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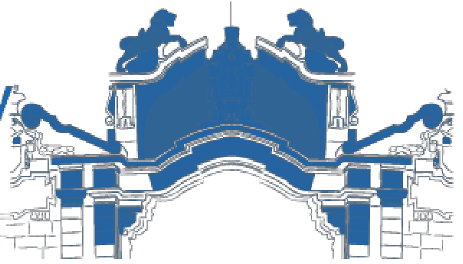

EVALUATION OF ENDOPHYTES ASSOCIATED WITH MAIZE AND ENTOMOPATHOGENIC FUNGI AGAINST THE SPOTTED STEM BORER, *CHILO PARTELLUS*

By:

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A Thesis submitted to the School of Graduated Studies, Department of Microbial, Cellular and Molecular Biology, in partial fulfillment of the requirements for Degree of Master of Science in Applied Microbiology

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Declaration

I, Denberu Kebede Aragaw, hereby declare that the research work presented in the thesis entitled: " EVALUATION OF ENDOPHYTES ASSOCIATED WITH MAIZE AND ENTOMOPATHOGENIC FUNGI AGAINST THE SPOTTED STEM BORER, *CHILO PARTELLUS* " is based on the original research work, except for reference to other peoples' work which has been duly acknowledged, carried out by me at the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, in partial fulfillment of the requirements for the Degree of Master Science and the thesis or no part thereof has been presented for the award of any other degree.

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List of Abbreviations and Acronyms

ANOVA	Analysis of Variance
API	Analytical Profile Index
APPRC	Ambo Plant Protection Research Center
BARC	Bako Agricultural Research Center
Bt	*Bacillus thuringiensis*
CFU	Colony Forming Unit
CM	Corrected Mortality
CSA	Central Statistics Agency
EIAR	Ethiopian Institute of Agricultural Research
EBI	Ethiopian Bio-diversity Institute
EPF	Entomopathogenic Fungi
FAO	Food and Agricultural Organization
Flw	Flowering
GFP	Green-Fluorescent-Protein
IPM	Integrated Pest Management
ISR	Induced Systemic Resistance
KOH	Potassium Hydroxide
LLR	Local Land Race
LSD	Least Significant Difference
LV	Late Vegetative
MA	MacConkey Agar
MARC	Melkasa Agricultural Research Center
NA	Nutrient Agar
OPVs	Open Pollination Varity
PDA	Potato Dextrose Agar
SAS	Statistical Analysis Software
SDA	Sabouraud Dextrose Agar
SSA	Sub Saharan Africa

Abstract

Larvicidal effect of maize endophytes isolated from root and stem of maize and pathogenicity of selected entomopathogenic Beauveria spp. and Metarhizium spp. isolates against C. partellus were studied. Among 39 maize endophyte isolates screened for insecticidal activity, 11 isolates (8 bacteria and 3 fungi) showed larvicidal activity against C. partellus. Pathogenicity of crude extracts of the 11 isolates significantly varied in mortality ranging from 4.79% to 50.26%. The Isolate aaueb-11 under the genus Bacillus produced the highest mortality (50.26%) which was at the same significance level to the positive control Bt. Kurtsaki (60.13%). Eight EPF isolates were selected from 27 isolates and there was a significance variation in causing mortality against larvae of C. partellus. EPF isolates APPRC-34GM, S#10H and APPRC-44BC were the most virulent strains with mean percent mortality of 81.39%, 90.00% and 90.00% respectively. Lethal time and lethal concentrations required to kill 50% and 90% of the experimental insects of the most virulent EPF strains were also determined. The isolate S#10H showed relatively short time to kill 50% of the population where the highest time recorded was 6.15 days. There was a general increase in LT_{50} *with decrease in conidial concentration. The highest dose of the isolate S#10H, required to kill 50% and 90% of the larval population were* $5.95x10^4$ *and* $2.2x10^7$ *conidia per ml, respectively. The effective isolates could be a promising biocontrol agent against larvae of C. partellus. However, the specific compound contributing on the insecticidal activity from the crude fermentation extracts of endophytes need insecticidal spectra study and further isolation and purification to incorporate in microbial based biopesticide development. Thus, the active ingredients and compound structures should be further studied.*

Key words: Maize endophyte; *Chilo partellus*; Entomopathogens; Insecticidal activity

1. Introduction

Maize *(Zea mays*), is one of the oldest human domesticated plant which is believed to have originated at least 7000 years ago in Central Mexico. It is the third largest planted crop next to wheat and rice where they together account 94% of all cereal consumptions used for human food sources (FAO 2014; Ranum *et al.*, 2014). Maize in its different processed forms is an important food for large numbers of people in the developing world, providing significant amounts of nutrients, in particular calories and protein. Maize in Africa is also a vital cereal crop, which serves as a staple food for millions of people (Ackuaku, 2009) where the consumption ranges from 52g/person/day in Uganda to 328g/person/day in Lesotho (Ranum *et al*., 2014).

The global maize production reaches more than 960 million tons during 2014-2015 (Kasim *et al.,* 2016). According to the International Grain Council Report (2017) the world's maize production in the year 2017/18 is estimated to be 1017 million tons. Ray *et al* (2013) indicated that the demand for maize in the developing world will double in 2050. Likewise, FAO (2014) estimate that over the next 35 years, farmers will need to increase the annual production of maize, rice and wheat to 3 billion tones, in the faces of droughts, new pest and disease threats, and extreme weather events provoked by climate change. Maize provides at least 30% of the food calories of more than 4.5 billion people in 94 developing countries including 900 million poor consumers for whom maize is the preferred staple food. In Ethiopia maize is cultivated by more than 9 million smallholder households (Tsedeke Abate *et al.,* 2015). However, maize consumption in Ethiopia is 94g/person/day (Ranum *et al*., 2014); it accounts the second largest cereal crop in land coverage and total production after teff (CSA, 2013).

Production of maize gets reduced by a number of both biotic and abiotic agents. Among the biological constraints of maize production infectious diseases and insect pests causes considerable yield loses. More than 40 infectious diseases have been recorded in Ethiopia that infects maize (Tewabech Tilahun *et al*, 2001). Maize production also suffers attack of Lepidoptera stem borers which feed inside plant stem causing yield lose as high as 80% in maize alone in Africa (Mashwani *et al,* 2015). Likewise, lose due to stem borers in Ethiopia ranges from15%-100% varying with crop and borer species as well as agro-ecosystem (Melaku Wale *et al*, 2006). Muluken *et al.* (2016) indicated that insect pests are the major constraints of maize and sorghum production in eastern Ethiopia following drought and leads an estimated 10% to 50% yield loses.

Among the various insect pests attacking cereal crops such as maize in Africa, *Chilo partellus* which invaded the continent from India is widely distributed and competitively displacing indigenous stem borers in East and Southern Africa. Maize is most vulnerable to *C. partellus* which causes severe yield lose where infestation level varies among varieties and growth stage. Maize yield in East Africa were reduced by 15-45% by *C. partellus* alone (Sylvain *et al*., 2015). *C. partellus* had proven to be a highly competitive colonizer in many areas of eastern and southern Africa, becoming the most injurious stem borer and displacing native species such as *C. orichalcociliellus* (Lepidoptera: Crambidae). It is expected that *C. partellus* will partially displaced *Busseola fusca* and become predominant stem borer in the continent (Sylvain *et al*., 2015). Lepidopterous stem borers, *Busseola fusca* and *C. partellus* are generally considered to be the most damaging insect pests of maize and sorghum in Africa. African invasive pest *C.*

partellus was first recorded in Malawi in 1930 and now it is found in Eastern and Southern Sub-Saharan countries.

Various approaches have been made to control maize stem borers including cultural, physical, biological, chemical and development of host resistance. However, each option for managing the damaging effect of stem borers on maize has its own limitations. Cultural methods are easy to use and do not involve operational costs, but require skill and may not be applicable for large scale farms. Pesticides have been frequently used to kill insect pests with its demerits especially of damaging natural enemies. Biological control methods are cost effective and environmentally safe, but are inefficient in maintaining pests' population below economic injury level. Biological control agents such as egg, larval and pupal parasitoids, predators and microbial pathogens have been used to manage infestations of *C. partellus*. *Trichograma chilonsi*, *Cotesia sesamiae* and *Pediobius furvus* are some of the parasitoids used for egg, larval and pupal stages of *C. partellus* respectively (Jiang *et al.*, 2004; Jalali and Singh, 2006). However, the performance of larval parasitoids for example percentage of produced cocoon of *C. sesamiae* is affected by host plant, stage and temperature (Jiang *et al*, 2004; Setamou *et al.*, 2005).

Microbial pathogens including bacteria, fungi, nematodes, protozoa and viruses are used to control stem borer (Cherry *et al.*, 1999; Esfandiari and Motamendi, 2013). Microbial pesticides based on the soil born bacterium *Bacillus thuringiensis* is among the most widely used groups of biopesticides. Likewise, Entomopathogenic fungal species, *Beauveria* and *Metarhizium*, have been studied for their role in the development of microbial biopesticides.

Tissue inhabiting endophytic microorganisms (bacteria or fungi or actinomycetes) which have a symbiotic association with its host are now becoming effective biocontrol agent used alternative to chemical control (Nair and Padmavathy, 2014). Different studies indicated that extensively used and known biological agents such as *Trichoderma* spp (Amin, 2013), *Bacillus* sp., and *Pseudomonas* sp. promotes plant growth and degrades pathogenic substances of plant pathogens, were endophytically isolated from maize roots and seed (Orole and Adejumo, 2011; Liu *et al*., 2012).

Endophytic microorganisms are recognized to have a potential for protecting the hosts against pests; including insects, nematodes and plant pathogenic fungi and bacteria (Vidal and Jaber, 2015). The well-known microbial pathogens of cereal stem borer insect pests such as *B. thuringiensis*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Lacancillium lecanii* endophytically colonize plant internal tissues (Vidal and Jaber, 2015; Sauka, 2017). Thus, knowledge from studying the endophytic microorganisms will facilitate the search for bacteria and fungus capable of exerting antagonism to pathogenic infections, insect pests and detection of biological plant growth enhancers. However, regardless of the importance of endophytes in plant and animal health protection, research data on endophyte in Ethiopia is limited. Thus, this study is conducted to explore effective maize endophytes (fungi and bacteria) so that selected entomopathogenic fungi (*Beauveria* spp and *Metarhizium* spp) and maize endophytes could be incorporate into integrated pest management (IPM) of *C. partellus*.

2. Objectives

2.1. General objective

- ✓ To assess maize endophytes and selected entomopathogenic fungi towards the control of the Spotted Stem Borer, *Chilo partellus*.

2.2. Specific objectives

- ✓ To isolate and characterize maize endophytic fungi and bacteria.
- ✓ To evaluate the larvicidal activity of the maize endophytes against the Spotted Stem Borer, *Chilo partellus*.
- ✓ To evaluate selected Entomopathogenic fungi (*Beauveria* and *Metarhizium*) isolates against larvae of the spotted stem borer, *Chilo partellus.*

3. Literature review

3.1. Economic value of maize

Maize is the third widely cultivated crop following wheat and rice. Maize is mostly used and traded as food and feed crop and has a wide range of industrial applications from food processing to fuel ethanol production (Ranum *et al.*, 2014). Maize in Africa is a vital cereal crop, which serves as a staple food for millions of people with varying consumption from country to country (Ackuaku, 2009; Ranum *et al*., 2014). Although maize production varies in yield, it is grown throughout the world. According to the report of Central Statistics Agency (CSA) (2017) of Ethiopia, in 2016/17 main croping season, maize accounted 16.98% (about 2,135,571.85 hectare) of the total grain crop area which covers the second largest production area next to teff which took 24% (about 3,017,914.36 hectare). However, maize comes the first in grain production which made up 27.02% (78,471,746.57 quintal) of total grain production following teff and wheat (CSA, 2017). Maize in Ethiopia is now becoming the foremost cereal crop in distribution and number of holders.

However, the global maize production in 2014-2015 attained more than 960 million tons (Kasim *et al.,* 2016) and, its demand in the developing world will be doubled in 2050 and become the crop with largest production globally. Despite the increased need of maize, its production gets biotic constraints such as Lepidoptera stem borers which feed inside plant stem. Of the various insect pests attacking cereal crops such as maize in Africa, *Busseola fusca* Fuller (Noctuidae), the pink stalk borer, *Sesamia calamistis* Hampson (Noctuidae); the African sugar-cane borer, *Eldana saccharina* Walker (Pyralidae), the ear borer *Mussidia nigrivenella* Rogonot (Pyralidae),

and the spotted stalk borer, *C. partellus* (Swinhoe) (Crambidae) are the main maize stalk borers with major economic importance (Tadele Tefera, 2004).

