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Evaluation of Biological Control Agents against Bacterial Wilt Pathogen (*Xanthomonas campestris* pv. *musacearum*) of Ensete (*Ensete ventricosum*)

By

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Applied Microbiology

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List of Symbols and Abbreviations

AUBB	Addis Ababa University Bacterial Biocontrol
AUFB	Addis Ababa University Fungal Biocontrol
Ant	Antagonist
Bact	Bactericide
HCN	Hydrogen Cyanide
IAA	Indole acetic acid
ISR	Induced systemic resistance
PGP	Plant growth promoting
P	Pathogen
SI	solubilization index

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Abstract

The present study was designed with the objective of isolating, screening, evaluating and characterizing antagonistic isolates against bacterial wilt of ensete from rhizosphere and phyllosphere of the host. A total of 361 bacterial and 145 fungal colonies were isolated. The *in vitro* antagonistic test on the pathogen showed that 17 fungal isolates and 3 bacterial isolates were screened to be active against the pathogen on the appropriate media with inhibition diameters ranging from 5mm to 40 mm. This indicates that the bacterial antagonists were more effective than fungal antagonists to inhibit the test microorganism with inhibition diameters ranging from 32mm to 40mm whereas the fungal antagonists were more diverse in their effectiveness than bacterial antagonists with inhibition diameters from 5mm to 21mm. The most effective antagonistic isolates (AUFB1, AUFB2, AUFB2, AUFB11, AUFB17, AUFB15, AUbB, AUbB1 and AUbB2) were characterized and identified to their genus level. The isolates AUbB1, AUbB3 and AUbB3 (bacteria) were identified as *Pseudomonas*. The fungal isolates AUFB2 and AUFB11 were categorized into *Trichoderma*, isolates AUFB1, AUFB15 and AUFB19 were classified as *Penicillium* and the isolate AUFB3 was identified as *Verticillium*. These isolates were further tested for antagonistic and plant growth promoting properties. It has been found that two bacterial isolates AUFB1, AUFB2 produced indole acetic acid, protease and solubilize inorganic phosphate whereas the isolate AUbB3 (bacterium) produced hydrogen cyanide, indole acetic acid and solubilize inorganic phosphate. It was also recorded that two fungal isolates AUFB15 and AUFB11 produced HCN and protease but the isolate AUFB15 was also produced indole acetic acid and solublize inorganic phosphate. *In vivo* under green house condition four effective isolates AUbB3, AUbB1, AUbB2 (bacteria) and AUFB11 (fungus) were evaluated for their efficacy against the pathogen. The data showed that the isolates AUbB1, AUbB3, AUbB2 and AUbB11 reduced the disease severity of ensete wilt with DSR (disease severity reduction) 74.8, 62.4, 57.4, and 56.4%, respectively. The isolate AUbB1 (bacterium) showed highest DSR whereas the isolate AUbB11 (fungus) showed least DSR. The isolates which showed significance pathogen reduction *in vitro* test and under green house conditions should be checked under field trials to further confirm their efficacy and to use them as antagonists. The result will serve as base line data for future endeavor of utilizing biological control system of ensete to increase productivity into low-input agriculture of the region and the country at large.

Key words: Ensete, *Xanthomonas campestris* pv. *musacearum,* Fungal and bacterial antagonist

1. INTRODUCTION

Ensete *(Ensete ventricosum* Cheesman) is typically a multi-purpose crop cultivated mainly as food and fiber in Ethiopia (Brandt, 1996). It belongs to Musaceae (banana family) and is an indigenous cultivated plant in southwestern Ethiopia (Bezuneh, 1984). However, wild ensete is widely distributed in central and eastern Africa in a number of countries including Congo, Mozambique, Uganda, Tanzania and Zambia (Brandt, 1996).

Morphologically it resembles a banana plant, which is often referred as "False Banana". Taxonomically banana is classified into a separate genus *Musa*. Both *Ensete* and *Musa* have a large underground corm, a bundle of leaf sheaths (pseudostem), and large, paddle-shaped leaves (Besrat *et al.*, 1979).

The main product of ensete is starch that is extracted from the underneath corm and the leaf sheaths and used for food. Ensete is also used as animal forage, fiber production which accounts more than 30%, construction materials, as an ornamental, and for its medicinal values to human and domestic animals (Brandt *et al*., 1997). Some researchers demonstrated that it has antimicrobial activity against viral, bacterial, fungal and nematodes, and human diseases (Holscher and Schneider, 1998).

It is estimated that more than 15 million populations in Ethiopia depend on ensete as staple and co-staple food source (Karin, 2002). The total area under ensete in Ethiopia is 224,400 hectares, whereas the total area under this crop in Southern Nations, Nationalities and Peoples Regional State (SNNPRS) and Oromyia is 145,800 and 78,600 hectares, respectively (Quimio and Mesfin, 1996; CSA, 1997). It is the main food source in Gurage, Kembata, Sidama, Gedio, Jemjem, and Arero. It is a second important crop as co- staple food in Wolayta, Gofa, Kafa zones and Yem special woreda (Kidist, 2003). *Ensete ventricosum* is an economic crop due to its high yielding potential, excellent

caloric and nutrient source. It also known for its adaptability, drought resistance, and multifunctional usage it is considered an economic crop plant in Ethiopia (Besrat *et al.*, 1979).

Several environmental (biotic and abiotic) and management factors such as drought, diseases, insect pests and population pressure affect the yield and productivity of ensete (Birmeta *et al*., 2004). Some of diseases are fungal diseases of corm rot, sheath rot and dead heartleaf rot and nematode diseases of root knot, root lesion and black leaf streak. There are also viral diseases of ensete known as mosaic and chlorotic leaf streak diseases. In addition, insects such as Jassid, fly, spider, mites, mealy bugs and some vertebrate pests' damage ensete plant and reduce its yield (Quimio and Mesfin, 1996). However, based on the distribution and the damage incurred on ensete production, ensete bacterial wilt disease caused by *Xanthomonas campestris* pv. *musacearum* is known to be the most threatening and important problem to ensete production system. The pathogen is very destructive as it kills the plants at all growth stages and regularly causes total losses (Bizuayehu, 2008 and Kidist, 2003).

Ensete wilt pathogen was first reported in Ethiopia in 1960s (Yirgou and Bradbury, 1968). Recently it has been reported that it is also banana infecting pathogen with significant damage of the yield in western Ethiopia, Uganda and Democratic of Republic Congo (Addis *et al*., 2004; Tushemereirwe *et al*., 2004). It is now recognized as a national problem and spread into most ensete and banana growing agro-ecology zones of the country. It is mainly spread through infected farm tools, infected planting materials, and repeated transplanting that damage the corm and roots. Animals fed infected plants and possibly insects feeding on the foliage are also incriminated with bacterial wilt disease (Dereje, 1985).

Previous study has been reported that there is no effective control measure against the bacterial wilt of ensete (Ssekiwoko *et al.,* 2006). In addition, no bactericide has been recommended against

ensete bacterial wilt (Kidist, 2003). However, there are different cultural strategies that have been employed for controlling ensete wilt disease such as planting healthy and disease-free suckers, planting resistant clones, destruction and controlled movement of diseased plants, cleaning of equipment that has come in contact with diseased plant material (Dereje, 1985; Agrios, 2005).

The impact of chemical control, lack of effective chemical control and acquisition of resistance, environmental pollution by pesticides, and damage to human health also necessitate alternative methods to control plant diseases. Biological control method is an alternative method of controlling plant diseases. Controlling plant diseases which caused by *Xanthomonas campestris* strains using antagonistic microorganisms has been reported. For instance, it has been reported in Mexico, USA and Barbados (Adaskaveg and Hine, 1985; Ritchie and Dittapongpithch, 1991; Ward and O'Garro, 1992) that biological control method using antagonists is a promising disease controlling method (Monteiro *et al.*, 2005).

Recently, the incidence of the disease found to be reduced by the treatment with *Pseudomonas putida* and *Pseudomonas syringae* (Byrne *et al*., 2005). According to Wulff *et al.*, (2002) inoculation of *Bacillus subtilis* into Brassicas crops like cabbage, rape, cauliflower and broccoli controlled the disease caused by *Xanthomonas campestris pv. campest*ris during dry and short rain seasons. *In vitro* test of *B. subtilis* and *B. megaterium* pv. *cerealis also* showed antagonistic effect against crucifers black rot (*Xanthomonas campestris Pv. campest*ris) (Monteiro *et al.,*2005).

Major microbial mechanisms by which biocontrol may protect plants against soil borne pathogens include competition for niches (Paulitz, 2000), antibiosis and the induction of systemic resistance (Pieterse *et al.,* 1996).

Bacterial wilt pathogen of ensete (*Xanthomonas campestris* pv. *musacearum*) is one of the pathogens of ensete which mainly affects its production and yield. Some studies in Ethiopia were undertaken to evaluate the severity of the pathogen. Although the pathogen has been controlled with cultural methods, it causes significance ensete yield loss (Kidist, 2003). Accordingly, alternative method of ensete wilt control is needed to be studied.

Therefore, this research was conducted in order to isolate, characterize and evaluate biological control agents against ensete wilt pathogen, *Xanthomonas campestris pv .musacearum, in vitro* and *in vivo* under green house condition.

OBJECTIVES

General objectives are:

To isolate, characterize, evaluate biological control agents against bacterial wilt disease (*Xanthomonas campestris* pv. *musacearum*) of ensete (*Ensete ventricosum).*

Specific objectives of the study are:

1. To isolate biocontrol antagonists agents against bacterial wilt from the rhizosphere and phyllosphere of ensete.
2. To study morphological, biochemical and physiological characters of biocontrol agents.
3. To evaluate potential biocontrol antagonists against bacterial wilt (*Xanthomonas campestris* pv. *musacearum)* of ensete (*Ensete ventricosum) in vitro* and *in vivo* under green house condition.

2. LITERATURE REVIEW

2.1 Taxonomy of Ensete

Ensete belongs to the order Scitaminae, family Musaceae. About 20 species were previously recognized in the genus ensete (Cheesman, 1947) but Simmonds (1962) placed *E. ventricosum* in the genus ensete together with only 5 other species, namely: *E. gilletti*, *E. homblei* , *E. perrieri* , *E. glaucum* and *E. superbum*, and noted that *E. glaucum* and *E. ventricosum* perhaps should be treated as a single species. *Ensete superbum* and *E. glaucum* grow in Asia, whereas *E. ventricosum*, *E. gilletti* and *E. homblei* occur in sub-Saharan Africa and *E. perrieri* in Madagascar (Birmeta *et al*., 2004).

Both ensete and banana have an underground corm, a bundle of leaf sheaths that form the pseudostem and large leaves. Ensete, however, is usually larger than banana, reaching up to 10 meters and with a pseudostem up to one meter in diameter and 2 to 5 meters in length. The leaves are more erect than those of a banana plant, have the shape of a lance head and may be five meters long and nearly one meter wide. The pseudostem and leaf midribs color vary considerably: some are purple to dark red but most are light green with variegated brown patches (Bezuneh, 1984).

In Ethiopia, wild ensete (*E. ventricosum*) occurs in the highlands (1100 to 3100 m above sea level) in the southwestern part such as Bonga, Omo river, and others but cultivated ensete grows in a wider area comprising the central, south and southwestern parts of Ethiopia, but mainly at higher altitudes ranging from 1,500 to 3,100 m (Birmeta *et al*., 2004). Most ensete growing areas have an average annual rainfall of 1,100 to 1,500 mm, a mean temperature of 10-21^{0}C and a relative humidity of 63-80%. Cattle manure is used as the main organic fertilizer. The ideal soils for ensete

cultivation are moderately acidic to alkaline (pH of 5.6 to 7.3) and contain two to three percent organic matter (Brandt *et al.,* 1997).

2.2 Uses of Ensete

In Ethiopia, ensete is primarily cultivated for food and used for starchy foods and beer. Different types of food obtained from ensete are *Kocho,* which is the bulk of fermented starch product; *Bulla* that is a small amount of water-insoluble starchy product and *Amicho* which is the boiled ensete corm, usually of a younger plant. The most important characteristic feature of ensete is its productivity of food per unit of land and its storage for a long period without spoilage (Seifu, 1996).

The fiber obtained from ensete is used to make bags, ropes, cordage and mats. Ensete leaves are used for wrapping materials, plate for serving food and cattle feed in dry season. The dried midribs and petiole are used for making mats and rope (in place of nail) in house construction and as fuel. Some ensete clones are used as local medication for different illness and damage such as bone fracture, bone breakage and diarrhea for both human beings and animals (Brandt *et al.,* 1997).

