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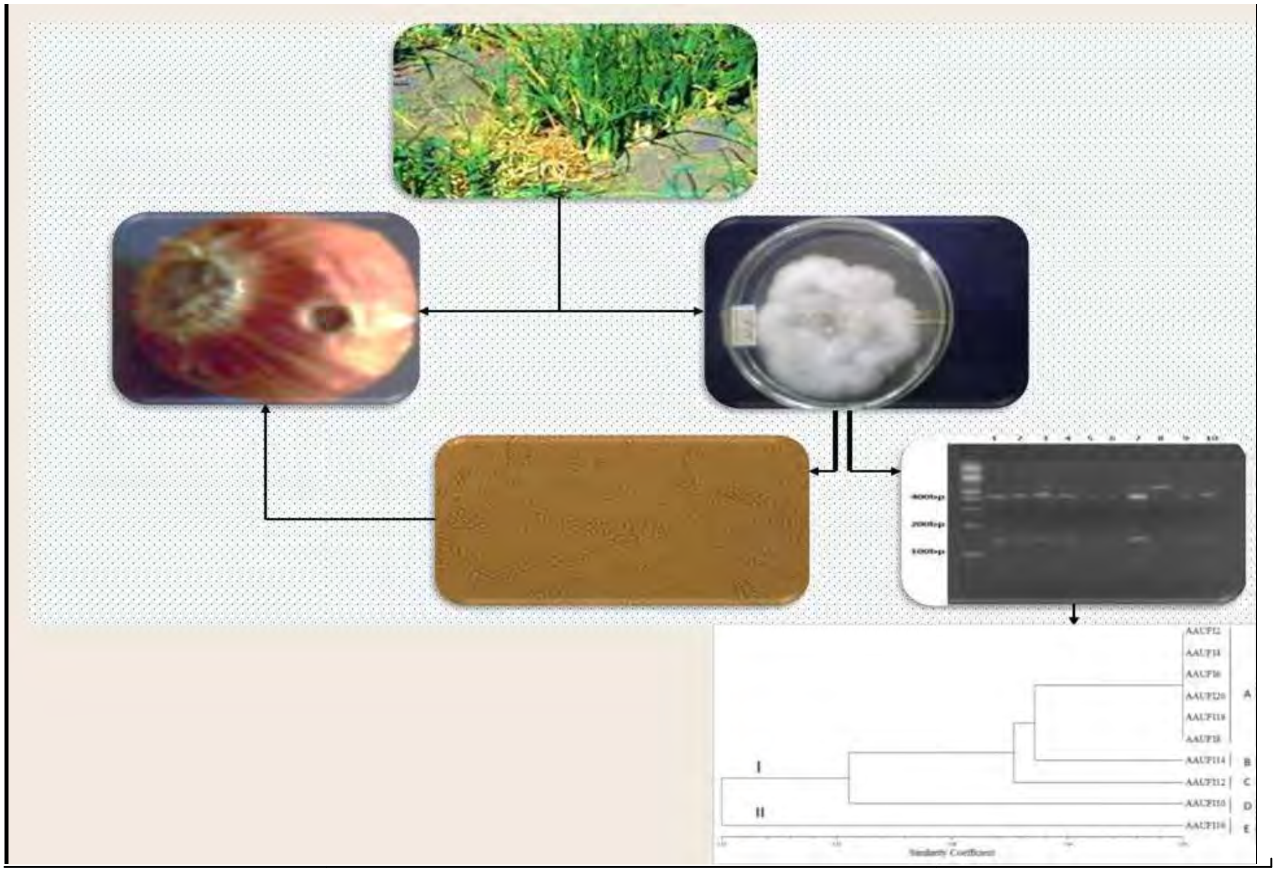
Diversity of Onion Basal Rot (*Fusarium* Isolates) and Their Management Using *Bacillus* Isolates Under Laboratory and Glasshouse Conditions.



By: Abreham Chebte

October 2015

Addis Ababa, Ethiopia.

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A thesis Presented to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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ABSTRACT

Diversity of Onion Basal Rot (*Fusarium* Isolates) and Their Management Using *Bacillus* Isolates Under Laboratory and Glasshouse Conditions.

Abreham Chebte
Addis Ababa University, 2015

In Ethiopia onion has an immense economic, nutritional and medicinal value among all vegetables. The crop is also proved to be income generating for smallholder farmers of east Shewa. However, basal rot disease has put a major hindrance for the productivity of onion in the area. Therefore, This study was conducted to isolate and characterizes Fusarium isolates of onion and to evaluate the antagonism effects of Bacillus isolates to control the disease. For isolation of Fusarium species a total of 43 diseased plant samples were collected randomly from three onion growing districts of east Shewa Zone. Biocontrol agents were isolated from the rhizosphere soil of onion. According to morphological characters, all isolates were identified as Fusarium oxysporum, Fusarium solani and Fusarium proliferatum. Pathogenicity of the isolates were tested under in vitro and glasshouse conditions. Rot lengths measured on cross section of onion bulbs caused by Fusrium isolates ranged from 0.23 cm (AAUFI2 and AAUFI6 each) to 2.1 cm (AAUFI16). The shortest root length (22.6 mm) was produced by isolates AAUFI16 while the shortest shoot length (138 mm) and the smallest dry weight (0.5 g) were produced by isolate AAUFI2. Percentage incidence of the isolates ranged 58.3% to 100%. Bacillus isolates were effective against Fusarium isolates. In vitro mycelial growth inhibition percentage by Bacillus isolate ranged from 53.6% (in AAUFI20) to 72.3% (in AAUFI4). Incidence of basal rot disease was significantly reduced by up to 41.5% (average) after treated with Bacillus isolate while root length, shoot length and dry weight increased significantly compared to the positive control. Percentage of disease control by isolate AAUBI3 ranged 33.3% to 66.67%. Based on the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) restriction fragment length polymorphism (RFLP) analysis, ten Fusarium isolates were divided into two major groups with similarity coefficient ranged from 0.38 to 1.0. Effects of basal rot disease on onion crop can be reduced by using this bioagents with other control methods.

Keywords: *Allium cepa L., Antagonism***,** *Bacillus, Fusarium, Genetic diversity, Pathogenicity.*

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List of Abbreviations/Acronyms

CSA	Central Statistical Agency
CZDA	Czapek Dox's Agar
FAO	Food and Agricultural Organization of the United Nations
FBR	*Fusarium* Basal Rot
HCN	Hydrogen Cyanide
IAA	Indoleacetic Acid
IDM	Integrated Disease Management
IGS	Intergenic Spacer
ISSR	Inter Simple Sequence Repeat Markers
ITS	Internal Transcribed Spacer
LSU	Large Subunit
MEA	Malt Extract Agar
NAM	Nutrient Agar Medium
OBEA	Onion Bulb Extract Agar
PCR-RFLP	Polymerase chain reaction with restriction fragment length polymorphism
PDA	Potato Dextrose Agar
PGPR	Plant Growth Promoting Rhizobacteria
PSM	Phosphate Solubilizing Microorganism
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal Deoxyribonucleic Acid
RFLPs	Restriction Fragment Length Polymorphisms
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolution per minutes
SCAM	Starch Casein Agar Medium
SDW	Sterile Distilled Water
SMC	Simple Matching Coefficient
SSU	Small Subunit
TCP	Tricalcium Phosphate
Tef	Translation elongation factor-alpha
UP-PCR	Universally Primed-Polymerase Chain Reaction

UPGMA	Unweighted Pair Group Method With Arithmetical Mean
WA	Water agar

1. INTRODUCTION

Onion is one of the most important herbaceous, monocotyledonous, biennial, cross pollinated and cool season vegetable crop (Saleem and Ghaffoor, 2003). The crop is originated in Central Asia (Baloch, 1994), and since ancient time it has been used as a condiment all over Latin America, Africa and Asia (Wang *et al*., 2006). Now onion becomes an important vegetable crop worldwide and ranked third in its economic importance among all vegetables next to potato and tomato (Food and Agricultural Organization of the United Nations (FAO), 2011). Consumption of onion has been increasing significantly in the world because of its medicinal and nutritional values (Wang *et al*., 2006). In 2011 production year a total of 4.3 million hectares of land was under onion production worldwide (FAO, 2011). The production was about 85.4 million tones and yielding 19.9 tones/ha (FAO, 2011). The pungency stuff of onion is due to allylpropyldisulphide and alinase. Onions also contain chemical compounds with potential anti-inflammatory, anticholesterol, anticancer and antioxidant properties (Slimestad *et al*., 2007).

In Ethiopia, the crop is one of the most important and greatly produced vegetable mainly as a source of cash income and for flavoring the local stew ‘wot’ (Lemma and Shemelis, 2003). In the country, a total of 22,777.88 ha of lands were under onion production and yielding 2,307,451.89 quintals (Central statistical Agency of Ethiopia (CSA), 2015). It accounts about 19.64% of all vegetable crops production (CSA, 2015). East Shewa zone of Oromia region is one of the major onion producing area in the country where cultivation is mainly carried out using irrigation (Negasi *et al.,* 2013). Onion is proved to be income generating for small holder farmers (Fekadu and Dandena, 2006). Onion production in east Shewa zone was about 181,582.94 quintals in 2014/2015 production year (CSA, 2015).

Despite expansion in production over years, increase in the productivity per unit of area has been very low in the area. Various fungal, bacterial, viral and nematode pathogens attack foliar, bulb and root of this crop. Most important diseases of this crop include pink root (*Pyrenochaeta terrestris*), *Fusarium* basal rot (*Fusarium* species), downy mildew (*Peronospora destructor*), *Botrytis* bulb rot (*Botrytis* species), black mold rot (*Aspergillus* *niger*), iris yellow spot virus, and bacterial soft rots (*Pectobacterium, Dickeya,* and *Burkholderia* spp) (Lowell *et al*., 2012; Mishra *et al*., 2014**).** All over the world, these diseases lead to severe production losses (Schwartz and Mohan,1995). One of these diseases, *Fusarium* basal rot (FBR) also known as *Fusarium* wilt is one of the harmful disease of onion with damage rate of more than 50% (Mishra *et al*., 2014). In the study area incidence of the disease reached 50-60% at the time of harvesting (Tesfaye Alemu (PhD), personal communication, 2013). It causes severe losses in the productivity both in the field and storage (Lager, 2011). Infected seeds and soil are the source of dispersal. The fungus causes infection at the basal stem of the onion bulb and degrades it; finally destroy the whole plant infections in dormant bulbs during storage causing secondary infections to occur (Cramer, 2000).

The ultimate objective of disease diagnosis and identification of responsible pathogens is to design cost effective and environmentally friendly disease management strategies. Different crop management measures have been proposed to control basal rot disease of onion including application of fungicide (Coskuntuna and Ozer, 2008). However, application of fungicides and other pesticides are leading to accumulation of pesticides in the environment and that affects human and animal health, environmental pollution and subsequently, leads to the development of pesticides resistance (Ferencz and Balog, 2010).

As a result, supplementary to chemical control of plant diseases by the use of plant growth promoting rhizobacteria (PGPR) able to antagonize fungi is considered as a more environmentally sound process (Deepa *et al*., 2010). They can antagonize pathogens through competition, auxin and hydrogen cyanide (HCN) production, increased uptake and availability of phosphorus (Muleta *et al*., 2007; Deepa *et al*., 2010), induction of systemic resistance (Bargabus *et al.,* 2003), and production of antibiotics, or secretion of lytic enzymes (Van Loon, 2007) that make them a potent tool for reducing damages through preventing deleterious effects of phytopathogens. The main PGPR are representatives of the genera *Pseudomonas, Bacillus,* and *Streptomyces* (Kloepper *et al*., 2004). *In vitro* and *in vivo* testing showed that the bacterium *Pseudomonas cepacia* protected onion seedlings from damage by *Fusarium oxysporum f. sp. cepae* (Kawamoto and Lorbeer, 1976). Studies in Iran showed that species of *Bacillus* were antagonistic to *Fusarium* on onion in field conditions, and that *Bacillus subtilis* was the most antagonistic of all tested (Tehrani and Riseh, 2004). Besides PGPR, antagonism of *Fusarium oxysporum f. sp. cepae* by *Trichoderma* spp. both in vitro and in green house conditions has been also investigated , and *T. virens* was the most effective (Rajendran and Ranganathan, 1996). Therefore, this work has been initiated to isolate and characterizes *Fusarium* isolates as limiting agents of onion plants collected from east Shewa Zone using morphological and molecular methods and determine the aggressiveness of pathogenic isolates on onion bulbs and seedlings, and to evaluate the antagonistic effects of *Bacillus* isolates to control the disease.

2. OBJECTIVES OF THE STUDY

2.1. General Objective

The overall objective of this study was to isolate and characterizes *Fusarium* isolates (basal rot) of onion collected from east Shewa Zone and to evaluate antagonistic activities of *Bacillus* isolates against them.

2.2. Specific Objectives

- To study the morphological, cultural and molecular characteristics of *Fusarium* isolates of onion
- To conduct pathogenicity test of the isolates on onion bulbs and leaves under laboratory and glasshouse conditions
- To evaluate the antagonistic activity of *Bacillus* isolates against *Fusarium* isolates in laboratory and glasshouse conditions

3. LITERATURE REVIEW

3.1. Economic Importance of Onion

Onion (*Allium cepa L.*) belongs to the genus *Allium* of the family Liliaceae or Amaryllidaceae. In Ethiopia it is an important cash crop (Negasi *et al.,* 2013). It has also quite considerable economic and nutritional importance in East Shewa zone. Almost all spicy dishes contain onion as one of the most important ingredient used for culinary purposes. People consider it as an indispensable part of human diet and are commonly used both by rich and poor. It is grown as spice and used for flavoring local dishes, and contributes to the national economy as export commodity (Fekadu and Dandena, 2006). Onions have medicinal properties because it contains starch, sugar, some protein, and vitamins A, B and C (Baloch, 1994). Onion is known for prevention or treatment of many diseases such as coronary heart diseases (Lanzotti, 2006), cancers (Shutenko *et al*., 1999) and diabetes (Sheela *et al*., 1995). Sulfur containing secondary metabolites alliins (cysteine sulfoxides) cause taste and sharpness, and are criteria for pharmaceutical quality of onion (Bloem *et al*., 2005). Another sulfur containing compound thiosulfinates exhibit antimicrobial properties (Peter, 2003). Compounds such as sugars and organic acids can also contribute to the organoleptic experience. It is an important source of income and employment for the farming community of East Shewa. In the area many people's also make their living by transporting to market this crop (FAO, 2010).

