CHARACTERIZATION OF *METARHIZIUM ANISOPLIAE* ISOLATED FROM ORGANIC FARMS AND ITS EFFICACY AGAINST THE INSECT PESTS UNDER LABORATORY CONTROLLED CONDITION

A

Dissertation

Submitted to the **Department of Microbiology,**

Central Campus of Technology

Tribhuvan University, Dharan, Nepal

In partial fulfillment of the requirements for the Award of

Degree of Master of Science in Microbiology

(Agriculture)

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CERTIFICATE OF APPROVAL

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ABSTRACT

In agriculture, insect pests are one of the most constrictive aspects resulting in greater losses in crop quality and quantity if not treated promptly. So, focusing on numerous novel solutions for the management of insect pests that must be environmentally benign is of critical importance. As a result, the primary goal of the study was to characterize *Metarhizium anisopliae* and to investigate its insecticidal activity against insect pests in laboratory controlled conditions. Altogether, 60 soil samples were collected from randomly selected four organic farms of Basantatar, Khanar, Tarhara, and Buddhachowk, Nepal, and the isolates were isolated and conventionally identified to confirm that they were *Metarhizium anisopliae*. After then, the isolates SK13 & SC1 were confirmed as strains of *Metarhizium anisopliae* by molecular method 18S rRNA gene sequencing. The isolates were then bioassayed against *S. frugiperda* and *Myzus persicae*. For this objective, a conidial suspension of the isolates (1.76×10^7spores/ml) was prepared and fed to the insects housed in disposable plastic glass by direct dipping the leaves of maize and beans before providing them to the relevant insect. Following the bioassay, In the case of *S. frugiperda*, isolates SK13 and SC1 had the same efficacy, however in the case of *Myzus persicae*; SK13 had a higher efficacy (91.79%) than isolate SC1 (86.66%). The results show that using this isolated fungus against insect pests will become an appealing alternative and a long-term solution for plant protection, resulting in decreased chemical pesticide exposures and automatically relieving our agricultural system of its detrimental consequences.

Keywords: Insect Pests, Pesticides, Biocontrol agent, *Metarhizium anisopliae*, Mycotoxins, 18S rRNA.

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LIST OF ABBREVIATIONS

CFC	: Conidia Forming Cell
EMP	: Entomopathogen
EPF	: Entomopathogenic Fungi
FD	: Fungal Death
IPM	: Integrated Pest Management
LP	: Lactophenol
OD	: Other Reason Death
PDA	: Potato Dextrose Agar
RARS	: Regional Agricultural Research Station
S	: Survival
SC1	: Isolated *Metarhizium anisopliae* SC1
SK13	: Isolated *Metarhizium anisopliae* SK13
SM	: Selective Media
UV	: Ultra Violet
FAW	: Fall Army Worm

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CHAPTER-I
INTRODUCTION AND OBJECTIVES

1.1 Background

Agriculture has always been plagued by numerous insect pests, which diminish crop yield by altering the quality and quantity of the crops involved. Pests can cause direct damage to crops or plants by eating them or weakening them, as well as indirect damage by spreading infections. Pests and diseases are widely believed to be the cause of crop losses of 20% to 40% worldwide. Savary et al. (2019) found that the average yield losses for wheat, rice, maize, potatoes, and soybeans were 21.5%, 30%, 22.5%, 17%, and 21.5%, respectively. They also demonstrated that the biggest losses were associated with countries with food shortages and rapidly expanding populations, as well as those with new or re-emerging pests and diseases. Khanal et al (2021) estimated that insect infestations and postharvest management result in the loss of 15–20% of total grain production in Nepal.

Many chemically synthesized pesticides and insecticides have been used to tackle these insect pests, but their misuse and excess use have resulted in insecticidal resistance and lingering consequences, including numerous health issues (Pilkington et al, 2010). So, biological control was introduced as an alternative to chemically manufactured insecticides for a variety of economic insect pests, with an emphasis on consumer health, varied environmental issues, crop quality, and quantity, and IPM systems (Kavallieratos et al, 2014).

Among various, fungal infections have received a lot of interest as microbial biological control agents in recent years since they are low in mammalian toxicity, occur naturally, and may easily infect insects by touch (Kavallieratos et al, 2014). They also control bugs that have piercing and sucking mouthparts, which are the most common pests in the crop (Wraight et al, 2001). The potential for entomopathogenic fungi to manage these insect pests in agriculture is immense. Fungi have an advantage over other biopesticides since they have a greater host range and can control a variety of insects and

pests with a single isolate (Lee et al, 2018). Fungi begin to grow when they become attached to the target insects and use the insects as a source of energy. The fungus releases spores that germinate on the surface of its host and then spread throughout its body to infect it. This causes the insects' outer membranes to degrade, resulting in desiccation and death (Rustiguel et al, 2017).

All across the world, entomopathogenic fungi are utilized to manage a variety of important agricultural pests. They may be the most adaptable biological control agent, aiding in the eco-friendly suppression of pest populations (Khadka, 2019). Depending on the species of fungus and the quantity of infecting spores, death occurs between 4 and 10 days after infection. After death, the fungus on the dead body produces thousands of new spores, which scatter and allow them to continue their life cycle on new hosts.

Metarhizium anisopliae is a prevalent entomopathogenic fungus that infects a wide range of insect hosts (Dean et al, 2002). It is named "green muscardine fungus" because it produces green, cylindrical conidia that contain a large number of spores (Moore et al, 1996). It has been demonstrated to control over 200 different insect pests from several orders, including Lepidoptera, Orthoptera, Hemiptera, Coleoptera, Dermoptera, etc. It is extensively recognized as a biocontrol agent for a wide range of insect pests (Reddy et al, 2014) such as termites, whiteflies, corn borers, locusts, aphids, mealy bugs, and thrips (Keppanan et al, 2018).

Because of its extensive distribution ability, high lethality, and wide range of infectivity against a variety of insect pests, *Metarhizium anisopliae* is regarded as one of the most promising mycoinsecticides (Ypsilos & Magan, 2005). The mass production methods are much easier, safer, and less expensive than Bt and NPV. Because they infect insect pests directly through the epidermal cells rather than through ingestion like bacteria and viruses do, most of the insects can be impacted. *Metarhizium anisopliae* infects the host by direct contact with propagules such as conidia, blastospores, or hyphae; however, subsequent infections were caused by the horizontal spore diffusion from mycoses cadavers (Shan & Feng, 2010).

When a conidium has a chance to adhere to the first-line barrier of the host, infection will begin. It is adaptive to produce different toxins when conditions are favorable for healthy growth. *Metarhizium* kills insects by depriving them of nutrients, invading and destroying tissues, and releasing toxins into the vulnerable host as well as by producing proteolytic enzymes such as proteases, chitinase, lipases, and other enzymes that aid in the breakdown of cell wall components (Lubeck et al, 2008). Then parasitize the entire muscular body of the susceptible host, causing flaccidity and, eventually death.

There are a variety of insect pests of several orders that cause major crop damage. They undermine food security at the household, national, and international levels and inflict significant economic losses. *Myzus persicae* (Green Peach aphid) is a severe insect pest that causes direct and indirect damage to healthy plants by consuming plant nutrients and distributing various viruses (Diaz et al, 2009). The introduction of this pest might harm crop quality. Aphids consume sap from growing leaves and flower buds (Gahatraj, 2019). And the leaves that are infected may curl downward, turn brown, and eventually fade away. On immature plant tissue, *Myzus persicae* can gather in extremely high concentrations, causing water stress, wilting, and retarded plant and slowing the plant's development pace. A prolonged aphid infection can significantly reduce the output of root and foliar crops (Thomas et al, 2018). When infestations are bad enough, aphid damage might even kill plants. It can also reduce plant health, growth, and productivity.

Similarly, *Spodoptera frugiperda* (maize fall armyworm) is the most typical insect pest, and it is polyphagous, feeding on over 85 different host species (Kandel & Poudel, 2020). They are responsible for serious direct ear injuries and leaf-feeding damage (Bessin, 2019), with younger larvae causing the most damage. Even though it was only reported in 2016, it has now become prevalent in over 100 countries in a relatively short duration (Kandel &Poudel, 2020).

Metarhizium anisopliae kill the insect pests just through contact not requiring ingestion for infection. It shows its efficacy is eco-friendly as well as there is no residual effect on the soil, which helps to improve soil health. It also doesn't have harmful consequences on the environment and living beings. And by observing the results, we recommend that the isolated strains of *Metarhizium anisopliae* Sk13 & SCl were the best choice for the control of *S. frugiperda* and *M. persicae*, which is a current experiment-based knowledge exigency in various agriculture-based research centers as well as to the general public.

1.2 Objectives

1.2.1 General objectives

The overall aim of this study was to characterize *Metarhizium anisopliae* isolated from soil samples of organic farms and its efficacy against *Spodoptera frugiperda* and *Myzus persicae* under laboratory-controlled conditions.

1.2.2 Specific Objectives

a. To isolate *Metarhizium anisopliae* from soil samples of organic farms.
b. To identify *Metarhizium anisopliae* by using 18s rRNA sequencing.
c. To perform rearing of *Spodoptera frugiperda* & *Myzus persicae* under laboratory-controlled conditions.
d. To determine the insecticidal property of the identified fungus against the insect pest taken.

CHAPTER-II

LITERATURE REVIEW

Plant pathogens, which include fungi, bacteria, viruses, and nematodes, as well as weeds, arthropods (mainly insects and mites), molluscs, and a few vertebrates, are examples of agricultural insect pests (Bahadur, 2018). About 10,000 species of insects of different orders attack crops that are used as food, but only 10% of all identified pest species are considered significant crop plant pests (Dhaliwal et al, 2007). They cause about 40% of the crop loss in the world, which is a significant hindrance to agricultural production.

Various emerging and exotic insect pests pose a threat to food security (Rathee & Dalal, 2018). Insect orders such as Lepidoptera, Coleoptera, Hemiptera, Dermoptera, and some others pose significant damage to agriculture. Insects cause the majority of plant damage by eating plant parts found above and below the earth directly, causing wilting of shoots and branches, a reduction in photosynthesis-available leaf surface, distortion of new shoots, reduction of the plant's proliferation and vitality (Pedigo & Rice, 2006). They also cause damage indirectly to healthy plants by transmitting fungal, bacterial, or viral infections. Furthermore, extensive oviposition of insects into plant tissue can result in the death or dieback of stems or branches of crops and plants (Barbercheck, 2011).

Additionally, it is anticipated that as the climate warms, pest insect losses would rise. For instance, Deutsch et al (2018) predicted that with each degree of global mean surface warming, yield losses of cereals (wheat, maize, and rice) will raise by 10 to 25%. This will be most noticeable in temperate zones, where the majority of grain is grown because heat accelerates pest population growth and metabolic rates.

2.1 Biological management of insect pests

In agriculture, insect pests are one of the major constraints, resulting in higher crop quality and quantity losses if not addressed promptly. A large volume of chemical pesticides is in practice to control these pests. And the continued and inappropriate use of these chemicals alters natural enemy dynamics, leads to pest resistance to these compounds, and is hazardous to users (Togola et al, 2018).

So the hunt for new alternate eco-friendly biological control agents such as entomopathogenic fungi became imperative (Anand et al, 2009). Various Nations like Switzerland, Austria, New Zealand, and Australia have successfully used microbial control to combat these insect pests (Keller, 2000). The most effective alternative control method that offers environmentally safe and long-term plant protection is biological control, which has been seen as a good option (Assefa & Ayalew, 2019).