Ensete farming could attain sustainable food production and its Kocho can be stored for longer time. Ensete has much water in cell-like structures which enhances its drought resistant as compared to cereals. According to Bezuneh and Feleke (1966), it can withstand relatively long period of drought (about 5 months). In addition, it is capable to maintain and enrich soil fertility.

2.3 Ensete Diseases

The various numbers of fungal, nematode, viral and bacterial diseases were reported to cause infection and damage at different degrees of intensity and reduce its yield (Quimio, 1992). Among these pathogens, bacterial wilt of ensete caused by *Xanthomonas campestris* pv. *musacearum* was first reported from Ethiopia by Yirgou and Bradbury (1968) and is currently found in all ensete

growing regions. Surveys conducted in the 1980s in major ensete growing zones of Ethiopia revealed the occurrence of ensete bacterial wilt disease in all zones with different degree of severity (Dereje, 1985). It is very destructive disease which kills ensete plants at all growth stages including 4 to 7 years old plants ready to harvest (Quimio and Mesfin, 1996). Once it appears in a field, it is easily transmitted from infected ensete plant to healthy plants through different mechanisms and in some areas where the severity of the disease and loss is high, farmers is obliged to abandon the whole field and replace it with another crop (Kidist, 2003).

According to Dagnatchew and Bradbury (1974) bacterial wilt of ensete affects banana under natural conditions and epidemics of the disease was reported in the former Kaffa province. The bacterial wilt of ensete symptom was also observed in banana through artificial inoculation of the disease (Dereje, 1985). Recently, a screening trial on 45 banana cultivars for resistance to bacterial wilt disease of ensete revealed that all cultivars were found susceptible (Awassa Agricultural Research Center Progress Report, 2000).

Bacterial wilt pathogen of ensete and banana (*Xanthomonas campestris* Pv. *musacearum)* is mainly spread through contaminated farm tools and infected planting materials. It also can be transmitted during several transplanting operations which increase the possibilities for infection through damaged or wounded corms and roots, with animals feeding on infected plants and possibly by insect vectors feeding on the foliage (Dereje, 1985). Bacteria cannot enter plants via intact cuticles but entry is either through wounds or natural openings such as hydathodes and stomata (Dereje, 1985; Awassa Research Center Progress report, 2000). Once the bacteria enter into the plant multiply in the intercellular space and move through the tissues so that cell death of the plant may follow due to toxins or pectolytic enzymes produced by the bacteria (Manners, 1993).

The rate of spread of the disease depends up on the rate of multiplication of the pathogen, its motility and ability to produce pectolytic enzymes and the structure of the host, which are also

affected by the environmental conditions especially temperature and the production of stimulants or inhibitors by the host agents (Manners, 1993). According to Wolde-Michael *et al.,* (2008) *Xanthomonas campestris* pv. *musacearum* survive in soil up to 9 days whereas in petioles and leaf sheaths for up to 3 months. Its Initial symptoms in infection of ensete include the presence of bacterial ooze in the leaf petioles and leaf sheaths, yellowing at the edge of leaf and progressive wilting of the leaves (Wolde-Michael *et al.,* 2008). The yellowing at the edge of the leaf was recorded at 15 and 21 days after inoculation in ensete plants which were inoculated at 6 and 12 months after transplanting respectively (Wolde-Michael *et al.,* 2008).

2.4 Management of Bacterial Wilt of Ensete

2.4.1 Cultural Control

According to Agrios (2005) sanitary measure is the first control measure which should be taken against bacterial diseases. The measure includes the use of disease free suckers as planting material, uprooting and burying of diseased plants far from the field, cleaning and flaming of equipment that has come in contact with diseased plants, rotation of crops and plots should be allowed for more than 3 months if the damage is severe. In addition, infected ensete plants tissues should not be used or transported to other locations, as they are a likely source of bacteria (Brandt *et al*., 1997; Wolde-Michael *et al.,* 2008).

2.4.2 Reaction of ensete clones to pathogen

Ensete farmers know that certain ensete clones such as Yeshirekinke in Gurage, Mezia in Wolaita, Ado and Genticha in Sidama, Siskela and Gimbo in Hadya and Nobo in Keficho have relatively high tolerance against bacterial wilt. This was partially confirmed in screening trials by Dereje (1985) and Awassa Agricultural Research Center (Awassa Agricultural Research center progress report, 2000).

2.4.3 Chemical Control of *Xanthomonas campestris*

Although controlling plant diseases with chemicals is common method, some plant diseases such as ensete wilt lack effective chemicals to manage the disease .However, some chemicals have been reported that against *Xanthomonas campestris* strains. Various *in vitro* trials were done on antibiotics and plant extracts against *Xanthomonas campestris* pathovars that cause diseases in different crops. It was reported that streptomycin was effective against black rot pathogen of cauliflower, *Xanthomonas campestris* pv. *campestris*, followed by oxytetracycline in *vitro* test (Lenka and Ram, 1997). However, tolerance to streptomycin and copper compounds for the control of *Xanthomonas axonopodis* (syn: *X. campestris*) pv. *vesicatoria* has been reported in Mexico (Adaskaveg and Hine, 1985), in USA (Ritchie and Dittapongpithch, 1991) and Barbados (Ward and O'Garro, 1992).

It was reported that plant extracts are potential in the control of diseases caused by *X. campestris* in several important crop plants *in vitro* test. According to Akhtar *et al.*, (1995) diffusates from various parts of *Phyllanthus emblica, Acacia nilotica, Sapindus mukorossis* and *Terminalia chebula* showed antimicrobial effects against *Xanthomonas campestris* pv. *citri* and an inhibition zone of 4.83-6mm. Extracts from *Acacia arabica*, *Achras zapota*, and other 6 higher plants were also found inhibitory to various pathovars of *Xanthomonas campestris* (Satish *et al*., 1999).

2.4.4 Disadvantage of Chemical Control

Although chemical compounds have been applied to control plant diseases, they result in negative impact on wide range of organisms. The increasing use of agrochemicals is negatively perceived by consumers and supermarket chains. Chemical pesticides contaminate groundwater, enter food-chains, and pose hazards to animal health and to the spraying personnel of the chemical pesticides. Several members of the European Union (EU), such as Sweden, Denmark, and Netherlands have

decided in the mid-late 1980s to decrease the chemical input in agriculture by 50% within a 10-year period (Butt *et al.,* 2001; Matteson, 1995).

Frequent applications of copper-based bactericides amended with an ethylenebisdithiocarbamate fungicide (maneb or mancozeb, class B2 carcinogens) provide some disease suppression of *Xanthomonas* leaf blight and other bacterial diseases of onion in Colorado, but they must be applied regularly eight or more times to be effective and suppress disease per season (Schwartz and Otto, 1998). This approach to the management of disease onion is expensive for onion growers. In addition, Copper resistance has been reported in Barbados (Paulraj and O'Garro, 1993).

2.5 Biological control

Biocontrol organisms offer environmentally friendly alternatives to chemical control methods to manage plant diseases or pests. Biological control agents could be used where chemical pesticides are banned (organ chlorines) or being phased out (methyl bromide) or where pests or pathogens have developed resistance to conventional pesticides or to grow organic food to satisfy consumer perception (Butt *et al*., 2001). It has been reported that some bacterial species can serve as biological control agents against soil-borne pathogens (Arfaoui *et al*., 2007).

Biological control depends up on the establishment and maintenance of a threshold population and viability below that level may eliminate the possibility of biological control (Xu and Gross, 1986). According to Byrnea *et al.,*(2005) *P. putida* B56, *P. fluorescens* A506 and *P. syringae* Cit7 provided significant reductions in disease severity of bacterial spot of tomato (*Xanthomonas campestris* pv. *vesicatoria*) under greenhouse conditions. Similarly, foliar application *Pseudomonas syringae* consistently provided significant disease suppression in control of bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria*. In addition, seed and root application of

Pseudomonas fluorescens and *Bacillus pumilus* provided significant suppression of bacterial spot in the field trials conducted in Alabama (Ji *et al.,* 2006).

According to Byrnea *et al*. (2005) controlling of bacterial spot of tomato, which is caused by *Xanthomonas campestris* pv. *vesicatoria*, with *P. syringae* Cit7 and *P. putida* B56 antagonists in field test was promising.

2.5.1 Potential Advantages of Biological Control Agents

Biological control method has potential to control crop diseases while causing no or minimal detrimental environmental impact (Haggag *et al.,* 2007). It has been used as alternative control method of chemical due to harmful effects of some pesticides to human health and the environment (Cook, 1993). Controlling plant disease with biocontrol microorganisms reduce environmental pollution and resistance development as compared to chemical method. This is because they produce degradable chemical in low amounts at targeted locations. This approach fits well in the worldwide strategy to grow healthy plants in a sustainable way and, therefore produce high quality food (Haggag *et al.,* 2007).

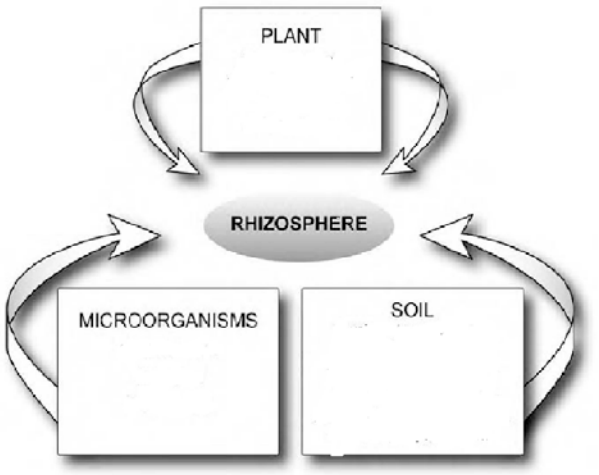
2.5.2 Limitation of Biological Control Agents

Although biological control agents have shown to protect crop plants from disease under experimental conditions, inconsistent performance between under experiment conditions and field locations has been reported. Biocontrol is less consistent than chemical control in field condition. Variation in consistence and performance of biological control agents has been attributed to many factors like biotic and ecological factors (Kamilova *et al*., 2005). Moreover, the survival and efficacy of biocontrol agents affected by host plant genotype, agricultural practices, mutation of biocontrol organism and resistance of pathogen to biocontrol mechanisms (Ownley and Windham, 2006). Biological control may also competitively displace non-target organisms (Cook *et al.*, 1996).

Unintended effects would occur if this competitive displacement were to seriously affect a non-target organism; perhaps even leading to its extinction or in some other way detrimentally affecting a component of the ecosystem. Furthermore, some fungi that intended for biological control could infect a wide variety of hosts, which sometimes include mammals. Thus, evaluation of potential microbial control agents must include an evaluation of their virulence towards non-target organisms (Goettel *et al*., 2001).

2.6 Rhizosphere Microbiology

Growing plant roots influence the activities of soil microorganisms in the adjoining volumes of soil known as rhizosphere (Lynch, 1982). In other words, rhizosphere is a narrow zone of soil (soil–plant interface) subject to influence of living roots, where root exudates stimulate or inhibit microbial populations and their activities. The rhizoplane or root surface provides a highly favorable nutrient base for many species of microorganisms. In essence, the rhizosphere can be regarded as the interaction between soil, plants, and microorganisms (Brimecombe *et al.,* 2007) (Figure 1).



Source: Brimecombe *et al.,* 2007

Figure 1.The Rhizosphere interaction

The root rhizosphere is the place of an intense microbial life and a high microbial activity (Mohamed, 2009). The composition and number of microorganisms present in the rhizosphere of different plants may differ due to variations in the quantity and quality of compounds exuded by the different plants (Aldén *el al*., 2001). Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and by serving as selective growth substrates for soil microorganisms (Kremer *et al*., 1990; Miller *et al.,* 1989).

The root colonizing ability is an essential prerequisite for the success of rhizobacteria. The introduced microorganisms in plant roots as biofertilizers, biocontrol agents (Weller and Cook, 1983) and plant growth promoters have generally shown a progressive decline in population size leading to limit their effectiveness (Compeau *et al.,* 1988). So, the inoculants microbes must be able to establish themselves in the rhizosphere at population densities sufficient to produce beneficial effects. Therefore, efficient biocontrol agents should survive in the rhizosphere, make use of nutrients exuded by the plant roots, proliferate, be able to efficiently colonize the entire root system and highly demonstrate rhizosphere competence with indigenous microorganisms (Mohamed, 2009).

