ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

DEVELOPMENT OF MYCOPESTICIDE FOR THE MANAGEMENT OF SORGHUM CHAFER, *Pachnoda interrupta* (Olivier) *(*COLEOPTERA: SCARABAEIDAE) IN ETHIOPIA

BELAY HABTEGEBRIEL

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Board of examiners

Name of external examiner…….

Comments……

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Signature …….

Name of internal examiner…….

Comments……

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Signature ……..

Advisers	**Signature**
1. Dr. Emana Getu	**……**
2. Dr. Mattias Larsson	**…….**
3. Dr.Mohamed Dawd	**…….**
4. Dr. Ylva Hillbur	**……**
Chairperson	**Signature**
Name ……	**…….**

ABSTRACT

*Pachnoda interrupta (*Olivier*) (*Coleoptera: Scarabaeidae) is a damaging pest of sorghum, other cereals and horticultural crops such as maize, wheat, barley, bananas, guava etc. in Africa which at times results in complete crop losses. Current control strategies heavily rely on chemical pesticides. Controlling adult beetles through application of insecticides, in addition to being detrimental to the environment and the ecology, does not provide long lasting control.Thus, efficient biological control agents that can control the pest in the breeding sites need to be developed.There is evidence that microbial pesticides can effectively control *P. interrupta*. Entomopathogenic fungi isolated from soils and insects in Ethiopia were evaluated under laboratory and field conditions with the objective of developing a myco-pesticide against *P. interrupta*. Evaluations were conducted in three phases. In the first phase 116 *Metarhizium* spp. and *Beauveria* spp. were evaluated based on high viability as measured by percentage germination on SDA media. Fourty-seven isolates with greater than 70% viability were selected and further evaluated using conidial suspensions applied to *Galleria mellonella* (Fabricius) (Lepidoptera, Pyralidae) larvae at a standard concentration of $1x10^8$conidia/ml. Four *Beauveria* spp. and three *Metarhizium* spp. isolates which caused over 60% mortality were selected for final virulence assay on *P. interrupta* adults at a dose of 1mg dry conidia/10 beetles. Highest mortality of beetles (82%) was recorded from the *Metarhizium* spp. isolates PPRC51 followed by PPRC2 (80%) and the *Beauveria* sp. MP3POST (80%) which were not significantly different. The three isolates were selected as candidates for additional tests to develop myco-pesticide against *P. interrupta.*

Molecular identification of the seven entomopathogenic fungal isolates used in the study was carried out using PCR amplification of the ITS regions of Ribosomal DNA and chitinase gene primers *(chi1* and *chi4)* and resulted in effective identification of three isolates as *M. anisopliae* and four isolates as *B. bassiana*. *In-vitro* characterization of the selected isolates using four artificial solid media at five distinct temperatures showed that two *M. anisopliae* isolates (PPRC51 and PPRC2) exhibited better radial growth, germination, and sporulation at temperatures of 20^{o}C, 25^{o}C and 30°C while these characteristics were limited at 15^{o}C and 35°C. These two isolates were selected as best candidates for further tests.

Field studies on fungal auto-dissemination device development from locally available materials conducted over three feeding and two mating seasons resulted in two efficient traps baited with a five compounds blend lure which were not significantly different in catch performance with the standard Japanese beetle trap. The two selected virulent isolates of *M. anisopliae* (PPRC51 and PPRC2) were tested for field efficacy using these two designs of locally affordable auto-dissemination traps loaded with 1gm of dry conidia. PPRC51 induced 41% field mortality on *P. interrupta* under high temperature and low relative humidity conditions. Field viability of the two isolates was 36 % and 40 % in five days for PPRC51 and PPRC2, respectively. Based on the efficacy data, PPRC51 is a promising candidate for development of a myco-insecticide against *P. interrupta* at commercial level in the form of augmentation biological control in the pest's natural habitat as a component of *integrated* pest management against *P. interrupta*.

Three liquid phase media were evaluated against the selected *M. anisopliae* and *B. bassiana* isolates in order to develop mass production and preservation protocols using the diphasic fermentation method and to evaluate their effects on virulence to *P. interrupta* using rice as sold substrate. PPRC2 produced highest number of spores ($4.57x10^{10}$ spores/g and $3.85x10^{10}$ spores/g) when using LB and MYE as liquid phase media respectively which were not significantly different. The highest spore yield per kg of rice substrate (119.72 g/kg) was obtained from the commercialized isolate IC69 which was mass produced by using MYE as a liquid phase medium followed by PPRC2 (43 g/kg) on LB and PPRC51 (48 g/kg) on MYE liquid media. Spore production was significantly affected by isolate, but not by liquid media.

Farmers in the study area have low perception on use of biological control as a component of IPM for *P. interrupta* and more than 70% of the interviewed farmers are inclined towards using chemical pesticides against the pest.

DEDICATION

To my family: my wife Freweini Mebrahtu and our children Furtuna and Abnet.

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ACRONYMS

ADT= Auto-Dissemination Trap

ANOVA= Analysis of Variance

CSA = Central Statistics Authority

DLCO = Desert Locust Control Organization

DNA = Deoxy ReboNucleic Acid

EPF = Entomo-Pathogenic Fungi

EARO= Ethiopian Agricultural Research Organization

FAO = Food and Agriculture Organization

ITS = Internal Transcribed region

IPM = Integrated Pest Management

JBT = Japanese Beetle Trap

LAT = Locally Affordable Trap

LB = Lenneoux Broth

LD_{50} = Lethal Dose to kill 50% of experimental animals

LT_{50} = Lethal Time to kill 50% of experimental animals

MOA = Ministry of Agriculture

MYE = Molasses Yeast Extract

MEA = Malt Extract Agar

MBCA = Microbial BioControl Agent

PPRC = Plant Protection Research Center

PCR = Polimerase Chain Reaction

SAS = Statistical Analysis Software

SDA = Sabouraud Dextrose Agar

SDAY = Sabouraud Dextrose Agar Yeast

SWBY = Sucrose Waste-Brewer's Yeast

ULV = Ultra Low Volume

UV = Ultra Violet

CHAPTER I

GENERAL INTRODUCTION

1.1. Background

Sorghum, *Sorghum bicolor* (L.) Moench, is the fifth leading cereal grain worldwide after wheat, rice, maize and barley with area coverage of about 42.7 million ha and a total production of 56.96 million metric tons and Ethiopia is the third largest sorghum producer in Africa after Nigeria and Sudan (FAO, 2004). It is the most important cereal crop in eastern Africa, in particular in Ethiopia (Birhane, 1986). Sorghum ranks third both in area coverage and total cereal production and it was cultivated on a total of over 1.7 million hectare of land in 2013 in Ethiopia with a total production of over 3.6 million tons from private peasant holdings in the main season (CSA, 2013).The center of origin and diversity of sorghum is believed to be Ethiopia (Thomas and Waage, 1966).

The production of sorghum in Ethiopia and elsewhere in Africa is threatened by a wide range of pests. These include insect pests (stem borers, army worm, sorghum shoot fly and sorghum chafer), birds (Quelea quelea, chestnut weaver, village weaver, starlings, doves and pigeons), weeds (Striga spp.) and storage pests (weevils, Angoumois grain moth) (Hiwot, 2000).

Sorghum chafer, *Pachnoda interrupta* (Olivier) has a wide host range with over 35 plant species; however, sorghum is the major host (Hiwot, 2000). It is the most destructive pest of sorghum in Ethiopia and during huge out breaks, the entire fields of sorghum can be destroyed at the milk stage (Tsedeke, 1988). *P. interrupta* is mainly distributed in Africa except for a few species reported from Arabia (Grunshaw, 1992). In Ethiopia, a

countrywide survey conducted in the 1970s reported nine species, one of which was represented by two subspecies (Clark and Crowe, 1978). *P. interrupta* was not reported as a significant pest for more than twenty years after the survey (Hiwot, 2000). However, starting from the year 1993 through 2005, the population density of *P. interrupta* increased dramatically with the species becoming a serious pest in the northeastern part of the country as it increased its geographic distribution and host range (MOA and EARO, 1999; Asmare and Yeshitila, 2014). From reported infestation of 1375ha in 1993, the total area infested by *P. interrupta* reached 113,739 ha in 2000 (Yeraswork, 2000). Yield loss of up to 70% was incurred by *P. interrupta* on sorghum in the Amhara region of Ethiopia (Yitbarek and Hiwot, 2000). Damage can reach up to 100% even on insecticide treated sorghum fields (Yeraswork, 2000).

According to Yitbarek (2008), farmers use a number of traditional methods to control *P. interrupta.* Some of the methods include hand picking adults during early morning hours, application of smoke by burning wood; burning compost heaps to kill the larvae and use of chemical insecticides as a final option. However, in most cases, these methods have not been fully successful in reducing beetle populations in the field because of continuous re-infestation, the height of the local sorghum varieties (above 2.5m) and lack of appropriate spraying equipment for such sorghum varieties. Thus, current control methods of *P. intterupta* entirely depend on direct spraying and baiting with insecticides although without much success (Seneshaw, 2002). For example, a total of 21,104.7 liters or kg of pesticides was used on sorghum fields during the 1994–1999 periods in the Amhara region of Ethiopia alone (Yeraswork, 2000). The high amount of pesticides used for control of *P. interrupta* means higher costs to the majority of the small holder farmers

and more environment and health related hazards. Since controlling of adult beetles through application of insecticides on scattered sorghum will not provide long lasting control, efficient biological control agents that can control larvae in the breeding sites should be developed (Seneshaw and Mulugeta, 2000). Despite the abundance and economic importance of *P. interrupta,* and heavy pesticide use, not many studies have been focused towards the development of bio-pesticides that can reduce pesticide use and their impacts on health and environment in Ethiopia.

Biological control agents such as entomopathogenic fungi can play an important role in integrated management of *P. interrupta*. Several entomopathogenic fungi have been isolated from dead cadavers of *P. interrupta* and other beetles in Ethiopia and were tested against western flower thrips under laboratory conditions and were found to be virulent (Seneshaw, 2001). In another study, evaluating the virulence of native fungal isolates, *Metarhizium* isolates PPRC-19 and PPRC-14 out performed other isolates in killing the larvae and adult beetles (Seneshaw, 2001), indicating the prospects of using the indigenous fungal isolates in the management of *P. interrupta*. However, no extensive studies have directly evaluated the field efficacy of the available native entomopathogenic fungi on *P. interrupta* in a systematic and scientific way.

Use of efficient traps and attractants may be helpful in improving control of *P. interrupta.* Synthetic plant compounds such as methyl salicylate, eugenol and isoamyl acetate have been proven efficient as attractants to *P. interrupta* (Yitbarek, 2008). Use of blends of chemicals can make trapping more effective. McGovern and Ladd (1984), reported more efficient trapping of *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae) with blends of chemicals. Some studies have found that locally produced traps could not catch

significantly higher *P. interrupta* than the commercially produced Japanese beetle trap (Wode-Hawariat *et al*., 2007) indicating the need for development of cheap and efficient ones. Efficient traps need to be re-designed for autoinoculation of *P. interrupta* with bio-pesticides so that the later can autodisseminate the inoculum to the pest population.

Efficient traps equipped with auto-dissemination device loaded with entomopathogenic fungi and attractants can be used for the control of *P. interapta* in an integrated way by attracting and infecting the beetle in a sustainable and environmentally safe method thereby eventually decreasing the population. In a similar strategy, using ‘attract and infect with pathogens’ method, Klein and Lacey (1999) obtained up to 97% mortality of adult Japanese beetle, *P. japonica* indicating the high potential of this method for beetle control.

Finally the best performing entomopathogenic fungi must be characterized and protocols for their mass production, formulation and preservation developed. Attempts should also be made to evaluate the newly developed and existing sorghum chafer management technologies on-farm using participatory methodologies so that an appropriate technology which is both effective and well accepted by farmers can be developed.

1.2. Objectives of the study

1.2. 1. General objective

- To develop effective, environmentally sound myco-pesticide and appropriate delivery system for the management of *P. interrupta.*

1. 2. 2. Specific objectives

- To evaluate the potential of native entomopathogenic fungi for development as myco-pesticides against *P. interrupta.*
- To characterize selected isolates of entomopathogenic fungi using molecular and cultural methods.
- To develop a low cost and effective trap, and an auto-inoculation device for attracting and infecting *P. interrupta* populations under field conditions
- To develop mass production and preservation protocols for selected isolates.
- To evaluate farmers' perception on the use of biological control against *P. interrupta.*

CHAPTER II

LITERATURE REVIEW

2.1. Biology and behavior of *Pachnoda interrupta*

The sorghum chafer, *P. interrupta* (Olivier) belongs to the order Coleoptera, family Scarabaeidae and subfamily Cetoniinae (Clark and Crowe, 1978; Grunshaw, 1992). About 130 species are recorded in the genus *Pachnoda* out of which nine species, one represented by two sub-species, have been reported in Ethiopia (Clark and Crowe, 1978; Krikken, 1994). *P. interrupta* is a univoltine insect and reproduces only once in a year giving one generation per year (Asmare and Yeshitila, 2014). It follows a complete metamorphosis and passes through all the egg, larva, pupa and adult stages during its life time.

Newly laid eggs of *P. interrupta* have average size of 1.40 x 1.23 mm, are white in color and ovoid to spherical in shape. The eggs, which are laid singly, are stratified in the oviposition medium and at eclosion reach a size of up to 2.0 x 1.77 mm, while turning brown in color and becoming swollen (Grunshaw, 1992). There are different reports about oviposition rate and duration of egg laying by a single female. Recent field based studies have shown that a single female laid an average of 1.22 eggs per day over a period of 11 days and dies soon after, while the eggs hatch in 15.7 days (Asmare and Yeshitila, 2014). Contrary to this, laboratory study by Seneshaw and Mulugeta (2002) showed that 1.8 eggs per day were laid and oviposition lasted about two months while adults could survive for six months after egg laying. However, Grunshaw (1992) reported that a single female laid up to 24 eggs in one night and hatching took seven to nine days.

Another report by Gebeyehu (2002) showed that 0.58 eggs were laid per day by a single female during a period of 25 days. According to Seneshaw and Mulugeta (2002), the average hatching period was reported to be 11.3 days, while Gebeyehu (2002) reported 9.6 days. The inconsistency in total number of eggs and hatching period has been attributed to factors such as: the diet of the beetles (e.g. uninterrupted laboratory diet compared to field conditions), constant disturbances of egg laying adults, soil type and moisture level, methodologies of egg collection and variation in laboratory and field experiments (Seneshaw and Mulugeta 2002; Asmare and Yeshitila, 2014).

The most recent study on the life cycle of *P. interrupta* by Asmare and Yeshitila (2014) indicated that larval duration range between 53 and 67 days with a mean of 59.58 days. But previous reports by Seneshaw and Mulugeta (2002) had shown that the larval stage lasts between 41 and 71 days with a mean of 55.7 days. Grunshaw (1992) also reported the mean time taken from egg eclosion to pupation to be 45.3 days. There are different reports about the duration of the larval stage. Previous studies (Grunshaw, 1992; Seneshaw and Mulugeta, 2002; Gebeyehu, 2002) indicated that the larval stage lasts for a mean of about 56 days. However Asmara and Yeshitila (2014) have recently reported variation in duration of the larval stage as affected by disturbance for data collection i.e. disturbed everyday (61days), every three days (62.5days), every six days (56 days) and undisturbed (59.83 days).

As described by Grunshaw (1992), the head width of the larva ranges from 1.17-1.24 mm in the tiny first instar to 3.05-4.05 mm in the well developed third instar. The head of the larva bears well developed chewing mouth parts and is smooth in shape and brownish-yellow in color with un-pigmented ocelli. The respiratory plate openings are postoriorly

directed and the size of the pro-thoracic spiracles ranges between 0.042 x 0.034 and 0.044 x 0.037 mm. The third instar larva which is distinct with cylindrical 6-8 terminal setae and transverse anal slit wrapped with hair is used as the identification feature of the scarabs. When the larva is ready to pupate, it makes an oval cocoon made from the soil cemented with larval saliva (Seneshaw and Mulugeta, 2002). The duration of the pupal stage is 18.9 days according to Seneshaw and Mulugeta (2002), 24.5 days according to Gebeyehu (2002) and this has recently been supported by Asmare and Yeshitila (2014) who found a mean of 18.3 days with a range of 13-25 days. These differences were apparently observed due to variations in temperature regimes used which is 28 ± 2°C (Seneshaw and Mulugeta, 2002), 25°C (Gebeyehu, 2002) and field conditions (Asmare and Yeshitila, 2014), respectively.

The body of the adult beetle of *P. interrupta* is flattish when viewed from above (Borror *et al.,* 1976).The adult is 12 to 16 mm long and a ventral shallow groove distinguishes males from females which have convex abdomen (Clark and Crowe, 1978). Adults can be easily distinguished by presence of yellow or red-brown spots and stripes on the elytra and the pronotum (Grunshaw, 1992; Mathews and Jago, 1993) although the overall color of the individual adults varies from black to pale yellow (Yitbarek, 2008). The mouth parts have no projections and are poorly developed: while antennae segments contain scape, pedicel and flagellomeres (Borror *et al.*, 1976).

Clark and Crowe (1978) reported that sexually matured adults mate and the females die soon after oviposition. In contrast, Seneshaw and Mulugeta (2002) demonstrated that the life span of sexually matured adults in the laboratory can be extended to six months after oviposition. The mean total number of days from egg to adults was recorded to be 93.8

with a range of 70–114 days under undisturbed field conditions (Asmare and Yeshitilla, 2014).

2.2. Host range and damage

The adult beetle of *P. interrupta* is the damaging stage. And adults feed on the flowers and suck all the contents of sorghum, maize and wheat grains at the milky stage (Grunshaw, 1992; Jago, 1993; Troure and Yehouienou, 1995). Over 37 crops and non-crop plants that belong to more than twenty families were identified as hosts (Hiwot, 2000). *P. interrupta* has a wide host range and feeds on flowers, nectar, pollen and fruits of wild and cultivated plants (Hill, 1989). Examples of host species recorded in Ethiopia, Mali, Cameroon, Nigeria, and Somalia include sorghum (*Sorghum bicolor)*, pearl millet (*Pennisetum glaucum)*, rice (*oryza sativa)*, maize (*Zea mays*), roses, cucumber, okra, sunflower, niger seed, sesame, guava and banana and trees and shrubs such as many *Acacia* spp., *Abutilon figarianum,Capparis tomentosa, Carissa edulis Ximentia americana, Zizyphus spinacristy, Berchemia discolor and Euclea schimperi* (Grunshaw, 1992; Troure and Yehouienou, 1995; Jago, 1995; Ratnadas and Ajayi, 1995; MOA and EARO, 1999; Yitbarek, 2007).

Yield loss due to *P. interrupta* on millet in cage experiments reached 45% (Grunshaw, 1992). Sorghum fields can be destroyed totally if infested at the milk stage especially during outbreaks (Tsedeke, 1988) and heavy infestations can result in 50% yield loss (Troure and Yehouienou, 1995). The adults cause grain abortion and panicle sterility incurring 70-100% yield loss on sorghum (Yitbarek and Hiwot, 2000; Yeraswork, 2000). During outbreaks between1993 to 2000, a single beetle observed on sorghum head was

considered as an economic threshold for control action (Hiwot, MOA 2012, Personal communication).

2.3. Distribution and occurrence

Official and scientific reports on the occurrence of *P. interrupta* in Africa date back to the 1950's. The beetle was recorded in many African countries such as Sudan and Cameroon (Risbec, 1950) and in Gambia, Burundi and Burkina Faso (Descamps, 1954), Senegal (Appert, 1957), Somalia (Schmutterer, 1969) and Mali (Grunshaw, 1992). Andemeskel (1987) reported *P. interrupta* as a major pest of sorghum and millet in Eritrea. It is widely distributed in Ethiopia where it is a destructive pest in sorghum dominated cropping systems at altitudes below 2000 m.a.s.l. (Clark and Crowe, 1978; Tsedeke, 1988). With the exception of a few records in Arabia and Madagascar, the genus *Pachnoda* appears to be restricted to Africa with many species of the cetoniinae widely distributed over the continent (Grunshaw, 1992; Giliomee and Donaldson, 1992).

The specific life activities of adult *P. interrupta* usually coincided with the onset of rainy seasons and flowering of plants (Clark and Crowe, 1978). According to Yeraswork (2000), the pest appears two times in a year. The first one occurs following the heavy rains in June during which the pest starts to fly and feed on different forage crops which have succulent flowers and fruits such as *Ximenia americana*, *Olea* sp and *Acacia* sp. The second flight of the pest occurs in September, which coincides with the flowering of the host plants including sorghum and maize.

P. interrupta is not a migratory pest, but during the day, active adults disperse in hot sunny weather exhibiting highest activity in search of food (Clark and Crowe, 1978;

Jago, 1993; and Jago, 1995). Availability of rainfall and vegetation may help localization of the movement and displacement of the beetles (Yitbarek, 2008).

The studies by Asmare and Yeshitila (2014) on the breeding and hibernating habitats and flight behaviors of *P. interrupta* have shown that adults that emerge in September feed up to December after which they remain quiescent for about 6 months. This enables the beetles to escape the harsh dry season and the associated food shortage. The study also revealed that the beetles prefer fertile and moist soils with 5 to 43 cm depth (depending on soil moisture and vegetation cover) which are found in river side and forests shaded by dense branches of trees and shrubs from different species. Hibernating and breeding altitudes range from 700 to 1660m and 519 to 1475 m above sea level, respectively. Soil and atmospheric temperatures range from 22^{o}C to 25^{o}C and 22.5^{o}C to 34^{o}C for hibernating and breeding, respectively. According to this study, the flight behavior of the beetles is not affected by wind direction.

2.4. Management of *P. interrupta*

Several control methods that include lure and kill techniques, chemical pesticides, biological agents and cultural control measures have been used by farmers to control the pest (MOA and EARO, 1999). Insecticides such as endosulfan and trichophone (Clark and Crow, 1978), synthetic pyrethroids (Grunshaw, 1992), sevin 85% WP and malathion 50% EC (MOA and EARO, 1999) have been used for the control of the sorghum chafer. However, poor efficacies, lack of spraying equipment, spray drifts to humans and adverse effects to bees have been reported (MOA and EARO, 1999; FAO-TCP, 2002).

Cultural control methods include early morning or late afternoon hand picking of adult beetles, bending of sorghum stalk to the ground, applying smoke to sorghum fields, growing none host or crops less preferred by the beetle and use of containers baited with residues of local brews or ripe mashed fruits mixed with pesticides. However, these cultural methods are not adequate to keep the pest population below economic threshold level (MOA and EARO, 1999; Hiwot, 2000; Yeraswerk, 2000). In Nigeria, early sowing of millet and sorghum integrated with resistant varieties and inter-cropping with cowpea, significantly reduced crop damage resulting in higher yields (Sastawa and Lale, 2000).

There are some indications of the potential use of indigenous entomopathogenic fungi as bio-control agents for the management of phytophagous scarabs such as *P. interrupta*. Seneshaw Aysheshim (2002) reported the potential of *Metarhizium* sp. isolates PPRC-19 and PPRC-14 in killing larvae and adult beetles in preliminary screening tests. Use of an 'attract and infect' method resulted in up to 97% field mortality of adult Japanese beetle, *P. japonica* (Klein and Lacy, 1999). It is therefore possible to use the same technique against *P. interrupta.*.

2.5. Types and use of biopesticides for pest management

Biopesticides can be developed from different sources using different methods and their scope includes various aspects of biological pest control. In general, the types of bio-pesticides can be grouped into three major categories: 1) Microbial biopesticides derived from micro-organisms (fungi, bacteria, virus, protozoa, algae and nematodes) or their secondary metabolites; 2) Biochemical pesticides including plant derived botanicals and naturally occurring substances such as pheromones (used for attracting or repelling pests , mating disruption, lure and kill strategies or monitoring), growth regulators and *Bacillus*

thuringensis (Bt) products, all of which use non toxic mechanisms to control pests; and 3) Plant incorporated protectants (PIP's) that are produced by introducing genes (e.g. Bt genes) into crops to transform the crops and express certain resistances against pests (Copping and Menn, 2000; Gupta and dikshit, 2010). The attention given to biopesticides in contemporary pest management is steadily increasing due to environmental and human and animal health concerns resulting from excessive use of synthetic pesticides. Biopesticides play an important role as a component of safe and eco-friendly integrated pest management (IPM) systems. The surge in interest to use biopesticides in IPM systems is mainly because of ecological benefits. Some of the benefits listed by Gupta and Dikshit (2010) include:1) safety to the environment, 2) host specificity3) quick decomposition and little or no pollution as only small quantities are required, 4) excellent compatibility with IPM systems, 5) cost effective and less exposed to pest resistance.

So far, the successful utilization of biopesticides and their role in the pesticide market is limited to agricultural niches that use largely *Bacillus thuringiensis* (Bt) based products (Copping and Menn, 2000). However, there is promising recent surge in demand, total production and use of biopesticides (Gupta and Dikshit, 2010). Among the biopesticides, microbial biopesticides are the most commonly used agents (Gupta and Dikshit, 2010). The microbial biocontrol agents (MBCA's) can destroy pests causing no detrimental effects to human health and the environment and pest resistance to MBCA's is less likely because of their complex mode of action (Khan *et al*., 2012).

2.6. Entomopathogenic fungi (EPF) as biocontrol agents

Fungi from over 90 genera and more than 700 species have been studied on insects (Khachatourians and Sohail, 2008, Khan *et al*., 2012). Fungus based biopesticides have

been given special attention among the entomopathogens because of their relatively common occurrence and ability to induce epizootic that helps to regulate insect populations (Butt and Goettel, 2000; Butt *et al*., 2001; Jaronski, 2010, Leger and Wang, 2010 and Khan *et al*., 2012). Fungus based microbial biopesticides such as myco-insecticides directly penetrate the insect cuticle making them most preferable for control of insects (Shipp *et al*., 2003) as opposed to bacteria, viruses and protozoa that must be ingested to cause infection (Lacey, 2012). Moreover, the life cycles of most entomopathogenic fungi often synchronize with environmental conditions and susceptible insect host stages (Shah and Pell, 2003). Over 170 commercial biopesticides from at least 12 fungal species have been commercialized over the last 50 years (Faria and Wraight, 2007). However, because of lack of consistency and slow action biopesticides have no significant market coverage despite their safety (Jackson *et al*., 2010). In addition to these, the production and formulation of candidate biopesticides requires substantial study on life history of the target insect, propagule viability and efficacy as well as ecological and environmental requirements and limitations (Jaronski, 1997; Vega *et al.*, 2009).

2.7. Mode of action of EPF

Except for a few taxa such as Culinomyces, entomopathogenic fungi have a unique mode of action as they infect insect hosts by directly penetrating through the external cuticle unlike other entomopathogens such as virus and bacteria that need to be ingested by the host (Inglis *et al*., 2001). Fungi possess hydrophobic conidial cell wall that enable the conidia to adhere to the insect cuticle through non specific adhesion mechanisms (Boucias *et al*., 1988; 1991). Attachment of conidia to insect cuticle is followed by

germination and formation of penetration structures such as appressorium and swelling of the germtube. However, germination does not necessarily entail penetration into the cuticle. Environmental factors such as inadequate moisture or components of insect cuticle (e.g. melanin and fatty acids) may prevent penetration of the germinated propagule into the cuticle. Enzymatic and mechanical mechanisms are involved in the penetration of entomopathogenic hyphomycete fungi in to the cuticle which is the first line of the host defense against infection which also determines host specificity (Gillespie, 2000, Roberts and St. Leger, 2004). The fungus *Metarhzium anisopliae* for example utilizes enzymes such as exoproteases, endoprotases, esterases, lipases, chitinases and chitobiases for penetration (Boucias and Pendland, 1998; Butt *et al*. 1998). After penetration, hyphae of the fungus grow in the haemocoel and the fungus produces toxins or immunomodulating substances to overcome the insect defense responses (Gillespie, 2000; Roberts and St. Leger, 2004). Examples of toxins produced by entomopathogenic hyphomycete fungi include beauvericin, bassianolide and oosporin (produced by *B. bassiana*) and destruxins (*M. anisopliae*) (Inglis *et al*., 2001). Toxins can cause titanic paralysis (Dumas *et al*., 1996) or suppress the immune system of the insect (Cerenius *et al*., 1990). Combined effects that include nutrient depletion, obstruction of organs and toxicosis ultimately cause death of the insect (Wraight *et al.*, 2007). Once the insect is dead, the fungus grows saprotrophically and produces metabolites that exclude competitive growth of other microorganisms in the cadaver allowing it to sporulate on the surface of the insect (Inglis *et al*., 2001).

Natural regulations of insect pests can be brought about by epizootics of hyphomycetous fungi. However, little is known about the factors inducing such epizootics which are

believed to result from complex interactions among the host, the environment, the pathogen and time or the disease tetrad (Inglis *et al*., 2001).

2.8. Development and application of EPF for pest management

There are many steps in developing EPF as biological control agents. These include isolation, identification, strain selection, interactions with the biotic and abiotic environmental factors, mass production and formulation, quality control and stability, application and efficacy tests, safety, registration and commercialization (Zimmermann, 1986). Several challenges must also be overcome to realize the use of entomopathogenic fungi for pest management. These challenges involve a number of complicated steps, from isolation and selection of an appropriate strain to field application of a formulated myco-insecticide, and failure to overcome the challenges can impede use of the products (Butt and Goettel, 2000; Jackson *et al*., 2010; Santi *et al*., 2011).

2.8.1. Isolation

According Inglis *et al.* (2012) entomopathogenic fungi can be isolated from insect cadavers, soil, plant tissue and plant surfaces. Isolation from insect cadavers involves harvesting spores from infected cadavers on to media. In cases of spores with no sporulation, cadavers may be put in suitable environments to encourage production of hyphae and conidia. Sometimes it may be necessary to homogenize the cadaver and plate the homogenate on to the surface of an agar medium. Selective media are important materials for entomopathogenic hypocreals. These media that inhibit bacterial growth use acids which encourage growth of fungi as fungal growth is generally favored on media with less than PH of 5. But most commonly bacterial inhibition is achieved using antibiotics such as tetracycline, chloramphenicol and streptomycin to amend the media.

Isolation of *B. bassiana* and *Metarhizium* spp. can also be done by amending media with the fungicide dodine (N-dodecyclguanide monoacetate) which selective for these fungi.

Selection of entomopathogenic fungi from soil is done using different methods such as 1) dilution spread plating that usually uses selective media, 2) direct plating which is direct application of soil on agar media and 3) insect baiting method which mostly uses larvae of the wax moth (*Galleria mellonella*).

2.8.2. Identification

One of the important steps in the development of microbial insect control agents in modern scientific studies is identification and characterization of selected microbial agents. Fungi can be identified using morphological characteristics but this method is not adequate due to ambiguous descriptions and limited availability of morphological keys (Fernandes *et al.,* 2009). Identification, characterization and analysis of genetic traits of fungi and other micro-organisms is facilitated by accurate and powerful molecular tools but would be difficult if not impossible with conventional morphological, physiological and biochemical methods (Inglis *et al*., 2012). Currently, molecular identification of potential entomopathogenic fungi is gaining acceptance as an important first step for successful development of myco-insecticides (Islam *et al.,* 2014). Particularly, molecular tools such as polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) and analysis of DNA sequences are being used widely as standard procedures for identification and phylogenetic comparisons between entomopathogenic fungi (Jensen *et al*., 2001; Destefano *et al*., 2004). Ribosomal DNA is also applied as a molecular marker for species identification (Islam *et al.,* 2014) as it reveals variations in

sequence for taxonomic studies (Fouly *et al*., 1997). Molecular techniques based on DNA are accurate tools for species identification (Driver *et al*., 2000; Entz *et al*., 2005).

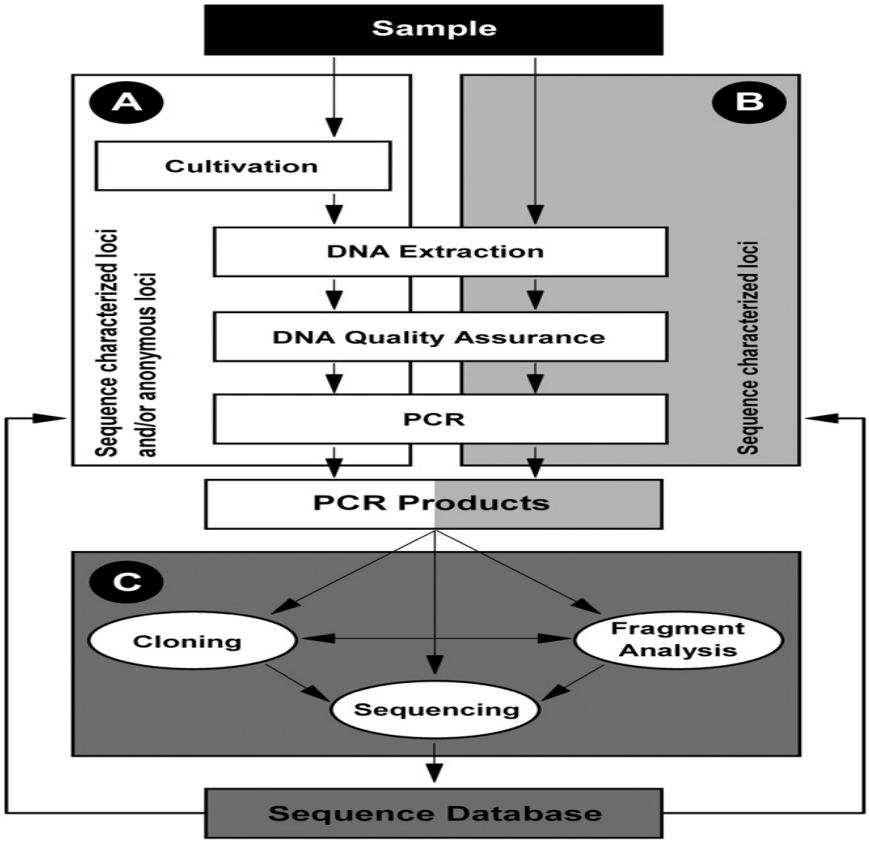

Figure 2. Schematic illustration of the steps and principles applied to study entomopathogenic hypocrean fungi using PCR based molecular techniques. (Modified from Enkerli &Wimer (2010) By Inglis *et al*.; 2012).

There are several steps in the molecular based analysis that should be handled as a full cycle (Amann *et al*.; 1995). PCR based identification of entomopathogenic fungi follows two major methods, namely cultivation dependent and cultivation independent methods (Enkerli &Wimer; 2010). As shown in figure 2, the steps involve DNA extraction, DNA quality assurance and running PCR followed by direct sequencing or sequencing after

cloning /fragment analysis of the PCR products. The results of sequencing are finally compared with sequence data bases. The molecular analyses are carried out on pure culture samples cultivated on artificial media in cultivation dependent methods. In contrast, in the cultivation independent methods, analysis is done directly on DNA samples extracted from samples such as insect cadavers and soil. Similarly, in a cultivation dependent analysis, PCR targets two basic loci 1) sequence characterized loci with known genes or DNA region such as ribosomal RNA (rRNA) gene and flanking spacer regions, 2) Anonymous loci with unknown genes scattered within the genomes that can be amplified simultaneously by PCR primers e.g. RAPD (randomly amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism). In a cultivation independent method, only sequence characterized loci are targeted.