In agricultural systems, there are various microscopic pathogens and lepidopterans bioactive constituents which have been used effectively (Pilkington et al, 2010). *Nomuraea rileyi*, BT, NPV, MNPV, *Metarhizium anisopliae*, *Beauveria bassiana*, and entomopathogenic nematodes (*Heterorhabditis*, *Steinernema*) are examples of potential microbes that could be used to treat FAW (Humagain et al, 2019).

2.2 Major hosts of entomopathogenic fungi

Entomopathogenic fungi occur naturally in soils from various habitats all over the world (Scheepmaker & Butt, 2010). They can transmit nitrogen from infected insects to host plants because they are rhizosphere competent, endophytic, and have “mycorrhizal” properties (Behie & Bidochka, 2014; Behie et al, 2015). About 1000 species of entomopathogenic fungi (EPF) have been reported from various taxonomical divisions of the fungal kingdom (Kaya & Vega, 2012). Fungi are significant entomopathogens because they are combative, transmit the infection to insects through contact, thrive in the environment for a long time, and have one of the broadest host ranges (Santharam, 2001).

Table 1: Common entomopathogenic fungi and their hosts (Source: Butt & Goettel, 2000).

Entomopathogenic fungus Division Deuteromycotina	**Invertebrate Host**
Aschersonia aleyrodis	Whiteflies,Scales
Beauveria bassiana	Wide host range
Beauveria brongniartii	Cockhafers and sugarcane borer
Culicinomyces Spp.	Mosquitoes
Metarhizium album	Homopteran insects
Metarhizium anisopliae	Wide host range
Metarhizium falvoviride	Orthopteran insects
Nomuraea rileyi	Lepidoptera
Paecilomyces farinosus	Coleoptera, Lepidoptera
Paecilomyces fumosoroseus	Wide host range
Tolypocladiu mcylindrosporum	Mosquitoes
Verticillium lecanii	Wide host rang**e**

2.3 *Metarhizium anisopliae* as a biological control

Many fungal endophytes are important for managing insect populations. The most thoroughly investigated insect-killing species is found in the genus *Metarhizium.* And the species of the genus *Metarhizium* that has undergone the most extensive research is *Metarhizium anisopliae*, an anamorphic fungus that belongs to the phylum Ascomycota (Tiago et al, 2014). From the arctic to the tropics, this fungus can be found all over the world in a dizzying array of habitats, including woods, grasslands, wetlands, coastal areas, and even deserts (Zimmerman, 2007).

It is named "green muscardine fungus" because it produces cylindrical conidia that contain a large number of spores typically which are a green color when conidia-ting on their arthropod hosts' corpses or in axenic culture (Moore et al, 1996). It is a common insect pathogen found in soils and has been studied and used as an insect pathogen for biocontrol (Clifton et al. 2018). However, in

addition to being an insect pathogen, this fungus easily colonizes the rhizosphere of plants (St. Leger et al, 2011).

Metarhizium anisopliae is regarded as one of the promising mycoinsecticides because of its wide distributing inability, strong lethality, and a broad array of infectivity against a variety of insect pests (Ypsilos & Magan, 2005). It exhibits an incredibly flexible metabolism. This metabolism allows them to thrive in a variety of environments, including those with limited nutrients (Rangel et al, 2008) and the presence of substances that are toxic to other fungi (Roberts & St. Leger 2004; Rangel et al, 2010).

When compared to Bt and NPV, the mass production techniques are much simpler, safer, and less expensive. Unlike bacteria and viruses, they infect insect pests directly via the epidermal cells and therefore do not require ingestion to infect them; hence sucking insects can be affected as well. *Metarhizium anisopliae* infect insects by invading their bodies and using a variety of enzymes, including proteases, chitinases, adhesins, actins, and hydrophobins, which must be expressed for the fungi to penetrate the insect cuticle (Cruz-Avalos et al, 2018). These enzymes are essential to the infection process because their inability to enter the insect hemocoel prevents the pathogen from developing further (Zhang et al, 2011).

GC and Keller (2003) found that *M. anisopliae*, in particular, is widely distributed in Nepal, both in agriculture and grassland. It has been used as a mycoinsecticide since Metchnikoff's time (Lord, 2005). Rijal et al (2008) discovered that after 10 days of treatment of *Helicoverpa armigera* with *Metarhizium* isolates, the larval mortality rate was over 85%.

According to Bohora et al (2018), indigenous *Metarhizium* isolates have higher white grub mortality than commercial isolates. Aktuse et al (2019) in their study to assess the efficacy of entomo-pathogenic fungi against eggs and second instar larvae, found that the *Beauveria* isolates caused 30% mortality of second instar larvae, while the *Metarhizium* isolates caused 87 % and 96.5 % mortality of egg and neonate larvae, respectively.

Metarhizium anisopliae (Daman) was found to be effective in reducing whitefly populations where there were few nymphs (NARC, 2011). The eggs and second-instar larvae of fall armyworm have also been successfully combatted by *Metarhizium anisopliae* and *Beauveria bassiana* (Komivi et al, 2019). Moderate mortality of 30% was brought on by *B. bassiana* in second-instar larvae while *M. anisopliae* caused egg mortalities of 79.5–87.0% in the lab condition. And also the cumulative mortality of eggs and newborns from *M. anisopliae* was as high as 96% under laboratory-controlled conditions.

2.4 Taxonomy of *Metarhizium anisopliae*

In the late 1870s, infected insects were used to develop the first known species of the *Metarhizium* Sorokin genus, which was discovered in Ukraine. It was given the generic name *Entomophthora anisopliae* at first. The scarab host was originally named *Anisoplia austriaca* (Metschnikoff, 1879), but the following year it was renamed *Isaria destructor* (Metschnikoff, 1880). Because both generic names were incorrect, Sorokin proposed a new generic name for this fungus that is *Metarhizium* (1883). And the current scientific name, a*nisopliae*, was retained. Three species described by Sorokin (1883) were rejected by Tulloch based on the morphology of the hyphal bodies formed within the hemocoel of their respective insect hosts and the conidial size of the respective species.

There were several species of *Metarhizium* described before 1976, but Tulloch (1976) only accepted *Metarhizium anisopliae* and *Metarhizium flavoviride*; all other species were synonymized or treated as varieties (Robert & Leger, 2004). The boundaries of *Metarhizium anisopliae* and *M. flavoviride* were widened by Driver et al. in 2000. Driver et al (2000) identified eight lineages as varieties and one unidentified species group (i.e. *M. flavoviride* "Type E") using the findings of a phylogenetic analysis of the nuclear ribosomal internal transcribed spacer (ITS).

Based on physiology and/or nucleic acid, additional identification and classification techniques have been tried. The taxonomy of the genus *Metarhizium* has recently undergone revision and the majority of the current review's attention is given to recent research on *M. anisopliae* strains that were

initially isolated from infected insects (Bischoff et al, 2009). About 30 *Metarhizium* taxa names have been included by CABI Bioscience et al (2007) in their Index Fungorum.

Table 2: General Taxonomy of *Metarhizium anisopliae* (Source: The CABI Bioscience Database of Fungal Names (Funindex) (Index Fungorum)

Kingdom:	Fungi
Phylum:	Ascomycota
Subphylum:	Pezizomycotina
Class:	Sordariomycetes
Subclass:	Hypocreomycetidae
Order:	Hypocreales
Family:	Nectriaceae
Genus*:*	*Metarhizium*
Species:	*Metarhizium anisopliae (*Metschn.) Sorokin

2.5 Morphology and biology of *M.anisopliae*

Metarhizium anisopliae is a well-researched facultative parasite for insect pest control that may infect a wide range of insects (Liu et al, 2007; Hoe et al, 2009). Tulloch (1976) reported that *M. anisopliae* appears white when young but transforms to dark green as the conidia mature. There are two different varieties of *M. anisopliae*: the short-spored *M. anisopliae var anisopliae* (conidia 3.5-9.0 m) and the long-spored *M. anisopliae var majus* (conidia 9.0-18 m).

Conidia are single-celled, smooth-walled, oval, aseptate, and cylindrical chains that form prismatic or cylindrical columns or a solid mass of parallel chains (Sinha et al, 2016). They are pale to bright green to yellow-green, olivaceous, sepia, or white in mass (Humber 1997).

The life cycle of *M. anisopliae* includes both a parasitic and a saprophytic phase. The parasitic phase develops when the conidia of the fungus make contact with the integument of the potential host. During the parasitic phase, both physical and chemical interactions are probably crucial for adhesion. According to Fargues (1984), molecular interactions and electrostatic forces may play a role in adhesion. Conidia firmly cling to insect cuticles, and it is believed that involve non-specific adhesion mechanisms facilitated by the hydrophobic nature of the conidial cell wall.

After that, the conidia germinate, developed into a germ tube and penetrate the hemocoel of the pests, and develop into it, preceded by a rapid growth of fungal cells that ultimately causes the death of the host (Aidross & Roberts, 1978). A single blastopore of the fungus that sprouts from the penetration mechanism circulates and grows in the insect hemocoel, depriving the host as its nutrients. Extracellular enzymes such as protease and lipase are produced by entomopathogens as part of their adaptive response, which ultimately kills insects.

2.6 Mode of infection of *Metarhizium anisopliae*

The infectious form is typically an asexual spore called conidia, which lives in the same environment as potential hosts. When conidia come into contact with the cuticle of a susceptible host, the infection process begins. The infection process includes the following steps.

2.6.1 Adhesion

The adhesion phase is critical for *M. anisopliae* to successfully infect the host (Leao et al, 2015). To enter the host through the cuticle, *Metarhizium anisopliae* develops a combination of cuticle-degrading enzymes and mechanical pressure (Barra-bucarei et al, 2016). Conidia, asexual spores of

Metarhizium species, are drawn to their hosts' waxy epicuticle by a combined action of passive hydrophobic forces, electrostatic forces, & protein interactions. Conidia, also known as rodlets, have an outer cellular layer composed of hydrophobins, proteins that aid in the conidia's adhesion to the hydrophobic epicuticle (Butt et al, 2013 & Tseng et al, 2014).

Conidia adhere to the cuticle based on the surface's hydrophobicity, the topography and chemical structure of the host's cuticle, the host's preferences for feeding, and the environment (Ment et al, 2010; Santi et al, 2010 & Greenfield et al, 2014). It is known that *Metarhizium anisopliae* carries the Mad1 and Mad2 genes, which help the insect, adhere to the target host's cuticle.

2.6.2 Germination of conidia

When exogenous carbon and nitrogen supplies are accessible, the process of conidial germination of *M. anisopliae* begins (Ment et al, 2010). During initial germination, trehalase, which uses trehalose found in the haemolymph of the respective host. Trehalase activity was thought to provide glucose for energy production (Blanford, 2012).

2.6.3 Differentiation of germ tube into appressoria

Just after germination, the spores enlarged and produce germ tubes that eventually differentiated into appressorium (Lovett & Leger, 2015). In place of hydrophobins, specific adhesins genes of *Metarhizium anisopliae* such as Mad1 and Mad2, more strongly secure the fungus to the cuticle and promote conidial germination, which leads to the formation of appressorium (Wang & Leger, 2007a).

The MPL1 gene, which encodes for MPL1, is involved in lipid homeostasis and appressorium development, and the ODC1 gene, which encodes for ornithine decarboxylase, was shown to be elevated during conidia germination and germ tube differentiation to produce appressorium (Maddrigal et al, 2011; Wang & St. Leger, 2007b). Appressorium also secretes a thin coating of mucilage to help the fungus adhere to the cuticle (Staats et al, 2014).

