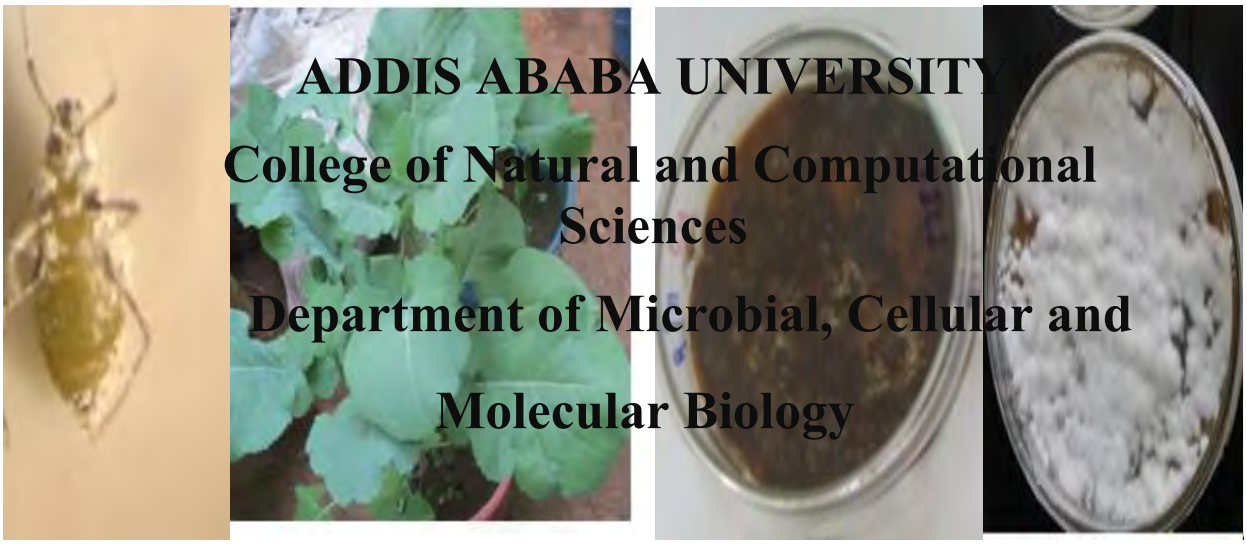

ADDIS ABABA UNIVERSITY

College of Natural and Computational Sciences

Department of Microbial, Cellular and Molecular Biology

Integrated Pest Management of Cabbage Aphid (*Brevicoryne brassicae* L.: Aphididae) on Ethiopian Mustard (*Brassica carinata* A. Braun) using Entomopathogenic Fungi and Selected Insecticides

By

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A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirement for the degree of Masters of Science in Applied Microbiology

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Abbreviation and Acronyms

AMPs	Antimicrobial peptides
DAT	Days after treatment
DH_2O	Sterilized distilled water
EC	Emulsifiable concentrate
FR	Field rate
ICM	Integrated Crop Management
IPM	Integrated pest management
LSD	List significant difference
OAM	Oatmeal agar medium
OPs	Organophosphates
PDA	Potato dextrose agar
POPs	Persistent Organic Pollutants
RPM	Revolutions per minute
RR	Recommended rate
SSF	Solid state fermentation
SC	Suspension concentrate
SP-IPM	System wide Program on Integrated Pest Management

ABSTARCT

Insect pests are responsible for most of the yield loss of horticultural plants, of which cabbage aphid (*Brevicoryne brassicae* (L.) is emerging as important sucking insect pest of cabbage crops. The indiscriminate use of insecticides against insect pests is becoming hazardous to environmental and human health, and ineffective because of the development of resistance against the chemicals. This necessitates the search for integrated pest management using living micro-organisms alone or with little or no application of chemicals to reduce chemicals and produce quality food. The present study was, therefore, designed with the objective of isolating and characterizing antagonistic local isolates of entomopathogenic fungi against aphid. Accordingly, isolates *Beauveria (*BEI1, BEI2) and *Metarhizium* (MEI1,MEI2) were collected from several forest soil samples and screened for their compatibility with different insecticides. The dose and time dependent antagonistic tests *in vitro* and *in vivo* against the aphid was observed. Accordingly, microbial inoculums of 1×10^{7} and 1×10^{8} conidia mL^{-1} showed high mortality (66.7-100%) of aphids after 6 days under laboratory conditions. Among the isolates, BEI1 isolate was the most virulent against adult and nymphs aphids. However, higher mortality was recorded on adult aphids (67-100%) as compared to nymphs (39-72%) after 4^{th}-5^{th} day of treatment. The *in vitro* compatibility test of the isolates with 3 chemical pesticides; Karate(50 ml ha^{-1}), Actara (20g ha^{-1}) and Radiant(100 ml ha^{-1}) at formulations of (Field rate, 0.75Field rate and 0.5Field rate) concentrations showed 70% -91% conidial germination rate by *M. anisopliae,* MEI1, MEI2 and 68%-98% conidial germination rate by *B. bassiana (*BEI1, BEI2). Although not significantly different, the insecticide Karate was more suppressive for spore germination followed by Actara and Radiant suggesting the possibility of using these insecticides together with the entomopathogens for integrated pest management. Similarly, the *in vivo* experiment also showed variations (P= 0.001) among the different treatments. Consequently, the treatment of cabbage aphid with 0.5Field rate karate with all entomopathogenic fungi showed mortality ranging from 21% -100% and 28.3% - 100% after 3-11 days compared with 45.6% -100% and 52.8-100% mortality rate of nymph and adult aphids respectively with treatments using field rate karate. The data also showed that effectiveness in the *in vivo* study took more days (11) than those *in vitro* studies (6)days. The combined application of insecticide sequentially with selective fungi proved to be synergistic given more control than the fungal isolates alone.

Keywords: Bio-pesticides, Entomopathogenic fungi, Compatibility, Germination, Mortality

1. INTRODUCTION

Brassica carinata A. Braun, commonly known as Ethiopian mustard is a amphi-diploid species that belong to the family Brassicaceae. It is believed to have originated from the Ethiopian highlands and its cultivation is thought to have started about 4000 years B.C. (Nigussie Alemayehu and Becker, 2002; Schippers,2002).

Traditionally, *B. carinata* seeds also called 'Gomenzer' in Amharic are used to grease clay pan used for baking traditional Ethiopian bread "enjera", to cure certain ailments or stomach upsets and to prepare some beverages. The leaves of young plants are good sources of vegetable relish (Nigussie Almayehu and Becker, 2002). In addition it is used in the farming systems, especially in large scale farms as a break crop for cultivation of cereals with comparable ecological amplitude (Oleszek, 1987).

It is cultivated as leaf vegetable and oilseed crop next in importance to noug crop (*Guizotia abyssinca* Casa) and Linseed (*Linum ustatismum* L) in many areas of the country with low productivity (Tesfaye *et al*., 2011). The farmer can expect an average leaf and shoot yield of 35 t/ha, but at research stations leaf yields of 50–55 t/ha have been reported, depending on production season and cultivar. In India and Canada farmers may get seed yields of 1200–1800 kg/ha in a good year (Mnzava and Schippers, 2007). Mustard production is constrained by different biotic and abiotic factors, the most being infestation by different insect pests like fungi, weeds and insects (Gupta and Dikshit, 2010).

Insect pests are responsible for most of the yield loss in horticultural plants of which the sap sucking aphids are the most important. The cabbage aphid (*B. brassicae* L.) is an important pest of *Brassica*s that can cause direct damage by piercing and sucking sap from attacked parts, and cause indirect damage by exposing tissues to bacterial, viral and fungal pathogens (Munthali and Tshegofatso, 2014). This necessitates implementing a control of the pests to prevent yield loses of crops.

The advent of chemical insecticides in the mid twentieth century created the concept that insect crop pests could be eliminated and consequently, more than 32 insecticides are currently used to control them (Jaronski, 2010). Application of chemical insecticides to control insects in results

in accumulation of high levels of toxic residues leading to health risk, contamination of the environment and destruction of non-target soil organisms, and microbial flora in the ecosystem. In addition, extensive and indiscriminate uses of chemical insecticides have resulted in the development of resistance in pests (Aziz *et al.,* 2013). Lowry *et al.* (1993) indicated that mobility and reproductive ability of aphids have contributed to reduced efficacy of chemical insecticides.

These days there is a public demand for organic and pesticide-reduced crops because of phytotoxicity, secondary pest outbreaks, and pesticide resistance as a result of the continuous use of chemicals (Hale and Elliott, 2003). To this end, the integrated approach of insect pest management through biological control, chemical control and cultural practices is well recommended (Ren *et al*., 2010). The hallmark of biological control is the development of microbial control agents for use in integrated control of insect pests (Akmal *et al*., 2013). All biological controls involve the use of natural enemies to suppress pest populations either permanently or temporarily.

Microbial control aims at suppression of insect pests by the use of entomopathogens like fungi, viruses, bacteria, protozoa and nematodes which usually possess the special features required for implementation of IPM system, host specificity, high virulence, safety to natural enemies of the target pest and ecologically non-disruptive (Loc *et al*., 2005). Entomopathogenic fungi are key regulatory factors in controlling/reducing insect pest populations in nature (Roy *et al*., 2010 ; Tkaezuk *et al*.,2014). Some of the Entomo-pathogenic fungi developed as promising biocontrol agents are members of the genera *Beauveria and Metarhizium* (Wang *et al*., 2003). These entomopathogens have been commercialized and used to control pest insects for more than 120 years (Zimmermann, 2007).

Both *B. bassiana* and *M. anisopliae* have a very broad insect host range capable of producing high concentration of aerial conidia (Jaronski,1997), produce many natural bioactive compounds (Mar and Lumyong, 2012), and produce proteases, chitinases, and lipases which can degrade insect cuticle (Charnley, 2003).

The use of entomopathogens together with other crop protection options, as part of the IPM to reduce the damage of major insect pests is well known (Loc *et al*., 2010). These biological

control agents can be used with low dose of commercial pesticides synergistically or sequentially to enhance the pesticide activity and minimize the risks of pesticides on the environment (Silva *et al*., 2013 ; Bitsadze *et al.,* 2013 ; Cuthbertson *et al.,* 2010 ; Malekan *et al*., 2012**)**.

Many studies showed that entomopathogens perform well in aphid management for different agricultural crops (Asi *et al.,* 2009; Ibrahim *et al*., 2011; Ujjan and Shahzad, 2012; Al-alawi and Obeidat, 2014). Although previous studies in Ethiopia showed that these antagonistic fungi are effective to control other agricultural crop pests such as desert locust (*Schistocerca gregaria*), pink stem borer (*Sesamia calamistis* Hampson) and African migratory locust (*Locusta migratoria*) (Senesshaw, 1998 : Tesfaye, 2012: Tsidat, 2014). There is little information about the effect of entomopathogenic fungi against adult and nymph stage of cabbage aphids.

OBJECTIVE OF THE STUDY

1.1. General objective

The general objective of the current study was : to isolate and characterize local *Beauveria bassiana* and *Metarihzium anisopliae* fungi and to evaluate their antagonistic activities alone and integrating with some selective insecticides against cabbage aphids infecting mustard under *In vitro* and *In vivo* conditions

1.2. The specific objective of the present work were to:

- Isolate indigenous *Beauveria bassiana* and *Metarihzium anisopliae* fungal species from suba forest soil samples
- Characterize and test the pathogenicity of *Beauveria bassiana* and *Metarihzium anisopliae* isolates to **(** *Brevicoryne brassicae* L.) aphids under laboratory and greenhouse conditions
- Study the compatibility of insecticides with entomopathogenic fungi for effective control of cabbage Aphids attacking mustard crop

2. LITRATURE REVIEW

2.1. Insect pests of *Brassica carniata* A. Braun (Brassicaceae)

A pest insect is one that is judged by man to cause harm to himself, his crops, animals or his property. In farming an insect may be classified as a pest when it reduces the yield and/or quality of the 'harvested product' by an amount that is unacceptable to the farmer. Insects may be classed as pests because they cause damage directly to harvestable products (Dent, 2000).

A huge number of insect pests are known to attack *Brassicas*, (Sibanda *et al*., 2000) and these insect pests are the major factors that reduce growth and crop yield of *Brassicas* (Kanrar *et al*., 2002). The vast majority of Insect pest species that commonly infest *Brassica* leafy vegetables include*: Plutella xylostella (L.) (*Diamond back moth*), Agrotis spp(*cutworm*),* Cabbage aphid (*Brevicoryne brassicae L.),* Cabbage moth *(Mamestra brassicae L),* Mustard aphid *(Lipaphis erysimi* Kaltenbach*),* Turnip aphid *(Hyadaphis erysimi* Kaltenbach*)* and Green peach aphid *(Myzus persicae* Sulzer) *(Valantin*-Morison *et al.,* 2007*;* Kazana *et al.,* 2007 : Cartea *et al.,* 2009; Dattu and Dattu, 1995 and Munthali *et al.,* 2004*)*.

2. 1. 1. Economic importance and distribution of Aphid pest

There are more than 4,000 species of aphids, and they are believed to have appeared more than 280 million years ago. Around 100 million years ago, there was an explosion in the variety of flowering plants, and the aphids were diversified to exploit this new abundance of plant species. Aphids cause major economic losses on almost all crops, and account for a large part of the 13% loss in agricultural output (Piper, 2007).

In contrast to many taxa, aphid species diversity is much lower in the tropics than in the temperate zones. They can migrate great distances, mainly through passive dispersal by winds, exemplified by the currant-lettuce aphid, Nasonovia_ribisnigri, that is believed to have spread from New Zealand to Tasmania (Courtney, 2005).

One of the most remarkable things about aphids is their reproductive ability. Consequently, aphids quickly colonize the host plant resulting in mottled leaves, yellowing, curled leaves, browning, wilting, low yields and death (Piper, 2007).

In addition to the impact of feeding, aphids also transmit 50% of all insect-borne plant viruses. Aphids also secrete a sticky substance called honeydew, which encourages the growth of saprophytic fungi like *Cladosporium spp* and *Penicillium spp.* which appear as sooty moulds blackening the leaf and limiting the leaf area available for photosynthesis (Williams and Dixon, 2007).

Among the aphid pests *Brevicoryne brassicae* (L.) (Homoptera: Aphididae), the cabbage aphid, attacks plants in the family *Brassicaceae* (Cruciferae), which includes such important crops such as oilseed rape, *Brassica napus* L., mustard, *Sinapis* (*Brassica) alba* (L.) and *nigra* (L.) and cabbage vegetables, *Brassica oleracea* L. *B. brassicae* can build up large colonies covering whole plants, and up to 80% yield of oilseed rape may be lost directly due to feeding by the aphid (Kift *et al*., 2000).

2.1.2. Biology and life cycle of *Brevicoryne brassicae*

Aphids display a diverse range of complicated life cycles. Each life cycle has three stages: egg, nymph and adult. There are two major types of aphid life cycle based on how they utilize their host plants: host alternating (heteroecious) and non-host alternating (monoecious or autoecious) (Williams and Dixon, 2007). Host-alternating aphids live on one plant species in winter (primary host), migrate to an unrelated plant species (secondary host) in summer, and migrate back to the primary host in autumn. Eggs are produced on the primary host after males and sexual females have mated. Non-host-alternating aphids remain either on the same host species or migrate between closely related species throughout the year; in other words, they can produce eggs on the same group of host plant species that is fed on by all of the parthenogenesis generations. Some aphid species never produce eggs, and these are known as anholocyclic. Some species show holocycly and anholocycly, but rarely both monoecy and heteroecy (Williams and Dixon, 2007).

Brevicoryne brassicae does not host-alternate, developing both holocyclic and anholocyclic populations on plants in the *Brassicaceae* family (Blackman and Eastop, 1985). The monoecious species that now live only on herbaceous plants have evolved from heteroecious species that no longer utilize their primary host. A diagram of the monoecious life cycle is shown in (Fig. 1) Typically, mating occurs in autumn, usually in a woody species. Eggs are laid and these

overwinter. In spring, the eggs hatch and usually give rise to a sequence of two highly fecund, apterous (wingless) morphs, the fundatrix, or stem mother, and the fundatrigenia. These produce spring migrants, where they reproduce parthenogenetically through the summer. During spring, these females change into winged forms, fly to seedlings of *Brassicas* and produce offspring nymphs adult can reproduce 4-12 nymphs per day. After several parthenogenesis generations, winged males and gynoparae are produced. The gynoparae produce sexual females (oviparae) which then mate with the males and produce the overwintering eggs (Williams and Dixon, 2007). Their short generation time and overlapping generations resulting from vivipary dramatically increases their reproductive potential during the parthenogenic phase (Blackman and Eastop, 1984).

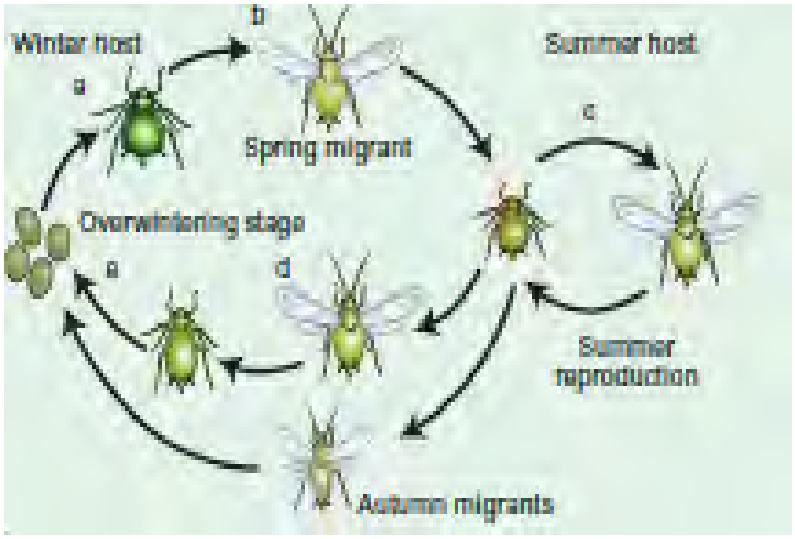

Fig1: life cycle of *Brevicoryne brassicae* **:-** Source: (AHDB, 2015). Encyclopedia of pests and natural enemies in field crops

2.1.3. Identification of aphids

Aphids are small insects, varying in size from 1 to 10 mm. They have soft bodies with long, spindly legs. In each species there are winged and non-winged forms. The mouthparts are formed into a long, thin structure called the *rostrum*, which is held under the body when the animal is not feeding. The eyes are small and relatively simple compared to other insects. Ants and other hymenopterans may be present (Piper, 2007). Generally, Honeydew-plant sap excreted through Cornicles. Sooty or Black mold (fungus) may be growing on honeydew. Found on underside of leaves and plant stems.

