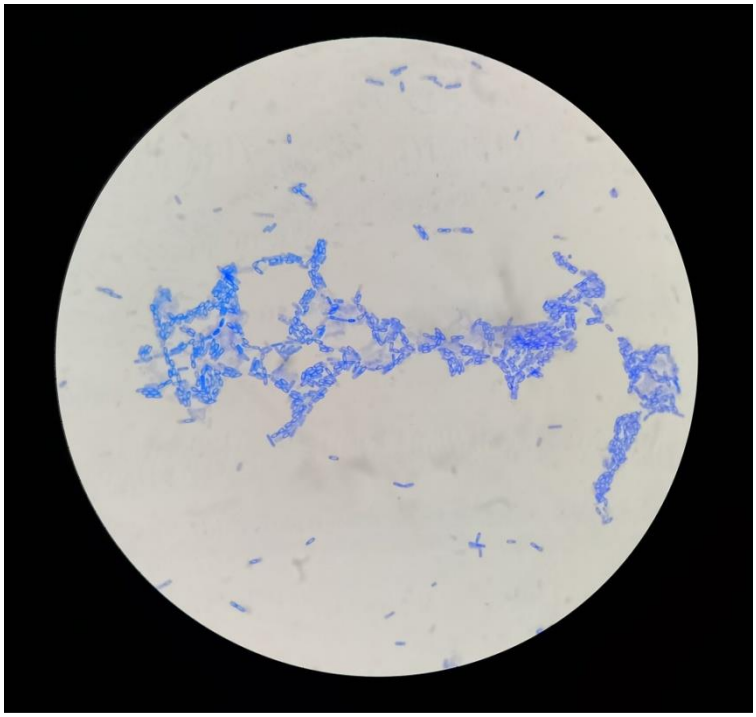
4.10 Efficacy of *B. thuringiensis* isolates on *Myzus persicae*

In comparison to other isolates, it was found that isolate SR1 had a better efficacy against *Myzus persicae* (93.22%).

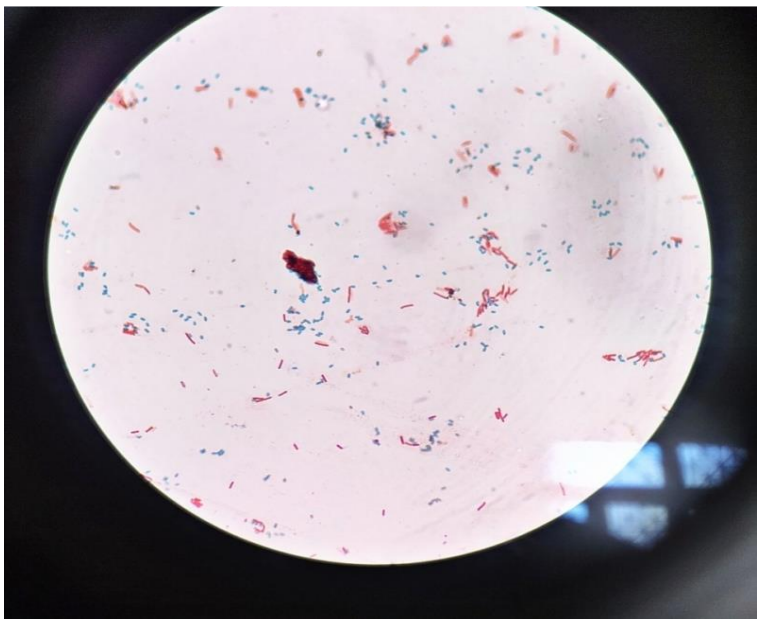
Table 4.9: Efficacy % of *B. thuringiensis* isolates on *Myzus persicae*

Treatment	Death % by Bt	Other reason death %	Survival %	Efficacy%
Control	0	21.33	78.67	-
SR1	76	18.67	5.33	93.22
SI7	80	13.33	6.67	91.52
ST9	76	16	8	89.83
SJ11	74.67	14.67	10.67	86.43

Photographs



Photograph 1: Crystal staining



Photograph 2: Spore staining

Photograph 3: Starch hydrolysis

Photograph 4: Gelatin hydrolysis

Photograph 5: Lecithin test

Photograph 6: Rearing of the insect pests

Photograph 7: Death of *Spodoptera frugiperda* by *Bacillus thuringiensis*

Photograph 8: *Myzus persicae* with no bacterial treatment

Photograph 9: Death of *Myzus persicae* by *Bacillus thuringiensis*

CHAPTER V

DISCUSSION

Plant protection frequently involves the use of chemical pesticides. This is due to the rise in insect resistance to several compounds included in plant protection products. Recently, the idea of using natural enemies, including entomopathogens, to control insect infestation has received more attention. Microorganisms called entomopathogenic microorganisms or simply entomopathogens could create new opportunities for reducing pest infestations. To replace pesticides in agriculture crops in an environmentally friendly way, entomopathogens are being produced. They can be employed as biological control agents to manage insect pests and improve agro-sustainability.

Pestilent insects cause significant agricultural loss all over the planet. In Nepal, uncontrolled use of insecticides to control insect pests is leading to a significant problem that cannot be solved. These insecticides pose a serious risk to human health since they can enter the body and cause immediate or long-term poisoning. It has negative consequences on health and the environment. Contrarily, beneficial natural diseases have drawn increasing attention as a result of the establishment and expensive modern synthetical insecticides. The effects of insect pests on agriculture include significant losses in crop, parasitized livestock, threats to farmers' livelihoods, decreased nutritional value of the product and risk to human health, etc.

According to Harrison *et al*., (2019), the FAW is currently the most devastating pest of maize, and its voracious eating habits have a big impact on food security. Both the FAW and its natural enemies are negatively impacted by the broad spectrum nature of the pesticides used to treat fall armyworms (Lewis *et al*., 2016). *M. persicae* can spread more than 100 plant viruses, making it very hazardous to different crops (Devi & Singh, 2007). Therefore, studies on the isolation, characterization, and bioassay of isolated strains of *B. thuringiensis* against FAW and *Myzus persicae* were carried out in the current inquiry. The present investigation deals with the insecticidal activity of

Bacillus thuringiensis against different insect pest of order Lepidoptera and Hemiptera including fall armyworm and green peach aphid respectively.

For the isolation, 60 soil samples were collected from different organic farms in Sunsari district, Nepal. A total of four organic farms from Dharan, Tarahara, Itahari, and Jhumka were selected randomly and the soil samples were selected by using a simple random technique method. For this, the area of each farm was divided into 150 blocks based on area and 10 integers from each were taken as a sample. The soil samples were collected, transferred to the laboratory and kept at 4ºC until they were processed. The isolation was done using sodium acetate selection method. This method prevents the germination of Bt spores through selective inhibition against non-spore forming bacteria. By morphological and biochemical analysis, four bacterial isolates from the study's 60 soil samples (SR1, SI7, ST9, and SJ11) were identified as strains of *Bacillus thuringiensis*.