2.8.3. Selection of appropriate strain

Selection of most pathogenic strains is the next step in finding an appropriate fungal pathogen following isolation and identification of potential entomopathogens. However, the selection process of an appropriate fungal pathogen for development as biopesticide can be complex and must evaluate the potential of the fungal isolate or strain in terms of forming a stable propagule for economic mass production, ease of application with available technology, and consistency in efficacy under environmental and ecological conditions (Wraight *et al.* 2001; Jackson and Schisler 2002; Jaronski, 2007; Jackson *et al*., 2010).

The field interaction of the entomopathogen and host or the pest cannot be easily predicted from laboratory or glass house experiments. And therefore, the skill and experience of experts can play important roles in selecting an appropriate strain that can

be developed to a biopesticide as highly adapted strains are neither abundant nor easy to isolate and culture (Hajek *et al*., 2007).

2.8.4. Interaction with abiotic and biotic factors

Biotic and abiotic factors affect the ability of myco-insecticides to survive, infect and kill the target pest. This includes persistence and transmission efficiency within and outside the host (Fuxa and Tanada, 1987). Therefore, environmental constraints must be identified and understood along with ecological requirements and life history of the target pest during production and formulation of candidate myco-insecticides to maintain its viability and efficacy (Wraight *et al*., 1997; Jaronski, 1997;Vega *et al*., 2009) both in standalone or integrated systems (Lacey and Shapiro, 2008). Although fungal ecology in crop systems have been studied in attempts to assess their potential as myco-insecticides (Hesketh *et al*., 2010), knowledge gaps still exist (Roy *et al.*, 2009). This necessitates assessment of the growth and virulence characteristics of candidate isolates under actual environmental conditions as a pre-requisite for successful development to myco-insecticides (Butt *et al.*, 2001; Kope *et al.*, 2008).Temperature, humidity and solar radiation are the most important parameters that affect insect fungal pathogens among other environmental factors (Inglis *et al*., 2001).

2.8.4.1. Abiotic factors

The temperature of the target eco-system can heavily influence growth and pathogenicity (Yeo *et al*., 2003) as well as speed of germination and kill (Migiro *et al*., 2010) of a fungal entomopathogen. Conidial germination is adversely affected by and rapidly slows in temperatures above 30^{o}C with the optimum being between 23^{o}C and 28^{o}C and most isolates reducing to half at 34- 37 ^{o}C (Jaronski, 2010). Instability of conidia at high

temperature leads to low thermo-tolerance (Fernandes *et al*., 2007).This necessitates the selection of isolates tolerant to the temperature range of the target ecosystem (Ferron *et al*., 1991) and appropriate formulation (Forgues *et al*., 1997) as a means to overcome the problem. For example, the LUBILOSA project (Lutte biologique Contre les Locuste et Sauteriaux) selected strains with pathogenic activity above 30^{o}C for biological control of the desert locust in west Africa (Mc Clatchie *et al.,* 1994).

The use of myco-insecticides for pest control is highly dependent on weather conditions especially higher ambient humidity (Ferron *et al.,* 1991). Luz and Fargues (1999), found that mortality due to mycoses (caused by *B. bassiana*) of first instar *Rhodnius pralixus* nymphs kept at constant humidity's of 75% to 95.5% at 25^{o}C did not exceed 50% within 10 days of treatment. However, when relative humidity was increased to 96.5%, 98.5% and 100%, all insects died over 10 days incubation time indicating that *B. bassiana* is more effective in higher humidity regimes.

Fungal propagules are highly susceptible to solar radiation (photo interaction) (Goettel and Inglis, 1997) and can be rapidly deactivated and lose persistence especially on the phyloplane (Furlong and Pell, 1997). However, susceptibilities of entomopathogenic fungal species and strains within species to solar radiation differ significantly (Braga *et al*., 2001). UV- A (320-400nm) and UV-B (280-320nm) components of sunlight are major factors of fungal propagule mortality and short persistence of myco-insecticides in the epigeal habitat limiting the half life of viable number of fungal conidia per unit area to 3-4 hours (Jaronski, 2010). Plant surfaces and insect cuticles can also be sterilized with UV radiation (Carruthers *et. al*., 1992). Formulations with UV blockers such as Tinpol can provide some protection (Inglis *et.al*., 1995).

2.8.4.2. Biotic factors

As entomopathogenic fungi infect the insect through the cuticle, epicuticular substances obviously stimulate spore germination although some insects posses fungistatic compounds (Jaronski, 2010). A good example was given by Smith and Grula (1982) who showed that conidial germination of *B. bassiana* was inhibited by *Helicoverpa zea* Boddie. Insect behavior may also affect ultimate sites of penetration (Butt and Goettel, 2000). Insect behavior studies with subterranean termites have shown that mutual grooming effectively removed fungal conidia readily attached to the cuticle (Yanagawa *et al*., 2008) indicating the need for rapidly germinating fungal propagules in grooming or frequently molting insects. Baverstock *et al*. (2010) described the ability of target insects to detect and avoid fungal pathogens and the transmission of fungal pathogens between insects as two processes that bear significant effects on insect-entomopathogen interactions. The behavior of insects (e.g. change in activity) can influence the probability of infection by the entomopathogenic fungi (Cory and Hoover, 2006). Behavioral avoidance or post contact responses such as grooming may provide selective advantage to insects against fungal infection (Chouvenc *et al.,* 2008). These responses may negatively affect the success of the fungus as a biological control agent (Baverstock *et al*., 2010).

2.8.5. Mass production and formulation

2.8.5.1. Mass production

Once a promising virulent isolate is identified, its amenability to mass production must be established. Development and commercialization of entomopathogenic fungi as biopesticides requires and is restricted to a species that can be economically mass produced *in-vitro* on cheap substrates (Wraight *et al*., 1997). Because amenability of a

microbial agent for mass production is a critical factor, species from the hypocreales have dominated almost all commercial products as they are easy to mass produce (Copping, 2004; Matthew and Read, 2007). Adequate amount of fungal propagules are critical for large scale applications. For example, biocontrol of folial insect pests requires spraying high concentration of fungal propagules that consequently demand cost effective production and formulation processes which are critical factors for commercial production (Jackson, 2007; Jackson *et al.*, 2010). In addition to these, nutritional, cultural (artificial and natural media) and environmental conditions during fungal growth can influence the virulence, efficacy, longevity, thermal tolerance and ecological fitness of mass produced fungal propagules (Mc Clatchie *et al*., 1994; Jackson, 1997; Ying and Feng, 2006).

Fungal propagules can be produced on artificial media using four general methods: 1) surface culture on solid media, 2) fermentation on semi-solid media, (3) submerged fermentation and 4) diphasic fermentation (Butt and Goettel, 2000). However, some fungi such as *Isaria fumosorosea* require light for conidial production and are difficult to mass produce on solid substrates (Sanchez-Murillo *et al*., 2004; Zimmermann, 2008). Some entomopathogenic fungi (for example *B. bassiana* and *M. anisopliae* var acridum (but not *M. anisopliae*) are dimorphic and can grow in liquid culture to produce balstospores or submerged microcyclic conidia under liquid fermentation which can be used for spraying after proper drying and formulation (Jenkins and Prior, 1993; Kassa *et al*. 2004). However, unlike aerial conidia, these conidia are not hydrophobic, and entail different challenges in formulation and use such as the need for inorganic nitrogen and high level of carbohydrates (Jackson, *et al.*, 2010).

Figure 1. Basic facilities and equipment for small scale mass production of EPF. A) Microbial bio-safety flow hood, B) Dehumidified room for sporulation and drying of inoculated bags, C) Digital incubator shaker for production of blastospores in the liquid phase and D) Plastic bowls for drying shown with an automatic dehumidifier.

Some solid substrates such as rice and other grains are commonly used for mass production process in autoclaved bags. The process yields in approximately 1-5 $\times 10^9$ spores/g dry weight of substrate with selected fungal strains (Alves, and Pereira, 1989) but may be substantially less (Lomer, 1993). This technology is adequate to support application rates of 1-5$\times 10^{12}$ spores/ha (Mondonca, 1992; Lomer, 1993).

Although this system is relatively simple, grains can be expensive and it is labor intensive which necessitates the use of more capital for automated production as mostly practiced for *Metarhizium* and *Beauveria* myco-insecticides (Barlett and Jaronski, 1988;; Santi *et al*., 2011). Some of the basic equipment and facilities necessary for small scale mass production of EPF is shown in figure 1 A-D. In addition to these, decreasing efficiency with increases in scale are important constraints of this system (Wraight and Carruthers, 2000).

2.8.5.2. Formulation

For large scale utilization of entomopathogenic fungal based biopesticides, appropriate formulation can be a fundamental factor that determines the successful delivery of the product to the target environment (Goettel and Roberts, 1992; Faria and Wraight, 2007). Basic components of most formulations include, in addition to the active ingredients (i.e. fungal spore), one or more of the following: a carrier, diluents, binder, dispersant, UV protectants and virulence enhancing factors (Moore and Caudwell, 1997). Fungal entomopathogens can typical be formulated as technical concentrates, wettable powders and oil dispersions compatible with lipophillic conidia (e.g. *M. anisopliae*) that can be delivered as atomized droplets using ultra low volume applicators (Buteman, 1997; Wraight *et al*., 2001; Faria and Wraight, 2007). However, the use of oil formulations that allow continuous aqueous phase suspensions of conidia is expensive and spraying equipment can be clogged with some types of oils (Wraight *et al*., 2001; Polar *et al*., 2005). To avoid interference of formulations with the infection process, use of compounds that enhance adhesion of spores to insect and plant surfaces is necessary (Butt and Goettel, 2000)**.**

Formulation and use of additives are critical to product stability (Butt and Goettel, 2000) and can influence efficacy (Friensen *et al*., 2006; Costa *et al*., 2008). Some oil formulations can cause detrimental effects on virulence and germination of the entomopathogenic fungal isolate impairing successful use of the product. For example, Polar *et al*.(2005), found that conidial germination of *M. anisopliae* in liquid paraffin oil and liquid paraffin emulsifiable adjuvant oil (EAO) were 91.6% and 86.0%, respectively, while the germination of conidia in 10% coconut EAO was only 59.2% in 48 hrs.

Persistence is another most important challenge on the use of entomopathogenic fungi as biopesticides. The active ingredient of a biopesticide e.g. myco-insecticide is a living organism, so there will be biological limits to its persistence and cannot be expected to be as persistent as long lasting insecticides. A fungal isolate that is only moderately pathogenic, but that persists and remains infectious, could ultimately be more useful as bio-pesticide than an isolate that is highly virulent, but requires application every two weeks (Thomas and Read, 2007).

2.8.4. Application of EPF for pest management

Successful utilization of biopesticides including entomopathogenic fungi in the field depends on effective delivery system (Bateman, 1999) which is an integral component of the whole process from isolation to commercialization of a product (Chapple *et al*., 2007). Field application results may not necessarily agree with laboratory and glasshouse application methods and results. Pittis and Russel (1994) reported that it is virtually impossible to predict field effects from glass house experiments. According to Chapple *et al.* (2007), these disparities may be caused by poor spray retention and distribution, environmental and microbial degradation, inter-microflora competition, and inactivation

within a target organism. Field verification of laboratory and glass house efficacies is therefore a compulsory step in the development of biopesticides.

There are different application or delivery techniques of entomopathogenic fungi against insects, each of which has relative advantages and disadvantages. These include broadcast application of granules against cryptic insect pests, ULV and high volume (inundative application) spray applications against range land and field crop pests, respectively (Wraight and Carruthers, 2000). Granule formulations for application can be developed by coating of dry spores on to grain or bran (Johnson and Goettel, 1993 draft proposal) drying and fragmentation of mycelium or mycellial pellets (MaCabe and Soper 1985; Rombach *et. al*.1988; Andersch *et. al.*1990) or encapsulating spores or mycelium in starch or alginate (Pereira and Roberts, 1991; Knudsen *et. al*., 1990).

Klein and Lacey (1999) used a modified version of the Japanese beetle trap (JBT) to infect adult Japanese beetles, *P. japonica* with dry conidia of *M. anisopliea* and *B. bassiana* mixed with wheat bran. Use of contamination devices to infect fruit flies (Dimbi *et al*., 2003) and Tsetse flies (Maniana, 2002) have been developed and successfully tested in the field (Ekesi *et al*., 2007; Migiro *et al*., 2010) indicating new prospects for application of entomopathogenic fungi for pest control.

Horizontal transmission of pathogens within the same host/target species is called autodissemination (Ignoffo, 1978) and the use of autodissemination traps has been suggested for bio-control of several insect pests by many authors (Lacey *et al.,* 1994; Furlong *et. al*., 1995; Klein and lacey, 1999; Dowd and Vega, 2003*).* In this technique, the target pest must be live trapped in substantial numbers and allowed to exit the trap

after contamination with the fungal spores to horizontally transfer the inoculums to the populations elsewhere (Lyons *et al*., 2012). Fungal auto-dissemination within a host population occurs as a result of activities and movements of the host (Scholte *et al*., 2004). Auto-dissemination devices have been developed that facilitate inoculation of insects with entomopathogens among con-specifics in the environment after they have been attracted to contaminated chambers (Vega *et al*., 2007). The rate of spread of entomopathogenic fungi within host populations and the potential of the pathogen for use as a microbial control agent is determined by transmission (Steinkraus, 2006). Transmission can occur horizontally (within a generation) and vertically (between generations) within a species, between species and from a local scale on a single plant to a landscape (Jason *et al*., 2010). Horizontal transmission between individuals of the same species (auto- dissemination) can occur through direct contact between contaminated and uncontaminated individuals or indirectly via conidia that have been deposited on the substrate (Quesada-Moraga *et al*., 2008).

Even though quantifying fungal transmission is relatively simple, determining the mechanisms is more challenging (Jason *et al*., 2010). Direct transmission between contaminated and uninfected individuals is less variable and more efficient than indirect transmission via conidia that have been deposited on substrate, and can lead to high mortality rates even when the number of contaminated individuals is low (Dekesel, 1995). For example, transmission of *B. bassiana* conidia from a single contact between treated male and untreated female adult spruce bark beetle, *Ips typographus* (L), was found to result in 75% mortality rate in a ratio of 1:20 (treated to untreated beetles) and was increased to 90% when the ratio was 1:1 (Kreutz *et al*., 2004).

2.8.5. Shelf life (storage) of EPF

Fungal viability and efficacy is greatly affected by storage conditions. Spores of myco-pesticides can initiate germination and die slowly in the storage environment (Jaroniski, 1997). Moisture content of spores and formulation can also influence shelf life of the bio-pesticide (Moore *et al*., 1995). Although a minimum of 12–18 months of shelf life is required for a successful biopesticide (Burges, 1998), most can only be stored for 6 –12 months at room temperature (Copping, 2004). Thus, keeping the fungal spores dormant for a satisfactory period while, maintaining viability and efficacy is a major challenge (Jackson *et al*., 2010).

2.8.6. Compatibility of EPF with semiochemicals

Some plant extracts, volatiles substances and pheromones used for attraction of insects can have detrimental effects on myco-insectides used simultaneously. For example, neem oil had been reported to inhibit germination, colony diameter and conidio-genesis by 45.3%, 36.6%, and 84.9%, respectively, in *B. bassiana* (Hirose *et al*., 2001). Similarly, Nana *et al*. (2011) reported inhibition of growth parameters (germination, radial growth, mycelia dried weight and conidial yield) of *M. anisopliae* by attraction, aggregation and attachment pheromone (AAAP) used as attractant for ticks.

2.9. Effects on non target organisms

For successful development of fungal entomopathogens as biopesticides, high efficacy against the target pest and low virulence to non target insects are important factors (Thungrabeab and Tongma, 2007). Non-target organisms are all living things aside from pests being targeted for control. However, since by definition entomopthogens live by parasitizing insects, they do not commonly attack outside the class insecta. Taking this

into consideration, for each specific instance, only those species occurring in the area of interest would normally be considered as non targets. The effect of entomopathogens on commercially important non-target organisms such as bees and silkworms as well as on non targets important for pest control (such as natural enemies of insects) must also be seriously considered during studies of potential non target impacts (Hajek and Goettel, 2007).

CHAPTER III

EVALUATION OF INDIGENOUS ENTOMOPATHOGENIC FUNGI *Beauveria bassiana* AND *Metarhizium anisopliae* FROM ETHIOPIA FOR THE DEVELOPMENT OF BIO-PESTICIDE AGAINST THE SORGHUM CHAFER, *Pachnoda interrupta (OLIVIER)* (COLEOPTERA; SCARABAEDIEA) UNDER LABORATORY CONDITIONS

3.1. Introduction

Sorghum chafer, *P.interrupta*, is one of the most destructive polyphagous insect pests of sorghum and over 35 other important crops in Africa in general and in Ethiopia in particular (Grunshaw, 1992; Hiwot, 2000). It can destroy sorghum fields completely during huge out breaks especially at the milk stage (Tsedeke, 1988). Some of the crops and host species damaged by the adults of *P. interrupta* recorded in Ethiopia, Eritrea, Mali, Cameroon, Nigeria, and Somalia include cereals such as sorghum (*Sorghum bicolor)*, pearl millet (*Pennisetum glaucum)*, rice (*Oriza sativa)*, maize (*Zea mays*), ornamentals such as roses, vegetables such as cucumber and okra, oil crops such as sunflower, niger seed and sesame; fruits such as guava, banana, mango and papaya and flowers of trees such as *Acacia* spp. (Andemeskel, 1987; Grunshaw, 1992; Troure and Yehouienou, 1995; Jago, 1995; Ratnadas and Ajayi, 1995; MOA and EARO, 1999; Sastawal and Lale, 2000; Hiwot, 2000).

In Ethiopia, starting from 1993 up to 2005, the population density of *P. interrupta* increased dramatically, becoming a serious pest in the northeastern part of Ethiopia as it has increased its geographic distribution and host range (MOA and EARO, 1999; Asmare and Yeshitila, 2014). For example, the area wide infestation of *P. interrupta* was 1375ha in 1993, while it was 113,739 ha in 2000 (Yeraswork, 2000). In the mean time grain abortion and panicle sterility caused by the pest resulted in yield losses ranging from 70 –

100% even on insecticide treated sorghum fields (Yitbarek and Hiwot, 2000; Yeraswork, 2000).

The current control methods of *P. intterupta* mainly depend on direct application of sprays and baits of synthetic insecticides (Seneshaw, 2002). A total of 21,104.7 liters of pesticides were used on sorghum fields from 1994–1999 in the Amhara region of Ethiopia alone (Yeraswork, 2000). The high amount of pesticides used for control of *P. interrupta* incurs higher costs to the majority of the small holder farmers and entails more environment, human and animal health related hazards. In addition to this, controlling of adult beetles through application of insecticides on scattered sorghum fields does not provide long lasting control. Thus, alternative control methods that can contribute to integrated management of this destructive pest must be sought. One of the possible methods is to develop efficient biological control agents that can control larvae and adults at the breeding sites (Seneshaw and Mulugeta, 2000). Biological control agents, which include, naturally occurring microbial bio-control agents (MBCAs), have great potential for controlling pest populations with little or no detrimental effects on human health and the environment (Khan *et al*., 2012). Among MBCAs, entomopathogenic fungi are most preferred because of ease of production, application, mode of action that does not need to be ingested by the target pest (Butt, 2002; Wang and St. Leger, 2007; Thomas and Read, 2007). Entomopathogenic fungi are especially important when used with IPM programs as they are compatible with pesticides (Lacey and Goettel, 1995; Wraight *et al*, 2007). These fungi have also restricted host ranges and thus cause little or no harm to non-target organisms such as parasitoids and predators (Goettel and Hajek, 2001; Vestergaard *et al*, 2003).

Despite the abundance and economic importance of *P. interrupta*, and heavy pesticide use, not many studies have been focused towards the development of microbial bio-pesticides in Ethiopia. Microbial biopesticides can reduce chemical pesticide use and their impacts on health and environment, while contributing to the sustainable sorghum production and safe management of the pest. The objective of this study was therefore to evaluate the potentials of native entomopathogenic fungi for the development of myco-insecticide against *P. interrupta*.

3.2. Materials and methods

3.2.1. Sources of isolates: The sources and genus of isolates used for the experiments are given in Table 1. Isolates were either obtained from collections in Ethiopia or directly isolated from soils and infected *P. interrupta*. A total of 116 isolates of *Beauveria* spp. and *Metarhizium* spp. were obtained and sub-cultured on fresh sabrouad dextrose agar (SDA) media and incubated at 25^{O}C for three weeks to allow for sporulation. The viable isolates were then stored at 4^{O}C until used.

Table 1. Source, genus and number of isolates used for the experiment

No	Source	Genus		Total isolates obtained
		Metarhizium isolates	*Beauveria* isolates	
1	Addis Ababa University	3	----	3
2	*DLCO-EA	13	42	55
3	**PPRC- Ambo	20	30	50
4	Isolated from soils of Rassa area/Ethiopia	---	6	6
5	Isolates from *Pachnoda interrupta* cadavers from soils of Rassa area /Ethiopia	1	1	2
	Total	37	79	116

*The Desert Locust Control Organization of East Africa

** Plant Protection Research Center, Ambo, Ethiopia

3.2.2. Screening for viability: All the 116 isolates were subjected to germination test to select the ones with reasonable viability (Figure 3, A). The germination tests were done according the standard techniques developed by Goettel and Inglis (1997). Spores were harvested from the surfaces of media using a sterile metal spatula and added to test tubes containing 10 ml of sterilized 0.01% Tween 80 to make a stock suspension. The concentration of the stock suspension was adjusted to $3x10^6$ conidia/ml using an improved neubaour heamocytometer and 100 µl of the suspension was then spread plated on SDA media in 90 mm diameter Petri-dishes. Germination was stopped after 24 hrs of

incubation at 25^{O}C by pouring 1ml of 70% alcohol and a sterile cover slip was put on each Petri dish and percentage germination was determined by counting at least 300 conidia under a compound microscope at x400 magnification. A conidium was considered germinated if it showed a germ tube growth as big as its size. Three replicate Petri dishes were used for each isolate. Isolates with >70% germination were considered viable and selected to be used for pathogenicity tests.

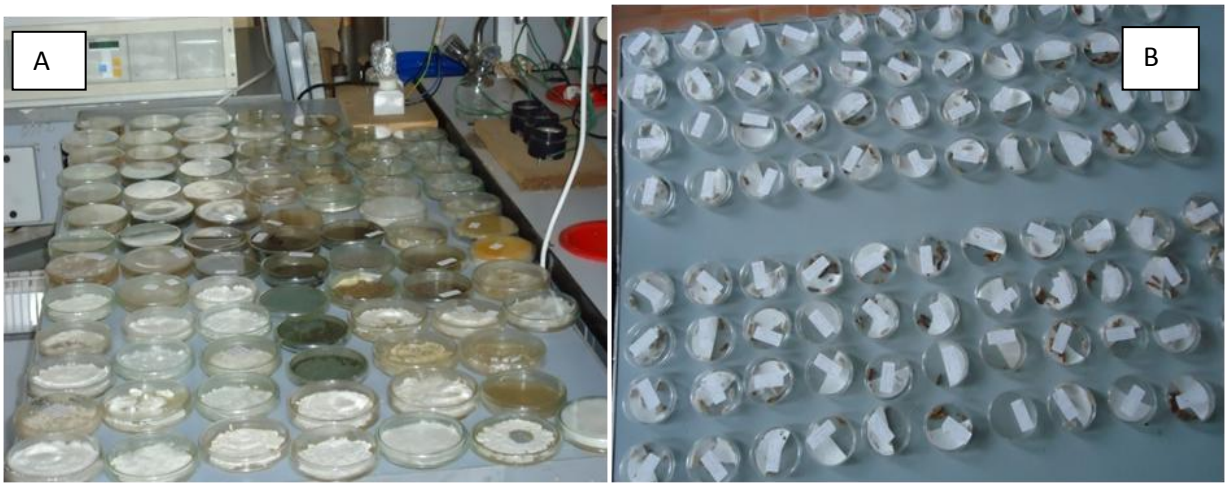

Figure 3. The screening process A) isolates being screened for viability on SDA media B) selected isolates being screened on *G. mellonella* larvae.

3.2.3. Rearing of *G. mellonella*

To obtain larvae, adult moths were collected in 500ml flasks containing folded tissue paper impregnated with water and honey. When eggs were laid, the tissue paper was removed from the flasks and put in plastic rearing boxes containing 180g, 50g and 180g of honey, wheat bran and glycerol, respectively as feed for the larvae. The boxes were incubated in the dark at 20^{O}C for four weeks. The resulting third to fourth instar larvae were used for the bioassays.

3.2.4. Pathogenicity test: the isolates selected from the initial screening (Table 2) were further evaluated for virulence against the wax moth, *G. mellonella* larvae (Figure 3, B).

Stock suspensions were prepared from the respective isolates as for the initial screening and spore concentration was adjusted to 1x 10^8 conidia/ml. The *Metarhizium* spp. isolates were bio-assayed in two batches, while the *Beauveria* spp. isolates were bio-assayed in three batches. For each isolate, 10 larvae were immersed in 10 ml conidial suspension for 10 seconds in a sterile beaker after which the contents of the beaker were passed through a sterile muslin cloth. The larvae were then transferred into 55mm diameter sterile plastic Petri-dishes containing filter paper and incubated at room temperature (22-26^{O}C). Control plots were treated with 10 ml solution of 0.01% Tween 80 only. The experiment was conducted in a completely randomized design (CRD) with four replications per isolate.

Mortality of the larvae was assessed every day for 10 days. Dead larvae were surface sterilized by briefly immersing in 70% of alcohol and quickly rinsing with sterile distilled water twice. Finally the larvae were transferred to a sterile Petri-dish containing wet filter paper, sealed with parafilm and incubated at room temperature to check for mycosis and confirm death due to the tested isolates.

3.2.5. Germination speed tests: The germination speed of all the four *Metarhizium* spp. and four *Beauveria* spp. isolates (selected from pathogenicity assay) was tested before being used in the virulence assays. Germination speed was evaluated in the same way as for viability test except that percentage germination was counted after 16 and 24 hours. A completely randomized design with three replications was used.

Table 2. Source (host /habitat), place of collection, genera and germination of isolates selected from the initial screening and used for bioassay on *G. mellonella* larvae

No.	Isolate code	Host/Habitat	Place of collection	Genera	% germination
1	PPRC 2	*P. interrupta*	Ashan	*Metarhizium*	93
2	9604	*Aceraea acerata*	Bugae (Arbaminch)	*Beauveria*	99
3	MP3POST(B)	Soil	Metahara	*Beauveria*	98.5
4	PPRC 51	*P. interrupta*	Shewa robit	*Metarhizium*	96
5	DLCO 131	N/A*	N/A	*Metarhizium*	99
6	MELKE 29	Soil	Melke/Tokekutaye	*Beauveria*	88.3
7	MELKE 36	Soil	Melke/Tokekutaye	*Beauveria*	96.4
8	9609	*Blosyus rugulosus*	Mugundo (Dilla road)	*Beauveria*	72
9	MELKE 4	Soil	Melke/Tokekutaye	*Beauveria*	78.2
10	MELKE 11	Soil	Melke /Tokekutaye	*Beauveria*	76.4
11	DLCO 54	Grasshopper	Wikro	*Beuveria*	72.1
12	PPRC 56	*P. interrupta*	Berbere	*Beauveria*	79
13	9605	N/A	N/A	*Beauveria*	70.4
14	DLCO 26	Grasshopper	Harmokale	*Metarhizium*	71.2
15	PPRC 27	*P. interrupta*	Sefi beret	*metarhizium*	73
16	DLCO 31	Grasshopper	Biyo	*Metarhizium*	70.5
17	Ke 43,s/no 03	Soil		*Beauveria*	79.4
18	PPRC 21	*P. interrupta*	Berbere	*Metarhizium*	72.3
19	PPRC 72	N/A	N/A	*Metarhizium*	70.6

20	DLCO 137	Soil	Wondogenet	*Metarhizium*	70.8
21	DLCO 78	Grasshopper	Maitimket	*Beauveria*	75.1
22	PPRC 67	*P. interrupta*	Kewet	*Metarhizium*	76.4
23	**9515	Spider	Awassa	*Beauveria*	73.9
24	DLCO 81	N/A	N/A	*Metarhizium*	70.9
25	F/no 1261A	Soil	Fincha	*Beauveria*	81.2
26	DLCO 8	*P. interrupta*	Shewarobit	*Beauveria*	73
27	PPRC 46	*P. interrupta*	Dedeaa	*Metarhizium*	70.5
28	DLCO 66	Grasshopper	Ibab	*Beauveria*	74.3
29	DLCO 44	Grasshopper	Wikro	*Beauveria*	70.6
30	PPRC 19	*P. interrupta*	Rufea kure	*Metarhizium*	83.4
31	DLCO 46	N/A	N/A	*Beauveria*	71.3
32	MELKE 19	Soil	Melke/Tokekutaye	*Beauveria*	83.8
33	DLCO 6	Soil	Shewarobit	*Beauveria*	80.1
34	DLCO 73	Grasshopper	Fura	*Beauveria*	70.4
35	DLCO 93	Grasshopper	Shelle	*Beauveria*	71.3
36	F/no 17A	Soil	Fincha	*Beauveria*	85.3
37	DLCO 56	Grasshopper	Fura	*Beauveria*	72.3
38	PPRC 29	*P. interrupta*	Gobenaytu	*Metarhizium*	84.3
39	MP3POST(M)	Soil	Metahara	*Metarhizium*	77.4
40	PPRC 6	*P. interrupta*	Kewet	*Metarhizium*	79.5
41	EE	Crustacean	Alamata	*Metarhizium*	70.5
42	9505	N/A		*Beauveria*	71.3

43	DLCO 135	Grasshopper	Ziway	*Metarhizium*	73.8
44	**MM	N/A	N/A	*Metarhizium*	70.4
45	Ke 42 s/no 06	Soil	Fincha	*Beauveria*	89.3
46	M2E	N/A	N/A	*Metarhizium*	70.1
47	ICIPE 30	*Busseola fusca*	Kendu bay(Kenya)	*Metarhizium*	74.2
48	PPRC 61	*P. interrupta*	Awaketu	*Metarhizium*	79.8
49	**DLCO 65	Grasshopper	Kobo	*Beauveria*	70.5
50	**DLCO 61	Grasshopper	Maitimket	*Beauveria*	72.4
51	**DLCO 105	Grasshopper	Shelle	*Beauveria*	70.4
52	**DLCO 39	Grasshopper	Qoriso	*metarhizium*	70.6
53	**DLCO 48	Grasshopper	Wikro	*Beauveria*	70.5
54	**DLCO 88	Grasshopper	Qoriso	*Beauveria*	70.8
55	**PPRC 14	*P. interrupta*	Dedeaa	*Metarhizium*	81.2
56	B42,3c	Soil	Fincha	*Beauveria*	86.2

* N/A = host information not available, ** not used in the anlaysis because of high control mortality

3.2.6. Virulence test: Three *M. anisopliae* and four *B. bassiana* isolates were selected from the pathogenicity assay for final bio-assay against *P. interrupta*. Adult beetles were collected during the mating season of 2013 from breeding areas around Mendubo village (10°50'N, 040°05'E; altitude 1206 m.a.s.l.) in Oromia zone of the Amhara Regional State in Ethiopia and kept in plastic baskets containing moistened sterile soil collected from the same area. The baskets had side openings for aeration and the tops were covered with

muslin cloth to prevent beetles from escaping. Collected beetles were fed with slices of ripe banana and observed for any natural infection for 10 days before being used for bio-assays.

Conidia of the selected isolates were re-isolated from sporulating cadavers of *G. mellonella* larvae and grown on SDA media at 25^0C. After 2-3 weeks of incubation, conidia were harvested with sterile metal spatulas and collected in sterile Petri-dishes. To remove excess moisture, the conidia were oven-dried at 30^0C overnight before being used for the bioassays. The number of spores/mg of the isolates were $3.03x10^8$ (9604), 2.9 x 108(9609), $3.5\ x10^8$ (MP3POST), $2.9\ x10^8$ (Melke36), $2.0\ x10^8$ (DLCO131), $1.4x10^8$ (Green Muscle), $1.7x10^8$ (PPRC51) and $3.9x10^8$ (PPRC2). The virulence test was conducted according to Lacey *et al*. (1994) with some modifications. Ten beetles were put in sterile 300ml plastic tubs with perforated lid. A 1mg spore sample of each isolate was added on top of the beetles in each of the plastic tubs and the beetles were allowed to move in the tubs for 30 minutes. The beetles were then transferred to 120mm diameter plastic Petri-dishes containing moist filter paper and incubated at room temperature for 10 days. During incubation, beetles were fed with slices of ripe banana changed every other day. To provide adequate moisture, 1ml of sterile distilled water was added to the Petri-dishes every day. Mortality was assessed every day and dead beetles were removed and surface sterilized with 70% ethanol and rinsed three times in sterile distilled water. The surface sterilized beetles were then transferred to sterile Petri-dishes containing moist filter paper and incubated at 25^0C to check for sporulation and to confirm death due to fungal infection. For this purpose, a completely randomized design (CRD) was used. There were four replications per isolate including control. Controls of each isolate were

treated with the respective spores killed at 80^0C in an oven for 48 hrs. The bioassay was repeated after 75 days using beetles from the October 2013 population from Rassa area (09^o 57'N and 040^0 04' E) and freshly sub-cultured and harvested spores as in the first bio-assay. Mean percentage mortality from the two experiments were used as measures of virulence. Comparison among selected most virulent isolates was done using their respective lethal time to kill 50% and 90% of the experimental animals (LT_{50} and LT_{90} values). The commercialized myco-insecticide Green Muscle containing *M. anisopliae* spores as an active ingredient was used as a standard in the two bioassays.

Table 3. Selected fungal species used for the virulence test and their origins

Isolate	**Fungal species**	**Origin of isolate (Host or Habitat)**	**Locality/Country**
9604	*B. bassiana*	*Aceraea acerata* (Lepidoptera: Nymphalidae)	Arbaminch/Ethiopia
9609	*B. bassiana*	*Blosyrus rugulosus* (Coleoptera: Curculionidae)	Dilla/Ethiopia
MP3POST	*B.bassiana*	Soil	Metehara/Ethiopia
Melke36	*B. bassiana*	Soil	Tokekutaye/Ethiopia
PPRC51	*M. anisopliae*	*Pachnoda interrupta* (Coleoptera :Scarabaeidae)	Shoarobit N.Shoa/Ethiopia
PPRC2	*M. anisopliae*	*Pachnoda interrupta* (Coleoptera :Scarabaeidae)	Ashan N.shoa/ Ethiopia
DLCO131	*M. anisopliae*	N/A	*N/A
GreenMuscle	*M. anisopliae*	*Schistocerica gregaria*	West Africa

*N/A= Host information not available

3.2.7. Dose response tests

Using field collected beetles from July 2013 mating season, a dose response bioassay was conducted on the three best isolates (PPRC51, PPRC2 and MP3POST) obtained from the first virulence assay. To determine the range of conidial doses used for the test, initial bioassay was conducted with six different doses (0.05, 0.1, 0.25, 0.5, 0.75, and 1 mg/10 beetles) without replications. The two lower doses (0.05 and 0.1 mg/10 beetles) did not result in any mortality within 10 days and therefore were excluded from the dose range. Thus, only four doses were used for the dose response tests. Dry conidia were prepared as for the virulence assay and weighed on a sensitive balance (Adventure ™ USA). All the procedures, design and replications used for the virulence assay were repeated exactly except that four different doses were applied.