3.2. *Chilo partellus*

3.2.1. Distribution and biology

Chilo partellus (swinohae), also known as the spotted stem borer, belongs to the order Lepidoptera which is the second largest order of insects. African invasive pest *C. partellus* was first recorded in Malawi in 1930 and now it is found in Eastern and Southern Sub-Saharan countries (Sylvain *et al.*, 2015). The pest invaded the African continent from India and now inhabits much of the eastern and southern Africa with a potential of displacing indigenous related stem borer species including *B. fusca* (Hutchison *et al.*, 2008). The occurrence of the spotted stem borer is highly influenced by altitude and moisture. In Ethiopia the abundance of *C. Partellus* was higher in semi-arid eco zones between 1200 to 1985 masl (Tadele Tefera, 2004; Melaku Wale *et al.*, 2006). Ntir *et al.* (2016), reported that, larval survival and relative growth rate of *C. partellus in vitro* at higher temperature (30°c) was higher than survival of *B. fusca* and *S. calamistis* which indicated the future increasing temperature due to climate change likely to confer an advantage on over dominance of *C. partellus* over other lepidopterans**.**

Chilo partellus has complete metamorphosis passing though egg, larva, pupa and adult stages with more than 2 generations per year depending the location and availability of host plants. The female moth lays 100-160 oval shaped eggs in cluster on the whorls of host plant and hatches within 7-10 days. The larvae then penetrate the stem tissues to feed and form tunnels in the stem and maize ears and pupate within the stem. The first three instars feed on leaf whorls while the last three instars bore into the stem (Kfir, 1992).

3.3. Economic importance of stem borers

3.3.1. Damage and yield lose

Lepidoptera, stem borers, with various cereals attacking species, are the most important cereal pests of Sub Saharan African (SSA) countries which constrain the efficient production of cereal crops (Midega *et al.*, 2015). However maize and sorghum are the primary hosts of *C. partellus,* the pest attacks a variety of crops and plants including sugarcane, several millets and several grass species (Hutchison *et al.*, 2008).

Among cereals, maize is damaged more by stem borers because it has more amino acids, sugars, and emits volatile compounds than the other gramineous hosts (Sylvain *et al.*, 2015). Larval stage of *C. partellus* is the damaging stage which can attack the entire part of the plant except the root. The larvae of stem borers initially feed by scrapping the leaf whorls producing characteristically window-panning and pine-holes and feed on the developing grain which exposes the plant for secondary infections (Taneja and Nwanze, 1990)**.**

Yield loss due to stem borers in Africa reaches as high as 80% in maize alone (Mashwani *et al.,* 2015). Lose due to stem borers in Ethiopia ranges from15%-100% vary with crop and borer species as well as agro-ecosystems (Melaku Wale *et al.*, 2006). Lepidopterous stem borers, *B. fusca* and *C. partellus* are generally considered to be the most damaging insect pests of maize and sorghum in Africa.

3.4. Management of *Chilo partellus*

Various approaches have been followed to control maize stem borers including cultural, physical, biological, chemical and host resistance.

3.4.1. Cultural method

Cultural methods are easy to use and not involve operational costs but require skill and may not be applicable for large scale farms. Intercropping, crop rotation, manipulation of planting date, post-harvest tillage and using crop residue for livestock feed were some of cultural practices used for stem borers' management in Eastern part of Ethiopia (Muluken Goftishu *et al.*, 2016). Kavita *et al.* (2016) obtained the best yield of maize by intercropping with cowpea which reduced the number of pinholes per plant caused by *C. partellus*.

3.4.2. Chemical control method

Pesticides have been frequently used to kill insect pests with its demerits especially of eradicating natural enemies. Insecticides such as Methamedaphos, Carbofuran, Cypermethrin, emmamectin, deltamethrin and thiamethoxam, etc. have been used to reduce maize stem borer infestations including *C. partellus* with response variations (Khan and Amjad, 2000; Iqbal *et al.*, 2017; Rauf *et al.,* 2017). Rauf *et al.* (2017), indicated that chemical insecticides such as carbofuran and fipronil with enhanced toxicity for *C. partellus* showed significant eradication in natural enemies. In eastern Ethiopia most of the farmers were unable to use synthetic chemicals due to lack of capital and non- availability of chemicals (Muluken Goftishu *et al.*, 2016).

3.4.3. Biological control methods

Biological control of stem borers includes the use of parasitoids, predators and microbial pathogens. Biological control methods are cost effective and environmentally safe, but it could not provide an immediate action on pest population like pesticides.

3.4.3.1. Parasitoids and predators

Different egg, larval and pupal parasitoids have been identified and used for the management of *C. patellus*. In Kenya the introduction of larval parasitoid *C. flavipes* Cameron (Hymenoptera:

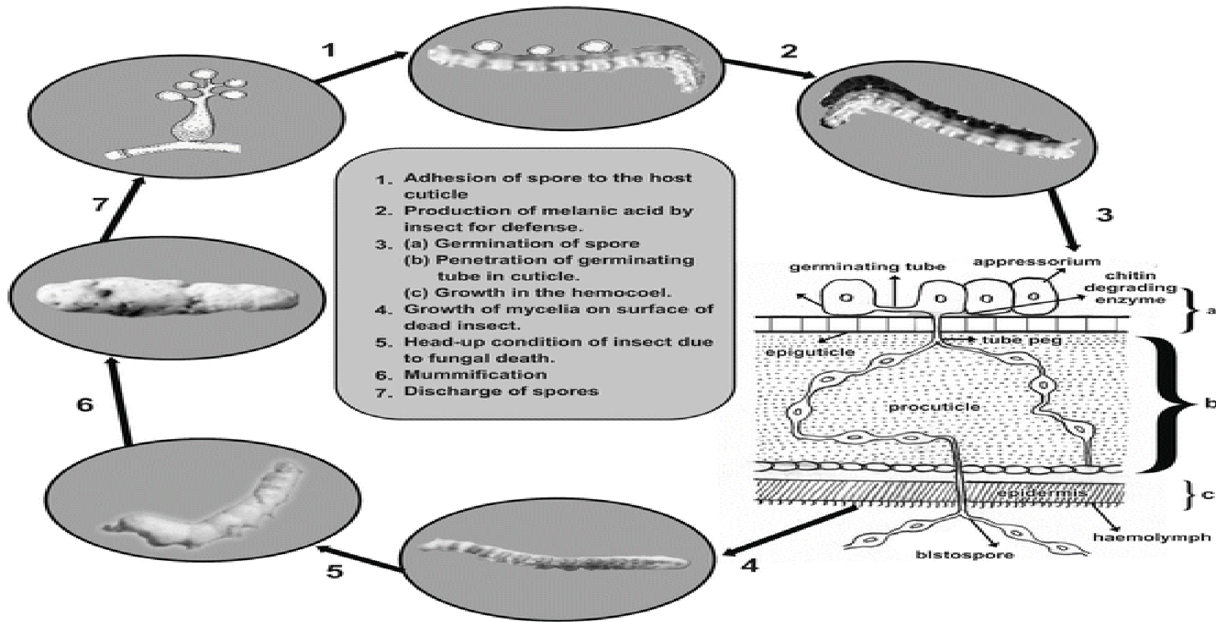
Braconidae) in 1993 protected 47.3% of maize yield loss through reducing stem borer infestation by 33.7% with a linear increase in parasitism year after year reaching 20% parasitism in 2004 (Kipkoech *et al.*, 2006). Natural occurrence of larval parasitoid *C. flavipes* was reported and *C. partellus* density in the Semi-arid eco zones of Ethiopia were reduced due to parasitism (Melaku Wale *et al.*, 2006). *C. flavipes* in lowlands areas of Ethiopia was found attack larvae of three lepidopteran stem borers including *C. partellus, B. fusca* and *S. calamistis* (Tadele Tefera, 2004). However, the performance of parasitoids was affected by host plants. Weight of *C. partellus* larvae were affected by host plants which causes reduced percentage of cocoons of C. *flavipes* produced later affected parasitism performance (Setamou *et al.*, 2004).

3.4.3.2. Pathogens

Research on the discovery and formulation of microbial biopesticides with various entomopathogens including fungi, bacteria, nematodes etc. have got attention to reduce risks joined with synthetic chemicals such as insect resistance development, distraction of non-target entomofauna such as predators, parasites and pollinators (Ruiu *et al*, 2013). However, *B. thuringiensis* is the well-known and commercialized bio pesticide (Sauka, 2017), various bacterial groups other than *Bacillus* have been studied for entomopathogenic activity at different level. Mampallil *et al.,* (2017) indicated, while majority of the entomopathogenic bacteria belong to the families *Bacillaceae*, *Pseudomonadaceae* and *Enterobacteriaceae*, members of *Streptococcaceae* and *micrococcaceae* families also exhibit entomopathogenic activity to a lesser extent. The disadvantage of entomopathogenic bacteria is that, the agents have to be ingested to produce virulent toxins to cause insect death.

Of the 750 species of fungi belonging to 90 genera documented to date to be pathogenic, only a few of the species have been developed as pathogenic against insect pests (Rai *et al.*, 2014).

Entomopathogenic fungi especially of the genera *Beauveria* and *Metarhizium* have been widely studied. The direct adhesion and penetration activity of entomopathogenic fungi (Figure 1.) made them most favorable than other entomopathogenic microbes (San Aw and Hue, 2017).



source: (Sandhu *et al*., 2017)

Figure 1. Mechanism of action of Entomopathogenic fungi (EPF).

3.5. Endophytes

In natural ecosystems plants are potential hosts for a broad spectrum of microbes (usually bacteria and fungi) that can live on the surface as epiphytes or colonize plant tissues as endophytes (Linar, 2010). Nearly 300,000 plant species exist in the world and each species harbors one to hundreds of endophytes having their own functions in protecting their host from various factors (Begum and Tamilselvi, 2016). Endophytic microorganisms spend the whole or part of their life cycle with the plant inter- or intra-cellularly without causing any pathogenic symptom although interacting biochemically and genetically which may function as plant growth

and defense promoter (Schneider and Barka, 2012; Dutta *et al.*, 2014). The activities of endophytes may be direct where the influence is in natural condition or indirect where the isolated endophyte with its character is re-inoculated to the host plant. Endophytic microorganisms are now attracting great interest from researchers as an alternative source for the control of plant pests.

3.5.1. Allocation/distribution of endophytes

The type and number of endophytic microorganisms fluctuates among different parts of the host plant and endophytes can be isolated from root, stem, leaf, bark and even from needles of plants (Findlay *et al.*, 1995). The distribution and population structure of endophytes can be considerably affected by factors such as genetic background, age, environmental conditions of their host plant and plant inhabiting life strategies. Hence, particular conditions such as temperature, humidity and geographic location that determined the distribution ranges of host plants in return determine the species of endophytic fungi and their spore germination, growth, reproduction, and metabolism (Jia *et al.,* 2016). At the same time continues systemic movement of endophytic organisms within the plant after penetration and colonization of the vascular ssystem and allocation of resources significantly affect the fixed plant part distribution. The transport of endophytes from seeds into plant root and tissues; from injected stem into roots and rhizosphere can be detected using Green-Fluorescent-Protein (GFP) labeled endophyte (Gaiero*et al.*, 2013) and indicates the presence of continuing translocation of endophytes throughout the plant microbiome.

3.5.2. Role of endophytes in plant physiology and plant protection

The hidden endophytic interaction offers several advantages to the host plant. Plant growth promotion, protection against surface feeding insects, fungal, bacterial and nematode infections and increased nutrient access are among the benefits of endophytes to their host plant (Begum and Tamilselvi, 2016; Dutta *et al*., 2014). Under natural conditions endophytes function as plant growth and defense promoter by producing important compounds such as Indol acetic acid, siderophore, by increasing phosphate solubilization, by producing biosurfactants, and producing precursors for secondary plant metabolites as well as source for new bioactive compounds (Begum and Tamilselvi, 2016; Schneider and Barka, 2012). Endophytes have superior biocontrol performance over symbionts commonly found in the soil. *Botryosphaeria quercum* endophytically isolated from Cocoa tree (*Theobroma cacao* L.) displayed slightly higher biocontrol indexes against phytopathogens isolated from healthy pods of cocoa over *Trichoderma viride* strains isolated from rhizosphere soil of cocoa production farms (Villamizar-Gallardo *et al.,* 2017).