The isolated *B. thuringiensis* colonies in this study had a variety of morphological and colony traits, including a round, white, slimy shape, smooth borders, and elevated elevation that surfaced in the petri plates following incubation. These isolated Bt characteristics are comparable to those described in Bergey's Manual of systematic Bacteriology, Volume 2, 1986, regarding the morphological characteristics of *Bacillus thuringiensis.*Bt is known to produce endospore and crystallinebodies. Hence, staining of the spore and crystal protein provided visual confirmation which is the strong evidence that the isolated organism is *Bacillus thuringiensis*. SiceBt can degrade catalase, a catalase test was performed by exposing the isolate to hydrogen peroxide and monitoring for oxygen production. Each isolate showed positive results for citrate, VP, motility, sugar utilization, gelatin hydrolysis, starch hydrolysis and Lecithinase activity.

After 48 hours to 5 days of incubation, Bt spores were produced in the present investigation. A laboratory bioassay was performed to determine how well the spores produced by the bacterial isolates (SR1, SI7, ST9, and SJ11) affected populations of *S. frugiperda* and *M. persicae* from the agricultural fields of Dharan-17 (Railway), Tarahara, Itahari, and Jhumka, respectively. The spores

were harvested by centrifugation, and the resulting mixture containing the spores was used for the bioassay. The spore suspension was then adjusted to the concentration of 1mg/ml. This study used five different treatments (Control, SR1, SI7, ST9, and SJ11) to carry out the bioassay. Three replications of each treatment in sterile water (D/W) served as the control. The bioassay was carried out using the leaf dipping technique (Nazir *et al*., 2019). For each replication, the mortality and survival rates were reported daily for *S. frugiperda* and weekly up to the second week for *Myzus persicae*.The duration and toxicity mechanism required to kill the insect pests depend upon the amount, structure and size of protein crystals consumed by the pests (Handayani *et al*., 2023). However, the number, shape and size of protein crystals that reach the insect pest's digestive tract are unclear in this study. It is reported that, toxicity of *Bacillus thuringiensis* depends upon the size and abundance of crystal protein found in the bacteria (Rana *et al*.,2002).

In bioassay, all isolates, SR1, SI7, ST9, and SJ11, showed the same mortality (100%) of *S. frugiperda* after three treatments, while the control had a mortality of 40%. Larval mortality caused by the bacteria was recognized as inert, wrinkled, curved, dry and blackish color of the dead insect.Moreover, 94.67%, 93.33%, 92% and 89.33% in the case of *Myzus persicae*, compared to 21.33%, in case of control. Death caused by bacteria for aphid was identified as fatal without any harn to other body parts.Fungi and other factors (bacteria, nematodes, mechanical injury, etc.) are to blame for the death of insects for other reason because the aphids that resulted in death from other reasons had broken wings, physical injury, separated body parts, fuzzy death that might have been caused by fungi, etc. Chilcott & Wigley (1993) showed that the percentage of isolates obtained from soil with toxicity against lepidopteran larvae ranged from 37% to 88%. Similarly, Iriarte *et al*., (1998) reported that most of the *B. thuringiensis* isolates showed insecticidal activity (above 25% mortality) against some lepidopteran species. According to Mahmud (2022) 70-80% of FAW death was caused by *B. thuringiensis* while according to another study, administration of *B. thuringiensis* suspension caused 88% mortality of the FAW (Indraini & Pujiastuti 2020). According to the mortality bioassay using *B. thuringiensis* as

the bioinsecticde for the green peach aphid, the strain showed mortality rates of more than 80% at a dosage of 10mg/ml (Torres *et al*., 2022).

During the bioassay, it was discovered that the cause of mortality was different. In this experiment, the number of *S. frugiperda* that died as a result of bacterial infection was found to be greater in isolates SR1 and SJ11 (SR1 & SJ11=14), followed by isolate SI7 and ST9 (SI7 & ST9=13), while no *S. frugiperda* were found to have died as a result of bacterial infection in the control (C=0). This finding suggests that SR1 and SJ11 are more virulent than SI7 and ST9 and have higher bacterial mortality/bacteriosis when it comes to controlling S. frugiperda (BD% of SR1 & SJ11= 93.33%) and came to the conclusion that there was a significant difference between treatment and bacterial death ($p>0.05$), which means the null hypothesis is rejected.

After the second week of *B. thuringiensis* inoculation, *Myzus persicae* was found to have died from bacterial isolates SR1 (SR1=71), followed by isolates SI7, ST9, and SJ11 (SI7=70, ST9=69, and SJ11=67) but no *M. persicae* was discovered to have died from bacteria in the control (C=0). According to this finding, SR1 is more virulent than other isolates and have greater rates of bacterial death and bacteriosis when it comes to controlling *M. persicae*. After discovering that ($p>0.05$) and rejecting the null hypothesis, we came to the conclusion that there was a significant difference between treatment and bacterial death.

To evaluate an organism's effectiveness, records from both dead and live bioassay are required. During the bioassay, neither of the chosen insect pests was alive, indicating that there may have been another cause, such as fungi, nematodes, or mechanical harm. In this investigation, isolates of *S. frugiperda* included SR1 (OD% =6.67%), SI7 (OD% =13.33%), ST9 (OD% =13.33%) and SJ11 (OD% =6.67%) exhibited lower rates of other-cause mortality than the control group (OD% =40%). In case of *Myzus persicae*, SR1 (OD% =18.67%) had a higher rate of other reason death mortality than the control (21.33%) and other *B. thuringiensis* isolates, and it was discovered that p-value is more than 0.05 ($p>0.05$), which suggests that there is no significant difference between the treatments and other reasons death.

Our bioassay results revealed that 23 *Myzus persicae* were still alive and unaffected by any treatments. SR1 (S% of SR1=5.33%), SI7 (S% of SI7=6.67%), ST9 (S% of ST9=8%), and SJ11 (S% of SJ11=10.67%) all showed lower survival rates than the control group (S% of control =78.67%). Because of the decreased survival rate, it is better able to control insect infestation. According to our research, SR1 had a reduced aphid survival rate, indicating that it was more successful in keeping *Myzus persicae* under control. All *B. thuringiensis* isolates in the *S. frugiperda* case had identical survival rates (0%) but the control had a greater survival rate (60%) therefore, it may be concluded that both isolates of *S. frugiperda* were equally susceptible to it. Survival and treatment had a statistically significant link ($p>0.05$).

When a precise count on the live and dead insects in all five treatments was (Control, SR1, SI7, ST9, and SJ11) available, we applied the Abbott formula to assess the real efficacy of insecticides against the treated insects. The portion of the original killed by the treatment is calculated as the difference between the percentage of living scales in the Control check and the percentage of living scales in the treated check. Using Abbotto's formula, the effectiveness of all four bacterial isolates (SR1, SI7, ST9, and SJ11) against *S. frugiperda* and *Myzus persicae* was finally determined from the outcomes of the bioassay (Abbotts, 1925). In this study, the corrected mortality (efficacy%) of all the *B. thuringiensis* isolates SR1, SI7, ST9, and SJ11 had the same efficacy (100%) against *S. frugiperda*, and for *M. persicae*, the efficacy % of *B. thuringiensis* isolates SR1(93.22%), SI7(91.52%), ST9(89.83%), and SJ11(86.43%) within 1mg/ml was found to be 91.79% and 86.88%, respectively.