3.3. Statistical analysis

All mortality data were adjusted for control mortality using Abbot's formula (Abbot, 1925) and arcsine transformed (Steel and Torrie, 1980) to normalize the data before being subjected to the ANOVA procedure of SAS statistical package version 9. Viability and germination speed data were also analyzed using the same package. Means were separated according to LSD and Tukey's Honestly Significant Difference (HSD) for Pathogenicity and virulence bioassays, respectively. For the three selected most virulent isolates, the responses of the days of each replicate were used for estimation of LT_{50} and LT_{90} with probit analysis for correlated data and comparison was done using ANOVA. Means were separated by LSD. The responses of the doses of each replicate were analyzed for estimation of lethal dose to kill 50% of the experimental animals (LD_{50}) of

the selected isolates as for LT_{50} and LT_{90}. Probit analyses were done using SPSS software Version 17 for windows (SPSS Inc.2002).

3.4. Results

3.4.1. Screening for viability: The percent germination of the 116 isolates evaluated for viability (Table 1) ranged from nearly 0% to maximum of 99%. Isolates with greater than 70% germination were considered viable and 47 isolates were selected for further screening (Tables 4 & 5).

3.4.2. Pathogenicity Tests: The 47 isolates selected in the initial screening were further screened against larvae of *G. mellonella*. Corrected percent mortality of larvae due to fungal isolates significantly varied among isolates of each of *Beauveria* spp. and *Metarhizium* spp. evaluated in 3 and 2 batches, respectively (Tables 4 & 5). The *Beauveria* spp. isolates caused between 0.19 % to 63%, 0.03% to 78% and 10% to 73% mortality in the first, second and third batches of bioassays, respectively. Similarly, the *Metarhizium* spp. isolates caused 0.03% to 83% and 6.68 % to 67% mortality in the first and second batches of the bioassays, respectively. Based on mortality, seven isolates (3 *Metarhizium* spp. vz. PPRC51, PPRC2, DLCO 131 and 4 *Beauveria* spp. vz. 9604, 9609, MP3POST and Melke 36) that caused 60% or more mortality on *G. mellonella* larvae were selected for the virulence assays against *P. interrupta* adults.

Table 4. Mortality of *G. mellonella* larvae caused by *Beauveria* spp isolates from Ethiopia in three batches of bioassay

First batch		**Second batch**		**Third batch**	
Isolate	**% Mortality ± SE**	**Isolate**	**% Mortality ± SE**	**Isolate**	**% Mortality ± SE**
9605	37.05 ± 16.22 abc	9505	0.03 ± 0 d	9615	23.33 ± 4.92 bcd
DLCO6	16.68 ± 10.65 bc	9604*	77.78 ± 13.75 a	DLCO31	33.33 ± 15.00 ab
DLCO8	16.68 ± 11.07 bc	9609*	60 ± 18.20 ab	DLCO54	45.83 ± 4.83 abcd
F/2NO17A	10.01 ± 7.57 c	DLCO135	0.03 ± 0.00 d	DLCO56	10.01 ± 7.57 d
F/2NO1261	16.68 ± 11.07 bc	DLCO44	13.34 ± 9.34 dc	DLCO81	16.68 ± 9.70 dc
Ke43S/NO	26.68 ± 13.18 abc	DLCO46	33.33 ± 9.34 bc	DLCO93	10.01 ± 7.57 d
MELKE29**	63.33 ± 3.93 a	DLCO61	13.33 ± 2.71 dc	MELKE11	53.33 ± 13.06 abc
MELKE36*	63.33 ± 7.22 a	DLCO73	10.01 ± 7.57 dc	MELKE19	33.33 ± 4.22 abcd
MELKE4	59.26 ± 15.77 ab	DLCO78	29.17 ± 7.08 bc	MP3POST*	73.33 ± 13.18 a
Ke42S/N0	0.19 ± 0 c	PPRC56	40 ± 9.96 bc		

* Indicate isolates selected for further screening. Means in the same column followed by similar letters are not significantly different according to LSD test at α=0.05.

** Not selected for not showing sporulation on *G. mellonella* larvae

Table 5. Mortality of *G. mellonella* larvae caused by *Metarhizium* spp isolates from Ethiopia in two batches of bioassay

First batch		**Second batch**	
Isolate	**% Mortality ± SE**	**Isolate**	**% Mortality ± SE**
EE	3.72 ± 6.19 bc	DLCO137	25.93 ± 13.70 b
M2E	0.03 ± 0.00 c	DLCO26	37.04 ± 5.87 ab
ICIPE30	0.03 ± 0.00 c	DLCO131*	66.67 ± 8.07 a
MP3POSTM	12.52 ± 12.29 bc	DLCO66	13.34 ± 9.34 b
PPRC2*	83.33 ± 11.07 a	PPRC19	13.34 ± 8.55 b
PPRC21	25 ± 0.00 b	PPRC27	36.67±3.93 ab
PPRC46	14.81 ± 2.89 bc	PPRC29	6.68 ± 8.55 b
PPRC67	22.23 ± 12.02 bc	PPRC51*	66.67 ± 14.70 a
PPRC72	23.82 ± 12.15 bc	PPRC6	7.42 ± 9.08 b

* Indicate isolates selected for further screening. Means in the same column followed by similar letters are not significantly different according to LSD test at α=0.05.

3.4.3. Germination speed tests: The germination speed varied significantly among the isolates (Table 6). Isolates 9604, MP3Post, DLCO 131 and PPRC 51 showed greater than 50% germination in 16hrs reaching 100 %, 98%,100 % and 94 % in 24hrs, respectively. Isolate Melke36 did not achieve 50% germination in 16 hrs, but its final germination in 24 hrs was 94.9 % which was not significantly different from the other isolates. The highest germination (100 %) in 24hrs was recorded from isolate 9604 and DLCO 131 followed by MP3Post, Melke 36 and PPRC 51 which scored 97.9 %, 94.9 % and 94.4 %, respectively. The least germination in 24 hrs (70.23 %) was recorded from the standard

isolate, Green muscle. Isolates 9609 and PPRC 2 scored 70.73 % and 70.6 % germination in 24 hrs, respectively and were not significantly different.

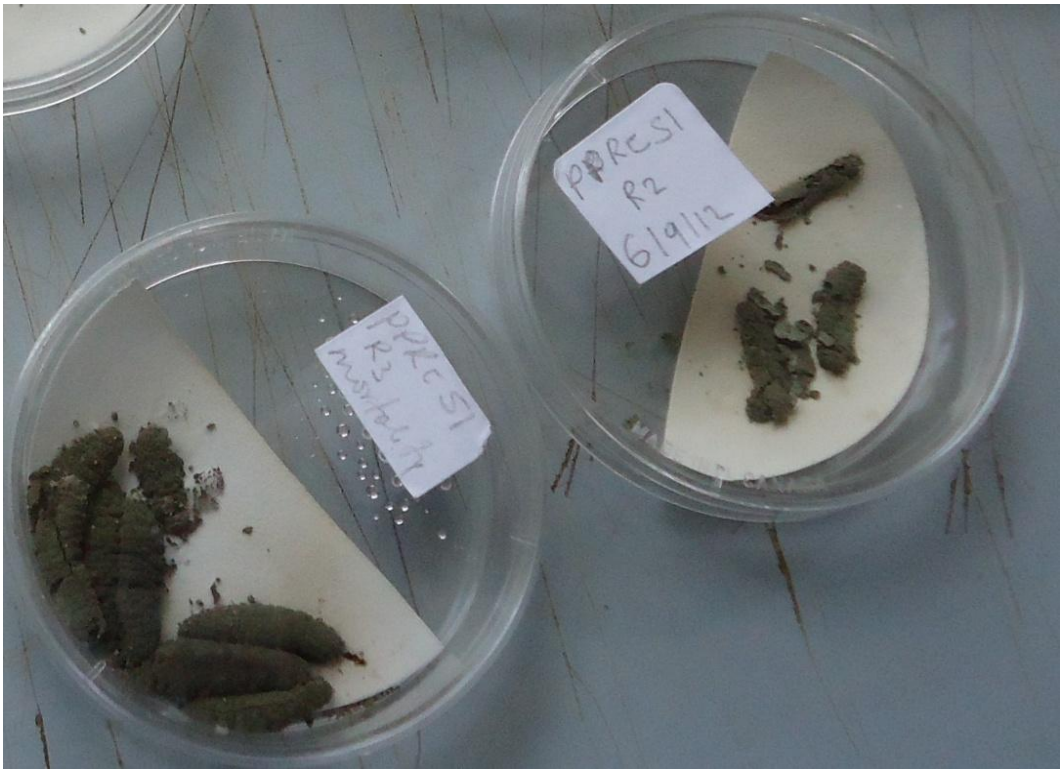

Figure 4. PPRC51 sporulating on *G. mellonella* larvae.

3.4.4. Virulence tests: There was a significant variation among the isolates (P=0.0003, df = 7, 16, F= 8.01) in causing mortality to *P. interrupta* (Figure 6). The lowest mean percent mortality was caused by the *B. bassiana* isolate 9604 (14.08 %) which was not significantly different from *B. bassiana* isolate 9609 (27.97 %), Melke36 (15.75 %), *M. anisopliae* isolates DLCO131 (25%) and GreenMuscle (20.38 %). The highest mortality to *P. interrupta* was caused by PPRC51 (82.40 %) which did not significantly differ from the *B. bassiana* isolate MP3POST and the *M. anisopliae* isolate PPRC2 which caused 79.63% and 77.14 % mortality, respectively.

Table 6. Mean percentage germination on SDA at 16hrs and 24 hrs post inoculation of *B. bassiana* and *M. anisopliae* isolates from Ethiopia selected for virulence assay against *P. interrupta*

Species	**Isolate**	**16hrs**	**24hrs**
Beauveria bassiana	9604	61.03±1.28 b	100±0 a
Beauveria bassiana	9609	11.2±0.46f	70.73±1.73b
Metarhizium anisopliae	DLCO131	83.6 ±1.76a	100±0 a
Metarhizium anisopliae	Green Muscle	25.07±1.43e	70.23±0.25b
Beauveria bassiana	MP3POST	62.63±0.81b	97.93±0.64 a
Beauveria bassiana	Melke36	32.63±1.42d	94.87±5.13 a
Metarhizium anisopliae	PPRC2	28.37±1.65e	70.6±2.80b
Metarhizium anisopliae	PPRC51	51.67±1.68c	94.43±1.19 a

Means in the same column followed by similar letters are not significantly different according to LSD test at α=0.05

Based on the results of the virulence assays, two *M. anisopliae* isolates (PPRC51 and PPRC2) and one *B. bassiana* isolate (MP3POST) which caused more than 75 % mortality agaist *P. interrupta* adults were selected. The time taken by the three selected isolates to cause death to 50% and 90 % of the experimental insects (LT_{50} and LT_{90} days) is shown in Table 7. The LT_{50} (days) of the selected isolates varied significantly (P = 0.0087, df= 2, 6, F= 11.6) with the lowest (5.33 days) recorded from PPRC51 followed by MP3POST (5.7 days). The highest (6.92 days) was recorded due to PPRC2 which was significantly different from both isolates. However, there was no significant difference (P= 0.057, df =2, 6, F= 4. 78) in the LT_{90} (days) of the three isolates.

Figure 5. *P. interrupta* infected with *M. anisopliae* isolates PPRC51 and PPRC2.

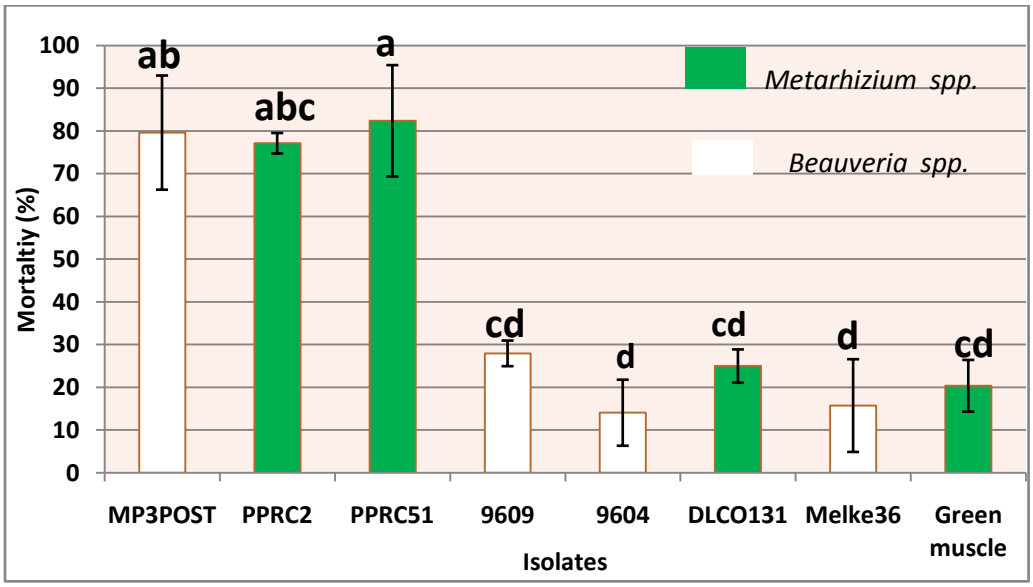

Figure 6. Mortality ± SE of *P. interrupta* due to *B. bassiana* and *M. anisopliae* isolates from Ethiopia. Bars containing similar letters are not significantly different according to Tukey's HSD test (α=0.05, df =7, 16, P=0.0003, F=8.01).

Table 7. Mean LT50 and LT90 (days ± SE) of the three selected isolates on *P. interrupta* adults 10 days post treatment

Isolate	LT_{50} ± SE	LT_{90} ± SE	Slope ±SE
PPRC2	6.92 ± 0.21 a	16.14 ± 1.17 a	3.56 ± 0.32 a
MP3POST	5.7 ± 0.25 b	12.59 ± 1.35 a	4.38 ± 0.59 a
PPRC51	5.33 ± 0.27 b	11.63 ±0.59 a	3.85 ± 0.34 a

Means with similar letters in the same column are not significantly different according to LSD test at α=0.05

3.4.5. Dose response tests

Mortality of *P. interrupta* adults due to the selected *M. anisopliae* and *B. bassiana* isolates at different doses is shown in figure 7. There were no significant mortality differences within each dose except for the dose of 0.5mg in which the *B. bassiana* isolate MP3POST showed significantly lower mortality ($P = 0.039$, $F = 5.78$, $df = 2, 6$) than the two *M. anisopliae* isolates. The mean LD_{50} of the three isolates also varied significantly ($P=0.025$, $F=7.12$, $df=2,6$) among the isolates with isolate MP3POST requiring higher dose (0.8mg/10 beetles) to kill 50% of the experimental insects than PPRC2 (0.62mg/10 beetles) and PPRC51 (0.55mg/10beetles) isolates which were not significantly different from each other. There were no significant differences in the mean LD_{90} among all the three isolates ($P= 0.41$, $F= 1.04$, $df = 2, 6$) (Table 8).

Table 8. Mean LD_{50} and LD_{90} (mg/10 beetles ± SE) of the three selected isolates on *P. interrupta* adults 10 days post treatment and slope of the regression line ± SE. Means with similar letters in the same column are not significantly different according to LSD test at α=0.05

Isolate	LD50 ±SE	LD90 ± SE	Slope ± SE
PPRC2	0.62 ± 0.075 b	1.72 ± 0.21 a	2.89 ± 0.18 a
PPRC51	0.55 ± 0.029 b	1.83± 0.17a	2.53 ± 0.33 a
MP3POST	0.8 ± 0.029 a	2.22± 0.34 a	3.13 ± 0.48 a

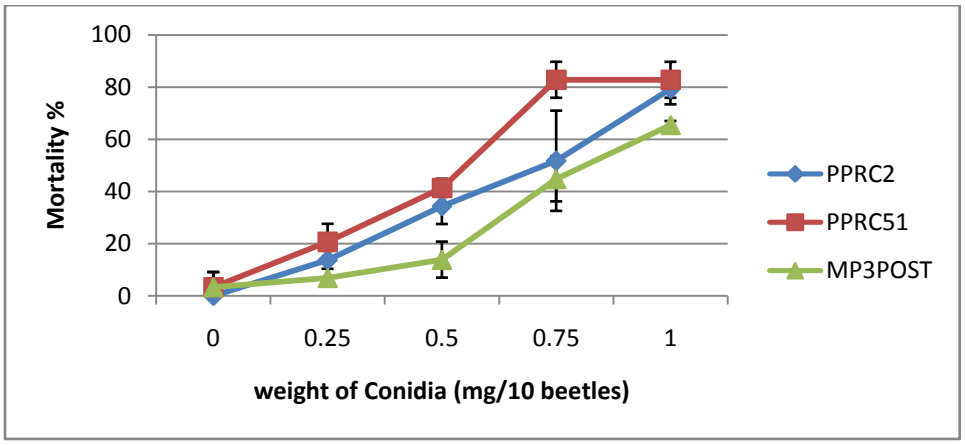

Figure 7. Mean percentage mortality ± SE caused by the selected isolates of *B. bassiana* and *M. anisopliae* on *P. interrupta* adults at different doses per 10 beetles (10days after application).

3.5. Discussion

Although there is no published research information on the use of *M. anisopliae* and *B. bassiana* against *P. interrupta*, research on another scarab beetle *P. japonica* (Lacey *et.al*., 1994; Klein and lacey, 1999) have shown the potential of these fungi for control of coleopterous insects. Similarly, this study has demonstrated the importance of these entomopathogenic fungi as potential microbial biocontrol agents for *P. interrupta*. Kassa *et al*., (2002) used similar multiphase screening approach to allow evaluation of many

isolates of entomopathogenic fungi. This method allows evaluation of many isolates in shorter period and economizes time. Among 116 isolates initially collected from sources in the country, 60 were excluded from further evaluation for exhibiting less than 70% viability on SDA media, while 9 were excluded from the analysis due to high control mortality. Thus, evaluation of 47 isolates on *G. mellonella* larvae resulted in identification of 7 (4 *Beauveria* spp. and 3 *Metarhizium* spp.) candidates (Table 3).

From among the seven isolates, the *M. anisopliae* isolate PPRC51 which caused 82.40 % mean mortality on field collected *P. interrupta* adults was selected as the most promising candidate for the management of *P. interrupta*. As shown in figure 4 and figure 5, PPRC51 sporulated profoundly on *G. mellonella* larvae and on *P. interrupta* adults. This isolate also took the shortest time (5.33 days) to kill 50 % of the experimental insects. The *B. bassiana* isolate MP3POST and *M. anisopliae* isolate PPRC2 which caused 79.67 % and 77.14 % mortality, respectively, were also selected for field evaluation.

The findings of this study have indicated the potential use of entomopathogenic fungi against *P. interrupta* that can be used for dissemination of virulent strains in the natural habitat of the pest for gradual decrease of its population over time. The virulent isolates PPRC51 and PPRC2 are recommended as potential candidates for development of myco-pesticide against *P. interrupta* for integrated management of the pest. However, field verifications of the laboratory findings are highly recommended.

CHAPTER IV

MOLECULAR IDENTIFICATION AND CULTURAL CHARACTERISTICS OF ENTOMOPATHOGENIC FUNGAL ISOLATES FROM ETHIOPIA TESTED FOR VIRULENCE AGAINST *Pachnoda interrupta*

4.1. Introduction

Sorghum chafer (*Pachnoda interrupta* (Olivier) (Coleoptera: Scarabaeidae) is a serious pest of sorghum with a wide host range attacking over 37 crops and non crop plants that belong to more than twenty families in Africa in general and in Ethiopia in particular (Grunshaw, 1992; Hiwot, 2000). *P. interrupta* destroys sorghum fields completely during the out breaks (Tsedeke, 1988). The adult beetle of *P. interrupta* is the damaging stage and feeds on the flowers and sucks all the contents of sorghum, maize and wheat grains at milk stage (Grunshaw, 1992; Jago, 1993; Troure and Yehouienou, 1995). Yield losses ranging from 70–100 % were recorded even on insecticide treated sorghum fields (Yitbarek and Hiwot, 2000; Yeraswork, 2000). During the huge outbreaks, between 1993 to 2000, in Ethiopia, a single beetle observed on sorghum head was considered as an economic threshold to take action (Hiwot, MOA, Personal communication).

The current control methods of *P. interrupta* mainly depend on direct application of sprays and baits of synthetic insecticides (Seneshaw, 2001). Use of pesticides is not a sustainable method of pest control because of the detrimental effects on the environment and the ecology. Repeated use of pesticides may also induce pesticide resistance which in turn stimulates more frequent sprays at even higher concentration leading to more aggravated situations (Watkins *et al*., 2012).

Developing and using biopesticides in an integrated pest management system has a potential to avoid the detrimental effects of pesticides. Among the biopesticides, microbial biopesticides which include the ones developed from organisms such as fungi, bacteria, viruses, protozoa and nematodes are the most commonly used agents (Gupta and Dikshit, 2010). The microbial bio-control agents (MBCA's) can destroy pests causing no detrimental effects to human health and the environment and pest resistance to MBCA's is less likely because of their complex mode of action (Khan *et al*., 2012). Entomopathogenic fungi are most preferred MBCA's because of wide host range, ease of production and application, mode of action that does not need ingestion of the entomopathogen by the target pest and improvements in formulations (Butt, 2002; Wang and St. Leger, 2007; Thomas and Read, 2007).

Developing microbial biopesticides in modern scientific studies demands identification and characterization of selected microbial agents. Fungi can be identified using morphological characteristics but this method is not adequate due to ambiguous descriptions and limited availability of morphological keys (Fernandes *et al.,* 2010). Identification, characterization and analysis of genetic traits of fungi and other micro-organisms is facilitated by accurate and powerful molecular tools whereas it would be difficult if not impossible with conventional morphological, physiological and biochemical methods (Inglis *et al*., 2012). Moreover, molecular identification of potential entomopathogenic fungi is gaining acceptance as an important first step for successful development of myco-insecticides as it makes taxonomic studies easier (Islam *et al.*, 2014). Particularly, molecular tools such as polymerase chain reaction (PCR) based analysis of DNA sequences are being used widely as standard procedures for

identification and phylogenetic comparisons between entomopathogenic fungi (Jensen *et al*., 2001; Destefano *et al*., 2004). Ribosomal DNA is also used as molecular marker for species identification (Islam *et al.*, 2014) as it reveals variations in sequence for taxonomic studies (Fouly *et al*., 1997). Molecular techniques based on DNA are accurate tools for species identification (Driver *et al*., 2000; Entz *et al*., 2005).

The objective of this study was to conduct molecular identification of selected entomopathogenic fungal isolates pathogenic to *P. interrupta* and to characterize the same using measurable *in-vitro* parameters on different artificial media and across five temperature regimes.

4.2. Materials and methods

4.2.1. Molecular identification

4.2.1.1. DNA extractions

The Entomopathogenic fungi from Ethiopia were cultured on solid saboroud dextrose agar (SDA) media. For each fungal isolate, 50-100 mg of the mycelia were scrapped and DNA extracted using the Isolate II Plant DNA Kit from Bioline, following the manufacturer's protocol. The resultant DNA was eluted in 50 µL of the elution buffer and stored at -20^{o}C, to be used later. The extracted DNA quality was then checked using Nanodrop 2000/2000c Spectrophotometer.

4.2.1.2. PCR amplification of the ITS4 and ITS5 gene region

Amplifications were carried out for the rDNA region of the fungal isolates using the ITS primers (White *et al*., 1990). The PCR was carried out in a total volume of 30 µL containing 0.2 µM of each primer, (ITS 5; 5'GGA AGT AAA AGT CGT AAC AAG G

3’ and ITS 4; 5’ TCC TCC GCT TAT TGA TAT GC 3’, respectively), 5X My *Taq* Reaction Buffer (Bioline), 1.25 mM $MgCl_2$, 1 unit My *Taq* DNA polymerase (Bioline) and 3 µL of genomic DNA template. Typical cycle conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 59 °C for 40 sec, and primer elongation at 72°C for 1 min followed by a final extension at 72 °C for 10 min done in an Arktik programmed thermalcycler.

4.2.1.3. PCR amplification using chi 1 and chi 4 primers

The results from the ITS gene region amplification above were also confirmed by amplifying the chitinase gene region using redesigned c*hi* 1 and *chi* 4 primers. In this regard, markers were designed from the chitinase gene to discriminate between the two species through gelelectrophoresis. The PCR was carried out in a total volume of 30 µL containing 0.2 µM of each specific primer, 5X My *Taq* Reaction Buffer (Bioline), 1unit My *Taq* DNA polymerase (Bioline) and 3 µL of DNA template. *Chi* 1 primer set gave a target region of 800 bp while the *Chi* 4 primer set had a target size of approximately 1000 bp. Typical cycle conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 sec, specific annealing for 15 sec, and primer elongation at 72°C for 10 sec followed by a final extension at 72°C for 10 min that was done in an Arktik programmed thermal cycler.

4.2.1.4. Detection and analysis of the PCR products

The amplified PCR products were resolved through a 1% agarose gel stained with ethidiumbromide (10 mg/mL) and subjected to electrophoresis set at 70 volts for 1 hr (Bio-Rad model 200/2-0 power supply and wide mini-sub cell GT horizontal

electrophoresis system, Bio-Rad laboratories, Inc., USA), followed by visualization of the DNA under UV-illumination. The gel photo was analyzed and documented using the KETA GL imaging system from Wealtec Corp.

4.2.1.5. Gel extraction and purifications

All successfully amplified PCR products for each of the targeted gene region were excised and purified using the Isolate II PCR and Gel Kit from Bioline following the manufacturer's instructions. A total of 17 purified PCR products were sent to Macrogen Inc, Europe Laboratories, the Netherlands for bi-directional sequencing.

4.2.1.6. Sequencing data analysis

This was done using bioinformatics tools and software. The sequences obtained were cleaned up and edited by Chromas Lite version 2.1.1 software and the consensus sequences from both the forward and reverse strands generated. For conclusive identification, the consensus reads generated were queried through BLASTN, at the GenBank data base hosted by the National Centre of Biotechnology Information (NCBI). This was also to check for similarity with organisms already identified.Table 9 illustrates the characterized EPF from Ethiopia. Furthermore, the consensus sequences generated were multi-aligned using ClustalX software (version 2.1) program. The multiple alignments created were used to generate a phylogenetic tree by use of Mega software program (version 6.06).

4.2.2. Cultural characteristics

The cultural characteristics of five isolates of entomopathogenic fungi i.e. three *Metarhzium* spp. (PPRC51, PPRC2, IC69) and two *Beauveria* spp. (MP3POST, IC279)

were compared using the measurable parameters of germination, radial growth and sporulation in four artificial media at 5 different temperatures. Out of the five isolates characterized *in-vitro*, IC69 and IC279 were selected based on their performance on other coleopterous insects to serve as standards for comparison with the isolates from Ethiopia and were not tested for virulence against the target pest *P. interrupta*. The three isolates from Ethiopia (PPRC51, PPRC2 and MP3POST) were selected from the seven isolates on the basis of their virulence to *P. interrupta.*

4.2.2.1. Radial growth: Potato dextrose agar (PDA), saboroud dextrose agar (SDA), saboroud dextrose agar yeast (SDAY) and malt extract agar (MEA) were used. Conidial suspensions of the respective isolates were prepared by scrapping conidia from the surfaces of three weeks old SDA cultures of the respective isolates with a sterile metal spatula and suspending it in 10 ml sterile water containing 0.05% Triton – X – 100. The concentration of the conidial suspensions was adjusted to 1 X 10^8 conidia/ml and 5 µl of the respective suspensions was transferred to the center of each 8.5 cm Petri-dish containing the respective media immediately after preparation. The back of the Petri-dishes was marked with two perpendicular diameters that served as reference points for radial growth measurements. Each Petri-dish served as a treatment and there were three replications per treatment. The inoculated media were then incubated at 15^oC, 20^oC, 25^oC, 30^oC, and 35^oC. The radial growth of each fungus isolate was measured every 3 days for 12 days by taking the average growth in millimeters along two pre-drawn perpendicular reference lines on the back of the Petri-dishes.

4.2.2.2. Germination assay: Media were prepared as in the radial growth assessment and 100µl of a $1X10^6$ conidia/ml suspension of the respective isolates was transferred to each medium and uniformly spread throughout the Petri-dish with a sterile glass rod. These were then incubated at different temperatures as in the radial growth assessment. Germination was stopped after 24 hrs by applying 1ml of lactophenol blue and three sterile cover slips were put in each medium. Percentage of germination was computed by counting at least 200 germinated and un-germinated spores under x400 magnification of a compound microscope.

4.2.2.3. Spore production: Media and conidial suspensions of the respective isolates were prepared as in radial growth and germination assay respectively. Suspension of the respective isolates containing $1X10^6$ conidia/ml was transferred to each medium at 100µl per Petri-dish and uniformly spread throughout the Petri-dish with a sterile glass rod. These were then incubated at the different temperatures as in the radial growth evaluation. After three weeks of incubation, spores were harvested from the fully sporulated cultures by scrapping conidia from the surfaces of three weeks old cultures of the respective isolates and media first with a sterile metal spatula and collecting in a sterile flask. This was then followed by washing the surfaces with two rounds of 50ml of 0.05% Triton – X – 100 to fully dislodge the conidia to the flask. The harvested conidia were counted using an improved neubaur heamocytometer after vortex-shaking the suspensions for two minutes.

4.2.2.4. Statistical analysis: Completely randomized design (CRD) with a 3x5x5 (media X isolate X temperature respectively) factorial arrangement was used and three way analysis of variance (ANOVA) was conducted on

all the data from the experiments. Germination percent data were arcsine transformed to normalize the variance before being subjected to the ANOVA procedure of SAS statistical package version 9. Means were separated according to Tukey's Honestly Significant Difference (HSD) test.

4.3. Results

4.3.1. Molecular identification

The internal transcribed spacer region (ITS) was successfully amplified in all of the isolates as shown in Figure 8. Figures 9 and 10 show the amplification of the two fungal genera; *Metarhizium* and *Beauveria*, respectively using c*hi*1 and *chi*4 genes. The BLAST searches corresponded to sequences registered under *M. anisopliae* and *B. bassiana* and were provided with accession numbers as depicted in Table 9. This table confirms the identities of the 7 isolates. Three of the samples were linked to *M. anisopliae* and four to *B. bassiana* in the GenBank database.

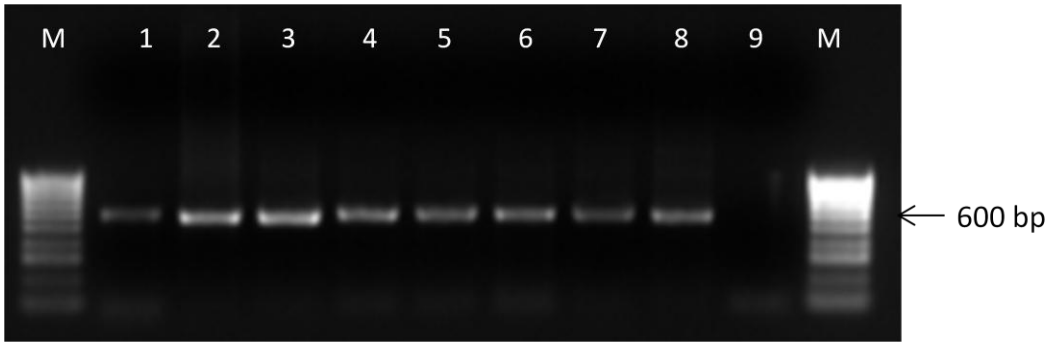

Figure 8. 1% agarose gel electrophoresis for fungal isolates from Ethiopia, using ITS primers. M=1Kb DNA ladder, Lane 1= IC 279, Lane 2=PPRC5, Lane 3= PPRC2, Lane 4= MP3POST, Lane 5= Melke36, Lane 6= 9609, Lane 7= 9604, Lane 8= DLCO131, Lane 9= Negative control.

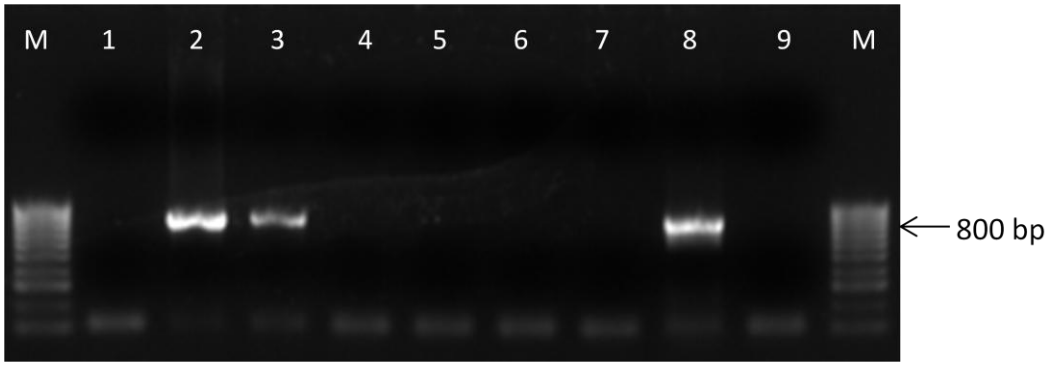

Figure 9. 1% agarose gel electrophoresis for fungal isolates from Ethiopia, using *chi* 1 primers. M= 1Kb DNA ladder, Lane 1= IC 279, Lane 2= PPRC51, Lane 3= PPRC2, Lane 4= MP3POST, Lane 5= Melke36, Lane 6= 9609, Lane 7= 9604, Lane 8= DLCO131, Lane 9= Negative control.

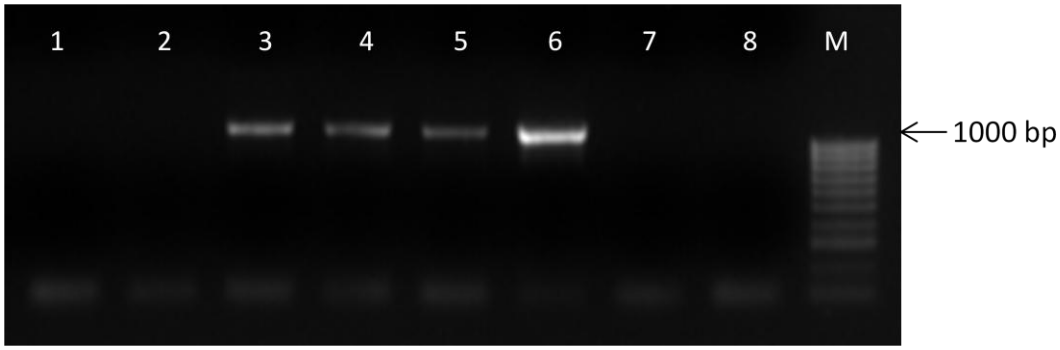

Figure 10. 1% agarose gel electrophoresis for fungal isolates from Ethiopia, using *chi* 4 primers. M=1Kb DNA ladder, Lane 1= PPRC51, Lane 2= PPRC2, Lane 3=MP3POST, Lane 4= Melke36, Lane 5= 9609, Lane 6= 9604, Lane 7= DLCO131. Lane 8= Negative control.