In aphids, there are two small pipes called cornicles or siphunculi (tailpipe-like appendages) at the posterior end that can be seen if you look with a hand lens. The cornicles of the cabbage aphid are relatively shorter than those of other aphids with the exception of the turnip aphid *Lipaphis erysimi* (Kaltenbach). These short cornicles and the waxy coating found on cabbage aphids help differentiate cabbage aphids from other aphids that may attack the same host plant (Carter and Sorensen 2013: Opfer and McGrath, 2013) cited by Gill, *et al*., (2013). The cabbage aphid is difficult to distinguish from the turnip aphid (*Lipaphis erysimi* (Kaltenbach)). The cabbage aphid is 2.0 to 2.5 mm long and covered with a grayish waxy covering, but the turnip aphid is 1.6 to 2.2 mm long and has no such covering (Carter and Sorensen, 2013).

2.1.4. Feeding behavior of Aphid

Host-plant selection by aphids is achieved by a sequence of responses from landscape to cellular scale. All responses represent behaviors with search-and-locate cycles in which sensory stimuli of different modalities play a role. Though initially visual cues seem to play an important role, olfaction and taste increase in importance after plant contact. Plant penetration can be distinguished in three distinct phases; pathway, xylem, and phloem phases. The intercellular pathway is frequently interrupted by brief intracellular punctures. The xylem phase can be considered as drinking to relieve water stress and the phloem phase comprises the main feeding, but this is always preceded by sieve element salivation, presumably to suppress phloem wound responses. During feeding, the composition of the phloem is continuously monitored and feeding can be ended on the basis of composition changes or signal molecules. Changes in phloem sap can be caused by plant ageing, diurnal as well as seasonal changes (changed source–sink relations), but also by feeding of specific and nonspecific insects (phloem feeders or not) and pathogens (Pettersson *et al*., 2007).

Most species of aphids feed on phloem sap which they obtain by tapping the phloem elements with their stylets. Phloem cells are living cells which are located at some depth within a plant. The contents of these elements (phloem sap) are rich in sugars and relatively poor in amino acids, especially those that are essential for growth (Dixon, 1998). Once a phloem vessel is punctured, the sap, which is under high pressure, is forced into the aphid's food canal. Occasionally, aphids also ingest xylem sap, which is a more dilute diet than phloem sap as the concentrations of sugars and amino acids are 1% of those in the phloem (Spiller, 1990).

2.2. Insect pest management techniques

2.2.1. Host Plant Resistance and Biotypes

Host plant resistance can affect the development rate of herbivorous insect pests. To minimize or replace the use of dreadful chemical pesticides alternative pest management strategies are given importance in recent times. Botanicals are generally more compatible with the environmental components than synthetic pesticides in pest control program due to biodegradable nature, less toxic to non-target organisms and multiple modes of action. In view of these, efforts were undertaken for assessing repellent and insecticidal activities of *Mentha piperita* plant extracts against cabbage aphid. Repellent activity of the plant extracts at higher concentration and exposure period percent increased repellent activity. (Mersha Wubie *et al.*, 2014). According to Cortesero *et al.*, (2000), host-plant effects on the efficiency of insect natural enemies can occur in various ways, such as by mediating host/prey accessibility and availability, providing host/prey finding cues, influencing host/prey suitability and providing supplemental food resources.

Plant species, on which insect pest development is delayed, increase the vulnerability of pests to natural enemies and their rates of parasitism (Benrey and Deno, 1997). Plants which are more vulnerable to pests are called susceptible plants and those which are able to strongly tolerate attacks are considered resistant. There are three general types of mechanisms for resistance i.e. antibiosis, antixenosis and tolerance. Antibiosis is defined as the adverse effect that a plant may have on the pest because of chemicals or structures the plant possesses. Antixenosis resistance involves behavioral factors that cause an insect not to choose the plant for feeding or laying its eggs, while tolerance is a characteristic of some plants that enable them to withstand or recover from insect or disease damage (Van Emden, 2007).

2.2.2. Cultural control

Cultural control is the use of various agricultural practices to make a habitat less suitable for reproduction and/or survival of pests. It is a long-established method of pest control. Cultural control aims, therefore, to reduce rather than eradicate pest populations and is typically used in conjunction with other control methods. However, in some instances, cultural practices alone may affect almost complete control of a pest. The agricultural practices used either may have a

direct effect on the pest or may act indirectly by stimulating population buildup of a pest's predators or parasites, or by making plants and animals more tolerant of pest attack. An essential prerequisite for effective cultural control is detailed knowledge of a pest's life history so that its most susceptible stages can be determined.

For crops, crop rotation prevents buildup of pest populations and planting or harvesting out of phase with a pest's injurious stage(s), which is especially important against species that have a limited period of infestation or for plants with a short period of susceptibility reduce the population of pests. Furthermore, the use of trap crops on which a pest will concentrate, that enables us to make an easy and subsequent destruction. Other traditional methods are soil preparation to bury or expose a pest, or increase the crop's strength so that it can more easily tolerate a pest, clean culture, the removal, destruction, or ploughing under of crop remains to deprive pests from hibernation, crop diversity, that is, reversal of the current practice of monoculture (growing a single crop over a wide area) (Gillott, 2005).

2.2.3. Chemical control

The use of chemical either to kill or to repel insect pests is the oldest method of pest control. (Pfadt, 1985) notes that the Greeks used sulfur against pests almost 3000 years ago and the Romans used asphalt fumes to rid their vineyards of insect pests. The Chinese used arsenic compounds against garden pests before 900 A.D., though arsenic was not used in the Western world until the second half of the 17^{th} century. Chemical pesticides have been used since the early 1940's to control insects that are either harmful to food crops or transmit human diseases (Gillott, 2005).

Management of aphids has been done primarily by using chemical methods; however environmental and health problems have arisen due to this practice. Widespread use of insecure synthetic chemical pesticides and demanding crop production causes several socio-economic problems throughout the world. For example, more than twenty aphid species together with *Myzus persicae* (Harrington and Emden, 2007) have showed resistance to a number of carbamate, pyrethroid and organophosphate based insecticides.

Chemical control is the most efficient and quickest method for the control of aphids. Insecticides belong to different groups like organochlorines, organophosphates and pyrethriods have been

used to control aphids on *Brassicas* (Dewar, 2007). However, many are associated with undesirable traits such as failure in controlling aphids, high mammalian toxicity, persistence and deposition of oil, and contributing to the development of insecticide resistance.

Natural and synthetic chemicals are traditionally used to kill pests and have been used as major methods of pest control for about 90 years. However, these chemicals have created three serious problems such as a great increase in the resistance of pests to the chemicals, the death of many beneficial insects as a result of the chemicals' non-specific activity and pollution of the environment (Gillott, 2005).

2.2.3.1. Mode of Action of some of the chemicals

Lambda-cyhalothrin ($C_{23}H1_9CIF_3NO_3$) is a pyrethroid insecticide. Pyrethroids are axonic poisons that affect the nerve fiber by binding to a protein that regulates the voltage-gated sodium channel. Normally, this gate opens to cause stimulation of the nerve and closes to terminate the nerve signal. The channels are pathways through which ions are permitted to enter the axon and cause excitation. When the channels are left open, nerve cells produce repetitive discharges and eventually cause paralysis (Bradbury and Coats 1989; Shafer and Meyer 2004). Pyrethroids bind to this gate and prevent it from closing normally, which results in continuous nerve stimulation and tremors in poisoned insects. Poisoned organisms lose control of their nervous system and are unable to produce coordinated movement.

Because of the lipophilic nature of pyrethroids, biological membranes and tissues readily absorb them. Specifically, lambda-cyhalothrin penetrates the insect cuticle, disrupting nerve conduction within minutes; this leads to cessation of feeding, loss of muscular control, paralysis, and eventual death (He, *et al*,. 2008).

Thiamethoxam ($C_8H_{10}CIN_5O_3S$) is the first commercial neonicotinoid insecticide from the thianicotinyl subclass. The most prominent member of this class of insecticides is thiamethoxam (Nauen *et al*., 2003). Thiamethoxam acts by binding to nicotinic acetylcholine receptors of the insectnervous system. It exhibits exceptional systemic characteristics and provides excellent control of a broad range of commercially important pests, such as aphids, jassids, whiteflies, thrips, rice hoppers, Colorado potato beetle, flea beetles and wireworms, as well as some lepidopteran species (Maienfisch *et al.*, 2001).

Spinetoram($C_{41}H_{65}NO_{10}$) is provides long lasting control of a broad spectrum of insect pests in a variety of crops. It is applied at low rates and has low impact on most beneficial insects. Spinetoram causes excitation of the insect nervous system by altering the known binding sites of other classes of insecticides such as of neonicitinoids, fiproles or avermectins (Mertz and Yao, 1990)

2.2.4. Problems associated with pesticide use.

2.2.4.1. Effects of chemical insecticides on non- target organism

Some pesticides are harmful to pollinators such as wild bees, certain wasps, honeybees, and other insects causing direct losses of the insect populations and indirect losses of crop yield because of the lack of adequate pollination. In addition, the most obvious effects of pesticides are direct poisoning of on aquatic organisms like fish and wildlife. In many cases, however, the indirect effects of pesticides, such as causing dissolved oxygen depletion. They also kill birds in several ways, including direct ingestion of granules, baits, treated seeds, and direct exposure to sprays. Indirect bird kills may result from consumption of treated crops, contaminated water, or feeding on contaminated prey. Inert ingredients in pesticide formulations may also be capable of causing phytotoxicity (Fishel, 2005).

Many chemical pesticides have broad spectrum of action and therefore kill beneficial insects that serve in the recycling of organic materials that increase soil fertility. Furthermore, some of the insects killed are natural enemies of the target pest, which are often more sensitive to the pesticide than the pest itself (Van Driesche and Bellows, 1996; Schepers, 1989).

2.2.4.2. Development of insect resistance to chemical control

The resistance of insects to insecticides has been a problem ever since synthetic insecticides have been introduced inappropriate and excessive use of pesticides has led to acquisition of resistance to over 500 species of insects and mites. Resistance in an insect population can be defined as a reduction in the degree of control given by the pesticide compared to what is expected, when factors like application problems, extreme temperatures and many others are not included or implicated. Resistance occurs when a pest alters a target site in its body or enhances its ability to metabolize toxins, or change its behavior to avoid exposure to the pesticide (Clarke *et al.,* 1997). Over the past 20 years, rapid advances have been made in the characterization and understanding

of such adaptations. Of the thousands of aphid species that exist globally, only a few have been reported as having developed insecticide resistance.

For example, some strains of aphids have developed distinct types of resistance to carbamate insecticides, organophosphates (OPs) by over producing one of two closely related carboxylesterases (E4 and FE4) that degrade the insecticide esters before they reach their target sites in the nervous system of the aphid. Another mechanism is development of a modified acetylcholinesterase which confers resistance to dimethyl carbamates, pirimicarb and triazamate (Clarke *et al.,* 1997; Foster *et al.,* 2007*).*

2.2.4.3. Impact of chemical pesticide use on public health

Although the use of chemicals in modern agriculture has significantly increased productivity, it has also significantly increased the concentration of pesticides in food and in the environment. The severity of any adverse effects from exposure to a pesticide depends on the dose, the route of exposure, rate of absorption, and its accumulation and persistence in the body. The toxic effect also depends on the health status of the individual, malnutrition and dehydration (WHO, 1990).

According to WHO-UNDP (1989) report there has been about 1 million, mainly occupational, incidents of pesticide poisoning. Even though the long-term health implications associated with exposure to pesticides and their residues are not as severe for the general public, the main source of exposure is either residues in contaminated food (fruit and vegetables.), from drinking water, physical contact or through respiration (Skinner *et al*., 1997).

There is now a widespread alarm among the general public about the possible effects of exposure to pesticides. These include: neurotoxin disorders, immunodisfunction, mutagenesis, teratogenesis and carcinogenesis (Andersson *et al.,* 2014: Vale *et al*., 2003). These fears have, of course, had their impact on legislation concerning pesticides.

2.2.4.4. Effects of chemical insecticides on the environment

Chemical insecticides have adverse effects on the environment (water, soil and air contamination from leaching, runoff, spray drift, wildlife, fish, plants, and other non-target organisms), many of these effects depend on the toxicity of the pesticide, the measures taken during its application,

the dosage applied, the adsorption on soil colloids, the weather conditions prevailing after application, and its long persistence in the environment (Dam Alas and Eleftherohorinos, 2011).

When insecticide is applied to control pests, a considerable amount of it reaches to the soil that has adverse effects on soil micro flora and fauna (Van der Werf, 1996). The pesticides are not normally found in nature and tend to accumulate in food chains as a result of their relative recalcitrance in their biochemical stability. The effect to aquatic life is widespread, with fish death as the major indicator. Pesticide contamination and toxicity is now measured with biological indicators of algae, crustaceans and fish as representative of food chain tropic levels (Van der Werf, 1996).

2.2.4.5. Rationale for seeking alternatives to chemical insecticides

There are a number of reasons people are looking for alternatives to chemical insecticides. Some of the contact insecticides have become ineffective because of wide-spread resistance in insect populations (Fields, 2004).

Agricultural scientists started to develop alternative crop management systems to minimize the negative effects of farming (based mainly on pesticide use for crop protection) to the environment and to human health. In particular, the Integrated Crop Management (ICM) includes guidelines to enforce actions for production of safe agricultural products with simultaneous respect to the environment.

Generally, due to insecticide resistance, pest resurgence, safety risks for humans and domestic animals, contamination of ground water, decrease in biodiversity, and other environmental concerns, there is a need to encourage research for the development of environmentally benign strategies for pest control including the use of biological control agents (Abid *et al*., 2012).

2.2.5. Biological control

Biological control is a cornerstone of pest management in many parts of the world. Use of entomophagous as biological control agents has resulted in reducing damage from pest species and in sustaining environmental health, particularly through reductions in pesticide use. These attributes indicate that use of entomophagous biological control agents will continue and even

grow. However, debate is increasing on the need for greater regulatory oversight of biological control agents, including entomophagous species (Mason *et al*., 2003).

In the simplest terms, biological control is the reduction of pest populations brought about through the actions of other living organisms, often collectively referred to as natural enemies or beneficial species. Virtually all insect and mite pests have some natural enemies, although not all are effective in suppressing pest populations. Effective biological control often requires a good understanding of the biology of the pest and its natural enemies (Michaud *et al*., 2008).

Biological control is a key component, though often underappreciated, in agriculture pest management. It may take many years to succeed and it can be safest cheapest and most effective approach to long term management of a pest. The first step in biological control of aphids and other pests is to evaluate the effectiveness of natural enemies by identifying those species that have high potential for use (Irshad, 2001).

2.2.5.1. Microbial pesticides

2.2.5.1.1. History of microbial control

In the history of microbial pesticides, most attention was given on pests of agriculture and on the vectors of human and animal disease. Pests include some of the agriculturally important taxa such as beetles, weevils and termites. However, there are many taxa including cockroaches, flies and mosquitoes that have agricultural, medical, and veterinary importance (Prior, 1996).

The use of micro-organisms to control pests was first developed by Agostino Bassi who demonstrated that muscardine of the silkworm is caused by the fungus *Beauveria bassiana* (Bals). This achievement was followed by the illustrious work of Pasteur work on two other diseases (pebrine and flacherie) of the silkworm that prompted him further to be interested in the microbial diseases of other animals and of man. In recent years there have been a rapid development of the field of insect pathology, and the knowledge of microbial diseases of insects is fast accumulating. Moreover, the use of microorganisms in the control of insect pests appears to be entering a renaissance (Steinhaus 1956).

Similarly, Elie Metchnikoff, who initiated his studies on cellular immunity by observing the activity of phagocytes in a crustacean (Daphnia) suffering from a yeast infection, is

acknowledged to have begun his researches on infectious diseases such as his investigation of a fungus disease (green muscardine) of the wheat cockchafer. So, from an historical standpoint at least, the insect pathologist or insect microbiologist should feel as at home here among his fellow microbiologists as do his colleagues concerned with the microbial maladies of other forms of life (Steinhaus, 1960).

2.2.5. 2. Advantages of biological control agents

Using organisms for biological control agents reduces exposure to potentially toxic pesticides by the producer, applier and consumer. Biological control agents have no harmful residues and therefore pose an extremely low risk of environmental pollution when used. In addition, the toxic action of microbial insecticides is often specific to a single group or species of insects, and this specificity means that most microbial insecticides do not directly affect beneficial insects (including predators or parasites of pests) in treated areas so increased biodiversity in the ecosystem and increased activity of natural enemies. For example, various fungi such as *B. bassiana, M anisopliae* and *Verticillum lecanii* (Zimm) Viegas that have been developed for commercial use as biological control agents have shown no infectivity to man or other vertebrates. They also enhance the root and plant growth by way of encouraging the beneficial soil micro flora (Usta, 2013).