Root exudates in the rhizosphere offer a carbon-rich diet to the rhizosphere microorganisms: organic acids (such as citrate, malate, succinate, pyruvate, fumarate, oxalate and acetate) and sugars (such as glucose, xylose, fructose, maltose, sucrose, galactose and ribose) constitute the major exudates, whereas variable amounts of α-amino acids, nucleobases and vitamins (such as thiamin and biotin) provide the minor exudates. The ability of Rhizobacteria to use organic acids as carbon sources correlates with rhizosphere competence (Goddard *et al.,* 2001). In essence, rhizosphere is relatively rich in nutrients due to the loss of as much as 40% of plant photosynthesis from the roots

(Lynch and Whipps, 1991). Consequently, rhizosphere microbes benefit because plant roots secrete metabolites that can be utilized as nutrients. Thus, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth. Beneficial rhizosphere microbial populations like Plant growth promoting microorganisms maintain root health, nutrient uptake, and tolerance of environmental stress (Cook, 2002).

Beneficial microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens. Rhizosphere microorganisms antagonize pathogens before and during primary infection and also during secondary spread on the root (Weller, 1988). One of the beneficial microorganisms that can grow in the rhizosphere is plant growth promoting Microorganisms.

2.6.1 Plant Growth Promoting Microorganisms (PGPM)

Plant growth promoting microorganisms (PGPM) are a heterogeneous group of microbes that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth. They exert beneficial effects on plant development via direct or indirect mechanisms. The exact mechanisms by which PGPR (Plant growth promoting Rhizobacteria) promote plant growth are not fully understood, but are thought to include: the ability to produce or change the concentration of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene; biofertilization (Boddey and Dobereiner, 1995); antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982); antibiotics (Shanahan *et al*., 1996) and cyanide (Flaishman *et al*., 1996); solubilization of mineral phosphates and other nutrients (De Freitas *et al*., 1997; Gaur, 1990); rhizoremediation (Kuiper *et al.,* 2004), induction of systemic resistance and competition for nutrients and niches (Lugtenberg and Kamilova, 2009).

2.6.2 Rhizosphere Competence

Biological control agents must not only have appropriate mechanisms for biocontrol but they should also be able to compete and persist in the environment where they must operate, and they should establish themselves in the rhizosphere and compete to acquire rhizosphere competence (Harman, 1996). Rhizosphere-competence is defined as colonization of root in the soil where the introduced microorganisms compete with indigenous soil microorganisms. Rhizosphere competence and root colonization of microorganisms depend on degradable substrates such as root and seed exudates, chitin, cellulose and pectins (Baker, 1991). The increased growth response of plants caused by rhizosphere microorganisms depends mainly on the ability of these microorganisms to survive and develop in the rhizosphere (Kleifield and Chet, 1992).

Rhizosphere competence is important because a biocontrol agent cannot compete for space and nutrients if it is unable to grow in the rhizosphere. For example, *Trichoderma* species, either added to the soil or applied as seed treatments, grow readily along with the developing root system of the treated plant (Howell, 2003). Competitive root tip colonization plays an important role in various mechanisms of biocontrol (Lugtenberg and Dekkers, 1999).

2.7 Mechanisms of Biological Control of Plant Pathogens

The different mechanisms by which biological agents control plant pathogens and deleterious microbes are through the production of antibiotics, lytic enzyme, hydrogen cyanide and siderophore or through competition for nutrient and space. In so doing, they can significantly improve plant health and promote growth by increasing of seedling emergence, vigor and yield (Antoun and Kloepper, 2001). The following mechanisms are the most important ones that determine effectiveness of biocontrol agents against pathogens.

2.7.1 Antibiosis

Antibiosis is the inhibition or destruction of one organism by a metabolite produced by another organism. Antagonists may produce powerful growth inhibitory compounds that are effective against a wide variety of microorganisms (Ownley and Windham, 2006). These are compounds are low molecular weight organic substances that are produced as secondary metabolites by certain groups of microorganisms at low concentration (George, 2002). These metabolites may have acidic (killing) effect or a static (inhibiting) effect on the growth of the target pathogen, and this is the best-known mechanism by which microbes can control plant diseases (Lecrere *et al*., 2005; Dowling and O'Gara, 1994; Fravel, 1988). The best-known antibiotics produced by gram-negative bacteria are phenazines, 2, 4- diacetylphloroglucinol (Bangera and Thomashow, 1999), pyrrolnitrin (Pfender *et al.,* 1993), pyoluteorin (Kraus and Loper, 1995) and oomycin A (Gutterson, 1990). Some biocontrol *Bacilli* produce the antibiotics zwittermycin A and kanosamine (Silo-suh, 1994). It was also reported that *Trichoderma* and *Gliocladium* produce antimicrobial compounds such as gliovirin and gliotoxin (Howell *et al*., 1993).

Different antibiotics have different mode of action on bacterial pathogens .These include: prevent proper cell wall formation; inhibition of protein synthesis and memberan integrity; disruption of plasma and outer memberan function and inhibition of DNA synthesis (Walker *et al.,* 2001).

2.7. 2 Induced Systemic Resistance (ISR)

Induced systemic resistance (ISR) is a mechanism that biocontrol agents induce a sustain change in the plant and increase its tolerance to infection of plant diseases. ISR protects the plant systemically following induction with an inducing agent to a single part of the plant (Brimecombe *et al.,* 2007). The action of ISR is based on plant defense mechanisms that are activated by inducing agents (Kloepper *et al.,* 1992).

Induced systemic resistance makes susceptible plant resistant to a wide array of subsequent pathogen attack (Kloepper *et al.,* 1992; van Loon and Glick, 2004) by activation of host responses that directly attack the pathogen through enzymatic action, production of plant produced antibiotic substances or by lignifying cell walls such that pathogen movement is slowed. ISR activates multiple potential defense mechanisms, which include increases in activity of chitinases, ß-1,3-glucanases, peroxidases, and other pathogenesis-related proteins (Lawton and Lamb,1987) ; accumulation of antimicrobial low molecular-weight substances such as phytoalexins (Kuc and Rush,1985); and the formation of protective biopolymers, such as lignin, cellulose, and hydroxyproline-rich glycoproteins (Hammerschmidt *et al.,*1984). A single inducing agent can control a wide spectrum of pathogens. In cucumber, it has indicated that treatment of the first leaf with a necrosis-forming organism protects the plant against at least 13 pathogens, including fungi, bacteria, and viruses (Dean and Kuc, 1985).

2.7.3 Enzyme Production

Biological control microorganisms attack pathogens by excreting lytic enzymes. It has reported that chitinase produced by *Serratia plymuthica* inhibited spore germination and germ-tube elongation in *Botrytis cinerea* (Frankowski *et al.,*2001); β -1,3-glucanase synthesized by *Paenibacillus* sand *Streptomyces* sp lyse fungal cell walls of *F. oxysporum* f. sp. *cucumerinum* (Singh *et al.,*1999). *B. cepacia* synthesizes β-1, 3-glucanase that destroys the integrity of *R. solani*, *S. rolfsii*, and *Pythium ultimum* cell walls (Fridlender *et al*., 1993). Lysis of pathogen by hydrolytic enzymes is often characteristic of mycoparasitism. This has been demonstrated for several *Trichoderma* species that control fungal pathogens (Harman *et al.,* 2004). Chitinase and β-1, 3 glucanase (laminarase) are particularly important enzymes secreted by fungal mycoparasites capable of degrading the fungal cell wall components, chitin, and β-1, 3 glucan (Schroth and Hancock, 1981).

2.7.4 Competition for Nutrients and Ecological Niche

According to Elad and Chet (1987) the competition of available carbon and nitrogen sources may account for observed disease reduction. They found competition for nutrients between germinating oospores of *Pythium aphanidermatum* and bacterial biocontrol strains significantly correlated with suppression of the disease. It appeared that bacteria were competing with germinating oospores for available carbon and nitrogen and by eliminating these resources, the bacteria effectively reduced oospores germination. Wilson and Lindow (1993) demonstrated that *P. fluorescens A* 506 colonized by competing for a limiting nutrient, thus making this nutrient unavailable to *Erwinia amylovora.* Niche exclusion is an alternative mechanism involved in biocontrol agents. *Pseudomonas* strains, for example, are able to establish on roots from inoculated seeds relatively easily (Brimecombe *et al.,* 2007).

2.7.5 Competition for Iron and Role of Siderophores

Rhizobacteria produce siderophores that chelates the available iron and prevent the iron nutrition of respective phytopathogens, thereby restricting the proliferation and colonization by phytopathogens (Lemanceau and Albouvette, 1993). Siderophores are iron chelating ligands which can be beneficial to plants by increasing the solubility of ferric iron (Fe III), which otherwise is unavailable for plant nutrition (Renshaw *et al*., 2002).

Siderophore-producing microorganisms are also known to impart induced systemic resistance to plants (Pieterse *et al.,* 2001) and suppressiveness to the soil (Mazzola, 2002) and have been implicated in the biocontrol of several plant diseases (Sindhu, 1997; Sayyed *et al*., 2005). Siderophore-based biological control agents provide iron nutrition; thereby promote the plant growth (Sayyed *et al*., 2007). It is assimilated by root cells in the reduced form (Fe II); however, especially in sufficiently aerated soils, the oxidized state (Fe III) is predominant and needs to be reduced to be taken up by plants (Hoyos-Carvajal *et al.,*2009). *Trichoderma* species can reduce Fe

III through chelators such as siderophores, and plants can take up chelated iron by reductases on the plasma membrane (Altomare *et al*., 1999; Jalal *et al*., 1986). Unlike bacterial siderophores, the production of *Trichoderma* metabolites able to chelate iron is constitutive and does not require Fe deficiency (Renshaw *et al*., 2002).

2.8 Factors Influencing Rhizosphere Interactions

It has been found that many environmental factors influence the amount and composition of root exudates and hence the activity of rhizosphere microbial populations. Microbial composition and species richness at the soil–plant interface are related either directly or indirectly to root exudates and thus vary according to the same environmental factors that influence exudation (Brimecombe *et al.,* 2007). Root exudates can serve as chemo attractants leading to root colonization (Chet and Baker, 1991). The presence of organic compounds, released by the plant roots, stimulates the microbial activity in the rhizosphere (Bacilio-ménez *et al.,* 2003). The microbial activity is generally higher in rhizosphere, and the population size of 10 to 100 fold higher was found in this zone as compared to the surrounding bulk soil (Weller and Thomashow, 1994).

Root exudation and microbial colonization have both been shown to change with plant age and stage of development. The quantity of both proteins (Juo and Stotzky, 1970) and carbohydrates (Hamlen *et al.,* 1972) released by herbaceous plants has been shown to decrease with increasing plant age (Liljeroth and Bååth, 1988). Microbial numbers in the rhizosphere increase over time, reaching a peak around the time of flowering and then decreasing (Schonfeld *et al., 2001*).

Moreover, the quantity, concentration and chemical composition of the root exudates vary with plant genotypes (He *et al.,* 2004). The amount and composition of root exudates entering the soil can be affected by multiple factors such as light intensity, temperature, nutritional status of the plants, activity of retrieval mechanisms, various stress factors, mechanical impedance, and sorption characteristics of growth media and microbial activity in the rhizosphere (Naher *et al*., 2009).

The activity of biological control agents is also influenced by in the environmental factors like climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil (Baker, 1986). Many soil edaphic factors including temperature, soil moisture, pH, and clay content also influence the survival and establishment of the biocontrol agents and their interaction with the pathogen. In addition, native microorganisms influence activity of biocontrol by increasing root exudation (Prikryl and Vancura, 1980).

3 MATERIALS AND METHODS

3.1 Sample Collection

Rhizosphere soil samples and leaves of ensete (*Ensete ventricosum* Cheesman) were collected from enset growing areas: Sidama (Hawassa, Wojgra woreda, and Hagerselam woreda), Jimma and Woilata (Areka). Roots with adhering soils of healthy ensete plants were collected, sub-sampled and transferred into sterile plastic bags and taken to Applied Microbiology Laboratory, Addis Ababa University, for experimental analysis.

3.2 Pathogen of Ensete

The culture of ensete bacterial wilt, *Xanthomonas campestris* pv. *musacearum*, was obtained from Ambo Plant Protection Research Center. It was maintained on YDC (Glucose Yeast Extract Calcium Carbonate Agar) slant containing 10.0 g of yeast extract, 20.0g of D-glucose, 20.0g of $CaCO_3$, 15.0g of agar per 1 liter of distilled water (Goszczynska *et al.,* 2000).