A high degree of FAW larval mortality was demonstrated by *Bacillus thuringiensis* isolated from organic agricultural farms of Dharan-17 (Railway), Tarahara, Itahari, and Jhumka. Similar to this, *B. thuringiensis* isolated from Dharan-17 (Railway) organic agricultural farm showed a high degree of aphid mortality, followed by *B. thuringiensis* isolated from Tarahara, Itahari, and Jhumka organic agricultural farms. 1% of the market for agrochemicals, which includes insecticides, fungicides, and herbicides, is currently made up of *B.*

thuringiensis-based products (Ortiz & Sansinenea, 2022). *B. thuringiensis* solutions dominate the market for bacterial products in the microbial pesticide category, with close to 70% of the overall share (Dunham & Trimmer, 2018). Today, *B. thuringiensis* is a favored biological resource for managing lepidopteran, dipteran, and coleopteran populations.

B. thuringiensis is therefore regarded as the most effective bio-pesticide due to its high level of specificity, quick decomposition, ability to kill organisms other than their intended targets, and ability to boost population of beneficial organisms, which increases crop output. *B. thuringiensis* is also safe for people to be exempted from certain restrictions that apply to highly modern synthetic pesticides. It is not just effective against the intended pests. By destroying the insect pest and preserving the crop, the bacteria release proteins called toxins. Crops productivity rises in response to declining pest populations. Beneficial insects aren't affected, only pests that are destructive to crops are eliminated. Chemical pesticides have a wide range of negative consequences on both the environment and living things. *B. thuringiensis* has been used to increase crop output in addition to being a microbial pesticide and a bacterium that promotes plant development (Kumar *et al*., 2021).

Accidental inhalation of *B. thuringiensis* is still harmless because the protein's toxicity primarily affects the alkaline digestive system, whereas exposure to chemical pesticides harms people and may even be fatal because they are carcinogenic. *B. thuringiensis* however, is thought to be incredibly safe in terms of chemical pesticides because the human gut is acidic in nature. One explanation for this is the host spectrum's specificity, which makes it an alternative to the broad range of chemically manufactured pesticides (Schnepf *et al*., 1998). Low-income household may find synthetic pesticides to be an expensive management tool, and the majority of mechanical or cultural control alternatives require a lot of time and labor. Hence, one of the accepted ecological strategies is the biological control mechanism against insect pests on agricultural crops. The field of microbial pesticides offers a singular opportunity for conducting prospective and predicative research in the field of pesticides.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

As a result of the increased use of chemical insecticides, resistance to chemical has developed to manage insect pests throughout time. Microbial pesticides like *Bacillus thuringiensis* could be a practical and effective alternative to conventional insecticides. The goal of this study was to assess how well the bacterial isolates worked against *S. frugiperda* and *Myzus persicae*.

We may conclude from the study that *B. thuringiensis* have the potential to have an effect that kills insects. Since the toxin is produced constantly for a longer period of time, it is more effective and affordable in terms of application and field management than chemical pesticides. Due to its pre-existing presence in soil and the fact that *B. thuringiensis* toxins are only effective against certain insect species, *B. thuringiensis* is regarded as an environmentally beneficial insecticide.

6.2 Recommendations

Based on the above concluding remarks, the following recommendations are made:

1. *B. thuringiensis* can be potentially used as bio-pesticide.

2. *B. thuringiensis* must be examined for virulence and effectiveness in various species.

3. Confirmation of *B. thuringiensis* must be done using molecular techniques.

4. For enhanced efficacy in some species, *B. thuringiensis* in combination with other bio-insecticides may be taken into consideration.

5. It can be deployed as an effective alternative to chemical insecticide.

REFERENCES

Abbott WS (1925).“A method of computing the effectiveness of an insecticide.” Journal of Economic Entomology, **18**: 265-267.

Abd El-Ghany A &Abd El-Ghany N (2017).Molluscicidal activity of *Bacillus thuringiensis* strains against *Biomphalariaalexandrina*sanails. Beni-Seuf University Journal of Basic and Applied Sciences; **6**:391-393.

Aceves-Diez AE, Estrada-CastañedaKj, Castañeda-Sandoval LM (2015).Use of *Bacillus thuringiensis* supernatant from a fermentation process to improve bioremediation of chlorpyrifos in contaminated soils. J Environ Manag**157**:213-219. doi:10.10.1016/j.jenvman.2015.04.026

Aynalem B (2018). Tomato Leafminer, *Tutaabsoluta* (Povolny) (Lepidoptera: Gelechiidae) and its Current Ecofriendly Management Strategies: A review Journal of Agricultural Biotechnology and Sustainable Development. **10**(2):11-24. doi:10.5897/JABSD2018.0306

Bahadur A (2018). Entomopathogens : Role of insect pest management in crops. Trends in Horticulture.1. 10.24294/th.vli4.833.

Basukala AP &Subedi IP (2014).Aphid Pests of *Viciafaba* L. and Their Management in Bhaktapur Municipality, Nepal.*Central Department of Zoology, Tribhuwan University, Kritipur, Kathmandu*.**2**(1):13

Ben-Dov E (2014). *Bacillus thuringiensis* subsp. *israelensis* and Its Dipteran-Specific Toxins.*Toxins*;**6**:1222-1243.

Benson MF (2017). Fall armyworm. http://doi.org/10.5962/bhl.title.123807

Bergey’s manual of systematic bacteriology – Volume 2. Baltimore, London, Los Angeles, Sydney: Williams and Wilkins, 1986. Pp. 965-1599

Berliner E. Âoeberdieschlafsucht der mehlmottenraupe. Z *GesamteGetreidewes*. 1911;3;63. (Ita).

Berry C (2012). The bacterium, Lysinibacillussphaericus, as an insect pathogen.*J. Invertebr. Pathol*;109:1-10. doi: 10.1016/j.jip.2011.11.008.

Bhusal K &Bhattarai K (2019). A Review on Fall Armyworm (*Spodoptera frugiperda*) and its Possible Management Options in Nepal, Journal of Entomology and Zoology Studiewsa, **7**(4): 1289-1292.

Biag DN &Mehnaz S (2010). Determination and Distribution of cry-type genes in halophilic*Bacillus thuringiensis* isolates of Arabian Sea Sediments Rocks. *Microbiological Research*.**165**(5):376-383.

Brar S, Verma M, Tyagi R, Surampalli R, Bernabe S, Valero J (2007). *Bacillus thuringiensis* proteases: Production and role in growth, sporulation and synergism. Process Biochem.**42**:773-790.