Since biological control agents do not kill non-target insects, little or no development of resistance by the target organism [however, resistance has developed in some target insects to *Bt*, *Bacillus sphaericus* and baculoviruses. Compatibility with other biological control agents, possibility of long-term control, secondary pest outbreaks are controlled, recycling organic materials is maintained thus increasing soil fertility (Tanada and Kaya, 1993).

2.2.5.3. Disadvantages of biological control agents

The disadvantage of bio-control compared with chemical pesticides include the activity of biological control agents, particularly microbial control agents are dependent on environmental conditions such as humidity, temperature, ultraviolet radiation and pH which cannot be readily controlled in the field and therefore, short field persistence. As a result, their action can be inconsistent when these factors change. Due to these problems, many biological control agents have not yet met the expectations of modem agriculture where farmers want predictable and

consistent results of pest control. In addition, specificity only to target organism, strict timing of application for maximal effect, long period of lethal infection (*i.e*., little or no "knock-down" effect), development of resistance by target organisms, especially to *Bt* and *B. sphaericus* and uneconomical except for niche markets (Lacey and kaya, 2007).

Another disadvantage of biological control agents is that some of them are not compatible with other pesticides. For example, *V. iecanii,* which is used to control the glasshouse whitefly, *Trialeurodes vaporar;omm* (Westw) is not compatible with the fungicides tolyfluanid and dichlofluanid (Rodgers, 1993).

Because a single microbial insecticide is toxic to only a specific species or group of insects, each application may control only a portion of the pests present in a field and garden. If other types of pests are present in the treated area, they will survive and may continue to cause damage. Conventional insecticides are subject to similar limitations because they too are not equally effective against all pests. This is because of selectivity indeed and this negative aspect is often more noticeable for both general predators, chemicals and microbial (Usta, 2013). Review On the other hand predators and chemicals may be danger for other beneficial insects in threatened area. Because several microbial insecticides are pest-specific, specificity is desirable from the environmental perspective, so that non-target and beneficial organisms are not harmed, but such specificity limits the market for the product, to the extent that it may not be economically viable. End-users often want to treat a variety of pests with a single application, which is not possible if the product is very specific. This situation may not apply in the case of urban pests, where treatments are often required for a single, specific pest problem, such as cockroaches, termites or flies (Prior, 1996)**.**

2.2.5.4. Entomopathogenic nematodes

Although there are numerous nematode taxa that have shown potential in biological control, the entomopathogenic nematodes (EPN), Rhabditida: Steinernematidae and Heterorhabditidae, have been most successful and have received the most attention (Grewal *et al*., 2005). EPNs kill arthropod hosts via a mutualistic symbiosis with bacteria, Xenorhabdus spp. and Photorhabdus spp. for steinernematids and heterorhabditids, respectively (Poinar, 1990). Infective juveniles (IJs), the only free-living stage, enter hosts through natural openings (mouth, anus, and

spiracles), or in some cases, through the cuticle. After entering the host's hemocoel, nematodes release their bacterial symbionts, which are primarily responsible for killing the host within 24–48 h, defending against secondary invaders, and providing the nematodes with nutrition (Dowds and Peters, 2002).

With few exceptions, example Steinernema scarabaei (Koppenhöfer and Fuzy, 2003). entomopathogenic nematodes have a wide host range. Some nematode species have been reported to infect dozens of insect species across five ormore orders (Poinar, 1990), and certain nematode species are used commercially against 12 or more insect species. Entomopathogenic nematodes are amenable to mass production using in vivo (infected insects) or in vitro (solid or liquid fermentation) methods (Shapiro-Ilan , *et al*,.2002).

2.2.5.5. Entomopathogenic Viruses

Viral infections are not restricted to humans, as many viruses infect other animals, plants, insects and microorganisms, some with shocking effects. Viruses have demonstrated to be important biological control agents, in many cases. The main features of the most important insect viruses are:-

2.2.5.5.1. *Baculovirus*

The Baculoviruses are the most widely exploited virus group for biocontrol: they are very different from viruses that infect vertebrates and are considered very safe to use. The mode of pathogenesis and replication of entomopathogenic viruses varies according to the family, but infection nearly always occurs by ingestion. Virions then bind to receptors in the gut and penetrate epithelial cells. In the Baculoviruses, the infection often spreads to the haemocoel and then to essential organs and tissues, particularly fat bodies. Baculoviruses make up a family of insect viruses and are grouped into two main groups or genera: *Nucleopolyhedrovirus* or NPVs; and *Granulovirus* or GVs. Both groups contain circular double-stranded DNA genome of approximately 80-180 kb, which is condensed within nucleocapsids and are predicted to encode from about 90 to 180 genes (Van Frankenhuzen, 2007).

2.2.5.5.2. *Entomopoxvirus*

The Poxviridae family is separated into two subfamilies: the *Entomopoxvirinae,* which comprise poxviruses of insects; and the *Chordopoxvirinae*, which comprise poxviruses of vertebrates

(Goodwin *et al*, 1991). The first subfamily or *Entomopoxvirus* (EPV), comprise three genera based on host insect and virion morphology. in addition to these additional entomopathogeniv viruses like Cypovirus and Iridovirus have been present.

2.2.5.6. Entomopathogenic bacteria

An enormous number of bacterial species have been reported from pest and beneficial insects (Jurat-Fuentes and Jackson, 2012). But a relatively small number of entomopathogenic bacteria have been commercially developed for control of insect pests of crops, forests, humans, and livestock. These include several *Bacillus thuringiensis* (Bt) sub-species, *Lysini bacillus* (Bacillus) sphaericus, Paenibacillus spp. and Serratia entomophila. The most widely used bacteria for control of numerous insect pests are Bt subspp. (Glare and O'Callaghan, 2000).

2.2.5.7. Entomopathogenic Fungi (EPF)

It has been estimated that the kingdom fungi consists of 1.5 million species (Hawksworth, 2001) with approximately 110,000 described species (Kirk et al. 2008). Of these, 700 species in 90 genera are recognized as insect pathogens and approximately 170 pest control products have been developed. The growing demand for reducing chemical inputs in agriculture and increased resistance to insecticides have provided great impetus to the development of alternative forms of insect-pest control.

Entomopathogenic fungi are widely distributed in a wide range of habitats including aquatic, forest, agricultural and pasture habitats (Chandler *et al.,*1997). However, epizootics caused by entomopathogenic fungi in agricultural habitats are more numerous, particularly in temperate regions, than those in other habitats. They are among the first organisms to be used for the biological control of pests. Most of them are found within the Deuteromycetes and entomophthorales. Some entomopathogenic fungi have restricted host ranges, for example, *Aschersonia aleyrodes* infects only scale insects, and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific to target pests. Entomopathogens such as *M. anisopliae* and *B. bassiana* are well characterized in respect to their pathogenicity to several insects, and they have been used as agents for the biological control of agriculture pests worldwide (Sandhu *et al.,* 2012*)*.

Entomopathogenic fungi are potentially the most versatile biological control agents due to their wide host range. These fungi comprise a diverse group of over 90 genera with approximately 750 species, reported from different insects (Rai *et al*.,2014). These fungi provide an environmentally responsive substitute to chemical pesticides. They are natural, easy to formulate, less toxic to mammals, with no residual activity and less chance to develop resistance (Prior,1996). Entomopathogenic fungi are a major component of integrated pest management techniques as biological control agents against insect pests and other arthropods and are an integral part of mycoinsecticides in horticulture, forestry and agriculture (Inglis *et al*., 2000). Various additional unexpected roles have been reported for fungal entomopathogens, including their presence as fungal endophytes, plant disease antagonists, rhizosphere colonizers and plant growth promoting fungi (Saikkonen *et al.*, 2006). Although entomopathogens very diverse, the most popular pesticides the major entomopathogenic fungi used as mycoinsecticides and mycoacaricides and used for inundative and inoculative applications are derived from 12 species (De Faria and Wraight, 2007).

2.2.5.8. Biology and taxonomy of entomopathogenic fungi

The life cycle of EPF is composed of the spore which geminates into mycelia and the mycelia in turn produce spores (spore–mycelia–spore phases). The life cycle of most entomopathogenic fungi consist of two phases: a normal mycelia growth phase mostly outside the host body and a yeast like budding phase mostly in the hemocoel of host. The yeast-like, dimorphic mode of growth in *Beauveria bassiana* was described by Alves, *et al,* (2002); and the production of oblong blastospore- like propagules in *M. flavoviride*was described by Fargues *et al,* (2002). *Beauveria bassiana*in the absence of a specific insect host grows through an asexual vegetative life cycle consists of germination, filamentous growth and formation of sympoduloconidia (Wang *et al*,.2004).

Entomopathogenic fungi were among the first organisms to be used for the bio-control of pests. More than 700 species of funge from around 90 genera are pathogenic to insects (Khachatourians and Sohail, 2008). Most EPF species are from the fungal divisions *Ascomycota* and *Zygomycota*. The *Ascomycete* fungi were previously divided into two groups, the *Ascomycota* and the *Deuteromycota.* The Fungi Imperfecti of *Deuteromycota* was known for having no sexual stage

was known called as. But later on, cultural and molecular studies have demonstrated that some of these "imperfect fungi" (formally class Hyphomycetes in the Deuteromycota) were in fact anamorphs (asexual forms) of the Ascomycota within the order Hypocreales, and Clavicipitaceae family (Fukatzu, 1997 ; Krasnoff, 1995). Within the Zygomycota, the most entomopathogenic species are in the order Entomophthorales (Roy *et al*., 2006).

2.2.5.9. *Beauveria bassiana* and *Metarhizium* and their Morphological Characterizations

2.2.5.9. 1. *Beauveria bassiana*

The genus *Beauveria* contains at least 49 species of which approximately 22 are considered pathogenic (Kirk, 2003). *Beauveria bassiana* a white muscardine fungus, is the most historically important of the commonly used fungi in this genus. Originally known as *Tritirachium shiotae,* this fungus was renamed after the Italian lawyer and scientist Agostino Bassi who first implicated it as the causative agent of a white (later yellowish or occasionally reddish) muscardine disease in domestic silkworms (Furlong and Pell, 2005; Zimmermann, 2007) cited by Rai *et al.,* 2014).

Beauveria bassiana, is a fungus that grows naturally in soils throughout the world and acts as a pathogen on various insect species, (Sandhu *et al*., 2001). It can easily be isolated from insect cadavers or from soil in forested areas by using media as well as by baiting soil with insects. Morphologically surface of the colony is white to cream and fluffy to powdery. The fungal hyphae are tubular, narrow, septate fragile filaments; it is usually white becoming slightly yellowish over time. The hyphal width differs from 2-3 μm. Conidiogenous cells are nearly flask shaped with a narrow zigzag terminal extension cell length and width respectively 8.7, 2.9. They also proliferate sympodially. Conidia are single-celled and globose or ovate in shape, and usually germinate on a geniculate or at each bend point of zigzag rachis (Hussein *et al*., 2010).

2.2.5.9.2. *Metarhizium* spp.

Metarhizium (Metschnikoff) (Ascomycota: Hypocreales) is also a cosmopolitan genus of soil borne EPF(entomopathogenic fung). It was first isolated in the 1880s by Elie Metchnikoff, a Russian microbiologist, from his grain beetle research and named it the green muscardine fungus, *Entomophthora anisopliae* (Lord, 2005). *Metarhizium* species causes a disease in insects and have a wide range of virulence and are known to infect more than 200 different insect

species, many of which are major agricultural pests, such as sugarcane stem-borers, Aphids, scarab grubs and grasshoppers (Bidochka & Small, 2005).

The fungus is easily identified because it forms green chains of cylindrical conidia that densely compact on the infected host, causing green muscardine disease. Fungal colonies are initially white or creamy mycelium, becoming shades of green/yellow to shades of dark green during sporulation. Conidiophores are simple or double- branched. The range of conidial size is from 0.79 -7.2 μm in length, and from 0.54 -3.5 μm in width (Hussein *et al*., 2010).

2.2.5.9.3. *Verticillium lecanii* spp.

Verticillium lecanii is a widely distributed fungus, which can cause large epizootic in tropical and subtropical regions, as well as in warm and humid environments (Nunez *et al*., 2008). It was reported by Kim *et al*. (2002) that *V. lecanii* was an effective biological control agent against *Trialeurodes vaporariorum* in South Korean greenhouses. This fungus attacks nymphs and adults and stucks to the leaf underside by means of a filamentous mycelium (Nunez *et al*., 2008).

2.2.5.9.4. *Nomuraea* spp.

Nomuraea rileyi another potential entomopathogenic fungi is a dimorphic hyphomycete that can cause epizootic death in various insects. It has been shown that many insect species belonging to Lepidoptera including *Spodoptera litura* and some belonging to *Coleoptera* are susceptible to *N. rileyi* (Ignoff, 1981). The host specificity of *N. rileyi* and its ecofriendly nature encourage its use in insect pest management. Although, its mode of infection and development have been reported for several insect hosts such as *Trichoplusiani*, *Heliothis zea*, *Plathypena scabra*, *Bombyx mori*, , and *Anticarsia gemmatalis*.

2.2.5.9.5. *Paecilomyces* spp.

Paecilomyces fumosoroseus is one of the most important natural enemies of whiteflies worldwide, and causes the sickness called “Yellow Muscardine” (Nunez *et al*., 2008). Strong epizootic potential against *Bemisia* and *Trialeurodes* spp. in both greenhouse and open field environments has been reported. The ability of this fungus to grow extensively over the leaf surface under humid conditions is a characteristic that certainly enhances its ability to spread rapidly through whitefly populations (Wraight *et al*., 2000). Kim *et al*. (2002) reported that *P. fumosoroseus* is best for controlling the nymphs of whitefly. These fungi cover the whitefly's

body with mycelial threads and stick them to the underside of the leaves. The nymphs show a "feathery" aspect and are surrounded by mycelia and conidia (Nunez *et al*., 2008).

2.2.5.10. Mode Action of entomopathogenic fungi

Unlike the other infectious agents, that requires routes of entry for infection of insect hosts, (viruses, bacteria, protozoans,) entomopathogenic fungi infect the host directly through the host cuticle. Infection begins with attachment of single-celled dispersive forms of the fungus, e.g., conidia or blastospores, to the insect cuticle (Urquiza and Nemat Keyhani**,** 2013). Once a propagule attaches insect cuticle, it may germinate and produce penetration structures (e.g. germ-tube swelling, appressorium or extracellular sheath) from which penetration hyphae are formed (Sandhu, 1995). The entomopathogen further employs a combination of enzymatic and mechanical mechanisms to penetrate the cuticle. However, sometimes even if germination does occur, the fungus may not be able to penetrate the cuticle, due to inappropriate environment (e.g. conditions of moisture) and/or the presence of inhibitory factors, such as fatty acids or melanin, within the cuticle. The production of exoproteases are important in penetration by *Metarhizium anisopliae* and other fungi, but other enzymes, such as endoproteases, esterases, lipases, chitinases and chitobiases, are involved as well (St Leger, 1993; Boucias and Pendland, 1998; Butt *et al.*, 1998a). Once the fungus reaches the haemocoele, it grows as hyphal bodies, (Fig.2) which are single- or multicelled structures that lack a formal cell wall but do contain a thin, fibrillar layer on the plasma membrane (some forms are often referred to as 'blastospores') Before the fungus can proliferate in the haemocoele, it must often overcome the insect's defence response, and the production of toxins by the fungus can debilitate the efficacy of the defense response mounted by the insect (Inglis *et al*., 2001).

Insect death may result from a combination of actions, including depletion of nutrients, physical obstruction or invasion of organs and toxicosis. For example, *Beauveria bassiana* produces a number of toxic compounds, including beauvericin, bassianolide and oosporein. Perhaps the best-studied toxins produced by entomopathogenic Hyphomycetes are the destruxins produced by *M. anisopliae*. Several of these are known to induce tetanic paralysis (Dumas *et al.*, 1996), while others can be immunosuppressive (Cerenius *et al.*, 1990). In the initial infection stages, no considerable behavioral symptoms are observed. But, some days before death, symptoms such as

reduced co-ordination, feeding and activity start to appear followed by behavioral fever, and increased feeding, (Noma and Strickler, 2000).

Following death, the fungus often grows saprotrophically within the host, to release metabolites (oosporein are produced by *B. bassiana* and *Beauveria brongniartii*) to exert competitive exclusion of competing microorganisms from the cadaver. Soon after host death, and under favorable conditions, hyphae emerge from the cadaver; they produce conidiogenous cells, sporulation occurs on the host surface and the conidia are liberated and dispersed passively by wind and rain (Inglis *et al*., 2001). Many hyphomycetous fungi (*Beauveria, Metarhizium* and *Paecilomyces*) produce conidia possessing hydrophobic properties due to cysteine-rich proteins called hydrophobias within the rodlet layer of the cell wall.