3.3 Isolation of Antagonists

3.3.1 Isolation of Bacterial Antagonists

For each sample treatment, root adhering soil was carefully brushed off and the roots were gently washed with sterile distilled water to extract root associated bacteria. Ten gram of each of the adhering soil samples was separately mixed in 90 ml of root washed water in 250 ml flasks. The flasks were shaken in a Gallenkamp-orbital shaker for 30 minutes at 120 rpm to make homogenize solution. One milliliter of each sample was serially diluted with sterile normal saline solution (85% of NaCl) to provide 1:10 w/v dilution (10^{-2}-10^{-6}). One hundred micro liter of the suspension from $10^{-4,}$ 10^{-5} and 10^{-6} were uniformly spread on Nutrient Agar Media (Difco) and incubated at 30^0C for 2 days. Colonies with different morphology were picked from Nutrient Agar plate, sub-cultured to have pure colonies and preserved at 4^0C for further tests.

3.3.2 Isolation of Fungal Antagonists

Ten gram of the adhering soil samples were separately mixed in 90ml of sterilized distilled water in 250ml flasks. The flasks were shaken in a Gallenkamp-orbital shaker at 120 rpm for 30 minutes to homogenize the soil suspension. One milliliter of each sample was serially diluted with sterile normal saline solution (85% of NaCl) to provide 1:10 w/v dilution (10^{-2}-10^{-6}). One hundred micro liter of the suspension from 10^{-3}, 10^{-4}, and 10^{-5} was uniformly spread on PDA (Potato Dextrose Agar) (Difco) supplemented with 25 µg/ml chloramphenicol as a bacteriostat and incubated at 25 ^{0}C for 3 days. Colonies with different morphology were picked, purified and preserved at 4^{0}C for further work (Watanabe, 2001).

3.4 Screening for Antagonistic Agents

3.4.1 Agar-diffusion test

One hundred micro liter of 24 hr culture of pathogen suspension containing 10^8 cfu/ml was spread on KB plates and four holes of 9 mm diameter punched into the agar. In these holes 24 hr old bacterial isolates suspension of each test antagonist (10^9 cfu/ml) was added and the plates incubated at 28^{0}C for 48 h. Inhibition of pathogen growth was assessed by measuring the radius of inhibition zone (mm) after incubation for 48 h at 28 ^{0}C (Monteiro, 2002).

3.4.2 Dual Culture assay

Fungal isolates were screened for their antagonistic activity against *Xanthomonas campestris pv. musacearum* in-vitro dual culture assay on KB (Suarez-Estrella *et al.,* 2001; Fokkema, 1973). One hundred micro liter of 24h old culture (10^8cfu/ml) of *Xanthomonas campestris* pv.*musacearum* was swabbed on the whole surface of the tested media. Then 5mm diameter agar blocks of 7days old pure fungal culture was cut and inoculated at edge of plates in three replications. The zone of growth inhibition was recorded after 5 days of incubation at 25^{0}c.

3.4.3 Evaluation of Antagonistic Isolates for Protease Activities

Protease activity of the screened biocontrol isolates was tested on Casein Digest Agar Medium. The medium contains g/liter: skim milk 100.0, trypitcase soy 25.0, and agar 15.0. 24h old cultures were inoculated in to the medium and incubated at 30^0c for 5 days. A clear zone of skim hydrolysis was recorded as positive test an indication for protease activity (Booth, 1971).

3.4.4 Fluorescent Pigment Production

The ability of bacterial isolates to produce yellow- green fluorescent pigments or flourescein siderophore was tested by culturing the bacterial isolates on KB medium. Twenty four hours old culture was inoculated into the medium and incubated at 30^0c for 48hrs. The fluorescent pigments were observed for the fluorescence under 366 nm UV light after 48hr incubation (Stieglitz and Weimer, 1985).

3.4.5 HCN Production

The production of HCN from glycine was examined by using the method of Bakkers and Schippers (1978). This test was carried out by using King's B medium amended with 4.4g per liter of glycine. Twenty four hour old culture was inoculated into the plate and placed inside the Petri dish lids with Whatman No.1 filter paper that has been soaked with 0.5% (w/v) picric acid and 20% (w/v) sodium carbonate. The Petri dishes were fixed with parafilm and incubated at 28^0c for 48hrs. A change of the filter paper from yellow to orange, red, brown, or reddish brown was recorded as indicator of weak, moderate, or strongly cyanogenic potential, respectively.

3.5 Evaluation for Plant Growth Promoting (PGP) Activity

3.5.1 Production of IAA

Production of indole acetic acid (IAA) by antagonistic isolates was assayed by using Luria –Bertani (LB) medium. The medium contains trypton 10g, yeast extract 5g and NaCl 5g per 1 liter of distilled water according to the medium of Gordon and Weber (1951). A 24 hr old culture (10^6cfu/ml) broth of each isolate was centrifuged at 10,000 for 15 minutes in three replications. Two milliliter of the supernatant was transferred to another tube to which 2 to 3 drops of O-phosphoric acid and 4ml of ferric chloride-perchloric acid reagent (1 ml of 0.5 M $FeCl_3$ + 50 ml of 35 % perchloric acid) was added. The development of pink color after 25 minutes, showed the production of IAA.

3.5.2 Phosphate Solubilization Activity

The antagonistic isolates were tested *in vitro* for their phosphate solubilization activity using Pikovskaya Agar Medium containing 10.0 g of glucose, 5.0 g of Ca_3 $(PO_4)_{2,}$ 0.5 g of yeast extract, 0.5 g of $(NH_4)_2SO_{4,}$ 0.2 g of NaCl, 0.2 g of KCl, 0.1 g of Mg $SO_4.7H_2O$, 0.0002 g of Fe SO_4.7 H2 O and 18.0 g of agar with pH 7.2. Twenty four hours old cultures were spot plated and incubated for 5 days at 30^0c. The presence of clearing zone around colonies was measured and used as indicator for positive phosphate solubilization. Solubilization index (SI) was calculated based on formula of SI = colony diameter + halo zone diameter/ Colony diameter (Pikovskaya, 1948).

3.6 Characterization of Antagonistic Microorganisms

3.6.1 Characterization of Bacterial Isolates

3 .6.1.1 Colony and Cellular Morphology

Culture characteristics of selected biocontrol isolates were determined on Nutrient Agar Medium. Twenty four hours old broth cultures were streaked on plates and incubated for 2 days. The colonies were characterized by colony appearance and pigmentation (Christopher and Bruno, 2003). The shape of the cells of the isolates was determined by preparing a wet mount from a young culture and examined with the oil immersion objectives.

Gram reaction of the isolates was tested by using KOH test, according to the method of Suslow *et al*., (1982). A single colony was picked from a 24hr old culture of Nutrient Agar Plates and mixed with 1-2 drops of 3 % (w/v) KOH solution until an even suspension was obtained. The formation of mucoid tread of slime on lifting up the loop was indicate as gram negative bacteria where as the appearance of watery solution, with no slime formation was considered as gram positive bacteria.

3.6.1.2 Motility Test

Motility of antagonists was determined by stabbing isolates in test tubes with straight wire loop during oxidation –fermentation test. Motile isolates showed diffused growth whereas non motile isolates showed non-diffused/ straight growth (Collins and Lyne (1976).

3.6.2 Biochemical Test of Bacterial Antagonists

3.6.2.1 Cytochrome Oxidase Test

The presence of cytochrome oxidase system in the isolates was tested by using the method of Collins and Lyne, (1976). Small piece of Whatman filter paper was soaked with 1 %(w/v) N, N, N,

N-tetramethly-p-phenylenediamine dihydrochloride. A loop full of 24 hr old cultures were scrapped and rubbed on the filter paper. Appearance of blue color within 10 and 30 seconds was considered as a positive test for cytochrome oxidase.

3.6.2.2 Catalase Test

The presence of Catalase activity in the isolates was checked by flooding a drop of 3% H_2O_2 on pure colonies of 24 hr cultures over the slide, immediate effervescence of gas bubble was recorded as a positive result (Harisha, 2007).

3.6.2. 3 Oxidation –fermentation test

The oxidative and fermentative of a carbohydrate by isolates were determined on oxidation - fermentation medium by the method described by Aneja (2005). The medium contains: 2.0 g of peptone, 1.0 g of yeast extract, 5.0 g of NaCl, 0.2 g of K_2HPO_4, 0.08 g of bromothymole blue per 1 liter of distilled water and pH was adjusted to 7.1.

The medium was freshly prepared and 5 ml of broth medium was dispensed in each test tube. After sterilization, the medium was cooled to about 35^0C with tap water to avoid dissolution of oxygen in the medium to which 10% (w/v) of sterilized glucose was added. Two test tubes inoculated by stabbing with straight wire loop. One of the tubes was sealed the surface with a thin layer of paraffin oil to create anaerobic condition whereas the other tube was incubated aerobically.

Acid production associated with color change from green to yellow in the aerobic tube was indicative of oxidation whereas the color change from green to yellow in the both tubes showed fermentation.

3.6.2. 4 Indole Production Test

Indole production from isolates was determined by using 1% tryptone broth and incubated for 48hrs at 30^{o}c. About 1ml of kovac's reagent (diethylamide benzaldehyde) was added to each test tube and agitated gently to extract the indole. The development of bright- pink color on the top layer indicated indole production (Aneja, 2005).

3.6.2. 5 Starch Hydrolysis

The ability of isolate to metabolize starch was tested by using starch minimal medium containing 0.5% of solute starch, 0.3% of Mg SO_4, 0.02% of $CaCl_2$, 1% of K_2HMPO_4 and 20 g of agar per liter of distilled water. Clear zone was detected around colonies after flooding with Lugol's iodine is indicative of starch hydrolysis (Grimont *et al*., 1977).

3.6.3 Characterization of Fungal Isolates

3.6.3.1 Slide culture

The morphology of spores and mycelia of fungal isolates were examined by growing them on slide according to Stevens (1974). A bent glass rod was placed inside the Petri dish layered with filter paper. A slide was put on the glass rod with soaked cotton pad on the corner of the Petri dish. The set up was covered with the lid and autoclaved at 121^{0}C and 15 lb. Sterilized PDA from a different Petri dish was cut into $1cm^2$ blocks and put at the center of the slide on the beat glass rod. Fungal isolates were inoculated at the center of four sides of agar block and covered with sterile cover slip under aseptic condition and incubated at 25^{0}C for 5 days. After 5 days the cover slip was lifted up carefully and placed on another clean slide. Likewise, a clean cover slip was placed on the slide after agar block was discarded. The slides were then stained with Lactophenol cotton blue and mounted on a microscopic (Gallenkamp) and observed under high power objection (400X). The spore type, mycelia morphology were recorded.

3.6.4 Ecophysiological Test

3.6.4.1 Temperature Tolerance

The temperature tolerance of bacterial and fungal cultures was tested by growing them on Nutrient Agar Medium and Potato Dextrose Agar, respectively. They were incubated at different temperatures of 4^0c, 10^0c, 20^0c, 30^0c, 37^0c, 45^0, 55^0c and 80^0c for 3 days.

3.6.4.2 pH Tolerance

The ability of isolates to grow on acidic and alkaline media was checked by inoculating the bacterial culture in 100 ml of Nutrient Broth Medium in 250 ml flasks and adjusted to pH levels of 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 by using pH meter with addition of diluted HCL and CaCO3. After incubation on Gallenkamp-orbital shaker at 120 rpm for 3 days, the biomass of bacterial isolates was filtered through Millipore size filter paper, dried at 65^0C for 48hrs and measured. Likewise, fungal culture was also inoculated in 100 ml of Potato Dextrose Broth in 250 ml flasks to pH levels of 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 to check their growth. The biomass of fungi was filtered through filter paper, dried at 65^0c for 48hrs and measured after 10 days of incubation on Gallenkamp-orbital shaker at 120 rpm (Ying and Feng, 2006).

3.6.4.3. Salt Tolerance

The salt tolerance of the bacterial and fungal antagonistic isolates to different salinity level was tested on Nutrient Agar and Potato Dextrose Agar plates, respectively .The media supplemented with NaCl at concentration of 1% ,2%, 3%, 4%, 5% ,6% ,7% ,8%, 9%(w/v). The colony growth was recorded after 3 days of incubation at 28±2^{o}C (Abdel-Latif, *et al.,* 2006).

3.7. Selection of Isolates for Pot experiment

Effective isolates were selected for in vivo evaluation (pot experiment) and their effectiveness in controlling of wilt pathogen on ensete seedlings.

3.7 .1 Inoculum Preparation

Selected bacteria antagonists were cultured in Nutrient Broth at 28 ± 2^0c on orbital shaker. Twenty four hours old cultures were taken and transferred to 100ml of the medium and incubated on orbital shaker at 120 rpm for 48hrs. Then the cultures were adjusted to 10^7 cells/ml using sterile water according to Stromberg *et al.,* (2004).