Bravo A, Gill SS, Soberon M (2005). *Bacillus thuringiensis* mechanisms and use. In: Gilbert LI, Latrou K, Gill SS, editors. Comprehensive Moleuclar Insect Science. Elsevier; pp. 175-206.

Bravo A, Gomez I, Conde J, Munoz-Garay C, Sanchez J, Miranda R, Zhuang M, Gill SS, Soberon M (2004). Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochem.Biophys.Acta-Biomembr*. 1667, 38-46.

Bravo A, Gill SS, Soberon M (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control.Toxicon.**49**(4):423-435.

Broderick NA, Goodman RM, Raffa KF &Handelsman J (2000). Synergy between zwittermicin A and *Bacillus thuringiensis* subsp. Kurstaki against gypsy moth (Lepidoptera: Lymantriidae). *Environ. Entomol*.**29**, 101-107.

Buchanan RE, Gibbons NE (1974).*Bergey's Manual of Determinative Bacteriology*. Vol. 7. Baltimore: Williams and Wilkins Co.

Bulla LA, Bechtel DB, Kramer KJ, Shethna YI, Aronson AI, Flitz-James pc (1980).Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*.*Crit Rev Microbiol*;**8**:147-204.

CABI (2017).*Spodoptera frugiperda*. Retrived from Invasive Species Compendium: http://www.cabi.org/isc/datasheet/29810

Campbell NA & Reece JB (2002). Biology 6thed.). Pearson Education.611-670. ISBN 978-0-201-75052-6.

Capalbo DMF, Valicente FH, Moreas I de O and Pelizer LH (2001). Solid-state Fermentation of *Bacillus thuringiensis toloworthi* to control fall armyworm in maize. EJB.**4**, 1-4.

Capinera, John L (2005).”Featured creatures”.*University of Florida website – Department of Entomology and Nematology*.University of Florida.Retrived 2009-09-07.

Chilcott CN & Wigley PJ (1993). Isolation and toxicity of *Bacillus thuringiensis* from soil and insect habitats in New Zealand. Journal of Invertebrate Pathology, 61(3), 244-247.

Crickmore N, Zeigler DR, Feitelson J, et al (1998). Revision of the nomenclature for the *Bacillus thuringiensis*pesticidal crystal proteins. Microbiology and Molecular Biology Reviews;62**(3)**:807-813.

Capinera JL (2017). “Fall Armyworm, *Spodoptera frugiperda* (J.E. Smith) (Insecta: Lepidoptera: Noctuidae)”, Extension Service, University of Florida.

Crickmore, N (2006). Beyond the Spore – Past and Future Developments of *Bacillus thuringiensis* as a Biopesticide. J. Appl. Microbiol. 101, 616-619

deMaagd RA, Bravo A, Crickmore N (2001). How B*acillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet;**17**:193-199.

Delgado PAM, Murica OP (2011) Hongosentomopathogenos: Uma alternative para la obtencion de Biopesticidas.Ambi-Agua 6:77-90

Dhaliwal GS, Dhawan AK, Singh R (2007). Biodiversity amd ecological agriculture: Issue and perspectives. Indian J Ecol**34**(2):100-109.

Dhaliwal GS, Jindal V, Mohindru B (2015) Crop losses due to insect pests: global and Indian scenario. Indian J Entomol**77**:165-168

Diaz BM, Oggerin M, Lopez Lastra CC, Ruboi V, Fereres A (2009). Characterization and Virulence of *Lecanicilliumlecanii* against different aphid species, Biological Control, 54:825-835.

Dunham W, Trimmer M (2018). *Biological Products around the World, Bioproducts Industry Alliance Spring Meeting & International Symposium (BPIA.Org)*; 2BMonthly Newsletter: Nairobi, Kenya.

du Rand Nicolette (2009). Isolation of Entomopathogenic Gram Positive Spore Forming Bacteria Effective AgainstColeoptera (PhD thesis). Pietermartizburg, South Africa: University of KwaZulu-Natal.

Early R, Gonzalez-Moreno P, Murphy S & Day R (2018).Forcasting the global extent of invasion of the cereal pest *Spodoptera frugiperda*, the fall armyworm. Neo Biota, **40,** 20-25.

FAO (2017).*Fall Armyworm*. Retrived from Food Chain Crisis: http://www.fao.org/food-chain-crisis/how-we-work/plant-protection/fallarmyworm/en/

Feitelson JS, Payne J, Kim L (1992). *Bacillus thuringiensis*: Insect and Beyond. Nature Biotechnol.**10**, 271-275.

Fernando H, Valicente EDS, Tuelher, Maria Isabelle Santos Leite, Fernanda LF, &Corina MV (2010). Production of *Bacillus thuringiensis*biopesticides using commertal laboratory medium and agricultural by-products as nutrient sources.*RevistaBrasileria de Milho e Sorgo*, 9-(1): 1-11.

Gahatraj S (2019). Integrated management of green peach aphid *Myzus persicae*Sulzer (Hemiptera: Aphididae), International Journal of Entomology Volume 4(2): 42-45. www.entomologyjournals.com

Garbeva P, van Veen JA, van Elsas JD (2003). Predominant Bacillus spp. in agricultural soil under different management regimes detected via PCR-DGGE. *Microb Ecol*. 2003;**45**:302-316.

Goergen G, Kumar L, Sankung SB, Togola A, Tamo M (2016). First report of outbreak of the fall armyworm *Spodoptera frugiperda* (J E Smith) (Lepidoptera, Notuidae), a new alien invasive pest in West and Central Africa.PLoS ONE, 11 (10),Pp.1-9.

Giacomodonato MN, Pettinari MJ, Guadalupe IS, Beatriz SM, Lopez NI (2001). A PCR-based method for the screening of bacterial strains with antifungal activity in suppressive soybean rhizosphere.*World J Microbiol Biotechno*:**17**:51-55.

Gordon RL, Haynes WC, Pang CHN (1973). *Agricultural Handbook*. 427 Washington DC: Agricultural Research service, US Department of Agriculture. The Genus Bacillus.

Guedes BAM, Zanuncio JC, Ramalho FS, Serrão JE (2007). Midgut morphology and enzymes of the obligate zoophytophagous stinkbug *Brontocoristabidus* (Signoret, 1863) (Heteroptera: Pentatomidae) *Pan-Pacific Entomologist*;83(1):66-74.

Handaayani K, Janah SM, Ekowati CN &Kanedi M (2023).Larvicide effects of *Bacillus*sp isolated from soil against fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae). GSC Biological and Pharmaceutical Sciences, 22(03), 028-034.

Hardke JT, RE Jackson & BR Leonard (2014). Opportunities to manage fall armyworm (Lepidoptera: Noctuidae) on Bollgard IIVR cotton with reduced rates of insecticides. J. Cotton Sci. **18**: 59-67

Hegedus D, Erlandson M, Gillott C, Toprak U (2009). New insights into peritrophic matrix synthesis, architecture, and function.*Annual Review of Entomology*;**54**:285-302.