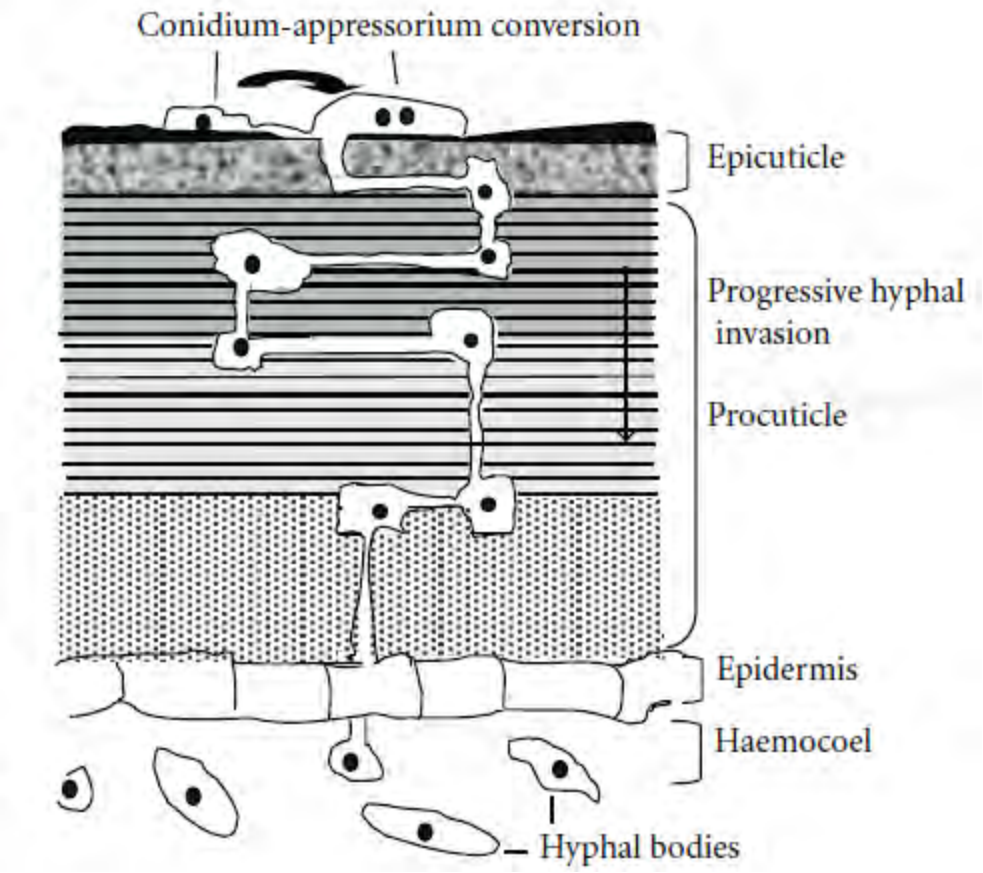

Figure-2: structure of the insect cuticle and mode of penetration of fungal hyphae. Source: **(**Sandhu *et al*., 2011)

The time of death of an infected insect varies from 2-15 days post infection depending on the fungal strain and species, but more particularly on the characteristics of the host (Boucias and Pendland, 1998).

2.2.5.11. Factors affecting the efficacy of entomopathogenic fungi

A co-evolutionary arms race occurs between insects and their pathogens. Whereas selection on the pathogen is for greater exploitation of the host, selection on the host is for greater exclusion of the pathogen (Roy *et al*., 2006).

Pathogenicity is the qualitative ability of a pathogen to cause disease and it is determined by a variety of factors, including the physiology of the host (e.g. defense mechanisms) and physiological and morphological factors influence the susceptibility of insect pests to entomopathogenic fungi include population density, behavior, age, nutrition, genetics and exposure to injuries caused by mechanical, chemical or non-microbial agents, the physiology of the fungus (e.g. pathogenicity factors, such as the production of enzymes and toxins) and the environment (Inglis *et al*., 2001).

Aside from immune responses, insects have evolved a number of mechanisms to keep pathogens away that include: The production of epi-cuticular antimicrobial lipids, proteins, and metabolites; by shedding of the cuticle during development; and behavioral-environmental adaptations such as induced fever, burrowing, and grooming, as well as potentially enlisting the help of other microbes. All these activities are intended to stop the pathogen before it can breach the cuticle. Cuticular lipids, however, can also promote or inhibit fungal attachment to cuticle and such attachment can be affected by nutritional requirements and can be enhanced via formulation that can result in increased efficacy of the fungal agents against target insects (Urquiza, and Keyhani, 2013).

In addition to cuticular lipids, surface antifungal defenses include small molecule toxins (including peptides) and proteins. While it remains unclear whether any small compound antifungal peptides are actually secreted to the insect surface, a number of insect-derived chitinase and protease inhibitors as well as a wide range of antimicrobial peptides (AMPs), some of which demonstrate the hallmarks of co-evolutionary pressures, have been reported. Simple

molting may provide a means for avoiding infection. Rapid ecdyses in aphids may be an important contributing factor to poor outcomes in applications of entomogenous fungi (Vandenberg, *et al.,* 1998: Kim and Roberts, 2012).

When mass producing a fungal pathogen, it is essential that the strain is well maintained. It must be free from all contamination and carefully conserved so that it not only remains viable in agar culture, but also retains its virulence. If fungal strains are sub-cultured too often on artificial media, they can lose their virulence. This can be prevented by maintaining isolates for storage on Potato carrot agar (PCA) slopes, which may be kept in the refrigerator for between six months and one year. The spores from these cultures can then be used to inoculate working cultures on Sabouraud dextrose agar (SDA) slopes (the recipes for these media can be found below). In the longer term, fungal strains should be cycled through the original host or closely related species periodically (Seema, *et al*,. 2013).

2.2.5.12. Integrated pest management (IPM)

Integrated pest management provides alternative way to manage crop pests and disease, instead of using Persistent Organic Pollutants (POPs) and other harmful agrochemicals. IPM builds on traditional, appropriate pest and disease management strategies that farmers have used for centuries, and combines them with new technologies based on ecologically sound principles, to manage pests and disease effectively with minimum impact on the environment (SP-IPM. 2006).

Undesirable effects and difficulties in controlling pests with broad spectrum pesticides demand the development of integrated pest management strategies. Chemical control in an IPM system focuses on the use of reduced-risk pesticides. The availability of such techniques will not only help manage pests, it will also reduce exposure to pesticides (Panda and Khush, 1995).

There are three phases in the development of an IPM strategy: problem definition, research, and implementation, of which the first is the most important, and to be most effective, IPM requires the input of as much information as possible, not only about the agro ecosystem, but also about the socioeconomic framework of the farming system in which the pest problem occurs. Thus, the collaboration of experts from a wide range of disciplines is necessary and if conducted properly, IPM leads to considerable financial saving and a great improvement in environmental quality (Gillott, 2005).

Under the Ethiopian context, however, such savings are insignificant as pesticide use is very low. A major objective of IPM here would be not to reduce pesticide use, but to prevent (or at least to delay) breakdown of the agro-ecosystem that has existed for centuries, and to prevent unnecessary stockpiling of pesticides and the inevitable consequences of accumulating obsolete pesticides. Subsistence farmers in Ethiopia, and elsewhere in Africa, traditionally use a combination of several pest management practices (such as cultural control, habitat manipulation, mechanical and physical control, natural biological control, host plant resistance, use of locally available materials) such that regular insect pest outbreaks of the magnitude experienced in commercial agriculture are rare (Abate and Ampofo 1996: Abate *et al*., 2000).

Biological control will only reduce the population of a pest and not completely eliminate it. This means that there are some pests left on the crop which can do damage. This may not be economically damaging to the farmer in terms of yield, but may reduce the cosmetic quality of the produce (Mcleod *et al.,* 1998). With the difficulties outlined above, it may be more practical to considerably reduce the use of pesticides rather that completely discontinue their use. This could be done by combining the use of pesticides with biological control in an Integrated Pest Management (IPM) program.

2.2.5.13. Formulation and mass production of Entomopathogenic fungi

Production and formulation are critical to the commercial development of a fungal biocontrol agent. The formulation of propagules of entomopathogenic fungi for use in biocontrol has been guided by the need to improve product shelf-life, biocontrol efficacy, and/or the physical characteristics of the product for application (Wraight *et al*., 2001).

Production and formulation strategies for potential mycoinsecticides must consider the environmental and ecological requirements and limitations (Vega, *et al*., 2009). From a biotechnology standpoint, a variety of fungal propagules can be produced using solid-substrate and deep-tank fermentation by altering nutritional and environmental conditions. Likewise, formulations can be employed that alter the chemical and physical attributes of fungal propagules for improved insecticidal activity under varied environmental conditions. Formulations developed with living, fungal entomopathogens for use in inundation biocontrol

must take into account the environmental and ecological life histories of the target insect while maintaining propagules viability and efficacy (Subramanian and Punamalai, 2013).

2.2.5.13.1. Conidia production using solid substrate fermentation

The primary infective form of most fungal entomopathogens is the conidium and, in fact, the solid substrate production of aerial conidia is the most widely used production method for the mycoinsecticides *Metarhizium* and *Beauveria* (Faria and Wraight 2007). The solid substrate phase of mass production provides a physical support for the fungus to produce aerial conidia (the infective propagules which are best suited to storage and formulation in oil). Usually, the substrate is all cereal or cereal by-product such as rice, millet, maize or wheat bran (Seema *et al*., 2013).

Solid state fermentation (SSF) allows several types of fungi to produce hardy and healthy conidia. SSF has additional advantages compared to submerged fermentation; SSF is more simple and productive, requires lower capital and energy, uses simpler fermentation media, does not require rigorous control of fermentation parameters, uses less water, produces less wastewater, easily controls bacterial contamination, and has cheaper downstream processing (Pandey, 1994).

2.2.5.13.2. Submerged conidia production

Both *B. bassiana* and *M. anisopliae* var *acridum*, but not *M. anisopliae*, will produce submerged or micro cycle conidia under certain liquid fermentation conditions (Kassa *et al*., 2004). These submerged conidia are not hydrophobic, unlike aerial conidia, and thus present different challenges in formulation and use. The micro cycle conidia of *B. bassiana* are produced after 96 h of fermentation only in the presence of inorganic nitrogen, as nitrate, and with very high levels of carbohydrate. Submerged conidia are morphologically different from aerial conidia on an ultra structural level, lacking one layer to their cell walls (Hegedus *et al*., 1990).

A liquid stage in the mass production system encourages rapid mycelial growth of the fungal culture which can then be used to inoculate the second, solid stage in the production process. The advantages of this procedure are that liquid cultures of fungi are fast growing and are therefore the most economical method of production. Where possible, industrial production of fungus is

carried out in liquid culture in large fermentation vessels which have electronic controls and monitoring (Seema *et al.*, 2013). We recommend a liquid culture of blastospores for inoculation of solid substrate. Blastospores are vegetative fungal propagules that are the preferred mode of growth for many entomopathogens in the haemocoel of infected insects (Shimuzu *et al*., 1993; Sieglaff *et al*., 1997). Yeast-like growth allows the fungus better access to the nutrients within the insect. Numerous entomopathogens of the genera *Isaria, Beauveria, Lecanicillium*, and *Metarhizium* can be induced to grow in a ‘‘yeast-like’’ fashion in submerged liquid culture.

The liquid medium used in the first stage of production should contain a supply of carbohydrate (for energy) and nitrogen (in the form of proteins or amino acids or as inorganic nitrogen such as KNO3, from which proteins can be synthesized) which are essential for growth. Any microbiological medium must supply these two components in one form or another. A cheap and effective liquid medium for the mass production of fungi can be prepared using sugar (sucrose) as the carbon source and dried, waste brewers' yeast as the nitrogen source (Seema, *et al*,. 2013).

2.2.5.14. Compatibility of entomopathogenic fungi with pesticides

Chemical insecticides, herbicides and fungicides are usually applied in conventional farming practices. Knowledge about compatibility of biocontrol agents with an array of chemicals in the form of pesticides, fungicides and botanicals in the agro environments is a prerequisite for deployment of the biocontrol agent. Combined utilization of selective insecticides in association with fungal pathogens can increase the efficiency of control by reduction of the amount of applied insecticides, minimizing environmental contamination hazards and pest resistance (Naren Babu *et al*.,2014). Fungal biological control agents and selective insecticide may act synergistically increasing the efficiency of the control, allowing the lower doses of insecticides, preservation of natural enemies and decreasing the likelihood of development of resistance to either agent (Ambethgar, 2009).

There are numerous examples where the application of chemical pesticides has enhanced the efficacy of entomopathogens against insect pests. For example, *P. fumosoroseus* was not effective against greenhouse infestations of the aphids *Aphis gossypii* and *Macrosiphoniella sanborni*, but when applied with azadirachtin (Margosan-O®) efficacy was enhanced and good

control of these aphids was attained, thereby increasing the cost-effectiveness of the chemical control strategy (Lindquist, 1993).

Entomopathogenic Hyphomycetes will not supplant the need for chemical pesticides in all commercial production systems. For example, insecticides may be needed to suppress a rapidly expanding pest population or to control pests not targeted by fungi. Various agrochemicals or pesticides have been worked out and revealed to inhibit or affect on germination of conidia as well as development of mycelia of entomopathogenic fungi depending upon the chemical class (Inglis *et al*., 2001). The inhibition of growth of entomopathogenic fungi by chemicals also vary on class and strains of fungus (Anderson *et al*., 1989). Therefore, it may be possible to select genotypes which are naturally less susceptible. Another approach is to 'genetically engineer' resistance to pesticides.

In this strategy, a gene conferring resistance to a specific pesticide is introduced into a susceptible entomopathogen. Chemical showing compatibility in the laboratory must be selective under field conditions. However, pesticides that are inhibitory in the laboratory do not always exhibit the same action in field environments. This can be a function of pesticide concentration or due to compartmentalization (i.e. the entomopathogen may never come in contact with the pesticide) (Inglis *et al*., 2001). Although pesticides affect growth and germination of mycopathogens however, conidial germination is more severely affected than mycelia growth (Amjad *et al*., 2012). The insecticidal concentration in the media is also main factor to determine toxic effects (Amjad *et al*., 2012). It may be possible, in many cases, to devise strategies that minimize negative interactions. Some companies recommend that antagonistic chemicals be applied 2–3 days before or after applications of their fungus-based products (Faria and Wraight, 2001).

3. MATERIAL AND METHODS

3.1. Study Area Description

The present study was conducted in Welmera Wereda, Oromia National Regional State, central high lands of Ethiopia (Fig. 3). The study forest is located at about 30 km west of Addis Ababa. The forest is known to have gradient of altitude and is situated approximately between $08^0$57'48"- $08^0$57'53" N and $038^0$35'44" - $038^0$35'45"E. The altitudinal range of the study area varies from 2200 - 3000 m above sea level. The annual rainfall in the area is estimated to be around 1225 mm and the mean monthly temperature ranges from 12 to 22.5^{0}C.

3.2. Soil sample collection

Five soil subsample each contained 200g were collected from 5 points a few meters apart by a digging to a depth of 2-10 cm. The five subsamples were mixed (composited) to one plastic gives 1 kg Sample soil (Fresquez, 1990). Thus, a total of ten composite soil samples each containing 1kg were randomly collected from suba forest sites (Fig 3). Geographic Positioning Systems (GPS) was used to locate sampling sites (Table 1). At the laboratory, the samples were passed through a 2 mm mesh sieve breaking soil lump and dried up to a moisture content of approximately 25–30% (which was optimal for fungal growth (Wraight *et al*., 2007). And stored in plastic container at room temperature until processing.

3.3. Collection and rearing of Cabbage aphid (*Brevicoryne brassicae)*

The original cabbage aphid colonies (*Brevicoryne brassicae*) were collected from highly infested leaves of field grown cabbage and mustard crops from Akaki area, Misrak Shoa, Oromia, Region. They were placed in sterile plastic containers (30×15×8 cm L×W×H) provided with small holes for ventilation. Samples were transported to the Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University. The aphids were cultured in the pots planted with Ethiopian mustard 10 cm height in the greenhouse at 25 ± 5°C. The aphids were kept under these conditions for two weeks for acclimatization before beginning the bioassay.

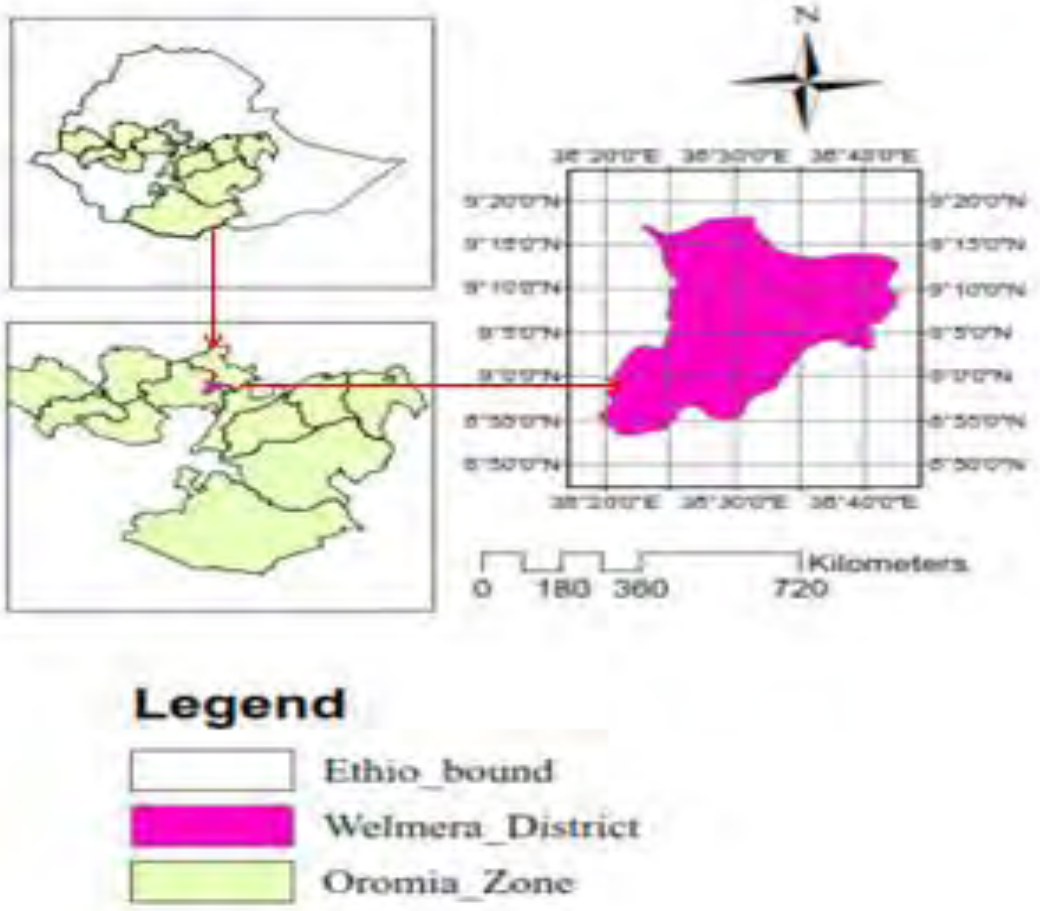

Figure 3- Site of Soil sample collection from suba forest

3.4. Isolation and cultivation of entomopathogenic fungi

Potato dextrose agar (PDA) and oatmeal agar medium (OMA) were prepared in Petri plates according to the specifications of the manufacturer (Gerber Products Co., MFR, MI 49412). In both media chloramphenicol was added at 25 mg/L to inhibit bacterial growth. According to Chase *et al*. (1986) 0.46 g/L of Dodine added to the oatmeal filtrate while stirring. Entomopathogenic fungi from soil samples were isolated using soil dilution plating method (Ingles *et al*., 2012). Soil samples were prepared to appropriate dilutions (10^{-3} -10^{-4}) by first diluting with (1g/9 ml water and 0.01 ml of a solution of Triton X-100). From the dilutions 0.5 ml was inoculated and spreading into oat meal agar plates in triplicates and incubated at 25°C and for 8-10 days. Colonies were purified by repeated sub culturing and those with different

colony size, shape and color were picked with an inoculation loop, and transferred to PDA plates and incubated at 25˚ C for 11 days. Single colonies were then transferred to new PDA plates to establish pure cultures (Stranne, 2014).