One of the most important implications behind endophytic microorganisms is that they confer broad-spectrum host resistance to insect herbivores. Rashid and Chung (2017), demonstrated that root drenching of *Arabidopsis* with *B. velezensis* YC7010 suspension resulted in the establishment of an Induced Systemic Resistance (ISR) against Green peach aphid. Soil is among the major sources/origins of endophytic infection of plants. Rashid and Chung (2017) also reviewed the induction of systemic resistance against insect herbivores in plants by beneficial soil microbes. They indicated root colonization by soil microbes can induce biosynthesis of plant defense-related compounds such as phenolic compounds against insects and the colonization of plant roots by rhizobacterium provokes the expression Jasmonic Acid and Ethylen dependent genes which induces ISR to leaf chewing insects. Kidd *et al*. (2013) also reported endophytic

bacteria under the genus *Pseudomonas* isolated from fumonsin-producing *Fusarium verticillioides* resistance inbred corn line shows strong growth inhibition against fumonsin-producing *Fusarium spp.* which may have role in the resistance of the corn. Similarly, volatile organic compounds emitted by soil born endophytic microbes can influence the defense responses of their hosts. Alessandro *et al* (2014) demonstrated healthy maize seedlings emit considerable amount of isomeric mixture of 2,3-butanediol due to endophytic *Enterobacter aerogenes* which attracts larval parasitoid *C. marginiventris*.

3.5.3. Application of endophytes in insect pest control

Knowledge from studying the endophytic microbial diversity will facilitate the search for bacteria and fungus capable of exerting antagonism to pathogenic infections, insect pests and detection of biological plant growth enhancers. A different scholar shows the effect of endophytic microorganisms on insect pests of different plants. *Aspergillus flavus* and *Verticillum albo-atrum* isolated from insect are suggested biopesticides (Ackuaku, 2009) against *E. saccharina* a stem borer pest of maize and sugarcane (Kfir, 2014). Likewise, treatments of *C. partellus* with *B. bassiana* and *M. anisopliae* shown an endophytic relationship decreases mean daily maize leaf consumption of second and third instars larvae (Tadele Tefera and Pringle, 2004).

The well- known and first commercialized biopesticide, *B. thuringiensis,* which has a proud and successful history in insect pest management for more than 80 years has an endophytic relationship with its host plant and protects form insect pest attack (Sauka, 2017). Likewise, entomopathogenic fungi such as *B. bassiana, M. anisopliae*, and *L. lecanii* colonizes plant internal tissue (Vidal and Jaber, 2015) and the endophytic growth of *B. bassiana* is common in

corn cropping system in USA where a zero to more than 60% of sampled plants exhibited entomopathogenic as an endophyte.

Bio-active compounds produced by endophytic microorganisms have also wide range of application in the agricultural sector. Bio-active secondary metabolites originated from endophytes have been recognized for their antagonistic activity towards plant disease causing pathogens (bacteria and fungi), insect pests and nematodes (Latch, 1993). N-formayl loline, Epoxy-janthitrem I-Naphtalene and Nodulisporic acid A are some of the well characterized anti-insect secondary metabolites produced by endophytic fungi (Lugtenberg *et al.*, 2016).

3.5.4. Endophytes associated with maize

Endophytic microorganisms, usually bacteria and fungi, have been isolated from maize (Souza *et al.,* 2004; Kidd *et al*, 2013; Thanh and Diep, 2014; Szilagyi-Zecchin et *al.*, 2015). *Trichoderma* spp., *Fusarium* spp, *Acremonium* spp, *Aspergillus* spp, *Penicillium* spp, and *Botryodiplodia* spp and bacteria such as *Bacillus* spp, *Pseudomonas* spp, *Sphingomonas* spp, *Halomonas* spp and *Pantoea* spp, which are known for promoting plant growth and degrading pathogenic substances, were endophytically isolated from maize roots and seed (Amin, 2013; Orole and Adejumo, 2011; Liu *et al*., 2012). Szilagyi-Zecchin *et al.*, (2015) also reported *Bacillus* spp. isolated from maize were potential inoculants for plant growth promotion by encouraging root length.

4. Materials and Methods

4.1. Maize planting

The maize varieties (local land race (LLR), hybrid and Open Pollinated Variety (OPVs)) were obtained from Ethiopian Bio-diversity Institute (EBI) and Ambo Plant Protection Research Center (APPRC). Local land race from EBI were collected around West Gojam in 1978, while the hybrid, *Jibat* and the OPV, *Hora*, were sourced from APPRC. The obtained varieties were planted in three locations in Ethiopian Institute of Agricultural Research (EIAR), namely, Melkasa Agricultural Research Center (MARC; 1555 masl), Bako Agricultural Research Center (BARC; Altitiude:1650masl) and Ambo Plant Protection Research Center (APPRC; altitude 2419masl) as they represent three different Agro-ecological zones; low land, midland and highland altitudes respectively. Maize planting was done during the main cropping season June to July in 2017. Twenty to twenty-five seeds of each genotype were planted using a spacing of 25 cm X 75 cm in plot of around 45 m^2 at each experimental station. All agronomic recommendations for maize including fertilizer applications and weeding were performed. No pesticide was applied to avoid any possible interference with the experiment.

4.2. Sample collection

Healthy looking maize plants within two plants interval over the raw of each planted genotype were uprooted and collected from each site at different growth stages: knee height stage, late vegetative stage and flowering stages. Two maize plants of each genotype were taken during every sampling. Sampling at the knee height stage and flowering stage were missed at Melkassa site due to some security problems. A total of 42 healthy maize plants having different growth stages were collected from the three sites and the samples were brought to APPRC for analysis

of fungal and bacterial endophytes. The collected samples were stored in a refrigerator until processed and the analysis was done within 24hr after collection.

4.3. Isolation of Fungal and Bacterial Endophytes

The sampled plants were washed with running tap water to remove clay particles and separated into stem and root parts. Surface disinfection was done according to (Amin, 2013). Each plant part was then cut into pieces (5-6cm) and surface disinfected by immersing in 70% ethanol for 2 minutes and 2.5 % sodium hypochlorite for 5 minutes. This was followed by three times washing with sterile distilled water for1 minute each time to remove residuals of the disinfectants and the tips of each pieces were removed using sterile surgical blade.

For isolation of endophytic fungi, the surface disinfected stem and root samples were cut in to ~5mm pieces and dissected using sterile surgical blade. Three to four pieces of the cut plant tissues were placed down on pre-dried Petri dish (9cm diameter) containing sterilized Potato Dextrose Agar (PDA) (Compositions: Potatoes infusion from: 20g/l, dextrose: 20g/l and Agar: 15g/l, CONDA) medium supplemented with Chloramphenicol (100mg/l) to suppress bacterial growth. The plates were sealed with paraffin and incubated at 26°c for 10 to 15days and examined every day. About 200µl of aliquots from the final washing were also spread plated on PDA and incubated at 26°c to check the effectiveness of the surface sterilization processes. The outgrowths of fungal mycelia from the tissue were considered as endophytes after surface disinfection process were confirmed to be effective. The emerging mycelium was sub cultured to a fresh PDA media for purification and the pure isolates were maintained at 4°c using PDA slants.

The same maize plant samples used for isolation of endophytic fungi were used for analysis of endophytic bacteria. Approximately 0.5-cm pieces (2 gram) of surface disinfected root and stem samples as described above (Amin 2013) were macerated separately using flame sterilized mortar and pestle and incubated at room temperature inside a bio-safety hood for 30-60 minutes to allow the complete release of endophytic microorganisms. One ml of the aliquots were serially diluted (four fold) in sterilized peptone water (peptic digest of animal tissue: 20g/l, sodium chloride 5g/l: final PH 7.4±0.2, HiMEDIA) and 200µl were spread plated using bend glass rod on pre dried Nutrient Agar (NA) (compositions: peptic digest of animal tissue: 5.00g/l, beef extract: 1.5g/l, yeast extract 1.5g/l, sodium chloride 5g/l: final PH7.4±0.2, HiMEDIA) and incubated for 3-5days at 30°c. About 200µl of the aliquot used in the final rinse were also inoculated on nutrient agar and incubated for up to three days at 30°c to confirm the success of sterilization. Bacterial isolates were considered as endophytes after the success of disinfection process were confirmed and the isolates were purified and preserved using 20% glycerol.

4.4. Identification of endophytic Fungi and bacteria

Endophytic fungi

Genus level identification of the obtained endophytic fungi were done based on their microscopic features using manuals of Barnett (1960) and Watanabe (2010). For microscopic morphological study, the slide cultures of each isolate were prepared to see the intact morphology of the fungus and permanently fixed for observation under light microscope (Olympus, USA) at 40x magnification.

Slide culture preparation: A 9cm diameter Petri dish containing filter paper and bent glass rod with glass slide were sterilized by autoclaving. A one by one cm block of water agar (15g/l) were cut with sterile surgical blade and placed at the center of the glass slide. Each fugal isolate was inoculated on the four sides of the agar blocks using sterile inoculating needle and sterile coverslip were put over the block. The filet paper was moistened with 2ml of sterile water and the plates were sealed with parafilm and incubated at 26°c. After 72hr incubation the agar blocks were discarded and the slides were wetted with a drop of 97% ethanol and allowed to heat fix by passing on alcohol lump. The slides were stained with a drop of lactophenol blue and covered with a coverslip later sealed with nail polish. The slide was then observed under compound light microscope at 40x magnification to see the intact conidiophores structure. (Rosana *et al*.,2014).

Genus *Trichoderma* were microscopically characterized by unverticillated conidiophore and three to four verticillated phialides possessing oval conidia at the head. Ellipsoid vesicles at the apex bearing spore heads composed of chainlike conidia borne on uniseriate phialides were microscopic keys for genus *Aspergillus*. For identification of genus *Penicillium* single conidiophore raised from the mycelium, branched conidiophore (usually 3-4) at the apex which forms a brush like shape and biserated vesicles bases for phialides bearing a chain of three to five ovoid conidia were recognized. Similarly, genus *Fusarium* was identified considering seriated (2-5) conidiophore, curved macroconidia at the end and monophialidic conidiophore. Genus *Botriodiplodia* characteristically possessed 2-celled elliptical conidia constricted at the center and uniserated vesicle (Barnett, 1960; Watanabe, 2010).

4.5 Biochemical and physiological identification of endophytic bacteria

The obtained endophytic bacteria were gram stained and focused under compound light microscope to determine Gram type and cell morphology. In addition to gram staining, gram type was also confirmed with KOH (3%) solubility test and growing on the selective MacConkey Agar (MA) medium. Gram negative rods were then tested for cytochrome oxidase enzyme production and oxidase positive strains were seeded on MA media to differentiate lactose fermenters from non-lactose fermenters. Bacteria strains with pinkish color were considered as Lactose fermenter while colorless strains were determined as non-lactose fermenters. Gram positive rods were further tested for endospore production, motility test, penicillin sensitivity and starch hydrolysis (Goszczynska *et al*, 2010).

Biochemical tests

KOH solubility test: KOH solubility test was carried out by mixing a 24-48hr old bacterial culture into a drops of potassium hydroxide solution (KOH) (3%w/v) on a glass slide until an even suspension was obtained. The formation of mucoid thread when the loop is lifted from the slide indicated the bacterium is gram negative.

Starch hydrolysis test: 5g soluble starch was added in nutrient agar (NA) and a 24hr old bacterial culture were streaked at the center using inoculating loop. The plates were incubated at 30°c for 2-5 days. The plats were flooded with iodine solution and a crear zone were recorded for amylase activity.

Endospore production: This was done by treating the broth bacterial suspension at 85°c for 15 minutes inside a water bath and streaked back on plates containing nutrient ager. The plates were observed for the growth of bacteria after incubated at 30°c for 24-48hr.

Motility test: A test tube containing semi solid motility agar media were stab inoculated using sterile inoculating needle. The spreading of bacteria from the stab line were visually observed after 48-72hr incubation period at 30°c.

Oxidase test: Cytochrome oxidase enzyme activity were detected by smearing a 24-48hr old bacteria colonies using a wood stick on whatman No. 1 filter paper moistened with a drops of fresh oxidase reagent (1% NNN'N-tetramethyl-p-phenylene-diamine-dichloride). Color changes to dark purple in 30-60 seconds were recorded as oxidase positive.

Penicillin sensitivity test: Disc diffusion method were used to determine penicillin sensitivity. A 24hr old bacteria suspensions were streaked on MA media and standard penicillin discus were loaded at the center and incubated for 24hr at 30°c.The plates were observed for zone of inhibition after 24hr of incubation.

All Gram-negative rods were identified to species level using Analytical Profile Index (API) kites where each kit has 20 microtubes containing dehydrated substrates for biochemical tests. Two API kits API 20E and API 20NE kit system were used. API 20E were used for gram negative lactose fermenting rods while API 20NE were used for non-lactose fermenting gram negative rods. The API kit identification was done following the manufacturer's procedure as described in the manual. For identification of the strains, the obtained result after 24hr of incubation were red by referring the reading table given a numerical value having a 7-digit

numerical profile for the 20 tests. Interpretation and identification of the isolates were done using the apiweb™ identification software (V4.1.).

4.6. Analysis of Species diversity and richness

Variability among endophytic strains from maize genotypes, growth stage and plant parts were determined using Shannon and Simpson's diversity (H) and richness (D) Indexes after calculating using the formula below (Magurran, 2004; Kim *et al*, 2017).