3.2. Onion Production and Production Constraints in East Shewa Zone

Onion can be grown under a wide range of climatic conditions but is more successful under mild season without extremes of heat or cold and excessive rainfall (Saleem and Ghaffoor, 2003). In Ethiopia, the alliums group (onion, shallot, garlic) are the important bulb crops produced by smallholders and commercial growers for both local use and for export. Among these crops, onion is rapidly becoming a popular vegetable among consumers. The wide range of altitudes has given the country a variety of agro-climatic conditions which is suitable for the production of a broad range vegetables and flowers, allows successful production of onion crop (FAO, 2010). An altitude between 700 and 1800 m above sea level is the best for growing of onion crops under Ethiopian condition (Lemma and Shimelis, 2003). In Ethiopia Shallots are the traditional crop but now onions are becoming more widely grown vegetable crops. Ever since the crop is distributed to different parts of the country, and is now become an important vegetable crop for consumption and markets of people of east Shewa zone. A lion's share of 95% of the vegetables and fruits produced in the country comes from the smallholder sector (Negasi *et. al.,* 2013).The area under onion is increasing from time to time mainly due to its high profitability per unit area and ease of production, and the increases in small scale irrigation areas. Increasing onion production also contributes to commercialization of the rural economy and creates many off-farm jobs. In the area it is cultivated both under rainfed in the *meher* season and under irrigation in the off season for its bulb (Figure 1a) and seed production (Figure 1b).

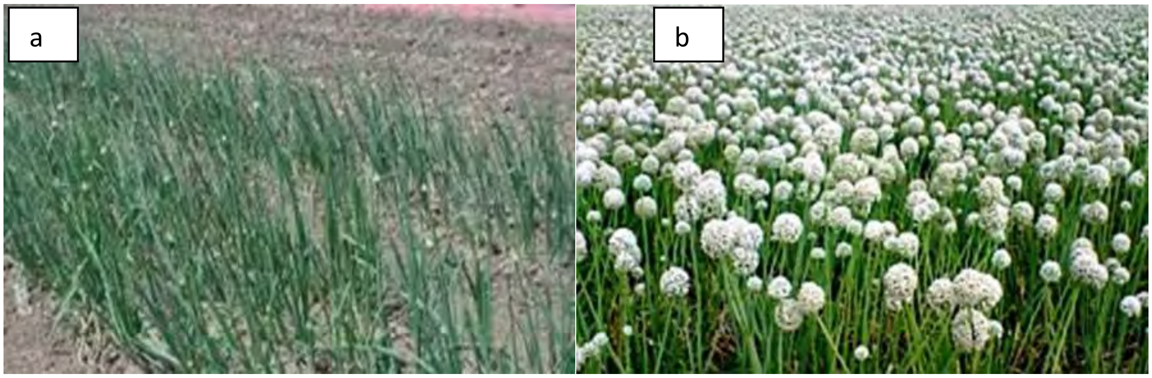

Figure 1. Onion bulbs and seeds production in the off season (using irrigation) by small holder farmers in East Shewa Zone, Oromia region, Ethiopia (Photos by Abreham Chebte).

In the area onion production faces different biotic and abiotic constraints. Among the major problems of onion production in the area low or depleting soil fertility, poor agronomic practices such as imbalanced fertilization, weed damage, insect pests, lack of proper postharvest handling and absence of appropriate crop management practices (Negasi *et al.,* 2013) seriously affect plant growth and onion production in all the study sites. A variety of diseases and disorders affect onion and related crops in the areas both at field and in storage). It is important that onion diseases be recognized early in their development so that effective management strategies can be implemented. Accurate disease diagnosis is an important part of an integrated disease management (IDM) program that assists identification of onion diseases that occur in the field and storage. But there is a shortage of information and disease diagnosis, monitoring and management guidelines in the area as well as in the country (Fekadu and Dandena, 2006). Careful and regular monitoring of the crop can provided this timely information (Ekundayo *et al*., 2011).

3.3. Fungal Diseases of Onion

Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products annually (Schwartz and Mohan, 1995). There are a number of fungal pathogens that attack onion plant throughout its developmental stages and significantly reduce the crop yield (Mishra *et al*., 2014). Neck rot (*Botrytis* spp.), basal rot (*Fusarium* spp.), downy mildew (*Peronospora destructor)*, black mold (*Aspergillus niger),* blue mold rot (*Penicillium* species), damping-off (*Pythium* spp., *Phytophthora* spp.*, Rhizoctonia solani* and *Fusarium* spp.*)*, powdery mildew (*Leveillula taurica (anamorph: Oidiopsis sicula)*, purple blotch (*Alternaria porri*), Botrytis brown stain (*Botrytis cinerea*), Botrytis leaf blight (*Botrytis squamosa)* and Smut (*Urocystis cepulae*) are the major fungal pathogens reported on onion (*Allium cepa*) in the world (Harold, 1932; Lowell *et al*., 2012; Mishra *et al*., 2014).

*Fusarium*s species are the major soil borne fungal pathogens of most vegetable crops including onion, and cause economically significant losses (Schwartz and Mohan, 1995). Among fungal disease of onion caused by *Fusarium* species basal rot is an important disease (Lowell *et al*., 2012). Several soil borne species of *Fusarium* are responsible for this disease. Most often, *Fusarium oxysporum* f. sp. *Cepae* causes the rot of a basal plate (Lowell *et al*., 2012; Mishra *et al*. 2014). *Fusarium solani* in early growth stage and *Fusarium proliferatum*, as mycotoxins producing specie (Fuentes *et al*., 2013) also reported on onion. *Fusarium proliferatum* was reported on onion in Italy (Mannerucci *et al.,* 1987) and in Mexico in garlic (Fuentes *et al*., 2013), in Germany as mycotoxin producing fungus in garlic (Seefelder *et al*., 2002), in Serbia, the presence of *F. proliferatum* on garlic and white onion varieties was observed in 2000 (Stankoia *et al*., 2007) and Dugan *et al*. (2003) reported it as a pathogen of serious impact on garlic and onion in the Pacific North West. In recent time *Fusarium redolens* also reported on onion in Iran (Ghanbarzadeh *et al.,* 2014).

3.3.1. Basal rot disease of onion

3.3.1.1. Biology and taxonomy of the pathogen

Pathogens of onion basal rot disease are belongs to Kingdom: Fungi, Phylum: Ascomycota, Class: Sordariomycetes, Order: Hypocreales, Family: Nectriaceae, Genus: *Fusarium* (Nelson *et al.,* 1981). The major pathogen causing basal rot disease of onion is *Fusarium oxysporum* f. sp. *Cepae* (Lowell *et al.*, 2012). The pathogen produces chlamydospores, macroconidia,and microconidia. Chlamydospores are round, thick walled,and formed abundantly in soil(Brayford, 1996). They are the primary source of inoculum underfield conditions. Macroconidia are short to mediumin length, falcate, thin-walled, slightly tapered at the ends, and usually 3-septate. Microconidia are usually non-septate, oval to reniform in shape, and abundant in culture (Burgess *et al.*, 1994).

3.3.1.2. Disease symptoms

The visual symptoms of FBR of onion caused by *F. oxysporum f. sp. Cepae* can be observed on plant leaves, roots, basal stem plate, and bulb scales of small seedlings, mature plants, and dormant bulbs (Figure 2) (Cramer, 2000; Lowell *et al.*, 2012).The first signs of the disease appear on the leaf tips, which turn yellow and begin die back as the plant nears maturity (Brayford, 1996). In addition, it can cause delayed seedling emergence (Davis and Reddy, 1932), seedling damping off (Lowell *et al.*, 2012), and stunted growth of seedlings (Entwistle, 1990). Below ground roots rot are replaced by a mass of white moldy growth. A noticeable symptom of FBR is the separation of roots from the bulb at the stem plate during uprooting. Within the basal plate, *F. o.* f. sp. *cepae* causes a brown discoloration of the basal plate tissue (Cramer, 2000). Bulbs that appear to be free of symptoms at harvest but are infected can decay in storage (Brayford, 1996).


Foliar symptoms showing withering and necrosis of leaves.

Foliar symptoms and extensive root loss on infected seedlings.

White mycelial growth on the basal plate.

Bulb and basal plate rot.

Basal plate rot.

Figure 2. *Fusarium* basal rot affects the roots and bulbs of onions. Source Lowell *et al.* (2012)

3.3.1.3. Epidemiology of basal rot (*Fusarium* species) of onion

This disease can be found wherever onions have been grown long enough to build up sufficient inoculum to cause disease (Mishra *et al*., 2014). Infected debris, infected soil, irrigation water, farm equipment, onions seed and onion transplants can be a major source of inoculums (Everts *et al*., 1985). The pathogen can also persist in the soil for several years due to its production of chlamydospores that are long-term survival structures (Brayford, 1996). Other *Allium* species such as chives, garlic and shallot also affected (Havey, 1995). *F. o.* f. sp. *cepae* also affects different field and other vegetable crops such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), soybean (*Glycine max* Merr.), pea (*Pisum sativum* L.), cucumber (*Cucumis sativus* L.), squash (*Cucurbita pepo* L.) and Tomato (*Lycopersicon esculentum* Mill) (Tsutsui, 1991).

They invade the onion through wounds or roots scars at the base of the bulb (Shalaby and Struckmeyer, 1966). The fungus entry can be through releasing of pectic enzymes to break down pectin in the cell wall of the onion (Holz and Knox Davies, 1985). In addition root maggot feeding injuries may serve as a major entry sites for the fungus (Everts *et al*., 1985).

Infection occurs at soil temperatures of 15°C to 32°C; with an optimum at 28°C - 32°C, because infection and disease development is favored by high soil temperature (Mishra *et al*., 2014). In storage, decay progresses rapidly at higher temperatures. Basal rot usually appears in mid to late summer as the crop approaches maturity. The optimum pH for growth is 6.6 but growth can occur at a pH range of 2.2 to 8.4 (Everts *et al*., 1985).

3.4. Detection of Fungal Plant Pathogens

3.4.1. Conventional Methods

All disease management programs need a simple, safe, accurate, rapid and cost effective method of pathogen detection. Visual identification of plant disease is the most rapid and cost effective conventional method of disease diagnosis, but it is difficult for inexperienced person (Bock *et. al*., 2010). Other conventional method of pathogen identification is based on observed morphological characteristics by microscopic examinations of the diseased tissue. Shape and size of macro and microconidia, presence and absence of chlamydospores, and appearances, pigmentations and growth rates on agar media (Leslie and Summerell, 2006). This method also require highly specialized taxonomists, which are now rarely available. Various problems associated with microscopically detection of plant pathogens can be overcome by protein and/ or Nucleic acid based detections (Yogendra *et al*., 2013).

3.4.2. Molecular Identification Methods

Molecular biology has offered a number of insights to the detection and enumeration of fungal pathogens and information on identifying unknown species from their DNA sequences (Paminondas and Paplomatas, 2004). In recent years, there has been vast progress in the development of molecular biological tools and technologies for diagnosis of plant diseases (Kumar, 2014). Each technique provide important information on genetic relationships, taxonomy, population structure and epidemiology associated with fungi (Cooley, 1991).

Study of genetic diversity in plant pathogens have been made feasible, beyond use of differential hosts, through diverse molecular techniques (O'Donell, 1992). Different molecular markers have been used in characterization of the genetic diversity of fungal plant pathogens. Random amplified polymorphic DNA (RAPD), universally primed-polymerase chain reaction (UP-PCR), inter simple sequence repeat (ISSR) markers, simple sequence repeat (SSR) marker, and PCR- restriction fragment length polymorphisms (RFLP) of rDNA-IGS and rDNA-ITS regions, large submit RNA gene and translation elongation factor-alpha (tef) (Zhang *et al.,* 2006) are the most common molecular techniques for genetic diversity study of different fungal plant pathogens. The amplification of DNA sequences through the polymerase chain reaction (PCR) has found the most widespread application in the diagnosis and detection of fungi (Louis *et al.,* 2000). Unlike fungal culture approaches, PCR does not require the presence of viable organisms for implementation and may be performed with very small quantities of biological material (Zhang *et al.,* 2006).

Ribosomal DNA possess characteristics that are suitable for the detection of pathogens at the species level (O'Donell, 1992). These rDNA sequences are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992). The rDNA sequences also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats (Bruns *et al*., 1991). Each repeat consists of 18S small subunit (SSU), 5.8S, and 28S large subunit (LSU) genes (Figure 3). A rRNA gene also includes variable regions such as the internal transcribed spacers (ITS) that are formed between the subunits. It used to identify closely related species of a fungal genus (Hennequin *et al*., 1999). ITS region is probably the most widely sequenced region of DNA in fungi. rDNA-ITS region show a higher degree of diversity than other ribosomal regions such as small subunits (SSU) and large subunits (LSU) (Brasileiro *et al*., 2004). The more conserved 18S and 28S regions tend to combine the more closely related species into the same taxonomic group, but ITS regions tend to split groups into distinct species (O'Donell and Gray, 1995).

The rDNA has been utilized for species determination in a wide variety of yeasts and fungi (Zhang *et al.*, 1998). The most successful methods of species determination have employed PCR amplification of target sequences. These methods rely on the conserved nature of rDNA such that isolates from the same species maintain the same sequence whereas the more phylogenetically diverse the species is, the greater is the difference in the sequences of rDNA (Farr *et al.*, 2002). PCR–RFLP of ITS+5.8S have been used by Suga *et al.* (2000) in distinguishing formae speciales (f. spp.) of *F. solani* and Lee *et al.* (2000) for comparing genetic relationships between 12 *Fusarium* species from different areas.