2.6.4 Penetration of cuticle

Entomopathogenic fungi access the nutrients needed for their growth and reproduction by penetrating the insect body through the cuticle. Both mechanical pressure and enzymatic degradation are required for entry. Penetration sites were frequently visible as dark, melanocytic lesions in the epicuticle (Zacharuk, 1973).

A serine protease that functions similarly to subtilisin and starts the degradation of proteins appears to be the primary enzyme driving this growth (St. Leger et al, 1996). During penetration, *M. anisopliae* produces several proteins including trypsins, carboxypeptidases, subtilisins, and chymotrypsins (Rajula et al, 2021). These proteins cause procuticle damage in the target host. The fungus colonizes the host as soon as it enters and begins to produce destruxins, which suppress the immune system of the insect.

2.6.5 Colonization of haemolymph, sporulation, and extrusion

After successfully penetrating the insect's defenses, the fungus disperses into the hemolymph by producing blastospores or by existing as a distinct yeast-like structure, reaching the respiratory system of the insect where it can absorb the most nutrients. Insect death is caused by a variety of factors, such as nutrient depletion, mechanical injury introduced by tissue invasion, and toxin production inside the insect's body (Sinha et al, 2016).

M. anisopliae produces destruxins, particularly the more insecticidal destruxins A and E, which suppress the host's cellular and humoral defense systems. The spores would then be covered by the host hemocytes to protect them from any attack meant to wipe them out. Finally, when the green mycelium forms on the insect carcass, sporulation takes place and hyphae extrusion is seen (Hubbard et al, 2014).

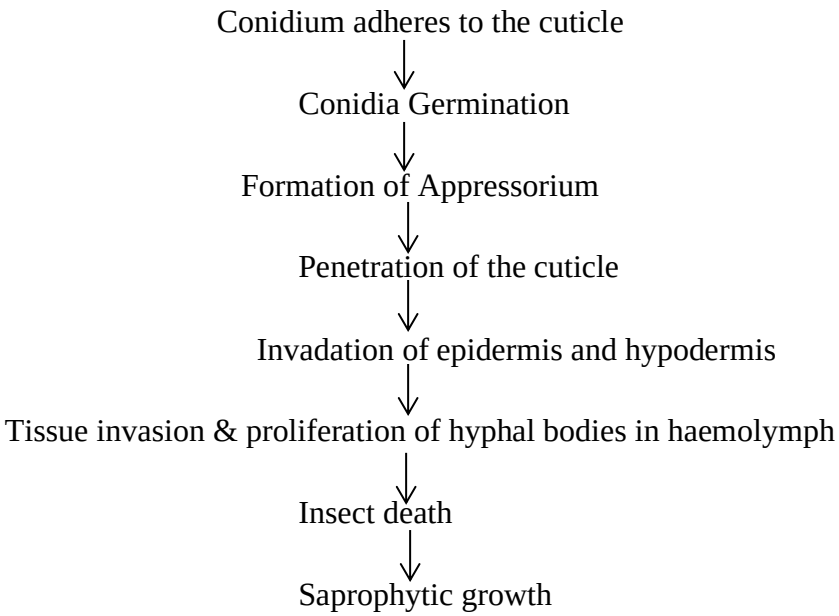


Fig 1: Diagrammatic representation of Infection steps.

2.7 Toxins produced by *Metarhizium anisopliae*

In 2000, Strasser et al. reported that secondary metabolites and toxins produced by *Metarhizium* may be present in bio-control formulations for the control of insect pests and were examined as a safe alternative because it doesn't show any adverse effects on non-target species. A conidium is the one that starts infection when it gets an opportunity to attach to the host's first-line barrier. Then, various hydrolytic enzymes such as chitinases, proteases, and lipases are responsible for the disintegration of the cuticle and invasion into the host insect.

When it is favored to grow properly, it is adaptive to produce various toxins. Destruxins (Dxs), a group of cyclic depsipeptides with insecticidal, antiviral, and phytotoxic properties, are also being investigated for their potential cytotoxic effects in cancerous cells. Destruxin A, E, and B (DA, DE, and DB) are Dx that have insecticidal properties (Thomsen & Eilenberg, 2000). Brassica plants were toxic to DB and desmethyl-DB (Saharan et al, 2003). When injected into *Galleria mellonella larvae*, DB results in titanic paralysis.

2.8 *Myzus persicae* (Green peach Aphid)

Aphids are widespread vegetable 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). It is a highly polyphagous species with a host range of more than 400 species in 40 distinct plant families, including many commercially significant crop plants. It prefers peaches but also affects fruits, vegetables, sunflowers, tobacco, and sugar beet. The best cultural techniques are removing crop leftovers and weed hosts.

2.8.1 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 and 12 days for an entire generation and over 20 generations reported annually (Gahatraj, 2019).

The aphid undergoes cyclical parthenogenesis, in which several generations of apomictic parthenogenesis are followed by a single sexual generation, to reproduce regularly. In the spring and early summer, *Myzus persicae* reproduces parthenogenetically on peaches, and in the autumn, it reproduces sexually (Karagounis et al, 2006). 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. 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 for 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.

2.9 *Spodoptera frugiperda* (Fall armyworm)

Spodoptera frugiperda (Lepidoptera: Noctuidae), a deadly pest with a ravenous appetite, makes a huge impact on over 80 cereal and vegetable crop host species (Goergen et al, 2016). It causes significant economic damage to cultivated crops such as maize, rice, sorghum, sugarcane, and wheat as well as other vegetable crops and cotton. Leaf whorls in their early stages, ears, and plumes are regarded as crucial feed, leading to significant maize damage and, in certain cases, total yield loss.

This pest emerged in the tropical and subtropical zones of the United States (CABI, 2017) and has now evolved into a novel intruding pest (Goergen et al, 2016). Georgen et al. discovered the first official report of the pest from Africa in 2016; it has spread to more than 40 other African countries (FAO, 2018). The pest was first discovered in Asia in May 2018 in the Shivamogga district of the Indian state of Karnataka (Sharanabasappa Kalleshwaraswmy et al, 2018; Ganiger et al, 2018) in Andhra Pradesh (EPPO, 2018). This pest has recently been reported in Sri Lanka (FAO, 2019), Bangladesh (FAO, 2019), Myanmar, Thailand (IPPC, 2018), Vietnam, Myanmar, China, Taiwan, South

Korea (IPPC, 2019), Indonesia, Japan (IPPC, 2019) and islands of Saibai and Erub in Torres Strait (IPPC, 2020).

The Nepal Agriculture Research Council (NARC) has reported the first spotting of fall armyworms in the Nawalparasi, district (N 27o 42'16.67", E 084○ 22'50.61") Nepal, called phaujikira locally (Pokharel, 2019).On August 12th, 2019, NPPO Nepal formally declared the pest's invasion. According to Montezano (2018), FAW larvae can feed on 353 different plant species, but have a strong preference for maize.

The fall armyworm may be able to produce many generations in a single season and is probably going to become endemic due to the optimal climatic conditions present in Africa and Asia as well as the number of suitable host plants. And the climate of Nepal was also proved to an ideal for the development of this pest, hence crop losses of up to 100% in maize are expected if fall armyworm is not controlled or there is no natural biological control (Bhusal & Bhattarai, 2019).

The potential loss of yield brought on by a fall armyworm infection relies on several variables. And how the crop responds to *S.frugiperda* infestation is greatly influenced by the presence of biological enemies, the timing of the infection, microbes that can naturally reduce population sizes, and the health and vigor of the maize plant.

According to Baudron et al (2019), maize infestation ranged between 26.4% and 55.9% and had a yield impact of 11.57%. Chimweta et al (2019) have also noted that damage to leaves, silk, and tassels ranging from 25 to 50% caused a 58% decrease in grain yield. When the weather is warm and humid, the migrating infesting *S. frugiperda* is known to seriously harm maize crops (Ayala et al, 2013 & Clark et al, 2007). According to Zebdewos Salato (2017) sowing corn in the fields during the relatively warm and more humid summer months creates an environment that is favorable for this insect to quickly multiply and spread to more places.

2.9.1 Life cycle of *S. frugiperda*

The length of the life cycle of *S. frugiperda* varies according to environmental and climatic conditions. For instance, the life cycle lasts 30 days in the summer, 60 days in the spring and the autumn, and possibly 80 to 90 days in the winter (James and Engelke, 2010). It includes four stages; Egg, six instars larval stage, Pupa, and Adult.

2.9.1.1 Egg

When a male searches for a female to mate with, she responds by releasing species-specific pheromones such as (z)-7-dodecane-1-ol acetate, (z)- 9-tetradecen-1-ol, (z)-11-hexadecenal, and (z)-11-hexadecen-1-ol acetate. This process usually occurs during the night (Luginbill, 1928). After mating, the female laid small, circular, dome-shaped eggs, which have a diameter of 4mm and height of 3mm, in clusters of about 50-150 are glued on the abaxial surface of the leaves, usually close to the base junction of the leaf and stem (Jarrod et al, 2015).

Moreover, the female covers and fills up the spaces between the eggs with scales, giving the pile of eggs a hairy or moldy appearance. A female will typically produce 1500 eggs, however, this number might go up to around 2000. According to Sparks (1979), eggs are laid in masses ranging in size from a few to hundreds, and they hatch in 4 days under ideal conditions. But it will quickly transform into larvae within a few days in the summer season.

2.9.1.2 Larva

The fall armyworm typically has six instars and each has unique structural and physical characteristics. The duration of the larval stage ranges from 14 to 30 days which depends on the temperature of the atmosphere (Smith, 2017). When the first instar larvae hatched, they are green with a black head and black spots. The larvae are green to dark brown with longitudinal stripes. The length of larvae measures about 3.2-3.5 cm when they are fully grown (Deole, 2018). According to Hardke et al (2015), a late instar of *S. frugiperda* with an inverted "Y" suture on its head capsule and four black dots arranged in a square pattern on its eighth abdominal segment. Due to the presence of

biting mouthparts of larva, this stage of development is called the most destructive.

2.9.1.3 Pupa

The caterpillar pupates at a depth of 2 to 8 cm on the ground after 14 days (Prasanna et al, 2018). The cocoon pupation period lasts between 7-37 days. During pupation, the larva constructs a loose oval silk cocoon measuring about 20 to 30 mm in length, by tying together particles of soil with silk (CABI, 2017b). A reddish brown colored pupa 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). Both the male and female pupae measure about 1.3 to 1.5 cm in length. The pupa stage lasted between 6-8 days. The duration and sustenance of the pupa stage are strongly affected by the climate's heat (Sparks, 1979).

2.9.1.4 Adult

Adults are naturally nocturnal and the majority of their activity happens on warm and humid nights (Kandel, & Poudel, 2020). Since it cannot develop at temperatures below about 10°c, warm, humid growing seasons with lots of rainfall are beneficial for its survival and population growth (Stokstad, 2017). Female moths are larger than male moths in general. The wingspan of an adult FAW moth ranges between 32 and 40 mm. Female adult forewings are dark brown, and grey, and are marked with light and dark colors, while male adult forewings are straw-colored, light brown, and grey (Deole, 2018). Male moths have mottled forewings with triangular white spots on the tip and Centre. The adult stage lasts between 7 and 21 days. Adults can travel great distances in the air, and they migrate at a startlingly rapid rate of 300 miles (483 km) per generation (Sparks, 1979).