$(H= -\sum_{i=1}^{s} pi \ln pi)$, $D= \frac{1}{\sum_{i=1}^{s} pi2}$

Where p is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N), **ln** is the natural log, Σ is the sum of the calculations, and **s** is the number of species.

4.7. Insect rearing

4.7.1. Rearing of *Galleria mellonella*

Rearing of *Galleria mellonella* was done at Biocontrol Laboratory of APPRC. The adult moths were maintained in 500ml flasks containing folded tissue paper infused with water and honey to allow mating and lay eggs. Tissue papers with laid eggs were picked from the flask and inserted in plastic rearing boxes containing 80g, 50g and 180g of honey, wheat bran and glycerol, respectively, as feed for the larvae. The boxes were incubated in the dark at 20°c for up to four weeks to obtain appropriate instars used for screening of entomopathogenic fungi EPF isolates (Mohamed and Coppel, 1983).

4.7.2. Rearing of *Chilo partellus*

The test insect, *C. partellus* was collected during off season around irrigated maize farms of lowland areas of the rift valley. The collection was done following the damage symptoms on the plant and the hole plants were uprooted and transported to APPRC. The *C. partellus* larvae and pupae were retrieved after dissecting the maize stems. Dead, parasitized and deformed looking field collected larvae and pupae were discarded. Parasitoid and diseases free larvae and pupae were used for rearing. Larvae were reared using its natural host, maize, in the lab at APPRC (Bons, 2011).

The larvae were transferred to new maize plant and the fresh food was changed every three days until pupation. The pupae were kept in moistened Petri dishes until adult emergence. The adult moths were maintained inside a rearing cage with 3-4 weeks old maize plant grown on a pot and allowed to lay eggs. Eggs were collected daily and kept in a sterile Petri dishes. Newly hatched larvae were transferred to a plastic jar with fresh maize leaves. To feed the larvae regularly, maize plants were grown in a plastic pot and kept in a glass house with photoperiod of 12hr: 12hr light and dark. Second and third instars larvae were used for the test.

4.8. Evaluation of maize endophytes against larvae of the spotted stem borer, *Chilo partellus*

4.8.1. Larvicidal screening of crude fermentation metabolites of maize Endophytic fungi and bacteria

4.8.1A. Crude filtrate preparation and prescreening

Larvicidal effect of endophytic fungi and bacteria were tested using their crude fermentation filtrate. Maize endophytic fungi and bacteria cultures were grown on a sterilized fermentation

broth media containing soluble starch (20g/l), Soy meal (15g/l), Sodium chloride (4g/l), Yeast powder (5g/l), peptone (2g/l), and calcium carbonate (4gr). The final PH of the medium was adjusted to 7.0-7.2 using PH meter (PHS-3D-02, China) and 100ml of the broth suspension were transferred to 250ml Erlenmeyer flask and inoculated with fungi and a 24hrs old bacterial cultures (Shi *et al.*, 2013).

The inoculated broth was incubated at 25°c for 8 days at 120 rotations per minute. Uninoculated fermentation broths were also kept to check the presence of insecticidal impact of the media composition. After 8 days of incubation the broth suspensions were centrifuged at 6000 rpm for 30 minutes to avoid media parts and the crude supernatant filtrates were collected to check the presence of insecticidal antibiotics. Crude fermentation extracts from 39 maize endophytic bacteria and fungi isolates (27 bacteria and 12 fungi) (Table 5.) were used to check the presence of insecticidal effects. For larvicidal screening, from the 81maize endophyte all the *Bacillus* isolates and representative isolate of each other bacterial and fungi genus were used. The entire genus *Bacillus* was selected because bacterial groups under the genus Bacillus such as *B. thuringiensis* have been proven for their larvicidal activities.

Pre-screening

A three weeks old maize plant leaf grown in a greenhouse (12:12 photoperiod) were cut in an estimated 4cm long and 1cm wide size and surface sterilized by dipping in 70% alcohol for five minutes followed by 3 times washing with sterile distilled water for one minute. The surface disinfected leaf pieces (2 to3 pieces) were transferred to 9cm diameter sterile glass Petri-dish. Two ml of the microbial fermentation crude extracts were sprayed using atomized hand sprayer. Five late second instars larvae were introduced to each Petri dish and allowed to feed on the

treated leaf pieces for 48hrs to check the presence of insecticidal compounds. After 48hrs of ingestion of the microbial filtrates through the feed, the Petri dishes were assessed for the presence of dead and live larvae. Leaf pieces treated with Diazinon and *B. thuringiensis Kurstaki* were used as a positive check. Larvae were fed with maize leaves treated with sterilized distilled water used as negative control. The suspension filtrates from uninoculated fermentation broth were also tested to cross check the larvicidal activity of the media components. Mortality data were collected daily for 7 days. Strains that caused larval death after adjusting for natural death were selected for pathogenicity test against *C. partellus* larvae (Shi et al., 2014; Tadele Tefera and Pringele, 2004)

4.8.1B. Larvicidal pathogenicity of Endophyte fermentation filtrates

Crude fermentation extracts of eleven endophytic isolates (8 endophytic bacteria and 3 endophytic fungi) selected from the prescreening were used for larvicidal pathogenicity study using leaf spray method. Pre-weighted three weeks old maize leaf were surface sterilized as described above and allowed to dry through placing on sterile filter paper. Second to late second instars larvae of *C. partellus* were obtained by dissecting maize plants from the rearing cage established at APPRC. Two ml of the test solution were sprayed on surface sterilized maize leaves using atomized hand sprayer inside autoclaved glass Petri dishes, and then 10 larvae were added on the surface of the leaves and allowed to feed for 48 hrs. The damaged leaves were replaced with new leaves. The negative controls were treated with two ml of sterile distilled water and leaves treated with *Bt. kurstaki* and Diazinon were used as positive controls. The larvae were also separately treated with solutions of uninoculated fermentation broth to check the fermentation media was free of insecticidal compounds. Each treatment was replicated three

times and maintained at room temperature (average temp. 25°c) and mortality was recorded daily for 7 days (Tadele Tefera and Pringele, 2004).

4.9. Pathogenicity and selection of Entomopathogenic fungi Isolates on *Galleria mellonella* larvae

4.9.1. Source of EPF Isolates

EPF isolates used for the experiment were obtained from APPRC Bio-control Laboratory and Addis Ababa University Mycology laboratory (Table 1). A total of 27 entomopathogenic *Beauveria* spp. *and Metarhizium* spp. were sub cultured on Sabouraud Dextrose Agar (SDA) media and incubated at 26°c for three to four weeks to allow sporulation and maintained at 4°c for further usage. All the EPF strains were isolated from soil samples collected from different parts of the country isolated using a Galleria bait method (Table 2).

Table 1. Source of Entomopathogenic fungi (EPF) isolates

No	Source	Genus		Total isolates obtained
		Metarhizium spp.	*Beauveria* spp.	
1	APPRC	6	16	16
2	Addis Ababa University	2	3	5
	Total	8	19	27

4.9.2. Entomopathogenic fungi isolates viability screening

All the 27 isolates were subjected to germination test to assess viability of the conidia following the procedures described by Belay Habtegebriel *et al* (2016). Three to four weeks after incubation on SDA, the conidia of each fungal isolates were obtained by scraping with sterile metal spatula and suspended in a test tube containing 10ml sterile water with Tween 80

(0.01%V/V) to make stock suspension. Conidial concentration of the stock suspension was adjusted to $3x10^6$conidia/ml with an improved Neubaour heamocytomter using a light microscope (Olympus-CH30RF200, Japan) (40x magnification power).

About 100µl of the suspension was spread plated on SDA media in 90mm diameter Petri dish and 1ml of 70% alcohol was spread on each Petri dish after 24hrs of incubation at 26^oc to stop over germination. Each Petri plates were pseudo triplicated by putting three sterile cover slips on each Petri-dish and percentage of germination were determined by counting at least 300 conidia under a light microscope (40x magnifications) and a conidium was considered as germinated if it showed a germ tube growth as big as its size. Germination test for Each isolate was replicated three times. Percent of viability was calculated by dividing number of germinated spores with total number of spores examined multiplied by 100.

4.9.3. Screening of EPF isolates on *Galleria mellonella* larvae

Larvae of *G. mellonella* were obtained from Bio-Control laboratory of APPRC for EPF screening to select relatively virulent strains. Conidia of each *Beauveria* and *Metarhizium* isolates were harvested from a 2-3 weeks old culture plate and the conidial concentration were adjusted to $1x10^8$ conidia/ ml as described above. Ten third to fourth instars larvae were immersed to a sterile beaker containing the spore suspension for 10-30 seconds and placed on sterile filter paper to prevent suffocation of the larvae by water. The larvae were then transferred to 9cm diameter sterile Petri dishes and placed at room temperature (average room temperature 25^oc) for ten days. The controls were immersed in sterile distilled water for similar exposure time. Each treatment were replicated three times. Mortality were daily recorded, and the dead insects were surface disinfected by immersing in 3% Sodium Hypochlorite and 70% alcohol for 3 and 2 minutes respectively followed by three times washing with sterile distilled water and

placed on moist filter paper at room temperature to confirm fungal outgrowth from the cadaver (Belay Habtegebriel *et al.,* 2016).

Table 2. List of Entomopathogenic fungi isolates used for the experiments

No.	Isolate code	Genus	Host/habitat
1	S#34	*Beauveria* spp	soil
2	DS-51-1	*Metarhizium* spp	soil
3	S#048BC	*Metarhizium* spp	soil
4	DS-86-2	*Metarhizium* spp	soil
5	DS-37-1	*Metarhizium* spp	soil
6	S#44BC	*Beauveria* spp	soil
7	S#10H	*Beauveria* spp	soil
8	DS-51-2	*Beauveria* spp	soil
9	S#05	*Beauveria* spp	soil
10	S#53	*Beauveria* spp	soil
11	APPRC-34GM	*Metarhizium* spp	soil
12	DS-52-2	*Metarhizium* spp	soil
13	AF2	*Beauveria* spp	soil
14	GF4	*Beauveria* spp	soil
15	BF4	*Beauveria* spp	soil
16	B4	*Beauveria* spp	soil
17	APPRC-44BC	*Beauveria* spp	soil
18	DS-35-2	*Beauveria* spp	soil
19	M1	*Beauveria* spp	soil
20	APPRC-27	*Beauveria* spp	soil
21	KF3	*Beauveria* spp.	soil
22	B1	*Beauveria* spp	soil
23	S#41	*Beauveria* spp	soil
24	B7	*Beauveria* spp	soil
25	S#45	*Beauveria* spp	soil
26	APPRC-40GM	*Metarhizium* spp	soil
27	M2	*Metarhizium* spp	soil

4.9.4. Pathogenicity of selected EPF isolates against larvae of *Chilo partellus*

Eight EPF isolates screened above were used to evaluate their pathogenicity on the larvae of *C. partellus.* The isolates were selected based on their viability and virulence on larvae of *G. mellonella* as obtained above and some phenotypic characteristics such as sporulation potential.

The spore suspensions of each isolates and the desired conidial concentration ($1x10^8$ conidia/ml) were prepared and two ml of the suspension were sprayed on 10 second to late second instars larvae of *C. partellus*. The control groups were treated with two ml of sterile distilled water with a drop of Tween 80. Fresh three weeks old maize leaves were offered to the larvae after it was surface disinfected. Each treatment was replicated three times and maintained at room temperature. Mortality were recorded daily for 11 days and the dead larvae were examined for mycosis development to confirm fungal infection (Tadele Tefera and Pringle, 2004).

4.9.5. Dose response study of selected EPF isolates against larvae of *C. partellus*

Suspension preparation

Three-week-old conidia of three EPF isolates (Two *Beauveria* spp and one *Metarhizium* spp) were used for multiple concentration assays to determine their lethal concentration and lethal time to kill 50% and 90% of the given animal population. This was done following the procedures described by Tadele Tefera and Pringle (2004). Briefly, stock conidial suspensions of sporulating fungal cultures were prepared by mixing the conidia in a test tube containing 10ml sterile distilled water with Tween 80 (0.01%V/V) using vortex mixer. The stock conidial concentration was determined using an improved neubaour hemocytometer and conidial concentrations were dawn adjusted to four concentration levels $1x10^8$, $1x10^7$, $1x10^6$ and $1x10^5$ conidia/ml.

In each experiment, a batch of 10 *C. partellus* larvae were placed in a sterile Petri dish (9cm diameter) and treated with 2ml of each conidial suspension using a hand-held spray atomizer. Maize leaves (3 weeks old) were washed with 3% sodium hypochlorite for 3 minutes and rinsed 3 times with sterile distilled water to remove the disinfectant. Pre-weighed pieces of leaves,

approximately 5cml long and 2cm wide, were offered for the larvae in a 9cm diameter Petri dish and allowed to feed for 24hours before replacing the leaves with a new one. The negative controls were treated with sterile distilled water with Tween 80 (0.01%V/V). The experiment was kept at room temperature for 11days. Each treatment was replicated four times. Measurements were made daily and the dead larvae were examined for mycosis development to confirm fungal infection (Tadele Tefera and Pringle 2004).