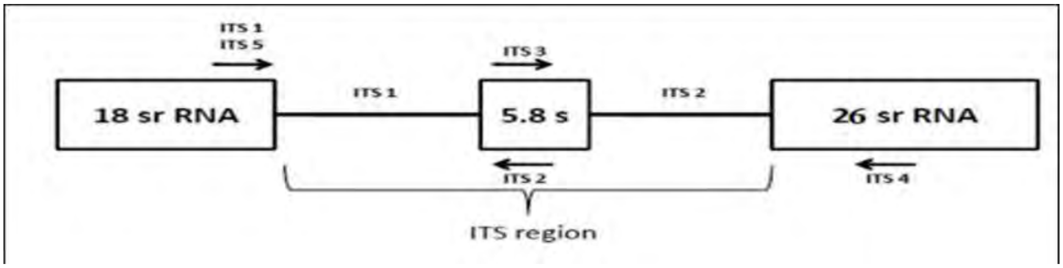

Figure 3. Schematic diagram of internal transcribed spacer (ITS) region. Source Weider *et al*. (2005)

Genes encoding 18S, 5.8S and 28S ribosomal RNA subunits are separated by the internal transcribed sequences 1 (ITS1) and 2 (ITS2) that are spliced after transcription. The primers ITS1 and ITS4 have been used to amplify this rDNA clusters from all fungi species so far, producing a species-specific fragment.

Variation among individual rDNA repeats can sometimes be observed within the ITS region because of their high degree of variation, compared to the 18S and 28S rRNA coding regions of rDNA (Berthier *et al.*, 1996). Identical ITS sequences do not automatically mean that the fungi are conspecific (belonging to the same species) but that they are closely related. Differences in the nucleotide sequence composition of the variable ITS regions have been successfully employed to design specific primer sets that amplify DNA selectively among and within species of plant pathogens (Moricca *et al.*, 1998). This region was sequenced with the M13 forward and reverse sequencing primers to develop a genus-specific PCR assay for the rapid identification of *Fusarium* (Abd-Elsalam *et al.*, 2003).

3.5. Management of Basal Rot Disease of Onion

Management practices generally employed for onion basal rot disease control include cultural practices, resistant cultivars, chemical applications and biocontrol method. However, incorporation of integrated management provides a better opportunity to manage plant diseases (Chandel and Deepika, 2010).

3.5.1. Cultural practices

Soil inoculums levels and onion bulb loss by FBR will reduce in the following year through crop rotation with a crop like maize or wheat (Higashida *et al.,* 1982). Four years crop rotation with a non susceptible host is recommended before planting another onion crop in that field (Havey, 1995). The incidence of *Fusarium* spp. caused basal rot disease can also decrease with field solarization (Katan *et al.*, 1980). The process involves covering moist soil with polyethylene sheets for several weeks during the hottest time of the year. The effects of solarisation may last for more than one season, and subsequent crops will benefit from the reduced pathogen population (Katan *et al.,* 1980). Natural reinfestation by *Fusarium* species was slower in solarised soil than in soils treated with other methods, such as fumigation (Katan *et al.,* 1980). Avoid aggravating plant stresses such as soil compaction, root pruning during cultivation, pink root infection, weeds infestation, moisture extremes and use of nitrate fertilizers reduced disease severity (Entwistle, 1990).

3.5.2. Resistant varieties

Breeding for resistance to *Fusarium* species offers another means to minimize yield loss due to this pathogen. In Ethiopia, onion cultivars having a resistance to *Fusarium* spp. have not been screened so far (Abreham Chebte, personal communication, 2014). In order to develop lines that are resistant to FBR, numerous screening methods have been developed that involve field or greenhouse screening of seedlings, mature plants, or dormant bulbs (Holz and Knox-Davies, 1974;Tsutsui, 1991). Mature plants show the greatest resistance to FBR followed by seedlings and dormant bulbs (Holz and Knox-Davies, 1974).

Some of this resistance may be attributed to plant vigor and the ability of the plant to withstand infection. Plant vigor would be the greatest for mature plants. The resistance has been since incorporated into intermediate and long day commercial hybrids. However, FBR resistance is currently lacking in short-day onion cultivars (Goldman, 1996). In a study involving *F. o*. f. sp. *cepae* inoculation of onion transplants of different ages, older transplants showed no signs of disease symptoms whereas the youngest transplants exhibited disease symptoms even though the fungus was isolated from inoculated transplants at each growth stage (Stadnik and Dhingra, 1997).

Onions that are screened for *F. o*. f. sp. *cepae* resistance should be screened both during field growth and during the post harvest period. The seeds or bulbs are inoculated with *F. o*. f. sp. *cepae* that was grown in culture, and then the percent of germinated seeds or the percent of infected bulbs are recorded (Somkuwar *et al*., 1996). A positive correlation of results has been shown between bulbs screened in the greenhouse and bulbs screened in the field (Krueger *et al*., 1989). In some cases, the inoculated onions are grown until leaf drop, and plants are screened for infection at harvest (Retig *et al*., 1970). The harvested bulbs are then screened for up to forty days following the date of harvest (Retig *et al*., 1970). Screening methods and selection have been shown to improve the keeping time and slow the infection by *F. o. f. sp. cepae* in long day onion varieties (Retig *et al*., 1970). The resistance has been since incorporated into intermediate and long day commercial hybrids. However, FBR resistance is currently lacking in short-day onion cultivars (Goldman, 1996).

3.5.3. Chemical control

Numerous chemical methods exist for the control of FBR. Soil fumigation with methyl bromide ormetam sodium has proven effective for control of FBR (Jaworski *et al*., 1978). The cost of soil fumigation may be excessive for some growers but fumigation can provide other benefits like pink root and weed control. Sets and transplants can be treated with a fungicide such as benomyl and losses to FBR can be reduced (Koycu and Ozer, 1997). In addition, seeds have been treated with benomyl, carbeddazim carboxin hydroxyquinoline and iprodione (Abd-El-Razik *et al*., 1990; Barnoczkine-Stoilova, 1988), with reduction in FBR infection. Four fungicides i.e. Antracol, Carbendazim, Copper oxychloride and Kingmil MZ with 10, 100, 1000 and 10000 ppm concentrations were evaluated against *F. oxysporum* under *invitro* as well as *in-vivo* condition, and Carbendazim, followed by Antracol appeared as the most effective fungicides (Behrani *et* *al*., 2015). Moreover, applications of benomyl+thiram (1.50 and 0.45 g ai/kg seed) inhibited the growth of *F. oxysporum*, and reduced the post-emergence damping-off of onion seedlings. Prochloraz (1.35 cc ai/kg seed) was the most effective seed treatment for controlling *F. oxysporum* induced damping-off in infested soil (Ozer and Koycu, 1998).

3.5.4. Biological control

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide (Handelsman and stabb, 1996). As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection (Helene *et al.,* 2011). However, the high effectiveness and ease of utilization of these chemicals can result in development of pathogen resistance to the applied agents, environmental contamination and the presence of pesticide residues on food (Ferencz and Balog, 2010). Consequently, there is an increasing demand from consumers and officials to reduce the use of chemical pesticides. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Handelsman and stabb, 1996).

Biological control is a term used to describe the " reduction of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man" (Baker and Cook, 1974). Soil is an excellent niche for growth of many microorganisms: protozoa, fungi, viruses, and bacteria. Some microorganisms are able to colonize soil surrounding plant roots, the rhizosphere, making them come under the influence of plant roots (Kennedy, 2005). Plant associated bacteria have a potential in stimulating plant growth and managing soil and plant health (Verma *et al.,* 2010). Plant growth promoting bacteria (PGPB) are associated with many plant species and commonly present in many environments (Kloepper *et al*., 1989). The most widely studied group of PGPB are plant growth promoting rhizobacteria (PGPR) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere (Bakker *et al*., 2007).

PGPR are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Kennedy, 2005). Many of these PGPR are able to transcend the endodermis barrier, crossing from the root cortex to the vascular system, and subsequently thrive as endophytes in stem, leaves, tubers and other organs (Kloepper *et al*., 1989). The extent of endophytic colonization of host plant organs and tissues reflects the ability of bacteria to selectively adapt to these specific ecological niches. Consequently, intimate associations between bacteria and host plants can be formed without harming the plant(Verma *et al.,* 2010).

They can enhance plant growth through direct or indirect mechanism (Arzanesh *et al*., 2011). PGPR stimulate plant growth directly either by synthesizing phytohormones or by promoting nutrition processes such as nitrogen fixation, phosphate solubilization, by accelerating the mineralization processes and siderophore production, which facilitate iron uptake from soil (Muleta *et al*., 2007; Verma *et al.,* 2010). In addition to this the widely recognized mechanisms of biocontrol mediated by PGPR are competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens and/or abiotic stresses(Arzanesh *et al*., 2011).Their application as crop inoculants for biofertilization would also be an attractive option to reduce the use of chemical fertilizers (Vessey, 2003). Among PGPRs *Pseudomonas* and *Bacillus* species constitute, together with *Streptomyces* species, the most bacteria often found in the rhizosphere of many crop plants and behave as PGPRs (Kennedy, 2005).

Under in vitro conditions, fungal antagonists, *Trichoderma viride*, *T. harzianum*, *T. hamatum*, *T. koningii*, and *T. pseudokoningii*, and bacterial antagonists, *Pseudomonas fluorescens* and *Bacillus subtilis* were effective against *F. o.* f. sp. *cepae* (Rajendran and Ranganathan, 1996). A combination of *T. viride* and *P. fluorescens* were most effective for reducing FBR incidence under pot and field conditions (Malathi, 2015). Isolates of *Trichoderma* sp., *T. harzianum* (TH 3) gave the greatest (83%) inhibition and *Pseudomonas* sp. (Pf 12) exerted significantly the greatest (75%) reduction of mycelial growth of *F. oxysporum* f. sp. *cepae;* based on the laboratory analysis, effective biocontrol agents were evaluated in glasshouse and field conditions (Malathi, 2015). *Trichoderma viride* and Pseudomonas fluorescens have antagonistic capacity against plant pathogens which enhance systemic acquired resistance and plant growth promoting character. *Pseudomonas fluorescens* plays an important role in phosphate solubilization which improves the soil and plant health. Scanty information is available on quality production of onion by approaching integrated crop health and disease management (Gupta *et al.*, 1987).

3.5.4.1. *Bacillus* species

Bacillus species are able to form endospores that allow them to survive for extended periods under unfavorable environmental conditions(Saharan and Nehra, 2011). This trait is relevant in their relative durable viability when stored for a relatively long period (shelf-life). Bacillus based biocontrol agents are quite important in the management plant diseases (Jacobsen *et al.,* 2004). Mechanisms involved in Bacillus eliciting plant growth promotion include auxin production, increased availability of phosphorus (Deepa *et al.* 2010), biocontrol abilities, and induction of systemic resistance (Kloepper *et al.,* 2004).

There are a number of *Bacillus* strains that used to control plant diseases through the production of metabolites (Charest *et al.*, 2005). It strongly affects the environment by increasing nutrient availability of the plants (Barriuso *et al.*, 2008). For example, isolates of *Bacillus subtilis* inhibited *S. cepivorum* in vitro, and were able to suppress the incidence of onion white rot, leading to an increased onion emergence and yield (Utkhede and Rahe, 1980). Members of *Bacillus* were reported as producers of antibiotics inhibiting various phytopathogens including *F. oxysporum f. sp. ciceri* (Kumar, 1999) and *Fusarium solani* (Caroline *et al*., 2013). *Bacillus licheniformis* when inoculated on tomato and pepper shows considerable colonization and can be used as a biofertiliser without altering normal management in greenhouses (Garcia *et al.*, 2004). *Bacillus megatorium* also very consistent in improving length and dry matter content of root in mint (*Mentha piperita* L) (Kaymak *et al.*, 2008). Research showed that different *Bacillus* strains were also effective against coffee wilt disease caused by *Gibberella xylarioides* (Melkamu *et al.*, 2013).

Investigations have shown the large possibility of using biological agents but not applied on large scale. However, there are some commercial biocontrol products from *Bacillus* for the control of different plant pathogens. The most common are Companion from *B. subtilis* for the biocontrol of Root rots (*Aspergillus*, *Golovinomyces* *cichoracearum,Fusarium oxysporum*, *Fusariumnivale*, *Magnaporte poae,Phytophthora*, *Pythium, Rhizoctoniasolani, Sclerospora graminicola,Sclerotinia minor*), leaf spot (*Alternaria, Botrytis cinerea,Colletotrichum orbicular,Colletotrichum*, *Didymella bryoniae*, *Erwinia carotovora, Erwini tracheiphila, Plasmodiophorabrassicae, Podosphaera xanthi,Pseudomonas syringae, Xanthomonascampestris*) (Helene *et al*., 2011).

The other common product is Serenade Gray mold for the biocontrol of Botrytis (*B.cinerea*), black Sigatoka (*Mycosphaeraella fijiensis*), early blight (*Alternaria solani*), late blight (*Phytophthora infestans),* powdery mildew (*Leveillula taurica, Oidiopsistaurica, Erisiphe chichoracearum,Erysiphe* spp., *Sphaerothecamacularis, Sphaerotheca* spp., *Podosphaera clandestina, Podosphaeraleucotricha, Uncinula necator*), downy mildew (*Bremia lactucae,Peronospora* spp.), early leaf spot (*Cercospora* spp.), Botrytis neck rot (*Botrytis* spp.), scab (*Venturia* spp.), leaf-drop (*Sclerotinia* spp.), bacterial spot (*Xanthomonas* spp.), walnut blight (*Xanthomonascampestris),* fire blight (*Erwiniaamylovora*), anthracnose (*Colletotrichum*), white mold (*Sclerotinia sclerotiorum*). The mode of action of these biocontrol products comprise antibiosis, competition, growth, promotion and resistance induction (Helene *et al*., 2011).