Table 9. Characterization of the Entomopathogenic fungi selected from Ethiopia

Sample Name	Synonymous isolate from NCBI	Accession no.	% identity
BL2 (PPRC51)	*Metarhizium anisopliae*	JF495775.1	99
BL3 (PPRC2)	*Metarhizium anisopliae*	FJ545313.1	99
BL4 (MP3POST)	*Beauveria bassiana*	KC461112.1	100
BL5 (Melke 36)	*Beauveria bassiana*	KC461112.1	100
BL6 (9609)	*Beauveria bassiana*	KC461112.1	100
BL7 (9604)	*Beauveria bassiana*	GU233705.1	99
BL8 (DLCO131)	*Metarhizium anisopliae*	JN256674.1	99

The sequences from the ITS gene region were also used to generate a phylogenetic tree (Figure 11). The evolutionary history was inferred using the Neighborjoining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.19117607 is shown in figure 11. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site.

The analysis involved 7 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 518 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013). The tree clustered the *Metarhizium* and *Beauveria* species into two clusters, as expected.

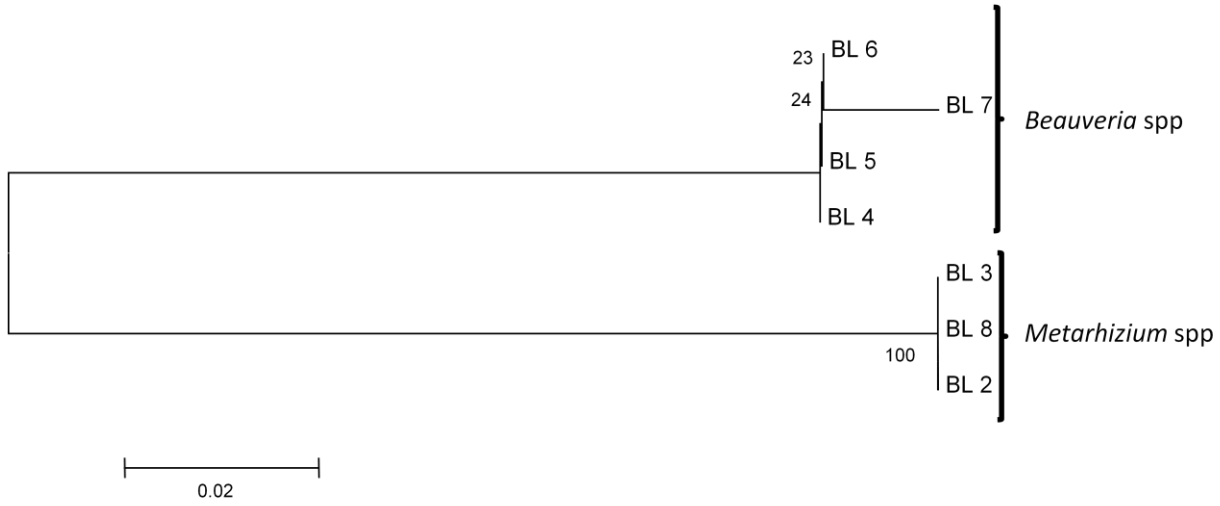

Figure 11**:** Evolutionary relationships of the Ethiopian EPF using the ITS gene region. BL2 = PPRC51, BL3 = PPRC2, BL4 = MP3POST, BL5 = MELKE36, BL6 = 9609, BL7= 9604.

4.3.2. Cultural characteristics

4.3.2.1. Germination assay: There was a highly significant difference in germination of the spores among all the isolates ($P<0.0001$, df = 4, 200, $F=69.72$) across all the temperatures ($P<0.0001$, df= 4, 200, $F=1438.69$) and all the media ($P<0.0001$, df= 3, 200, $F=31.92$). The interactions between isolates and media ($P<0.0001$, df= 12, 200, $F=30.59$) isolates and temperatures ($P<0.0001$, df= 16, 200, $F=58.44$) and among all the three factors ($P<0.0001$, df= 60, 200, $F=17.21$) were also significant.

As shown in figure 14, none of the isolates achieved 100% germination on any of the media at temperatures of 15^{o}C and 35^{o}C except for MP3POST which did so at 35°C on MEA. All the isolates achieved between 60-100% germination on all the media and at temperatures of 20^{o}C, 25^{o}C and 30^{o}C except PPRC2 which germinated generally below 40% on SDA, PDA and SDAY. At 15^{o}C, all the isolate on all media showed below 27%

germination except for PPRC51 which achieved 94% germination on SDA media. Relatively better germinations were recorded at the highest temperature (35^{o}C) than at the lowest temperature 15^{o}C. At 35^{o}C Generally greater than 50% germination was achieved by all the isolates on all media except for IC-69 (38% on MEA), IC-279 (21% on SDAY and 34% on MEA) and MP3POST (39% on PDA, 38% on SDA and 20% on SDAY).

4.3.2.2. Radial growth: The cumulative radial growths of the tested isolates of *B. bassiana* and *M. anisopliae* varied significantly among all the isolates ($P<0.0001$, df = 4, 200, $F=132.92$) across all the temperatures ($P<0.0001$, df= 4, 200, $F=817.9$) and all the media ($P<0.0001$, df= 3, 200, $F=115.3$). The interactions between isolates and media ($P<0.0001$, df= 12, 200, $F=21.74$) isolates and temperatures ($P<0.0001$, df= 16, 200, $F=48.62$) and among all the three factors ($P<0.0001$, df= 60, 200, $F=18.2$) were also significant.

Figure 14 shows the mean values of radial growths of the isolates at different temperatures and media. The radial growth of PPRC51 on SDA and PDA media at the respective temperatures is shown in Figure 12. The highest (31.17mm) and lowest (0.33mm) radial growths were recorded from MP3POST on SDA at 20^{o}C and SDAY at 35^{o}C respectively. All the isolates generally showed less than 10mm of radial growth at the lowest (15°C) and the highest (35°C) temperatures in all the artificial media except IC-69 (14.17mm on SDA) and MP3POST (22.5 on SDAY). In contrast, temperature ranges of 20 °C - 30°C supported better vegetative growth of the isolates in all the media. The second highest radial growth was recorded from PPRC51 (30.83mm) at 30°C on

SDAY media. This isolate also had better vegetative growth at this temperature on all the other media. Similarly, PPRC 2 showed the highest vegetative growth (19.83mm) on the same medium (SDAY) at 25^{o}C.

4.3.2.3. Evaluation of spore production: the sporulation capacity of the isolates was evaluated based on the number of spores/ml. As in the radial growth and germination assay, highly significant variations were observed in spore production among all the isolates ($P<0.0001$, df = 4, 200, $F=7.62$) across all the temperatures ($P<0.0001$, df= 4, 200, $F=1525.25$) and all the media ($P<0.0001$, df= 3, 200, $F=27.34$). Significant variations were also observed in the interactions between isolates and media ($P<0.0001$, df= 12, 200, $F=8.32$), isolates and temperatures ($P<0.0001$, df= 16, 200, $F=45.93$) and among all the three factors ($P<0.0001$, df= 60, 200, $F=5.26$).

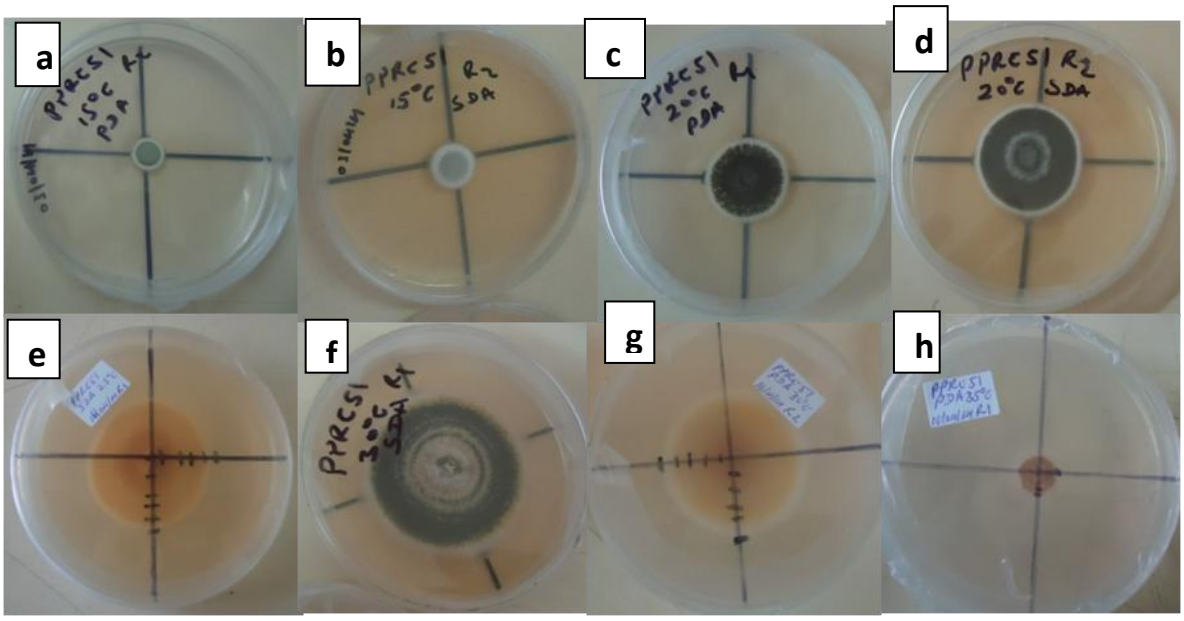

Figure 12: Radial growth and sporulation of PPRC51 isolate of *M. anisopliae* on SDA and PDA media at 15^{o}C (a and b), 20°C (c and d), 25°C (e), 30°C (f and g), 35°C (h) in 12 days.

The number of spores/ml produced at 15^{o}C and 35^{o}C were below $0.13x10^{10}$ and $1.2x10^{10}$ respectively. High spore production was observed at temperatures of 20^{o}C, 25^{o}C, and 30^{o}C with all the isolates producing above $2.23x10^{10}$ spores/ml (figure 15). The highest number of spores was produced by IC-279 at 25°C on SDA medium ($8.77x10^{10}$ spores/ml) followed by the same isolate and temperature on SDAY ($8.65x10^{10}$ spores/ml). The lowest number of spores ($3.13x10^{10}$ res/ml) at this temperature was obtained from PPRC2 on MEA. At 30°C, the same isolate produced the lowest number of spore ($2.23x10^{10}$ spores/ml) on MEA medium. On SDA media, PPRC51 produced the highest spores/ml ($7.67x10^{10}$ spores/ml) followed by IC-69 ($7.58X10^{10}$ spores/ml), while PPRC2 produced $7.6\ x10^{10}$ spores/ml at the same temperature on SDAY media. Two of the isolates from Ethiopia (PPRC51 and PPRC2) produced higher number of spores at 25^{o}C and 30^{o}C compared to other temperatures and isolates. However, MP3POST produced comparable numbers of spores at 20^{o}C.

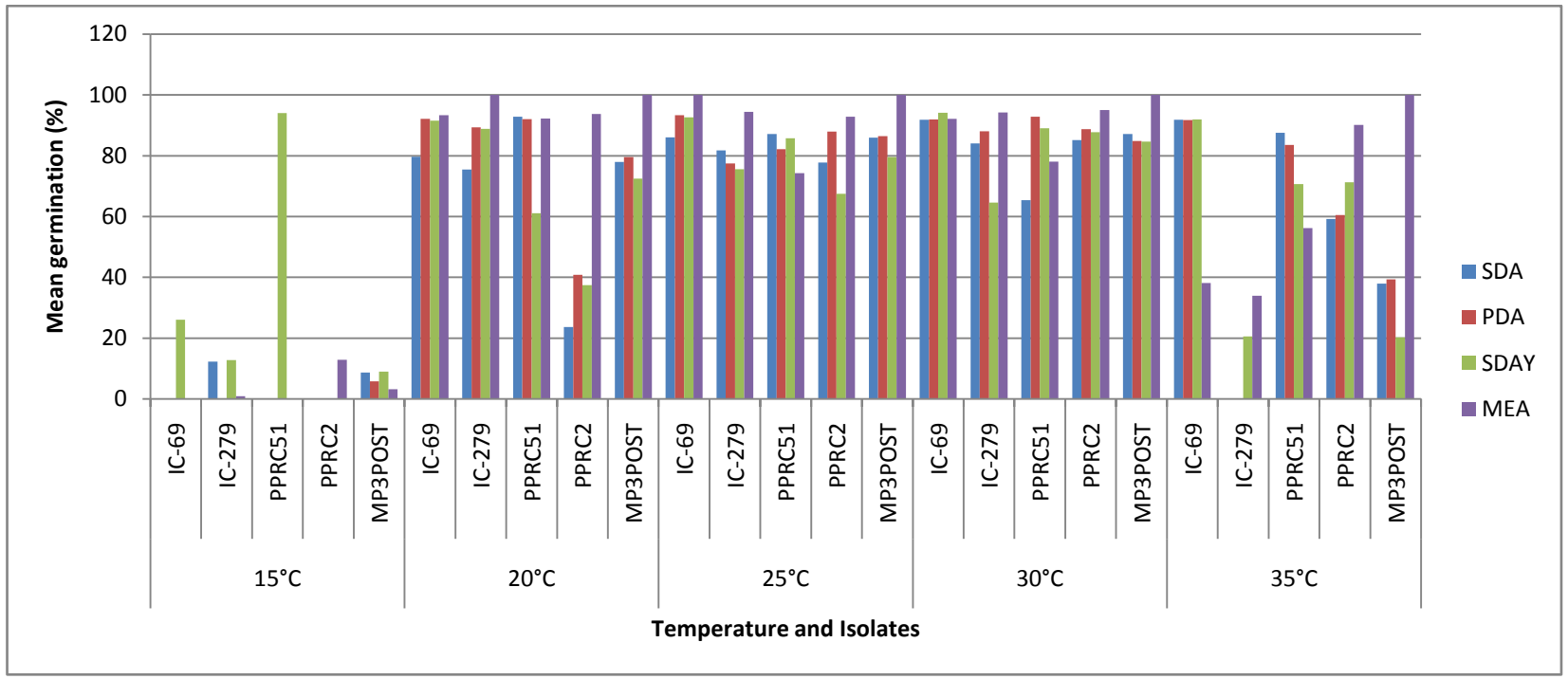

Figure 13. Germination of 2 *B. bassiana* and 3 *M. anisopliae* isolates on different artificial media and temperatures.

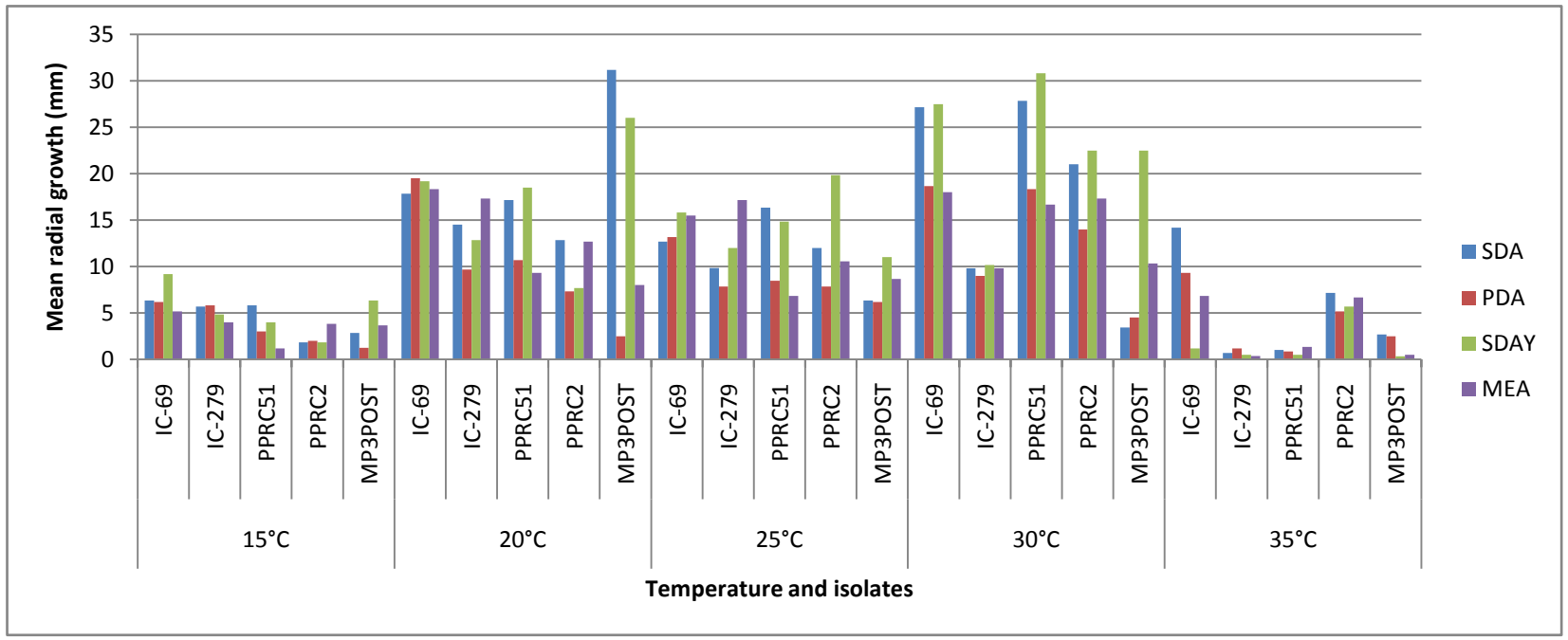

Figure 14. Radial growth of 2 *B. bassiana* and 3 *M. anisopliae* isolates on different artificial media and temperatures.

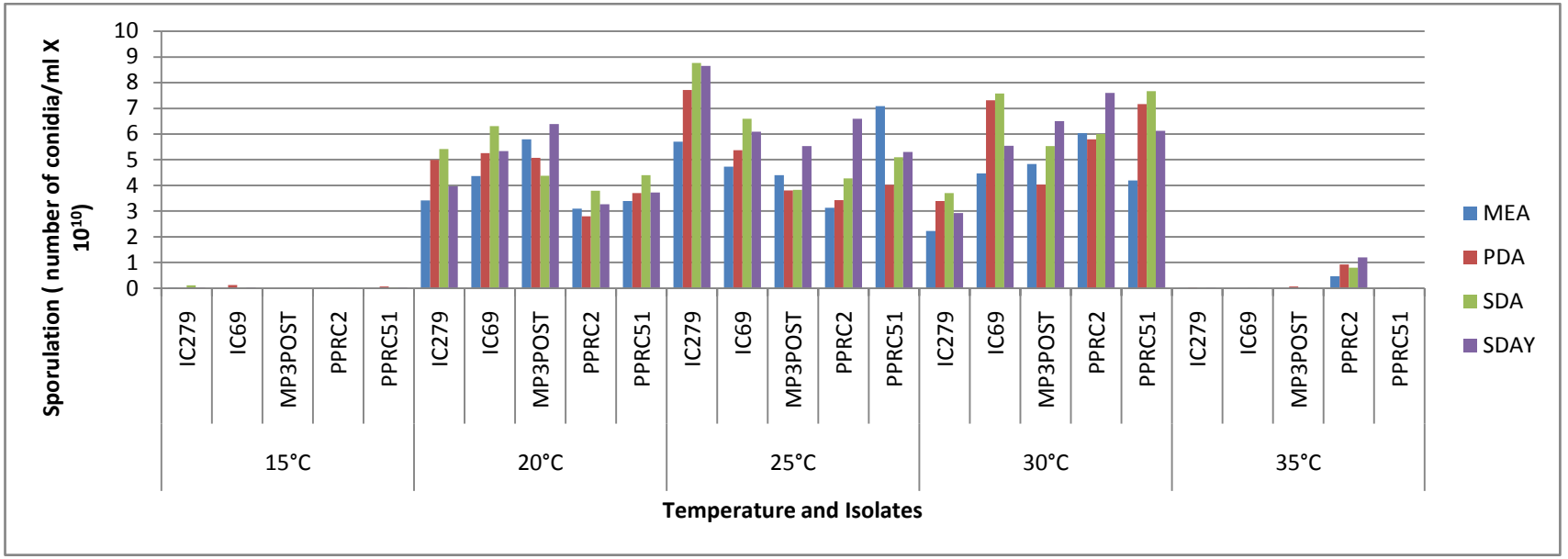

Figure 15. Sporulation of 2 *B. bassiana* and 3 *M. anisopliae* isolates on different artificial media and temperatures.

4.4. Discussion

The entomopathogenic fungi from Ethiopia used in the study were effectively identified as *M. anisopliae* and *B. bassiana* by the PCR amplification of the ITS regions of rDNA and chitinase genes. ITS PCR primers are used to amplify DNA from a wide range of fungi (Kendall and Rygiewicz, 2005).These molecular phylogenetic tools are also being used frequently for accurate taxonomic identification of entomopathogenic fungi. ITS1 and ITS4 primers amplify ITS1 and ITS2 sequences around the 5.8s coding sequence lying between the small sub unit (SSU) sequence and the large sub unit (LSU) sequence of the operon (White *et. al.,* 1990). The ITS region which is a highly conserved rDNA sequence is commonly used as a sole tool or supplemented with other universal sequences (e.g. tubulin and actin) for identification, characterization and phylogenetic analysis of fungal isolates (Balazy *et al*., 2008).

In this study ITS5 and ITS4 were used as forward and backward primers and were able to successfully identify the fungal isolates. In comparison to the present study Islam *et al*. (2014) used the rDNA of fungal isolates for PCR amplification of ITS1 and ITS4 regions and confirmed their taxonomic identity as *M. anisopliae***.** Similarly, Chitinase genes were used as molecular markers for *M. anisopliae* (Bogo *et al*., 1998 ; Kang *et al*., 1999; Enkerli *et al*., 2009). Niassay *et al*. (2013) reported no differences in nucleotide sequences of *chi2* and *chi4* genes and protein structures of 13 *M. anisopliae* isolates regardless of the origins of the isolate indicating the importance of these chitinase genes as molecular markers for *M. anisopliae* although genotyping may be difficult using these genes. Similarly, in this study *chi4* and *chi1* primers were used as molecular markers to supplement identification of the *B. bassiana* and *M. anisopliae* isolates respectively.

Germination, sporulation and radial growth have been used in understanding pathogenicity (Liu *et al*., 2003) and characterization of entomopathogenic fungi (Almeida *et al*., 2005). Direct relationships between speed of infection of the host and increased radial growth have been suggested (Feijo *et al*. 2007). In addition to these, high spore production by the entomopathogen is an important factor for dispersal and epizootics (Mitchell, 2003).

In this study the tests for germination, radial growth and sporultion at different temperatures and media have shown variations that indicated the relative thermal and nutritional preferences of the isolates. In general germination was fairly high between 20°C and 35°C on all the artificial media. Similar findings were reported by other studies (Tefera ane Pringle, 2003; Dimbi *et al*., 2004, Bugeme *et al*., 2008). However, germination of the *B. bassiana* isolate IC279 was relatively lower at 35°C which is in agreement with the findings of Bugeme *et al*., (2008) who reported that *B. bassiana* isolates including IC279 exhibited lower germination at this temperature as compared to *M. anisopliea isolates.* In contrast, Ekesi *et al*. (1999) reported no significant difference in germination between isolates of *B. bassiana* and *M. anisopliae* at 15^{o}C, 20^{o}C, 25^{o}C, and 30^{o}C while differences occurred only at 35^{o}C. In this study however, significant differences were observed at these temperatures across all the media. This can be attributed to nutritional and strain variability. Conidial germination, spore production and other *in-vitro* characteristics of entomopathogenic fungi are known to be affected by strain variability and nutritional compositions of the media such as carbon sources, carbon : nitrogen ratio and their respective concentration (Leland *et al*., 2005; Shah, *et al*., 2005).

In addition to these, conidial germination is adversely affected by and rapidly slows in temperatures above 30^{o}C with the optimum being between 23^{o}C and 28^{o}C and most entomopathogenic fungi isolates reducing to half at 34- 37 ^{o}C (Jaronski, 2010). Instability of conidia at high temperature indicates low thermo-tolerance (Fernandes *et al*., 2007). This necessitates the selection of isolates tolerant to the temperature range of the target ecosystem (Ferron *et al*., 1991). The two *M. anisopliae* isolates (PPRC51 and PPRC2) have exhibited relatively high germination at 35°C which is a necessary characteristic for development of biopesticide against *P. interrupta* which inhabits and breeds in similar temperatures if the isolates can kill the pest at that temperature.

However, better radial growth was observed at temperatures of 20^{o}C, 25^{o}C and 30^{o}C while growth was limited at 15 and 35^{o}C. Similar observation was also reported by Ekesi *et al*. (1999) who reported low growth of *B. bassiana* and *M. anisopliae* at 15 and 35^{o}C without significant differences among isolates. Other studies (Millner *et al*., 2002; Tefera and Pringle, 2003; Rodriguez *et al*., 2009) have also reported limited radial growth of *B. bassiana and M. anisopliae* isolates at 35^{o}C on different media. Radial growth was generally below 19 mm in 12 days (1.58 mm/day) at 25°C in all isolates despite use of different media in the study. In contrast, Senthamizhlselvan *et al*. (2010) reported that *B. bassiana* achieved 65.22 mm (6.52 mm/day) growth on PDA and 68.02 mm (6.8 mm/day) on SDA at 25°C within 10 days. Similarly, Soundrapandian and Chandra (2007) found that *M. anisopliae* achieved radial growth of 27 mm on SDA and PDA media, while it reached 25 mm on MEA media at 25°C which is slightly higher than that observed in this study. Another contrasting result by Petlamul and Prasertan (2012)

showed a 37.10mm (3.71 mm/day) growth at 29±3 °C on Czapeck Dox Agar (CDA) for *B. bassiana*. While this latter difference may be attributed to differences in media, the first two contrasting results could have been obtained due to strain variability. Several studies have confirmed that the best radial growth of *M. anisopliae* and *B. bassiana* is generally achieved at temperatures between 20 - 30°C (Ekesi *et al*., 1999; Tefera and Pringle, 2003; Dimbi *et al*., 2004) which is the case in the present study as well. In general, *M. anisopliae* isolates IC69 and PPRC51 showed better radial growth across all media at 30°C.

Spore production was also restricted at 15^{o}C and 35^{o}C across all media. Relatively higher sporulation was observed at 30°C compared to 20^{o}C and 25°C, although there were variations within the isolates across the different media. Similar variations were observed within isolates of *M. anisopliae* (Petlamul and Prasertan, 2012; Niassy *et al*., 2012). In the present study, the *M. anisopliae* isolates PPRC51, PPRC2 and IC69 scored the highest spore yield at 30°C exhibiting better tolerance to higher temperature.

The present study concludes that four of the entomopathogenic fungal isolates used in this study have been effectively confirmed as *B. bassiana* and three of the isolates as *M. anisopliae* by the molecular identification tools. It is also concluded that *chi*1 and *ch*i4 primers can be used as an important supplementary identification tool to ITS primers.

The *in-vitro* characterization has indicated the best media and temperature for germination, radial growth and sporulation of the selected isolates. Thus, it is concluded that all the selected isolates exhibit best germination, radial growth and sporulation between temperatures of 20^{o}C-30°C on all the tested media. Maximum germination can

be achieved using MEA media at 25°C but, for cultivation of the isolates, use of SDA and SDAY media at 30°C is most preferable as it supports better radial growth followed by substantial spore yield.

The two *M. anisopliae* isolates (PPRC51 and PPRC2) that have shown good performance in terms of *in-vitro* characteristics are recommended as candidates for development of mycoinsectide against *P. interrupta.* Further investigations are recommended to identify factors that enhance thermal tolerance and infectivity of the isolates under field conditions.

CHAPTER V

DEVELOPMENT OF AUTO-INOCULATION DEVICE FOR FUNGUS BASED BIO-PESTICIDE FOR MANAGEMENT OF THE SORGHUM CHAFER; *Pachnoda interrupta* (OLIVEIER) (COLEOPTERA: SCARABAEDIEA).

5.1. Introduction

Sorghum chafer, *Pachnoda interrupta*, is the most destructive pest of sorghum in Ethiopia destroying the entire fields at the milk stage (Tsedeke Abate, 1988). During out-breaks, the pest can cause 70 –100% yield loss (Yitbarek and Hiwot, 2000; Yeraswork 2000). Current control methods of *P. interrupta* entirely depend on direct spraying and baiting with insecticides. However, since controlling of adult beetles through application of insecticides on scattered sorghum will not provide long lasting control, efficient biological control agents that can control the pest in the breeding sites need to be developed (Seneshaw and Mulugeta, 2000).

Traps equipped with inoculation devices are important alternative methods to spread entomopathogens into insect pest populations. The use of autodissemination traps has been suggested for bio-control of several insect pests by many authors (Lacey *et al.*, 1994; Furlong *et. al*., 1995; Klein and lacey, 1999; Dowd and Vega, 2003*).* In this technique, the target pest must be attracted to an auto-inoculation trap in substantial numbers and allowed to exit the trap after contamination with the fungal spores to horizontally transfer the inoculums to the populations elsewhere (Lyons *et al*., 2012). Fungal auto-dissemination within a host population occurs as a result of activities and movements of the host (Scholte *et al*., 2004). Auto-dissemination devices facilitate inoculation of insects with entomopathogens horizontally (within a generation) among

con-specifics in the environment and vertically (between generations) within a species, between species and from a local scale on a single plant to a landscape after they have been attracted to contaminated chambers (Vega *et al*., 2007, Jason *et al*., 2010). Transmission which determines the rate of transmission and potential of the pathogen (Steinkraus, 2006) can occur through direct contact between contaminated and uncontaminated individuals or indirectly via conidia that have been deposited on the substrate (Quesada-Moraga *et al*., 2008).

Direct transmission between contaminated and uninfected individuals is less variable and more efficient than indirect transmission via conidia that have been deposited on substrate, and can lead to high mortality rates even when the number of contaminated individuals is low (Dekesel, 1995). Auto dissemination traps with attractant lures are important tools for application of entomopathogenic fungi in augmentation and inoculation pest management strategies and developing efficient AIT systems is a key in using these strategies for successful control especially in inaccessible breeding or overwintering larval and adult habitats (Klein and Lacey, 1999). Use of AIT to disseminate entomopathogenic fungi for pest control can also be regarded as a low-input approach in conditions where conventional control means raises economic feasibility questions as using chemical sprays can be costly (Dowd and Vega, 2003). Although fungal ecology in crop systems have been studied in attempts to assess their potential as myco-insecticides (Hesketh *et al*., 2010), knowledge gaps still exist (Roy *et al.*, 2009).This necessitates assessment of the growth and virulence characteristics of candidate isolates under actual environmental conditions as a pre-requisite for successful development to myco-insecticides (Butt *et al.,* 2001; Kope *et al.,* 2008*). P. interrupta* are

known to aggregate in plant hosts such as sorghum and acacia trees but also aestivate in the soil in over wintering habitats (Welde-hawariat *et al*., 2007; Bengtsson et al., 2009). This behavior can be exploited for autodissemination (autoinoculation) of microbial biopesticides such as entomopathogenic fungi to its natural breeding habitats in augmentation strategy for sustainable management of the pest. Use of autodissemination traps can facilitate the process of infection of beetles with virulent entomopathogenic fungi. The objectives of the experiments were therefore to develop an efficient trap equipped with fungus autodissemination device and to evaluate the field performance of the selected *Metarhizium anisopliae* isolates in infecting adult *P. interrupta* beetles using these traps.

5.2. Materials and Methods

5.2.1. Description of the study sites

The field experiments were conducted in two zones of the Amhara Regional State of Ethiopia. One of the sites is located in Kewot district (wereda) Rasa village at Lewtegn (09 ^{o}57' N and 040 ^{0}04' E) and at Ayele ager (09^{o}55' N, 10^{o}40' E) in North Showa zone of the Amhara Region, Ethiopia (figure1). These study sites are located 255 km northeast of Addis Ababa, at altitudinal range of 1300- 2600 m.a.s.l (meters above sea level). The area has a bimodal rainfall pattern with short rains observed between March and May and main rainy season between July to October. Unevenly distributed average annual rainfall of 500 to 700 mm and annual temperature range of 8^{o}C to 40^{o}C characterize the semi-arid ecological zone where the sites are located. Major crops grown in the area include: sorghum, teff, maize and cowpea. Kewot woreda is one of the areas where *P. interrupta* is most prevalent. The second site is located in Dewe-Harewa district at Mendubo village

(10°50'N, 040°05'E; altitude 1206 m.a.s.l.) and in Bati district at Abuare village ($10^0$57'N, $040^0$03'E; altitude 1383 m.a.s.l.) of the Oromia zone of the Amhara region with a distance of 340 km and 355 km from Addis Ababa. These sites also have similar characteristics as the above mentioned sites. The distance between the two zones is approximately 150 km.

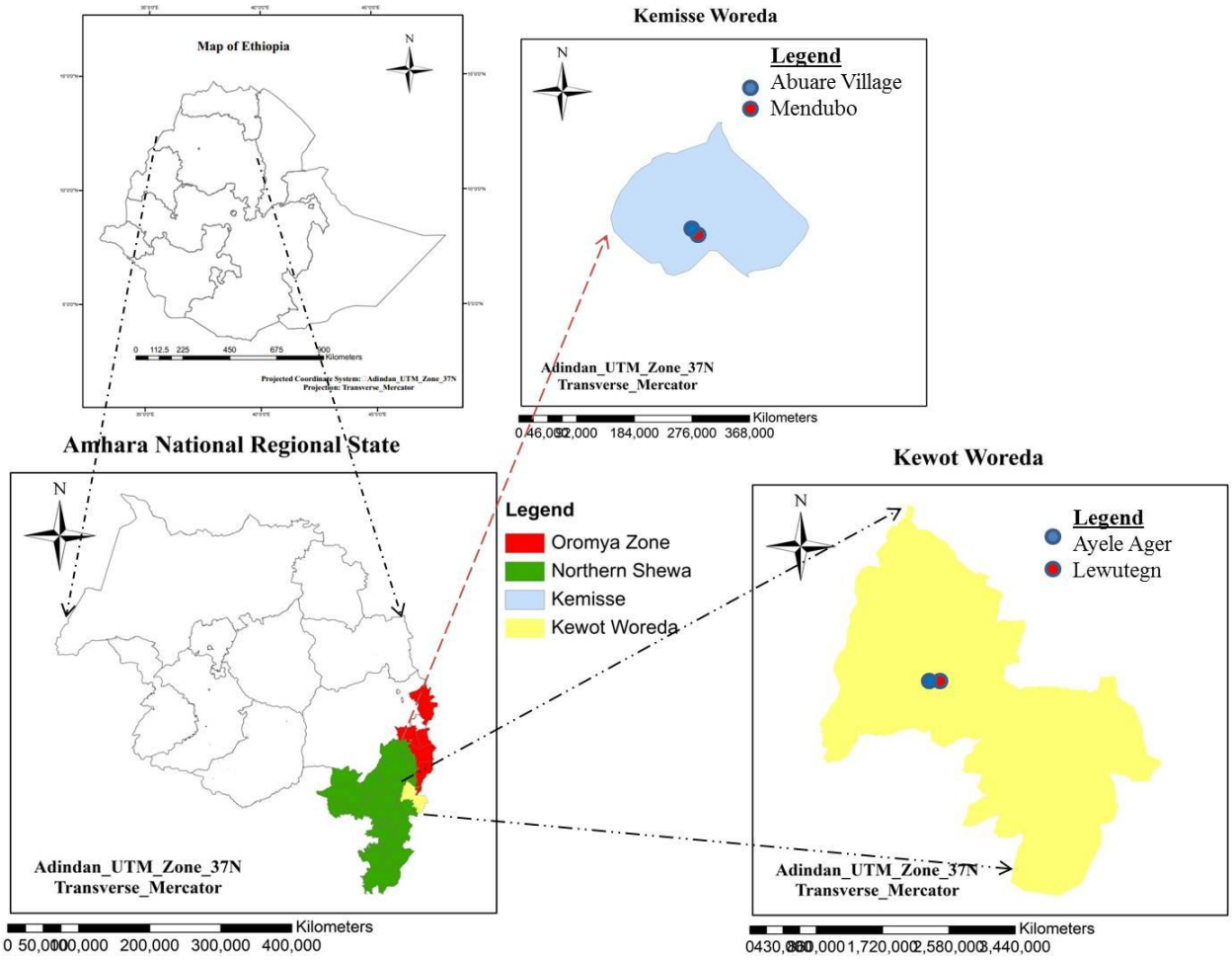

Figure 16. Location of the study sites on the map of Amhara Regional State of Ethiopia.
* Woreda refers to a district in a zone (Map adapted from Getnet, 2014)

5.2.2. Evaluation of trap catch and field efficacy test in October, 2012

This field experiment was conducted in Amhara regional state; Oromia Zone Batti District at Abuare village of Burka kebele. The experimental site was located at $10^0$57'468''N and $040^0$03'839''E. The altitude of the area was 1383m above sea level.