CHAPTER-III

MATERIALS AND METHODS

3.1 Study design

A cross-sectional study was carried out in the Microbiology and Molecular Biology lab at the Central Campus of Technology. The isolates were sent to Humanizing Genomics Macrogen, Korea for molecular identification.

3.2 Laboratory setup

The Microbiology laboratory of the Central Campus of Technology in Dharan was used for the isolation of *M.anisopliae* and rearing of *S. frugiperda* and *Myzus persicae* as well as their bioassay against isolates was performed in the Entomological Laboratory of the Regional Agricultural Research Station, Nepal Agricultural Research Center (NARC), Tarhara, Nepal. The major equipments and glass wares used during this work are listed in Appendix A.

3.3 Site justification and planning

A Simple random sampling technique was used to select the sampling site randomly from the four different organic farms (Basantatar, Khanar, Buddhachowk, & Tarhara) 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 into 100 blocks and 10 blocks with a range of 10 numbers was picked from each site.

3.4 Sample collection site

The Soil samples were collected from the agricultural field of the Organic farms of Basantatar, Khanar, Buddhachowk & Tarhara, Sunsari, Nepal whereas the insect pests were collected from the various infected agriculture fields of Tarhara, Nepal.

3.5 Study period

The research was completed within 6 months of the period from December 2021 to June 2022.

3.6 Sampling

The soil samples of this study were collected randomly from selected organic farms in Sunsari, Nepal, using adequate sampling procedures for soil collection, processing, and analysis. A total of 60 soil samples were collected from the four different organic farms in Sunsari, district. Soil samples were taken inside the plastic bags using sterile gloves after being dug out with a garden spade that had been washed with 70% ethanol. Each sample was properly labeled. About 10g of the soil samples were taken from a depth of 3-5cm and deposited aseptically in clean plastic bags(25*35 cm), which were tied with rubber before being transported to the laboratory (Ravindran et al, 2016). All the soil samples were sieved by sieving mesh (10mm) to remove small stones, twigs, and roots and kept at 4°C in the laboratory until further processing (Zimmermann, 2007). In the case of insect pests, the trapped *S. frugiperda* & *Myzus persicae* were collected in sterile plastic containers and transported to the Entomology laboratory of the Regional Agricultural Research Station under a controlled situation for the rearing process.

3.7 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.

3.8 Laboratory work

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

3.8.1 Isolation of *Metarhizium anisopliae*

Antibiotic-rich selective media (Strasser et al, 1997) was used for selective isolation of the entomopathogenic fungus *Metarhizium anisopliae*. The fungus was isolated using the soil dilution plating method (GC, 2006). About 10 g of each soil sample was weighed and added to 90 mL of sterile 0.1 % (w/v) Tween-80 individually. To release the spores stuck to soil particles, samples were mixed thoroughly for 60 minutes with a magnetic stirrer. Then, about 100µl aliquot of each sample was spread onto a selective medium containing (g/L) Peptone, 10; Glucose, 20; Agar, 18; Streptomycin, 0.6; Tetracycline, 0.05; Cyclohexamide, 0.05; and Dodine, 0.1 mL; pH 7.09. The plates were incubated for 3-7 days at 28°C (Tupe et al, 2017). Finally, to obtain pure cultures, individual sporulating colonies were selected and subcultured by using the single-point inoculation method on the same medium.

3.8.2 Identification of *Metarhizium anisopliae*

A pure culture of fungus on a selected medium was used for morphological identification. A basic procedure outlined in medical mycology guidelines describes the preparation of cellophane tape for the microscopic examination of fungal colonies (Forbes et al, 2002). With forceps, a 5-inch (40mm) piece of clear cellophane tape is wrapped back on itself, sticky side out. The loop is gently pressed to the surface of the mycelium then lifted and unfurled onto a glass slide (25 - 75 mm) having three or four drops of Lactophenol cotton blue dye. The slide will attach to the sticky tape with fungi on it. The biological stain is a coating of Lactophenol cotton blue. The transparent cellophane tape can be used to view the morphology of the fungus. Then, a compound microscope was used to examine the slide (Humber, 1997).

3.8.2.1 Microscopic Examination

The isolates were examined under a light microscope and a compound microscope to determine the structure of colony morphology, appearance, hyphae color, spore size, arrangement, and color.

3.8.2.2 DNA extraction and sequencing

To extract fungal genomic DNA from hyphae, a slightly modified chemical lysis technique was utilized (St. Leger and Wang, 2009). The fungus was inoculated into a 1.5 ml microtube with PD (potato dextrose broth) and agitated at 250 rpm at 25oC for 3-4 days. Mycelia were pelleted and resuspended in 400 µl of fungal DNA extraction buffer (0.2 M Tris-Cl (pH 7.5), 0.5 M NaCl, 10 mM EDTA (pH 8.0), and 1% (w/v) SDS) after centrifugation at 10,000 rpm for 10 minutes. After adding 400 µl phenol-chloroform-isoamyl alcohol, the liquid was vortexed for 5 minutes (25:24:1). After 8 minutes of centrifugation at RT/10,000 rpm, the aqueous upper layer was transferred to a new micro-centrifuge tube. After incubating the sample for 30 minutes at 37ºC with 1µl of RNase solution (20 mg/ml, Sigma), it was purified again using phenol-chloroform-isoamyl alcohol (25:24:1). 2.5 liters of 100 % ethanol was used to precipitate the DNA in the aqueous phase. The pellet was rinsed with 70% ethanol, dried, and resuspended in 50 µl of ddH2O after centrifugation at 4ºC/12,000 rpm for 10 minutes. Then, the isolated DNA was the solution was used for further identification through 18s rRNA sequencing performed in a molecular laboratory in Macrogen, Korea. ITS1-forward (TCCGTAGGTGAACCTGCGG) and ITS4-reverse (TCCTCCGCTTATTGATATGC) primers were used during identification (White et al, 1990).

3.8.3 Rearing of insect pests

Insect pest larvae were reared at the entomological laboratory of the Regional Agriculture Research Station (RARS), Nepal by providing the necessary nutrients and recreating the appropriate habitat for the respective insect pest in the quarantine which was collected randomly from the affected zone before. The quarantine room was darkened to protect it from direct sunlight and UV light.

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

First, maize seeds, red beans, and black beans were sown into flower pots (maize in 12 pots and beans in 12 pots) with adequate organic manure, and the pots were preserved in the Tarhara farm's glass house. Watering was done at 2 or 3-day intervals. The larvae of the fall armyworm and aphids were collected from the affected field after the plants began to grow. They were retained on the plants until the pure fungal culture was ready. Then, the collected larvas were then taken inside the entomological laboratory of RARS to be examined for mechanical injury. After that, a jar has a capacity of 1000 ml was taken and the collected larvae were stored inside it by supplying fresh maize leaves. The container was secured with a muslin cloth to prevent the larvae from escaping. Similarly, *Myzus persicae* was given fresh leaves of beans. Both leaves were replaced daily. These tasks were completed in the laboratory at room temperature and 70% relative humidity (Ramanujam et al, 2020).

3.8.4 Preparation of spore suspension of *Metarhizium*

After 12–15 days of incubation of the fungal strain in selective media, the spores were harvested by scrapping the contents of each Petri plate. After that, the scrapped spores were homogenized with distilled water containing 0.1% of Tween-80 to produce *a Metarhizium* spore suspension of about 1×10^7 conidia/ml. A Haemocytometer was used to count the number of conidia.

3.9 Bioassay of the fungal pathogen

The bioassay was carried out using the Leap-dip bioassay approach established by Nazir et al (2019). During the bioassay, *S.frugiperda* and *Myzus persicae* from the orders Lepidoptera and Hemiptera was exposed to *Metarhizium* conidial suspension and their reactions were observed. Three replicates of the experiment were performed. In the case of *S. frugiperda*, 5 larvae were collected per replication, but for *Myzus persicae*, 25 aphids were collected per replication in a disposable plastic glass with a porous cotton cloth lid that was tightened with rubber.

A 10ml spore suspension with a spore concentration of 1×10^7 conidia/ml was taken in a tiny beaker. The fresh maize and bean leaves were then soaked in

the suspension for 15 seconds before being supplied to the insect pests. The leaves were solely submerged in sterile water containing 0.1% Tween 80 for the controls. The glasses containing *S. frugiperda* and *Myzus persicae* were then incubated under strict conditions (65±5% RH and 25±2℃). Insect mortality caused by fungi was detected and compared to a negative control in terms of insect numbers.

3.10 Statistical analysis

The data recorded from the bioassay was noted and tabulated. The data (FD OD and S) within three treatments (Control, SK13, and SC1), each having three replicas were statistically analyzed by using IBM SPSS Statistic 20 Statistical software. For this, a two-way ANOVA was performed on mortality data for insects (death occurred due to fungus and other reasons). Tukey's honestly significant difference (HSD) post hoc test was used to further compare treatment averages at the conventional level of probability (0.05).

Flow chart for the study

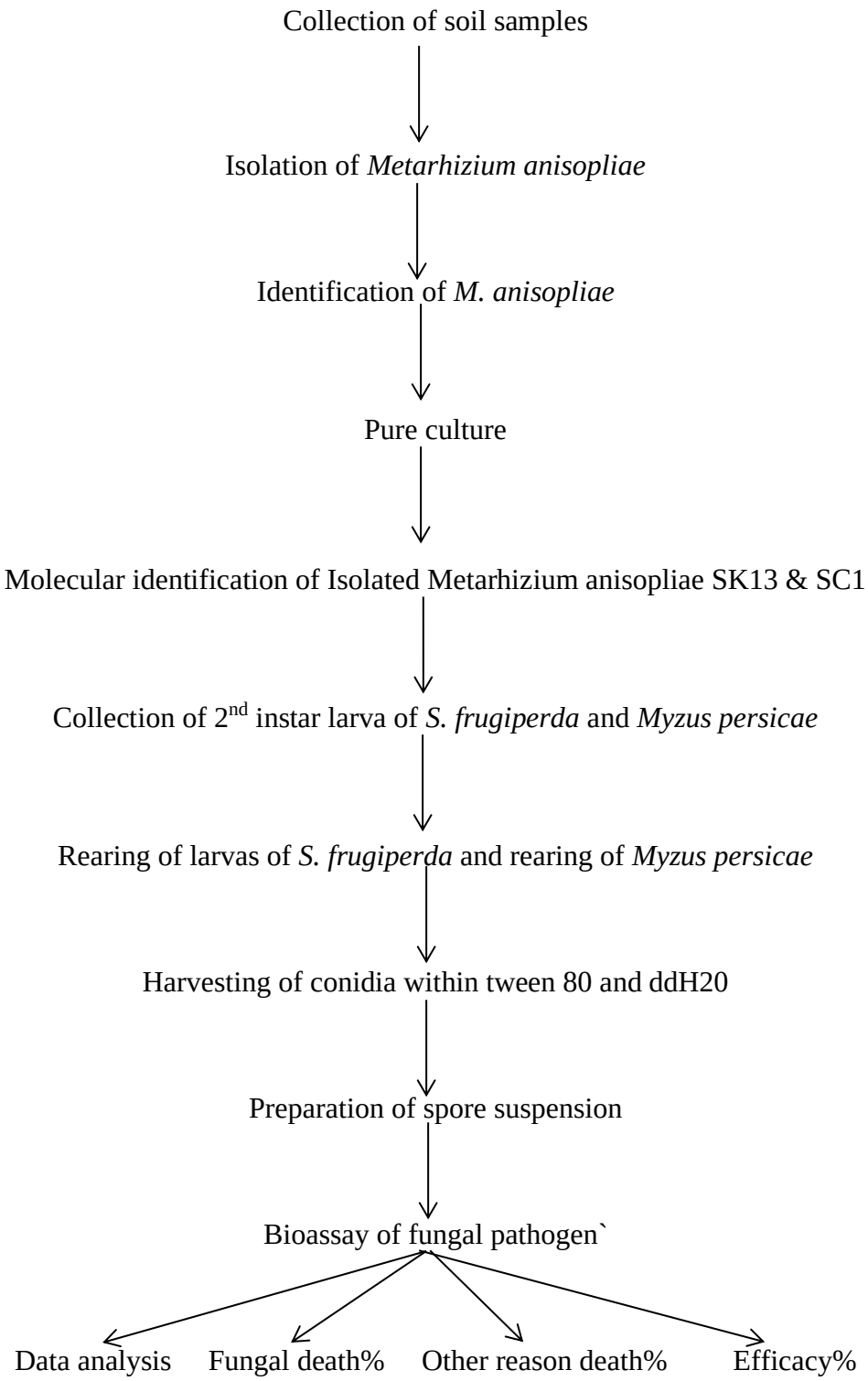


Fig 2: Outline of the study

CHAPTER-IV

RESULTS

4.1 Sampling of soil

In total, 60 samples were collected, 15 from each of the four sampling locations: Buddhachowk, Dharan, Basantatar, and Khanar, Nepal.