3.5.4.2. Actinomycete species

Actinomycetes are gram-positive, aerobic and filamentous bacteria. The majority of this group is saprophytic and found widely distributed in the soil that plays important ecological roles in soil nutrient cycling (Elliot and Lynch, 1995). These bacteria are also an important source of antibiotics, vitamins, enzymes and source of diverse antimicrobial metabolites (Terkina *et al*., 2006). In the antagonism of actinomycetes against fungi an important role is played by chitinases, enzymes which hydrolyze their cell wall. Among *Actinomycetes* genera *Streptomyces* and *Micromonospora* are largely described. Soil *Actinomycetes* particularly *Streptomyces* spp. enhances soil fertility and have antagonistic activity against wide range of soil-borne plant pathogens (El-Tarabily *et al*., 2008). The efficacy of antagonistic *Actinomycetes* in biological control has been shown against many plant pathogenic fungi such as *Fusarium oxysporum,Fusarium culmorum* and

Rhizoctonia solani causing damping-off of pine seedlings (Patrycja and Hanna, 2013). But *Fusarium oxysporumf.sp.vasinfectum* has a resistance to the antagonizing effects of three genera were isolated from Central Sudan. Nineteen different species were belonging to *Streptomyces*, five species belong to *Actinomyces* and one species belong to *Arachnia* formerly *Actinomyces* (Shami and Saadab, 2014).

There are biopesticides from *Actinomycete* species commercialized for the biocontrol of different plant pathogens. Examples of commercial biocontrol products from *Actinomycetes* are Mycostop (*Streptomyces griseoviridis* K61) which has antibiosis, competition, parasitism, growth promotion mode of action against root rot, damping-off, and wilt caused by *Fusarium*, *Alternariabrassicola, Phomopsis*, *Botrytis,Pythium, Phytophthora* and *Rhizoctonia* of several crops. The other one is Actinovate (*Streptomyces lydicus*) which has a mode of action of antibiosis, enzymes, competition, growth promotion against damping-off and root rot (*Pythium,Rhizoctonia*, *Fusarium*, *Phytophthora*, *Verticillium*), powdery mildew (*Erysiphe*, *Oidium*, *Podosphaera*, *Sphaerotheca*), downy mildew (*Pseudoperonospora*, *Peronospora*), gray mold (*B. cinerea*) and alternaria blight (*Alternaria* spp.) of vegetables, Ornamental plants, forest species (El-Tarabily and Sivasithamparam, 2006).

4. MATERIALS AND METHODS

4.1. Study Areas

The sampling sites are located at three onion producing districts (Adama, Adami Tullu-Jido Kombolcha and Dugda Bora) of east Shewa Zone of Oromia Regional State, Ethiopia (Figure 4). Adama district is situated in Rift Valley with latitude of 8° 33'N and longitude of 39° 16' E with an average elevation of 1712 meters above sea level (m a.s.l.). It is the administrative center of Oromia Regional State, and located 99 km South-East of Addis Ababa. The average annual rainfall is 809 mm while average the maximum and minimum temperature of the area is 20.5°C and 13.3°C, respectively. It has a relative humidity of 59.8% . The soil is sandy and clay loam, and pH of 7.26.

Adami Tulu-Jido Kombolcha district is located in the mid rift valley, 167 Km South of Addis Ababa in Oromia Regional State. It lies at latitude of 7°9'N and longitude of 38°7'E, and at an elevation of 1650 m a.s.l. It has a relative humidity of 60%, and receives average annual rainfall of 760.9 mm with minimum and maximum average annual temperatures of 12.7°C and 27.2°C, respectively. The soil is fine loam with sandy silt clay in proportion of 34, 48 and 18%, respectively, and pH of 7.48.

Dugda Bora is one of the district found in east Shewa Zone of Oromia Regional State, 134 km South of Addis Ababa along road to Hawassa. It is located latitude 8° 10′ N and longitude 38° 50′ E. The average altitude is 1650 m a.s.l., with an average rainfall of 716mm. The mean minimum and maximum temperature in the area ranges from 14 to 27ºC, respectively. The mean relative humidity is 61.3%. Crop production is rain fed with limited irrigation in some villages. The soils are moderately fertile sandy and sandy clay loams, and pH of 7.1.

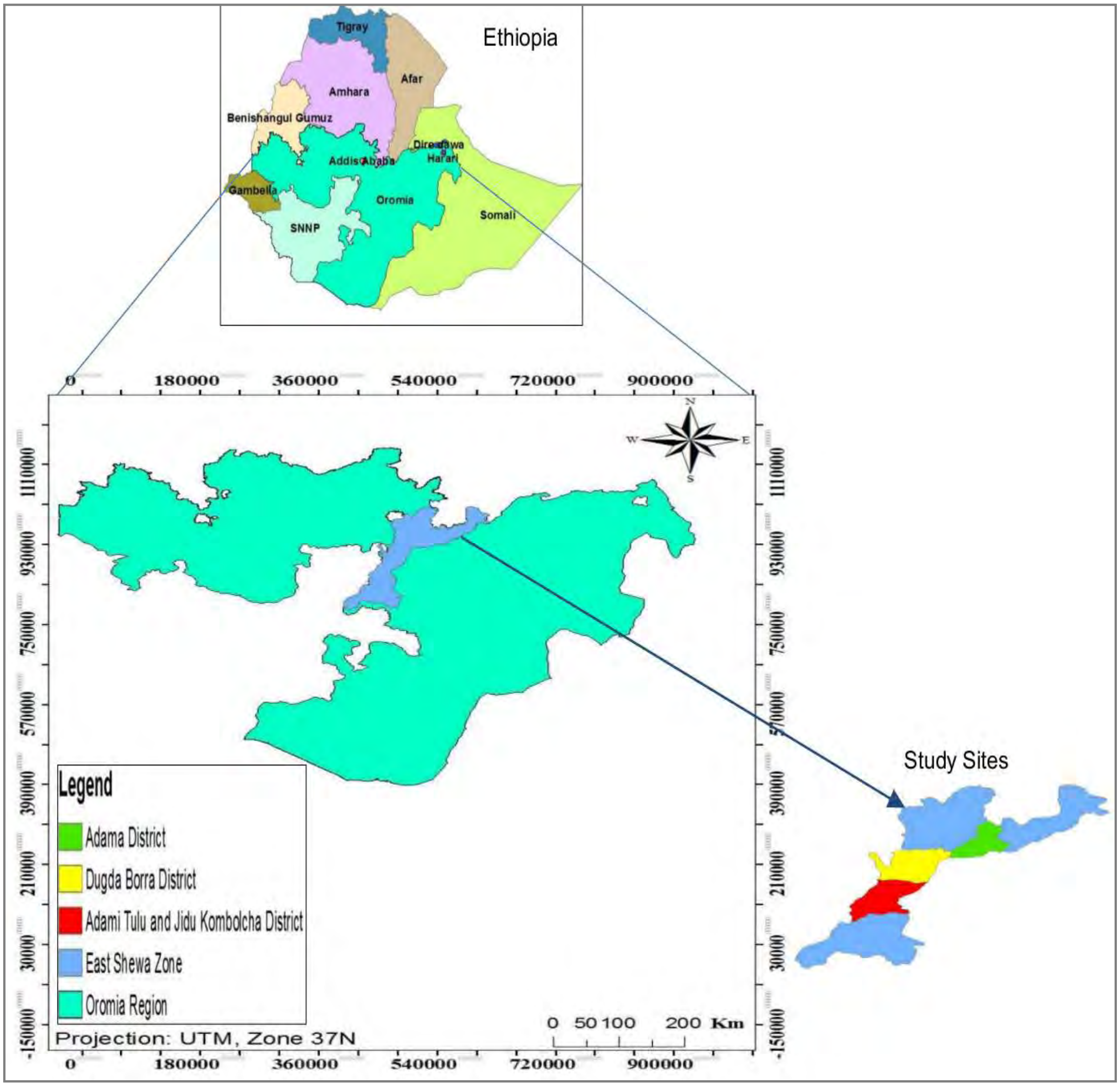

Figure 4. Map of Ethiopia showing the study areas.

4.2. Samples Collection

A total of 43 onion plant samples showing disease symptoms such as curving , yellowing and wilting leaves were collected from standing crops in the field during summer 2013 using simple random sampling technique. Six soil samples were also collected from rhizosphere soil of onion. Samples of the soil were taken with an auger (up to 10 cm depth) after removing 3 cm of the soil surface. The diseased onion and soil samples were brought to Mycology Laboratory, Department of Microbial, Cellular and Molecular

Biology, College of Natural Sciences, Addis Ababa University. Then the onion samples were kept at 4^{o}C for 7 days (till the isolation started).

4.3. Isolation and Identification

For isolation of *Fusarium* isolates; outer scales of the bulbs were detached to remove saprophytic fungi. Diseased tissues (4–6 mm) from leaf and basal plate areas of onion bulbs were surface sterilized in 1% sodium hypochlorite (NaOCl) for two minutes, rinsed thoroughly with sterile distilled water three times, dried in blotted paper and placed in Petri dishes containing potato dextrose agar (PDA) medium amended with chloramphenicol. The Petri dishes were incubated for 7 days at 25^{o}C. All isolates showing *Fusarium* like growth on PDA medium were picked and transferred to PDA medium, in order to purify *Fusarium* isolates. The isolates were identified as *Fusarium* by using the slide culture technique (Tuite, 1969) which allows the direct microscopic observation of morphological structures of taxonomic value. The technique was consisted of inoculating a bit of isolates at the sides of a small cube of carnation leaf agar (1 cm^{2}) maintained in the center of a slide and covered with a glass cover. The slide cultures were kept on a support to avoid direct contact with the humid base of the Petri dish. After incubating at 25^{o}C for 7 days, the micro cultures were examined under microscope and the image were captured. All monoconidial isolates were marked based on their region and the field of sampling and kept in slants at 4 °C for further study.

4.4. Morphological Characterization

For taxonomic identification of *Fusarium* isolates, the classification methodology of Leslie and Summerell (2006) was used. Morphological characteristics like front and back side colony color on PDA, size and shape of macro conidial and micro conidial and number of septa of the isolates were examined. Macro conidial and micro conidial lengths were observed and measured using Olympus System Microscope (OLYMPUS, BX51, BX2 series, Japan) coupled to a 12-bit QImaging Retiga Camera System. Images were taken using the same microscope with 40X objectives. Phase contrast images of the macroconidia and microconidia were taken using the same microscope.

4.5. Cultural Characteristics of *Fusarium* Isolates

Fusarium isolates were inoculated on four different media: Potato dextrose agar (PDA), Malt extract agar (MEA), Czapek Dox's agar (CZDA) and Natural medium/onion bulb extract agar (OBEA) to evaluate their culture media preference. OBEA was prepared from onion bulbs (200g/l) (outer scales of the bulbs were removed, and washed with SDW), dextrose (20g/l) and agar (solidifying agent) (20g/l) in 1000 ml SDW. Each of the medium was sterilized and poured into sterilized Petri dishes. A 5 mm diameter agar disc was excised from pure culture of each of the *Fusarium* isolates and placed at the center of each agar plate. The plates were incubated at 28^{o}C for 7 days. Each treatment was replicated three times.

4.6. Isolation of *Bacillus* Isolates

Six soil samples were dried separately at 45 °C for 45 minutes in a hot air oven to kill saprophytic fungi and then cooled to room temperature. For isolation of *Bacillus*, serial dilution agar plate method was used (Aneja, 2005). In serial dilution agar-plate methods, 10g of soil sample was suspended in a conical flask containing 90 ml of sterile distilled water. The flasks were shaken for 30 minutes on shaker and their contents designated as stock cultures. A series of culture tubes containing 9 ml of sterile distilled water were taken, and serial dilutions 10^{-2}, 10^{-3}….10^{-7} were made by pipetting 1 ml volume. Finally, 1ml aliquot of various dilutions are added to sterile Petri dishes (triplicate for each dilution) to which were added 20 ml of the sterile, cool, molten (45^{0}c) media (Nutrient agar (HIMEDIA)) for *Bacillus* isolation. The dilutions 10^{-4} to 10^{-7} were selected for enumerations of *Bacillus* isolates. The plates inoculated were incubated at 30°C for 3 days.

4.7. *In vitro* Pathogenicity Test on Onion Bulbs

Eighteen *Fusarium* isolates were screened for pathogenicity test based on the morphological and cultural characteristics, such as rate of growth, pigment production in PDA, colour and appearance of aerial mycelia. Onion bulbs of "Adama" variety, used for the pathogenicity test were brought from Melkasa Agricultural Research Center, and pathogenic isolates of *Fusarium* basal rot was determined using the protocol of Toit *et al.* (2003) with the following modifications. After removing the outer scales of onion bulbs as well as the roots and disinfecting with 70% ethanol. The basal stem was pierced with 2 mm diameter sterile cork borer to a depth of approximately 1 mm. Finally, each hole was inoculated with 0.1ml of conidial suspension having a concentration of $1x10^6$spores/ml prepared by diluting 7-day-old *Fusarium* cultures in sterile distilled water (SDW). The concentration of the pooled suspension was adjusted to $1x10^6$conidia/ml by using a heamocytometer. Control bulbs were inoculated only with SDW. After two weeks of incubation, bulbs were cut off from the inoculation sites and measured with a ruler for rot development in the tissue. The test was conducted with two trials and three replications for each isolate. The isolates were validated by re-isolating from intentionally inoculated onion bulbs with corresponding *Fusarium* isolates used for inoculation.