5.2.2.1. Source of fungus: the *Metarhizium* sp. isolate (PPRC51) used for the experiment was originally isolated from *P. interrupta.* The isolate was selected from evaluation of 56 isolates of *Beauveria* spp. and *Metarhizium* spp. against larvae of the wax moth, *Galleria mellonella* under laboratory conditions. For the field experiment, pure spores of the best performing *Metarhzium* sp. isolate PPRC 51 were harvested from 17days old culture of the isolate grown on quarter strength Sabroud Dextrose Agar Yeast (SDAY) medium using sterile metal spatula and dried at 30^0 C in Petri-dishes for 12hrs to remove excess moisture (Lacey *et al*. 1994). Spore quantification was done using an improved neubauer heamocytometer to determine the number of spores in a gram of the spore powder. The spores were then sealed in plastic bags and transported to the field in an ice box.

5.2.2.2. Traps and application of fungal spores: two designs of auto-inocualtion traps (AIT) were developed from a big (5 litres) plastic water bottle with cut top (bucket trap). The designs of the auto-inoculation chambers (figure 18 and 19) were developed from small (1/2 litres) plastic water bottles.The diameter of the canister opening was 15cm and had a height of 21cm. The open plastic bucket trap was initially selected because it had better performance in terms of beetle catch in the previous three seasons of evaluation of traps (Getnet, unpublished data). The inner walls of the inoculation chamber of the auto-inoculation traps were covered with a velvet cloth to allow retention of fungal spores. One of the traps had one inoculation chamber with a ladder-like structure to guide the

beetles to the chamber and one outlet, while the other trap had two inoculation chambers and two outlets (figure17a - d). The traps and the auto-inoculation chambers were painted green from the outside. For each of the traps, approximately 0.6gm of the spores was applied on the walls of the inoculation chambers using sterile metal spatula and dispersed with a clean camel's hair brush. In the case of the trap with two infection chambers, 0.3gm of spores was applied to each chamber. Traps not treated with the fungus and the Japanese beetle trap (JBT) served as untreated and standard controls respectively. The viability of the spores was determined before and after application to the inoculation chambers of the bucket traps; hereafter called auto-inoculation traps (AIT*a*) (with one out let) and AIT*b* (with two outlets)). In addition to this, viability was checked every day afterwards for three days (until the end of the experiment).

Figure17. Auto inoculation traps used for the October, 2012 field experiments a) inoculation chamber with a ladder b) outlet covered with plastic bag, c) trap with two outlets and d) Inoculation chamber lined with velvet cloth.

5.2.2.3. Lures: a blend of 5 compounds (Phenylacetaldehyde, 2,3-butanediol, Methylsalicylate, Eugenol, Isoamyl acetate) was used as an attractant. One thousand micro-liters (1000µl) of each of the compounds was loaded on to a separate 4 ml glass vial (45 × 14.7 mm, clear, Skandinaviska GeneTec AB) dispenser with cotton roll (3.9 cm long and 0.9 cm in diameter, Top Dent®, Dental rolls) using micro pipette and mounted on one side of each of the traps (five vials per trap). The release rate of each of the compounds was adjusted to 0.5-1mg/hr or approximately 25mg/day and had a longevity of at least 1 week.

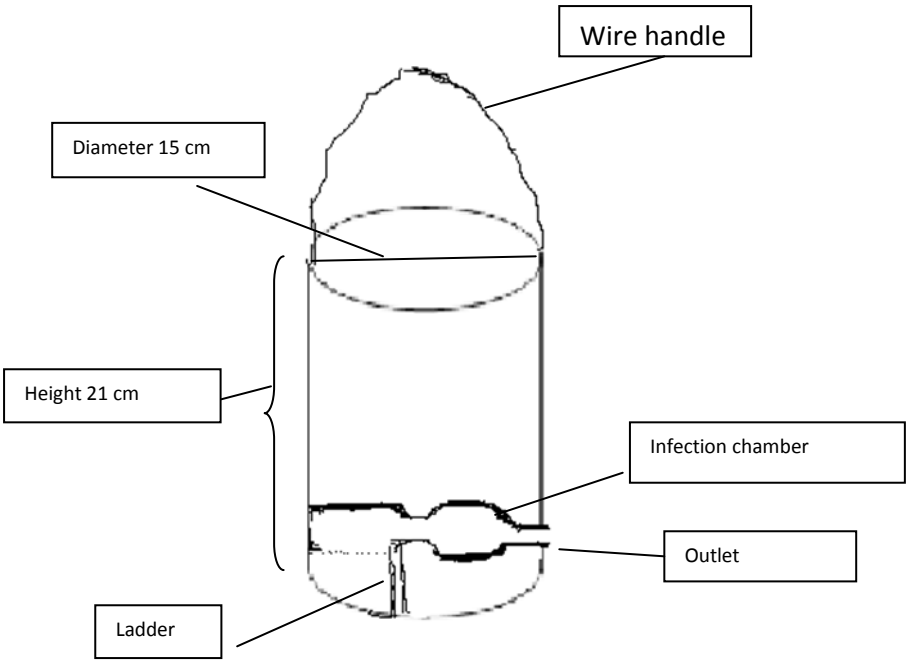

Figure 18. Design of the auto-inoculation trap with a ladder and one outlet (AIT*a*) used in the October, 2012 field experiments at Burka.

5.2.2.4. Treatments*:* there were seven treatments with five replications. The treatments are**:** T1=AIT*a* with one outlet +fungus + 50ml of water, T2= AIT*a* with one outlet

+fungus only, T3= AIT*a* with one outlet only (no fungus), T4= Japanese beetle trap (JBT), T5=AIT*b* with two outlet +fungus + 50ml of water, T6= AIT*b* with two outlets +fungus only, T7= AIT*b* with two outlets only (no fungus).

Collection bags were tied to the outlets of the traps to collect beetles which cross the inoculation chambers. Treatments were arranged in a randomized complete block design (RCBD) in sorghum fields and hung on tall sorghum stalks with the head of the sorghum removed. The distance between blocks and traps was 50 m and 10 m respectively. Collected beetles were separately kept in labeled Petri-dishes containing filter papers, fed with small slices of pilled banana and observed for mortality. Dead beetles were surface sterilized with 70% alcohol and rinsed with sterile water. These were then transferred to sterile Petri-dishes containing wet filter paper and kept at room temperature to observe sporulation for confirmation of death due to fungal infection.

5.2.3. The effect of auto-inoculation of *P. interrupta* adults with a *Metarhizium* isolate in July 2013 on the October 2013 adult population using a modified auto-inoculation trap

Two breeding sites near sorghum fields separated by a distance of 500 m were purposively selected around the Haro and Mendubo areas near Dire village for the experiment. The study area was purposively divided into treated and untreated sites. The main village road was used as a dividing border between the two sites. Thus, the area to the right of the road as one drives from Dire village to Haro was treated with fungal spores inside auto-dissemination traps while the area to the left of the road was designated as untreated with wheat bran only inside the auto-inoculation traps to serve as a control.

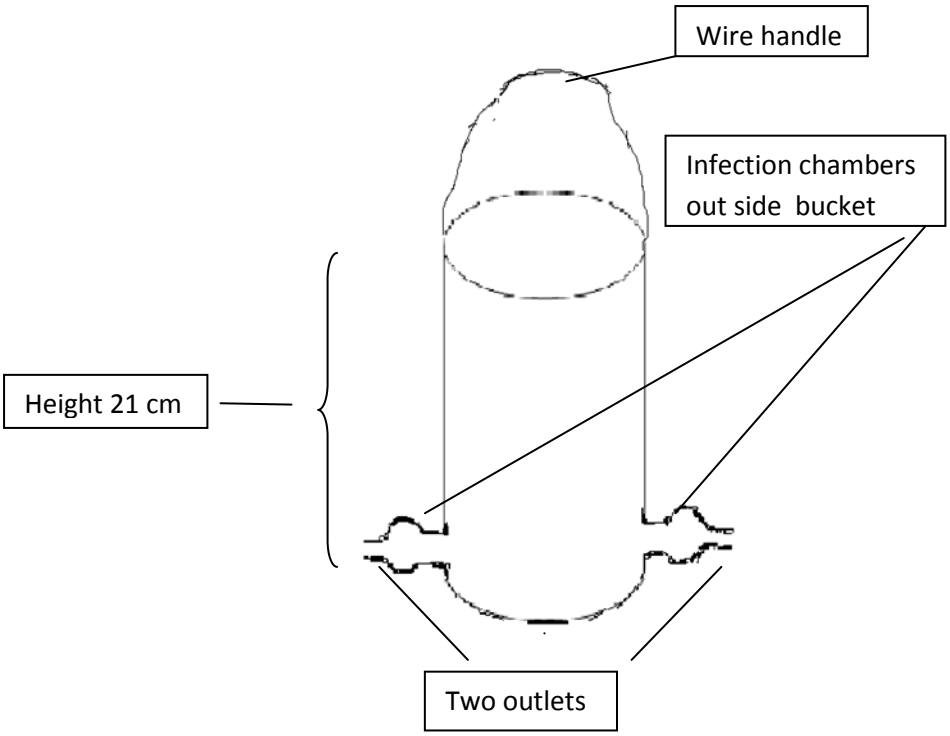

Figure 19. Design of the auto-inocualtion trap with two outlets (AIT*b*) used in the October, 2012 field experiments at Burka.

Traps and application of fungus: the trap used in this experiment was a modified version of the traps used in the October, 2012 field experiment. It was made from the same plastic water bottles (5 liter and 1 liter volume for the big and small respectively) with the bottom of the big bottle cut out and the bottle inverted upside down. It contained vanes cut from the sides of the canister (the inverted bottle) and pushed inwards from four directions (Figure 20 a - c). The auto-inoculation chamber was made of a small plastic water bottle perforated on one side to allow drainage of water. The inoculation chamber of the trap was painted green to decrease impact of UV light on spores. Finally the inoculation chamber was fitted into the bottom of the inverted plastic bottle forming the auto-inoculation trap (AIT).

The AIT's in the treated site were loaded with a 1gm: 5gm ratio mixture of spore (*M. anisopliea* isolate PPRC 51) and sterilized wheat bran respectively. The fungus used was found to be the best of the isolates evaluated under laboratory conditions against the target insect *P. interrupta.* The auto dissemination traps in the control site were loaded with 5gm of wheat bran only and were otherwise handled the same way as the treated site. There were 10 replicate sites (sub-sites) for each of the treated and untreated sites. Within each sub-site, five ADT's and three JBT's were randomly set-up vertical to the road at a distance of 50m within the row starting 10 m from the road. The JBT's in the sub-sites served as surveillance traps to estimate the back ground level of beetle population, fungi carried by the beetles (if any) and monitor natural deaths within the sub-site. Beetles collected from these traps were also kept for breeding purposes and laboratory work. Beetles entering the AIT's were allowed to leave the traps. Each sub-site was used as a block and the experimental design was a randomized complete block design (RCBD). The blend of the five compounds (Phenylacetaldehyde, 2,3-butanediol, Methyl salicylate, Eugenol, and Isoamyl acetate) was used as a lure in both sites and all traps. For observation of natural mortality or mortality due to naturally occurring fungi, five beetles from each JBT caught in the first day of the experiment were randomly selected and put in Petri-dishes containing moist filter paper. The Petri-dishes were kept at room temperature for 10 days.

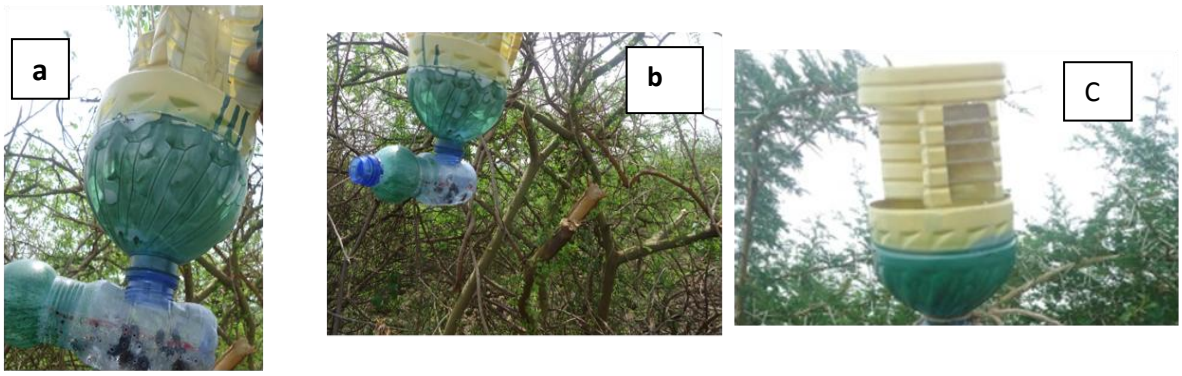

Figure 20. AIT used in July, 2013 field experiments a) AIT hanging from acacia tree with captured beetles inside the auto-inoculation chamber b) showing the outlet of the chamber c) showing the vanes and funnel.

5.2.4. Evaluation of catch performance and field efficacy of a *Metarhizium* isolate using a modified auto-inoculation trap in October, 2013

The field experiment was carried out from October 06-10, 2013 at Rassa area of the Northern Shewa Zone of the Amhara region 35 Km from the town of Shewa robit. The experimental site was located at a specific area called Dowhada (Lewetegn) at N 09^{o} 57' 22.3'' and E 040 ^{o}04 '05.8''. The initial plan was to conduct the experiment at Mendubo village of Dewe harewa district (near Kemisse) area where the July, 2013 field experiment were carried out to evaluate the effect of auto-dissemination of fungi on the population of the beetles. However, the beetles did not appear in the area throughout the feeding season. Thus, the experiment was conducted in the mentioned location although some modifications were introduced to the auto-inoculation trials.

The AIT used during the July, 2013 field experiment was used for the auto-dissemination experiment in this season (October, 2013). The trap was made of one 5 liter and two 1 liter plastic water containers fitted together (Figure 21-b) and was painted yellow and green as in the standard JBT. There were three treatments and 10 replications for each of the auto-inoculationnand catch evaluation experiments (Tables 10 and 11). For the

inoculation evaluation, treatments consisted of spores of *M. anisoplia* isolate PPRC 51 and sterilized wheat bran flour as a carrier at a ratio of 1: 2.5gm and 1:5gm in a pouch of muslin cloth. The control treatment consisted of 5gm of carrier and trap with a pouch of muslin cloth only.

For catch evaluation, treatments were a locally affordable trap (LAT) (with no auto-inoculation chamber) and auto-inoculation trap (AIT1) (with auto-inoculation chamber) and the JBT as a control (figure 21 a, b and c respectively).

For the treated replications, the traps with auto-inoculation chamber were loaded with the spores of the fungus. A small pouch of muslin cloth was used to put the blend of spores and carrier inside the chamber so that spores fall on the beetles' bodies as the trap moves while the beetles pass through the auto-inoculation chamber. The spores were protected from UV light and rain water inside the chamber. A randomized complete block design in 10 replications was used. The traps were randomly suspended on sorghum plants in sorghum fields at a distance of 10m within each block. The distance between blocks was about 50 m. The experiment was conducted from October 7-10, 2013. Traps were emptied everyday and beetles collected from each trap were kept in separate Petri-dishes and fed with slices of ripe banana. Mortality of beetles was observed for 15 days. Dead beetles were transferred to other Petri-dishes with moist filter paper and observed further to confirm mortality due to fungi. Only the beetles which showed visible signs of fungal growth after incubation were included for mortality analysis. The viability of the spores before and after the experiment was 81% and 68 % respectively. For quantification of spores picked up from the auto-inocualtion chamber by individual beetles, one beetle from each treatment in a block was randomly selected and separately kept in a Petri-dish.

The individual beetles were put in glass vials containing 1ml of Tween 80 (0.01%) and vortex shaked for 2 minutes. Spores were then counted using an improved neubaur heamocytometer. The mean daily temperature and relative humidity during the experiment period were 30.47 ^{o}C and 42.83% respectively as recorded between 11:00 am and 2:00 pm.

5.2.5. Evaluation of two auto-inoculation traps and field efficacy of three *M. anisopliae* isolates in July, 2014

The field experiment in the July, 2014 mating season aimed at evaluation of catch performance of the previously designed auto-inoculation trap (AIT1) as compared to a further modified version (AIT2) (Figure 22 A&B) and comparison of efficacy of two selected *M. anisopliae* isolates (PPRC51 and PPRC2) together with a standard commercialized isolate (ICIPE 69) using the two AIT's. Field viability of the tested isolates inside the AIT's was also assessed.

The previous version of the trap used for the July and October, 2013 field experiments contained vanes and its auto-inoculation chamber is attached from the outside. The chance of the inoculation chamber's exposure to UV light is high and sometimes rainwater leaked into it. Some modification was done to this trap as shown in (figure 22 B). In addition to this, a further modified version with the auto-inoculation chamber from the inside was prepared (Figure 22 A). Details of the further modified trap are given in (Figure 23).The trap was made of the same 5 litre plastic water bottle with the top part cut. The cut part was then inverted and put on the top open part of the cut bottle to act like a funnel. The autoinoculation chamber was made of a 1litre plastic water bottle and fitted to the bottom of the bottle under the funnel. This trap had no vanes. Instead it

contained a partly cut small (1/2 liter) plastic water bottle in the middle of the canister above the funnel suspended with wires. The two trap designs were tested in July, 2014 for efficiency in inoculating the beetles with the fungi and for catch performance.

Table 10. Treatments used for autodissemination experiment at Dowhada (Lewetegn) site around Rassa during October 7 – 10, 2013

Code	Treatments	Remarks
T1	1:2.5 gm (Fungi :wheat bran flour)	Auto-inoculation trap one (AIT1)
T2	1:5 gm (Fungi :wheat bran flour)	Auto-inoculation trap one (AIT1)
T3	5 gm of wheat bran flour only (control)	Auto-inoculation trap one (AIT1)

Table11**.** Treatments used for catch performance experiment at the Dowhada (Lewetegn) site around Rassa during October 7 – 10, 2013

Code	**Treatments**	**Remarks**
T1	AIT1	Auto-inoculation trap
T2	JBT (control)	Standard Japanese beetle trap
T3	LAT	Locally affordable trap with no auto-inoculation chamber

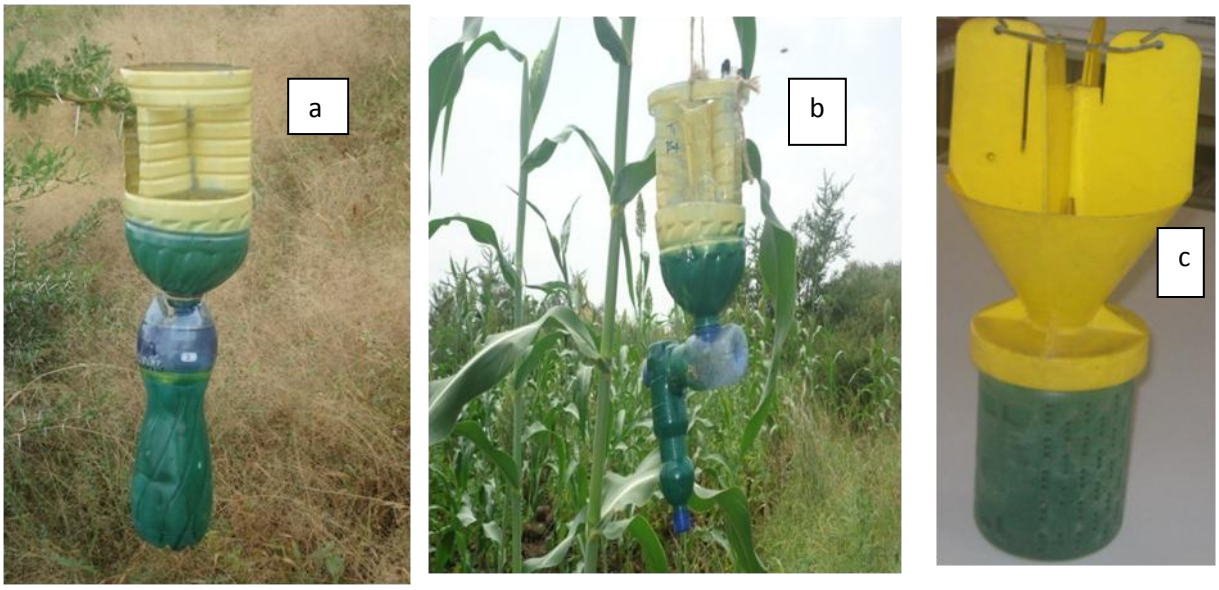

Figure 21. Traps used in the October, 2013 field experiments (a) Locally affordable trap (LAT) with no auto-inoculation chamber (b) Auto-inoculation trap1 (AIT1) (c) JBT.

Three *M. anisopliae* isolates (PPRC51, PPRC2 and IC69) were tested with the traps for field level efficacy and catch performance. Two of the isolates were the results of bioassays on *P. interrupta.* The isolate PPRC51 was tested in October, 2012, July, 2013 and October, 2013. The isolate PPRC2 was not significantly different from PPRC51 during bioassays on *P. interrupta* and was tested as a back-up isolate in this season. The isolate IC69 is a commercialized isolate from ICIPE which was used as a standard. The field viability of the spores of the three isolates used was also checked out.

i. Catch performance of auto-inoculation traps (AIT's)

The experiment was set up to evaluate the catch performance of two AIT's. The two traps were used as treatments with the JBT as a standard comparison trap. The experimental design was randomized complete block (RCBD). There were 5 replications of each trap and the traps were randomly assigned to each block. The five compound blend was used as a lure as in previous experiments. Traps were emptied every day for catch counts.

ii. Evaluation of field efficacy

Three *M.* anisopliae isolates (PPRC51, PPRC2 and ICIPE 69) and two versions of AIT's were used for the experiments. There were 8 treatments including two controls (Table 12). The experimental design was randomized complete block with 5 replications. Distance within blocks and between blocks were 10 m and 50m respectively. The treatments were randomly assigned to each block. The five compound blend was used as a lure as in previous experiments. Each of the inoculation chambers of the auto-inoculation devices (treatments) were loaded with 1gm of the pure spores of the respective fungal isolates mass produced on rice. The control AIT treatments were loaded with killed spores. Beetles caught passing through the auto-inoculation traps were collected every day, counted and put in plastic containers for observation for up to 15 days. Dead beetles were incubated in Petri-dishes with moist filter paper to confirm death due to fungus. Weather data (Temperature, Relative humidity and rainfall) was collected during the experimental period.

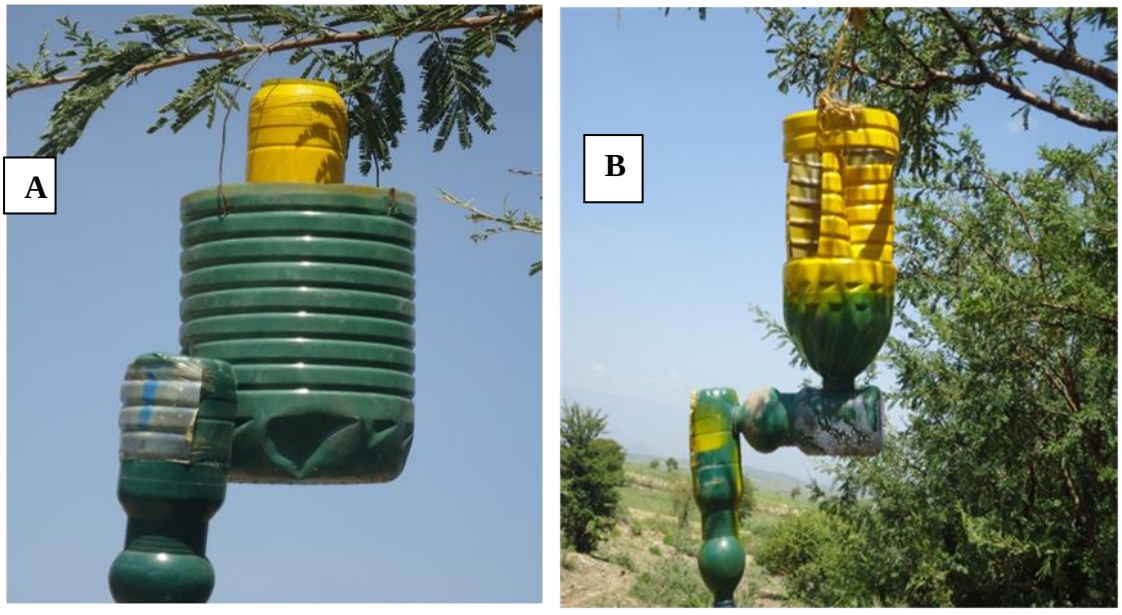

Figure 22. Locally affordable auto-inoculation traps A) Auto-inoculation trap 2 (AIT2), (B) Auto-inoculation trap 1 (AIT1).

iii. Determination of field viability of spores

The combination of the treatments used for the experiement are given in table 12.The field level viability of the spores before loading in to the auto-inoculation device and every day thereafter was checked. Spores were picked up from the inoculation chambers of the AIT's using clean cotton buds and put in 1.8 ml sterile cryovials and kept at 4 ^{o}C until processed. In the laboratory, 1ml of sterile 0.01% Tween 80 solution was added to the vials and vortex shaken for 2 minutes to dislodge the conidia from the cotton buds. Spores were counted using an improved neubaur heamocytometer and the concentrations adjusted to $1x10^{6}$ conidia/ml. Finally 100 µl of the spore suspension was transferred and spread on SDA media and incubated at room temperature for 24hrs. Viability of spores was determined by counting at least 300 spores and calculating the percentage of germinated spores. The mean temperature and relative humidity of the area during the experiment period were 36.5℃ and 18.33% respectively.

iv. Determination of the number of spores picked up by individual beetles

Determination of the number of spores picked up by individual beetles from each of the inoculation chambers of the auto-inoculation traps was conducted in the green house at Ambo Plant Protection Center. Each of the two AIT's was loaded with 1gm of the respective fungal spores. *M. anisopliae* isolates (PPRC2, PPRC51 and IC-69) were used. There were three replications for each of the isolates and the traps. Field collected beetles were fed with slices of ripe banana for 10 days before being used for the experiments to observe any signs of natural infection. Ten healthy and strong beetles were then manually

put into the traps loaded with spores of the respective isolates one by one and collected from the exit of the traps and put individually in sterile test tubes. Ten milliliter of sterile 0.01% Tween 80 was added to the test tubes and vortex shaken for 2 minutes to dislodge the conidia from the beetles. Spores were then counted using an improved neubaur heamocytometer.

Table 12. Combinations of *M. anisopliae* isolates and auto-dissemination traps used for determination of the field viability of spores in July, 2014

Treatment No.	Description	Remarks
T1	PPRC51 + AIT1	Auto-inoculation device from outside
T2	PPRC51 + AIT2	Auto-inoculation device from inside
T3	PPRC2 +AIT1	Auto-inoculation device from outside
T4	PPRC2 +AIT2	Auto-inoculation device from inside
T5	ICIPE69 +AIT1	Auto-inoculation device from outside
T6	ICIPE69 +AIT2	Auto-inoculation device from inside
T7	AIT1 ONLY	Control (killed spores)
T8	AIT2 ONLY	Control (killed spores)

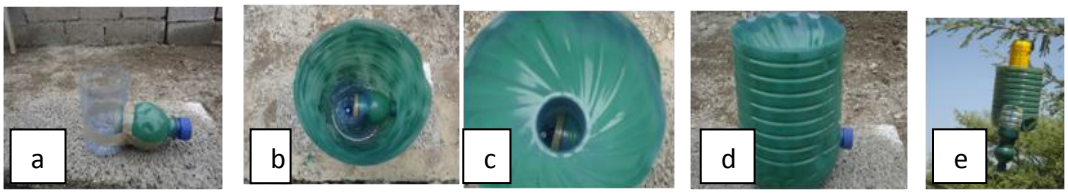

Figure 23. Detailed parts of AIT2 a) Auto-inoculation chamber, b) Chamber fitted into canister, c) funnel put on top of canister, d) canister with protruding outlet, and e) trap with small water bottle suspended on top of canister and hang on a twig of an acacia tree.

5.2.6. Evaluation of field viability of spores in october, 2014

Since the beetles did not appear, only spore viability test inside the auto-inoculation traps was conducted. The procedure was similar to that of the determination of field viability of spores conducted in July, 2014 (iii above).

5.3. Statistical analysis

Mortality data were corrected for control mortality using Abbot's formula before transformation. Percent germination and mortality data were arcsine transformed before analysis. Count data on trap catch were square root ($\sqrt{x+0.5}$) transformed. Analysis of variance (ANOVA) followed by mean separation was conducted on all transformed data using SAS software version 9.2.

5.4. Results

5.4.1. Evaluation of trap catch and field efficacy test in October, 2012

The viability of the fungal spores before application to the inoculation chambers of the ADT's was 98.9% . Viability of the spores after 24, 48 and 72 hrs was 97.6%, 96.2 % and 96 % respectively showing no significant difference over three days (figure 24). The mortality of the beetles captured was observed for10 days after collection and varied significantly (Figure 25). Mortality ranged from 0 to 13.72% (~14%). The highest

mortality was recorded from the fungus treated bucket trap with one outlet containing 50ml of water (T1) and was significantly different (P= 0.017, F= 3.25, df=6, 24) from all the other treatments. The rest of the treatments did not differ significantly from each other.

There was no significant difference (P=0.40, F=1.08, df =6, 24) in the mean catch of all the autodissemination traps and the JBT used as a control (figure 26).

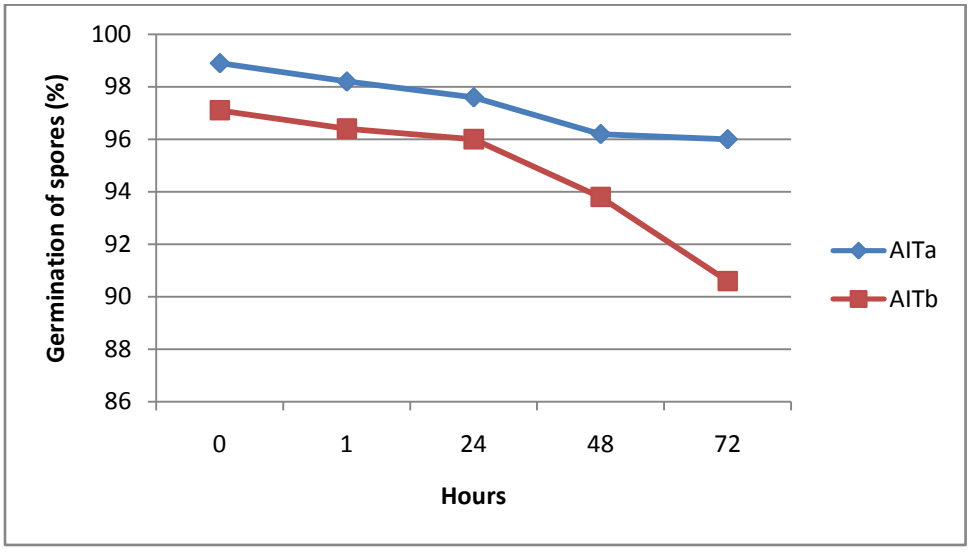

Figure 24. Field viability of *M. anisopliae* isolate PPRC51 in two auto dissemination traps in October , 2012 at Burka. AIT*a* trap with one outlet, AIT*b* trap with two outlets.

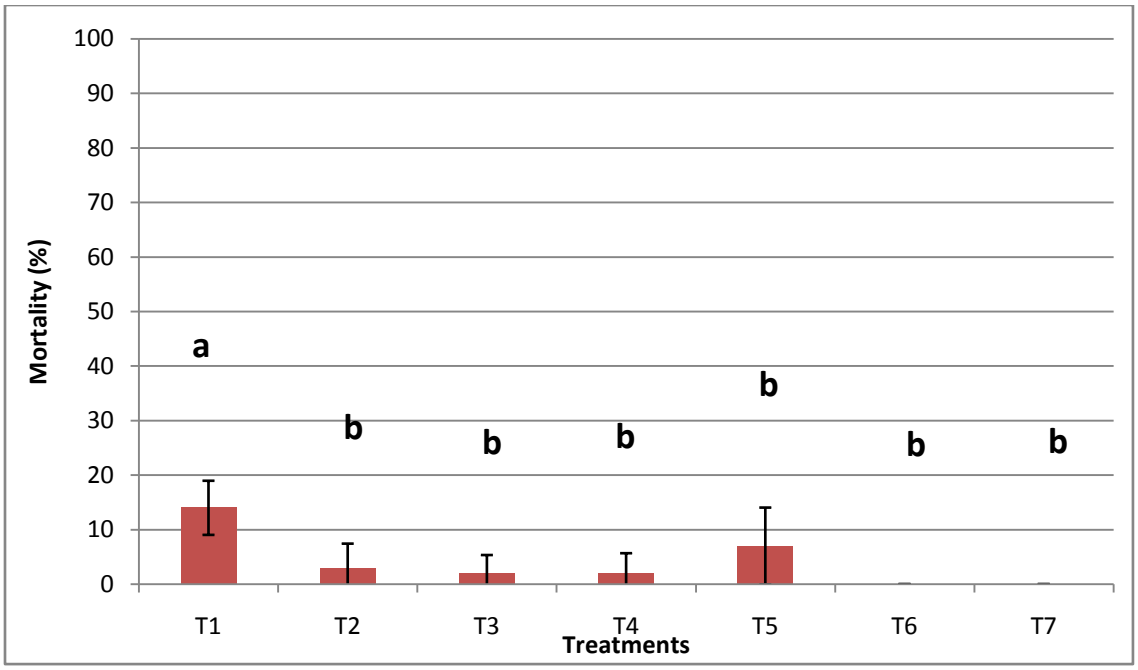

Figure 25**.** Mortality of beetles caught from fungus treated and untreated traps over three days at Burka in October, 2012.