3.5. Identification of Entomopathogens

Conidial suspensions of each colony were separately inoculated into PDA, incubated at 25˚ C for 11 days as before. Cultural characteristics (size of colony, texture, pigmentation on the front sides of the plates was recorded. Microscopic slide mounts were prepared according to Steven (1974). The slide cultures were stained with lacto phenol cotton blue (Formula per 100 ml phenol (20.0 g), lactic acid (20.0 ml), glycerol (20.0 ml), cotton blue (0.05 g) and distilled water (20 ml). The slides were mounted under a compound microscope (10x40 magnification) to determine the conidial and mycelia structures of the entomopathogens (Humber, 2012). Standard and known cultures of entomopatogens from the department were also prepared for comparison.

Table 1. The study site GPS coordinates and selected entomopathogens isolated from soil in the different sampling sites in Suba Forest ,Welmera Wereda, Ethiopia

No	Fungal isolates	Latitude(N)	Longitude(E)	Altitude(M)
1.	*Metarhizium*(MEI1)	$08^0$57'53"	038^0 32 ' 44"	2488
2.	*Metarhizium*(MEI2)	$08^0$57'49"	038^0 32 '45"	2500
3.	*Beauveria (BEI1)*	$08^0$57'48"	038^0 32'46.1"	2493
4.	*Beauveria* (BEI2)	$08^0$57'48.8"	$038^0$32 '45.7"	2498

3.6. Laboratory and greenhouse bioassay *Metarhizium anisopliae* and *Beauveria bassiana* isolates against cabbage aphids (*Brevicoryne brassicae*)

3.6.1. Entomopathogenic fungi

All entomopathogenic isolates were maintained on Potato Dextrose Agar (PDA in the Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University.

The PDA medium was used for sub culturing and *in vitro* spore harvesting from the surface of 10-14 days old culture, sealed with parafilm and incubated at 25 ± 2 ºC.

3.6.2. Aphids

To obtain different developmental stages of *Brevicoryne brassicae* adult aphids were inoculated onto fresh leaf discs of mustard leaf in Green house and allowed to reproducing parthenogenetically. Rearing was carried out to ensure different developmental stages were obtained at the same time so that treatments could be performed concomitantly.

3.7. Spore concentrations of the fungal isolates

Dry conidia were harvested from 2–3 week old surface cultures of entomopathogenic fungi by scraping with a sterilized glass rod of which 10 and 30 g, respectively were suspended in approximately 100 and 300 ml sterilized distilled water (DH2O) supplemented with 0.01 % Triton X-100 in sterile 500 ml Erlenmeyer flasks and were sealed and kept on 140 rpm shaker to produce a homogenous suspension for the laboratory and greenhouse bioassay, respectively.

The conidia suspension was filtered through sterile cheesecloth to remove mycelium. Conidial suspensions were first adjusted to 1×10^8 conidia/ ml using Neubauer haemocytometer (Ingles *et al*., 2012) under a compound microscope (400x magnifications) and the subsequent doses were prepared from this for bioassay experiments.

3.8. Conidia Viability Tests

Conidial viability was assessed according to Inglis *et al*. (2012) by spread-plating 0.1 ml of spore suspension (1×10^7 spores/ml) on Potato-Dextrose Agar (PDA) plates in triplicates. The plates were sealed with Parafilm membrane and incubated at 25°C under complete darkness for 18 h. Lacto-phenol Cotton blue was then added to terminate germination and stain the spores for enumeration of germinated/non-germinated spores. One sterile microscope glass cover slip was then placed on the surface of the 18 h old culture in each plate. Percentage germination was determined by counting 100 spores, categorized as germinated and non-germinated, under each cover slip on each Petri plate under the light microscope (400 x magnifications). A conidium was considered to have germinated if the germ-tube was at least twice the size of the spore (Ingles *et al*., 2012)

3.9. *In vitro* compatibility of entomopathogenic isolates with pesticides

3.9.1. Pesticide for compatibility test with entomopathogenic fungi

The insecticides chosen for the compatibility bioassay with entomopathogenic isolates were the ones that are frequently used by vegetable farmers and described in Table 2. The test concentrations were according to the field rate recommendations of the manufacturers instruction (Dow agro sciences, 2008; Syngenta E.A.Ltd ; Syngenta Crop protection AG). The field recommendations rate (FR), 0.75FR and 0.5×FR (FR-50%) .The concentration tested for each product was calculated based on the spray volume rate of 100 L ha^{-1} of water. These values were chosen since they cover all concentrations recommended for the tested products, providing information that could be used in field compatibility tests.

Table 2. Insecticides for the compatibility tests with *entomopathogenic* isolates based on the spray volume rate of 100 L ha^{-1} of water on *Brassica* plants

Tread name	Formulation	Chemical group	Technical name	Concentration of active ingredient	Recommended Dose
Insecticide					
Actara,Syngenta	25WDG	Neonicotinoid	Thiamethoxam	250 g/kg WG	20 g/ha
Karate	5EC	Pyrethroid	L-cyhalothrin	50 g/l	50 ml/ha
Radiant®	12Sc	Spinosyn	Spinetoram	120g/l	100 ml/ha

Formulation types :- Emulsifiable concentrate (EC), suspension concentrate (SC), water dispersible granules (WDG), Volume of application = 100 L of water per hectare.

3.9.2. Effect of Pesticides on Conidial germination

To determine the effects of insecticides on conidial germination, conidia were harvested from 14-day-old cultures on PDA by scraping the surface with a sterile scalpel and flooding the plates with sterile water containing 0.01% Triton x-100 (Gurulingappa, *et al*., 2011). The conidial suspension was filtered to remove the hyphal debris. The conidial concentration was adjusted as before i.e. 1 x 10^8 conidia/ml with sterile water. A suspension of 1 ml of 1 x 10^8 conidia/ml was added to 9 ml of each of the 3 replicates of the 3 insecticide sufficient to make a final volume of 10 mL per concentration (FR, 0.75FR and 0.5FR). For the control treatment, 1 ml of 1 x 10^8

conidia/ml was added to 9 ml of sterile water. Then, 100 µl of each suspension was inoculated to the surface of a fresh plate of PDA. The inoculated plates were incubated at 25°C for 24 h. The germination was assessed by placing cover slips on to the media inside the plates and examined under the microscope at 40X magnification. Conidia having germ tubes greater than their width were considered to be germinated (Inglis *et al.,* 2012). Germ-tube was at least twice the size of the spore .The percent spore germination was recorded using formula given by Kiraly *et al.* (1974) cited by Dar *et al*. (2013).

$$\text{Percent spore germination} = \frac{\text{Number of spores germinated}}{\text{Total number of spores examined}} \times 100$$

3.10. *In-vitro* bioassay with fungal isolates against nymphs and adult cabbage Aphids (*Brevicoryne brassicae*)

To test the efficacy of each of the fungi isolates on nymphs and adult aphids, fresh leaves of *Brassica carniata* were surface sterilized with 5% Sodium hypochlorite for 30 seconds and then rinsed with sterilized water two times. The treated leaves were dried to remove excess moisture and placed on Petri plate lined with sterile and moist whatman filter papers. Every two days, the old leaves were replaced with fresh leaves of *Brassica carniata*. Collected nymphs and adult aphids from the non-experimental *Brassica* plants from greenhouse brought to the laboratory with their host leaf.

The experiment was run under CRD (Completely Randomized Design) with five treatment, (four treatment with two conidial concentrations (1×10^7 and 1×10^8 conidia/ml) each for both nymph and adult aphids were prepared form BEI1,BEI2, MEI1 and MEI2 and 0.01 per cent X-T 100 solution served as control). Each concentration was replicated three times. In two separate experiments, spore suspensions of Beauveria bassiana isolate BEI1 and 2 and Metarhizium anisopliae isolate MEI1 and 2 (produced in solid medium) were sprayed on adult and nymph aphids. Approximately 6 adults and nymphs of aphid were placed into each glass Petri plate in three replicates for each treatment including the control. A total of 108 adult and nymph aphids were used in each treatment including the controls.

The sprayer was calibrated so that 900 µl of each concentration was used for 3 replicates (18 insects). Thus each insect was directly and individually sprayed with about 50 µl of the appropriate concentration. At first, the 0.01 per cent X-T 100 solution (control) treatment was applied and then the other treatments working from low concentration to high. The number of live and dead aphid adults and nymphs were assessed after 24, 48, 72, 96 , 120 and 144 hours of incubation. To determine mortality, individuals were gently prodded with a single bristle paint brush. An aphid that did not respond immediately was considered as dead (Kivett, 2015). The dead aphids which produced mycelial growth were considered for the mortality count. Neonate aphids were counted and removed daily from the seedlings.

3.11. Experiments under greenhouse Conditions:

3.11.1. Experimental design and Treatment

Ten treatments (Table 3) with three replicates were laid-out in Completely Randomized block Design (CRBD) in the greenhouse by including 8 treatments and two controls (the untreated and the standard controls, viz., sterile water (X-T 100 solution) and FR karate, respectively. Each fungal isolate was tested at one concentrations of 1×10^8 conidia/ml. The four treatments were combined with half recommended rate (0.5FR) of karate and fungal isolates. There were three benches (blocks) each measuring 130, 150 and 50 cm (L,H,W). The two blocks each divided into 4 plots, where 4 treatment combinations and the third one was divided in to two plots with 2 treatment combinations and 10 unit plots altogether.

Table 3. Experiment design and number of Treatment

Treatment No	Isolates	Crop	Insect
1	BEI1	*Brassica carniata*	Adult and Nymph Aphid
2	BEI2		
3	MEI1		
4	MEI2		
5	BEI1 + 0.5FR Karate		
6	BEI2 + 0.5FR Karate		
7	MEI1+ 0.5FR Karate		
8	MEI2+ 0.5FR Karate		
9	FR Karate(+ve control)		
10	X-T 100 solution(-ve control		

NB: Sample codes:- BEI1,BEI2,MEI1 ,MEI2 and 0.5FR impliy *Beauveria* isolate1,*Beauveria* isolate2, *Metarhizium* isolate1, *Metarhizium* isolate 2 and half field rate

3.11.2. Ethiopian mustard (*Brassica carinata* A. Braun) Production for greenhouse experiment

Brassica carinata A. Braun seeds were collected from market. They were in plastic pots (26 x18 x 24 cm height, width and diameter, respectively) containing a soil mixture of loam and sand (2: 1) in a greenhouse. Five seeds were planted per pot and were subsequently thinned to two plants per pot after five days from plant emergence. They were watered three times a week. The plants were kept in the greenhouse outside of the cage until it reached sufficient height (40 cm tall) or until the experiment started.

3.11.3. Bioassay with fungal isolates against nymphs and adult Aphids on potted plants

Each replication consisted of a pot with two plants. Pots (40 cm diameter) which were placed inside large cages at 25 ± 2 °C, while the water was supplied to the plants three times a week through the zip hole at the front of cages. Each plant was artificially infested with adult and nymphs of aphids (inoculums) from non-experimental infested plants in a greenhouse into cages. Thirty (30)

healthy adults and nymphs aphids each per seedling. Totally, 180 adults and nymphs aphids were used for each treatment. Then adult and nymphs of aphids were seeded on each plants for two days before the first conidial concentration application was made (Kivett, 2015). Both treatment and non-treatment leaves within plants were counted to determine if aphids migrated away from the original sites.

Then conidial concentration at the inoculum size of 10^8 conidia/ml(100ml/pot were sprayed on the nymphs and adult aphids seeded in each plant in a closed net cage and aphids sprayed with 0.01 per cent X-T 100 solution served as control. There are 3 replications. The spray was performed during late evening time. Thus, Mortality of aphids was recorded separately at 3^{rd}, 5^{th}, 7^{th} 9^{th} and 11^{th} of days. The replication mean was considered as the average of the aphids counted from 6 plants per treatment.

3.11.4. Bioassay of Combined effect of Fungal Isolates and insecticides -against nymphs and adult Aphids on potted plants

The experiment was run under five treatments against control treatment [T1- BEI 1+ 0.5 FR karate; T2-*BEI 2 + 0.5FR karate*; T3-MEI 1+ 0.5FR karate ; T4- MEI 2 + 0.5 FR karate; T5- FR karate alone ; T6 - Untreated check or 0.01 % X-T 100] treatment groups. The formulations of the karate were diluted with tap water based on the spray volume rate of 100 L ha^{-1} (Silva *et al*., 2013).

The combination treatments were done in a sequential treatments approach, in which karate followed by fungal pathogen. The concentrations employed were based on their field recommendations rate (FR) and 0.5×FR and one respective concentrations of all the fungal isolates (1×10^8 conidia/ml) were prepared for both nymphs and adult stages of aphids (Malekan *et al*., 2012). After two days inoculation the leaves on the plants sprayed with the half field recommendations rate of the chemical spray sequentially with fungal concentration while the full field recommendations rate (positive control as standard check) spray alone without fungal concentration from a 20 cm distances by hand sprayer with fine nozzle. The fungial suspensions were sprayed on the insect-infested plants one days after exposure to half field recommendations rate of the chemical karate, 50 ml each to ensure thoroughly wetting the upper and lower surface of the leaves. Aphids sprayed with 0.01 per cent X-T 100 solution served as negative control.

To prevent carryover effect among isolates, the potter tower was cleaned with 70% ethanol and sterile distilled water between spraying sessions. Mortality of aphids was recorded separately at 3^{rd}, 5^{th}, 7^{th} , 9^{th} and 11^{th} of days.

3.12. Confirmation of the fungal infection in Aphids

Dead insects were taken out of the cages daily. Cadavers are incubated in a high moisture environment like whatman moistened filter paper and, if the cadavers are subsequently colonized by the fungal, these insects are considered to have died from mycosis. Retrieved mycelia were cultured in PDA and were identified . Insects that did not produce mycelia were recorded as other mortality (Inglis *et al.,* 2012). Mortality until day 6 and 11 of the experiment was analyzed in laboratory and greenhouse, respectively).

3.13. DATA ANALYSIS

One-way analysis of variance (ANOVA) was conducted on the mortality data to test the level of significance of the difference in response between the treatments and comparison of means. Multiple comparisons were used to determine significant differences between the treatment (Tukey test). values were computed by using statistical computer program of Statistical Package of Social Sciences (SPSS version 20 software). Percent growth inhibition of each fungal isolate over untreated check was worked out for the tested pesticides and ANOVA was used to analyze germination measurements followed by comparison of means of total growth using the statistical program SAS version 9.3. Mean separation was calculated using the LSD test value when the F-test was significant at $p = 0.05\%$.

4. RESULTS

4.1. Isolation of entomopathogenic fungi from soil

Based on their morphological and cultural characteristics, the four isolates were identified into *Metarhzium* spp (species) MEI1 and MEI2 and *Beauveria* spp BEI1 and BEI2 (Table 4). Accordingly, the colony characteristics of *Beauveria* spp. showed white to cream color, flat powdery and cotton-like appearance on the front side of the PDA medium (Figure 5) whereas the cultural characteristics of *Metarhizium* spp on the front side of PDA showed dark herbage green or yellowish green, olivaceous colonies after 7 days of incubation (Fig. 6). The MEI1 colonies displayed much lighter green colonies than MEI2. The conidia of *Beauveria* were small, round and varied in width and length between isolates (Fig.7). The size of the conidia of isolates of BEI1 and BEI2 was in the range from Length x Width of 6- 8.5 x 7.5-9 and 6.5-7 x 5.5-8.5 µm in diameter, respectively (Table 4). Likewise, the conidia of *Metarhzium* spp were cylindrical in shape (Fig. 8). Isolates MEI1 and MEI2 showed conidial sizes Length x width of 7.5 - 16.5 x 4.5-8.5 and 6.5- 15.3 x 5.4- 9.5 µm, respectively (Table 4).

The conidial viability test of the isolates was in the range of 97.2-100% without showing any significant difference among the isolates. As a result, *Beauveria* isolates of BEI1 and BEI2 showed germination rate of 100 % and 99.2% whereas *Metarhizium* isolates MEI1 and MEI2 showed germination rate of 98.% and 97% respectively.