Likewise, selected fungal antagonist was cultured in potato dextrose broth at 25^0c on orbital shaker. 5mm diameter of mycelial blocks were taken from 10-day old cultures and transferred to 100 ml of the medium and incubated on orbital shaker at120 rpm for 7 days. The cultures were adjusted to 10^7spores/ml in sterilized water according to Sinha and Wood, (1968).

3.7.2 *In vivo* /Pot Experiment

A pot experiment was designed under greenhouse conditions using plastic pots containing 3kg of sterilized soil that was brought from the sampling site (Areka Agricultural Center). Disease free suckers of ensete plants from Areka Agricultural Center were planted in the pots. After the emergence of three leaves the plants were treated by stem puncture and leaf infection pin pricks with 1) sterilized water, 2) pathogen with inoculum of size 10^6 cfu/ml and 3) pathogen with inoculums size of 10^6 cfu/ ml and bacterial and fungal antagonists with inoculum size of 10^7cfu/ml and 10^7spores/ml, respectively. Three replicate pots were used for each treatment in a completely randomized experimental design (Morsy *et al*., 2009).

The percentage of disease severity (DS %) was evaluated 30 days after inoculation by estimating the percentage of leafs with lesion areas. The percentage of disease severity reduction (DSR%) was calculated according to Edginton *et al*. (1971): DSR (%) = [(DSc - DSt)/DSc)] x 100, where DSc = leaf area with lesions on the control plants that treated with only pathogen and DSt = leaf area with lesions on the treated with antagonist and pathogen.

4. DESIGNATION OF ISOLATES

The bacterial and fungal antagonistic isolates against ensete wilt pathogen were designated as AUFB (Addis Ababa University fungal biocontrol)), and AUbB (Addis Ababa University bacterial biocontrol).'AU', 'F', 'b', and 'B' represent Addis Ababa University, fungus, bacterium and biocontrol, respectively.

5. DATA ANALYSIS

Data of ensete wilt disease severity reduction by antagonistic isolates *in vivo* test analyzed by one-way ANOVA (Post Hoc tests) SPSS Version 15.0.

6 RESULTS

6.1 Screening of Antagonistic isolates against *Xanthomonas campestris* pv. *musacearum*

A total of 361 bacterial and 145 fungal colonies were collected from the rhizosphere and phyllosphere of ensete plant. They were isolated, screened and evaluated for their antagonistic activity against *Xanthomonas campestris* pv. *musacearum in vitro* test using dual culture assay. The results revealed that 17 fungal isolates and 3 bacterial isolates were found to be active against the pathogen *in vitro* test (Table 1).

Table 1. Screening and evaluation of antagonistic isolates against *Xanthomonas campestris* pv. *musacearum* isolated from the rhizosphere and phyllosphere of ensete.

Isolates	Total number of isolates	Number of antagonistic isolates	Proportion of antagonists
Fungal Isolates	145	17	12%
Bacterial Isolates	361	3	1%
Total	506	20	4%

It was noticed that the tested antagonistic isolates against test pathogen showed inhibition diameter ranging from 5mm to 20mm (Table 2). It was found that the inhibition diameters of bacterial isolates against the pathogen ranging from 32 mm to 40mm whereas inhibition diameters of fungal isolates were ranging from 5mm to 21mm. Among the fungal isolates, AUFB11 was found to display the largest inhibition diameter 21mm whereas isolates AUFB1, AUFB2 and AUFB3 showed inhibition diameter of 20mm. Fungal isolates were more diverse in their inhibition diameters ranging from 5-10mm (lowest group), 11-15mm (middle group) to 16-21mm (the highest inhibition group) against the test pathogen.

Table 2. Measurement of inhibition diameter of antagonistic isolates against *Xanthomonas campestris* pv. *musacearum in vitro* test

Isolates (Fungi)	Inhibition diameter (mm)	Isolates (Bacteria)	Inhibition diameter (mm)
		AUbB1	32
		AUbB2	40
		AUbB3	32
AUFB1	20		
AUFB2	20		
AUFB3	20		
AUFB4	5		
AUFB5	7		
AUFB6	5		
AUFB7	13		
AUFB8	9		
AUFB9	10		
AUFB12	16		
AUFB13	14		
AUFB14	7		
AUFB15	19		
AUFB16	7		
AUFB17	19		
AUFB10	17		
AUFB11	21		

Key**:** AUFB= Fungal Antagonists, AUbB= Bacterial Antagonists

6.2 Characterization and Identification of the selected Biocontrol Agents

6.2.1 The Fungal Biocontrol

Fungal isolates were characterized and identified into their group type based on their cellular characteristics, mycelia and spore morphology from PDA media. They were identified into their group based on Watanabe Manual of Pictorial Atlas of Soil and Seed Fungi: Morphologies of cultured fungi and key to species (Watanabe, 2002).

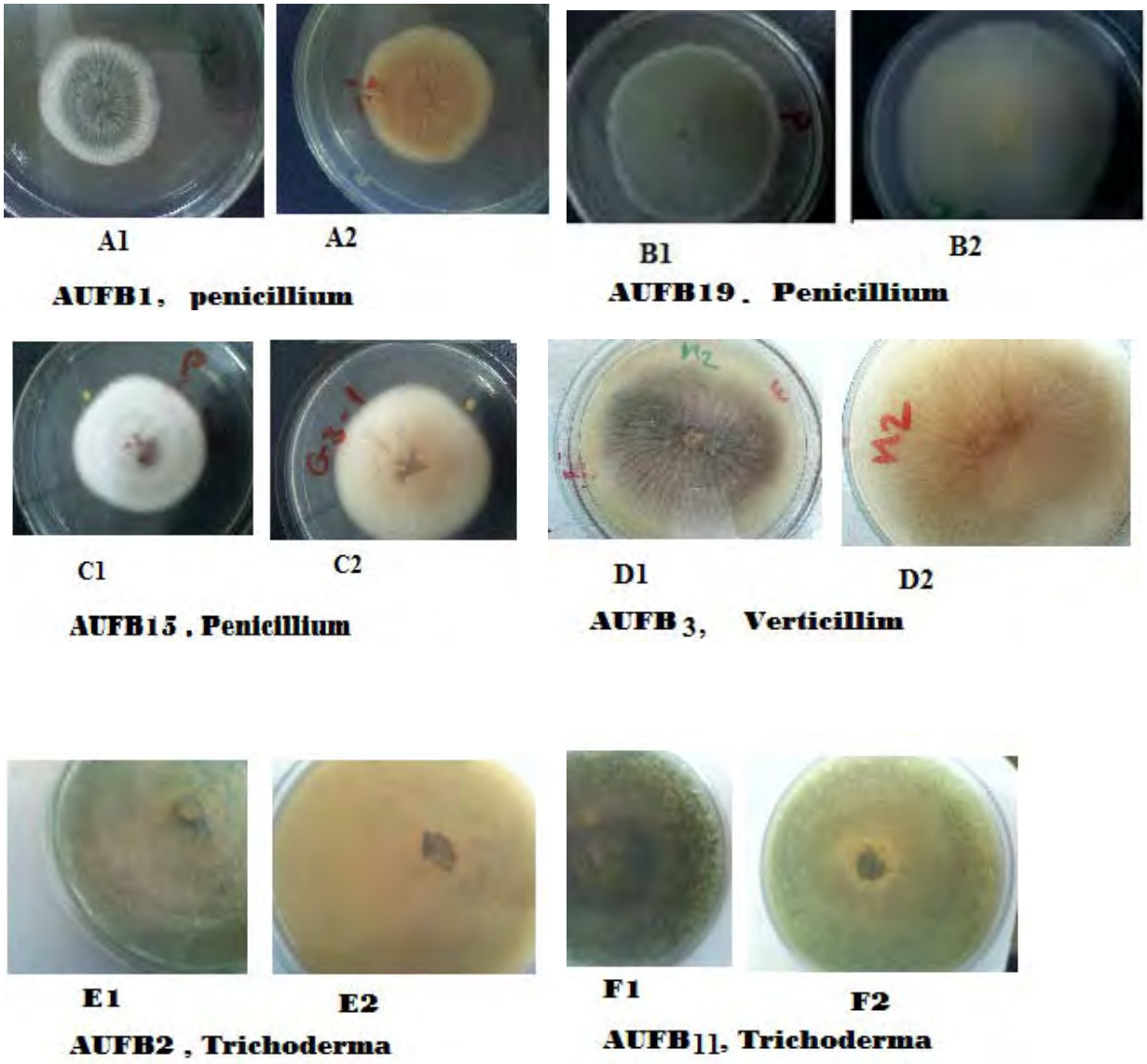
It was observed that the color of AUFB1 colony was green at the center with white at the periphery of front side with brown center and white periphery of backside (Fig. 2). It has septate hyphae, ovate spores and conidial heads composed of continual conidia. The color of isolate AUFB15

colony was white in front side with white brownish backside. It has septate hyphae with round spores held together continual. The colony color of isolate AUFB19 was found to be green in front side with white back side. It has septate hyphae with chain of round conidia on phialides.

Based on the Manual of Watanable, isolate AUFB1, AUFB15 and AUFB19 were identified to the genus *Penicillium*. Likewise, isolate AUFB2 showed green colony in front side with brown backside. In addition, it has septate hyphae with mass of spores on phialides. Isolate AUFB11 was found to be green in front side of colony color with light green in its backside. It has septate hyphae with mass of spores on hyphae. Based on the same manual they were identified to the genus *Trichoderma*. Likewise, isolate AUFB3 was found to be dark brown in front side and light brown in back side of the colony color with elliptical spores borne in clusters that are arranged in whorls. Based on the same Manual it was identified to genus of *Verticillium* (Watanabe, 2002).

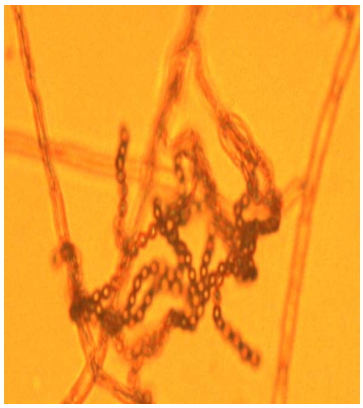
Table 3. Characteristics of Selected Fungal antagonistic isolates

Isolates	Colony Color		Spores arrangement	Mycelia	Identification
	Front side	Reverse side			
AUFB1	Green with white periphery	Brown	Condial heads composed of continual chains of condia	Branched condiophores, septate hyphae	*Penicillium*
AUFB2	Green	Brown	Mass of ovate spores on phialide	Branched condiophores, septate hyphae	*Trichoderma*
AUFB3	Dark brown	Light brown	Elliptical spores born in cluste	Branched condiophore	*Verticillium*
AUFB11	Green	Light green	Cluster of spores on each phialide	Networked condiophore, septate hyphae	*Trichoderma*
AUFB15	White	White brownish	Continual chain of condia	Branched condiophore, septate hyphae	*Penicillium*
AUFB19	Green	White	Chain of continual condia	Condiophore branched at the apex, septate hyphae	*Penicillium*

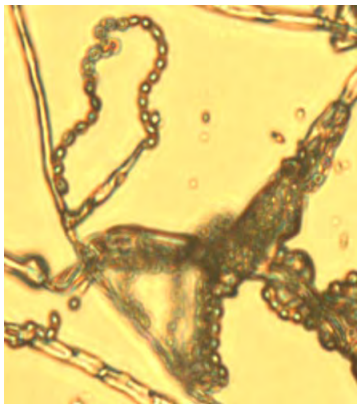


Key: 1=Front side of a colony, 2= back side of a colony

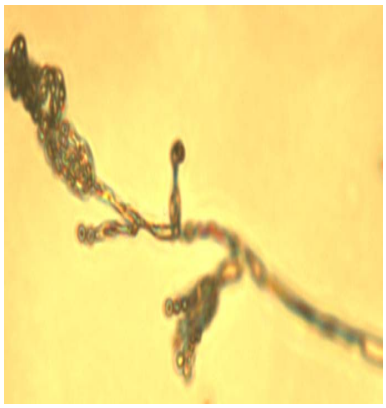
Figure 2. Fungal colony morphology on PDA media.