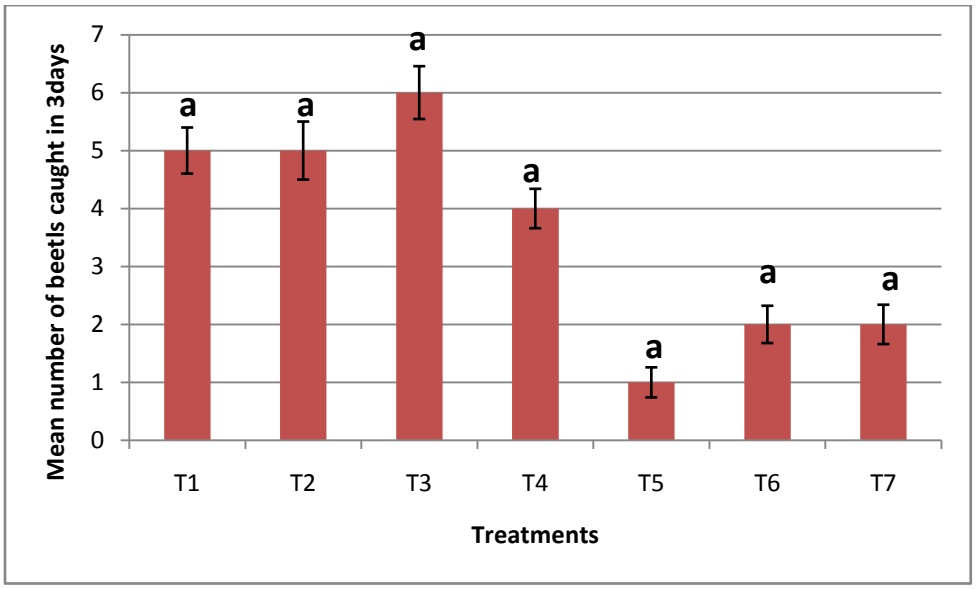

Figure 26. Mean catch of traps with one outlet (T1, T2 &T3), the JBT (T4) and traps with two outlets (T5, T6 &T7) in October, 2012 at Burka.

5.4.2. The effect of auto-inoculation of *P. interrupta* adults with a *Metarhizium* isolate in July 2013 on the October 2013 adult population using a modified auto-inoculation trap

The beetles entering the surveillance traps (JBT's) were counted to estimate the population of beetles in the specific sites where the AIT's were set up. Total catches per day in the untreated site (figure 27) and treated site (figure 28) showed differences in the number of beetles caught between the two sites. The number of beetles caught in the treated site replications was slightly higher than those from the untreated site replications. The cumulative catches per replication over five days also showed a similar trend (figure 29).

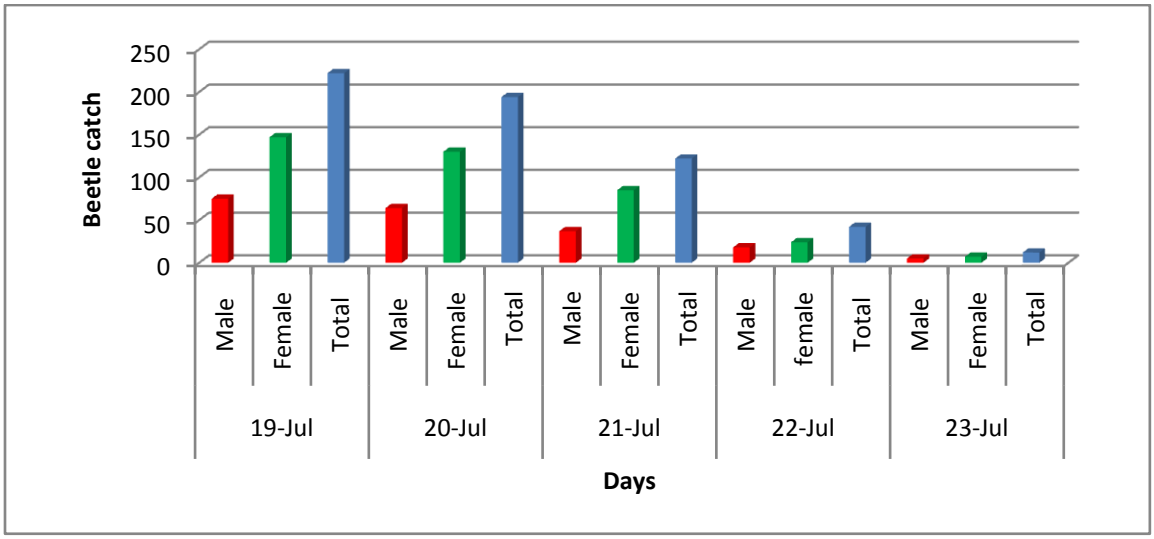

Figure 27. Total catches per day in untreated site observed for five days in July, 2013 from surveillance Japanese beetle traps (JBT's) starting after 24 hrs from trap deployment.

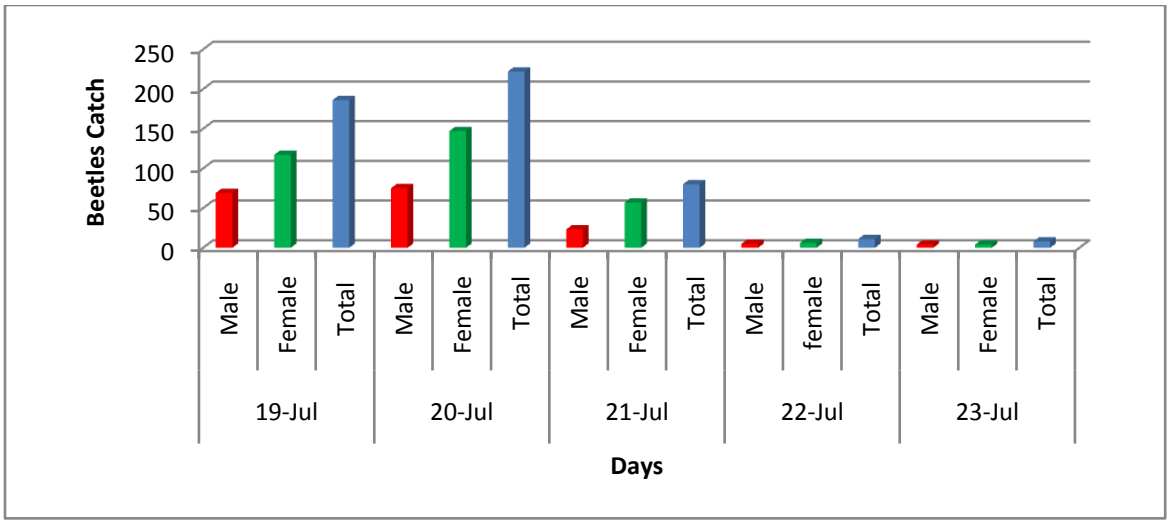

Figure 28. Total catches per day in fungus treated site observed in five days in July, 2013 from surveillance Japanese beetle traps (JBT's) starting after 24 hrs from trap deployment.

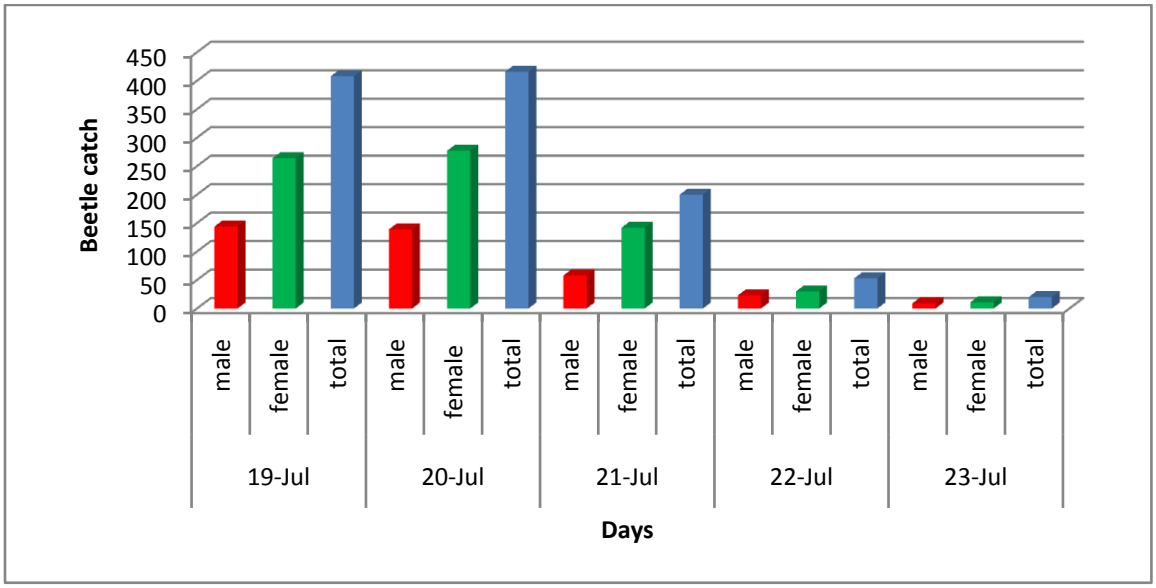

Figure 29. Total number of beetles caught per day over five days from surveillance JBT traps at Mendubo village in July, 2013 (fungus treated and untreated sites).

None of the beetles sampled to check for natural mortality died within 10 days of observation. This indicated that there were no natural infections with any fungi in the area prior to auto-inoculation trials.

5.4.3. Evaluation of catch performance and field efficacy of a *Metarhizium* isolate using a modified auto-inoculation trap in October, 2013

There was significant difference (P = 0.0001, F= 15.94, df= 2, 18) in beetle mortality between the control (T3) and fungus treated AIT's with auto-inoculation chamber (T1 and T2) (figure 30) although the later two did not significantly vary from each other. The JBT caught significantly higher number of beetles (P=0.016, F=5.21, df=2, 18) than AIT1 and the LAT which did not significantly differ from each other (figure 31). The mean number of spores carried by individual beetles passing through the fungus treated auto-inoculation chamber was 4.56 $x10^4$ and 4.7 $x10^4$ for T1 and T2 respectively.

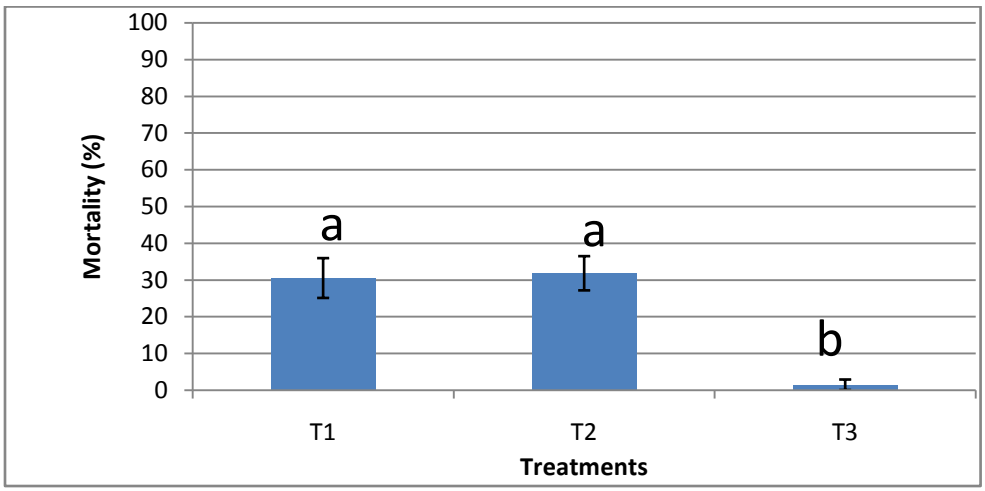

Figure 30. Mean percent mortality of beetles collected from fungus treated and control AIT's during the October, 2013 field experiment at Lewtegn (Rassa area).

5.4.4. Evaluation of two auto-inoculation traps and field efficacy of three *M. anisopliae* isolates in July, 2014

i. Catch performance of AIT's

The catch performance of the two locally affordable AIT's as compared to the JBT was evaluated and there were no significant differences among them (P = 0.1141, df =2, n= 5) (Figure 32).

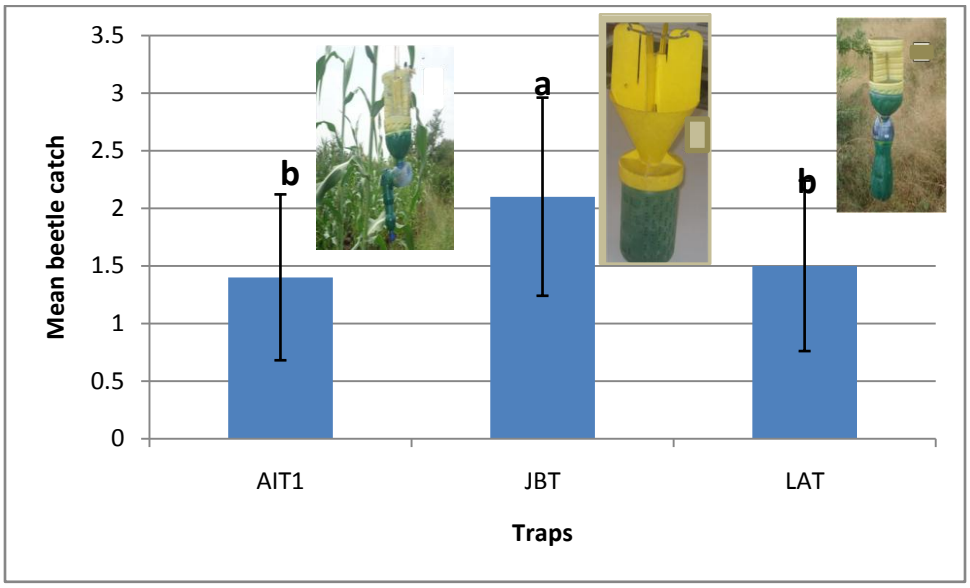

Figure 31. Mean cumulative beetle catches per trap in 3 days in the October, 2013 field Auto-inoculation trials at Dowhada (Rassa area).

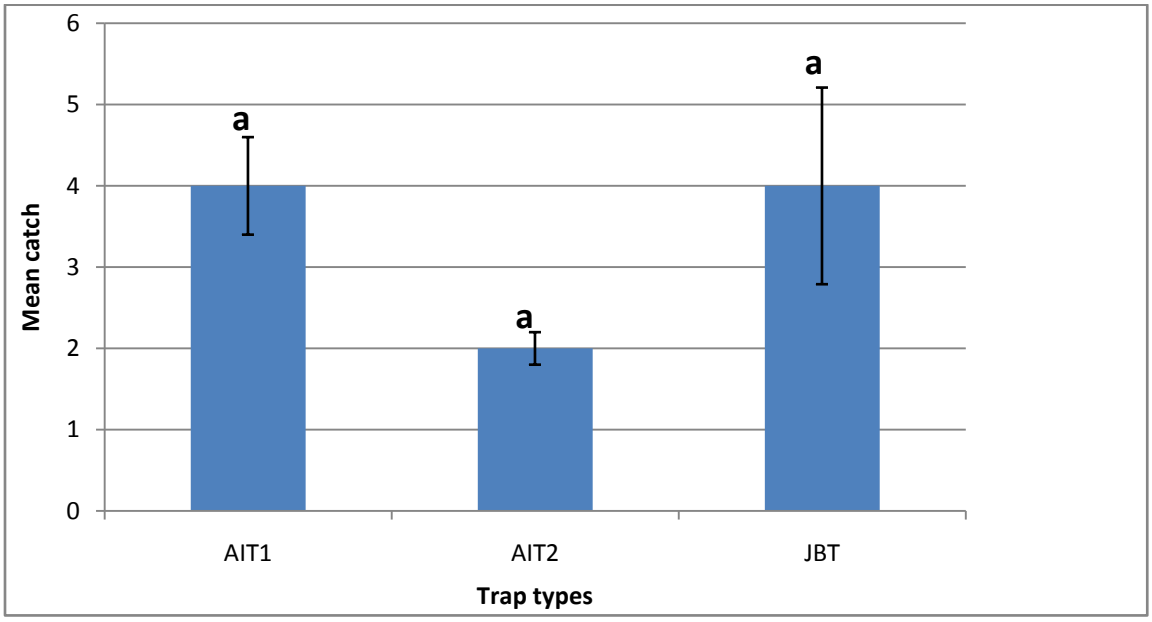

Figure 32. Mean beetle catch of two locally affordable auto-inoculation traps and the Japanese beetle trap in July, 2014 at Rassa.

ii. Field efficacy of fungal isolates

There were significant differences among the *M. anisopliae* treated and control traps (P< 0.0018, F= 6.24, df 4, n = 5) in observed mortality of adults of *P. interrupta* (figure 33). There was no significant difference between the two AIT's (P=0.06, F= 3.80, df 1, 32, n =5). The isolate PPRC 51 was associated with the highest mortality (40.73%) followed by PPRC2 and IC69 39.91% and 26.16% respectively when applied using AIT1. Using ADT2, observed mortalities were 25.02%, (PPRC51), 26.69% (PPRC2) and 15.03% (IC69). The interaction between isolates and AIT's was not significant (P =1.00, F= 0.16, df 3, 32, n=5).

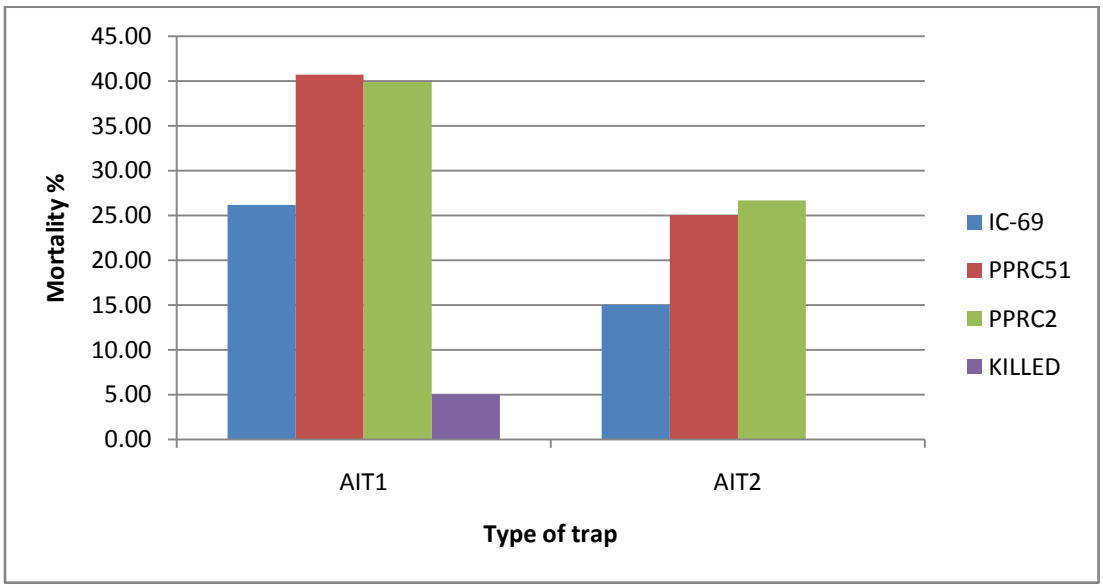

Figure 33. Field mortality of beetles caught by AIT1 and AIT2 treated with three isolates of *M. anisopliae* and killed spores in July, 2014 at Rassa.

iii. Field viability of spores

The mean field viability (as measured by %germination) of spores of the three *M. anisopliae* isolates over five days significantly varied between 82.12 (PPRC 51 in AIT1)

– 0% (IC69 in AIT1 and AIT2) (P= 0.0001 for day 0 – day 4 and P= 0.015 for day 5; F=28.26 ,29.15, 47.77, 101.40, 43.23 and 8.53 for day 0 – day 5 respectively with df=2,24) (figure 34). The mean initial viability (day o) of PPRC51 and PPRC2 were 82.12% and 84.5% respectively. The mean initial viability of isolate IC69 was (62.37%). As days passed viability decreased sharply especially for IC69 which dropped to 17.92% in the second day and to zero in day three. In contrast, the viability of PPRC51 and PPRC2 did not drop below 39.14% and 35.96% respectively in day five.

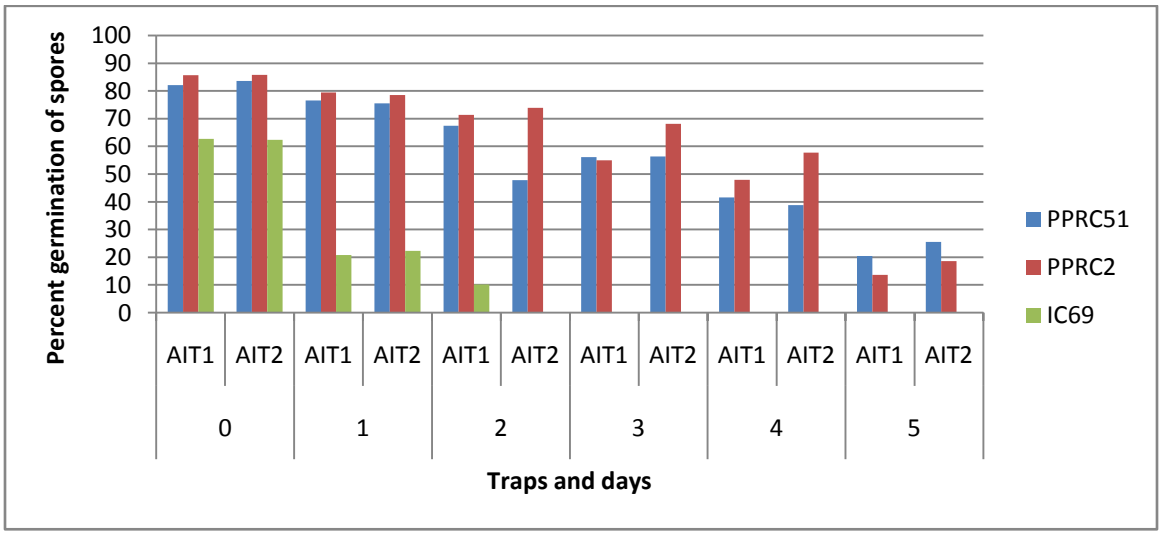

Figure 34. Mean percent germination of three *M. anisopliae* isolates applied in two auto-inoculation traps over 5days in July, 2014 at Rassa.

iv. Number of spores picked up by a single beetle.

The mean number of spores picked up by a single beetle range from $3.14x10^7$ (PPRC2) to $9.6x10^7$ (PPRC51) (figure 35).Significant variations were observed in the number of spores picked from the isolates ($P<0.0001$, df 2, n=10).There was no significant difference between using AIT1 or AIT2 and interaction effects between AIT's and isolates were not significant.

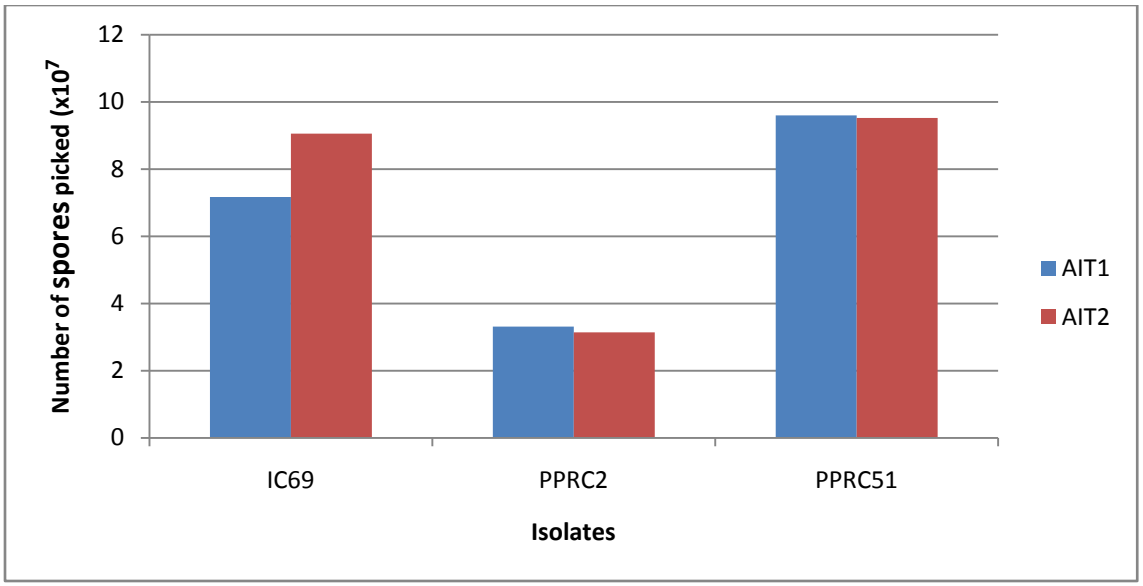

Figure 35.Number of spores picked up by single beetle from AIT's loaded by three isolates of *M. ansisopliae*, 2014.

5.4.5. Evaluation of field viability of spores in october, 2014

The field viability of spores of the three *M. anisopliae* isolates over five days showed a similar trend as that of the July, 2014 experiment (figure 36). The viability of the isolates varied significantly and ranged from 78.3% (PPRC2) to 0% (IC69) over the five days(P=0.0001 for days 0-5 with F= 15.34; 39.23; 127.18; 325.90; 17.04; 98.36 and 39.10 respectively at df = 2, 24). Isolates PPRC51 and PPRC2 did not significantly differ throughout the experiment days while significantly differing from IC69. Whereas the viability of PPRC51 and PPRC2 did not drop below 35.6% and 40.3% respectively in day five, IC69 could not stay viable beyond day 2 particularly in AIT2. In contrast, in AIT1, the isolate stayed viable up to day 5 with mean viability of nearly 32% at day 5.

There was a slight interaction effect between isolates and AIT's at day 3 (P=0.0001, F=17.04) and day 4 (P=0.03, F=3.73). Generally, the viability was relatively low in AIT2 than in AIT1.

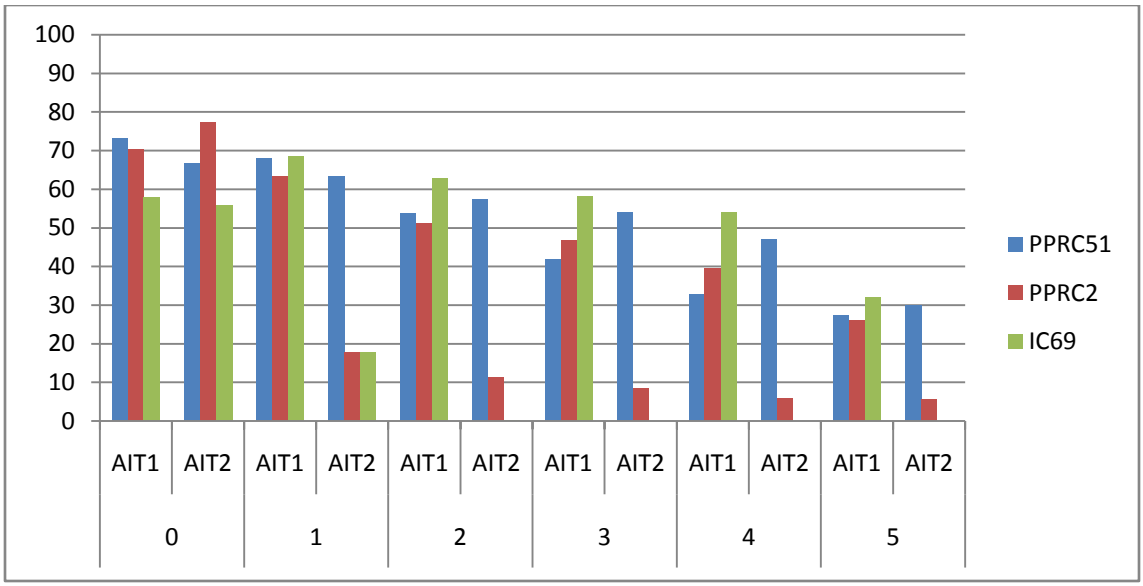

Figure 36. Mean percent germination of three *M. anisopliae* isolates applied in two autodissemination traps over 5days in October, 2014 at Rassa.

5.5. DISCUSSION

The strategy of attract, infect (contaminate) and release for insect control has been attempted on other pests. For example autodissemination devices have been developed to infect coleopteran insects such as sap beetles (e.g.*Carpophilus lugubris)* (Dowd and Vega, 2003), rhinoceros beetle (*Oryctes rhinoceros*) (Moslim *et al*., 2011) and emerald ash borer (*Agrilus planipennis)* (Lyons *et al*, 2012). Auto-inoculation traps have also been used on non coleopteran insects like fruit flies (*Ceratitis cosyra, C. fasciventris and C. capitata)* (Dimbi *et al*., 2003) and tsetse flies (*Glossina* spp.*)* (Maniana, 2002) offering new approaches for application of entomopathogenic fungi in the field for control of insects (Migiro *et al*., 2010). Klein and Lacey (1999) have also demonstrated the possibility of fungal autodissemination to *Popillia japonica* populations by modifying the standard Trece Catch Can Japanese beetle trap. But, use of standard commercial traps is costly and difficult to obtain for the subsistence farmers in Ethiopia. Thus, the field studies started in October 2012 by modifying cheap and locally available plastic water bottles which were used to develop two auto-inoculation traps and infect *P. interrupta* adults with *M. anisopliae*. In a similar low cost approach, auto-inoculation device made of locally available plastic water bottle materials was used to contaminate tsetse flies (Maniania, 1998).

The results of the study in 2012 showed that there was no significant difference in catch performance of the auto-inoculation traps and the JBT. This finding complied with the previous year's findings that the catch performance of the open bucket trap is equal to that of the JBT when the blend of the five compounds is used as a lure (Getnet, 2014). In general the number of beetles caught by the traps was very low. This can be attributed to

the low population of the beetles observed in the experimental area during the trial period. It can also be because of the beetles' behavior to continue feeding on the sorghum heads until fully depleted without flying to other sorghum heads (Yitbarek, 2008). This condition might have kept the beetles quiet for a long time decreasing the chance of being caught by the traps. Many beetles were observed escaping from the bucket traps before being collected during the experiment indicating the need for improvement of the traps. One possible improvement was introduction of an inverted funnel on top of the bucket trap to keep beetles inside, once trapped.

The mortality of the beetles was generally low. A possible reason for this can be beetles not entering the inoculation chamber as frequently as expected or not acquiring adequate amount of conidia. The number of conidia acquired by an insect depends on the formulation of the pathogen, the trap design, duration of contact with the conidia and on the size of the beetle (Kreutz *et al*., 2004). As observed during the experiment, most of the beetles were not retrieved from the collection bags. Instead, they tended to stay at the bottom of the bucket trap or escaped out even after entering the auto-inoculation chamber. Another reason may be that the amount of fungal spores was low and may have been blown by wind.

Taking the observations in the October, 2012 field experiments into account, the July, 2013 field experiments constituted an attempt to modify the traps and the inoculation chambers. As a result, the traps were modified to have vanes and preventive bottoms with holes fitted with funnel to prevent beetles from escaping and to allow entry of more beetles to the inoculation chamber. In addition to this, the modified trap was painted yellow and green. The experiments were setup to show impact of application of a fungal

entomopathogen (*M. anisopliae*) in the mating season (July, 2013) on the population of beetles in the feeding season (October, 2013). Unfortunately, *P. interrupta* beetles did not show up in October, 2013 in the experiment site and this objective was not addressed. The study team arrived at the experiment site on October 4, 2013. However, the beetles did not appear in the area throughout the feeding season. This was an unusual phenomenon as the number of beetles in the mating season was so high (fugures 27, 28 and 29) and *P. interrupta* is known to appear both in mating seasons and feeding seasons (Yitbarek *et al*., 2007). Why the beetles did not appear remains a puzzle. Could it be because of the fungus applied using auto-dissemination traps in July 2013? The study did not answer this question, but the disappearance of the beetles from the area can also be an indication of the unpredictable nature of *P. interrupta* which warrants further study in the behavior of the beetle. The number of beetles was high in July 2013 compared to all the seasons in the study period from July 2011 to October 2014. This condition also calls for further study as the number of beetles had been declining since the year 2000 following its surge in the early 1990's (Hiwot, 2000). On the other hand evaluation of the modified trap for catch performance was conducted on the same site in the July, 2013 mating season and was found to be as efficient as the JBT (Getnet, 2014) warranting its use for subsequent trials.

Although *P. interrupta* did not appear in the experiment site as expected in October, 2013, it appeared in Rassa area where it was not expected as it was not reported in the preceding mating season (July, 2013). Consequently, the experiments planned to be conducted in Mendubo area were conducted at Rassa with some modifications.The results of the October, 2013 field experiments also showed the efficiency of the modified

auto-inoculation trap both in terms of catch and inoculation of the beetles with the fungus. Mortality of the beetles caught was improved to 31.79% from the 14% mortality recorded in October 2012. The fungus was loaded in a mixture of wheat bran at two ratios and put in muslin cloth pouches in this experiment. This provided better way to spores and protection from being blown by wind. Use of either of 1:5 or 1:2.5 ratio (spore: wheat bran) did not cause significant variation in mortality of beetles. Klein and Lacey (1999) also obtained similar non significant effect of spore: wheat ratio on mortality of *P. japonica* adults using a modified auto-inoculation trap. The mean temperature and relative humidity were 30.47°C and 42.83%, respectively during the experimental period.

In the case of catch performance, the modified auto-inoculation trap caught significantly less number of beetles than the JBT. Yitbarek Wolde-Hawariat *et al*., (2007) also found the JBT to be more efficient than a locally affordable trap for *P. interrupta* field catching. However, in July, 2013 the catch performance of the modified trap was equal to that of the JBT (Getnet, 2014). These inconsistencies might have been caused due to the generally low beetle population observed during the experimental period.

In the July 2014 field experiments, the application of spores in pouches and in mixtures with wheat bran was too complex compared with the simple application of spores to inoculation chambers. Thus, use of pouches was omitted. The previous version of the trap used for the July and October 2013 field experiments contained vanes and its auto-inoculation chamber hangs on outside. The inoculation chamber is exposed to UV light and rainwater leaks into it very easily. The need for further improvement of the inoculation chamber for better protection of spores was emphasized. Therefore, an

additional auto-inoculation trap was further modified and tested along side with the previously modified auto-inoculation trap. For ease of reference, the first and the latter auto-inoculation traps were named as AIT1 and AIT2, respectively.

The performance of AIT1, AIT2 and the JBT in beetle catch was not significantly different in the July 2014 field experiments. This indicates that the locally affordable auto-inoculation traps are as efficient as the JBT in catching beetles and can be used for mass trapping as well. The population of the beetles was low in this season also compared to the July 2013 field experiments at Mendubo.

Mortality of *P. interrupta* adults showed improvements compared to the two previous auto-inoculation trials. The mortality of beetles passing through the auto-inoculation traps increased from 14% and 32% in October 2012 and October 2013 respectively to 41% in July 2014. PPRC51 caused the highest mortality as in the previous field trials indicating its relatively higher potential as mycoinsecticide. This isolate caused over 82% mortality in the laboratory bioassays. But, field situations are full of uncontrollable environmental factors, the most important of which include temperature, humidity and solar radiation (Inglis *et al*., 2001) that can decrease field mortality compared to laboratory bioassays. Use of either of the two ADT's did not cause any significant difference in mortality of beetles. The commercially available isolate of *M. anisopliae* (IC69) used as a standard caused less than 25% mortality significantly differing from the other two local isolates.