Table- 4. **Macroscopic and microscopic characters of *B. bassiana and M. anisopliae* isolates**

Isolates	Surface colour	Spore shape	Spore size
BEI1	White	round	6 - 8.5 x 7.5- 9 µm
BEI2	White	round	6.5-7 x 5.5 -8.5 µm
MEI1	Greenish	Cylindrical	7.5-16.5 x 4.5-8.5 µm
MEI2	Greenish	Cylindrical	6.5-15.3 x 5.4-9.5 µm

Plate 4: Isolates of *Beauveria* on PDA medium grow at 25°C for 14-21- days

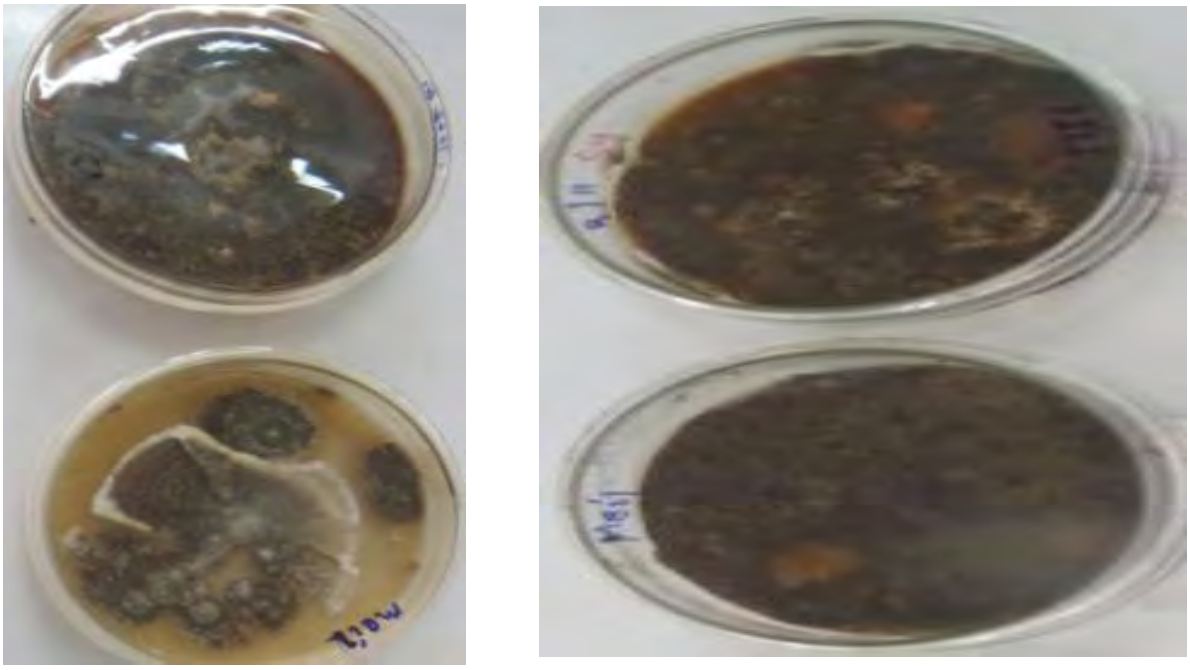

plate 5: Isolates of *Metarhizum* on PDA medium grow at 25°C for 14-21days

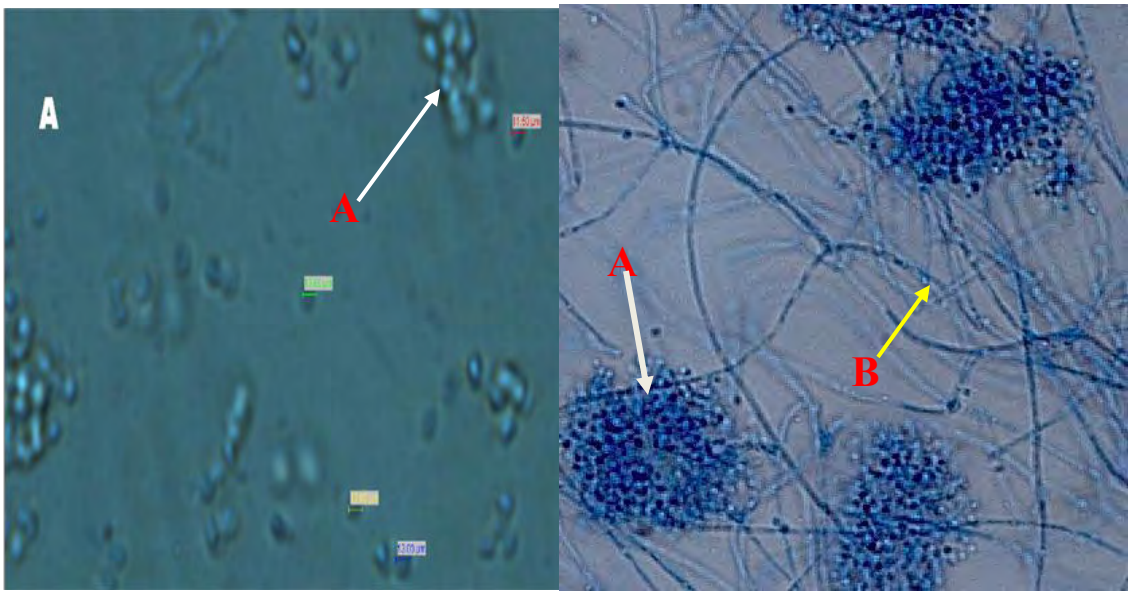

Figure 6. (A) Spore and (B) Mycelial structures of *Beauveria* isolates observed at 400 X of compound microscope

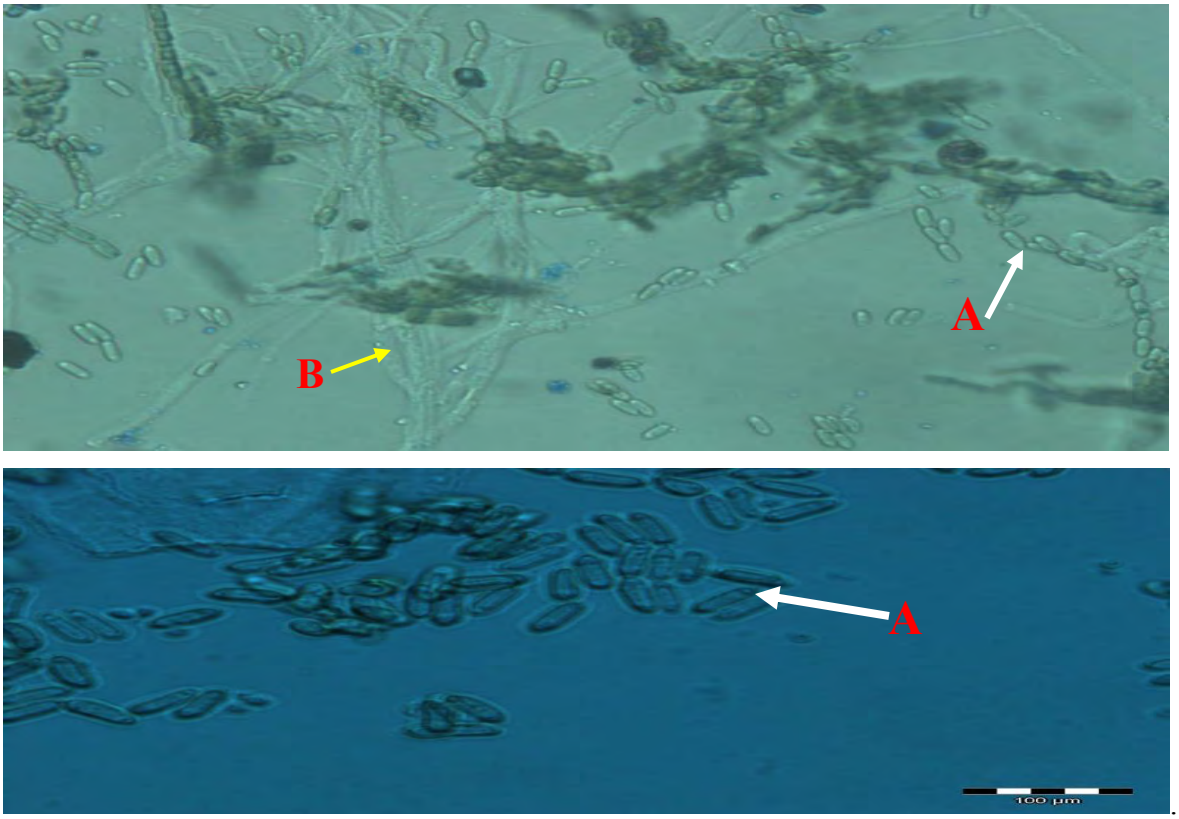

Figure 7 : (A) Spore and (B) Mycelial structures of *Metarhizium* isolates observed at 400 X of compound microscope

4.2. Percent mortality of adult Aphids treated with *Beauveria* and *Metarhizium* spp. isolates under Laboratory Conditions

The dose-dependent antagonistic effect of the four isolates; *Metarhizium* and *Beauveria*, showed no substantial mortality on adult aphids at a dose of 1 x 10^8 for the first two days (Table 5). However, at day 3 of treatment, a significant percent mortality of the adult aphids was caused by isolate BEI1 (38%) followed by MEI1 (27%), BEI1 (16.6%) and MEI2 (16.6%), without any significant mortality difference except BEI1 (at $p<0.008$) (Table 5). On day 4, infection rates of adult aphids (mortality rate) increased by 66.6 % by isolate BEI1 followed by isolates MEI1 (50%), BEI2 (44.45 %) and MEI2 (44.45%), respectively compared with negative controls (Table 5).

Generally, as time progressed, (on day 5) the percentage mortality of the aphids treated with BEI1 increased by 100%, whereas treating of the aphids with isolates MEI1, BEI1 and MEI2 caused mortality rates of 83%, 77 %, and 77%, respectively, and all of them achieved 94% mortality after the 6^{th} day of treatment**.** In general, the data showed that isolate BEI1 was superior to others and two of the isolates BEI2 and MEI2 were at par with each other at the treatment concentration of 10^8 spore/ml at day 5 whereas BEI2, MEI1 and MEI2 were similarly effective on day 6 (Table 5).

There was no mortality of adult aphids within 24 h, at a concentration of 10^7spores ml^{-1}. However, isolates BEI1, BEI2, and MEI1 showed mortality with a rate of 33.34%, 27%, and 39, % respectively after 4 days of fungal application (Table 5). Similarly, on day 5 there was a significant ($p= 0.001$) morality record with treatments of isolates BEI1, MEI1, BEI2 and MEI2 with percentage death of (72%, 61%, 61 % and 55%) respectively compared to the negative control. The 94% mortality rate of adult aphids reached was achieved with isolate BEI1 followed by isolates *BEI1 and MEI2* (83.3%) and MEI1 (77 %) after 6 days of incubation (Table 5).. The data in general, showed similar pattern of mortality at both concentrations without significant difference on the two treatment doses (Table 5).

Table.5. Dose dependent percentage mortality of adult aphids treated with different entomopathogenic fungal isolates

Isolates	Treatment	Day3	Day4	Day5	Day6
	Conidia ml-1	M (%) ± SE	M (%) ± SE	M (%) ± SE	M (%) ± SE
BEI1	10^7	16.7±9.62^{ab}	33.3±9.62^{ab}	72.2±5.56^{ab}	94.5±5.55^{a}
	10^8	38.9±5.55^{a}	66.7±0.00^{a}	100±0.00 ^{a}	-
BEI2	10^7	11.1±5.55^{ab}	27.8±11.11^{ab}	61.3±5.55^{ab}	83.3±9.6^{a}
	10^8	16.7±0.00^{ab}	44.5±5.55^{ab}	77.8±5.58^{ab}	94.5±5.55^{a}
MEI1	10^7	22.2±5.55^{ab}	38.9±5.55^{ab}	61.1±5.55^{ab}	77.8±11.11^{a}
	10^8	27.8±11.11^{ab}	50.0±9.62^{ab}	83.4±0.00^{ab}	94.5±5.55^{a}
MEI2	10^7	5.6±5.55^{bc}	16.7± 9.62^{bc}	55.6±11.11^{bc}	83.3±9.62^{a}
	10^8	16.7± 9.62^{ba}	44.5±14.69^{ab}	77.0±5.56^{ba}	94.5±5.55^{a}
Control	None	0±0.00^{bc}	0±0.00^{cd}	0±0.00^{d}	5.6±5.55^{b}
P value		0.008	0.001	0.001	0.001

Means followed by the same letter with in a column are not significantly different (Tukey's HSD test, $p< 0.05\%$)

Legend: M (%) = Percentage mortality

4.3. Percentage mortality of nymph aphids treated with *Beauveria* spp. and *Metarhizium* spp. isolates at different concentrations

Percentage mortality of nymphal aphids due to the treatment of different isolates at different concentrations was significantly different (P=0.0001) over time (Table 6).There was no significant increase in the death of nymphal aphids upon exposure for the first three days except a 27.8% mortality with treatment MEI1 at a concentration of 1×10^8conidia/ml followed by 16.7 % mortality by isolates BEI1 and BEI2. Later increased to BEI1 (77.8%) and BEI2 (72.2 %), MEI1 (66.7%) and MEI2 (55.6 %) after 5 days of incubation (Table 6). On the 6th day after exposure, BEI1 and BEI2 achieved 100% followed by 83.33% mortality rate by isolates MEI1 and MEI2, respectively. Generally, the mortality trend against nymphal aphids showed very slow mortality until day 4 with a substantial increase in nymph mortality between 4-6 days.

The treatment of nymphal aphids with a dose of 10^7 spores/ml on day 3 was less and non significant (P= 0.031) percent mortality of the nymphal aphids by isolate BEI1,(11%), BEI2 and MEI1 (16%). However, after 4 days of incubation there was significant (P= 0.009) mortality with death rate of 38.9% with isolate BEI1 followed by isolates, BEI2 and MEI2 with a morality rate of 27.8%. Similarly, on a day 5, significant (P= 0.0001) morality was recorded by all the isolates compared to the negative control(Table 6). The death rate reached 88.9%, by BEI1 and BEI2 and 72.2 % and 66.7% with treatments of MEI1 and MEI2, respectively after 6 days of exposure (Table 6).

Table. 6. Dose dependent percentage mortality of Nymph aphids treated with different entomopathogenic fungal isolates

Isolates	**Treatment**	**Day3**	**Day4**	**Day5**	**Day6**
	Conidia ml-1	M (%) ± SE	M (%) ± SE	M (%) ± SE	M (%) ± SE
BEI1	10^7	11.1 ± 5.55^{ab}	38.9 ± 5.55^{a}	77.8 ± 11.11^{a}	88.9 ± 11.11^{ab}
	10^8	16.7 ± 9.62^{ab}	38.9 ± 5.55^{a}	72.2 ± 5.55^{ab}	100.0 ± 0.00^{a}
BEI2	10^7	16.7 ± 9.62^{ab}	27.8 ± 11.11^{ab}	55.6 ± 5.55^{ab}	88.9 ± 11.11^{ab}
	10^8	16.7 ± 9.62^{ab}	38.9 ± 11.11^{a}	72.2 ± 11.11^{ab}	100.0 ± 0.00^{a}
MEI1	10^7	16.7 ± 0.00^{ab}	27.8 ± 5.55^{ab}	55.6 ± 5.55^{ab}	72.2 ± 5.56^{ab}
	10^8	27.8 ± 5.55^{a}	38.9 ± 5.55^{a}	66.7 ± 0.00^{ab}	83.3 ± 9.62^{ab}
MEI2	10^7	0 ± 0.00^{bc}	5.6 ± 5.55^{bc}	38.9 ± 5.55^{bc}	66.7 ± 0.00^{ab}
	10^8	5.6 ± 5.55^{bc}	22.2 ± 14.70^{ab}	38.9 ± 5.55^{bc}	83.3 ± 9.62^{ab}
Control	None	0 ± 0.00^{bc}	0 ± 0.00^{bc}	0 ± 0.00^{d}	0 ± 0.00^{c}
P value		0.031	0.009	0.0001	0.0001

Means followed by the same letter with in a column are not significantly different (Tukey's HSD test, $p< 0.05\%$)

Legend- M (%) = Percentage mortality

4.4 . Compatibility test of insecticides with entomopathogenic fungal isolates

All the insecticides caused different level of inhibition on conidial germination of *Beauveria* spp. and *Metarihizium* spp. (Table 7). All the fungal isolates showed variations in resistance at their

recommended doses FR, 0.5×FR and 0.75×FR) of insecticides after 24 hours of incubation. Consequently, all the insecticides suppressed germination of conidia except the insecticide Radiant which did not affect the germination rate of BEI1 and BEI2 with 98% and 92% germination rates, respectively. But all the fungal isolates were relatively sensitive to Karate and Actara insecticide at RR with germination rates of 68 - 79 % and 71- 82 % respectively.

Similarly, the *Metarhizium* isolates also showed germination rates with karate FR (75, 70%), 0.75FR (85%, 73%) and 0.5FR (85%, 78%) for MEI1 and MEI2, respectively. In the case of Actara the BEI1 and BEI2 isolates, did not show significant (LSD=9.5) difference at the smallest concentrations compared to the control (Table 7). However, the *Metarhizium* isolates showed significant difference in germination rate at all concentrations. For BEI1, Actara (FR) showed a significant reduction in germination compare to the two lowest concentrations. Similarly, in the MEI2 the RR showed significant reduction compared to the lower concentrations and the control (LSD= 9.5), while for MEI1, Actara treatment did not show significant difference among the three concentrations but were significantly different from the untreated control (LSD=9.5) (Table 7).

For *Beauveria* spp. the insecticide Radiant did not show significant difference in suppressing their germination at the three concentrations (0.5×FR, 0.75×FR and FR) compared to the other insecticide but FR showed significant inhibition on the isolates from the control treatment. Generally, the isolates of BEI1, BEI2 and MEI1 were resistant to radiant to the least concentration (0.5FR) compared to the untreated control (LSD= 10.2) (Table 7).