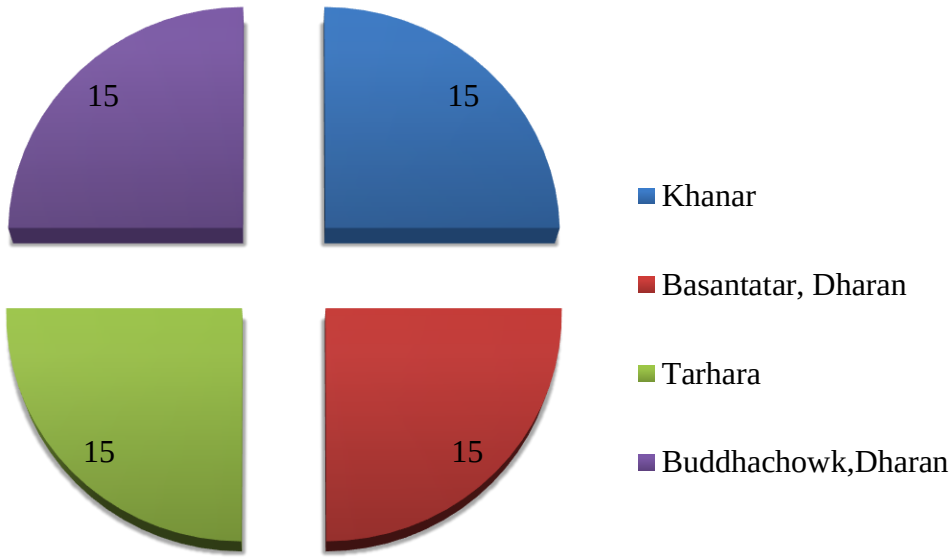


Fig 3: Soil Sampling Site for Soil

4.2 Identification of *Metarhizium anisopliae*

After proper incubation, the green colonies were examined under microscopic examination. A cellophane tape method was used for microscopic observation. Some morphological and cultural characteristics of isolated *M. anisopliae* (SK13 and SC1) are shown in Table 3.

Table 3: Morphological and cultural characteristics of fungal isolates (SK13 & SC1).

Fungal isolates	Colony morphology	No. of days for sporulation	Color of conidia	Microscopic view
SK13	Dark green	6	Dark green	Cylindrical green spores
SC1	Green	5	Green	Rice Grain shaped green coloured spores

4.3 Molecular identification

The two isolated samples were sent for Molecular identification at Macrogen Research Centre, in Korea. From the result obtained, we found that the isolated species SK13 and SC1 confirmed the required entomopathogen which was strains of *Metarhizium anisopliae*. The sample Sk13 was found 91% similar to *Metarhizium anisopliae* strain NHJ10578 18S ribosomal RNA gene and that of SC1 was found 92% similar to *Metarhizium anisopliae* strain 40B1ii 18S ribosomal RNA gene.

Table 4: 18S rRNA gene sequencing of the isolates.

Isolate code	Taxon name	Strains name	Similarity (%)
Sk13	*Metarhizium anisopliae*	*Metarhizium anisopliae* strain NHJ10578 18S ribosomal RNA gene	91.10%
SC1	*Metarhizium anisopliae*	*Metarhizium anisopliae* strain 40B1ii 18S ribosomal RNA gene	92%

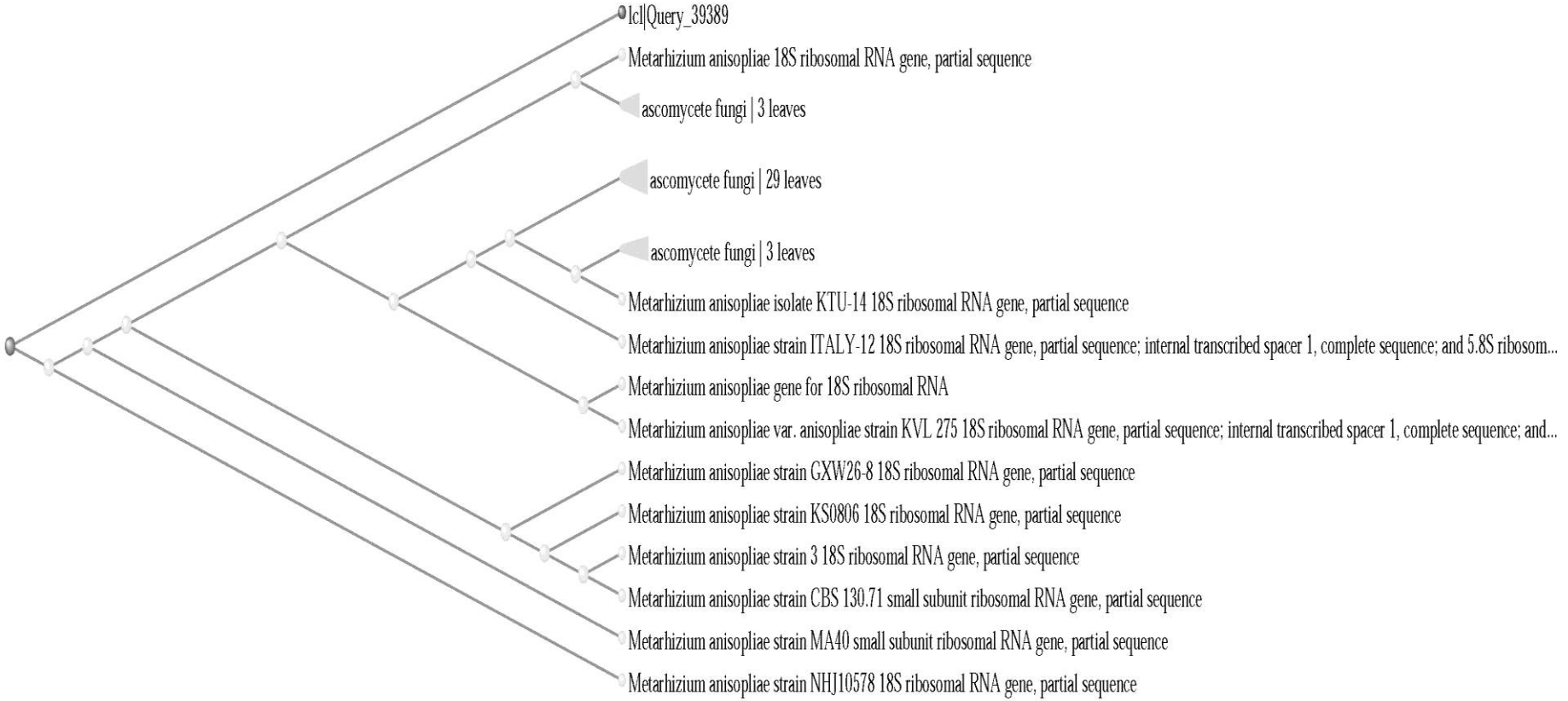


Fig 4: Phylogenetic trees based on 18s rRNA gene sequencing demonstrates a connection between *Metarhizium* strains SKC1 and other closely related *Metarhizium* strains. The neighbor-joining method was used to construct the tree. The scale bar represents a 0.09 nucleotide substitution per position.

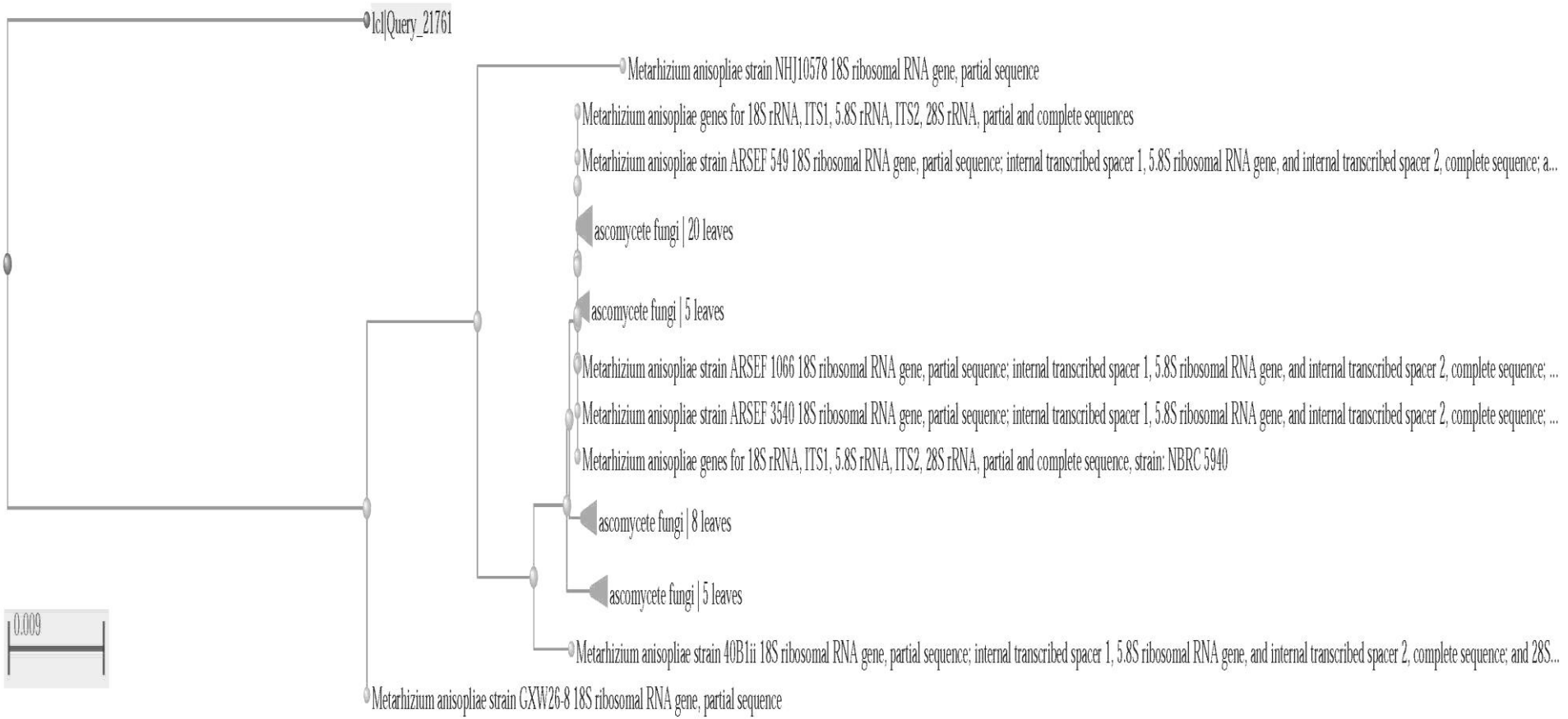


Fig 5: Phylogenetic trees based on 18s rRNA gene sequencing demonstrates a connection between *Metarhizium* strains SK13 and other closely related *Metarhizium* strains. The neighbor-joining method was used to construct the tree. The scale bar represents a 0.09 nucleotide substitution per position.