4.10. Statistical data analysis

Mortality data were corrected for the control mortality $\%CM=\frac{(\%T-\%C)}{(100-\%c)}*100$; where; CM is corrected mortality, C is mortality in the untreated larvae, and T is mortality in the treated larva (Abbott, 1925). Percent data of corrected mortality were arcsine transformed to stabilize the variance as needed. All mortality and germination data were subjected to ANOVA procedure of SAS software version 9 to determine the significant differences among treatments. For viability and pathogenicity tests of EPF isolates on *G. mellonella* the means were separated using Duncun's multiple range test at *P* value = 0.05. For pathogenicity of selected EPF and crud metabolites of endophytes against the larvae of *C. partellus*, Least Significant Difference (LSD) test was used to separate the means at *P* value= 0.05**.**

The LT_{50} and LT_{90} (Lethal time required to kill 50% and 90% of the treated insect population) was determined using probit analysis (IBM SPSS statics 20). The dose responses of each replicate were analyzed for estimation of lethal concentrations to kill 50% and 90% of the experimental insects (LC_{50} and LC_{90}) as for LT_{50} and LT_{90}. Analysis of variance was conducted and the means were separated by LSD.

5. Result

5.1. Isolation and identification of maize endophytes

A total of 81endophytic bacteria and fungi isolates (62 bacteria and 19 fungi) were obtained from the roots and stems of the sampled maize plants. Among the 62 endophytic bacteria isolates, 42 isolates (67.8%) were gram negative bacteria and 20 (32.2%) isolates were gram positive. Both LLR and OPVs maize varieties hold both Gram-positive and Gram-negative bacteria with different percentage (Figure 2). Among the bacteria isolates obtained from LLR 61% were Gram positive whereas contrasting this 65% of bacteria isolates from OPVs were Gram negative. All the obtained bacterial isolates from hybrid maize variety were Gram negative.

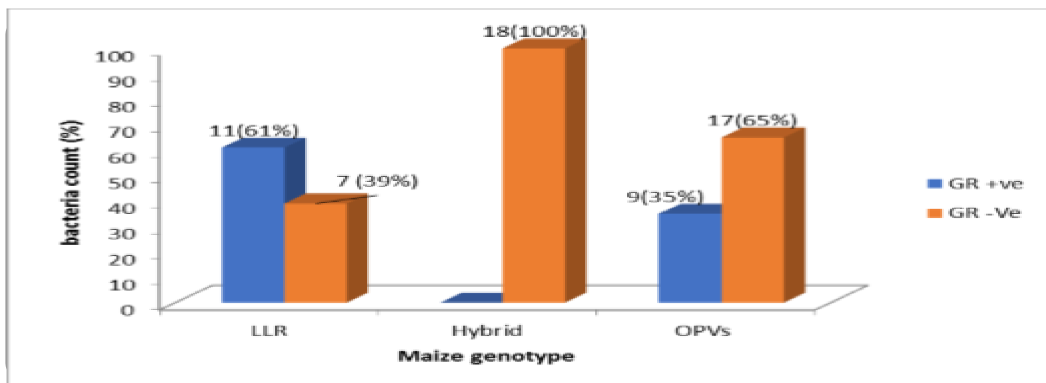


Figure 2. Distribution of Gram positive and Gram-negative bacteria in different maize genotypes. Where, LLR: local land race, OPVs: open pollinated variety, GR +ve: Gram positive and GR -Ve: Gram negative.

As the API identification results showed, gram negative endophytic bacteria were grouped into eight genera including *Enterobacter* spp., *Acinetobacter* spp., *Pseudomonas* spp. *Klebsiella* spp. *Stenotrophomonas* spp., *Sphingomonas* spp., *Burkholderia* spp., and *Salmonella* spp. The twenty-gram positive bacilli were grouped under the genus *Bacillus*. The endophytic bacteria were separated in to three major groups based on gram type and lactose fermentation. The first group was Gram positive rods and they were grouped under the genus *Bacillus* as per the

biochemical test results. The second groups were Gram negative rods and could ferment lactose and fall under the family *Enterobacteriaceae*. The third groups were Gram negative rods which did not digest lactose and grouped as *Non-Enterobacteriaceae*. API analysis identified members of group two as *Enterobacter cloacae, Klebsiella oxytoca, Salmonella* spp, and *Klebsiella pneumonia* and members of group three as *Pseudomonas fluoresces, Acinetobacter baumannii, Pseudomonas luteola, Pseudomonas oryzihabitans, Sphingomonas paucimobilis, Stenotrophomonas maltophilia* and *Burkholderia cepacia*.

Based on the microscopic conidial observation (Figure 3) all the endophytic fungi isolates were identified belonging to five genera; *Trichoderma* spp 6(32%), *Penicillium* spp 4 (21%), *Fusarium* spp 7(37%), *Botrydipolodia* spp 1(5%) and *Aspergillus* spp 1(5%) (Table 3). *Fusarium* spp and *Trichoderma* spp were the dominant endophytic fungal species with percent occurrence of 37% and 32% respectively.

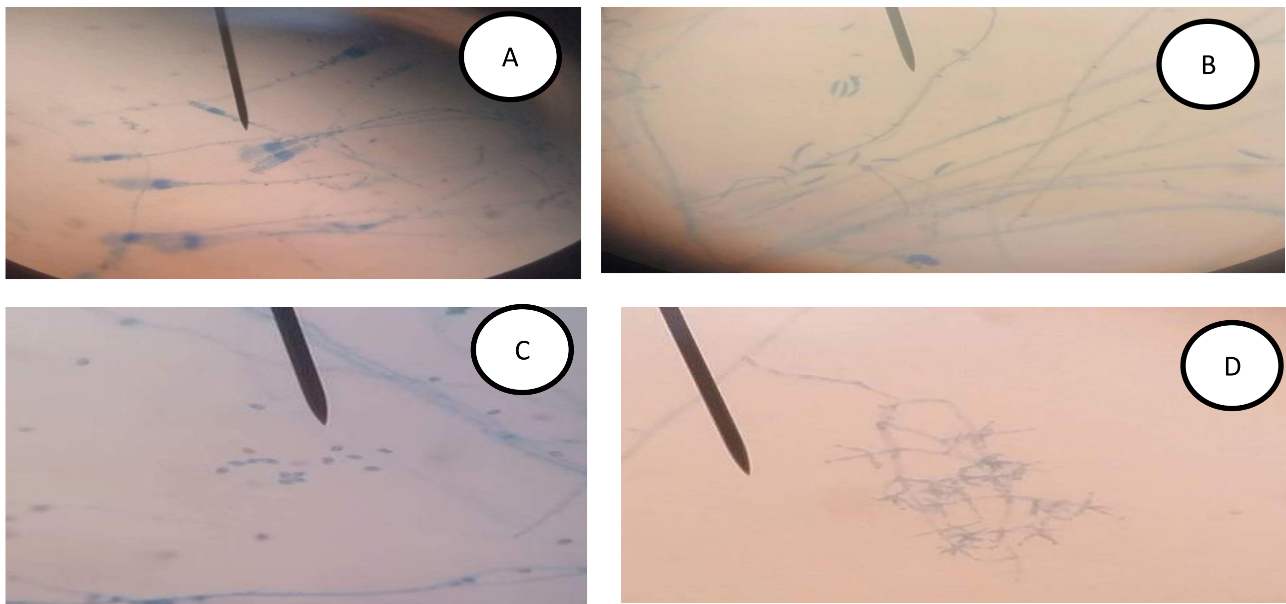


Figure 3: Microscopic conidial structure of endophytic fungi; A: *Penicillium* spp. B: *Fusarium* spp C: *Botrydipolodia* spp D: *Trichoderma* spp

Table (3). Percent occurrences of Endophytic fungi in maize genotype, part and growth stage.

Genus	total % of occurrence	Plant part		Growth stage			Maize variety		
		Root	Stem	Knee	LV	Flw	LLR	Hybrid	OPVs
Trichoderma Spp	6(32%)	4(31%)	2(33%)	0(0%)	3(30%)	3(38%)	5(38%)	0(0%)	1(20%)
Botryodiplodia Spp	1(5%)	0(0%)	1(17%)	0(0%)	1(10%)	0(0%)	0(0%)	1(100%)	0(0%)
Fusarium spp	7(37%)	6(46%)	1(17%)	1(100%)	2(20%)	4(50%)	5(38%)	0(0%)	2(40%)
Penicillium spp	4(21%)	2(15%)	2(33%)	0(0%)	4(40%)	0(0%)	2(15%)	0(0%)	2(40%)
Aspergillus Spp	1(5%)	1(8%)	0(0%)	0(0%)	0(0%)	1(13%)	1(9%)	0(0%)	0(0%)

Where LV: late vegetative stage, flw: flowering stage, LLR: local land race and OPVs open pollinated Variety

5.2. Species richness and diversity

It is evident from the results of this study that showed the distribution and population structure of maize were considerably affected with genotype, plant part and growth stage. The OPV exhibited relatively diversified endophytic bacteria composition and bacterial species diversity and richness were found H=1.65 and D=4.33 (Appendix 2A). The OPV inhabited 7of the bacterial groups and 3 of the fungal groups with the dominance of *Bacillus* spp. accounting 9 x10^5 CFU/g (Table 4). However, the presence of endophytic fungi was most diversified in LLR both in number and type. *Entrobacter* spp. and *Acinitobacter* spp. were isolated from the three maize genotypes. In terms of species diversity OPVs embraced all the identified species except *Pseudomonas* and *Salmonella* species. *Sphingomonas* spp and *Sentotrophominas* spp were only isolated from OPVs (Table 4).

(Table 4. Counts of each bacterial strain over maize plant part, growth stage and variety (CFU/g).

Bacteria genus	Plant part		Maize growth stage			Maize variety		
	Root	stem	Knee	LV	Flw	LLR	Hybrid	OPVs
Enterobacter spp	$5x10^5$	$5\ x10^5$	$6\ x10^5$	$3\ x10^5$	$1\ x10^5$	$5\ x10^5$	$1\ x10^5$	$4\ x10^5$
pseudomonas spp	$3\ x10^5$	$2\ x10^5$	$5\ x10^5$	N	N	$3\ x10^5$	$2\ x10^5$	N
Acinetobacter spp	$6\ x10^5$	$6\ x10^5$	$8\ x10^5$	$4\ x10^5$	$1\ x10^5$	$4\ x10^5$	$2\ x10^5$	$7\ x10^5$
Klebsiella spp	$6\ x10^5$	0	$7\ x10^5$	N	N	$5\ x10^5$	$1\ x10^5$	$1\ x10^5$
Salmonella spp	N	$1\ x10^5$	N	N	$1\ x10^5$	$1\ x10^5$	N	N
Burkholderia spp	$3\ x10^5$	N	$1\ x10^5$	$2\ x10^5$	N	N	$1\ x10^5$	$2\ x10^5$
Stenotrophomonas spp	$2\ x10^5$	N	$1\ x10^5$	$1\ x10^5$	N	N	N	$2\ x10^5$
Sphingomonas spp	$1\ x10^5$	N	N	$1\ x10^5$	N	N	N	$1\ x10^5$
Bacillus spp	$8\ x10^5$	$1.2\ x10^6$	$6\ x10^5$	$1.0\ x10^6$	$4\ x10^5$	$1.1\ x10^6$	N	$9\ x10^5$

Where LLR: Local land race, OPVs: Open pollinated variety, LV: late vegetative, Flw: flowering, CFU/g: colony forming unit per gram of sample and N; not isolated

The highest number of endophytic bacterial species diversity was recorded in maize root which occupy all the bacteria species except *Salmonella* (Table 4 and Appendix 2B). Genus *Bacillus* was dominantly isolated both in the stem and root at concentrations of 1.2 $x10^6$ CFU/g and 8 $x10^5$ CFU/g, respectively. In addition to this, the roots of OPVs maize genotype contained relatively divers bacterial groups (H=1.71 and D=5.16) compared to the above ground stem parts. Similarly, the type of endophytic fungi species diversity of maize root and stem were found to inhabit equal number of endophytic fungal specie (Table 3). However, the level of presence of endophytic fungi in root and stem varied. *Fusarium* spp and *Trichoderma* spp were the dominant endophytic fungal species in root accounting 46% and 31% respectively.