For the field efficacy study only the two *M. anisopliae* isolates were used with a commercialized *M. anisopliae* isolate (IC-69) as a standard. The *B. bassiana* isolate (MP3POST) was not used in the field efficacy study because the isolate could not be

amenable to mass production. Despite infection and the high mortality of *P. interrupta* caused by the selected isolates in laboratory trials, the field efficacies were relatively low. There can be several reasons for the low field mortality. One possible reason is the decrease in viability of the dry conidia throughout the experiment period. For example, as depicted in Figure 34, the viability of PPRC51 in AIT1 dropped to 46.35% by day 5 from initial viability of 82.12% at the start of the experiment (day 0) which is almost a 50% decrease. Another reason may be that the study was conducted using unformulated dry conidia harvested from mass production on rice substrate. This was done in an attempt to find an isolate more resistant to the environmental conditions. Appropriate formulations may enhance infectivity of the isolates. Use of formulated products such as oil formulations are known to increase infectivity of mycoinsecticides (used for inundative sprays and not for auto-inoculation) used for locust control under low relative humidity conditions (Bateman *et. al*., 1993; Lomer *et. al*. 2001; Thomas and Kooyman, 2004). Niassy et al. (2012) were able to demonstrate that kairomone LUREM-TR use for thrips monitoring and autodissemination had detrimental effect on conidial viability of ICIPE 69. A similar condition might have occurred during these experiments too.

During the experiments higher mortalities were observed in some replications indicating the potential of the isolates. Although higher mean mortalities were recorded, only cadavers showing visible signs of infection (sporulation) were considered for computing percent mortality.

The mean percentage relative humidity and temperature of the field trial sites was 18.33 % and 36.5 °C, respectively during the five days of data collection. The mean field efficacy of PPRC51 (40.73 %) is relatively high considering the hot climatic conditions

of the breeding areas of *P.interrupta***.** High insect host mortality due to entomopathogenic fungi can be attributed to strain genetic characteristics and high conidial viability (Soetopo, 2004). But several other factors also influence the field efficacy of entomopathogenic fungi including the behavior of the target pest in its natural habitat (Gindin *et al*., 2006) and the environmental factors such as solar radiation (UV light), temperature and low relative humidity (Wraight *et al*., 2007). For example, mycoinsecticides are highly affected by temperature which can limit development of entomopathogenic fungi below 15 ^{O}C or above 35 ^{O}C (Dimbi *et al*., 2003). In contrast, existence of microenvironment in the insect host's body surface and high humidity leaf zones may facilitate conidial germination after contact with the cuticle (Inglis *et al*., 2001; Shipp *et al*., 2003). However, the insect thermal behavior and environmental temperature exert strong influence on the speed with which the fungus kills the insect (Thomas and Blanford, 2003). These indicate that identification of the appropriate fungal pathogen for development as a myco-insecticide is complex as the interactions of the host, the pathogen and the environment under field conditions are not easy to predict or determine (Hajek *et al*., 2007; Jackson *et al*., 2010).

Viability of spores was assessed in July and October 2014 and in general, reasonable field viability of the spores could not be sustained for over 3 days in the two seasons study. Other studies have shown sustained field viability of spores for several days (Lyons *et al*., 2012) but, the spores were produced by live cultures on barley in cloth pouches. In contrast, the viability of the isolates used in this study declined quickly. This condition might be attributed to the high temperature and low relative humidity observed in the experiment sites. In similar study, Klein and Lacey (1999), found that the viability

of spores used in auto-inoculation traps for Japanese beetle dropped to less than 34% in six days. In the current study, the temperature and relative humidity were 36.5°C and 18.33%, respectively during the experimental period of July 2014. In October 2012 temperature and relative humidity were 31.3°C and 23% respectively while in October 2013 temperature was 30.7°C and relative humidity was 42.83%. The temperature of the target eco-system heavily influences growth and pathogenecity (Yeo *et al*., 2003) as well as speed of germination and kill (Migiro *et al*., 2010) of a fungal entomopathogen. It is also known that the use of myco-insecticides for pest control is highly dependent on weather conditions especially higher ambient humidity (Ferron *et al.,* 1991). In agreement with this study, Jaronski (2010) found that conidial germination was adversely affected by and rapidly slowed in temperatures above 30^{o}C. Conidia are generally instable at high temperatures (Fernandes *et al*., 2007).This necessitates the selection of isolates tolerant to the temperature range of the target ecosystem (Ferron *et al*., 1991) and appropriate formulation (Forgues *et al*., 1997) as a means to overcome the problem.

The mean number of spores picked up by individual beetles in the October, 2013 field trials was 4.56 $x10^4$ and 4.7 $x10^4$ for T1 and T2, respectively. The number of spores picked up by individual beetles was generally greater than 3.14 $x10^7$ for all isolates in the green house trial which is higher than the field experiment. This indicates that there is a great difference between field and laboratory trials and results must be carefully interpreted. The field spore pick up obtained in this study is comparable to the findings of Klein and lacey (1999) who found up to 1.12 $x10^8$ conidia per beetle in *P. Japonica* passing through an auto-inoculation trap. Another comparable result was reported by

Vega *et al*. (1995) who reported a spore pick up rate of $2.2x10^6$ CFUs of *B. bassiana* per beetle in the sap beetle *Carpophilus lugubris*.

In conclusion, although several attempts of use of AIT's for pest control in Africa and the world at large exist, no such studies are available in Ethiopia. This study has demonstrated a ground breaking development of autodissemination device for use in the management of *P. interrupta* in Ethiopia. Use of AIT's for pest control can be regarded as a low-input approach in conditions where conventional control means raises economic feasibility questions (Dowd and Vega, 2003). It has been reported that controlling *P. interrupta* on scattered sorghum fields using chemical pesticides poses difficulties (Sinshaw and Mulugeta, 2002). In view of these problems, use of traps for auto-inoculation of entomopathogenic fungi can be an important component for the integrated management of *P. interrupta*.

In view of the objective of the experiment, the nearly 41% field efficacy of the unformulated conidial application obtained from the study is substantial and the isolates PPRC51 and PPRC2 are recommended as potential candidates for development of myco-pesticide against *P. interrupta* for integrated management of the pest. In addition to these results, the field efficacy experiment in this study has indicated outlying challenges for similar future experiments. One of the challenges was for example the low population of beetles in the experiment sites. Therefore, additional field studies using appropriate formulation under high population conditions and more research on mass production characteristics, storage and effects of the isolates on non-targets are recommended. Studies involving population-scale behavioral responses of the target pest to EPF (Baverstock *et al*., 2010) and multitrophic interactions (Cory and Ericsson, 2010) are

important to fully establish the efficacy of EPF in the field. Therefore, additional studies to demonstrate or assess the dissemination of the fungi to breeding areas and to cause epizootics in larvae and adults in the breeding areas need to be carried out.

CHAPTER VI

EFFECTS OF DIFFERENT LIQUID PHASE MEDIA ON MASS PRODUCTION CHARACTERISTICS, SHELF LIFE AND VIRULENCE OF *Beauveria bassiana* AND *Metarhizium anisopliae* AGAINST *Pachnoda interrupta* USING RICE AS SOLID SUBSTRATE

6.1. Introduction

The sorghum chafer, *Pachnoda interrupta* is a major pest of sorghum and other cereal and horticultural crops that causes up to 100% crop loss on sorghum. As efficient and economical control measures are not available, control strategy heavily depends on use of chemical pesticides (Wolde-hawariat *et al*., 2007; Bengtsson *et al*., 2009). But, chemical pesticides are hazardous to the environment, the users and the end consumers of sorghum in addition to incurring high cost to farmers. Use of microbial biological pest control agents such as insect pathogenic fungi, bacteria, virus and nematodes has been accepted as safe alternative crop protection tools to chemical pesticides in modern agriculture (Rao *et al*., 2006; Anand *et al*., 2009). Several entomopathogenic fungi including *M. anisopliae* and *B. bassiana* have gained attention as microbial bio-control agents in the last 50 years (Jaronski and Jackson, 2012) with over 170 commercially available products throughout the globe (Faria and Wraight, 2007). Entomopathogenic fungi (EPF) based mycoinsecticides have been considered as safe alternative control strategy for several insect pests such as locusts and grass hoppers, white flies, termites, aphids, and beetles (Lomer *et al*., 1997; Klein and Lacey, 1999; Strasser *et al.*, 2000; Wraight *et al.*, 2001) and can also be used for control of *P. interrupta* as a component of integrated pest management system.

Production of inoculums of high quality with reasonable quantity on mass production substrates is an important milestone for the use of EPF as mycoinsecticides. The diphasic technique of mass production of entomopathogenic fungi whereby blastospores are produced in a liquid medium under continuous shaking and used to inoculate solid substrates for production of aerial conidia is the most viable method of mass production of EPF (Burges and Hussey, 1981; Lomer *et al*., 1997; Jaronski and Jackson, 2012). Nutritional, cultural (artificial and natural media) and environmental conditions during fungal growth can influence the virulence, efficacy, longevity, thermal tolerance and ecological fitness of mass produced fungal propagules (Mc Clatchie *et al*., 1994; Jackson, 1997; Ying and Feng, 2006). Moreover, for each fungus strain, a convenient liquid medium needs to be developed (Jaronski and Jackson, 2012).

The objective of this study was therefore to evaluate different liquid phase media for mass production of selected *M. anisopliae* and *B. bassiana* isolates in order to develop mass production and preservation protocols and their effects on virulence of the isolates to *P. interrupta* using rice as sold substrate.

6.2. Materials and Methods

Two separate experiments were conducted. The first one evaluated the spore production, biomass production and viability of the selected *M. anisopliae* and *B. bassiana* isolates on three liquid media. The second experiment evaluated the effect of these different liquid phase media on the quality of spores of the selected isolates mass produced on rice solid substrate and their virulence against *P. interrupta.* All the experiments were conducted at the International Center of Insect Physiology and Ecology (ICIPE) arthropod pathology unit laboratories in Nairobi, Kenya except the bioassay against *P.*

interrupta which was conducted at Ambo Plant Protection Research Center (PPRC) laboratories, Ethiopia.

6.2.1. Liquid media evaluation

6.2.1.1. Preparation of fungal cultures

Isolates of two *B. bassiana* (Mp3post, ICIPE 279) and three *M.anisopliae* (PPRC51, PPRC2 and ICIPE 69) were sub-cultured on SDA media and incubated for three weeks. After sporulation, conidia were scraped using sterilized metal spatulas from the surfaces of the media and put into universal bottles containing 10ml of 0.05% Triton -X- 100 solution to prepare conidial suspensions. These suspensions served as stock for appropriate dilutions to obtain required conidial concentrations.

6.2.1.2. Preparation and inoculation of liquid media

Three liquid broth media *viz.* sucrose /waste brewer's yeast (SWBY), molasses/yeast extract (MYE), and lennoux broth base (LB), were used. SWBY: (20g of sucrose and 20g of dried yeast extract (from Kenya brewers) was mixed in 1 liter of distilled water and brought to boil in the water bath for 15 minutes. Molasses yeast extract broth contained 20ml of thick (viscose) sugarcane molasses and 20g of yeast extract mixed in 1 liter of distilled water. Lennouex broth base was constituted by mixing 30g of glucose (Panreac), 30g of yeast extract (Fluka) and 10g of peptone (HIMEDIA) in 1liter of distilled water. All the prepared solutions were then separately steered for 10minutes using a magnetic steer to homogenize the media. One hundred milliliters of each media were then poured to 250ml flasks and sterilized in an autoclave for 20 minutes at 121^{o}C and 15 PSI. When the media cooled to approximately 50^{o}C, 25µg/ml of chloramphenicol was aseptically added into each flask and hand shaken for a minute. Conidia of *M. anisopliae* and *B.*

bassiana were harvested from 3 weeks old cultures of each of the isolates and suspended in universal bottles containing 10 ml of 0.05% Triton - X- 100. The media in each flask were finally inoculated with 1 ml of the suspension containing a 6 X 10^6/ml conidial concentration of the respective isolates. The inoculated liquid media flasks were kept in a non-shaking incubator at 25^{o}C for 14 days. After the incubation period, sporulated media were thoroughly mixed using a magnetic stir bar for 5 minutes. The mixture was then filtered through a double layer of clean muslin cloth. The volume was adjusted to 1000ml using sterile distilled water to dilute the mixture. Samples were taken for viability test and to calculate number of spores/ml. The filtered material (biomass) was carefully transferred to 9cm diameter Petridishes and oven dried in a ventilated oven at 65 °C for 72 hrs. Finally, the biomass was weighed on a Mettler balance.

Each of the treatments (table13) was replicated three times and each flask served as a replicate. The un-inoculated flasks from each medium with all the respective media contents except for conidia served as control treatments.

6.2.2. Effects of liquid phase media on quality and virulence of mass produced spores

6.2.2.1. Preparation and inoculation of liquid phase media

Liquid media were prepared and inoculated exactly as in section 6.2.1.2. except that in this case, the flasks contained 75ml of liquid media. In addition to this for inoculation of solid substrate, the prepared media were incubated in an incubator shaker at 25^{o}C and 100RPM for 7 days. The treatments were also the same as used for liquid media evaluation (Table 13).

Table 13.Treatments used for evaluation of liquid media

Treatment	Isolate	Species	Liquid media (broth)	Remarks
T1	PPRC 2	*Metarhizium*	MYE	Molasses and yeast extract
T2	PPRC 2	*Metarhizium*	SWBY	Sucrose and waste brewer's yeast
T3	PPRC 2	*Metarhizium*	LB	Lennoux broth
T4	MP3post	*Beauveria*	MYE	
T5	MP3post	*Beauveria*	SWBY	
T6	MP3post	*Beauveria*	LB	
T7	PPRC 51	*Metarhizium*	MYE	
T8	PPRC 51	*Metarhizium*	SWBY	
T9	PPRC 51	*Metarhizium*	LB	
T10	IC69	*Metarhizium*	MYE	
T11	IC69	*Metarhizium*	SWBY	
T12	IC69	*Metarhizium*	LB	
T13	IC279	*Beauveria*	MYE	
T14	IC279	*Beauveria*	SWBY	
T15	IC279	*Beauveria*	LB	
T16	Control	None	MYE	
T17	Control	None	SWBY	
T18	Control	None	LB	

Each of the treatments was replicated three times and each flask served as a replicate. The experimental design was completely randomized design (CRD). The un-inoculated flasks

from each medium with all the respective media contents except for conidia served as control treatments.

6.2.2.2. Preparation and inoculation of solid substrates

Whole rice (local variety (Pishori) from Mwae, kiriyaga county, Kenya) and locally produced sorghum were used as solid substrates. One kilogram of each of the substrates was washed with running water and pre-cooked by pouring boiled water (300ml) into plastic containers containing the substrates, closing the containers and soaking for 30 minutes. Once the substrate was cool, 20ml of soybean oil was added and thoroughly mixed with a clean fork to help reduce clamping. The well mixed substrate was sealed in Millner bags (polypropylene mushroom spawn autoclave bags) (60 cm long by 35 cm wide cut in to two) with gas-permeable vent patches and sterilized by autoclaving for 20 minutes at 121^{o}C and 15 PSI. The bags were allowed to cool overnight and inoculated with the respective media containing the blastospores of the *M .anisopliae* and *B. bassiana* isolates. Control treatments were inoculated with the control flasks containing the media only and additional 75 ml of sterile water. For inoculation of the substrates, 75 ml of additional sterilized water was used with each bag and bags were massaged to spread the inoculums and the water throughout the substrate. Samples were taken from the substrates to determine the moisture content immediately after inoculation of the bags with the respective isolate-liquid media combination. The bags were then sealed inside the laminar flow hood and transferred to shelves in a mass production room equipped with an automatic heater and dehumidifier at 24 ^{o}C. The mean moisture content of the rice and sorghum substrates was 56.18% and 56.04% respectively. The bags were checked after 24 hrs of incubation and mixed gently, and tapped after one week and left

for another two weeks. After full sporulation, the substrates were transferred to surface sterilized dry clean bowls and aerated to let dry and enhance sporulation for 5 days. Harvesting of spores was done using a 300µm sieve to separate substrate and spores.

6.2.2.3. Determination of spore quality

The quality of the mass produced spores was determined by taking measurements on weight of spores/kg of substrate, number of spores/gm of spore powder, viability of spores, moisture content of spores, and biological purity of spores. To measure weight of spores/kg of substrate, harvested spores were weighed on a Mettler balance. To determine the number of spores/gm of spore powder, 0.1 gm of harvested spores was added to universal bottles containing 10 ml of sterilized 0.01% Tween 80 to make a stock suspension. Appropriate serial dilutions were done and finally spores were counted using an improved neubaur heamocytometer. For determination of viability, harvested spores were added to universal bottles containing 10 ml of sterilized 0.01% Tween 80 to make a stock suspension. The concentration of the stock suspension was adjusted to 3×10^6 conidia /ml using an improved neubaur heamocytometer and 100 µl of the suspension was then spread plated on SDA media in 90 mm diameter Petri-dishes. Germination was stopped after 24 hrs of incubation at $25^{O}C$ by adding 1ml of lacto-phenol blue solution. A sterile cover slip was then put on each Petri-dish and percentage germination was determined by counting at least 300 conidia under a compound microscope at x400 magnification. A conidium was declared germinated if it showed a growth as big as its size. Three replicate Petri-dishes were used for each isolate.

To determine the moisture content of spores at harvest, 1gm of spore powder was added to pre weighed oven dried universal bottles and oven dried for 24hrs. The universal

bottles were then reweighed and moisture content was determined by using the formula (adapted from Lomer and Lomer, 1999):

$$\frac{wet\ weight - dry\ weight}{wet\ weight} x100$$

Biological purity of mass produced spores was determined by serially diluting a 0.1gm spore powder to a $1x10^8$ concentration and spreading plating 200µl of the suspension on SDA media in three replications for each isolate. The plates were then incubated at 25°C for 5days. The number of pure colonies/ml and contaminant colonies/ml were then counted and multiplied by the respective dilutions and the percentage of contaminant colonies was calculated to get the percent purity (% of contamination) of the sample dry spore powder. All experiments were replicated three times and a completely randomized design (CRD) was used as the experimental design.

6.2.2.4. Shelf life of isolates

To test the shelf life of the selected isolates, 5g of spores of the three *M. anisopliae* isolates mass produced on rice substrate were dried over night at 30°C to remove excess moisture and sealed in polyethylene bags and covered with aluminium foil. The moisture content of spores was between 6-7 % before being sealed. The bags were then stored at 4°C and at room temperature (22-24°C) in the laboratory at PPRC. Completely randomized design was used.Three replicate bags were prepared for each isolate. Prior to sealing, the initial percentage germination of each replicate spore was tested on SDA media and recorded before storage. The germination of the spores was checked every month for six consecutive months.

6.2.2.5. Bioassay for virulence against *P. interrupta*

The virulence of mass produced spores of three *M. anisopliae* isolates was evaluated. The isolates were selected on the basis of their amenability to mass production on rice substrate for final bio-assay against *P. interrupta*. Two of the isolates (PPRC51 and PPRC2) were isolated from *P. interrupta* in Ethiopia and had high virulence to *P. interrupta* and the third isolate was a commercialized isolate IC69 from ICIPE (isolated from soil in D.R. Congo) which was included as a standard for comparison. Adult beetles were collected during the mating season of 2014 from breeding areas around Rassa and kept in plastic baskets containing moistened sterile soil collected from the same area. The baskets had side openings for aeration and the tops were covered with muslin cloth to prevent beetles from escaping. Collected beetles were fed with slices of ripe banana and observed for any natural infection for 10 days before being used for bio-assays.

Ten beetles were put in sterile 300ml plastic tubs with perforated lid. A 1mg spore was added on top of the beetles in each of the plastic tubs and the beetles were allowed to move in the tubs for 30 minutes. The beetles were then transferred to 120mm diameter plastic Petri-dishes containing moist filter paper and incubated at room temperature for 10 days. During incubation, beetles were fed with slices of ripe banana changed every other day. To provide adequate moisture, 1ml of sterile distilled water was added to the Petri-dishes every day. Mortality was assessed every day and dead beetles were removed and surface sterilized with 70% ethanol and rinsed three times in sterile distilled water. The surface sterilized beetles were then transferred to sterile Petri-dishes containing moist filter paper and incubated at 25^0C to check for sporulation and to confirm death due to fungal infection.

6.3. Statistical analysis

All percentage data were arcsine transformed before analysis to stabilize the variance and normalize the data. A completely randomized design (CRD) with a 3x5 (media X isolate respectively) factorial arrangement was used in the case of liquid media evaluation. Similarly, a 3x3 (media X isolate) factorial arrangement was used in the case of liquid phase (blastospore) media evaluation. There were four replications per isolate including control. In the case of virulence evaluation, percent mortality data were corrected for control mortality using Abbot's formula (Abbot, 1925). A CRD with four replication was used and controls of each isolate were treated with the respective spores killed at 80^0C in an oven for 48 hrs. Mean percentage mortality of *P. interrupta* was used as measure of virulence. Data were analyzed using the ANOVA procedure of SAS statistical soft ware version 9.2.

6.4. Results

6.4.1. Liquid media evaluation

The number of spores produced in the three different liquid media showed significant variation among isolates and liquid media (P=0.02, F=3.28, df = 4, 30 and P=0.0001, F=12.63, df=2, 30 respectively). The interaction between isolates and liquid media also varied significantly (P=0.01, F=2.86 and df = 8, 30).

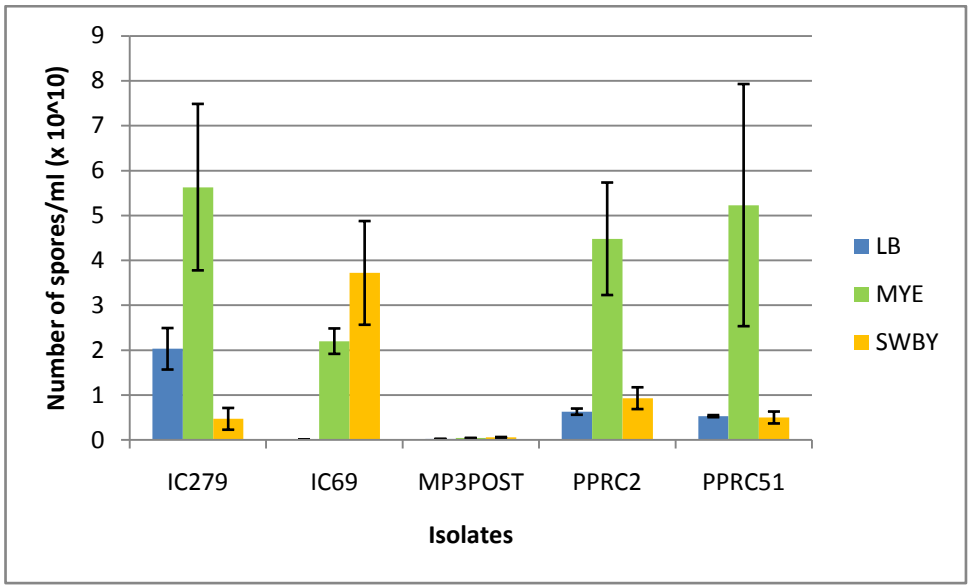

Figure 37. Number of spores/ml produced by two *B. bassiana* and three *M. anisopliae* isolates in three liquid media. Lines on top of bars show ± standard errors.

The ICIPE isolate IC279 fermented in MYE liquid media produced the highest number of spores/ml ($5.63x10^{10}$) followed by PPRC51 ($5.23x10^{10}$) on the same medium (figure.37.). PPRC2 produced $4.48x10^{10}$ spores/ml on this medium ranking third. The number of spores produce by isolates on all other media were less than half of the number of spores produced in MYE media except for IC69 which produced $3.72x10^{10}$ spores/ml with SWBY medium. MP3POST which produced below $0.05x10^{10}$ spores/ml in the three media was the lowest spore producer among all the isolates.

The viability of the isolates produced in the three liquid media also showed significant variations (P=0.002, F=5.43, df=4, 30). The different media used did not show any significant variations on spore viability (P=0.40, F=0.93, df=2, 30). However, the interaction between isolates and media was significant (P=0.03, F=2.43, df=8, 30). Highest viability was recorded from IC279 (100%) in MYE medium followed by PPRC51 (90.54%) in LB medium (Figure38) IC69, MP3POST and PPRC2 preferred

SWBY medium to other media and showed greater than 68% viability on the medium. PPRC51 showed better viability in LB and MYE media with 90.54% and 82.34 % respectively. The lowest viability (56.79%) was recorded from MP3POST in MYE medium.

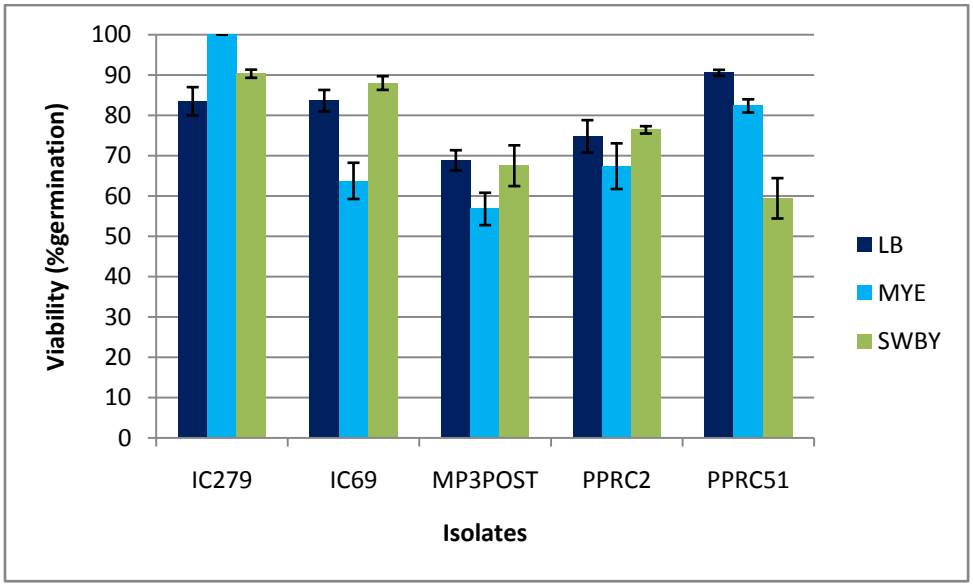

Figure 38.Viability of spores of two *B. bassian* and three *M. anisopliae* produced in three liquid media.

Biomass production significantly varied across all isolates and media with P=0.0001, F=119.9, df=4, 30 and P=0.0001, F=513.97, df=2, 30 respectively. The interaction between isolates and media was also significant (P=0.0001, F=37.02, df=8, 30). PPRC2 produced the highest (2.47g) biomass followed by PPRC51 (2.16g) in LB medium (figure 39). Generally most of the isolates preferred LB medium to the two other media for biomass production. The lowest biomass was recorded from MP3POST with 0.39g with MYE medium.

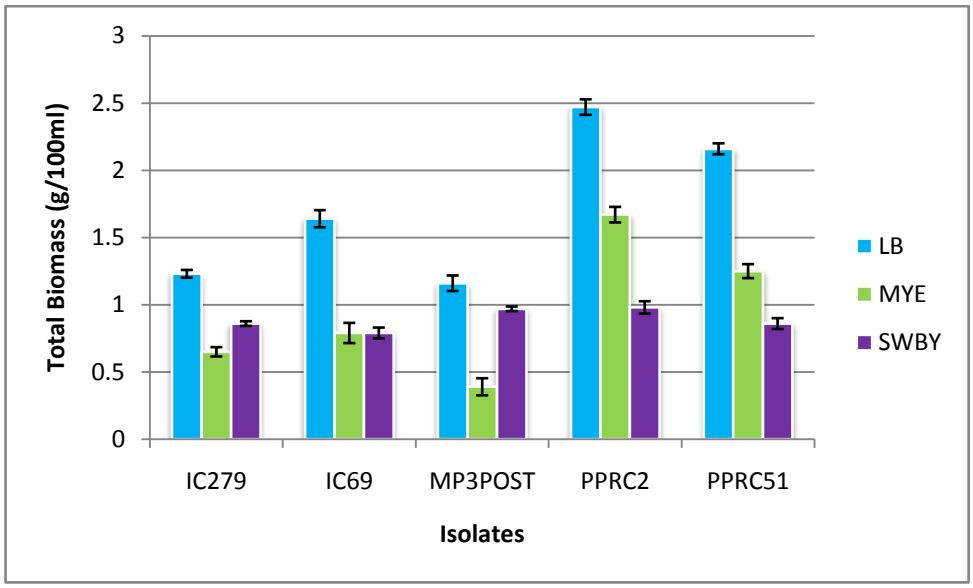

Figure 39. Biomass production of two *B. bassian* and three *M. anisopliae* produced in three liquid media.

6.4.2. Effects of liquid phase media on quality of mass produced spores

The criteria used to determine the quality of the mass produced spores were based on the product quality control parameters elaborated by Lomer and Lomer (1999) in the Lubilosa insect pathology manual. The effect of the liquid media on each of the parameters is given below.

Weight of spores/kg of rice substrate varied significantly across the three isolates (P=0.0001, F=582.66, df=2, 18). There was no significant difference among blastospore media and their interactions with isolates (P=0.09, F=2.63, df=2, 18 and P=0.18, F=1.75, df 2, 18 respectively).

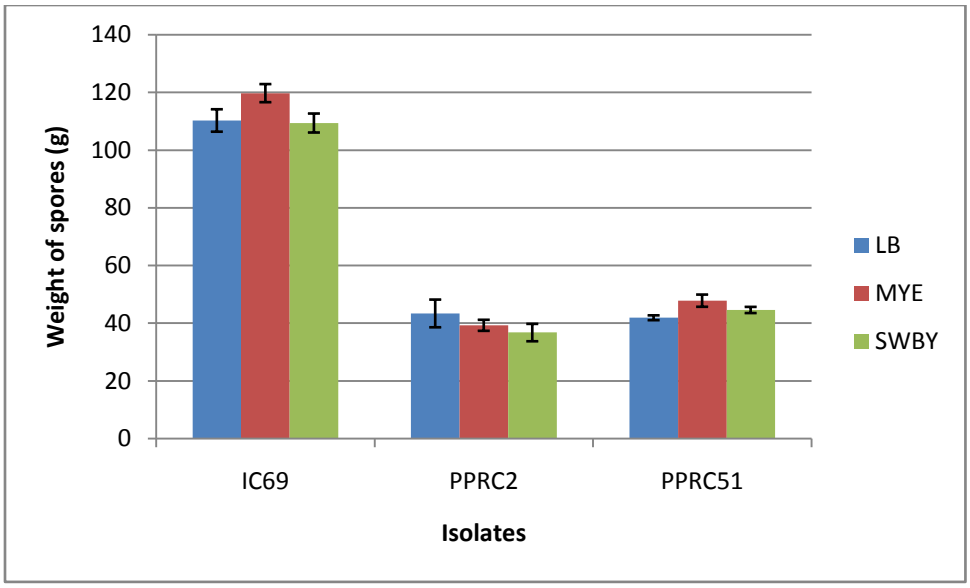

Figure 40. Weight of spores produced per kg of rice substrate by three *M. anisopliae* isolates using three different liquid phase media.

The highest weight of spores (119.72g/kg) was obtained from IC69 mass produced by using MYE as a liquid phase medium (figure 40). The isolate also produced spores of 110.25g/kg and 109.37g/km of rice with LB and SWBY liquid media respectively. PPRC51 and PPRC2 generally produced less than 48g of spore/kg of rice substrate when produced using all liquid phase media as source of blastospores with the lowest (36.78g/kg) produced from PPRC2 with SWBY medium.

The number of spores/g of spore powder also varied significantly (P=0.0018, F=9.21, df=2, 18) among the isolates. However, the liquid phase media and interaction between isolates and liquid phase media did not show any significant variations (P=0.37, F=1.03, df=2,18 and P=0.55, F=0.79, df=4, 18 respectively).

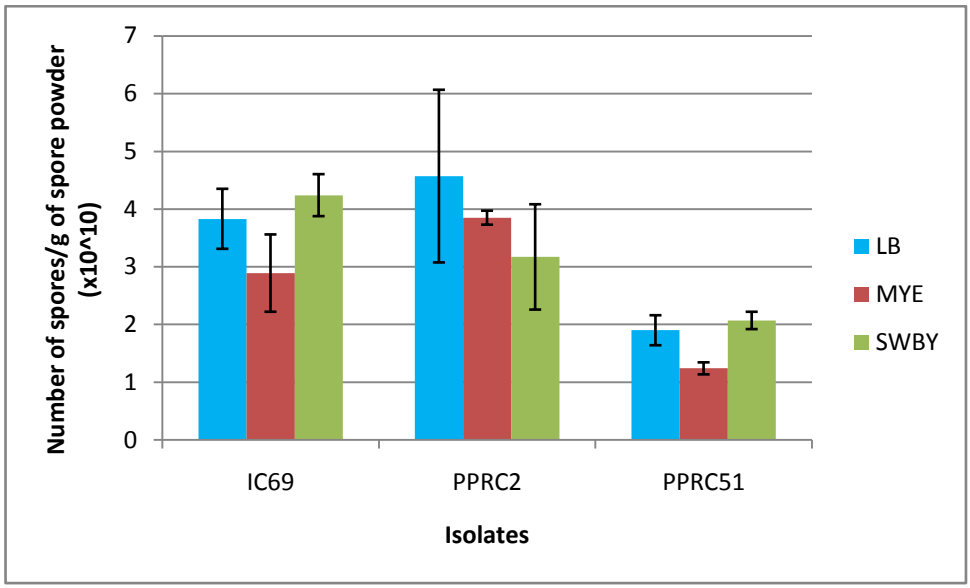

Figure 41. Number of spores per gm of spore powder produced by three *M. anisopliae* isolates on rice substrate using three different liquid phase media.

PPRC2 produced the highest number of spores ($4.57x10^{10}$ spores/g) when using LB as liquid phase medium (figure 41). The lowest number of spores was obtained from PPRC51 ($1.24x10^{10}$ spores/g) using MYE liquid phase medium.

The viability of spores after harvest also varied significantly among the isolates (P=0.0093, F=6.13, df=2, 18). There was no significant variation in affecting viability of spores among the different liquid phase media and their interaction with the isolates (P=0.14, F=2.25, df=2,18 and P=0.36, F=1.16, df=4, 18 respectively). All the isolates showed over 90% viability regardless of the source of the liquid phase media. Highest viability was recorded from IC69 with the use of MYE liquid phase medium (figure 42).