4.8. *In vitro* Antagonistic Activities of *Bacillus* Isolates

The test was carried out using dual culture technique to see the effect of *Bacillus* isolates on each isolates (Crawford *et al.*, 1993). *Fusarium* inhibition tests were performed by plate assay. A loop of *Bacillus* culture was streaked at one side of a PDA and each Petri dish was inoculated with 7 days old culture fungal agar block on the other side. Petri dishes were then incubated at 28°C for 7 days and examined for inhibition of *Fusarium* isolates growth by the *Bacillus* isolates. Development of clear zone around the *Bacillus* isolates were indicated the inhibition of *Fusarium* isolates growth. The width of cleared zones of antagonism were measured with a ruler after 7 days. Growth of biocontrol agents and each *Fusarium* isolate was observed in separate Petri dishes. Each experiment was replicated three times. The levels of inhibition were calculated by using the equation (Yuan and Crawford, 1995):

Inhibition (%) = [(growth diameter in untreated control - growth diameter in treatment) X 100] / growth diameter in untreated control

4.9. Antifungal Activities of *Bacillus* Isolates

4.9.1. Hydrogen Cyanide (HCN) production

Production of HCN was detected according to the method of Lorck (1948). Freshly grown cells were spread on 30 g tryptone soy agar which is supplemented with 4.5 g/l of glycine, and sterilized filter paper saturated with 1% solution of picric acid and 2% sodium carbonate was placed in the upper cover of the Petri dish. Then the plates were sealed with parafilm and incubated at 30°C for 5 days. A change in colour of the filter paper from yellow to reddish brown was an index of cyanogenic activity while no colour change represents no cyanogenic activity.

4.9.2. Determination of indole acetic acid production (IAA)

Indole acetic acid production of the isolates was determined by preparing eight grams of nutrient broth which was suspended in 500 ml of distilled water. Then loop of freshly grown *Bacillus* cultures were inoculated into 10 ml nutrient broth and 3g of tryptophan in each test tube and incubated at 30°C for three days . Four ml culture was removed from each test tube and centrifuged at 10,000 rpm for 15 min. An aliquot of 1 ml of supernatant was transferred into a tube containing 2 ml of Salkowski reagent (7.5 ml of 0.5 M $FeCl_3$ in 250 ml of dH_2O and 150 ml of H2SO4). Then the mixture was incubated at dark and room temperature for 25 minutes. As stated by Brick *et al. (*1991) the presence of indole acetic acid was determined by the development of pink colour.

4.9.3. Phosphate solubilization

Phosphate solubilization of *Bacillus* was determined on Pikovskaya's medium (Yeast extract: 0.5g/l, Dextrose: 10.0g/l, Tricalcium phosphate: 5.0g/l, Ammonium sulphate: 0.5g/l, Potassium chloride: 0.2g/l, Magnesium sulphate: 0.1g/l, Manganese sulphate: 0.0001g/l, agar: 15.0g/l and Ferrous sulphate: 0.0001g/l). Tricalcium phosphate (TCP) was added to enhance formation of halo zones around phosphate solubilizing *Bacillus* isolates. The medium was autoclaved at 121°C for 15 min and poured into Petri dishes. Then *Bacillus* isolates was spot inoculated on the Petri dishes, and incubated for 4 days at 28°C. As indicated by Pikovskaya, (1948) the presence of clear zone around the colonies showed phosphate solubilization.

4.10. Glasshouse Experiments

4.10.1. *In vivo* pathogenicity test

Inoculums for *in vivo* pathogenicity test were prepared by culturing ten pathogenic *Fusarium* isolates (from *in vitro* test) on PDA for 10 days in Petri dishes. Microconidial suspension of the isolates was prepared by pouring 1 ml of sterile water in each Petri dish, and scrapped with the aid of a sterile spatula to made the spores loosen from the medium. Then 1ml was made up to 20 ml in sterile bottles, and properly shaken in the rotary shaker at 8, 000 rpm for 10 minutes to dislodge the spores from the mycelia. Spore concentration was estimated using haematocytometer and adjusted to a final concentration of 1x10^6conidia⁄ml by diluting in sterile distilled water. Then, after roots of three weeks old onion seedlings were soaked in the adjusted spore suspension (Adebayo and Ekpo, 2005). The seedlings were transplanted into pot having 24cm diameter. Six onion seedlings per each pot which was filled with sterile local soil, sand soil and organic matter in the ratio of 3:2:1, respectively. The amount of soil in each pot was 4kg. Three replicates were performed for each isolate (Adebayo and Ekpo, 2005).

Pot experiment was carried out in the glasshouse of College of Natural Sciences, Addis Ababa University. Plants were watered daily in the glasshouse at 25°C and 60-90% relative humidity for 11 weeks. After 11 weeks, onion plants were harvested to assess the effect of *Fusarium* isolates on onion growth parameters. The growth parameters assessed were shoot length, root length and dry biomass of root and shoot. Randomly selected seedlings were used to determine each parameter per treatment. Finally, *Fusarium* isolates were confirmed by re-isolating from plants infected with isolates.

4.10.2. *In vivo* evaluation of antagonistic activity of *Bacillus* isolate

Bacillus isolate (AAUBI3) was selected for *in vivo* biocontrol activities test based on its high *in vitro* antagonism activities than other *Bacillus* isolates against *Fusarium* isolates, and it showed high IAA production. Inoculum preparation was carried out according to Cavaglieri *et al.* (2005). Two loopfuls of the bacterium from 3-day old cultures on tryptone soy agar (TSA) were transferred separately to 50 ml tryptone soy broth (TSB) medium, and incubated overnight at 30°C. Viability was confirmed by standard plate count method using tryptone soy broth plus 2% agar (TSBA). Inoculums for glasshouse experiment were prepared from a 48 h shaken culture at 10, 000 rpm of isolate incubated at 30°C shaker incubator. Tenfold serial dilution was carried out and the dilution series is plated with three replicates. *Bacillus* suspension containing 5×10^7 cfu/ml viable colonies were used as inoculants in the glasshouse. After 2 days of inoculation of the pathogenic isolates, by taking 2ml of the Bacillus suspension and diluting with 12 ml sterile distilled water and then injected into sterile soils in the glasshouse. *Bacillus* isolate was applied using three replications, and each pot comprised six plants. The treatments were *Bacillus* isolate alone , negative control (with no *Fusarium* isolate and *Bacillus* isolate), positive control (only *Fusarium* isolate) and both *Fusarium* isolate and *Bacillus* isolate.

4.10.3. Assessment of disease incidence and reduction

Disease incidence was recorded by counting the number of infected plants and dividing it with the total number of plants assessed in each treatment. The result obtained was converted to percentage using the formula, Percent of disease reduction was obtained by the formula below : (Haruna *et al.,* 2012):

$$\text{Disease incidence} = \frac{\text{Number of diseased plants}}{\text{Number of plants assessed.}} \times 100$$

$$\text{\% Disease reduction} = \frac{\text{diseased plant in positive control- diseased plant in treatment}}{\text{number of plants in positive control}} \times 100$$

4.11. DNA Extraction

All Fusarium isolates were grown in 100 ml of potato dextrose broth (PDB) added 10 µl of Tween 80 (keeps the fungal cells dispersed and helps in weighing the culture) before autoclaving and incubated in a rotary shaker at 180 rpm for 4 days at 30°C. The mycelium was then taken with the aid of a sterile spatula from the surface of the PDB. One hundred fifty (150) mg of fresh wet weight mycelium was used for genomic DNA extraction of each isolate. After vacuum filtration, the mycelium of each isolate was dried using liquid nitrogen, ground with sterile sea sand in a mortar and pestle (Sambrook *et al.*, 1989). Genomic DNA was extracted using a HiPurA^{TM} Fungal DNA Purification kit following the manufacturer's instructions. Then DNA quality and quantity was evaluated using nano drop and 1.0% agarose gel.

4.12. PCR Conditions

The ITS and 5.8S regions of *Fusarium* spp. was amplified with primers ITS1 (5’-TCCGTTGGTGAACCAGCG G-3‘) and ITS4 (5 ‘-TCCTCCGCTTATTGATATGC-3’). Amplification was performed in 25 µl of reaction mixture containing 2.5 µl of 10 × PCR buffer (500 mM KCl, 100 mM Tris HCl pH of 9.0, 1% Triton X-100), 2 µl of 2 mM MgCl2, 2 µl of template DNA, 200 µM of each dNTPs, 2.5 units Taq DNA polymerase and 10 pmol of both primers ITS1 and ITS2. The mixture was subjected to PCR in an Eppendorf Mastercycler (nexus). The PCR cycles began with an initial denaturation step for 5 min at 95^{o}C, followed by 35 cycles of denaturation for 40 seconds at 95^{o}C, annealing for 45 seconds at 55^{o}C and extension at 72^{o}C for 1 min. A final extension of 72^{o}C for 7 min was incorporated into the program. PCR products were separated by electrophoresis through 1.0% agarose gels. Gels were stained with Ethidium bromide and photographed under a UV transilluminator (Birren and Lai, 1993).

4.13. Restriction Enzyme Digestion

Aliquots of 10µl of PCR products were digested with 100 units of restriction enzymes *Eco*RI, *Hind*III and *Pst*I according to the manufacturer's instruction (MOLBIO^{TM} HIMEDIA). The restriction fragments were separated on 2.0% agarose gel, run for 105 min at 100 V, 400 mA and stained with Ethidium bromide. The restriction fragments were visualized under the UV visualizer and 100 bp DNA ladder was used to estimate the size of the restriction fragments (Chehri *et al.,* 2011).

4.14. PCR-RFLP Analysis

The molecular size of each fragment was estimated using standard curve of migration versus the log of the molecular size of 100 bp ladder. Each fragment was scored on the basis of the presence (1) or absence (0) of particular fragments. A data matrix was constructed based on the presence or absence of the fragments and converted to a similarity matrix. The similarity matrix was then subjected to the unweighted pair group method with arithmetical mean (UPGMA) cluster analysis based on simple matching coefficient (SMC) (Romesburg, 1994). The data analysis was performed by using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc, version 2.1) (Rohlf, 2000) to analyze the relationship among all isolates of *Fusarium*.

4.15. Data Analysis

For *in vitro* and glasshouse pathogenicity and antagonism test a completely randomized design with three replicates were used. Analysis of variance of repeated means, and a multiple comparison against the control was performed with Duncan’s multiple tests with the error probability of 5% using Statistical Package for Social Sciences (SPSS) version 20.

5. RESULTS

5.1. Morphological Characteristics of *Fusarium* Isolates

Based on the coloring obtained on PDA medium, a preliminary classification was conducted, and 10 pathogenic isolates were suggested as *F. oxysporum*, *F. solani* and *F. proliferatum* species (Table 1). Isolates having a colony color of white (dull white) from front view and brown (violet/cream) from reverse view on PDA medium were identified as *F. oxysporum*. Isolates which were identified as *F. solani* showed a colony color of white (yellowish pink) from front view and cream color from reverse view on PDA medium while *F. proliferatum* showed purple violate colony color from reverse view. Microconidia of isolate AAUFI6 (*F. proliferatum)* was short to moderate size (2.37 µm -13.49 µm), and globose (oval) in shape (Table 1). Macroconidia of isolate AAUFI6 (*F. proliferatum)* was thin walled with foot-shaped basal cell, and blunt apical cell. Some of the typical morphological characteristics of the10 isolates are summarized in table 1.

Table 1. Morphological characteristics of *Fusarium* isolates collected from onion growing areas

Isolates	**Color**		**Microconidia**		**Macroconidia**				**Suggested species**
	Front view	Reverse View	Shape	Size(µm)	Apical	Basal	Size(µm)	N<u>o</u> septa	
AAUFI2	Dull white	Brown	Oval with one septa , Oval	1.80-11.58	Hooked	Distinctly notched	48.33-73.15	3	*F. oxysporum*
AAUFI4	White	Violet	Oval with one septa	2.48-30.47	Blunt	Barely notched	65.54-74.03	3-4	*F. oxysporum*
AAUFI6	White	Cream	Oval, Oval with one septa	1.02-7.19	Papillate	Barely notched	53.16-79.63	3	*F. oxysporum*

AAUFI8	Dull white	Violet	Oval, Oval with one septa	2.75-22.09	Blunt	Foot shaped	56.24 -68.31	3-4	*F. oxysporum*
AAUFI10	Yellowish Pink	Cream	Oval, Globose, Oval with one septa	2.17-12.85	Blunt	Barely notched	57.46-89.24	3	*F. solani*
AAUFI12	White	Cream	Oval, Oval with one septa	1.99-14.14	Hooked	Foot shaped	51.68-63.81	3-4	*F. oxysporum*
AAUFI14	Dull White	Cream	Globose oval, Oval	1.88-13.81	Papillate	Barely notched	43.51-59.82	3	*F. solani*
AAUFI16	White	purple violet	Globose, Oval	2.37-13.49	Blunt	Foot shaped	56.49 -71.37	3	*F. proliferatum*
AAUFI18	Dull White	Brown	Oval	2.35-13.36	Papillate	Distinctly notched	54.46-78.24	3-4	*F. oxysporum*
AAUFI20	Dull White	Brown	Oval with one septa	2.59-14.84	Blunt	Barely notched	47.51-57.62	3	*F. oxysporum*
Fog*	White	Violet	Oval with one septa	2.62-26.2	Hooked	Distinctly notched	50.50-67.49	3	*F. oxysporum*

* Reference strain

5.2. Effects of Different Culture Media on Mycelial Growth of *Fusarium* Isolates

Inoculation of *Fusarium* isolates on different media showed each isolate had different ability to grow on different culture medium with different nutrition requirement and content (Figure 5). All the isolates generally exhibited higher growth activity on natural medium (Onion bulb extract agar medium) than PDA, MEA and CZDA media (Figure 5). In comparison with other media, CZDA medium gave the lowest mycelia growth of 10 isolates of *Fusarium*.