Heimpel AM (1967). A critical review of *Bacillus thuringiensis* var. *thuringiensis* Berliner and other crystalliferous bacteria.*Annu Rev Entomol*;**12**:287-322.

Höfte H & Whitely HR (1989).Insecticidal crystal proteins of *Bacillus thuringiensis.Microbiological Reviews*:53(2):242-255.

Hoy MA (2013). Insect Population Ecology and Moleculer Genetics.IN M.A Hoy, *Insect Molecular Genetics (Third Edition)* (pp.591-659).Academic Press.

Ibarra JE,Del Rincon MC, Orduz S et al (2003). "Diversity of *Bacillus thuringiensis strains* from Latin America with insecticidal activity against different mosquito species". *Applied and Environmental Microbiology*. 69(9):5269-5274.

Igyuve T, Ojo G and Ugbaa M (2018). Fall armyworm (*Spodoptera frugiperda*); it's biology, impact and control on maize production in Nigeria.' Journal of Crop Science **5**: 70-79

Indraini R and Pujiastuti Y (2020).Effectiveness of *Bacillus thuringiensis* as a bioinsecticide in maize crop (Zea Maize L.) against fall armyworm (*Spodoptera frugiperda*).Doctoral Dissertation the Sriwijaya University, Palembang.

Iriarte J, Bel Y, Ferrandis MD, Andrew R, Murillo J, Ferré J & Caballero P (1998). Environmental distribution and diversity of *Bacillus thuringiensis* in Spain. Systematic and applied microbiology, 21(1), 97-106.

Iriarte J, Porcar M, Lecadet MM, Caballero P (2000). Isolation and Characterization of *Bacillus thuringiensis* strains from aquatic environments in Spain. Curr.Microbiol; **40**: 402-408.

Jenkins JL & Dean DH (2001).Binding specificity of *Bacillus thuringiensis* Cry 1 Aa for purified, native *Bombyxmori*aminopeptidase N and cadherin-like receptors.BMC Biochemistery, 2:12.

Liebhold AM, Berec L, BrockerroffEG et al (2016). Eradication of invading insect populations: from concepts to applications. Annu Rev Entomol.doi:10.1146/annurev-ento-010715-023809

Lin SC, Lo YC (2004).Crystal structure and electron micrographs of fingalvalvotoxin A2.*J Mol Biol*;343:477-491.

Keavany BM, Rasko DA & Thomas MG (2009).Characterization of the complete Zwittermicin A biosynthesis gene cluster from *Bacillus cereus*.*Appl. Environ. Microbial*.**75**, 1144-1155.

Kleter GA, Bhula R, Bodnaruk K, Carazo E, Felsot AS, Harris CA, et al (2007). Altered pesticide use on transgenic crops and the associated general impact from an environmental perspective.*Pest Manag Sci*;63:1107-1115.

KoneckaE,Baranek J Hrycak A, Kanznowski A (2011). Insecticidal activity of *Bacillus thuringiensis* strains isolated soil and water. *Department of Microbiology, Faculty of Biology, Adam Mickiewiz university, Umultowska 89,61-614, Poland.*

Knowles BH (1994). Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins.Advances in Insect Physiology. 24:275-308.

Knowles BH, Dow JAT (1993). The crystal δ-endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. *BioEssays*;15(70:469-476.

Kumar P, Kamle M, Borah R, Mahato DK & Sharma B (2021). *Bacillus thuringiensis* as microbial biopesticide: uses and application for sustainable agriculture. Egyptian Journal of Biological Pest Control. **31**:95.

Lamanna C, Sakaguchi G (1971). Botulinal toxins and the problem of nomenclature of simple toxins.*Bacteriol Rev*;35:242-249.

Levy SM, Falleiros AMF, Gregório EA, Arrebola NR, Toledo LA (2004). The larval midgut of *Anticarsiagemmatalis* (Hübner) (Lepidoptera: Noctuidae): light and electron microscopy studies of the epithelial cells. *Brazilian Journal of Biology*;64(3B):633-638.

Li XQ, Tan A, Voegtline M, Bekele S, Chen CS &Aroian RV (2008). Expression of Cry5B protein from Bacillus thuringiensis in plant roots confers resistance to root-knot nematode. *Biol. Control*:97-102.

Liebhold AM, Berec L, Brockerroff EG et al (2016). Eradication of invading insect populatoins: from concepts to applications. Annu Rev Entomol.doi:10.1146/annurev-ento-010715-023809

Madigan MT &Martinko JM (2005). Brook Biology of Microorganisms (11thed.). Prentice Hall.ISBN 978-0-13-144329-7.

Maduell P, Armengol G, Llagostera M, Orduz S, Lindow S (2008). *B. thuringiensis* is a poor colonist of leaf surfaces. *Microb. Ecol*;55:212-219. doi: 10.1007/s00248-007-9268-4.

Mahmud M (2022). Efficacy of bioinsecticidefrom *Bacillusthuringiensis*Ernst Berliner for controlling *Spodoptera frugiperda* Je Smith (Lepidoptera: Noctuidae). Doctoral Dissertation theHasanuddin University. Madagaskar.

Mantzoukas S & Eliopoulos PA (2020).Endophytic entomopathogenic fungi: a valuable biological control tool against plant pests. ApplSci 10:360. https://doi.org/10.3390/app10010360

Meadows MP, Ellar DJ, Butt J, Jarrett P and Burges HD (1992).Distributors, Frequency and Diversity of *B.thuringiensis* in an Annual Food Mill.Applied and Environmental Microbiology, 58, 13344-1350.

Melo AL, Soccol VT, Soccol CR (2016). *Bacillus thuringiensis*: Mechanism of action, resistance, and new applications: review. Critical Rev Biotechnol 36:317-326. doi:10.3109/07388551.2014.960793

Monnerat RG, Bravo A. Proteins bioinseticidasproduzidaspela bacteria *Bacillus thuringiensis*: Mode de ação e resistência. In: Melo IS, Azevedo JL, editors. *ControleBiológico*. Jaguariúna, Brazil: Embrapa-CNPMA; 2000.pp. 163-200.

Morse RJ, Yamamota T, Stround RM (2001). Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure*;9:409-17.

Ohba M, Mizuki E, Uemori A (2009). Parasporin, a new anticancer protein group from *Bacillus thuringiensis. Anticancer Res*;29:427-433.

Okumura S, Ohba M, Mizuki E, Crickmore N, Coté JC, Nagamatsu Y, Kitada S, Sakai H, Harata K, Shin T, et al (2014). Parasporin Nomenclature.

Ortiz A, Sansinenea E (2022). *Bacillus thuringiensis* Based Biopesticides for Integrated Crop Management. In *Biopesticides*; Elsevier: Amsterdam, The Netherlands; pp. 1-6.