4.4 Death record of *S. frugiperda* during Bioassay

The death record of larvae of *S. frugiperda* during bioassay was recorded daily among three replications utilizing three treatments (Control, SK13, and SC1). In the bioassay, 5 larvae/replicas of *S. frugiperda* were taken in each treatment. After that, larvae were provided leaves of maize which were already prepared by drowning it in spore suspension (10ml) taken in a beaker. During the bioassay, larvae were discovered dead after daily monitoring for a variety of reasons, including death by relative fungus and death by physical injury and other pathogens such as bacteria and nematodes. In this investigation, no fungal death was detected in the control treatment; however, 14 larvae of *S. frugiperda* larvae died as a result of infection produced by isolated *Metarhizium* strains SK13.

Table 5 shows the death record of *S. frugiperda*, where TD = Total death occurred, FD = Death occurred due to fungus, and OD = Death occurred due to other reasons.

Table 5: Death record of larvae of *S. frugiperda*

Days	1st	2nd	3rd	4th	5th	6th	7th	TD	FD	OD
Treatments Replica	TD FD OD	TD FD OD	TD FD OD	TD FD OD	TD FD OD	TD FD OD	TD FD OD			
Control 1 2 3	 0 0 0	 0 0 0	 1 0 1 0 0	 1 0 1 1 0 1 2 0 2	 0 0 2 0 2	 0 0 0	 0 0 0	 2 1 3	 0 0 0	 2 1 3
							Total	6	0	6
Sk13 1 2 3	 1 0 1 0 0	 0 1 1 0 0	 4 4 0 1 1 0 1 1 0	 0 3 3 0 0	 0 0 1 1 0	 0 0 1 1 0	 0 0 2 2 0	 5 5 5	 4 5 5	 1 0 0
							Total	15	14	1
SC1 1 2 3	 0 0 0	 1 0 1 0 1 0 1	 1 1 0 1 1 0 3 2 1	 0 1 1 0 1 1 0	 3 3 0 1 1 0 0	 0 2 2 0 0	 0 0 0	 5 5 5	 4 5 3	 1 0 2
							Total	15	12	3

4.5 Death record of *Myzus persicae* during the bioassay

The death records of *Myzus persicae* were recorded weekly among three replications utilizing three treatments (Control, SK13, SC1). In this bioassay, 25 aphids/replicas were taken. After that, leaves of beans that were immersed into the spore suspension were fed to the aphids taken. During the bioassay, several insects were discovered dead after daily monitoring for a variety of reasons, including death by relative fungus and death by physical injury and other pathogens such as bacteria and nematodes. In this investigation, no fungal death was detected in the control treatment; however, it was reported that SK13 and SC1 had infected 60 and 52 aphids, respectively.

Table 6: Death record of *Myzus persicae*

Where TD = Total death occurred, FD = Death occurred due to fungus, and OD = Death occurred due to other reasons.

No. of aphids taken: 25 per treatment plate

Days	1st week	2nd Week	Total death	FD	OD
Treatments Replica	TD FD OD	TD FD OD			
Control 1 2 3	 5 0 5 3 0 3 4 0 4	 1 0 1 0 1 0 1	 6 3 5	 0	 6 3 5
		Total	13		13
SK13 1 2 3	 15 12 3 19 16 3 24 22 2	 6 4 2 6 6 0 0	 21 25 24	 16 22 22	 5 3 2
		Total	70	60	10
SC1 1 2 3	 18 15 3 21 16 5 16 9 7	 4 4 0 3 3 0 5 5 0	 22 24 21	 19 19 14	 3 5 7
		Total	67	52	15

4.6 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 isolated *M. anisopliae* (SK13) and *M. anisopliae* (SC1) had the same mortality rate (100%) within three treatments. Table 7 shows the detail of the study's specifics.

Table 7: Mortality % of *S. frugiperda* within three treatments

Treatments	Total no of larvae tested	No. of dead larvae of *S. Frugiperda*	Mortality (%)
Control	15	6	40
SK13	15	15	100
SC1	15	15	100

4.7 Mortality of *Myzus persicae* within three treatments

The mortality of *Myzus persicae* was computed using the bioassay data after the experiment, and it was discovered that the control exhibited a death rate of 17.33%, whereas the isolates (SK13 & SC1) had mortality rates of 93.33% and 89.33%, respectively. Table 8 shows the detail of the study's specifics.

Table 8: Mortality of *Myzus persicae* within three treatments

Treatments	Total no. of aphids tested	No. of dead aphids	Mortality (%)
Control	75	13	17.33
SK13	75	70	93.33
SC1	75	67	89.33

4.8 Death occurred due to fungus (FD %), other reasons (OD %), and Survival (S %) record of *S. frugiperda* during the bioassay

The bioassay record was used to calculate the FD%, OD%, and S%. In the control treatment, there is no fungal death, whereas the FD% of isolates SK13 was 93.33% and SC1 was found to be 80%. The control had a greater OD% (40%) than the two isolates SK13 and SC1 (6.67% and 20%, respectively). Similarly, the control had a higher survival rate (60%) than the isolates SK13 and SC1, which had no survival rate, indicating that all the larvae of *S. frugiperda* treated were found to die. Table 9 shows the study's findings.

Table 9: FD %, OD % & S % of *S. frugiperda*

Treatment	FD %	OD %	Survival %
Control	0	40	60
	0	20	80
	0	60	40
Total mean	0	40	60
SK13	80	20	0
	100	0	0
	100	0	0
Total mean	93.33	6.67	0
SC1	80	20	0
	100	0	0
	60	40	0
Total	80	20	0

4.9 Death occurred due to fungus (FD %), other reason Mortality (OD %), and Survival (S %) of *Myzus persicae*

The FD%, OD%, and S% were computed using the bioassay record. There was no fungal death in the case of control, while the FD% of isolates SK13 was 80% and SC1 was found to be 69.33%. The OD% of the control was found to be higher (18.67%) than that of the two isolates SK13 and SC1 (13.33% and 20%, respectively). Similarly, the survival rate was determined to be higher (81.33%) when compared to the survival rates of isolates SK13 and SC1 (6.67% and 10.67%, respectively). Table 10 summarizes the study's findings.

Table 10: FD %, OD %, and S % of *Myzus persicae*

Treatment	FD %	OD %	Survival %
Control	0	24	76
	0	12	88
	0	20	80
Total mean	0	18.67	81.33
SK13	64	20	16
	88	12	0
	88	8	4
Total mean	80	13.33	6.67
SC1	76	12	12
	76	20	4
	56	28	16
Total mean	69.33	20	10.67

4.10 Death records of *S. frugiperda* due to fungus during the bioassay

During the bioassay in this study, the SK13 was discovered to be more virulent than the other treatments. The detailed relationship between treatment and fungal death in *S. Frugiperda* is provided in Table 11.

Table 11: Death records of *S. frugiperda* due to fungus during the bioassay

Treatment	Replication	FD	P value
Control	1	0	
	2	0	
	3	0	
Total		0	
SK13	1	4	0.000
	2	5	
	3	5	
Total		14	
SC1	1	4	
	2	5	
	3	3	
Total		12	

4.11 Death records of *Myzus persicae* due to fungus during the bioassay

During the bioassay in this study, the SK13 was discovered to be more virulent than the other treatments. The detailed relationship between treatment and fungal death on *Myzus persicae* is provided in Table 12.

Table 12: Death records of *Myzus persicae* due to fungus during the bioassay

Treatment	Replication	FD	P value
Control	1	0	
	2	0	
	3	0	
Total		0	
SK13	1	16	
	2	22	0.000
	3	22	
Total		60	
SC1	1	19	
	2	19	
	3	14	
Total		52	

4.12 Death records of *S. frugiperda* due to other reasons (OD) during the bioassay

In our laboratory experiment, larvae of *S. frugiperda* were found to die from mechanical damage, Nematodes, and bacteria, but not from entomopathogenic fungus. Table 13 displays the precise association between other causes of mortality and treatment.

Table 13: Death records of *S. frugiperda* due to other reasons (OD) during the bioassay

Treatment	Replication	OD	P value
Control	1	2	
	2	1	
	3	3	
Total		6	
SK13	1	1	
	2	0	0.145
	3	0	
Total		2	
SC1	1	1	
	2	0	
	3	2	
Total		3	

4.13 Death records of *Myzus persicae* due to other reasons (OD) during the bioassay

In our laboratory experiment, larvae of *Myzus persicae* were found to die from mechanical damage, Nematodes, and bacteria, but not from entomopathogenic fungus. Table 14 displays the precise association between other causes of mortality and treatment.

Table 14: Death records of *Myzus persicae* due to other reasons (OD) during the bioassay

Treatment	Replication	OD	P value
Control	1	6	
	2	3	
	3	5	
Total		14	
SK13	1	5	
	2	3	0.489
	3	2	
Total		10	
SC1	1	3	
	2	5	
	3	7	
Total		15	

4.14 Efficacy of isolated *M. anisopliae* on *S. frugiperda*

The effectiveness or efficacy % of the isolates was calculated using a modified Abbotto's algorithm from the bioassay data record (Abbott, 1925). In this study, isolate SK13 and SC1 showed similar efficacy (100%) against larvae of *S. frugiperda*. The study's details are shown in Table 15.

Table 15: Efficacy % of fungal isolates on *S. frugiperda*

Treatment	Death % by fungus	Other reason death%	Survival%	sUtrt-strt%	Efficacy%
Control	0	40	60	_	_
SK13	93.33	6.67	0	60	100
SC1	80	20	0	60	100

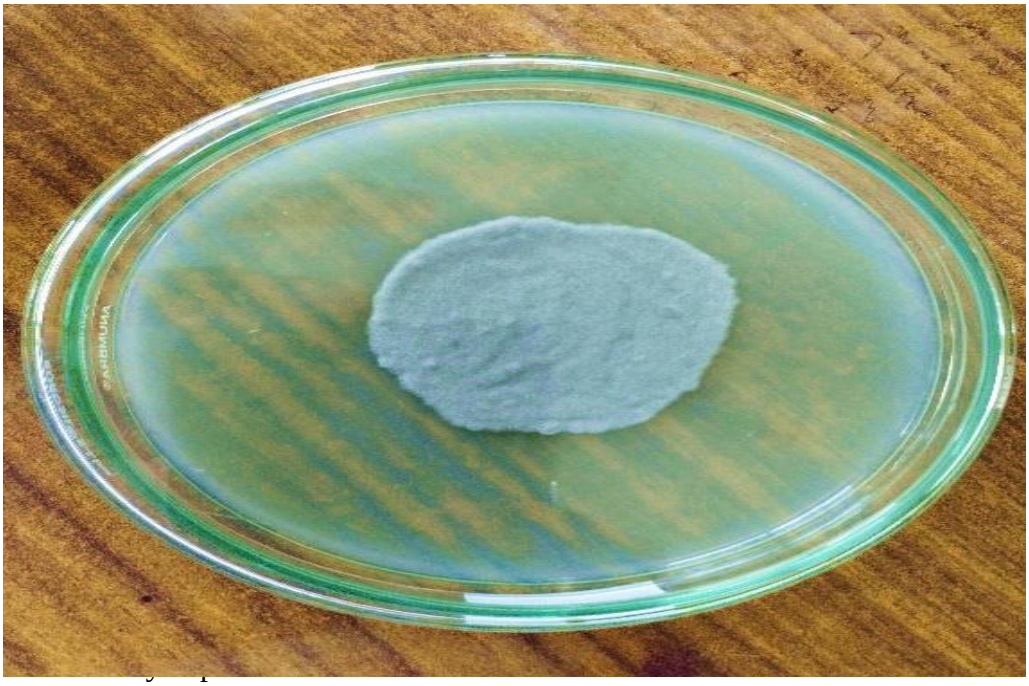
4.15 Efficacy of fungal isolates on *Myzus persicae*

It was found that isolate SK13 had a higher efficacy on *Myzus persicae* (91.79%) as compared to isolate SC1 (86.88%).