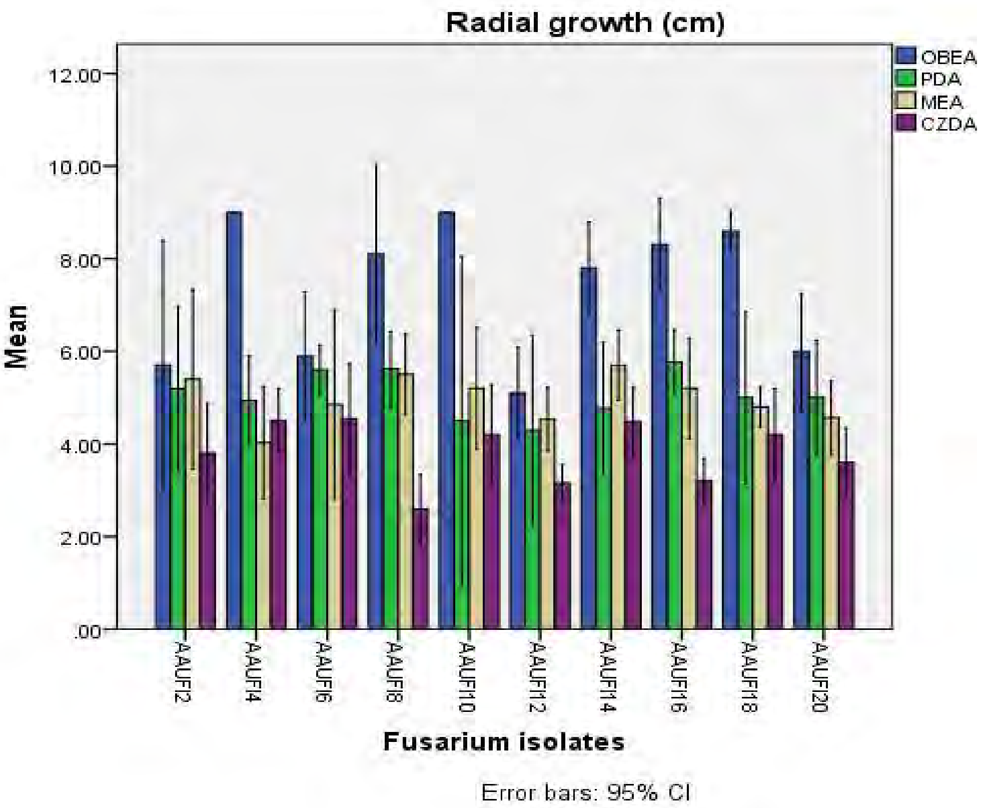

Figure 5. Effect of different culture medium on mycelia growth of *Fusarium* isolates.

5.3. *In Vitro* Pathogenicity Test on Onion Bulbs

The results of *in vitro* pathogenicity test revealed that ten isolates of *Fusarium* showed disease symptoms of wilt on onion bulbs (Figure 6). Symptoms which observed as basal rot of onion were brown discoloration and rot tissues on cross-sections of onion while control bulbs were remained symptomless (Figure 6). All the ten pathogenic isolates had different rate of virulence (Figure 7). Isolate AAUFI16 had the maximum rot length of

2.1 cm while isolate AAUFI2 and AAUFI6 had the minimum rot length of 0.23 cm each. These are statistically significant different from the control having zero rot length.

Figure 6. Pathogenicity test on onion bulb

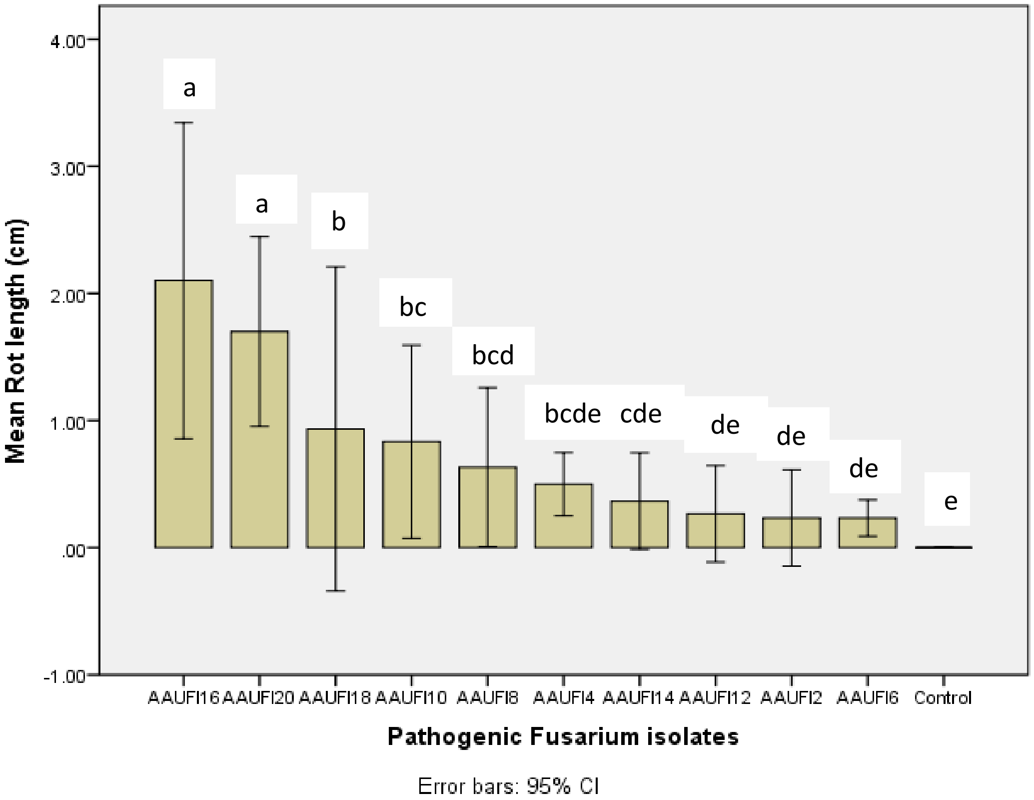

Figure 7. Effects of *Fusarium* isolates and their pathogenicity rate on onion bulbs.

5.4. Mechanism of Inhibition by *Bacillus* Isolate

Even if isolate AAUBI3 of the four *Bacillus* isolates was the most active, mechanisms of antagonism and inhibition of all the four *Bacillus* isolates were investigated (Table 2).

5.4.1. Phosphate solubilization

Bacillus isolates were examined for their ability to solubilize phosphate as an indirect mechanism of biocontrol by modifying the environmental conditions, and result sowed that all isolates except isolate AAUBI2 showed clear zones (Table 2).

Table 2: Mechanism of inhibition by four *Bacillus* isolates

Bacillus isolates	PGPR properties		
	Phosphate Solubilization	HCN	IAA
AAUBI1	+	-	+
AAUBI2	-	-	+
AAUBI3	+	-	++
AAUBI4	+	-	+

N.B. ++ = Strong positive response, + = Positive response and - = Negative response for HCN, IAA production and phosphate solubilization test.

5.4.2. HCN production

Based on *in vitro* evaluation of HCN production by *Bacillus* isolates using the picric acid assay, none of these isolates produced HCN (Table 2).

5.4.3. IAA production

Production of IAA by *Bacillus* isolates was detected through the development of pink color, It has been observed that all isolates produced this color (Table 2).

5.5. *In Vitro* Antagonistic Activity of *Bacillus* Isolates

Four *Bacillus* isolates were tested *in vitro* against *Fusarium* isolates of onion. The results revealed that from these *Bacillus* isolates, isolate AAUBI3 was highly active against *Fusarium* isolates (Figure 8). Result showed that inhibition of the isolates by *Bacillus* isolate AAUBI3 was statistically significant (Figure 9). Results revealed that the highest percentage of inhibition by *Bacillus* isolate was observed in isolate AAUFI16 (83 %) while in isolate AAUFI6 was the lowest (65%), and the highest mycelial growth was observed in isolate AAUFI4 (72.33%) whereas in isolate AAUFI20 was the lowest (53.58%) (Figure 9).

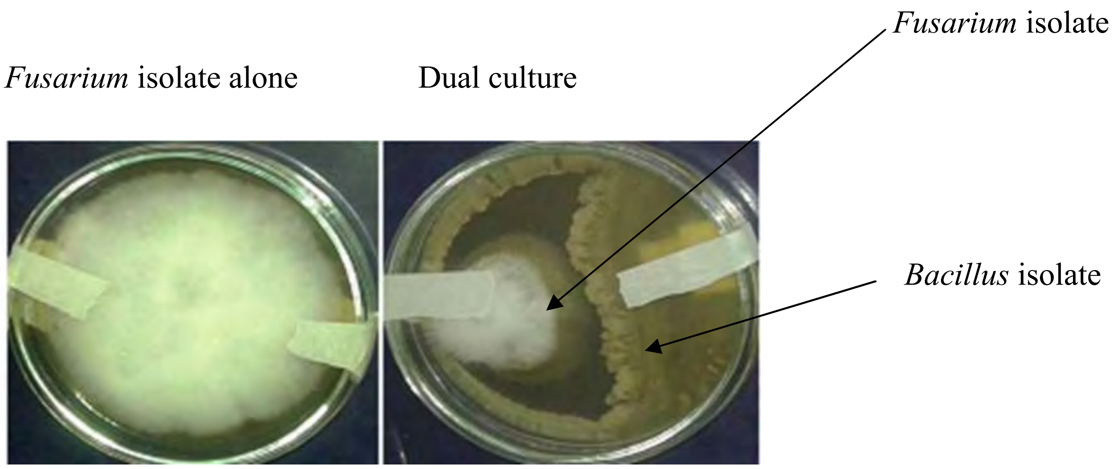

Figure 8. *In vitro* inhibitory effects of *Bacillus* isolate on mycelial growth of *Fusarium* isolate after 7 days of incubation on PDA medium .

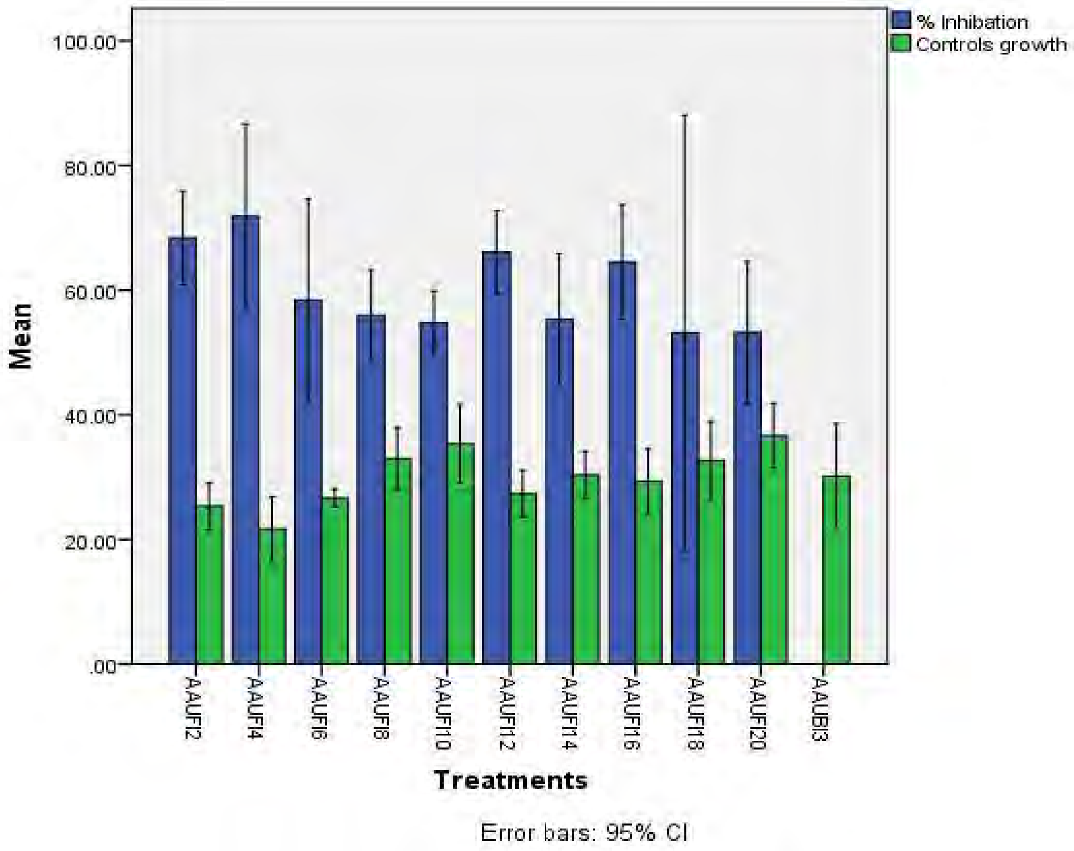

Figure 9. Percentage of mycelial growth inhibition of *Fusarium* isolates by *Bacillus* isolate AAUBI3.

5.6. Glasshouse Experiment

5.6.1. Pathogencity test of *Fusarium* isolates and evaluation of biocontrol potentiality of *Bacillus* isolate under glasshouse condition.

All the tested isolates were pathogenic and caused symptoms of basal rot disease (Fig. 9). Moreover, the tested isolates were varied according to their aggressiveness and pathogenicity characters under the bioassay conditions (Table 3).

5.6.1.1. Root length of onion seedlings

The result showed that root length of plants infected with *Fusarium* isolate AAUFI16 was the shortest and 22.6 mm in length (Table 3). It was statistically significant (P=0.05) different from the control which was 38 mm long and also from isolates AAUFI20, AAUFI18 and AAUFI12 having root length of 37 mm, 31mm and 30mm, respectively (Table. 3). But isolate AAUFI16 didn't not showed statistically significant difference from AAUFI2 (23 mm), AAUFI4 (26 mm long), AAUFI6 (28 mm), AAUFI8 (23 mm), AAUFI10 (25 mm long) and AAUFI14 (29 mm long). Root length of onion seedlings infected with each *Fusarium* isolates and treated with *Bacillus* isolate were statistically significant different from seedlings infected with positive controls (Table 3). The longest root length of 41.2 mm was measured from onion seedlings inoculated with only *Bacillus* isolate (Table 3).