Table.7. Effect of pesticides (compatibility test) at different concentration on conidial germination of entomopathogens

Fungal isolates	Pesticide concentration	Karte	Actara	Radiant
		G (%)	G(%)	G(%)
BEI1	0.5FR	86^{bc}	92^{abc}	98^{abc}
	0.75FR	77^{cde}	88^{bcd}	94^{abcd}
	FR	68e	74^{ef}	89^{bcdef}
BEI2	0.5FR	84^{cd}	84^{abc}	92abcde
	0.75FR	77^{cde}	79^{def}	86cdef
	FR	79^{cde}	77^{def}	85def
MEI1	0.5FR	85^{bc}	87^{bcd}	91abcde
	0.75FR	85^{bc}	82^{cde}	85def
	FR	75^{cde}	79^{def}	81^{f}
MEI2	0.5FR	78^{cde}	84^{cde}	87cdef
	0.75FR	73de	78^{cde}	85^{ef}
	FR	70^{e}	71^{f}	84def
Control	None	100^{a}	100^{a}	100^{a}
LSD		11.1	9.5	10.2

All the means within a column followed by the same letter are not significantly different at (p=0.05)

Legend - FR, 0.75FR and 0.5FR implies field rate, three- forth field rate and half field rates and

LSD- Least significant difference

G (%) - Percentage germination

Similarly Radiant formulation for *B. bassiana isolate BEI1* caused the least inhibition of conidia germination (98%) , with the lowest concentrations (0.5 × FR), followed by Actara formulation at the concentrations of 0.5 x FR (92%) for both BEI1 and BEI2 isolates (Table **7**). The formulations with Karate, Actara and Radiant induced various levels of germination inhibition at all concentrations ranging from 98 % BEI1 (radiant at half FR) to 68 % BEI1 (Karate at FR).

4.5 . Experiments under Greenhouse Conditions:

4.5.1. The effect of single and combined effects of entomopathogenic fungi with insecticide on nymph stages of Aphids on potted plants

Percentage mortality of the nymph aphids treated with the antagonistic fungi alone and selected insecticides showed significant (P= 0.001) difference among the treatments (Table 8) The data showed that maximum mortality rate (46%) was recorded from standard chemical (full concentration FR Karate) after 3 days of exposure compared to the un-inoculated negative control. Combined treatment of half FR concentration of karate together with fungal isolates showed mortality rate of (30.0%, 27 %, 25% and 21%) with treatment of BEI1 + 0.5FRkarate, BEI2 + 0.5FRkarate, MEI1+ 0.5 FR karate and MEI2 + 0.5FRkarte, respectively without showing significant difference among the treatments (Table 8).

After 5 days of treatment, however, the combined treatments with BEI1+ 0.5FRkarate, BEI2 + 0.5 FR karate, MEI1+ 0.5FRkarate and MEI2 + 0.5 FR karate increased the mortality of aphid nymphs by 64.4%, 61 %, 57% and 51%, respectively. The IPM treatments showed lower mortality than mortality (92%) recorded from the FR insecticide treatment, but better than the mortality rate (7%-23%) treated with the bio-control fungi alone. However, maximum mortality rates of 81% and 95% were attained by all combined IPM treatments comparable to the chemical (FR) treatment (100%) after 7 days of exposure. In general, combined IPM treatments achieved 90-100% mortality of aphids at their nymphal stage after 9 days of exposure (Table 8), where single inoculation with individual entomopahthogenic fungi achieved high mortality rate (73% - 94%) after 11 days of treatment (Table 8).

Table. 8. The combined and singular efficacy of 4 isolates of fungi at 1× 10^8 conidia/ml and insecticides at 50% field recommended doses against nymphs of aphids on potted Ethiopian mustered plants.

Isolate	Treatment	Day 3	Day 5	Day 7	Day 9	Day 11
	Conidia ml^{-1}	M(%)±SE	M (%) ± SE	M (%) ±SE	M (%) ± SE	M (%) ± SE
BEI1	10^8	10±0.55^{c}	23.3±0.96^{d}	41±3.64^{d}	59.4±13.06^{b}	93.9±0.55^{a}
BEI2	10^8	6.7±0.96^{c}	18.9±2.22^{d}	35±4.19^{d}	62.8±2.00^{b}	88.2±0.96^{bc}
MEI1	10^8	5.6±0.55^{c}	14±2.93^{d}	27±1.46^{de}	50±4.19^{b}	84.4±2.78^{cd}
MEI2	10^8	1±0.55^{cd}	7±1.46^{de}	16.7±0.96^{ef}	33.9±3.09^{bc}	72.8±3.38^{e}
BEI1 + Ka	10^8+0.5FR Ka	30±0.96^{b}	64±3.37^{b}	95±2.54^{a}	95.5±2.42^{a}	100±0.00^{a}
BEI2 + Ka	10^8 + 0.5FR Ka	27±0.55^{b}	60.6±1.56^{b}	91.8±2.61^{ab}	95.5±2.94^{a}	100±0.00^{a}
MEI1 + Ka	10^8 + 0.5FR Ka	25±2.88^{b}	56.7±3.46^{b}	86.7±2.53^{ab}	97.8±1.46^{a}	100±0.00^{a}
MEI2 + Ka	10^8 + 0.5FR Ka	21±4.004^{b}	50.8±4.84^{bc}	80.5±4.34^{bc}	97.8±0.55^{a}	100±0.00^{a}
+ve control	FRKa	45±3.37^{a}	92±1.47^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
-ve control	None	0±0.00^{cd}	0±0.00^{de}	0±0.00^{g}	0±0.00^{d}	0±0.00^{f}
P value		0.0001	0.0001	0.0001	0.0001	0.0001

Means followed by the same letter with in a column are not significantly different (Tukey's HSD test, $p< 0.05\%$)

Legend- M (%) = Percentage mortality, FRKa = Field rate karate

4.5.2. The effect of single and combined inoculation of entomopathogenic fungi with insecticide on adult stages of Aphids on potted plants

The antagonistic effect of fungal isolates on adult aphids alone, or in combination with selected insecticides was tested under greenhouse conditions (Table 9). Accordingly, the mortality rate of adult aphids with entomopathogenic fungi was not significant in the first 3 days of treatment. However, the combined treatment increased the mortality by BEI1 + 0.5FRka (74.4%), BEI2 + 0.5FRka (71.1%), MEI1+ 0.5FRka (64.4%) and MEI2+ 0.5FRka (62.8%) after 5 days of treatment compared to mortality of 99.4% with the treatment of standard chemical (FR concentration karate) treatment. Single inoculation with different entomopathogens alone showed mortality rate between 10-30% till 5 days of exposure.

After 7 days of exposure, the IPM treatments showed mortality rates ranging from 91.6% with treatment MEI2 +0.5FRka to 100% mortality with BEI1+ 0.5FRka (Table 9) comparable with standard chemical (karate) treatment FR(100). The single inoculation with fungal isolates; BEI1, BEI2, MEI1 and MEI2 showed mortality rate of 49.2%, 41.1%, 33.3%, and 22.2%, respectively. In general, there was a trend in highest percentage mortality rate of adult aphids (80-100%) with individual entomopathogen and IPM treatments after 11 days of exposure (Table 9) which is comparable to the standard check treatments.

Table. 9. The combined and singular efficacy of 4 isolates of fungi at 1× 10^8 conidia/ml and insecticides at 50% field recommended doses against adult of aphids on potted Ethiopian mustered plants.

Isolate	Treatment	Day 3	Day 5	Day 7	Day 9	Day 11
	Conidia ml^{-1}	M(%)±SE	M (%)±SE	M (%) ±SE	M (%) ± SE	M (%) ± SE
BEI1	10^8	11±1.11^{c}	29±1.46^{d}	49±1.44^{b}	81±1.15^{b}	97.5±0.48^{a}
BEI2	10^8	9±1.46^{cd}	23±0.02^{d}	41±1.12^{b}	69±2.95^{c}	92.2±2.01^{b}
MEI1	10^8	7.8±0.55^{cd}	19±2.00^{ed}	33±0.96^{bc}	57.8±3.09^{d}	85±1.53^{c}
MEI2	10^8	2.8±1.46^{cd}	10±1.92^{ef}	22±2.77^{d}	43±2.88^{e}	80±0.96^{d}
BEI1 + Ka	10^8+0.5FR Ka	36±1.66^{b}	74±3.37^{b}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
BEI2 + Ka	10^8 + 0.5FR Ka	35±1.66^{b}	71±2.00^{b}	97.8±0.55^{a}	100±0.00^{a}	100±0.00^{a}
MEI1 + Ka	10^8 + 0.5FR Ka	29±2.93^{b}	64±2.00^{bc}	93.9±1.11^{ab}	100±0.00^{a}	100±0.00^{a}
MEI2 + Ka	10^8 + 0.5FR Ka	28±2.34^{b}	62.8±3.88^{bc}	91.6±4.38^{ab}	99.9±0.03^{a}	100±0.00^{a}
+ve control	FR Ka	52.8±3.38^{a}	100±0.55^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
-ve control	None	0±0.00^{d}	0±0.00^{gf}	0±0.00^{e}	0±0.00^{f}	1.1±0.55^{e}
P value		0.0001	0.0001	0.0001	0.0001	0.0001

Means followed by the same letter with in a column are not significantly different (Tukey's HSD test, $p< 0.05\%$)

Legend- M (%) = Percentage mortality FRKa = Field rate karate

4.6 . Confirmation of the fungal infection in Aphids

Microscopic observations of aphids treated with entomopathogenic fungi isolates revealed that the insects had picked up the spores. Sporulation was observed usually 24 to 48 hours after death. Dead insects were covered with white and green powdery mass of spore on the external surface covering the insect body when the cadavers were placed in Petri dishes and compared with the original respective cultural characteristics of the different isolates and authenticated as original enthomopathogens.

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5. DISCUSSION

The present study shows the effectiveness of four entomopathogenic local fungal isolates; BEI1, BEI2, MEI1 and MEI2 from the two groups *Beauveria* spp. and *Metarhizium* spp. against aphids attacking Ethiopian mustard (*Brassica*) under both laboratory and greenhouse conditions. The study showed the isolates were effective against aphids at different concentrations and time of exposure (Table 5).The mortality was generally low on day 1-2 after treatment in all fungal isolates, and increased gradually with maximum mortality within 6 days of exposure. There was a slight, but not significant difference, in the overall virulence of the isolates, except isolate BEI1.

The study showed inoculation with $1x10^8$ conidia ml-1 of *Beauveria* spp. BEI1was the most effective with mortality rate of adult aphids ranging from 38.9% at the 3rd day of exposure up to 100% mortality after 5 days of inoculation (Table 5). However, the rest of the isolates achieved the same mortality rate of 94.5% after 6 days of inoculation ($p< 0.001$). Similarly Akmal *et al* .(2013) showed *B. bassiana* isolate BB-01 showed maximum mortality of (90%) on *Schizaphis graminum* and *(*100%) of brassica aphid on 6 and 7th days, respectively. The pattern of inhibition of the adult aphids by the same inoculums of local isolates after 6-days of exposure was similar with 87-97% and 52-81% mortality rates of adult cowpea aphids treated with best isolates of *B. bassania* and *Metarhizium anisopilae* from India (Saranya *et al.,* 2010). The effect of the local isolates of M. *anisopilae* on the mortality of cabbage aphids was better than the effect of the mortality rate of 20-62% of the same type of aphids inoculated with $1x10^7$ conidia ml-1 within 6 days (Asi *et al*., 2009).

On the contrary, the isolates in this study were not as effective as the isolates of *B. bassiana* PDRL 1187 and *M. anisopilae* PDRL526 from Pakistan that showed mortality rate of 88.2% and 72.6% on mustard aphids inoculated with $1 \times 10^7$with in 3 days of exposure under laboratory conditions respectively (Ujjan and Shahzad, 2012). Another isolate of *Beauveria* from Iran effectively controlled (75-100% mortality) rose adult aphids with treatments of 10^4-10^8 spores /ml within 4-5 days of exposure (Eidy *et al*., 2016).

The effectiveness of the local isolates on the aphid nymph mortality in 6 days was much better in achieving maximum mortality than the 39 and 80 % mortality recorded with a bio-control agent *B. bassiana* CKB-048 at a concentration of 2 $\times 10^8$ after 3 and 7 days of treatment, respectively (Maketon *et al*., 2013). The data, in general, showed that the adults were significantly more sensitive than the nymph at all concentrations. Consequently, maximum percent mortality of 100% was recorded after 5 days for adult whereas; the same mortality rate with nymphs was recorded after 6 days of treatment with a concentration of 1×10^8 ($P < 0.001$) (Table, 6).

Similarly, Murerwa *et al.,* (2014) showed higher susceptibility of five to seven day old aphids (41%) than immature 0-2 (20 %) day old nymphs to *Metarhizium anisopliae* isolate ICIPE 51 at a concentration of 1 $\times 10^7$ after 6 days of treatment. But less effective in terms of the long time they took to kill the aphid pests compared to local isolates. In contrast, Al-Salihi (2016) reported that nymphs and adults of *Supella longipalpa* treated by direct contact with 1x 10^8 spore/ml *B. bassiana* killed 64.29, 79.31 and 85.7 % of the adults, the fourth and second instars of nymphs after 5 days of treatment, respectively.

This indicates that the relative susceptibility of different development stages of a host depends on the host species and the type of the fungal isolates (Romana and Fargus, 1992). Ekesi and Maniania (2000) reported molting to be an important factor in arthropod resistance to fungal infection, especially in arthropods with short ecdysis intervals. If the host is in an immature stage, molting could reduce the effectiveness of the fungal entomo-pathogens, in part owing to the shedding of conidia attached to the molted cuticle**.**

The compatibility studies between the enthomopathogens and different chemicals are important tools in selection process before performing greenhouse tests within the context of integrated pest management (IPM). Accordingly, almost all isolates showed similar pattern of tolerance in terms of germination of conidia, except the isolate *Metarhizium anisopliae* (MEI2) which was sensitive to all insecticides. None of the isolates were resistant to the recommended rate of the tested agrochemicals (Table 7). However, the isolates were tolerant to all pesticides at half (50%) dilution of the recommended rates of application. This is similar to the report of Oliveira *et al.,*(2003) who showed that *B.bassiana* isolates were compatible with the treatment of Thiamethoxam at half and field recommended doses with conidial viability from 62% -93%.

Consequently, the isolates showed conidial germination of 68%-98% after treated with the insecticides Karate (lambda-cyhalothrin), Actara(Thiamethoxam) and Radiant at half the recommended rate indicating that these insecticides would be suitable for use in combination with *M. anisopliae and B.bassiana for* insect pest control as part of an integrated pest management strategy (IPM).

The compatibility of *M. anisopliae* with thiamethoxam and lambda-cyhalothrin was similar with the work of Silva *et al*. (2012) at FR concentration that showed a compatibility of *M. anisopliae* (strain CG 168) with thiamethoxam (81.8% germinatoin) and lambda-cyhalothrin (86.3% germination). However, this study showed thiamethoxam (MEI1 82 % germination) which was more compatible than lambda-cyhalothrin (MEI1 75% germination.

Niassy *et al.,* (2012) showed that L-cyhalothrin was toxic to the fungus *M. anisopliae* with percent germination of 31.7%, whereas thiamethoxam was compatible with 72.5 % germination. On the contrary, this study indicated that L-cyhalothrin was compatible at all concentrations with 70-85 percent germination.

In this study in the case of Actara, the *Beauveria (*BEI1) and *Meterahizium (*MEI2*)* isolates at (RR) treatment showed significant reduction compared to the lower concentrations and the control (74 and 71%) germination, respectively. In contrast, Neves *et al.,* (2001) reported that non-significant reduction compared to control with 86.6% and 94.6 % germination, respectively.

The greenhouse bioassays showed that entomopathogenic fungal strains BEI1, BEI2 MEI1 and MEI2 were effective in reducing the population of mustard aphids compared to the control (Table 8 and 9). All taken together, *B. bassiana* (BEI1) exhibited the greatest virulence, with mortality rates of 97.5% and 93.9% after 11days of treatment for adult and nymph aphids, respectively. This shows that the local isolates were less effective in terms of the long time they took to kill the aphid pests compared to the 7 days treatment (67%-100% mortality) with the same inoculums of ($1X10^8$ spores/ml) *B. bassiana* isolates (Ujjan and Shahzad, 2012; Selvaraj and Kaushik, 2014). Ujjan and Shahzad (2012) also reported that some isolates of *M. anisopliae* showed 72% virulence against mustard aphid population after 3 days *of* treatment, whereas in the present study, isolates MEI1 and MEI2 of *M. anisopliae* showed 7.8 and 2.8% mortality within 3 days respectively.

Treatment of green peach aphid adults with isolates of *B. bassiana* in the greenhouse resulted in 30.7 to 48.3% mortality rates with $1X10^8$ spores/ml concentration. The data on the mortality of 49.2% after 7 days treatment with *B. bassiana* (BEI1) showed similarity with the same mortality recorded with similar enthomopathogens by Al-alawi and Obeidat (2014).

In this study, *B. bassiana* isolates were more virulent than those of *M. anisopliae* isolates in killing both nymph and adult aphids under greenhouse conditions **(**Table 8 and 9**).** This result was different from the work of Loc *et al.,* (2010) who showed that the pathogenesis of *M. anisopliae* (72.4%) was slightly higher than *B. bassiana* (62.8 %) on nymphs of black citrus aphids after 7 days of treatment (DAT). But this study, showed that the strain *Beauveria* spp (41.1%) mortality was more effective in killing aphids than *Metarhizium* spp (27.2%) mortality after 7 days of treatment.

Generally, the virulence of the isolates *in vivo* was less than the test *in vitro* taking longer time in the former than the latter. For instance, BEI1 and MEI2 showed aphid mortality rates of 77.8% and 55.6% under laboratory conditions while they showed mortality rates of 30% and 18%) *in vivo*, within 5 days of treatment, respectively. These findings were in conformity with earlier reports (Kumar *et al.,* 2012) who indicated similar observations that the virulence of entomopathogenic fungal isolates decreased during *in vivo* bioassays against another group of insect pests**.** In contrast, Ujjan and Shahzad (2012) reported that virulence of *M. anisopliae* isolate PDRL711 during greenhouse trial increased to 70% as compared to 44.2% during laboratory bioassay at 10^7 spore ml-1 concentrations.