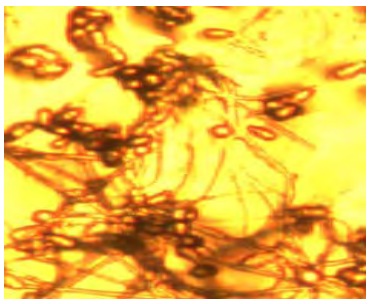
Mycelia and spores of AUFB1



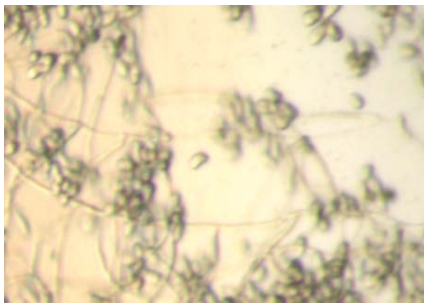
Mycelia and spores of AUFB15



Mycelia and spores of AUFB19



Mycelia and spores of AUFB11



Mycelia and spores of AUFB2

Figure 3. Mycelia and spores morphology of Fungal isolates

6.2.2 Ecophysiological Characteristics of Fungal Isolates

Data in the table 4 shows growth temperature, pH, and salt tolerance of the selected fungal antagonistic isolates against ensete wilt pathogen.

With regard to the temperature of their growth, all of them were found to grow with the range of 10^0C to 30^0C except isolate AUFB19 which showed growth temperature ranging from 10^0C to 37^0C. All isolates showed growth neither at 37^0C nor at 4^0C except AUFB19. It was observed that optimum growth temperature of isolates like AUFB1, AUFB3, AUFB11 and AUFB19 was at 25^0C whereas isolates AUFB2 and AUFB15 showed best growth at temperature 20^0C.

Table 4. Ecophysiological Characteristics of Fungal Isolates

Isolates	Growth range Temperature (^{0}C)	Optimum Temperature (^{0}C)	Growth range pH	Optimum pH	Salt (NaCl) tolerance (%)	Optimum growth salt (NaCl) (%)
AUFB1	10-30	25	4.5-9.5	6.5	1-4	1-2
AUFB2	10-30	20	4.5-8.5	4.5	1-4	1
AUFB3	10-30	25	3.5-8.5	6.5	1-4	1
AUFB11	10-30	25	4.5-8.5	7.5	1-4	1
AUFB15	10-30	20	4.5-8.5	4.5	1-5	1
AUFB19	10-37	25	4.5-8.5	5.5	1-5	1-2

With regard to pH of their growth, all of them were found to grow with the range of 4.5 to 8.5 (Table 4). The isolates like AUFB2, AUFB11, AUFB15 and AUFB19 showed growth pH of 4.5 to 8.5 whereas AUFB1 and AUFB3 were tolerated to grow pH of 4.5 to 9.5 and 3.5 to 8.5, respectively. All isolates showed growth neither at 3.5 nor at 10 except AUFB11 that showed growth at pH 3.5.

It was found that the isolates AUFB1 and AUFB3 showed the optimum growth at pH 6.5. The optimum growth pH of isolates like AUFB2 and AUFB15 was found at 4.5. The isolate AUFB11 had optimum growth pH at 7.5 whereas the isolate AUFB19 showed optimum growth at pH 5.5. In general, it was found that three tested isolates showed optimum growth pH 7.5-6.5 and three isolates pH 4.5-5.5.

The salt tolerance of six fungal antagonistic isolates against *Xanthomonas campesteris* pv. *musacearum* were observed *in virto* test. It was observed that four of them like AUFB1, AUFB2, AUFB3 and AUFB11 were able to grow in concentration of sodium chloride from 1-4% whereas two isolates like AUFB15 and AUFB19 showed growth from 1-5% of NaCl. It was also noticed that four tested isolates like AUFB2, AUFB3, AUFB11 and AUFB15 were able to grow best in

sodium chloride concentration of 1% whereas two isolates like AUFB1 and AUFB19 showed optimum growth with salt concentration from 1-2%

6.2.3 Bacterial Isolates

Table 5. Characterization and identification of bacterial antagonists against ensete wilt pathogen

Isolates	Colony appearance	Colony color	Gram reaction	Catalase	Motility test	Cell shape	Oxidase test	O/F test	Starch Hydrolysis	Indole production	Flouresceince production	Growth 45^0c or 4^0c	Identified Genus
AUbB1	s	W	Gm-	+	+	r	+	O+/F+	-	+	-	-	Pseudomonas
AUbB2	s	W	Gm-	+	+	r	+	O+/F+	-	+	-	-	Pseudomonas
AUbB3	s	W	Gm-	+	+	r	+	O+/F+	-	-	+	-	Pseudomonas

Key: Gm-=Gram negative, Gm+=Gram Positive, += Positive for the test, - =Negative for the test, O+=oxidative for glucose, F+= Fermentative for glucose, w=white, s= smooth, r=rod

The morphological and biochemical characteristic of bacterial isolates is presented in the table 5. The isolates were found to be gram negative and smooth in colony appearance, white in colony color, motile, both oxidative and fermentative, and rod in shape. With regard to Flourescein production only isolate AUbB3 was found to produce it. They were also found to be aerobic, catalase positive and not hydrolyze starch. Moreover, all of them showed neither growth neither at temperature of 4^0C nor 45^0C.

Based on Bergey's Manual of Systematic Bacteriology, the isolates were identified into genus of *Pseudomonas* (Garrity *et al.,* 1921).

6.2.4 Ecophysiological Characteristics of Bacterial Isolates

Data in the table 6 shows growth temperature, pH and salt tolerance of the three bacterial antagonistic isolates against ensete wilt pathogen.

Table 6. Ecophysiological characteristics of bacterial antagonistic isolates

Isolates	Growth range Temperature (^{0}C)	Optimum Temperature (^{0}C)	Growth range pH	Optimum pH	Salt tolerance (%)	Optimum growth salt (1%)
AUbB1	10-37	30	5.5-10	7.5	1-6	1-2
AUbB2	10-30	28	5.5-10	8.5	1-6	1
AUbB3	10-37	30	5.5-10	7.5	1-6	1

With regard to the temperature of their growth, two isolates AUbB1 and AUbB3 were found to grow with the temperature range of 10^0c to 37^0c whereas one isolate AUbB2 showed growth temperature of 10^0C to 37^0C. All isolates showed growth neither at 45^0C nor at 4^0C. It was found that bacterial isolates like AUbB1 and AUbB3 were showed optimum growth temperatures at 30^0C whereas the isolate AUbB2 had optimum growth temperature at 28^0C.

With regard to the pH of their growth, all of them were found to grow with the pH range of 5.5 to 10 (Table 6). They showed growth neither at pH 4.5 nor at 10. It was found that two isolates like AUbB1 and AUFB3 showed optimum growth at pH 7.5 whereas the isolate AUbB1 showed at 8.5.

The salt tolerance of three bacterial antagonistic isolates against *Xanthomonas campesteris* pv. *musacearum* were observed *in virto* test. They were able to grow from 1-6% of sodium chloride concentration. Two isolates like AUbB2 and AUbB3 were able to grow best in sodium chloride concentration of 1% whereas the isolate AUbB1 showed optimum growth with salt concentration from 1-2%.

6.3 Evaluation of Plant Growth promoting (PGP) and antagonistic Properties of Antagonists

With regard to PGP properties, four isolates were found to be active inorganic phosphate solubilizers with solubilization index (SI) of 1.6 and 2.75 (Table 7). Bacterial antagonists AUbB1, AUbB2 and AUbB3 were able to solublize inorganic phosphate with Solubilization index 1.83, 2.75

and 1.88, respectively (Table 7). However, only one fungal isolate AUFB15 was found to solubilize inorganic phosphate with SI 1.60. Among the tested antagonistic isolates AUbB2 showed highest inorganic phosphate solubilizers with solubilization index (SI) 2.75.

Table 7. Plant growth promoting (PGP) and antagonistic properties of antagonistic microorganisms

Isolates	HCN	IAA	Protease	Phosphate Solubilization index (SI)
AUbB1	-	+	+	1.83
AUbB2	-	+	+	2.75
AUbB3	+	-	-	1.80
AUFB2	-	+	-	0
AUFB3	-	+	-	0
AUFB11	+	-	+	0
AUFB15	+	+	+	1.60
AUFB19	-	-	+	0

+= Positive to the test, - = Negative to the test, HCN=Hydrogen Cyanide, IAA= Indole Acetic Acid

As far as the ability of isolates to indole acetic acid is concerned, five of the tested isolates were found to be positive for indole acetic acid (IAA). The isolates AUbB1 and AUbB2 (bacteria) and AUFB2, AUFB3 and AUFB11 (fungi) were capable of producing IAA in the growth medium (Table 7).

Some isolates were found to produce antibacterial substances of protease enzyme and HCN. Five out of among tested antagonistic isolates showed protease activity *in vitro* test (Table 7). It was found that two antagonistic isolates AUbB1, and AUbB2 (bacteria) and three fungal antagonistic isolates AUFB11, AUFB15 and AUFB19 (fungi) were capable of producing protease.

With regard to hydrogen cyanide production by antagonistic isolates, isolate AUbB3 (bacterium) and fungal isolates AUFB11 and AUFB15 were found to produce HCN (Table 7).

6.4 *In Vivo* Testing of antagonistic activities of isolates against Ensete Wilt Pathogen

It was noticed that the inhibiting effect of antagonistic isolates against bacterial wilt of ensete, *Xanthomonas campestris* pv. *musacearum*, in green house experiment with leaf and stem inoculation. Three bacterial antagonistic isolates AUbB1, AUbB2 and AUbB3 and one fungal isolate AUFB 11 were found to inhibit test pathogen. The inhibition effect of treated antagonists against the pathogen was obtained by measuring lesion development on each treated leaves after a month and then calculating disease severity reduction (DSR).

It has been observed that the leaves that were treated with sterilized water (negative controls) showed no lesion development on the other hand the leaves that were treated with only pathogen, *Xanthomonas campesteris* pv.*musacearum*, (positive controls) showed largest lesions developments (9.63cm) (Annex 7). The leaves that were treated with pathogen, *Xanthomonas campesteris* pv.*musaceurum,* and antagonists like AUbB1, AUbB2, AUbB3 and AUFB11 showed lesion development 2.5 cm,4.23 cm, 4.62 cm, and 4.2cm, respectively.

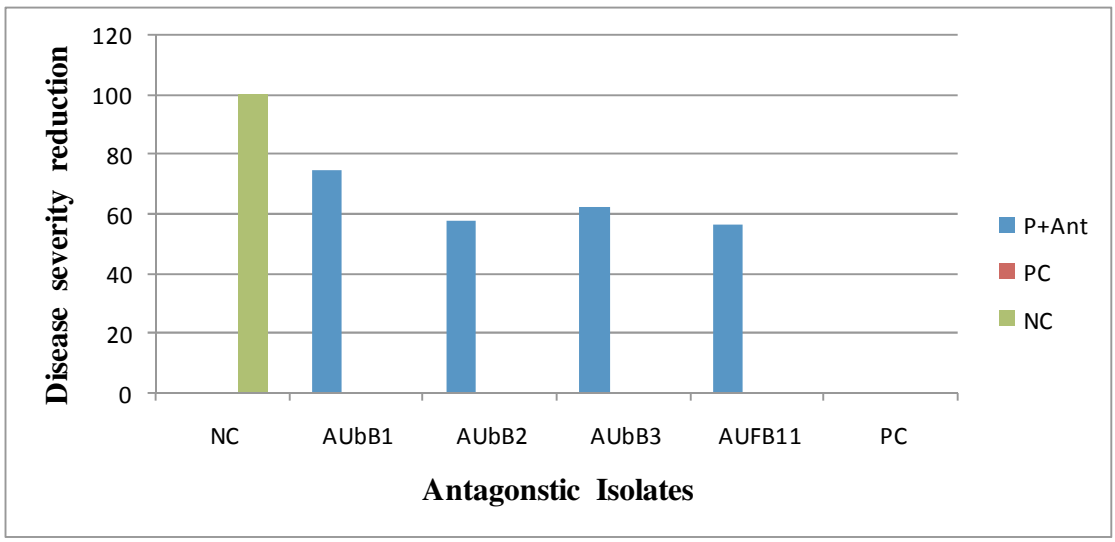
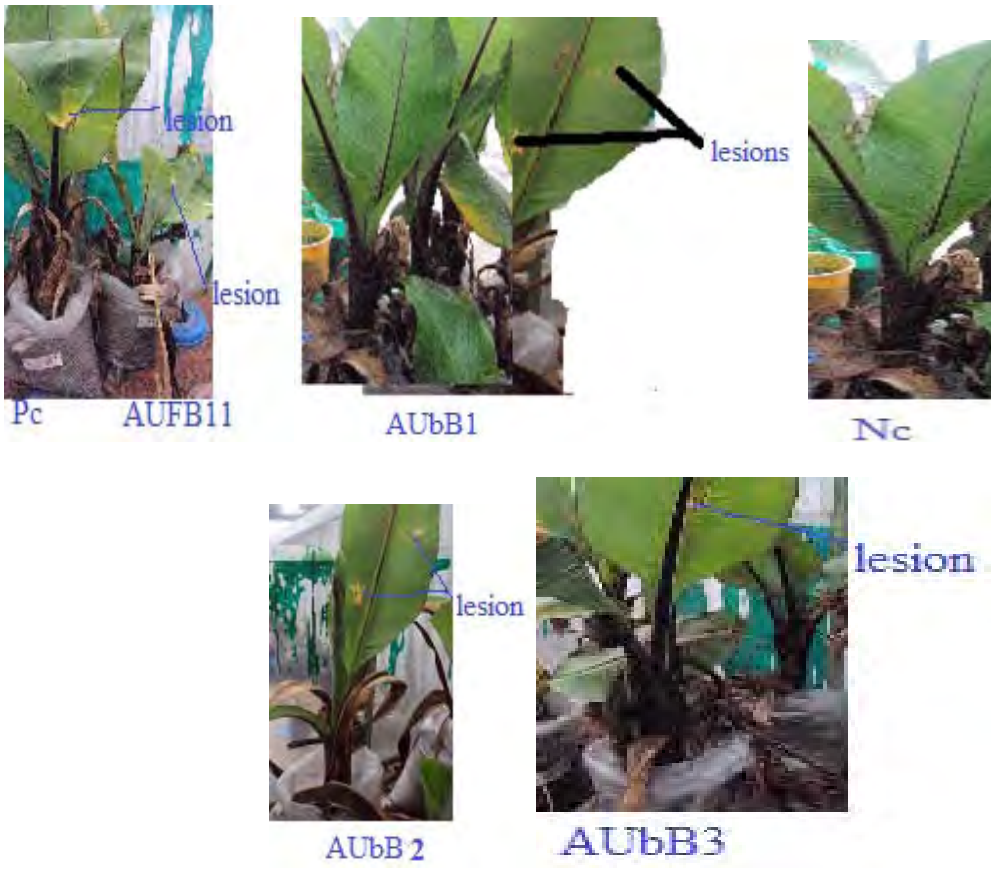