Table 16: Efficacy % of fungal isolates on *Myzus persicae*

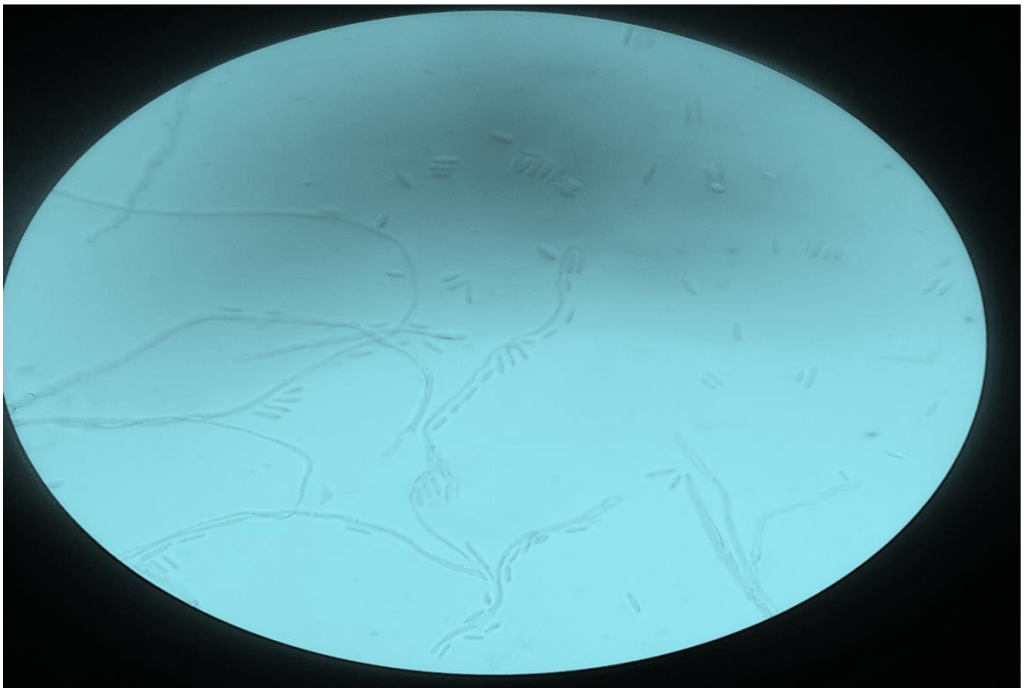
Treatment	Death % by fungus	Other reason death%	Survival%	sUtrt-strt	Efficacy
Control	0	18.67	81.33	_	_
SK13	80	13.33	6.67	74.66	91.79
SC1	69.33	20	10.67	70.66	86.88

Photographs



Photograph1: Pure culture of Isolated *Metarhizium anisopliae* on PDA

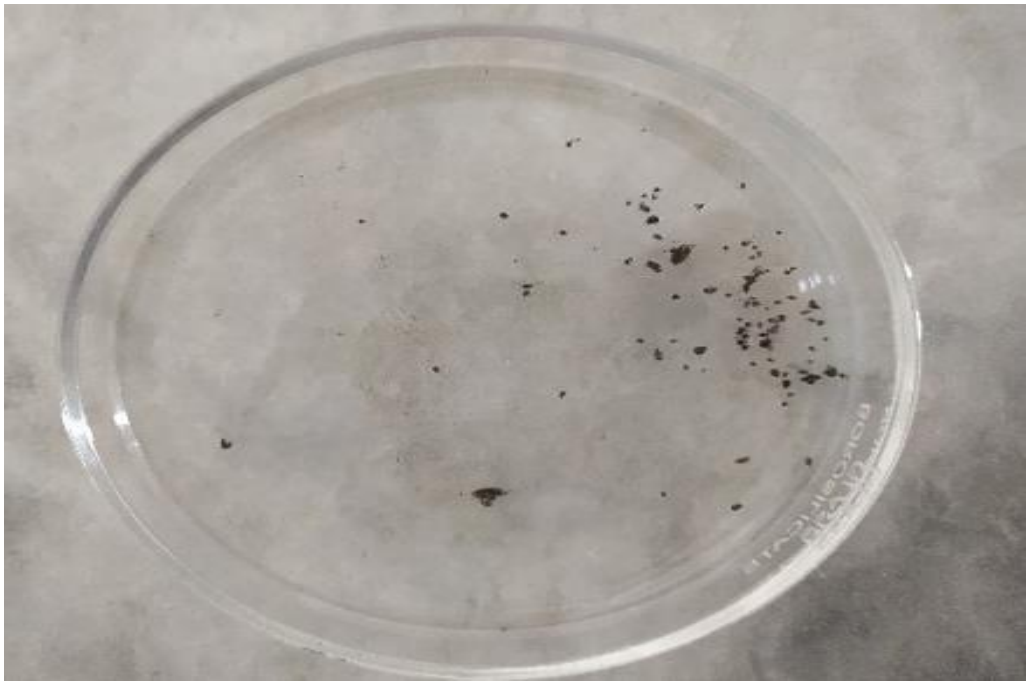
Photograph 2: Researcher observing the Spores of *Metarhizium anisopliae* through Microscope



Photograph 3: Microscopic view of cylindrical spores of Isolated *Metarhizium anisopliae*

Photograph4: Bioassay experiment

Photograph 5: *Spodoptera frugiperda* succumbed by *Metarhizium anisopliae*



Photograph 6: *M. persicae* succumbed by *Metarhizium anisopliae*

CHAPTER-V
DISCUSSION

Many insect pests have traditionally impacted agriculture, reducing agricultural productivity by changing the quality and quantity of the crops grown. Pests can harm crops or plants directly by devouring them or weakening them, as well as indirectly by spreading illnesses. The *Spodoptera frugiperda* is currently the most destructive pest of maize, and its ravenous eating habits have a significant influence on food security (Harrison et al, 2019). The broad spectrum character of the chemicals used to treat fall armyworms has negative effects on both the FAW and its natural enemies (Lewis et al, 2016). Due to this insect's favored habitat, primarily the whorl region, EPFs play a significant role in the environmentally responsible treatment of this pest. *M. persicae* can transmit over 100 plant viruses, making it particularly harmful to various crops (Devi & Singh, 2007). So Studies on the isolation, characterization, and bioassay of isolated strains of *M. anisopliae* against FAW and *Myzus persicae* were conducted in the current investigation. Entomopathogenic fungi (EPF) have attracted attention due to their limited host range, excellent lethality, non-target species safety, tolerance with some fungicides and many other types of insecticides, application convenience, and simplicity.

The study was done in the Microbiology laboratory of the Central Campus of Technology and the Entomology laboratory of the RARS, Tarhara. The study period was from December 2021 to June 2022. For the isolation of *Metarhizium anisopliae*, about 60 soil samples were collected from different organic farms in Sunsari district, Nepal. A total of four organic farms from Basantatar, Khanar, Buddhachowk, and Tarhara 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 100 blocks based on area and 10 integers from each was taken as a sample. About 10g of the soil sample from each site was taken at a depth of 3-5cm and deposited aseptically in clean plastic bags, which were tied with a rubber band before being transported to

the laboratory. All soil samples were transferred to the laboratory and kept at 4°C until they were processed.

The fungus was isolated using the soil dilution plating method (GC, 2006). About 10 g of each soil sample was weighed and added to 90 mL of sterile 0.1 % (w/v) Tween-80 individually. To release the spores stuck to soil particles, the samples were mixed thoroughly for 60 minutes with a magnetic stirrer. Then, about 100-µl to 200-µl aliquots of each sample were spread onto a plate containing a selective medium. Then, the plates were incubated for 3-7 days at 28°C (Tupe et al, 2017). However, Ghanbary et al (2009) used an artificial medium containing Macro and Micronutrients for the isolation of *Metarhizium anisopliae* from the cultural soil samples taken from different regions of Iran.

In this study, out of 60 soil samples, only two fungal isolates from Khanar and Buddhachowk, Sunsari were successfully grown and maintained on a selective medium and named SK13 and SC1 were confirmed as strains of *Metarhizium anisopliae* by 18s rRNA sequencing method. For molecular identification, the isolates were subcultured into PDA by the point inoculation method. When a pure culture was obtained, PDA slants with a pure culture of the fungus were prepared and properly covered in Parafilm paper to prevent contamination for sending the isolates to Macrogen, Korea. According to the findings, the strains known as SK13 and SC1 have been identified as *Metarhizium anisopliae* strains NHJ10578 18S ribosomal RNA genes and *Metarhizium anisopliae strains* 40B1ii18S ribosomal RNA genes, with similarity of 91.10% and 92% respectively.

In this work, isolated *M. anisopliae* colonies show several physical and colony characteristics that include green and dark green colonies with conidiogenous cells that appear in a dense layer on the Petri plates after incubation. Young *M. anisopliae* had a white appearance, but as they grew older, the conidia turned green and dark green. These properties of isolated *M. anisopliae* are comparable to those of the morphological features of *M. anisopliae* that Humber mentioned (1997).

In our investigation, the color of the conidia after 6 days of sporulation was found to be dark green in SK13, but the color of the conidia after 5 days of

sporulation was found to be green in SC1. The colony diameter at 10 days after inoculation in SK13 was 50mm and 41 in SC1. This outcome is fairly comparable to a study conducted in Dharwad, India, where the isolate's conidia were dark green and the colony diameter was 42.30 mm after ten days after inoculation (Talwar 2005).

Two fungal isolates (SK13 and SC1) that were obtained from an agricultural field in Khanar and Buddhachowk were subjected to a laboratory bioassay to see how well they affected populations of *S. frugiperda* and *M. persicae*. The conidia were collected by scraping off freshly grown and sporulated plates of isolated *M. anisopliae* using sterile bacteriological loops for the bioassay. The conidial suspension was then adjusted to 10^7conidia/ml with the help of a Haemocytometer in 10 ml of 0.1% Tween80 solution for both fungal isolates SK13 and SC1.

Three different treatments (SK13, SC1, and control) were employed in this study to conduct the bioassay. For the control, three replications of each treatment were carried out using sterile water (D/W) with 0.1% tween 80. The leaf dipping method was employed to conduct the bioassay (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*.