5.6.1.2. Shoot length of onion seedlings

The shortest shoot length was produced by isolate AAUFI2 (138mm) (Table 3). There was statistically significant difference from control, AAUFI16 and AAUFI20 having shoot length of 241 mm, 183 mm and 193 mm, respectively, while the largest shoot length was produced by isolate AAUFI20. But all treatments were statistically significantly different from the control (Table 3). Shoot length of onion seedlings infected with each *Fusarium* isolate and treated with *Bacillus* isolate showed statistically significant difference from seedlings infected with positive controls except AAUFI6+BaI, AAUFI8+BaI and AAUFI16+BaI treatments (Table 3). The longest shoot length of 236 mm was produced by AAUFI18+BaI while the shortest shoot length of 202.3 mm produced by AAUFI2+BaI (Table 3). These treatments showed statistically significant difference from positive control. The shoot length of onion seedlings infected with each

Fusarium isolate and treated with *Bacillus* isolate were not significantly different from negative control (Table 3).

5.6.1.3. Dry weight of onion seedlings

From Table 3, the lowest dry weight of 0.5 g was produced by *Fusarium* isolate AAUFI2, and it was statistically significant difference from control, AAUFI20, AAUFI18, AAUFI16,AAUFI12 ,AAUFI10, AAUFI8 and AAUFI6 having 5.2, 2.4, 1.4, 2.1, 2.1, 1.9, 1.6 and 2.0, respectively. Result of pathogenicity test in glasshouse showed that all isolates were statistically significant different from the control (Table 3).The lowest dry biomass of 1.6g was produced by AAUFI2+BaI. It showed statistically significant difference from negative control, BaI, AAUFI20+BaI, AAUFI18+BaI, AAUFI16+BaI, AAUFI14+BaI,AAUFI12+BaI, AAUFI10+BaI, AAUFI6 +BaI and AAUFI4+ BaI. Isolate AAUFI8 having dry biomass of 1.9 g was not statistically significant different from AAUFI2 (Table 3).

5.6.2. Disease incidence

The percent of disease incidence on onion seedlings infected with each *Fusarium* isolate and treated with *Bacillus* isolate were much lower than positive control (Table 3). The highest disease incidence of 100% was showed by isolates AAUFI2, AAUFI8 and AAUFI14. Results from onion seedlings infected with *Fusarium* isolates and treated with *Bacillus* isolate showed that AAUFI8+BaI had high disease incidence with 58.33%. Results showed that *Bacillus* isolate significantly reduced disease incidence in onion seedlings (Table 3). A negative correlation (P=0.01) of results has been shown between disease incidence and root length, shoot length and dry weight of onion seedlings.

5.6.3. Degree of disease reduction by *Bacillus* isolate under glasshouse

Results revealed that the highest disease reduction activity of 66.67% was observed in AAUFI2+BaI while in AAUFI16+BaI was the lowest (25%) (Table 3). Result has not been shown correlation between disease reduction by *Bacillus* and onion growth parameters assessed.

Table 3: Effects of *Bacillus* isolate on disease incidence and plant growth parameters of onion seedlings infected with *Fusarium* isolates.

Treatments	Root length(mm)	Shoot length(mm)	Dry weight(g)	% Disease incidence	% Disease reduction
AAUFI2+BaI	39.0 ± 0.58^{efg}	202.3 ± 41.16^{cdefgi}	1.6 ± 0.15^{bcde}	41.67^{bcd}	66.67
AAUFI4+BaI	40.7 ± 7.90^{fg}	225.8 ± 17.06^{ghi}	3.2 ± 0.45^{hi}	33.33^{abc}	50
AAUFI6+BaI	37.7 ± 0.88^{defg}	214 ± 1.35^{efghi}	2.9 ± 0.06^{ghi}	50^{bcde}	33.3
AAUFI8+BaI	38 ± 0.58^{defg}	204.8 ± 1.96^{defghi}	1.9 ± 0.24^{cdef}	58.33^{cde}	50
AAUFI10+BaI	41.3 ± 1.2^{g}	234.7 ± 8.69^{hi}	3.4 ± 0.09^{i}	16.67^{ab}	50
AAUFI12+BaI	42 ± 2.08^{g}	218.2 ± 4.33^{fghi}	3.0 ± 0.33^{hi}	16.67^{ab}	50
AAUFI14+BaI	38.7 ± 0.88^{efg}	205.6 ± 3.87^{defghi}	2.4 ± 0.08^{fgh}	50^{bcde}	50
AAUFI16+BaI	35.3 ± 0.88^{cdefg}	206 ± 2.79^{defghi}	2.9 ± 0.05^{hi}	33.33^{abc}	25
AAUFI18+BaI	41.1 ± 0.59^{fg}	236 ± 5.98^{hi}	3.5 ± 0.44^{i}	33.33^{abc}	50
AAUFI20+BaI	42.4 ± 5.33^{g}	229 ± 2.78^{hi}	3.1 ± 0.25^{hi}	16.67^{ab}	41.67
AAUFI2	23 ± 0.58^{ab}	137.7 ± 23.38^{a}	0.5 ± 0.00^{a}	100^{f}	-
AAUFI4	26 ± 2.90^{ab}	154.8 ± 6.12^{ab}	1.3 ± 0.00^{bc}	83.33^{ef}	-
AAUFI6	28 ± 2.65^{abc}	172 ± 10.12^{abcde}	2.0 ± 0.20^{cdef}	66.67^{cdef}	-
AAUFI8	23 ± 2.68^{ab}	162 ± 18.50^{abcd}	1.6 ± 0.21^{bcde}	100^{f}	-
AAUFI10	25 ± 1.35^{ab}	164.6 ± 4.59^{abcd}	1.9 ± 0.03^{cdef}	75^{def}	-
AAUFI12	30 ± 1.84^{abcde}	178.8 ± 10.71^{abcde}	2.10 ± 0.35^{cdefg}	66.67^{cdef}	-
AAUFI14	29 ± 4.15^{abcd}	158.7 ± 2.03^{abc}	1.0 ± 0.15^{ab}	100^{f}	-
AAUFI16	22.6 ± 1.4^{a}	183.3 ± 3.89^{bcdefg}	2.13 ± 0.49^{defg}	58.33^{cde}	-
AAUFI18	31 ± 2.16^{abcde}	177 ± 17.46^{abcdef}	1.4 ± 0.12^{bcd}	83.33^{ef}	-
AAUFI20	37 ± 0.78^{bcdef}	193.3 ± 12.33^{hi}	2.4 ± 0.29^{fgh}	58.33^{cde}	-
BaI	41.2 ± 1.16^{g}	239.3 ± 5.78^{i}	4.8 ± 0.50^{J}	0^{a}	-
Negative Control	38 ± 1.16^{defg}	241.4 ± 7.64^{i}	5.2 ± 0.50^{J}	0^{a}	-

Values are mean of 3 replicates ± SE. Each replicate had a total of 4 plants. Values with different letters in the same column are significantly different at *P*= 0.05 by Duncan's multiple tests.

5.7. Molecular Analysis

Results obtained from amplification of ITS+5.8S regions with primer pairs ITS1 and ITS4 showed that each *Fusarium* isolate, identified as *F. oxysporum, F. solani* and *F. proliferatum* produced approximately about 530 bp (Figure 10). ITS +5.8S regions were restricted with three different hexa cutter restriction enzymes (*EcoRI, PstI* and *HindIII*). Out of the three restriction enzymes, two enzyme (*EcoRI* and *PstI*) restricted the ITS region. Restriction enzyme *PstI* which had restriction sites in the ITS region revealed that extensive polymorphism in the isolates. But among three restriction enzymes, one enzyme, *HindIII* had no restriction site in the amplified ITS and 5.8S regions (Figure 10, d). Results revealed that band patterns from restriction enzyme *PstI* differentiated ten *Fusarium* isolates (Figure 10, c).

Digestion of PCR products with *EcoRI* generated two banding patterns in all *Fusarium* isolates (Figure 10, b). Digestion with *PstI* produced different band patterns for isolate AAUFI16 (*F. proliferatum*), the size of the fragment was approximately about 550bp while it produced two common band patterns for the other isolates except isolate AAUFI10 (*F. solani*) having only one band pattern. Fragment 150 bp was present in all isolates except isolate AAUFI16 (Figure 10, b).

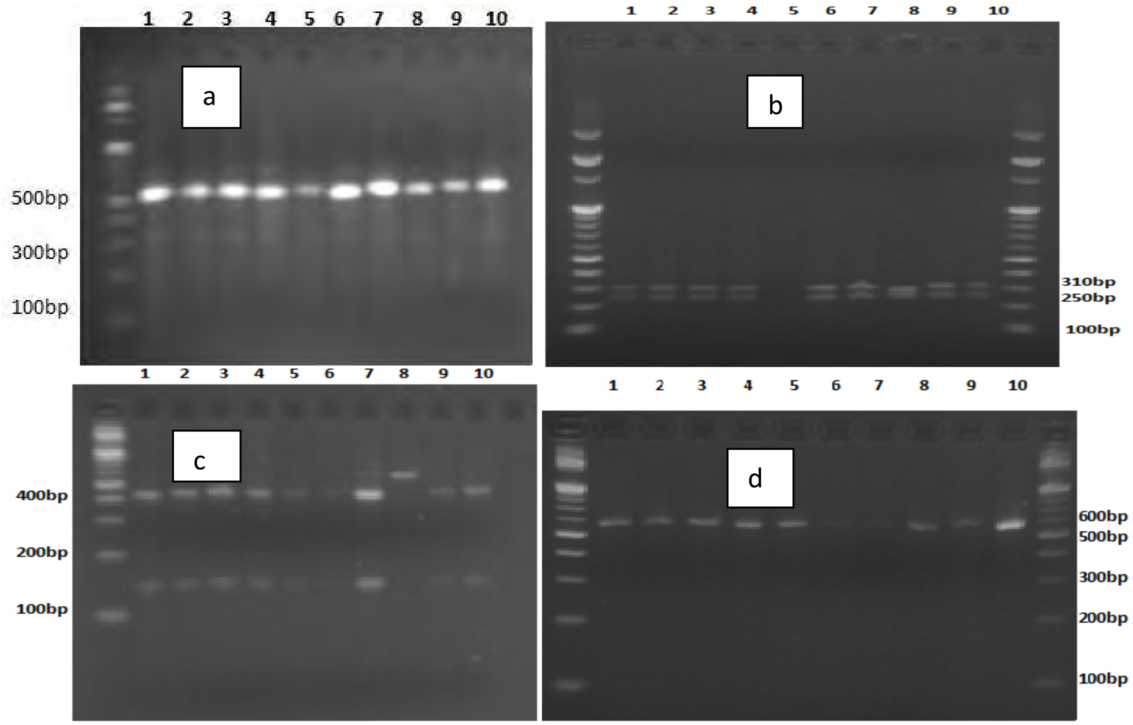

Figure 10. Agarose gels showing: a = amplification of the ITS region (ITS1, ITS2 and 5.8S) and restriction patterns of PCR-amplified rDNA digested with *EcoRI* (b), *PstI* (c) and *HindIII* (d). DNA size marker of 100 bp ladder, (1) isolate AAUFI2, (2) isolate AAUFI4,(3) isolate AAUFI6, (4) isolate AAUFI8, (5) isolate AAUFI10, (6) isolate AAUFI12, (7) isolate AAUFI14, (8) isolateAAUFI16, (9) isolate AAUFI18, (10) isolate AAUFI20.

Results from cluster analysis based on restriction band patterns showed that ten isolates were grouped into two major groups (I and II) and Five sub-groups with different genetic similarity coefficient ranged from 0.38 to 1.0 (Figure 11). The similarity coefficient within sub-group A was 1.0 (100% similarity) while in sub-groups B and C it was ranged from 0.69-0.84 (69%-84% similarity). On the other hand, the similarity coefficient of sub-group D was found in range of 0.53 to 0.69, and sub-group E (isolate AAUFI16) which is distinct to the other isolates was found with range of 0.38-0.53 similarity coefficient (Figure 11).

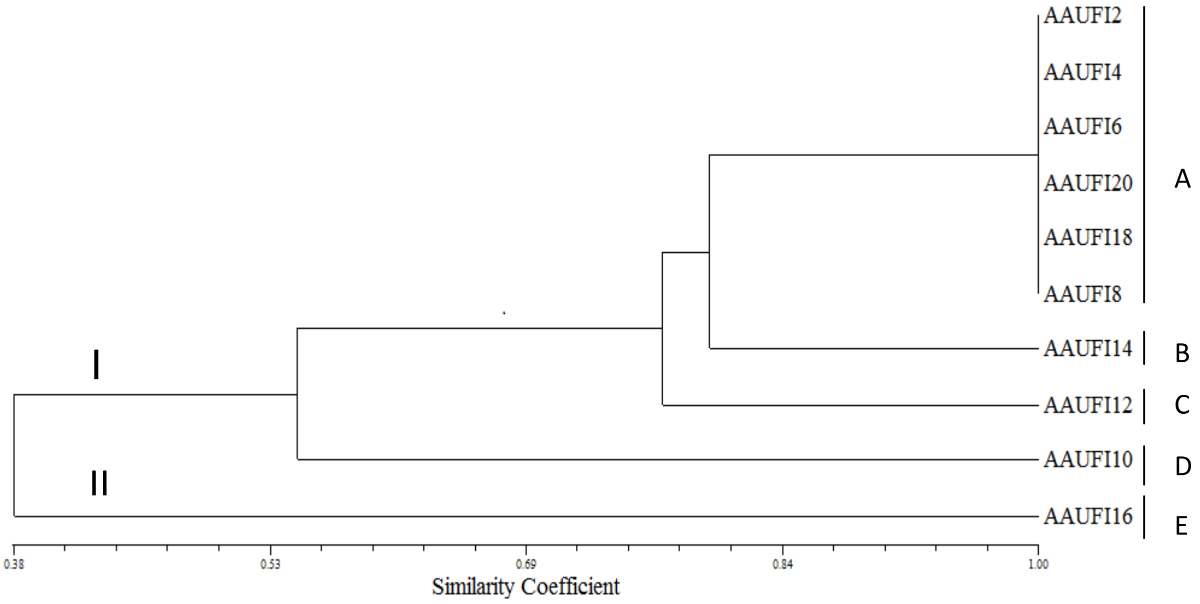

Figure 11. UPGMA dendrogram showing relationships among 10 isolates of *Fusarium* based on restriction site data of ITS-PCR products.