Figure 4. *In vivo* evaluation of bacterial ensete wilt (*Xanthomonas campestris* pv. *musacearum*) severity reduction (%) by inoculating with pathogen and antagonistic isolates (P+Ant).The positive control was inoculated with pathogen (PC) but negative control (NC) with distilled water.

It was determined that the DSR (disease severity reduction) that was obtained by using antagonists like AUbB1, AUbB3, AUbB2 and AUFB11 was 74.8, 62.4, and 57.4, respectively. It was noticed that the isolate AUbB1 showed the greatest disease severity reduction 74.8 while on the contrary isolate AUFB11 showed least DSR 56.4 %.

Bacterial antagonistic isolates AUbB1, AUbB3, AUbB2 showed disease severity reduction against the pathogen 74.8 %, 62.4 %, 57.4 %, respectively whereas fungal antagonistic isolate AUFB11 showed 56.4%.



Key: Pc= positive control, Nc= negative control, AUFB= fungal antagonist treated with the pathogen,AUbB= bacterial antagonists treated with the pathgen

Figure 5. Antagonisic effect of biocontrol isolates against *Xanthononas campesteris* pv.*musacearum* , under green house condition.

7 DISCUSSION

In this study, three bacterial and seventeen fungal antagonistic isolates against ensete wilt pathogen were screened and evaluated *in vitro* test. The antagonism property of the isolates was determined by measuring their growth inhibition zones around them. The relative effectiveness of the bacterial and fungal antagonistic isolates was found to vary according to their inhibition diameters.

It was noticed that the bacterial antagonistic isolates showed highest inhibition diameters (32mm to 40mm) whereas fungal isolates exhibited less inhibition diameters (5 mm to 21 mm). The bacterial antagonistic isolates were found to be more effective antagonists and showed promising antagonistic effects against test pathogen *in vitro* test. This is may be due to bacterial isolates could release more effective and diffusible metabolites that inhibit the growth of test pathogen. Accordingly, bacterial isolates inhibited growth of test pathogen of ensete wilt may be due to their effective growth inhibiting mechanism(s). Thus, these bacterial isolates have potential to be biological control agents against ensete wilt pathogen.

However, it was found that fungal isolates were found more diverse in their effectiveness to inhibit test pathogen than bacterial antagonistic isolates. The fungal isolates with inhibition diameters 5-10mm were determined as lowest inhibiting group, with inhibition diameters 11-15 mm as middle inhibiting group and with inhibition diameters 16-21 mm as highest inhibiting group. The largest inhibition diameter among the fungal isolates was found 21mm which is less than the least inhibition diameter (32mm) among the bacterial isolates. The fungal isolates were considered as less effective antagonistic isolates. This is may be due to faster growth of test pathogen over the fungal isolates and production of less effective and diffusible antagonistic metabolites.

It has been reported that some antagonists against *Xanthomonas campestris* strains were screened *in vitro* test. According to Visser *et al*., (1986) lactic acid bacteria showed growth inhibition against *Xanthomonas campestris* strains with inhibition diameters of 28mm to 33.9mm *in vitro* test. According to Massomo *et al*., (2004) *Bacillus* species exhibited antagonism property against *Xanthomonas campestris* pv. c*ampestris* in the agar plate test.

The result obtained from morphology and cellular characteristics of fungal antagonistic isolates supported identification of the isolates to their genus level. It was found that three fungal antagonistic isolates (AUFB1, AUFB15 and AUFB19) were identified to genus *Penicillium*, two isolates (AUBF2 and AUFB11) to *Trichoderma* and one isolate (AUFB3) to genus *Verticillium* (Table 3). This shows that these fungal isolates that were found to inhibit the growth of ensete wilt pathogen *in vitro test* were identified to genus *Trichoderma, Penicillium* and *Verticillium*. Accordingly, some isolates of genus *Trichoderma, Penicillium* and *Verticillium* were found to inhibit the growth of ensete wilt pathogen *in vitro* test.

The isolates that were identified to genus *Penicillium* showed growth temperature ranging from 10^0C-30^0C, growth pH from 4.5-8.5, and salt tolerance 1-4 with some exception. The isolates that were identified to genus *Trichoderma* displayed no variation in growth temperature (10-30^0C), in growth pH (4.5-8.5) and in salt tolerance 1-4 except optima of temperature and pH. Likewise, the isolate that were identified to genus *Verticillium* showed growth temperature ranging from 10^0C - 30^0C, growth pH 3.5-8.5 and salt tolerance 1-4.

Based on the result obtained from biochemical test and morphological characteristics of bacterial isolates (Table 5) as well as Bergey's Manual of Systemic Bacteriology (1984), the bacterial isolates were identified to genus *Pseudomonas*. This shows that some isolates of genus *Pseudomonas* were found to inhibit the growth of test pathogen (ensete wilt pathogen) *in vitro* test.

These bacterial isolates showed similar growth temperature (10^0C -37^0C), growth pH (5.5-10) and salt tolerance (1-6) except their optima. The host plant of test pathogen has a mean temperature for its cultivation ranging from 10^0C-21^0C (Brandit *et al*., 1997). All tested antagonistic isolates were capable to grow within this temperature range *in vitro* test (10-21^0C). Thus, the isolates may tolerate and capable to grow if they may be applied in the ensete growing field.

In addition to antagonistic properties of the isolates, they were found to show plant growth promoting properties. The result obtained showed that three bacterial and one fungal antagonistic isolates solublized inorganic phosphate *in vitro* test (Table 7). Bacterial isolate AUbB2 showed highest phosphate solubilization with solubilization index 2. 75 compared with the other tested antagonistic isolates. This shows that the isolates were found to solublize insoluble phosphate compound and convert it to in the form of available to plant. Thus, they were found to convert unavailable phosphate compound to available for plant use and enhance their growth and yield.

It has been reported that biocontrol microorganisms produce organic acids which are necessary to scavenge phosphates (Pi) from medium containing bound phosphorus (Kapri and Tewari, 2010). It has also been reported that the important genera *Bacillus* and *Pseudomonas* (bacteria) (Illmer and Schinner, 1992) and *Aspergillus* and *Penicillium* (fungus) solublized inorganic phosphate *in vitro* test (Seshadri *et al*., 2004; Wakelin *et al*., 2004). In addition, phosphate solubilizing biocontrols could serve as efficient biofertilizers that improve soil fertility, phosphate uptake by plants, growth and yield of crop plants (Algawadi and Gaur, 1988; Chen *et al*., 2006).

It has been observed that antagonistic isolates produced indole acetic acid (IAA) *in vitro* test. Among tested isolates two bacterial and three fungal isolates were capable to produce indole acetic acid (Table 7). IAA is one of the most physiologically active phythohormones found to be wide spread among root associated bacteria (Patten and Glick, 2002). It has been reported that

Pseudomonas spp. (Xie *et al.*, (1996) and fungal species like *Aspergillus niger*, *Penicillium citrinum* and *Trichoderma harzianum* produce IAA *in vitro* test (Yadav *et al*., 2010).

The result obtained showed that one bacterial (AUbB3) and two fungal isolates (AUFB11 and AUFB1) were capable to produce antimicrobial hydrogen cyanide volatile compound (Table 7). The antibacterial activity of these isolates may be due to production of HCN with other metabolites. It has been reported that some *fluorescent pseudomonads* produced volatile antimicrobial HCN, which helps to suppress black root rot (Voisard *et al.,* 1989) and wheat foliar pathogens (*Septoria tritici* and *Puccinia recondita* f.sp. *tritici*) (Flaishman *et al*., 1996). It has also been reported that HCN is volatile pathogen inhibitory compound which produced by biological controls (Haas *et al*., 2002).

It has been observed that two bacterial (AUbB1 and AUbB2) and three fungal isolates (AUFB11, AUFB15 and AUFB19) were found to produce protease enzyme (Table 7). The growth of the test pathogen inhibited around these antagonistic isolates may be due to protease enzyme that produced by the isolates. Thus, the antibacterial activity of these isolates may be due to production of protease with other metabolites. It has been reported that *Trichoderma* spp (Haggag and Abo-sedera, 2005) and *Pseudomonas fluorescens (*Anderson et *al*., 2004) produce protease enzyme that has great importance in the degradation of bacterial cells (Kredics *et al*., 2003).

Disease management options currently available for ensete production do not provide adequate control of ensete wilt pathogen and there is a need for new disease management strategy. In this study, it was observed the effect of three bacterial and six fungal antagonistic isolates against ensete wilt pathogen under green house experiment with leaf and stem inoculation. The ensete plants that were treated with both antagonists and pathogen sowed less lesion development as compared to positive control. It was noticed that the disease severity reduction ranging from 56.4 to 74.8%. This shows that the antagonistic isolates were capable to inhibit the growth of pathogen on ensete plant

and restricted the host damage by the pathogen. This is may be due to antagonistic isolates may produce antibacterial substances; enhance systemic resistance and their competence over pathogen. This shows that ensete wilt diseases severity was reduced by the effect of antagonistic isolates toward the pathogen. Accordingly, ensete yield loss by its wilt pathogen reduced by antagonistic isolates under green house condition.

The bacterial antagonistic isolates, that were identified to genus *Pseudomonas*, displayed disease severity reduction ranging from 57.4 to 74.8%.It has been reported that the disease severity reduction of bacterial speck of tomato *by Bacillus pumilus* and *Pseudomonas fluorescens* was 76.3 and 52.8% (Ji *et al.*, 2006), respectively. It has been also reported that the reduction of disease of *Xanthomonas campestris* strains incidence was obtained by using *Pseudomonas putida* and *Pseudomonas syringae* and *Bacillus subtilis* (Wulff *et al.*, 2002). *Bacillus subtilis* also controlled the disease caused by *Xanthomonas campestris pv. campest*ris in Brassicas crops like cabbage, rape, cauliflower and broccoli during dry and short rain seasons. *B. subtilis* and *B. megaterium* pv. *cerealis also* showed antagonistic effect against crucifers black rot (*Xanthomonas campestris Pv. campest*ris) *in vitro* test (Monteiro *et al.,*2005).