As the age of the maize plant increased, the composition and number of endophytic bacteria decreased (Table 4 and Appendix 2C). The maximum bacterial species diversity (H=1.65 and D=4.53) was recorded at the knee stage of OPVs followed by the knee stages of hybrid where H=1.33 and D=3.63. Endophytic bacteria were highly distributed at the knee height stage followed by late vegetative stage and, *Acinitobacter* spp, *Entrobacter* spp, *Klebsiella* spp, and *Pseudomonas* spp were the dominant strains at the knee height stage of maize whereas *Bacillus spp* were the only dominant groups isolated at the late vegetative stage (Table 4). In contrast to endophytic bacteria where the most diversity was obtained at the knee height stage; the presence of endophytic fungi was higher at the late vegetative growth stage of maize (Table 3). All the identified fungal groups except genus *Aspergillus* were found at the late vegetative stage of maize.

5.3. Larvicidal activity of maize endophytic isolates

Out of the 81 endophytes 39 isolates, all Bacillus species and one representative isolate of each genus, were selected for screening of insecticidal activity. Among the 39 maize endophyte isolates screened for insecticidal antibiosis activity 11 isolates (8 bacteria and 3 fungi) exhibited larvicidal activity against larvae of *C. partellus* and were selected for larval pathogenicity test (Table 5). The tested crud fermentation extracts of 11 maize endophyte isolates showed mean percent mortality ranging from 4.8% to 50.26% (Figure 4). The minimum mortality (4.8%) was recorded by aauef-3. The insecticidal virulence of endophytic fungi showed mortality rate not more than 21.32% and were grouped as less virulent strains. Isolate aaueb-11 under the genus *Bacillus* produced the highest mortality (50.26%) and was at the same significance level with the positive control *Bt. Kurstaki* (60.13%). Two of endophytic bacteria; isolate aaueb-21 and aaueb-11 showed relatively strong insecticidal activity 47.8% and 50.26% respectively.

Table 5. Prescreening of maize bacterial and fungal entophytes against the larvae of *C. partellus*

Genus of endophytes	Number of isolates screened (%)	Number of isolates with Insecticidal activity (%)	Total isolates selected
	Endophytic bacteria		
Bacillus	20/27 (74%)	29.6%	8
Pseudomonas	2/27 (7.4%)	0%	No
Acinetobacter	1/27 (3.7%)	0%	No
Klebsiella	1/27 (3.7%)	0%	No
Entrobacter	1/27 (3.7%)	0%	No
Salmonella	1/27 (3.7%)	0%	No
Burkholderia	1/27 (3.7%)	0%	No
Stenotrophomonas	1/27 (3.7%)	0%	No
	Endophytic fungi		
Penicillium	2/12 (16.7%)	0%	No
Fusarium	5/12 (41.7%)	16.7%	2
Trichoderma	3/12 (25%)	8.3%	1
Aspergillus	1/12 (8.3%)	0%	No
Botryodiplodia	1/12 (8.3%)	0%	No

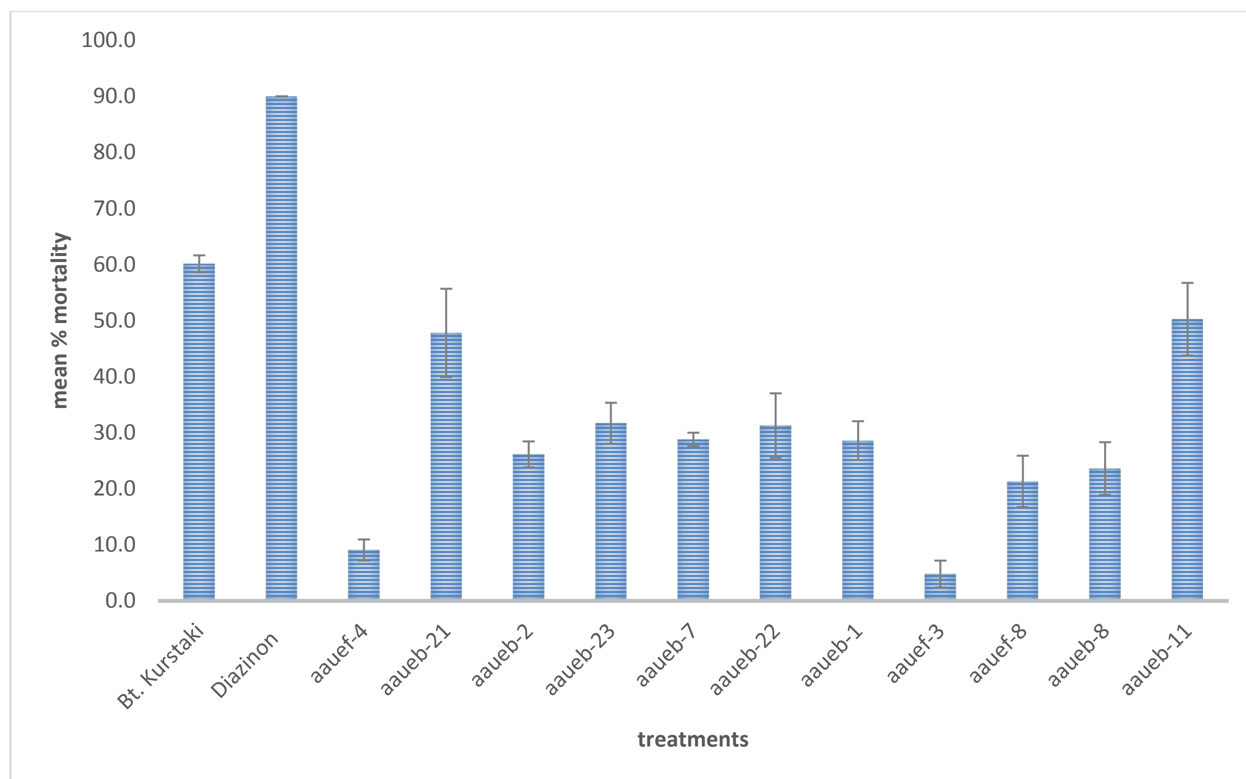


Figure 4. Mean percent mortality of larvae of *C. partellus* seven days after treatment with crude metabolite extract of maize endophytic bacteria and fungi; positive controls *Bt. kurstaki* and Diazinon.

5.4. Screening of EPF Isolates on *Galleria mellonella* larvae

5.4.1. Strain Viability and Pathogenicity tests

Viability of 27 EPF strains obtained from different sources (Table 2) were evaluated and percent of conidial germination exceeded 80% (Table 6). Pathogenicity of all the 27 EPF isolates was screened against larvae of *G. mellonella* was tested using galleria dipping method (Table 6). The percent mortality varied among the tested isolates causing mortality rates between 13.94% to 90.0%. The isolates S#048BC, M2 and DS-51-1 were the least virulent isolates causing mortality rates of 13.94%, 33.24% and 35.80% respectively. However, most of the isolates were moderately virulent inducing larval mortality greater than 40% and the top 8 isolates which

caused mortality of greater than 75% and which exhibited conidial germination greater than 85% were selected for virulence test against larvae of *C. partellus*.

Table 6. Viability and pathogenicity of EPF isolates against larvae of *Galleria mellonella*

Isolate code	Genus	% Mortality±SE	Mean % germination±SE
S#34	*Beauveria* spp	80.91±9.09ab	96.35±0.87ab
DS-51-1	*Metarhizium* spp	35.80±913de	94.73±0.66abcdef
S#048BC	*Metarhizium* spp	13.94±13.93e	84.41±3.74j
DS-86-2	*Metarhizium* spp	52.74±5.53bcd	95.06±1.72abcde
DS-37-1	*Metarhizium* spp	58.95±16.50bcd	91.86±1.97efgh
S#44BC	*Beauveria* spp.	73.94±16.06ab	91.73±2.03fgh
S#10H*	*Beauveria* spp.	90±0.00a	91.93±2.66efgh
DS-51-2*	*Beauveria* spp.	90±0.00a	88.99±1.99hi
S#05	*Beauveria* spp.	80.91±9.09ab	95.52±1.43abc
S#53	*Beauveria* spp.	90±0.00a	90.28±1.53ghi
APPRC-34GM*	*Metarhizium* spp	90±0.00a	90.95±1.83gh
DS-52-2	*Metarhizium* spp	77.02±12.98ab	96.72±0.34 a
AF2	*Beauveria* spp	90±0.00a	94.42±0.36abcdef
GF4	*Beauveria* spp	80.91±9.09ab	95.76±0.66abc
BF4*	*Beauveria* spp	90±0.00a	87.63±5.09i
B4*	*Beauveria* spp	90±0.00a	91.94±1.48 efgh
APPRC-44Bc*	*Beauveria* spp	90±0.00a	95.94±0.67abc
DS-35-2	*Beauveria* spp	90±0.00a	95.29±0.75abcd
M1	*Beauveria* spp	41.43±11.56cde	90.77±2.04 gh
APPRC-27*	*Beauveria* spp	90±0.00a	95.45±1.36 abc
KF3	*Beauveria* spp.	55.19±17.56bcd	89.67±0.59 hi
B1*	*Beauveria* spp	90±0.00a	97.65±1.11 a
S#41	*Beauveria* spp	90±0.00a	97.16±1.45 a
B7	*Beauveria* spp	65.32±13.92abc	93.18±1.58bcdefg
S#45	*Beauveria* spp	53.55±9.91bcd	94.93±0.68 abcdef
APPRC-40GM	*Metarhizium* spp	55.19±17.56bcd	92.19±0.42 defgh
M2	*Metarhizium* spp	33.24±11.63de	92.94±1.28 cdefg

Means with the same letter are not significantly different according to Duncan's multiple range test at $\alpha=0.05$.
** Indicate isolates selected for further screening on C. partellus.*

5.4.3. EPF Virulence study against larvae of *Chilo partellus*

The virulence of 8 EPF isolates (7 *Beauveria* and 1 *Metarhizium*) were evaluated and there were significant variations (Figure 5). The isolates were selected based on the percent of viability and virulence on *G. mellonella* larvae. Weak mean percentage mortality was recorded by isolate B4, DS-51-21 and B1 which scored 28.01%, 32.29% and 34.58%, respectively. Whilst isolates APPRC-34GM, S#10H and APPRC-44BC were the most virulent strains with mean percent mortality of 81.39%, 90.00% and 90.00%, respectively and were selected for multiple concentration assay.

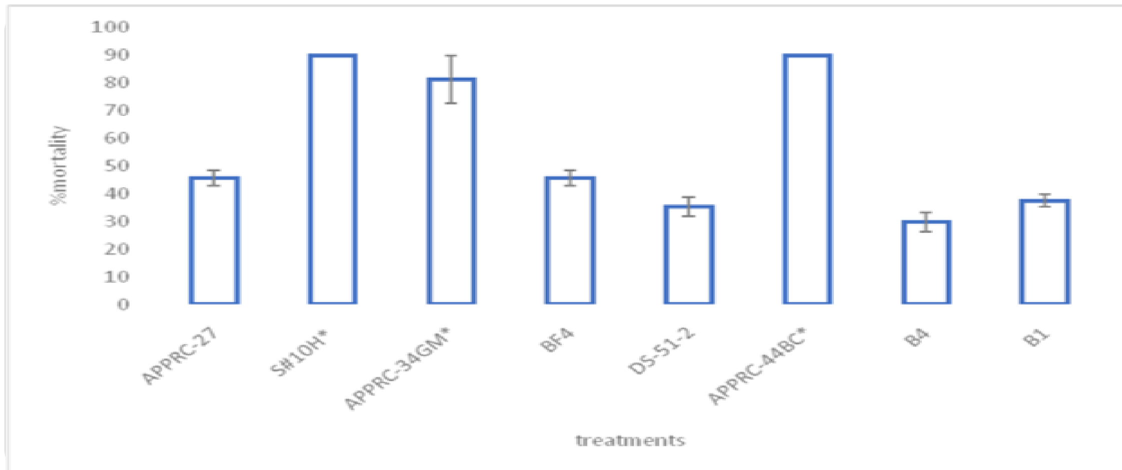


Figure 5. Mean percentage mortality of *Chilo partellus* larvae 11 days after treatment with selected *Beauveria* and *Metarhizium* isolates. Isolates with * were selected for dose response study.

5.5. Dose response study

There was a time difference between isolates in causing a 50% and 90% death of the give populations (Table 7). The LT_{50} ranged from 3.53 to 4.72 days and 6.15 to 9.05 days for higher and lower concentrations respectively. In terms of LT50, S#10H showed relatively short LT_{50} where the highest time recorded was 6.15 days. There was a general increase in LT50 with decrease in conidial concentration. The highest LT_{50} and LT_{90} 9.05 and 26.69 days respectively

were recorded by *Metarhizium* spp at the least conidial concentration. Similarly, the time required to kill 90% of the given animal population increased with decreased conidial concentration and at the highest conidial concentration ($1x10^8$conidia/ml) S#10H caused 90% mortality within 7.46 days which was almost equal to the time required by APPRC-34GM (7.42) days at conidial concentration of $1x10^6$conidia/ml. Therefore, S#10H is the best isolate to kill the given larval population with a relative short period of time.