During the current study, the combined treatment (IPM) showed higher mortality than the treatment of the fungi alone. Although, pesticides or EPF used by other researchers (Ambethgar, 2009: Amjad *et al.,* 2012: Malekan *et al.,* 2012 and Quintela *et al.,* 2013) were different but the current study revealed the basic idea that each antagonist (chemical and enthomopathogen) may weaken and make the pest vulnerable to disease or mortality (Khetan, 2001).

At the first two days, high mortality were registered from the FR chemical alone due to fast killing. But later, after the 7^{th} - 11^{th} day post treatment almost similar results were observed from the combination and chemical alone. In addition the combined applications of fungal with a

lower dosage of karate resulted in a significantly higher mortality levels compared to sole applications of fungal isolates.

The combined application of insecticides with highly efficacious entomopathogenic fungi in a sequential way proved to be promising with karate indicating synergistic action with highly potent fungal strains. These results are more or less in conformity with Niassy *et al.,* (2012) who used different insecticides Actara (Thiamethoxam) and Imidacloprid with *Metarhizium anisopliae* ICIPE 69, 91 and 82.2 % mortality, respectively in a sequential manner by using them against *Frankliniella occidentalis*. The karate combined with EPF (BEI1) gave the highest mortality on both adult and nymph aphids.

6. CONCLUSION AND RECOMMENDATION

6.1. CONCLUSION

Only limited number 4 isolates isolates were antagonistic against aphids from a relatively higher number of soil samples. The data showed the mortality of infected aphids with fungal isolates *in vitro* increased with increase in concentration of conidial suspensions and exposure time. The mortality observed was low on day 1-2 after treatment in all fungal concentrations, it increased gradually and maximum was obtained on 4-6 days. The compatibility test showed conidial germination rates of 75% - 91% with *Metarhizium* isolates and 77% - 98% with *Beauveria* isolates depending on the dose used (0.5 FR, 0.75 FR and FR) without showing significant difference between the two smallest concentrations. The insecticide in combination with all fungal isolates in sequence gave higher mortality than the fungal isolates alone against nymph and adult aphids.

6.2. RECOMMENDATION

Based on the result, the use of the isolates of *Beauveria* and *Metarhizium* spp. as microbial insecticides alone and combined with low dose of insecticides could be further tested in the field/greenhouse level so as to be used for Integrated Pest Management program for environmentally safe control of the *Brevicoryne brassicae* aphid and production of quality products (organic farming). Additional investigations are recommended for more effective EPF against different aphids with a relatively short time, and tolerance to higher dose of different insecticides. There is still a need for applying a new and innovative microbial control practices suitable for environmental conditions of each country.

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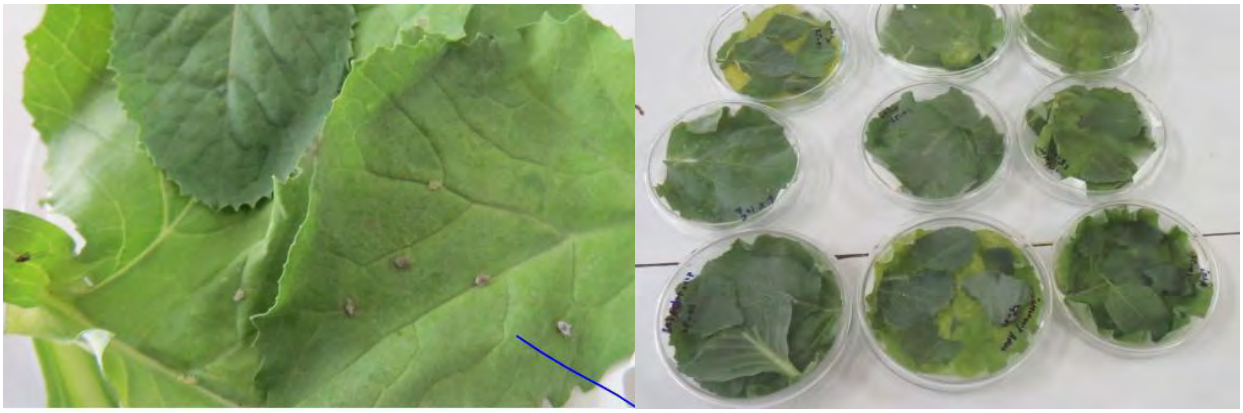
APPENDEXES

Appendix 1: Aphid colonies (*Brevicoryne brassicae)* were collected from highly infected leaves of mustard

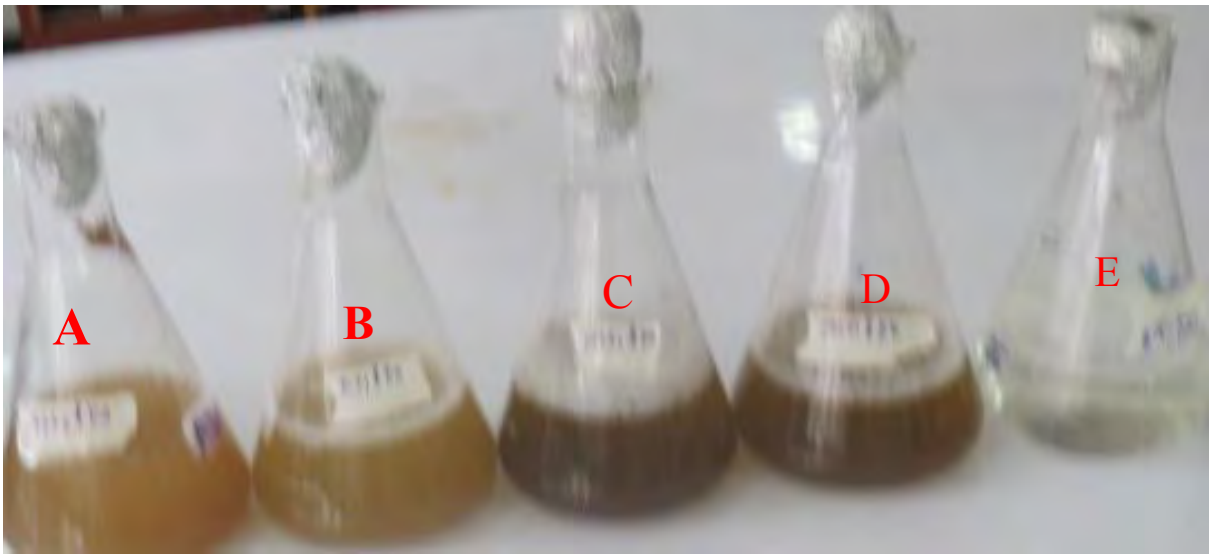
Appendix 2: Clear plates containing experimental Aphids feeding on mustard treated with different concentrations of various fungal isolates



Appendix 3 : A representative experimental cage 130, 150 and 50 cm (L,H and W) loaded with potted *Brassica carinata* seedling.

Appendix 4: Host plant used in the study after 11 days of planting

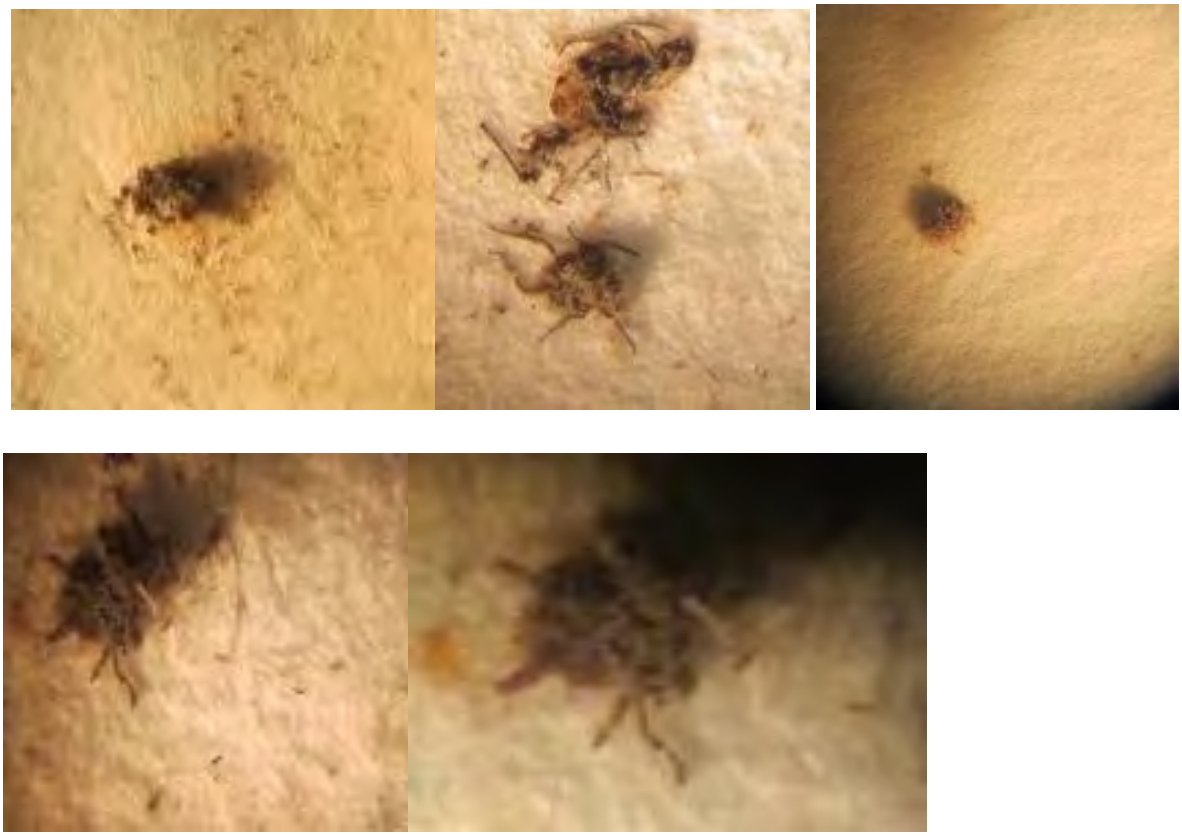
Appendix 5: Liquid media in 250 ml flasks which were inoculated with fungal conidia



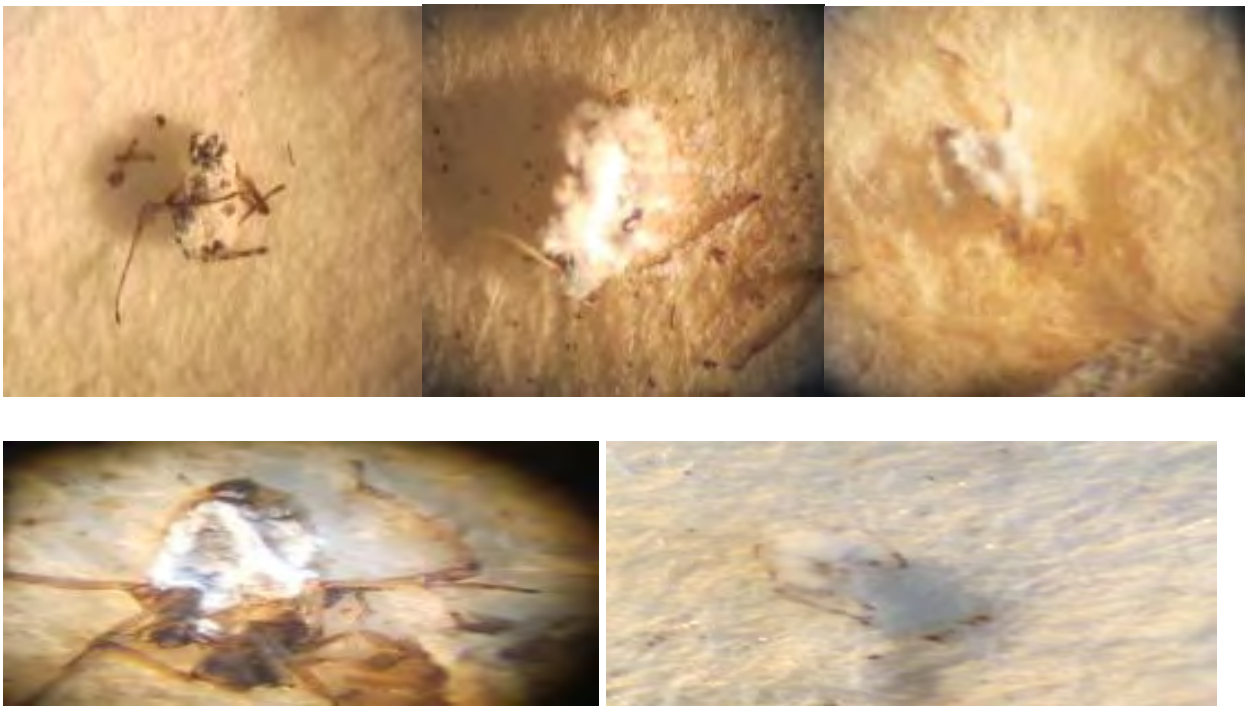
A. Conidia suspension of BEI1, B. Conidia suspension of BEI2, C. Conidia suspension of MEI1, D. Conidia suspension of MEI2 and E. Triton x-100 and distilled water as control

Appendix 6 : Healthy Aphids before treatment with *M. anisopliae* and *Beauveria bassiana*

Appendix 7 : Cadaver of Aphids mycosed by *Metarhizium anisopliae*



Appendix 8 : Cadaver of Aphids (mycosed by *B.bassiana*)

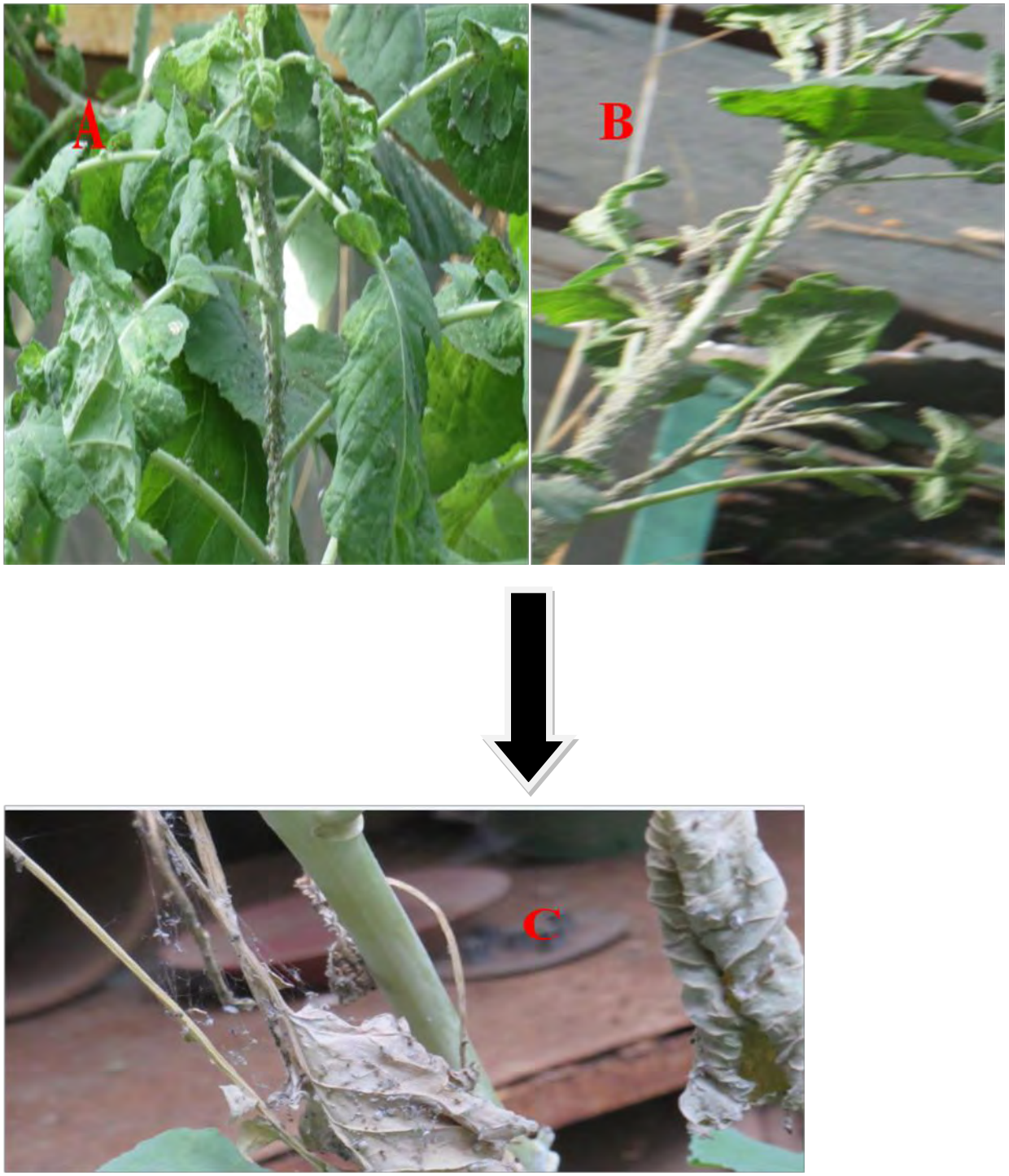


Appendix 9:(A-C) Biologically and combined treated Mustard performance at 11 days after inoculation

A) Host plant treated with fungal only after 11 day B) Host plant treated with combined application of fungi and insecticide

C) Host plant treated with insecticide only

Appendix 10 : Fig A and B untreated negative control Mustard performance at 11 days after inoculation



C) untreated negative control Mustard performance at 20 days after inoculation

Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for any other awards at this or other university and all the sources of material used for this thesis have been dually acknowledged.

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This MSc thesis has been submitted for examination with our approval as University Advisors.

1. **Fassil Asefa:- (PhD) AAU**

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