It was determined that the disease severity reduction of ensete wilt pathogen, *Xanthomonas campesteris* pv.*musacearum,* by fungal isolate AUFB11, which was identified to genus *Trichoderma*, was 56.4. Assis *et al*., (1999) reported that yeast antagonists showed disease, which caused by *Xanthomonas campestris* pv.*campesteris,* severity reduction ranged from 24.2 to 78.6% on cabbage phylloplane under greenhouse condition.

Accordingly, it was found that the isolates (AUbB1, AUbB2, AUbB3 and AUFB11) showed significance disease severity reduction under greenhouse conditions as compared to positive control (Annex 8).Thus, controlling of ensete wilt pathogen with bacterial antagonists (AUbB1,

AUbB2 and AUbB2) under green house condition was found to be promising. According to Byrnea *et al.,* (2005) controlling bacterial spot of tomato, which caused by *Xanthomonas campestris* pv. *vesicatoria*, with *P. syringae* Cit7 and *P. putida* in field was promising.

8 CONCLUSION AND RECOMMENDATION

8.1 Conclusion

The production and yield of ensete, which is drought resistant economic plant in Ethiopia, is mostly affected by ensete wilt pathogen (*Xanthomonas campestris* pv. *musacearum*). The management of the pathogen is very important in order to increase its production and reduce its yield loss. In this study, biological control agents against ensete wilt pathogen were screened, characterized and evaluated *in vitro* and *in vivo* indicated that the ability to reduce the growth and yield losses by the pathogen.

A total of 361 bacterial and 145 fungal colonies were isolated from rhizosphere and phyllosphere of ensete plant, there are only, three bacterial and seventeen fungal antagonistic isolates were potential to control the test pathogen *in vitro* evaluation. Fungal antagonist displayed inhibition diameter 5mm to 20mm whereas bacterial antagonist showed inhibition diameter 32 mm to 40 mm. A few bacterial antagonists evaluated/ tested against ensete wilt pathogen have showed more effective than fungal antagonists.

Three bacterial antagonists and six selected fungal antagonists were tested their Plant Growth promoting, antagonistic and ecophysiological properties had good performance. In addition, three bacterial antagonists (AUbB1, AUbB2, and AUbB3) and one selected fungal antagonist AUFB11 were tested their antagonistic effect against the pathogen under green house condition.

Regarding to plant growth promoting and antagonistic properties of antagonistic isolates, four antagonistic isolates (AUbB1, AUbB2, AUbB3 and AUFB15) were solublized inorganic phosphate, five antagonistic isolates (AUbB1, AUbB2, AUFB2, AUFB3 and AUFB15) produced indole acetic acid and three antagonistic isolates (AUbB3, AUFB11 and AUFB15) produced hydrogen

cynide (HCN). The bacterial isolates like AUbB1 and AUbB2 were found positive for indole acetic acid and phosphate solubilization. The isolate AUFB 15 (fungus) were noticed to positive for protease, HCN and phosphate solubilization whereas the isolate AUFB11 (fungus) positive for protease and HCN. All antagonistic isolates were capable to grow in temperature range 10-30^{0}C, pH range 5.5-8.5 and 1-4% of salt tolerance with some exception.

In pot experiment, four antagonistic isolates (AUbB1, AUbB2, AUbB3 and AUFB11) were tested to evaluate their effect against ensete wilt pathogen. It was observed that the isolates AUbB1, AUbB2, AUbB3 and AUFB11 showed DSR (disease severity reduction) 74.8%, 57.4%, 62.4% and56.4%, respectively.

Generally, the isolates showed significance diseases reduction relative to the positive control. Finally, controlling ensete wilt pathogen (*Xanthomonas campestris* pv. *musacearum*) with bacterial antagonists (AUbB1, AUbB2, and AUbB3) under green house condition was found to be promising.

8.2. Recommendation

Isolation and characterization of endophyte bacterial antagonistic agents against bacterial wilt of ensete (*Xanthomonas campestris* pv. *musacearum*) from different ensete and banana growing regions should be made to find effective isolates that can widely suppress bacterial wilt of ensete and banana.

The effective antagonistic isolates like AUbB1, AUbB2 and AUbB3 against ensete wilt pathogen *in vitro* test and *in vivo* under green house conditions should be checked under field experiment to evaluate and confirm their efficacy, potentiality and survival ability. In addition to these their antibiotic production and efficacy determination will be done further study.

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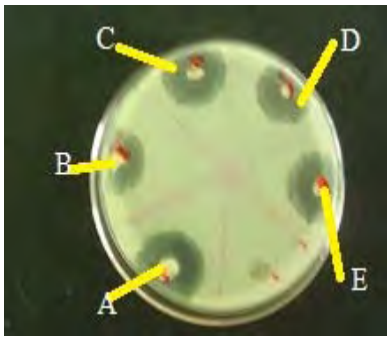
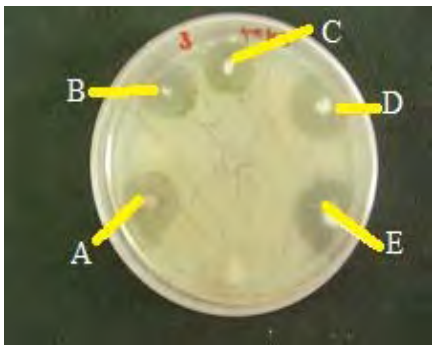
Annexes

Annex 1**.** Measurement of inhibition zone of isolates against ensete wilt pathogen *in vitro* test

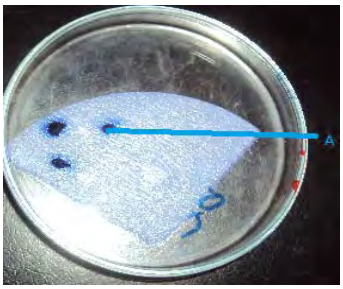




A) KB Medium B) PDA medium

Inhibitory effects of antagonists against pathogen of Bacterial ensete wilt. Inhibition zone was produced by antagonistic isolates: A=AUFB11, B=AUFB2, C=AUFB3, D=AUFB1 and E=AUFB15 on: A) KB medium and B) PDA medium

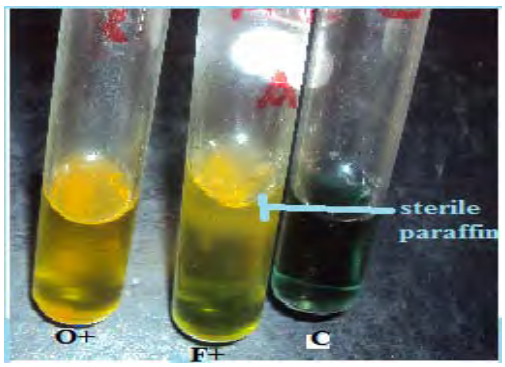
Annex 2. Phosphate solublization and cytochrome Oxidase test



B= positive colony **A= positive colony**

Phosphate solublization test Cytochrome Oxidase test

Annex 3. Oxidative – fermentative and motility test as well as pot experiment



O+=**oxidative postive,** F+=**Fermentative postive , C= control** Pot experiment

Annex 4**.** Phosphate solubilization activities

Isolates	Colony diameter (mm)	Diameter of colony and halo zone(mm)	Phosphate solubilization index
AAUBB	0.5	0.9	1.80
AAUBB1	0.6	1.1	1.83
AAUBB2	0.4	1.1	2.75
AAUGB1	0	0	0
AAUGB2	0	0	0
AAUGB3	0	0	0
AAUHB1	1.5	2.5	1.67
AAUHB2	0	0	0
AAUAB2	0	0	0

Annex 5. Measurement of leaf lesions and disease severity reductions by antagonists

Treatments	Measurements of lesion on leaves (cm)					DSR (%)				
	R1	R2	R3	R4	Mean	R1	R2	R3	R4	Mean
T1. Xc only (P)	10	10.5	9	9	9.63	0	0	0	0	0
T2. Xc +AUbB1	3.5	2.5	2	2	2.5	65	76.2	77.8	77.8	74.8
T3. Xc +AUbB2	4.5	5	3.5	3.5	4.62	55	52.3	61.3	61.1	57.4
T4. Xc +AUbB3	4.5	3.5	3	5.5	4.23	55	66.7	66.7	61.1	62.4
T5. Xc+ AUFB11	4.4	3.6	4.3	4.5	4.2	56	67.7	52.2	50	56.4
T9. Treated with DS (N)	0	0	0	0	0	100	100	100	100	100

DSR= Disease severity reduction, DS=distilled water, P= Positive control, N=negative control, R=replication, Xc= *Xanthomonas campestris* pv. *musacearum*, DSR%= [(DSc - DSt)/DSc)] x 100, where DSc = leaf area with lesions on the control plants that treated with only pathogen and DSt = leaf area with lesions on the treated with antagonist and pathogen.

Annex 6. Statistic analyses (descriptives and multiple Comparisons) of disease severity reduction in vivo antagonistic effects against ensete wilt pathogen

Descriptives

VAR00001

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
pathogen+AUbB1	4	74.2000	6.17954	3.08977	64.3670	84.0330	65.00	77.80
patngen+AUbB2	4	57.4250	4.49694	2.24847	50.2694	64.5806	52.30	61.30
pathogen+AUbB3	4	62.6250	5.85456	2.92728	53.3091	71.9409	55.00	67.70
pathogen+AUFB11	4	56.4750	7.88305	3.94153	43.9313	69.0187	50.00	67.70
negative control	4	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
positive control	4	.0000	.00000	.00000	.0000	.0000	.00	.00
Total	24	58.4542	31.01015	6.32992	45.3597	71.5486	.00	100.00

Multiple Comparisons

Dependent Variable: VAR00001

LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
pathogen+AUbB1	patngen+AUbB2	16.77500*	3.59198	.000	9.2285	24.3215
	pathogen+AUbB3	11.57500*	3.59198	.005	4.0285	19.1215
	pathogen+AUFB11	17.72500*	3.59198	.000	10.1785	25.2715
	negative control	-25.80000*	3.59198	.000	-33.3465	-18.2535
	positive control	74.20000*	3.59198	.000	66.6535	81.7465
patngen+AUbB2	pathogen+AUbB1	-16.77500*	3.59198	.000	-24.3215	-9.2285
	pathogen+AUbB3	-5.20000	3.59198	.165	-12.7465	2.3465
	pathogen+AUFB11	.95000	3.59198	.794	-6.5965	8.4965
	negative control	-42.57500*	3.59198	.000	-50.1215	-35.0285
	positive control	57.42500*	3.59198	.000	49.8785	64.9715
pathogen+AUbB3	pathogen+AUbB1	-11.57500*	3.59198	.005	-19.1215	-4.0285
	patngen+AUbB2	5.20000	3.59198	.165	-2.3465	12.7465
	pathogen+AUFB11	6.15000	3.59198	.104	-1.3965	13.6965
	negative control	-37.37500*	3.59198	.000	-44.9215	-29.8285
	positive control	62.62500*	3.59198	.000	55.0785	70.1715
pathogen+AUFB11	pathogen+AUbB1	-17.72500*	3.59198	.000	-25.2715	-10.1785
	patngen+AUbB2	-.95000	3.59198	.794	-8.4965	6.5965
	pathogen+AUbB3	-6.15000	3.59198	.104	-13.6965	1.3965
	negative control	-43.52500*	3.59198	.000	-51.0715	-35.9785
	positive control	56.47500*	3.59198	.000	48.9285	64.0215
negative control	pathogen+AUbB1	25.80000*	3.59198	.000	18.2535	33.3465
	patngen+AUbB2	42.57500*	3.59198	.000	35.0285	50.1215
	pathogen+AUbB3	37.37500*	3.59198	.000	29.8285	44.9215
	pathogen+AUFB11	43.52500*	3.59198	.000	35.9785	51.0715
	positive control	100.00000*	3.59198	.000	92.4535	107.5465
positive control	pathogen+AUbB1	-74.20000*	3.59198	.000	-81.7465	-66.6535
	patngen+AUbB2	-57.42500*	3.59198	.000	-64.9715	-49.8785
	pathogen+AUbB3	-62.62500*	3.59198	.000	-70.1715	-55.0785
	pathogen+AUFB11	-56.47500*	3.59198	.000	-64.0215	-48.9285
	negative control	-100.00000*	3.59198	.000	-107.5465	-92.4535

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

Declaration

I the undersigned, declare that this thesis is my original work that has not been presented for any degree award or academic qualification anywhere and all materials used for this thesis have been duly acknowledged.

Abayneh Tunasha Banata

Signature ----------

Date----------

This thesis has been submitted for examination with our approval as research advisors;

Fassil Assefa (PhD) Signature----------

Date----------

Tesfaye Alemu (PhD) Signature----------

Date----------