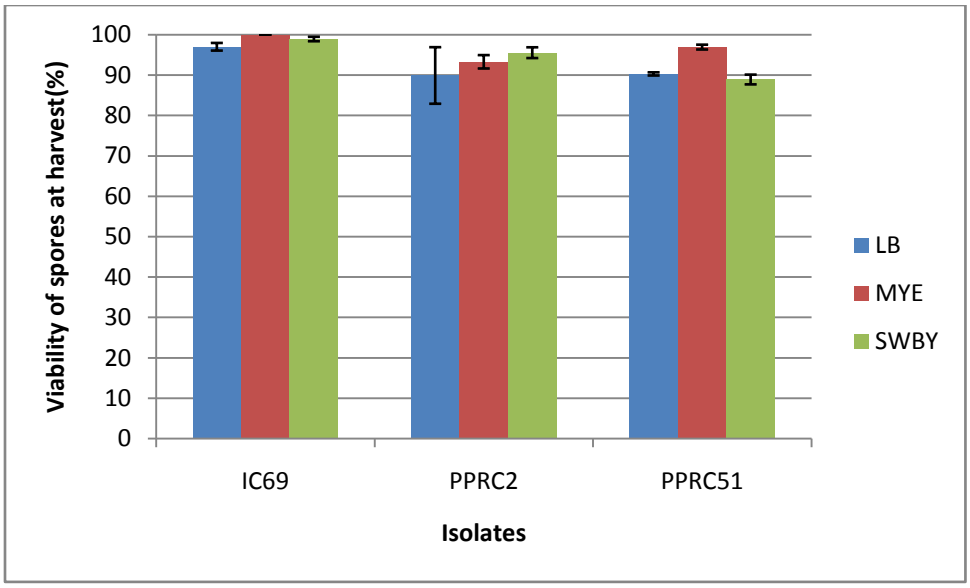

Figure 42. Viability of harvested spores of three *M. anisopliae* isolates mass produced on rice substrate using three liquid phase media.

The moisture content of spores at harvest did not show any significant variation among isolates when using the different liquid phase media as source of blastospores (P=0.17, F=1.93, df=2, 18 and P=0.26, F=1.45, df=2, 18 respectively). However, the interaction between isolates and liquid phase media exhibited a slight interaction (P=0.03, F=3.33, df= 4, 18). PPRC2 produced with MYE as a liquid medium contained the highest moisture (55.33 %) compared to IC69 and PPRC51 which contained 48.67% and 36.67% moisture at harvest respectively using the same liquid phase medium. The lowest moisture content (36%) was also recorded from PPRC2 produced with SWBY as liquid phase medium (Figure 43).

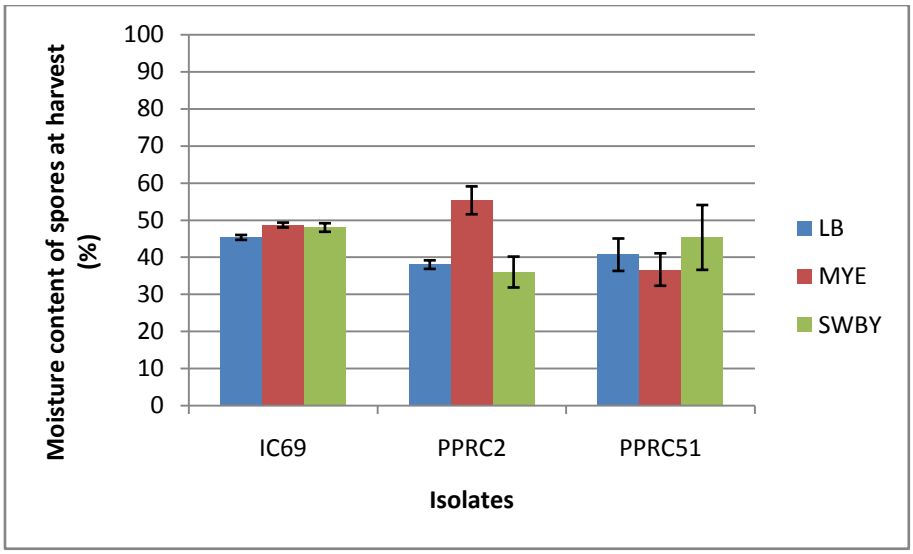

Figure 43. Moisture content at harvest of three *M. anisopliae* isolates mass produced on rice substrate using three different liquid phase media.

Biological purity did not show significant variation among the isolates and the liquid phase media (P=0.41, F=0.95, df=2, 18 and P=0.42, F=0.91, df=2,18 respectively). However, the interaction of isolates and liquid phase media was highly significant (P=0.0001, F=86.64, df= 4, 18).

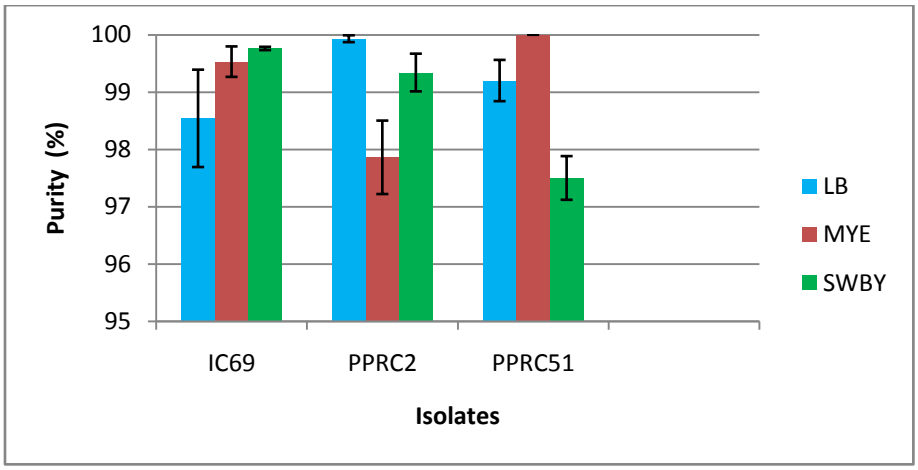

Figure 44. Biological purity of three *M. anisopliae* isolates mass produced on rice using three different liquid phase media.

PPRC51 produced using MYE as liquid phase medium produced spores with 100% purity (figure 44) while producing the lowest purity (97.5%) on SWBY medium compared to other media. Generally the biological purity of the spores produced using all the three liquid phase media was greater than 97%.

6.4.3. Shelflife of isolates

The shelflife of the spores of the isolates stored at 4°C over six months did not show any significant variations (generally P> 0.05) (figure 45(A)). However, the percent germination of the spores declined from the maximum initial of 87.2% for to 68.67% for isolate IC69. Maximum viability was observed on isolate PPRC51 (75%) while isolate IC69 showed minimum viability (68.67%) at the end of six months. Spores stored at room temperature (22-24°C) showed significant variations starting the first month through the third month (P= 0.04 (F=5.66), 0.01 (F=9.69), and 0.01(F=10.47) for months1-3 respectively) (Figure 45 (B)). There was no significant variation in viability among the three isolates after the third month (months 4-6) (P>0.05). The maximum viability after six months of storage at room temperature was recorded from the isolate PPRC51 (64.37%) while the minimum viability was recorded from the isolate PPRC2 (62.17%).

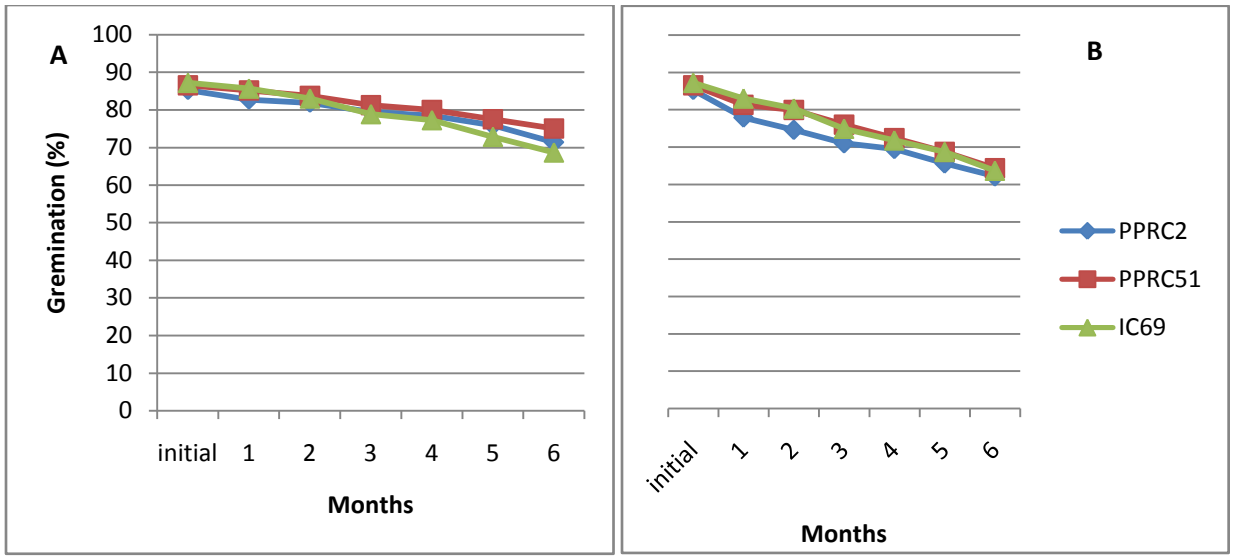

Figure 45. Shelflife of three *M. anisopliae* isolates over six months at 4°C (A) and 22-24°C (B)

6.4.4. Effects of liquid phase media on virulence of mass produced spores

Because of the low number of beetles collected from the field in July, 2014, only spores produced using MYE as liquid phase medium were used for the bioassay against *P. interrupta* adults. The virulence of the three isolates of *M. anisopliae* did not vary significantly (P=0.34, F=1.28, df=2, 6). PPRC2 caused the highest mortality (80.92%) followed by PPRC51 and IC69 which caused 77.75% and 57.14% mortality respectively (figure 46). The mean number of conidia per gram of each of the isolates used for the bioassay was $2.67x10^8$ (PPRC2), $9.2x10^7$(PPRC51) and $5.07x10^7$(IC69).

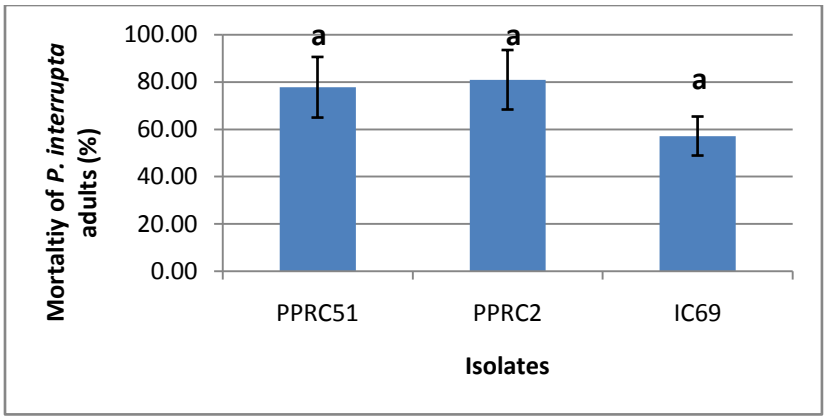

Figure 46. Mean percentage mortality of *P. interrupta* caused by three *M. anisopliae* isolates Mass produced on rice substrate using MYE as source of liquid phase medium.

6.5. Discussion

Initially the experiments were intended to include sorghum as a substrate for comparison with the most frequently used substrate i.e. rice. However, the sorghum variety that was used for the study appeared to be not convenient for mass production right from the beginning of the experiments probably because of poor cooking characteristic. It did not allow production of spores (sporulation) except for some mycelia growth. Consequently, it was excluded from the experiments and focus was only on rice substrate. An important characteristic of a selected strain of EPF (particularly *M. anisopliae* and *B. bassiana* strains) is its ability to conidiate *in-vitro* on solid substrates (Jaronski and Jackson, 2012). During this study, isolate MP3POST (*B. bassiana*) was not able to produce any spores on rice substrate despite diffuse mycelia growth. This isolate used to sporulate well on SDA medium in the laboratory. This is a well known phenomenon as the good sporulation of some strains on agar media may not be true indicator of good conidiation on solid substrates and can be misleading as the strains can only have mycelia growth with poor sporulation on solid substrates (Jaronski and Jackson, 2012). As a result, MP3POST and

IC-279, also a *B. bassiana* isolate which was included as a standard to compare the *B. bassiana* isolate (MP3POST), were excluded from the experiments. Thus the experiment focused only on the *M.anisopliae* isolates and rice substrate.

Investigating the nutritional requirements of EPF is important to improve inoculums supply through mass production and facilitate commercialization of biopesticides (Sun and Lui, 2006). The experiment on evaluation of liquid media in the current study showed that MYE significantly affected and supported production of highest number of spores/ml ($5.63x10^{10}$ IC279) and spore viability (100% IC279). Evaluating the effect of liquid media on viability, biomass production and number of spores using six different liquid media including MYE, Dirakhshan *et al*. (2008) also found superiority of MYE in all parameters for *Verticillium lecani* in which $8.33x10^{8}$ spores/ml and 91.5% germination were obtained. Another study by Latifan *et al.* (2013) also found a 2.4 $x10^{8}$ spores/ml of spore production and 99.5% germination of *B. bassiana* produced in liquid media supplemented by sugarcane molasses which agrees with the findings of this study. Similarly previous studies have confirmed that sugarcane molasses supplemented liquid media yielded highest blastospore and biomass production by *B. bassiana* and *M. anisopliae* (Hussain *et al*.; 2012) as well as *Paecilomyces farinosus* (Hotmskiold) and *P. lilacinus* (Thom.) Samson (Leena *et al*., 2003). The findings of this and previous studies indicate that liquid media supplemented by sugarcane molasses are very suitable for growth and production of blastospores that can be used for inoculation of solid substrates.

Although there are a range of varieties of grains investigated for use as solid substrates (Jenkins and Goettel, 1997; Sahayaraj and Namachivayam, 2008), the most commonly used substrates are rice (Oryza sativa) and flaked barley (Hardeum vulgare) (Jaronski and

Jackson, 2012). Sahayaraj and Namasivayam (2008) found $1.12x10^9$ conidia per 100g of rice substrate for *B. bassiana* by directly inoculating the substrate with conidial suspension without using liquid media. In this study, direct inoculation was not used for obvious advantages of the liquid phase media. Direct inoculation is not preferable to using liquid media because it does not allow detection of contaminants and may expose the substrate to be overwhelmed by bacteria which use the lag phase of the fungi as an advantage to proliferate (Jaronski and Jackson, 2012). In addition to avoiding contamination, use of liquid media in the diphasic mass production system for EPF has been reported to increase spore yield from solid substrates. Inoculating rice with conidia of *B. bassiana* produced in liquid substrate composed of crystalloid flour, potato and dextrose, Santoro *et al*. (2005) found spore yield of $2.7x10^{12}$/g of substrate increasing productivity by up to 1000 fold than produced on solid substrate without the liquid media.

Typically, a hypocrealean fungus can produce 20-150g conidia/kg of substrate, containing 1×10^{10} - 2×10^{11} conidia/g of solid substrate (depending on the fungus) (Jaronski and Jackson, 2012). The findings of this study also confer to this fact. For example PPRC2 produced the highest number of spores ($4.57x10^{10}$ spores/gm) when using LB as liquid phase medium and $3.85x10^{10}$ when using MYE liquid medium which were not significantly different from each other. Spore yield per kg of rice substrate also showed a similar trend with the highest weight of spores (119.72g/kg) obtained from IC69 mass produced by using MYE as a liquid phase medium and the lowest (36.78g/kg) produced from PPRC2 with SWBY medium. Use of any of the liquid media did not affect the number of spores/g of spore powder and the weight of spores/kg of substrate in

this study. It is to be noted that the commercialized isolate IC69 produced more than twice as high as the Ethiopian isolates (PPRC51 and PPRC2) indicating that strain selection is more important than liquid media selection for higher spore yield.

Santoro *et al*. (2005) suggested that sporulation was influenced by the source of nutrition in the liquid medium and caused reduction in production time while increasing productivity of *B. bassiana*. The source of liquid medium has also been reported to affect spore production of entomopathogenic fungi on solid substrates (El-Damir, 2006). In this study however, spore productivity appeared to be affected more by the strain than by the liquid media used. One of the reasons for this variability might be the fact that the experiments were conducted on different species of fungi and different types of liquid media. This can be regarded as an indication for the importance of developing specific mass production protocols for each specific species or strain of fungi intended to be used as biopesticides. The viability, moisture content at harvest and biological purity of spores of the tested isolates also did not vary significantly due to media except for slight and high interaction effects in moisture content and biological purity, respectively.

Mortality of *P. interrupta* caused by spores of mass produced isolates also did not differ very much from the mortality caused by spores produced on artificial media. Although statistical comparison was not done to avoid biases and erroneous conclusions, simple comparisons between the mortalities caused by spores of the isolates harvested from artificial media and solid substrates are not extremely different. PPRC2 caused mortality of 80.92% and 77.14% (chapter III) when harvested from rice substrate and SDA media respectively. Similarly, PPRC51 caused 77.75% and 82.40% (chapter III) mortality when harvested from rice substrate and SDA media respectively.

In conclusion, the current study has shown that liquid media supplemented by sugarcane molasses can enhance blastospore and biomass production and can be used to produce higher number of blastospores with substantial amount of biomass. In addition to this, the study has demonstrated that using either of the liquid media does not significantly affect all the quality parameters for mass production of the *M. anisopliae* isolates selected for control of *P. interrupta.* In view of these findings, molasses and waste brewer's yeast which are readily available from sugar industries and breweries in Ethiopia can be used to constitute liquid medium for successful mass production of EPF on rice substrate as a cheap source of liquid phase medium for these and other isolates.

CHAPTER VII

EVALUATION OF FARMERS' PERCEPTIONS ON BIOLOGICAL CONTROL OF SORGHUM CHAFER (*P. interrupta*) AT RASSA (SEFIBERET KEBELE) OF KEWET WEREDA IN NORTH SHOA ZONE

7.1. Introduction

Sorghum chafer, *P. interrupta* is a very destructive pest of sorghum and other important cereal and horticultural crops in Ethiopia. It was one of the limiting factors of sorghum production during the 1993–2003 out breaks although its population has notably decreased now. However, it continues to occur in sorghum fields and cause damage to sorghum production in some areas. It is particularly prevalent in the Amhara and Afar Regional States in some Kebeles. One of the most frequently affected kebeles is Sefiberet kebele of Rass area of Kewet wereda in the North Shoa Zone of the Amhara Regional State.

Control measures against *P. interrupta* have been and continue to be heavily dependent on use of chemical pesticides either in the form of baits or direct sprays. Other control measures include hand picking and killing of beetles, applying smoke and shaking off the beetles from sorghum heads. None of these measures have provided adequate control of the pest so far. Biological control has been suggested as environmentally safe alternative component of integrated management of the pest (Sinishaw and Mulugeta, 2002). The success of biological control of arthropod pests in sub-Saharan Africa particularly on annual crops has been limited due to poor involvement of farming communities and extension personnel in information dissemination (Nyambo and Löhr, 2005). In addition

to these, information on improved technologies including pest control strategies is an important factor of production (Sabo and Zira, 2009).

An important first step in ensuring participatory dissemination of new or improved technological outputs is to explore the perception of the end users on the outputs. Therefore, a preliminary study was conducted at Rassa (Sefiberet kebele) of kewet wereda in North showa zone with the objective of evaluation of farmers' perception on biological control of *P. interrupta* as a component of integrated pest management.

7.2. Methodology

A structured interview using a questionnaire containing four questions (Table 14) was used for the evaluation. A three days training on biological control of *P. interrupta* as a component of integrated management of the pest was given to participants including the farmers interviewed. A total of 84 and 71 farmers were randomly selected and interviewed before and after the training, respectively. Fourteen and 9 women farmers were involved in the interviews before and after the training, respectively. Data were analyzed using SPSS version 20 software.

Table 14.Questionnaire for evaluation of farmers' perceptions on biological control *of P. interrupta* as a component of integrated pest management (IPM)

Question 1. How threatening is *P. interrupta* to sorghum production nowadays?							
Not a threat		**High threat**		**Moderate threat**			
Question 2. Which one of the control measures for *P. interrupta* do you prefer?							
Cultural		**Chemical**		**Biological**		**IPM**	
Question 3. Have you ever heard of biological control of *P. interrupta*?							
Yes		**No**					
Question 4. Do you think that *P. interrupta* can be controlled using biological control methods?							
Yes		**No**					

7.3. Results

7.3.1. Pre-training interview

Out of a total of 84 farmers interviewed, 79 farmers (94.05%) had never heard of biological control (BC) of *P. interrupta* and 5(5.95%) had heard of BC of the pest .Only 7 (8.33%) of the farmers thought that biological control measures can control *P. interrupta*, while 91.67% thought otherwise. Asked which control measures they preferred, 83 (98.81%) of the farmers preferred chemical control methods to cultural (0%), biological (1.19%) or IPM (0%). With regard to the threat that *P. interrupta* poses to sorghum production nowadays, 68 (80.95%) of the interviewed farmers thought the pest posed moderate threat while 14.29% and 4.76% thought the pest posed no threat and high threat, respectively (figure 46).

7.3.2. Post training interview

After a three days training on biological control of *P. interrupta* as a component of IPM, 45 (63.38%) considered biological control as a possible control measure for *P. interrupta* 26 (36.62%) did not consider it as a possible control measure. However, when it comes to preference of control measures, 50 (70.42%) of the farmers still preferred chemical control measures to (figure 47 A and B) any of cultural (1.41%), biological (28.17%) or IPM (0%). Only question 2 and 3 were thought to be affected by the training and the answers to questions 1 and 4 were deliberately ignored.

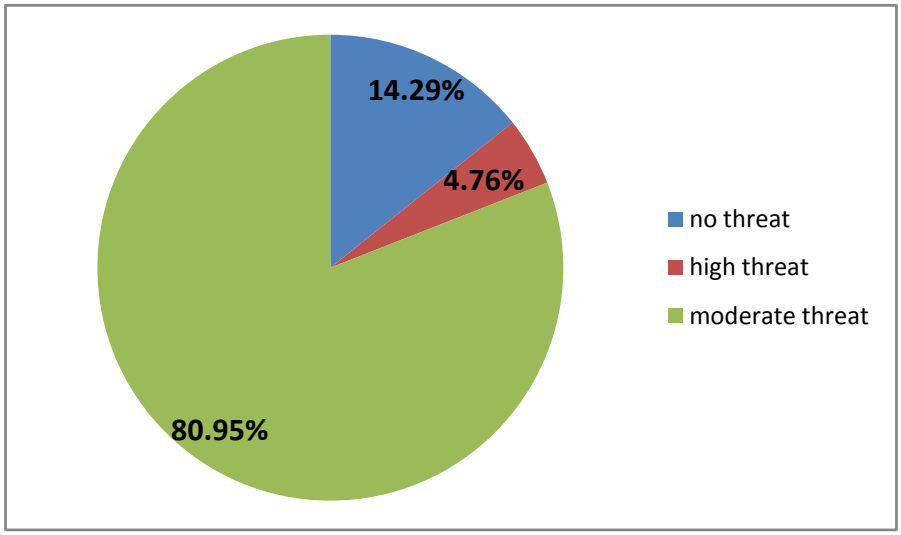

Figure 46. Farmers' evaluation of the threat of *P. interrupta* to sorghum production at sefiberet kebele of Kewet wereda of North shewa zone in Amhara Regional State.

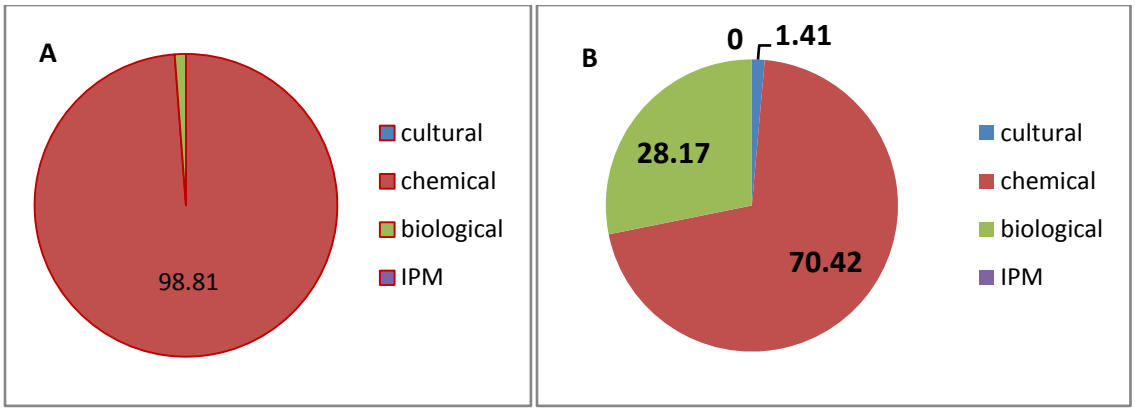

Figure 47. Farmers' preference (in percent) of control of *P. interrupta* at sefiberet kebele of Kewet wereda of North shewa zone in Amhara Regional State before (A) and after (B) a three days training on IPM for the pest.

7.4. Discussion

The results of the study showed that the perception of the farmers on use of either biological control or IPM of *P. interrupta* is very low. About 98.81% of the farmers preferred chemical pesticides for control of the pest before the training. After the training, interviewed farmers still preferred use of chemical pesticides when the pest appears (70.42% of the respondents) although the percent of farmers who considered (thought) biological control as a possible control measure increased from 8.33% before the training to 63.38% after the training. However, none of the respondents (0%) considered IPM as a measure of control both before and after the training which indicates the luck of knowledge on importance of IPM for control of the pest in the area. The study also showed that *P. interrupta* is considered to pose moderate threat (80.95% of the respondents) to sorghum production nowadays. The pest is considered as high threat by 4.76% of the respondents. These might show the concerns of the farmers about the unpredictable damage that could be caused by the pest at any time. Previous studies

showed that farmers considered *P. interrupta* as the most dangerous pest of all the pests in the area (Yeraswerk, 2000).

In conclusion, the study has shown the extremely low perception of the farmers on use of biological control as a component of IPM for *P. interrupta.* The study also indicated that the farmers are highly inclined to wards using chemical pesticides against the pest. It is also concluded that the pest still poses moderate threat to sorghum production in the area and farmers are concerned about its control.

Extensive research that includes more kebeles and details of socio-economic characteristics of the kebeles is recommended to clearly determine the perception of the farmers on use of IPM for the pest. More training on IPM, with special emphasis on the use of the fungal biopesticides and traps that resulted from this thesis work, need to be disseminated to more farmers in affected regions and kebeles.

8. GENERAL CONCLUSION

The findings of this study have indicated the potential use of native entomopathogenic fungi against *P. interrupta*. The *Metarhizium anisopliae* isolate PPRC51 is selected as a promising candidate for development of a myco-pesticide against *P. interrupta* at commercial level that can be used for autodissemination of virulent strains in the pest's natural habitat for gradual decrease of the pest population over time as a component of integrated pest management system. The *Metarhizium anisopliae* isolate PPRC2 can also be used in a similar way as a second choice candidate.

The entomopathogenic fungi used in the study were effectively identified as *M. anisopliae* and *B. bassiana* by the PCR amplification of the ITS regions of rDNA and chitinase genes. The ITS5 and ITS4 regions were used as forward and backward primers and were able to successfully identify the fungal isolates. Similarly, *chi4* and *chi1* primers can be used as molecular markers to supplement identification of the *B. bassiana* and *M. anisopliae* isolates, respectively.Therefore, the tools can be used for identification of *Metarhizium* and *Beauveria* spp. isolates for control of *P. interrupta.* The isolates have been provided with accession numbers in the Genbank and can be traced back in the future. Best cultivation media and temperature for the selected isolates are SDA (or SDAY) and 30°C, respectively.

This study has demonstrated a ground breaking development of traps equipped with autodissemination device made of locally available materials for use in the management of *P. interrupta* in Ethiopia. The developed AIT's can be regarded as a low-input approach as conventional control means using chemical pesticides poses difficulties and

raises economic feasibility questions for the subsistent farmers. The AIT's can be used for autodissemination of entomopathogenic fungi to remote breeding sites and can help reduce the population of *P. interrupta*.

The quantity, quality and virulence of spores of the selected isolates is not affected by use of any of the liquid phase media used and thus each of LB, MYE or SWBY can be used as alternative sources of liquid medium depending on availability for effective mass production of the isolates with rice as a solid substrate. The isolates can also be stored at room temperature for upto six months without significant loss of viability.

The study has shown the low perception of the farmers on use of biological control as a component of IPM for *P. interrupta.* It also indicated that the farmers are inclined towards using chemical pesticides against the pest. It can also be concluded that the pest still poses moderate threat to sorghum production in the study area and farmers are still concerned about its control.

9. RECOMMENDATIONS

Additional laboratory studies to determine the virulence of the isolates to the target insect under similar temperature regimes used for *in-vitro* characterization during the current study need to be conducted. Further investigations are also recommended to identify factors that enhance thermal tolerance and infectivity of the selected promising isolates under field conditions.

Additional field studies to demonstrate the ecological establishment of the selected isolates under high population conditions. In addition to this, more studies to demonstrate or assess population-scale behavioral responses of the pest, the dissemination of the fungi to breeding areas and to cause epizootics in larvae and adults in the breeding areas need to be carried out to fully establish the efficacy of the selected isolates in the field.

The *Metarhizium anisopliae* isolate PPRC51 is recommended as potential candidate for development of myco-pesticide against *P. interrupta* and attempts should be made to commercialize it for integrated management of the pest along with the obviously cheap (compared to the commercially available JBT) auto-inoculation traps developed during this study.

Molasses and waste brewer's yeast which are readily available from sugar industries and breweries in Ethiopia can be used to constitute liquid medium for successful mass production of EPF on rice substrate as a cheap source of liquid phase medium for these and other isolates. Studies on use of these liquid phase media on other cheaper substrates such as sorghum are recommended.

Extensive research that includes more kebeles affected by the pest and details of socio-economic characteristics of the kebeles is recommended to clearly determine the perception of the farmers on use of IPM against the pest. More training on IPM, with special emphasis on the use of the fungal biopesticides and traps that resulted from this dissertation work, need to be disseminated to more farmers in affected regions and kebeles for successful control of the pest.

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11. APPENDICES

Appendix 1. ANOVA for factorial analysis of Percent germination of EPFon different media and temperatures

Source	DF	SS	MS	F-Value	Pr>F
isolate	4	12324.1	3081.0	69.72	<.0001
media	3	4231.6	1410.5	31.92	<.0001
temprature	4	254310.7	63577.7	1438.69	<.0001
isolate*media	12	16221.4	1351.8	30.59	<.0001
isolate*temprature	16	41319.7	2582.5	58.44	<.0001
isolat*media*temprat	60	45638.8	760.6	17.21	<.0001
R-square = 0.98, C.V. =10.46 %, Tukey's (HSD) Test					

Appendix 2. ANOVA for factorial analysis of radial growth of EPF on different media and temperatures

Source	DF	SS	MS	F-Value	Pr>F
isolate	4	1454.6	363.6	132.92	<.0001
media	3	946.3	315.4	115.30	<.0001
temprature	4	8950.4	2237.6	817.90	<.0001
isolate*media	12	713.8	59.5	21.74	<.0001
isolate*temprature	16	2128.0	133.0	48.62	<.0001
isolat*media*temprat	60	2987.5	49.8	18.20	<.0001
R-square = 0.97, C.V. =16.24, Tukey's (HSD) Test					

Appendix 3. ANOVA table for factorial analysis of sporulation of EPF on different media and temperatures

Source	DF	SS	MS	F-Value	Pr>F
isolate	4	9.3	2.3	7.62	<.0001
media	3	25.0	8.3	27.34	<.0001
temprature	4	1860.9	465.2	1525.25	<.0001
isolate*media	12	30.5	2.5	8.32	<.0001
isolate*temprature	16	224.1	14.0	45.93	<.0001
isolat*media*temprat	60	96.2	1.6	5.26	<.0001
R-square = 0.97, C.V. =17.71, Tukey's (HSD) Test					

Appendix 4. ANOVA table for factorial analysis of viability of spores in different media

Source	DF	SS	MS	F-Value	Pr>F
isolate	4	3437.3	859.3	5.43	0.0021
media	2	295.8	147.9	0.93	0.40410
isolate*media	8	3081.9	385.2	2.43	0.037
R-square = 0.59, C.V. =16.37, Duncan’s multiple range test					

Appendix 5. ANOVA table for factorial analysis of number of spores in different media

Source	DF	SS	MS	F-Value	Pr>F
isolate	4	36.71240609	9.17810152	3.28	0.0242
media	2	70.76527004	35.38263502	12.63	0.0174
isolate*media	8	64.00294084	8.00036761	2.86	0.0001
R-square = 0.67, C.V. = 94.9, Duncan’s multiple range test					

Appendix 6. ANOVA table for factorial analysis of biomass production in different media

Source	DF	SS	MS	F-Value	Pr>F
isolate	4	3.45428889	0.86357222	119.90	<.0001
media	2	7.40349778	3.70174889	513.97	<.0001
isolate*media	8	2.13319111	0.26664889	37.02	<.0001
R-square = 0.98, C.V. = 7.32, Duncan's multiple range test					

Appendix 7. ANOVA table for factorial analysis of wt of spores produced /kg of rice substrate using different liquid phase media

Source	DF	SS	MS	F-Value	Pr>F
isolate	2	30195.04380	15097.52190	582.66	<.0001
media	2	136.20642	68.10321	2.63	0.0997
isolate*media	4	181.28971	45.32243	1.75	0.1832
R-square = 0.98, C.V. = 7.72, Tukey's Studentized Range (HSD) Test					

Appendix 8. ANOVA table for factorial analysis of number of spores /g of spore powder produced on rice substrate using liquid phase media

Source	DF	SS	MS	F-Value	Pr>F
isolate	2	24.71769074	12.35884537	9.21	0.0018
media	2	2.77251296	1.38625648	1.03	0.3760
isolate*media	4	4.24738148	1.06184537	0.79	0.5459
R-square = 0.57, C.V. = 37.56, Tukey's Studentized Range (HSD) Test					

Appendix 9. ANOVA table for factorial analysis viability of spores produced on rice substrate using liquid phase media

Source	DF	SS	MS	F-Value	Pr>F
isolate	2	231.8437240	115.9218620	6.13	0.0093
media	2	85.0686100	42.5343050	2.25	0.1341
isolate*media	4	87.5648675	21.8912169	1.16	0.3617
R-square = 0.54, C.V. = 4.6, Tukey's Studentized Range (HSD) Test					

Appendix 10. ANOVA table for factorial analysis moisture content at harvest of spores produced on rice substrate using liquid phase media

Source	DF	SS	MS	F-Value	Pr>F
isolate	2	192.8888889	96.4444444	1.93	0.1736
media	2	144.8888889	72.4444444	1.45	0.2602
isolate*media	4	664.8888889	166.2222222	3.33	0.0330
R-square = 0.52, C.V. = 16.13, Tukey's Studentized Range (HSD) Test					

Appendix 11. ANOVA table for factorial analysis biological purity of spores produced on rice substrate using liquid phase media

Source	DF	SS	MS	F-Value	Pr>F
isolate	2	0.64595556	0.32297778	0.95	0.4058
media	2	0.61768889	0.30884444	0.91	0.4214
isolate*media	4	18.51982222	4.62995556	13.60	<.0001
R-square = 0.76, C.V. = 0.58, Tukey's Studentized Range (HSD) Test					

DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in any other university, and that all sources of material used for the thesis have been duly acknowledged.

Name: Belay Habtegebriel

Signature: _____

Date: 2 July, 2015

Place: Addis Ababa