Patole SS (2017). Review on Beetles (Coleopteran): An Agricultural Major Crop Pests of the World. Department of Zoology.*Int. J. Life. Sci. scienti. Res.*, 3(6):1424-1432.

Peng D et al (2016). A novel metalloproteinase virulence factor is involved in *Bacillus thuringiensis* pathogenesis in nematodes and insects. Environ. Microbiol.**18**, 846-862.

Periyasamy A, Kkani P, Chandrasekaran B, Ponnusamy S, Viswanathan S, Selvanayagam P, Rajaiah S (2016). Screening and characterization of a non-insecticidal *Bacillus thuringiensis* strain producing parasporal protein with selective toxicity against human colon cancer cell lines. Ann microbiol:1-12. doi:10.1007/s13213-016-1204-8

Province H & Gardner P (1974). The Gram's stained smear and its interpretation. *Hosp. Pract*. **9**, 85-91.

Pokharel P (2019). Fall armyworm have come to Nepal from America, and they can trouble maize farmers here – OnlineKhabar.https://English.onlinekhabar.com/fall-armyworms-have-come-to-nepal-from-america-and-they-can-trouble-maize-farmers-here.html

Polanczyk RA, Zanúncio JC, Alves SB (2009). Relationship between chemical properties of the soil and the occurrence of *Bacillus thuringiensis*.*Ciênc. Rural*;39:1-5.doi: 10.1590/S0103-84782009000100001.

Porcar M, Gomez F, Gruppe A, Gomez-Pajuelo A, Segura I, Schroder R (2008). Hymenopteran specificity of *Bacillus thuringiensis* strain PS86Q3. Biological Control: Theory and Application in Pest Management. 45(3):427-432.

Pocar M & Juarez- Perez V (2003).PCR-based identification of *Bacillus thuringiensis*pesticidal crystal genes. FEMS Microbiology reviews, 26(5):419-432.doi.org/10.1111/j.1574-6976.2003.tb00624.x

Prabhakar A, Bishop AH (2009).Effect of *Bacillus thuringiensis naturally* colonizing *Brassica ampestris*var. *chinensis* leaves on neonate larvae of *Pieris.brassicae. J. Invertebr. Pathol*. 2009;100:193-194.

Prasanna BM, Huesing JE, Eddy R &Peschke VM (2018). Fall armyworm in Africa: A guide for integrated pest management. https://repository.cimmyt.org/handle/10883/19204

Ramanujam B, Poornesha B &Shylesha AN (2020).”Effect of entomopathogenic fungi against invasive pest *Spodoptera frugiperda* (J.E.Smith) (Lepidoptera: Noctuidae) in maize”, Egyptian Journal of Bioilogical Pest Control, 30:100 https://doi.org/10.1186/s41938-020-00291-4

Rana P, Sijapat J, Rana N & Regmi C (2002). Distribution of *Bacillus thuringiensis* in the Soils of Nepal and Their Bioassay. Nepal Journal of Science and Technology, 4(1).

Roy A, Mahata D, Paul D, Korpole S, Franco OL, Mandal SM (2013) Purification, biochemical characterization and self-assembled structure of a fengycin-like antifungal peptide from *Bacillus thuringiensis* strain SM1. Front Microbiol 4:10-3389.doi:10.3389/fmicb.2013.00332

Ruiu L (2015) Insect pathogenic bacteria in integrated pest management. Insects 6:352-367. https://doi.org/10.3390/insects 6020352

Sayed SM, Ali EF, Al-Otaibi SS (2019). Efficiency of Indigenous Entomopathogenic Fungus of *Beauveriabassiana (Balsamo)* Vuillemin Isolates against the Rose Aphid, *Macrosiohumrosae*, (Hemiptera: Alphididae) in Rose Production, Egypt Journal of Biological Pest Control, 29(19):1-7.

Sharanabasappa, Kalleshwaraswamy CM, MaruthiMS &Pavithra HB (2018). Biology of invasive fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) on maize. Indian Journal of Entomology, 80(3), 540.

Sharma R & Sharma P (2021). Fungal entomopathogens: a systematic review. Egypt J Biol Pest Control 31:57. https://doi.org/10.1186/ s41938-021-00404-7

Shashank PR, Twinkle S, Chandrashekar K, Meshram NM, Suroshe SS, Bajracharya ASR (2018). Genetic Homogeneity in South America Tomato Pinworm, *Tutaabsoluta*: A New Invasive Pest to Oriental Region. 3 Biotech 8, 350.http://doi.org/10.1007/s13205-018-1374-0

Singh M, Kumar P, Patel SK, Kalia VC (2013). Production of polyhydroxyalkanoate co-polymer by *Bacillus thuringiensis*.*Indian J Microbiol* 53:77-83.doi:10.1007/s12088-012-0294-7

Singh KS, Cordeiro EMG, Troczka BJ, Pym A, Mackisack J, Mathers, Thomas C, Duarte A et al (2021).”Global patterns in genomic diversity underpinning the evolution of insecticide resistance in the aphid crop pest *Myzus persicae*”. Communications Biology.**4** (1):847. doi:10.1038/s42003-021-02373-x

Soberon M, Lopez-Diaz JA, Bravo A (2013).Cyt toxins produced by *Bacillus thuringiensis*: A protein fold conserved in several pathogenic microorganisms. Peptides;41:87-93.doi: 10.1016/j.peptides.

Sousa MEC, Santos FAB, Wanderley-Teixeira V, et al (2010). Histopathology and ultrastructure of midgut of midgut of *Alabama argillacea* (Hűbner) (Lepidoptera: Noctuidae) fed Bt-cotton. *Journal of Insect Physiology*:56(12):1913-1919.

Speiser B (2002). “ Chapter 219. Molluscicides”.Encyclopedia of Pest Management. In: Pimentel D. 506-508.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998). *Bacillus thuringiensis* and its Pesticidal Crystal Proteins.*Microbiol. Mol. Biol. Rev*. 62, 775-806.

Tang QL, Ma KS, Hou YM, Gao XW (2017). Monitoring Insecticide Resistance and Diagnostics of Resistance Mechanisms in the Green Peach Aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) in China. Pest BiochemPhysiol 143:39-47.

Tanzini M, Alaves S, Setten A, Augusto N (2001). Compatibilidad de agent estensoactivoscom*Beauveriabassiana* y *Metarhiziumanisopliae*.ManejoIntegrado De Plagas 59:15-18

Thomas DJ, Morgan JA, Whipps JM, Saunders JR (2001). Plasmid transfer between *Bacillus thuringiensis* subsp. *israelensis* strains in laboratory culture, river water and dipteran larvae. *Appl Environ Microbiol*;67:330-338.

Torres-Quintero MC, Arenas-Sosa I, Zuñiga-Navarrete F, Hernández-Velázquez V, Alvear-Garcia A &Peña-Chora G (2022). Characterization of insecticidal Cry1Cb2 protein from *Bacillus thuringiensis* toxic to *Myzus persicae* (Sulzer).Vol**189**:107731.