Table 7. LT_{50} and LT_{90} of *Chilo partellus* after eleven days of treatment with different conidial concentration of selected *Beauveria* and *Metarhizium* isolates.

Conidia concentration ml-1	LT50 (days ±SE)			LT90 (days ±SE)		
	APPRC-44BC	S#10H	APPRC-34GM	APPRC-44BC	S#10H	APPRC-34GM
$1x10^5$	7.12±0.42	6.15±0.46	9.05±1.11	16.80±3.16	12.34±0.92	26.69±4.78
$1x10^6$	6.99±0.55	5.25±0.77	7.42±2.08	16.30±1.87	9.48±1.33	18.90±6.41
$1x10^7$	4.46±0.34	4.85±0.41	4.94±0.39	9.71±1.10	8.57±1.48	8.63±0.65
$1x10^8$	3.53±0.42	4.72±0.38	4.39±0.45	8.13±1.01	7.46± 1.16	8.22±0.86

The highest dose required to kill 50% and 90% of the given larval population $5.95x10^4$and $2.2x10^7$ conidia per ml respectively were recorded by S#10H. However, the lower LC_{50} and LC_{90} were recorded at APPRC-34GM $1.06\ x10^4$ and $1.73x10^6$ respectively. Hence, APPRC-34GM had the ability to made death with less conidial concentrations (Table 8).

Table 8. LC_{50} and LC_{90} of *Chilo partellus* after eleven days of treatment with different conidial concentration of selected *Beauveria* and *Metarhizium* isolates.

Isolate	Genus	LC_{50}	LC_{90}
APPRC44BC	*Beauveria spp*	$3.0x10^4$	$7.07x10^6$
S#10H	*Beauveria spp*	$5.95x10^4$	$2.20x10^7$
APPRC-34GM	*Metarhizium spp*	$1.06\ x10^4$	$1.73x10^6$

6. Discussion

Various endophytic bacteria and fungi had been isolated from different parts of maize in the current study (Table 3 and Table 4). Among the endophytic bacteria obtained in this work, *Bacillus spp*., *Enterobacter* spp., *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas* spp. and *Burkholderia* spp also endophytically reported from root and stem of maize (Thanh and Diep , 2014). Similarly, bacteria under the genera *Burkholderia, Enterobacter* and *Stenotrophomonas* were endophytically isolated from maize genotypes (Silva *et al.*, 2014). The endophytic presence of *Stenotrophomonas maltophilia* were also endophytically isolated from tomatillo *Physalis ixocarpa* proved for production of antifungal volatile organic compounds such as dimethyl disulfide (Rojas-Solís *et al.*, 2018).

The endophytic presence of fungal isolates obtained from the samples taken were also similarly observed with different studies. Orole and Adejumo (2011) isolated *Fusarium verticillioides* and *Trichoderma koninigii* from roots and grains of maize. Potshangbam *et al*. (2017) revealed the endosymbiotic presence *of Trichoderma* spp*, Fusarium* spp*, Penicillium* spp and *Aspergillus* spp from stem and root samples of maize. Among the six endophytic fungal genera isolated from roots of maize by Amin (2013), five of those genera except genus *Acremonium* were identified in the current study.

In the present work the distribution of maize endophytic bacteria and fungi were analyzed considering genotype, plant part and growth stage. However, the occurrences and distributions of endophytic bacteria and fungi were considerably varied with maize genotypes, parts and developmental stages. Such distribution variations may be related with plant phenology, soil types, soil PH and rhizosphere microbial density. Root exudates, a complex chemical cocktails that mediates interaction between rhizospheres and soil microbial communities, are greatly

influenced by environmental conditions and plant genotypes (Morel and Castro-Sowinski, 2013). The general occurrences of endophytes were greatly affected with growth stages and as the plant grows up tissue inhabiting microbes' diversity and richness were decreased.

The diversity was greater at the knee growth stages of maize. Park *et al*. (2012) reported fungal endophytes associated with roots of ginseng plant varied with the ages of the plant where the colonization frequency was decreased with increased age of the plant. In the current study endophytes have been considerably found at the knee stage of maze which is the most vulnerable stage for disease, stress and insect infestations. Hence the knee stage and late vegetative stages are the critical stages which harbor many of the different endophytic bacteria which may have physiological importance for the host and antagonistic effect towards plant diseases causing pathogens.

OPVs and hybrid maize genotypes were found to harbor relatively diversified tissue inhabiting microbes and the distribution were higher at the root parts. Pereira *et al*. (2011) reported that endophytic bacteria under the genus *Enterobacte*r, *Erwinia*, *Klebsiella*, *Pseudomonas* and *Stenotrophomonas* have a predominant association with roots of maize. Silva *et al*. (2014) indicated that transgenic maize genotype inhabits divers' endophytic bacterial communities than parental genotypes. Contrasting to this population size of *Bacillus subtilis* were not affected by plant genotype after Bt transgenic and non Bt transgenic maize were artificial infected (Sun *et al*. 2016).

Hence, the disturbance of endophytic population structure of maize was not only affected by plant genotype but also the infection process. Similarly, the distribution of tissue inhabiting microbes was highly fluctuated over parts of maize. Previous studies of Fisher *et al* (1992) on the

distributions of maize fungal and bacterial communities showed the plant part closer to the soil were heavily colonized by bacteria than the top parts of the plant. Hence, the results obtained that maize root inhabiting diversified groups of bacteria than stem agreed with this. However, it is difficult to critically decide that root always contains diversified bacterial groups than stem since Gaiero *et al*. (2013) detected the continuous translocation of endophytes from site of infection throughout the plant micro biome using Green-Fluorescent-Protein (GFP) labeled endophyte. Likely, growth stage of maize has great role in finding diversified tissue inhabiting microorganisms.

In the case of determining the larvicidal activity of endophytic microbes of maize, the current finding suggests there is a potential to use endophytic bacteria for the management of maize stem borers. However most of the endophytic bacteria other than genus *bacillus* were not found to produce any insecticidal compounds in the current study, crude fermentation of 11 maize endophyte isolates were showed larvicidal activity against larvae of *C. partellus* (Figure 4). Although there is no published research information on the use of maize endophytes against larvae of *C. partellus*, there are research works on the use of endophytes against insect pests.

An endophytic fungus *Phomopsis oblonga* isolated from the inner bark of dying elm protects elm tree against the beetle *Physocnemum brevillineum* a vector of Elm Dutch disease and was the first endophytic fungus showing the indication of endophytes for the control of insect pests (Webber, 1981). However, the mean percent mortality of *C. partellus* larvae byendophytic fungi *Trichoderma* and *Fusarium* isolated from maize was very low 4.8% and 21.3% of respectively, the study revealed the supplementary potential of endophytic fungi for the reduction of stem borer population. The reason for the lower larval mortality caused by the fungi may be due to the none closing activity of the insect spiracles by fungi metabolites. Fungal virulence on insect

larvae is supposed due to closing of the insect spiracles by fungal spores during germination not due to fungal toxicity.

In the current study *Fusarium* treatment showed killing effect on larvae of *C. partellus* and were relatively effective than *Trichoderma*. The insecticidal antibiosis activity of *Trichoderma* and *Fusarium* isolated from soil and insect cadaver respectively were also reported against *Periplaneta americana,* cockroach (Abdul-Wahid and Elbanna, 2012; Amatuzzi *et al*, 2018). Fusaric acid and cyclic peptides are reported for their ability to inhibit defensive enzymes of arthropods. Fusaric acid, picolinic acid, dipicolonic acid and cyclic peptides like Bcauvericin are produced by many *Fusarium* species (Abdul-Wahid and Elbanna, 2012). Hence the relatively higher larvicidal effect of *Fusarium* may be due to the presence of these lethal fungal metabolites which inhibit enzymatic activities of the larvae.

Among the crude extracts of 39 maize endophyte isolates screened only 11 (28.2%) possessed larvicidal activity against larvae of *C. partellus* with the maximum mean percent mortality of 50.26%. Majority of the tested isolates did not possess any insecticidal compounds from the fermentation extract. Endophytes obtained from maize have been reported for their importance in maize growth promotion, increased nutrient assimilation and suppression of disease-causing pathogens (Thanh and Diep, 2014; Kidd *et al*. 2013). There are research findings indicating the insecticidal activities of plant tissue inhabiting bacteria obtained from different plants. Shi *et al* (2014) demonstrated that crude extracts of more than 80% of endophytes isolated from drunken horse grass, *Achnatherum inebrians,* were caused aphid mortality of above 60%. However, in the current study the maximum mortality recorded by endophytic bacteria was 50.26% which is very less compared to their findings. Such a higher mortality may have been achieved due to the toxic components of the fermentation extracts were concentrated using different organic solvents. This

indicates the increased possibility to harvest insecticidal compounds from the crude fermentation of tested endophytic bacteria using solvent extraction techniques. Although several of the endophytic bacteria species isolated from maize in the current study such as *Entrobacter cloacae, Klebsiella oxytoca, Salmonella Spps.,* and *Klebsiella pneumonia* have been reported as pathogens for human and other mammals, they have been previously reported as pathogens of insects. Esfandiari and Motamendi (2013) isolated *Acinetobacter calcoaceticus* and *Acinetobacter baumanii* from the dead larvae of stem borer *Sesamia nonagrioides*. *Bacillus thuringiensis* which has a proud and successful history in insect pest management has also an endophytic relationship with its host plant and protects form insect pest attack (Sauka, 2017). Therefore, the fermentation extracts of isolate aaueb-11 and aaueb-21 were relatively toxic towards larvae of *C. partellus*, suggesting they are used for the development of endophyte driven insect pest resistance of maize.

This research work used viability and pathogenicity tests against larvae of *G. mellonella* for screening of 27 isolates to select the most virulent EPF strains. The present work on entomopathogenic fungi proved that there were significant variations among isolates in causing mortality against larvae of *C. partellus*. Such virulence variations among isolates may be directly related with production of insecticidal toxins, immune systems of the host and screening conditions. Tadele (2004) indicated the pathogenicity of some entomopathogens was affected by decreased temperature below 25°c. The infection process of entomopathogens were also affected by the insect cuticle which is a physical barrier affecting adherence and germination of fungal conidia by having lower water activity, shortage of readily available nutrients and producing antimicrobial compounds (Lu and Leger, 2016).

This study indicated that there was a time differences in killing 50% and 90% of the given larval population. Such variation may be directly correlated with conidial concentrations applied and the host immune systems. The difference in LT_{50} and LT_{90} values may also reflect the genetic and physiological variations among the tested isolates. In agreement with this Addis and Tadele (2009) indicated the increased time required to kill 50% of maize weevil population by *Beauveria* and *Metarhizium* with a decreased conidial concentration. Hence, the most virulent entomopathogenic fungi isolates S#10H, APPRC-44BC and APPRC-34GM tested against larvae of *C. partellus* are recommended as candidates for development of myco-pesticides for management of the test pest. However, filed verifications of the laboratory findings are needed.

7. Conclusions and recommendations

7.1. Conclusions

The study was aimed to isolation and larvicidal activity evaluation of maize endophytes and screening of some selected entomopathogenic fungi against larvae of *C. partellus*. Hence, it is concluded that maize harbors both bacteria and fungi endophytically and their distribution and diversity were critically affected by genotype, growth stages and plant parts. Endophytes have been most abundantly found at the Knee stages of maize and open pollinated variety was good in harboring relatively diversified endophytic microorganisms comparing local land race and hybrid once. The crude fermentation extracts of some of these endophytic bacteria and fungi caused mortality on larvae of *C. Partellus*. However, the crude metabolite extracts of endophytic fungi had poor larvicidal activity than bacteria. Two of endophytic bacteria isolates (isolate aaueb-11 and aaueb-21) under the genus *Bacillus* had relatively pathogenic fermentation component against larvae of *C. partellus*. It is also concluded that, the pathogenicity of entomopathogenic fungi isolates used had good virulence against larvae of *C. partellus* where three of the EPF isolates (S#10H, APPRC-44BC and APPRC-34GM) were most effective in killing larvae of *C. partellus*.

7.2. Recommendations

As the study in the use of maize endophytes in insect pest management is very limited and this is the first study in Ethiopia regarding effects of maize endophytes in maize stem borer, *C. partellus*, management the study gives the following suggestions for future research interventions.

- These results showed endophytic fungi and bacteria residing inside healthy maize plant tissues without causing any detectable disease symptoms to the host; the hidden diversity of endophytic fungi and bacteria in maize is a new area which requires further exploration on the role of these microbes in protecting of maize against insect pests.
- This study provided information that, crude fermentation extracts of two of the endophytic bacteria contained chemical composition compounds with insecticidal activity. However, the specific compound contributing on the insecticidal activity from the crude needed insecticidal spectra study and further isolation and purification to incorporate in microbial based biopesticide development. Thus, the insecticidal ingredients and compound structures should be further studied.
- The result also suggested that the most virulent endophytic bacteria and entomopathogenic fungi isolates could be good candidates used for reduction of stem borers infestation. However, greenhouse and field evaluations on the effects of endophytic bacteria and entomopathogenic fungi against feeding, survival and development of *C. partellus* should be studied for formulated product development.