In bioassay, both isolates, SK13 & SC1, showed the same mortality (100%) of *S. frugiperda* after three treatments, while the control had a mortality of 40%. Moreover, Sk13 and SC1 showed death rates of 93.33% and 89.33% in the case of *Myzus persicae*, compared to 17.33% in cases of control. Fungi and other factors (bacteria, nematodes, mechanical injury, etc.) are to blame for the death of insects. According to Ullah et al (2022) that *M. anisopliae* resulted in the greatest mean mortality of *S. frugiperda* (88%) and *M. persicae* (65%) at the highest spore concentration (1.0×10^9spore/ml).

The reason for mortality was revealed to be different during bioassay. In this experiment, fungal death was discovered to be larger in isolated *M. anisopliae* Sk13 (SK13=15), followed by isolated *M. anisopliae* SC1 (SC1 =15), but no *S. frugiperda* were found to be killed by fungus in the control (C= 0). This finding indicates that SK13 (FD% of Sk13= 93.33%) is more virulent than

SC1 (FD% of SC1 = 80%) and has higher fungal mortality/mycosis when it comes to controlling *S. frugiperda.* And found that (P<0.05), which means that the relationship between and within treatments and death of *S. frugiperda* occurred due to fungus are significant.

In the instance of *Myzus persicae*, fungal death by isolate SK13 (SK13=70) was detected after the second week of *M. anisopliae* inoculation, followed by isolate SC1 (SC1 =67), however, no *M. persicae* were reported to be killed by fungus in the control (C= 0). This finding indicates that SK13 is more virulent (FD% of Sk13= 80%) than SC1 (FD% of SC1 = 69.33%) and has higher fungal mortality/mycosis when it comes to controlling *M. persicae*. We concluded that there was a significant difference between treatments and death of *M. persicae* occurred due to fungus (p<0.05).

All dead and live bioassay records are necessary to assess the efficacy of an organism. Both the selected insect pests were found to be dead during a bioassay without fungal infection, suggesting that there may have been another cause, such as (bacteria, nematodes, or mechanical injury). In this study, the control group (OD% = 40%) had a higher rate of other-cause mortality than isolates SK13 (OD%=6.67%) and SC1 (OD%=20% in) for *S. frugiperda.* There is no statistically significant difference between treatments and death caused due to other reasons (p >0.05).

In the instance of *Myzus persicae*, SC1 (OD% = 20%) had a higher rate of other reason death mortality than the control (18.67%) and SC1 (13.33%). and found p-value is greater than 0.05 which means the death of *M. persicae* due to other reasons and treatments is not significant.

According to our bioassay results, 13 *Myzus persicae* were discovered to be alive and unaffected by any treatments. The control group had a higher survival rate (S% of control = 81.33%) than Sk13 (S% of SK13=6.67%), and SC1 (S% of SC1=10.67%). The lower survival rate indicates that it has a stronger ability to manage insect pests. In our investigation, SK13 exhibited a lower aphid survival rate, making it clear that this one was more effective at controlling *Myzus persicae*.

In the case of *S. frugiperda*, both isolates had the same survival rate (0%) however the control had a higher survival rate (60%) So, it may be concluded that *S. frugiperda* was similarly susceptible to both isolates.

When a precise count of the live and dead insects in all three treatments was (Control, SK13, and SC1) available, we can apply 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 two fungal isolates (SK13 and SC1) against *S. frugiperda* and *Myzus persicae* was finally determined from the outcomes of the bioassay (Abbotts, 1925).

In this investigation, the corrected mortality (efficacy%) of *Metarhizium* isolates SK13 and SC1 was 100% against *S. frugiperda*, while the efficacy% of isolates SK13 and SC1 within 10^7 conidia/ml was 91.79% and 86.88%, respectively, against *M. persicae*. Akutse et al (2019) also discovered that 1×10^8 conidia/ml of *M. anisopliae* strain ICIPE 41 produced the greatest death rate of 96.5% in FAW newborns.

CHAPTER-IV
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Metarhizium anisopliae is a fungus that naturally develops in soil all over the world and infects different insects by acting in an eco-friendly way. The aim of this research is to assess the effectiveness of two fungal isolates, SK13 and SC1, against *S. frugiperda* and *Myzus persicae* under laboratory-controlled conditions. We observed that isolate Sk13 had a higher efficacy (SK13=91.79%) than SC1 (SC1=86.88%) in *Myzus persicae*, but that both isolates had the same efficacy (100%) against *S. frugiperda*. These findings lead to the conclusion that, due to their higher efficacy, entomopathogenic fungi (*M. anisopliae*) may be a safe alternative to conventional and microbial controls for the management of *S. frugiperda* and *Myzus persicae*. In comparison to isolate SC1, SK13 may be better suited for large-scale management of *Myzus persicae* in fields, although both isolates would be the best choice for controlling *S. frugiperda.*

6.2 Recommendations

1. Insect Pests are a key contributor to agricultural losses. *M. anisopliae* can be used as a biological control agent to manage *S. frugiperda and Myzus persicae.*
2. *Metarhizium anisopliae* can be isolated from various agricultural fields using a selective medium.
3. The pathogenicity and efficacy of *Metarhizium anisopliae* must be investigated across a range of species.
4. *Metarhizium anisopliae* in combination with other bioinsecticides may be considered for improved efficacy in some species.

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APPENDICES

APPENDIX A

Materials and Equipments:

List of Materials

Glasswares

Beaker	Conical flask
Glass rods	Slides
Test tubes	Petri dishes
Micropipette	Micropipette tips
Polyvials	Pipettes
Measuring Cylinders	

Miscellaneous

Gloves	Sterile cotton swabs
Forceps	Labeling sticker
Tissue paper	Burners
Bacteriological loops	Test-tube racks and holders
Parafilm	

Equipments

Haemocytometer	Autoclave
Incubator	Refrigerator
Hot air oven	Compound Microscope

Reagents

Tween 80 0.1%	Lactophenol cotton blue
Lactophenol	75% alcohol
Lysol	Distilled water

Media and antibiotics

Potato dextrose agar Sabouraud dextrose agar (SDA)

Potato dextrose broth

Antibiotics

Streptomycin sulfate

Tetracycline

Cycloheximide

APPENDIX B

CALCULATION OF EFFICACY:

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

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

For, *S. frugiperda*,

We have,

Survival untreated (Control) = 60%

Survival treated (SK13) = 0%

Survival treated (SC1) = 0%

So,

$$\text{Efficacy of SK13} = 100 \times \frac{60 - 0}{60}$$

$$= 100\%$$

$$\text{Efficacy of SC1} = 100 \times \frac{60 - 0}{60}$$

$$= 100\%$$

For, *Myzus persicae*,

We have,

Survival untreated (Control) = 81.33%

Survival treated (SK13) = 6.67%

Survival treated (SC1) = 10.67%

So, $\text{Efficacy of SK13} = 100 \times \frac{81.33 - 6.67}{81.33}$

$$= 91.79\%$$

$$\text{Efficacy of SC1} = 100 \times \frac{81.33 - 10.67}{81.33}$$

$$= 86.88\%$$

APPENDIX C

Calculation of Spores using Haemocytometer

Number of conidia in a small square chamber of Haemocytometer:

4, 3, 6, 5, and 4.

The average number of conidia in a small square chamber = $\frac{22}{5}$

=4.4spores/per unit area.

The area of the smallest unit of the Haemocytometer (L×B) = 0.0025mm²(from Haemocytometer).

Height of all units of the Haemocytometer (H) = 0.100m

Thus the volume of smallest unit = (L×B×H) = 0.00025mm³

Here, 4.4 spores/smallest unit of Haemocytometer means 0.00025mm³ of suspension.

Or, 0.00025mm³ contains 4.4 spores

Or, 1mm³ contains 4.4/0.00025 spores

=17,600

= 1.76×10^4 spores

Or, 1000mm^3 contains $1.76 \times 10^4 \times 1000$ conidial spores/ml

= 1.76×10^7 spores/ml

(Note: 1ml=1000mm³)

APPENDIX D

Statistical analysis

Death Record of Fall armyworm due to fungus

ANOVA

Dependent variable: Fungal Death

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	38.222	2	19.111	43.000	.000
Within Groups	2.667	6	.444		
Total	40.889	8			

Interpretation: Here, the p-value of the test is found less than 0.05, and the null hypothesis is rejected. It concludes that there is a significant difference in both between and within groups (P<0.05).

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Death record due to fungus

LSD (Least Square Difference)

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	SK13	-4.66667*	.54433	.000	-5.9986	-3.3347
	SC1	-4.00000*	.54433	.000	-5.3319	-2.6681
SK13	Control	4.66667*	.54433	.000	3.3347	5.9986
	SC1	.66667	.54433	.267	-.6653	1.9986
SC1	Control	4.00000*	.54433	.000	2.6681	5.3319
	SK13	-.66667	.54433	.267	-1.9986	.6653

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

Death Record of Fall armyworm due to other reasons

ANOVA

Dependent Variable: Death occurred due to Other Reasons.

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	4.222	2	2.111	2.714	.145
Within Groups	4.667	6	.778		
Total	8.889	8			

Interpretation: Here, the p-value of the test is found greater than 0.05 which concludes that there is no significant difference in both between and within groups (P>0.05).

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Death occurred due to Other Reasons

LSD(Least Square Difference)

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	SK13	1.66667	.72008	.060	-.0953	3.4286
	SC1	1.00000	.72008	.214	-.7620	2.7620
SK13	Control	-1.66667	.72008	.060	-3.4286	.0953
	SC1	-.66667	.72008	.390	-2.4286	1.0953
SC1	Control	-1.00000	.72008	.214	-2.7620	.7620
	SK13	.66667	.72008	.390	-1.0953	2.4286

Death Record Of *Myzus persicae* due to fungus

ANOVA

Dependent Variable: Death occurred due to Fungus

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	707.556	2	353.778	52.197	.000
Within Groups	40.667	6	6.778		
Total	748.222	8			

Interpretation: Here, the p-value of the test is found less than 0.05, and the null hypothesis is rejected. It concludes that there is a significant difference in both between and within groups (P<0.05).

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Fungal Death occurred due to fungus.

LSD (Least Square Difference)

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	SK13	-20.00000*	2.12568	.000	-25.2014	-14.7986
	SC1	-17.33333*	2.12568	.000	-22.5347	-12.1320
SK13	Control	20.00000*	2.12568	.000	14.7986	25.2014
	SC1	2.66667	2.12568	.256	-2.5347	7.8680
SC1	Control	17.33333*	2.12568	.000	12.1320	22.5347
	SK13	-2.66667	2.12568	.256	-7.8680	2.5347

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

Death record of *Myzus persicae* due to other reasons

ANOVA

Dependent Variable: Death occurred due to Other Reasons

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	4.667	2	2.333	.808	.489
Within Groups	17.333	6	2.889		
Total	22.000	8			

Interpretation: Here, the p-value of the test is found greater than 0.05 which concludes that there is no significant difference in both between and within groups (P>0.05).

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Death occurred due to Other Reasons

LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	SK13	1.33333	1.38778	.374	-2.0624	4.7291
	SC1	-.33333	1.38778	.818	-3.7291	3.0624
SK13	Control	-1.33333	1.38778	.374	-4.7291	2.0624
	SC1	-1.66667	1.38778	.275	-5.0624	1.7291
SC1	Control	.33333	1.38778	.818	-3.0624	3.7291
	SK13	1.66667	1.38778	.275	-1.7291	5.0624