Vreysen MJB, Klassen W, Carpenter JE (2016). Overview of technological advances toward greater efficiency and efficacy in sterile insect-inherited sterility programs against moth pests. FlaEntomol. 99:1-12.doi:10.1653/024.099.sp102

Wei JZ, Hale K, Carta L, Platzer E, Wong C, Fang SC, Aroian RV (2003). *Bacillus thuringiensis* crystal proteins that target nematodes. Proc. Natl. Acad. Sci. USA, 100,2760-2765.

Yezza A, Tyagi RD, Valéro JR, Surampalli RY (2006). Bioconversion of industrial wastewater and wastewater sludge into Bacillus thuringiensis based biopesticides in pilot fermentor. Bioresour. Technol. 97, 1850-1857.

Zhang X, Candas M, Griko NB, Taussing R, Bulla LA Jr (2006). A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway induced by the Cry1Ab toxin of *Bacillus thuringiensis.Proc. Natl. Acad. Sci. USA.* 103, 9897-9902.

Zhu YC, Zeng FR, Oppert B (2003). Molecular cloning of trypsin-like cDNAs and comparison of proteinase activities in the salivary glands and gut of the tarnished plant bug *Lyguslineolaris* (Heteroptera: Miridae) *Insect Biochemistry and Molecular Biology*;33(9):889-899.

APPENDICES

APPENDIX I

MATERIALS AND EQUIPMENTS

List of mateu7frials

1. Glasswares

Petriplates
Micropipette tubes
Beaker
Conical flask
Testtubes
Micropipette
Eppendruff tube
Measuring cylinder
Glassslides

2. Equipments

Equipments	**Company name**
Microscope	Olympus
Incubator	FAITHFUL
Centrifuge	REMI
Refrigerator	LG
Hot air oven	Accumax India
Water bath shaker	Optics technology
Digital balance	SSI DTGOT

3. Chemicals and Reagents

Alcohol
Grams's iodine solution
Crystal violet
Iodine
Distilled water
Coomassic brilliant blue R-250
Saffranin
Malachite green
Lysol

4. Miscellaneous

Inoculating loop
Gloves
Cotton swabs
Forceps
Match box
Test tube rack
Bunsen burner
Marker Labeling sticker
Zip lock plastic bags
Parafilm tape

APPENDIX II

COMPOSITION AND PREPARATION OF DIFFERENT REAGENTS

1. Gram staining reagents

i. Crsytal violet Gram stain

Crsytal violet	20g
Ammonium oxlate	9g
Ethanol or methanol, absolute	95ml
Distilled water	1 lt

Preparation:

Crsytal violet was weighed and transferred to a clean bottle and absolute ethanol was added and stirred until dye dissolved completely. Ammonium oxlate was weighed and dissolved in 200 ml of distilled water. Then it was added to the stain and total volume was increased to 1 litre by adding distilled water and mixed well.

ii. Iodine solution

Iodine	1.0g
Potassium iodine	1.5g
Distilled water	150ml

Preparation:

Potassium iodine was weighed and transferred to a clean bottle. 30-40 ml of distilled water was added to potassium iodine and mixed until it was fully dissolved. Iodine was weighed and added to potassium iodine solution and mixed well. Final volume was made 150ml by adding distilled water and mixed well.

iii. Acetone-alcohol decoloriser

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25ml

Preparation:

To 25 ml distilled water, 475ml of pure alcohol was added, stirred and placed into a clean bottle. Then 500 ml acetone was added and mixed well.

iv. Counter stain reagent

Safranin	10gm
Distilled water	1 lt

Preparation:

In a piece of clean paper, 10 gm of safranin was weighed and transferred to a clean bottle. 1 liter of distilled water was added and mixed well until safranin dissolved completely.

v. Catalase reagent

Hydrogen peroxide solution	3ml
Distilled water	97ml

Preparation:

To 97 ml distilled water, 3 ml of Hydrogen peroxide solution was added and mixed well.

APPENDIX III

I. Culture media

1. LuriaBertani Broth

Ingredients	(Gram/Litre)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.00
Final pH at (25ºC)	6.8±0.2

2.5 gm of media was dissolved in 100 ml of distilled water and boiled. The media was autoclaved for 15 minutes at 15 lbs pressure at 121ºC.

2. Luria Bertani Agar

Ingredients	(Gram/Litre)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.00
Agar	15.00
Final pH at (25ºC)	6.8±0.2

2.5 gm of media was dissolved in 100 ml of distilled water. 0.75 g of agar was added and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121ºC for 15 minutes.

3. Nutrient Broth

Ingredients	(Gram/Litre)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.00
Final pH at (25ºC)	6.8±0.2

1.3 gm of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121ºC for 15 minutes.

4. Nutrient Agar

Ingredients	(Gram/Litre)
Beef extract	0.5
Peptone	2.5
Yeast extract	1.0
Agar	15.0
Distilled water	500 ml

1.3 gm of media was dissolved in 100 ml of distilled water. 0.75 g of agar was added and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121ºC for 15 minutes.

II. Biochemical media

1. Nitrate Broth

Ingredients	(Gram/Litre)
Beef extract	3.0
Peptone	5.0
Potassium nitrate	1.0
Sodium thiosulphate	0.025
Final pH at (25ºC)	7.2±0.2

To dissolve the medium, 0.9 gm was diluted in 100 ml of distilled water and boiled. The media was autoclaved for 15 minutes at 15 lbs pressure at 121ºC.

2. Carbohydrate fermentation medium

Ingredients	(Gram/Litre)
Peptone	10.0
Sodium chloride	5.0
Phenol red	0.018
Carbohydrates (Glucose, Sucrose, Fructose)	1%
Final pH at (25ºC)	7.2±0.2

3. MR-VP media

Ingredients	(Gram/Litre)
Peptone	3.50
Pancreatic digest of casein	3.50
Dextrose	5.0
Monopotassium phosphate	5.0

1.7 gm of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved for 15 minutes at 15 lbs pressure at 121ºC.

4. Simmons citrate agar

Ingredients	(Gram/Litre)
Magnesium sulphate	0.2

Ammonium dihydrogenphosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

2.42 gm of media was heated to dissolve in 100 ml of distilled water. The media was autoclaved for 15 minutes at 15 lbs of pressure at 121ºC.

5. Hydrolysis agar media

i. Starch agar medium

Ingredients	(Gram/Litre)
Nutrient agar	2.8
Starch	1.0
Final pH at (25ºC)	7.2±0.2

2.5 gm of powder was suspended in 100ml purified water and mixed thoroughly. Heated and boiled for 1 minute, autoclaved 121ºC for 15 minutes.

ii. Egg yolk agar

Ingredients	(Gram/Litre)
Nutrient agar	100 ml
Egg-yolk emulsion	8 ml

1.5 gm of nutrient agar powder was suspended in 100ml purified water and mixed thoroughly. Heated and boiled for 1 minute, autoclaved 121ºC for 15 minutes. Allowed it to cool until it reached the temperature of 70 ºC. After that the egg yolk was added to the medium and stirred well until it gets evenly distributed.

iii. Gelatin agar medium

Ingredients	(Gram/Litre)
Nutrient agar	2.8
Gelatin	1.0
Final pH at (25ºC)	7.2±0.2

2.5 gm of powder was properly stirred in 100ml purified water and mixed thoroughly. Heated and boiled for a minute before autoclaving at 121ºC for 15 minutes.