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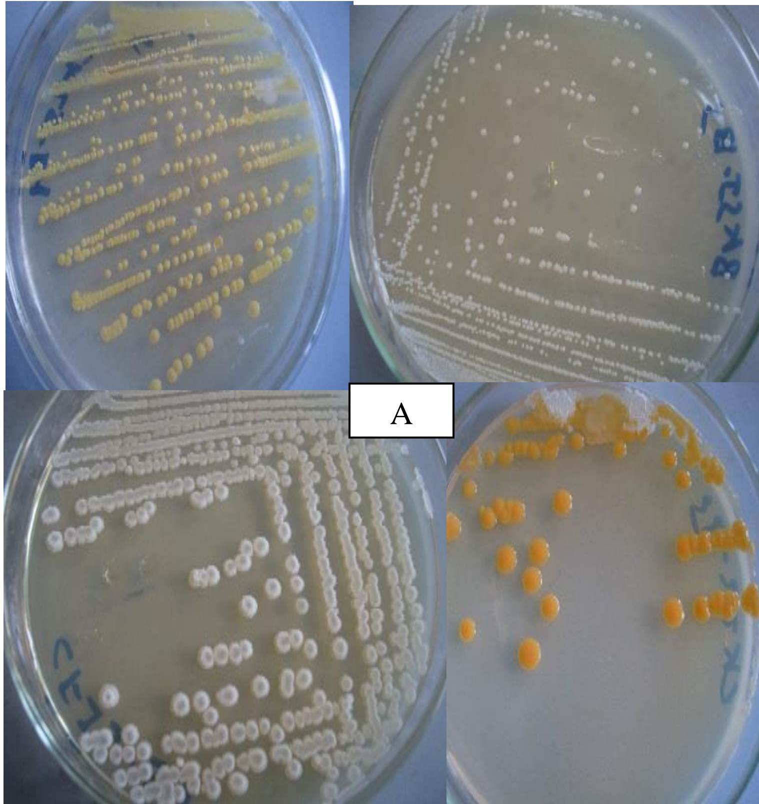
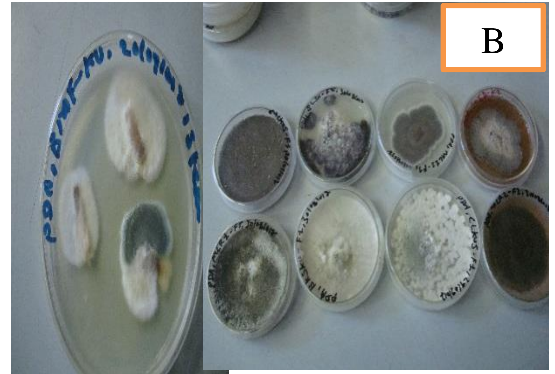
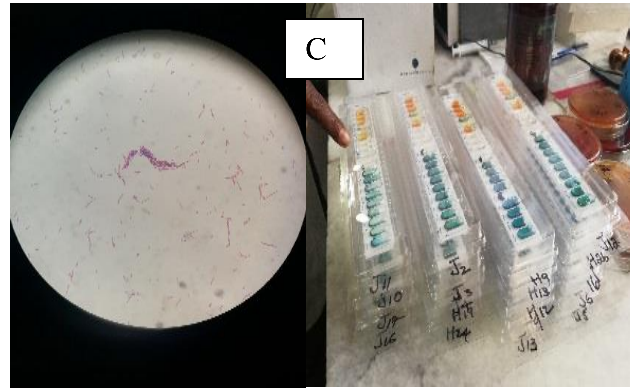
Appendixes

Appendix 1. representative isolates of endophytic bacteria and fungi

A; Pure cultures of endophytic bacteria; B. Pure cultures and outgrowth of endophytic fungi from maize tissues; C. cellular morphology and API identification of endophytic bacteria







Appendix 2. maize endophyte diversity Indexes

Appendix 2A. diversity indexes in maize genotype

Genotype	Shannon Index $(H= -\sum_{i=1}^{s} pi \ln pi)$	Simpson's Index (D) $D= \frac{1}{\sum_{i=1}^{s} pi2}$
LLR	1.15	2.13
OPVs	1.65	4.33
Hybrid	1.51	4.26

Appendix 2B. Endophyte diversity indexes over maize stem and root

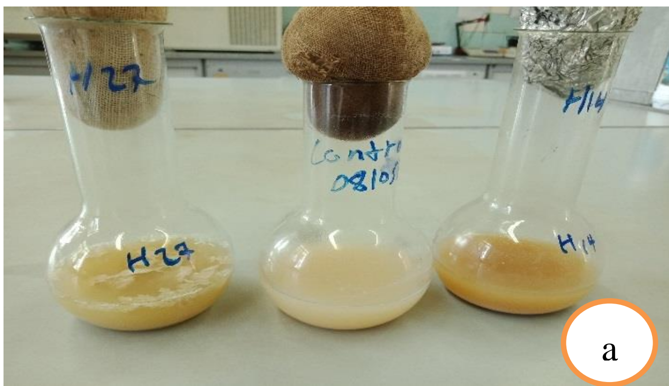
Genotype	Plant part	Shannon Index $(H= -\sum_{i=1}^{s} pi \ln pi)$	Simpson's Index (D) $D= \frac{1}{\sum_{i=1}^{s} pi2}$
LLR	Stem	0.38	1.28
	Root	1.61	4.17
OPVs	stem	1.24	3.13
	Root	1.71	5.16
Hybrid	stem	0.56	1.6
	Root	1.47	3.92

Appendix 2C. Diversity indexes of endophytes over maize growth stages

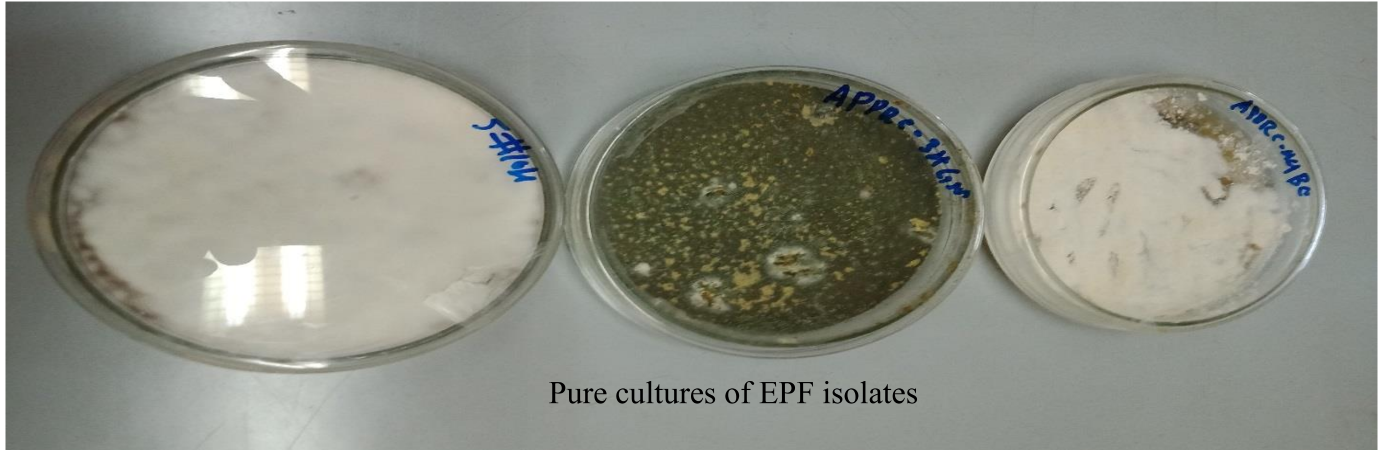
Genotype	Growth stage	Shannon Index $(H= -\sum_{i=1}^{s} pi \ln pi)$	Simpson's Index (D) $D= \frac{1}{\sum_{i=1}^{s} pi2}$
LLR	Knee	1.32	3.56
	LV	0.45	1.38
	Flw	0.56	1.6
OPVs	Knee	1.63	4.53
	LV	1.59	4.12
	Flw	0.00	1.00
Hybrid	Knee	1.33	3.63
	LV	0.69	2.00
	Flw	0.69	2.00

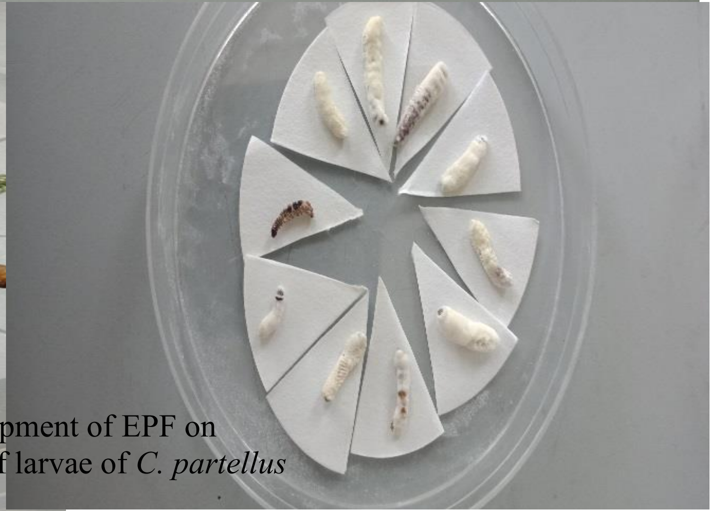
Appendix 3. extraction and evaluation of endophytic bacteria against larvae of *C. partellus*

a. growth of endophytes on fermentation broth media; b. crud fermentation extracts; c. dead *C.partellus* larvae due to crud fermentation extracts; d. feeding differences between treated and untreated larvae.



Appendix 4. Pure cultures of virulent EPF isolates and mycosis development on insect cadaver after treatment


Pure cultures of EPF isolates


Mycosis development of EPF on
Dead cadaver of larvae of *C. partellus*

Healthy larvae of *C. partellus* before Treatment

Dead cadavers of larvae of *C. partellus* after Treatment

Appendix 5. Maize endophytic bacteria and fungi used for pre-screening against larvae of *C. partellus*

Isolate code	Genus	Host variety	Insecticidal activity	Remark
Endophytic bacteria				
aaueb-1	*Bacillus*	LLR	Yes	Selected
aaueb-2	*Bacillus*	OPVs	Yes	Selected
aaueb-3	*Bacillus*	OPVs	No	
aaueb-4	*Pseudomonas*	Hybrid	No	
aaueb-5	*Acinetobacter*	Hybrid	No	
aaueb-6	*Bacillus*	LLR	No	
aaueb-7	*Bacillus*	OPVs	Yes	Selected
aaueb-8	*Bacillus*	LLR	Yes	Selected
aaueb-9	*Klebsiella*	LLR	No	
aaueb-10	*Bacillus*	OPVs	No	
aaueb-11	*Bacillus*	LLR	Yes	Selected
aaueb-12	*Enterobacter*	Hybrid	No	
aaueb-13	*Bacillus*	LLR	No	
aaueb-14	*Salmonella*	Hybrid	No	
aaueb-15	*Bacillus*	LLR	No	
aaueb-16	*Bacillus*	LLR	No	
aaueb-17	*Burkholderia*	OPVs	No	
aaueb-18	*Stenotrophomonas*	OPVs	No	
aaueb-19	*Pseudomonas*	Hybrid	No	
aaueb-20	*Bacillus*	OPVs	No	
aaueb-21	*Bacillus*	LLR	Yes	Selected
aaueb-22	*Bacillus*	OPVs	Yes	Selected
aaueb-23	*Bacillus*	LLR	Yes	Selected
aaueb-24	*Bacillus*	LLR	No	
aaueb-25	*Bacillus*	LLR	No	
aaueb-26	*Bacillus*	OPVs	No	
aaueb-27	*Bacillus*	OPVs	No	
Endophytic fungi				
aauef-1	*Penicillium* spp	LLR	No	
aauef-2	*Fusarium* spp	LLR	No	
aauef-3	*Trichoderma* spp	OPVs	Yes	Selected
aauef-4	*Fusarium* spp	LLR	Yes	Selected
aauef-5	*Fusarium* spp	LLR	No	
aauef-6	*Trichoderma* spp	Hybrid	No	
aauef-7	*Trichoderma* spp	OPVs	No	
aauef-8	*Fusarium* spp	LLR	Yes	Selected
aauef-9	*Fusarium* spp	OPVs	No	
aauef-10	*Aspergillus* spp	LLR	No	
aauef-11	*Botryodiplodia*	Hybrid	No	
aauef-12	*Penicillium* spp	OPVs	No	