APPENDIX IV

CALCULATION OF EFFICACY

The efficacy% was calculated by using modified Abbotto's formula given below:

$$\text{Efficacy \%} = \frac{\text{Survival untreated\% - Survival treated\%}}{\text{Survival untreated\%}} \times 100$$

For, *S. frugiperda*,

We have,

Survival untreated (Control) = 60%

Survival treated (SR1) = 0%

Survival treated (SI7) = 0%

Survival treated (ST9) = 0%

Survival treated (SJ11) = 0%

So,

$$\text{Efficacy of SR1} = 100 \times \frac{60 - 0}{60}$$

= 100%

$$\text{Efficacy of SI7} = 100 \times \frac{60 - 0}{60}$$

= 100%

$$\text{Efficacy of SI7} = 100 \times \frac{60 - 0}{60}$$

= 100%

$$\text{Efficacy of SI7} = 100 \times \frac{60 - 0}{60}$$

= 100%

For, *Myzus persicae*,

We have,

Survival untreated (Control) = 78.67%

Survival treated (SR1) = 5.33%

Survival treated (SI7) = 6.67%

Survival treated (ST9) = 8%

Survival treated (SJ11) = 10.67%

So,

$$\text{Efficacy of SR1} = 100 \times \frac{78.67 - 5.33}{78.67}$$

$$= 93.22\%$$

$$\text{Efficacy of SI7} = 100 \times \frac{78.67 - 6.67}{78.67}$$

$$= 91.52\%$$

$$\text{Efficacy of SI7} = 100 \times \frac{78.67 - 8}{78.67}$$

$$= 89.33\%$$

$$\text{Efficacy of SI7} = 100 \times \frac{78.67 - 10.67}{78.67}$$

$$= 86.43\%$$

APPENDIX V

Statistical Analysis

Death record of Fall armyworm due to bacteria

ANOVA

Dependent Variable: Death occured due to bacteria

Source	Sum of Squares(S.S)	df	Mean Square(M.S.S)	$F_{Calculated}$	Sig. (p-value)
Model	**243.333[a]**	**5**	**48.667**	**182.500**	**.000**
Treatments	**243.333**	**5**	**48.667**	**182.500**	**.000**
Total	**246.000**	**15**			

Interpretation:The null hypothesis is rejected because the p-value is found to be less than 0.05. The study finds that there is significant difference between and within groups (p>0.05).

Post Hoc Tests
(Controls, SR1, SI7, ST9, SJ11)

Multiple Comparisons

Dependent variable: Death occurred due to bacteria
Tukey HSD

(I) (Controls,SR1,SI7,ST9,SJ11	(J) (Controls,SR1,SI7,ST9,SJ11	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	SR1	-4.6667*	.42164	.000	-6.0543	-3.2790
	SI7	-4.3333*	.42164	.000	-5.7210	-2.9457
	ST9	-4.3333*	.42164	.000	-5.7210	-2.9457
	SJ11	-4.6667*	.42164	.000	-6.0543	-3.2790
SR1	Control	4.6667*	.42164	.000	3.2790	6.0543
	SI7	.3333	.42164	.928	-1.0543	1.7210
	ST9	.3333	.42164	.928	-1.0543	1.7210
	SJ11	.0000	.42164	1.000	-1.3876	1.3876
SI7	Control	4.3333*	.42164	.000	2.9457	5.7210
	SR1	-.3333	.42164	.928	-1.7210	1.0543
	ST9	.0000	.42164	1.000	-1.3876	1.3876
	SJ11	-.3333	.42164	.928	-1.7210	1.0543
ST9	Control	4.3333*	.42164	.000	2.9457	5.7210
	SR1	-.3333	.42164	.928	-1.7210	1.0543
	SI7	.0000	.42164	1.000	-1.3876	1.3876
	SJ11	-.3333	.42164	.928	-1.7210	1.0543
SJ11	Control	4.6667*	.42164	.000	3.2790	6.0543
	SR1	.0000	.42164	1.000	-1.3876	1.3876
	SI7	.3333	.42164	.928	-1.0543	1.7210
	ST9	.3333	.42164	.928	-1.0543	1.7210

*. **The mean difference is significant at the .05 level.**

Death record of *Myzus persicae* due to bacteria

ANOVA

Dependent Variable: Death occured due to bacteria

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	**4411.333[a]**	**5**	**882.267**	**426.903**	**.000**
Treatments	**4411.333**	**5**	**882.267**	**426.903**	**.000**
Total	**4432.000**	**15**			

Interpretation:The null hypothesis is rejected because the p-value is found to be less than 0.05. The study finds that there is significant difference between and within groups (p>0.05).

Post Hoc Tests

(Controls, SR1, SI7, ST9, SJ11)

Multiple Comparisons

Dependent Variable: Death occured due to bacteria
Tukey HSD

(I) (Controls,SR1,SI7,ST9,SJ11	(J) (Controls,SR1,SI7,ST9,SJ11	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	SR1	-19.0000*	1.17379	.000	-22.8630	-15.1370
	SI7	-20.0000*	1.17379	.000	-23.8630	-16.1370
	ST9	-19.0000*	1.17379	.000	-22.8630	-15.1370
	SJ11	-18.6667*	1.17379	.000	-22.5297	-14.8036
SR1	Control	19.0000*	1.17379	.000	15.1370	22.8630
	SI7	-1.0000	1.17379	.908	-4.8630	2.8630
	ST9	.0000	1.17379	1.000	-3.8630	3.8630
	SJ11	.3333	1.17379	.998	-3.5297	4.1964
SI7	Control	20.0000*	1.17379	.000	16.1370	23.8630
	SR1	1.0000	1.17379	.908	-2.8630	4.8630
	ST9	1.0000	1.17379	.908	-2.8630	4.8630
	SJ11	1.3333	1.17379	.785	-2.5297	5.1964
ST9	Control	19.0000*	1.17379	.000	15.1370	22.8630
	SR1	.0000	1.17379	1.000	-3.8630	3.8630
	SI7	-1.0000	1.17379	.908	-4.8630	2.8630
	SJ11	.3333	1.17379	.998	-3.5297	4.1964
SJ11	Control	18.6667*	1.17379	.000	14.8036	22.5297
	SR1	-.3333	1.17379	.998	-4.1964	3.5297
	SI7	-1.3333	1.17379	.785	-5.1964	2.5297
	ST9	-.3333	1.17379	.998	-4.1964	3.5297

***. The mean difference is significant at the .05 level.**