6. DISCUSSION

The ten pathogenic isolates were characterized morphologically, and identifying three species: *F. oxysporum, F. solani* and *F. proliferatum*. They had different morphological features of colony color, and size and shape of microcondia and macrocondia. Their morphological characteristic, identified as belonging to the genus *Fusarium*. Nelson *et al.* (1983) performed a morphological characterization using the coloration on PDA as indicating that each species of *Fusarium* has a specific color. *F. oxysporum* isolates had white mycelia, but some isolates produced violet pigment in the agar (this character was observed for AAUFI4 and AAUFI8 isolates). *F. solani* isolates had pinkish yellow and dull white mycelia. *F. proliferatum* with abundant white aerial mycelia which discolored to purple violet in the back side, producing violet pigments in the agar. Ghanbarzadeh *et al.* (2014) reported that these species caused basal rot in onion in East Azarbaijan province, Iran. These results also corresponded to those of previous studies regarded *F. oxysporum*, *F. proliferatum*, *F. redolens*, and *F. solani* as the important pathogenic species on onion plant (Schwartz and Mohan, 1995; Shinmura, 2002).

In this study, *F. oxysporum* was detected as the predominant *Fusarium* isolates in onion, comprising 70% of the total *Fusarium* isolates while *F. solani* was the second most important isolate, representing 20% of all isolates. The prevalence rates of fungal species were consistent with previous findings of Harun and Fatma (2010) who identified as *F. oxysporum* was the most dominant species in all investigated fields, comprising 66.57% of the total *Fusarium* species. *F. solani* was the second important species, representing 18.37% of all isolates, whereas *F. proliferatum* represented 2.71%. While Ghanbarzadeh *et al.* (2014) mentioned among 17 pathogenic isolates on onion, *F. solani* with the prevalence of 41% was the most dominant species, but surprisingly all the isolates had the least virulence. *F. oxysporum* included 23.5% and *F. proliferatum* included 29.5% of the identified *Fusarium* species and *F. redolens* was the least common species, comprising 5.8% of the isolates. Dissanayake *et al.* (2009) obtained 18 *F. oxysporum* isolates, 7 *F. verticillioides* isolates, and 7 *F. solani* isolates from wilted welsh onion plants collected from nine farms located in six regions of Japan. Similar results were obtained in previous studies performed by different researchers (Dugan *et al.,* 2003; Zlata *et al.,* 2008). Fuentes *et al.* (2007) detected *F. proliferatum* as the predominant fungal species in garlic fields in Mexico.

The effects of these isolates were evaluated using pathogenicity tests on onion bulbs and seedlings which can present their importance in store and field conditions, respectively. It is evident that from *in vitro* and under glasshouse pathogenicity test isolates were causing basal rot disease of onion plants. Results obtained from measurement of areas of infections (rots length on cross sections of bulbs), root length, shoot length and dry weight of onion seedlings showed that *Fusarium* isolates had different degrees of infection. The highest area of infections or rots length on cross section of onion bulbs were observed in isolate AAUFI16 (*F. proliferatum*).

Isolates of *F. oxysporum* were the most destructive agents of onion which caused severe bulb rot and growth parameters reduction. However, isolates of *F. proliferatum* (AAUFI16) was the most aggressive isolate on onion bulbs when compared with *F. oxysporum*, while it had moderate aggressiveness on onion seedlings growth parameters (root length, shot length and dry weight). Heterogeneity in aggressiveness among *F. oxysporum* isolates was observed. Similarly, Schwartz and Mohan (1995) showed heterogeneity in aggressiveness of *F. oxysporum*. Effects of *F. solani* isolates on onion bulbs and on seedlings growth were also considerable. In agree with other reports, *F. oxysporum* is a major limiting factor of onion that can be a great threat to all growth stages of onion bulbs both in farm and store conditions (Schwartz and Mohan, 2007; Ghanbarzadeh *et al.* (2014). Though, despite *F. oxysporum*, the yield loss of *F. proliferatum* at store is more noticeable than on farm (Southwood, 2012). Harun and Fatma (2010) and Ghanbarzadeh *et al.* (2014) studies revealed different results in the aggressiveness of *F. solani*, and found as weakly and least pathogenic isolate on onion. Results showed that the highest disease incidence of 100% was showed by isolates of *F. oxysporum* (AAUFI2 and AAUFI8) and isolate of *F. solani* (AAUFI14). The incidence of *Fusarium* isolates were consistent with previous findings of Shinmura (2002) who identified the highest incidence of 87.7% *Fusarium* species associated onion production.

In this research, *Bacillus* isolate successfully control the basal rot disease of onion. The treatment with the highest disease control activity was AAUFI2+BaI with 66.67%

disease control. *Bacillus* spp. from the rhizosphere have been reported to be effective against a variety of soil borne pathogens (Kloepper *et al.,* 2004).

None of the four *Bacillus* isolates produced HCN which is similar to Caroline *et al.* (2013) in whose research the *Bacillus* species were all negative for HCN. Cyanide is a toxic and degraded chemical produced by many rhizobacteria. Plant growth was enhanced *in vitro* by most of the rhizospheric isolated that produced HCN (Wani *et al.,* 2007). Reports have shown that HCN influences plant growth indirectly especially isolates from rhizosphere of chickpea, rice and mangrove (Shobha and Kumidimi, 2012). HCN produced by rhizospheric bacteria isolated from tomato rhizosphere also promoted plant growth directly and indirectly (Caroline *et al.*, 2013). Production of Hydrogen cyanide in *Bacillus* is about 50% in both rhizospheric soils and nodules compared to *Pseudomonas* that is over 80% (Ahmad *et al.,* 2008). According to Karuppiah and Rajaram (2011), most of the *Bacillus* isolates (*Bacillus* BA1, BA3, BA4, BA6, BA7, and BA8) from rhizosphere of vegetable plants produced HCN and so had antifungal activity against *Penicillium* spp, *F. oxysporum* and *Cercospora* spp. *B. amyloliquefaciens* produced HCN; improved plant growth and increased soybean yield (Sharma *et al.,* 2013). reserve

On the other hand, all the *Bacillus* isolates produced indole acetic acid from tryptophan to enhance plant growth. This is similar to production of auxin which is the commonest form of IAA by *B. amyloliquefaciens* KPS46 which also supported growth of soybean (Buensanteai *et al*., 2008). Most of the Bacillus strains isolated from the rhizosphere of potato crop (81%) were *B. amyloliquefaciens* strain, and they produced IAA (*Calvo et al*., 2010). Others are *B. amyloliquefaciens* AM1 and D29 and *B. subtilis* strain D16 (Almoneafy *et al.,* 2010).

All the *Bacillus* strains isolated from onion crop rhizosphere except isolate AAUBI2 solubilized phosphate. Similarly, *B. amyloliquefaciens* isolated from tomato crop rhizosphere solubilized tricalcium phosphate, where as *B. cereus, B. pumilus* and *B. subtilis* not solubilized tricalcium phosphate (Caroilne *et al*., 2013).

In vitro antagonistic activities against *Fusarium* isolates showed that *Bacillus* isolate inhibited the mycelial growth. The highest percentage of inhibition of 72.83% was observed. Similarly, *Bacillus* spp. have been reported as the best antagonists against *Fusarium* species of onion plants (Manimaran *et al*., 2011). This inhibitory activity of *Bacillus* species was reported to be as a result of antifungal compounds or metabolites like IAA production (Dihazi *et al.,* 2012). These results were more or less comparable with studies of in vitro antifungal activities of *Bacillus* spp against the mycelial growth of *F. solani* in varying degree ranging from 55.7% by *B. cereus* to 95.2% by *B. amyloliquefaciens* (*Calvo et al*., 2010). *B. subtilis* also inhibited the growth of *F. solani* by 82.1% *in vitro* (Caroilne *et al*., 2013). This result also agrees with Adebayo and Ekpo (2005), because *Bacillus* strains inhibited fungal growth *in vitro* and also promoted the growth of tomato plant in screen house trial. *Bacillus* species have been shown to have a broad spectrum of antimicrobial activities over diverse fungal and bacteria pathogen (Grover *et al.*, 2009).This inhibition fungal mycelial growth may be as a result of production of antibiotic, competition with pathogen for nutrients and direct antagonism (Akhtar *et al.,* 2010). Muleta *et al.* (2007) and Deepa *et al.* (2010) have also showed that *Bacillus* species inhibited the growth of different fungal species *in vitro* due to its antagonistic effects.

In this research onion seedling treatment with suspension of *Bacillus* isolate was effective in promoting onion growth which led to increase in the shoot and root length and dry weight compared to the positive control in the glasshouse. Treatment AAUFI20+BaI had the longest root length while treatment AAUFI18+BaI produced considerable shoot length and dry weight improvement. Gardener (2004) showed that *Bacillus* spp. or/and their by-products are applied to plants, the outcome is disease control. Similarly, *B. cereus* RS87 significantly promoted growth of root length, plant height and seedling emergence over control, and produced IAA (Deepa *et al.,* 2010)). Lamsal *et al.* (2012) also showed that shoot and root length were enhanced as well as increase in fresh biomass and total dry matter using rhizospheric *Bacillus* spp. for the biocontrol of anthracnose caused by *Colletotrichum acutatum* on pepper. The ability of *Bacillus* isolates to enhance growth and control plant disease could be as a result of production of plant hormones such as indole-3-acetic acid (IAA) and solubilized phosphate (Bloemberg and Lugtenberg 2001).

Inoculation of onion seedlings with *Bacillus* isolate greatly reduced percentage of disease incidence, and showed high percent of disease reduction on onion seedlings as compared to positive control. Thus, it was able to reduce disease incidence on seedlings by reducing basal rot symptoms by stimulating vegetable growth and root development. Similarly, Caroline *et al*. (2013) have found out that treatment of tomato plant with *Bacillus* species showed high percent of disease reduction and reduce disease incidence of *Fusarium* wilt. *Bacillus* isolate had higher efficacy in reduction of disease incidence. This efficiency might be related to the inhibition of pathogen by promoting plant growth through the production of IAA and increasing of availability of phosphors to the plant.

PCR-RFLP analysis of ITS+5.8S regions provided a better understanding of intra and interspecific variability among and within these pathogens. Result showed that digestion of ITS PCRs product with *PstI* produced variable restriction patterns. Differently, Datta and Lal (2013) showed that digestion of ITS+5.8S regions with *PstI* produced the same fragment lengths in different isolates of *Fusarium* species. In the present study, digestion of ITS+5.8S regions with *EcoRI* generated the same restriction patterns for all isolates. Similarly, Bayraktar *et al.* (2014), showed that digestion of ITS+5.8S regions with *EcoRI* produced similar fragment lengths, used for PCR-RFLP analysis of different *Fusarium* species associated with onion production. On the other hand, results from restriction digestion revealed that enzyme *HindIII* had not restriction sites on PCR–RFLP of ITS+5.8S regions of all isolates. It showed dissimilarity with the study of Datta and Lal (2013) that showed the enzyme has a restriction site, and produced different restriction patterns among different isolates of *Fusarium* species associated with onion production.

The cluster analysis based on restriction bands revealed high level of genetic diversity among these pathogens, and formed two major groups; I and II. Isolates of *F. oxysporum* and *F. solani* formed one group while isolates of *F. proliferatum* (AAUFI16) produced a distinct branch of dendrogram. Isolates of *F. oxysporum* were clustered into a single group with a similarity of 100% except isolate AAUFI12, whereas isolates of *F. solani* were clustered into two different groups with a similarity coefficient of 37.8% and

77.1%, respectively by PCR–RFLP analysis. Similarly, Galvan *et al.* (2008) used amplified fragment length polymorphism markers, grouping *F. oxysporum f. sp. cepae* isolates into two main clades, clade 2 and clade 3, described by ODonnell *et al.* (1998). Dissanayake *et al.* (2009) separated each of *F. oxysporum* and *F. solani* isolates obtained from welsh onion into two genetic groups with 61.6% and 40% similarity, respectively.

7. CONCLUSSION

Ten *Fusarium* isolates were isolated from bulb and leaf of naturally diseased onion plants grown in east Shea Zone, Ethiopia. Based on the morphological characteristics, the 10 isolates were identified as *F. oxysporum*, *Fusarium solani* and *F. proliferatum*. From the pathogenicity test, isolates of *F. oxysporum* was the major causal agents of basal rot of onion plants in the area. In general, results from *in vitro* and under glasshouse evaluation of *Bacillus* isolate against *Fusarium* isolates confirmed that *Bacillus* species are quite important and effective as biocontrol agents of basal rot disease of onion. In summary, PCR–RFLP of ITS+5.8S analysis used in this study provided a convenient tool for characterization and analyzing variations of *Fusarium* isolates associated with bulb rot of onion plants in east Shewa Zone. Results from cluster analysis based on restriction band patterns showed that ten isolates were grouped into two major groups, and isolate of AAUFI16 (*F. proliferatum*) formed a distinct cluster with the lowest similarity coefficient.

8. RECOMMNDATIONS

The following points have been forwarded as recommendation based on the above study.

- *Fusarium* species are the major onion production limiting factor in the study area. Therefore, the distribution and effects of the disease should be managed to increase onion yield in the area.
- *Bacillus* species helping in controlling basal rot disease of onion by antagonizing *Fusarium* species. Therefore, producers are better to use this bioagents with integration of other control methods to manage the disease. This will reduce effects of fungicide on the environment.
- The results obtained may assist in developing an integrated control program for *Fusarium* basal rot disease. However, more detailed investigations should be carried out on population structures of *Fusarium* species in the various regions of Ethiopia associated with onion crop, to design efficient, cost effective and environmentally sound disease management strategies.

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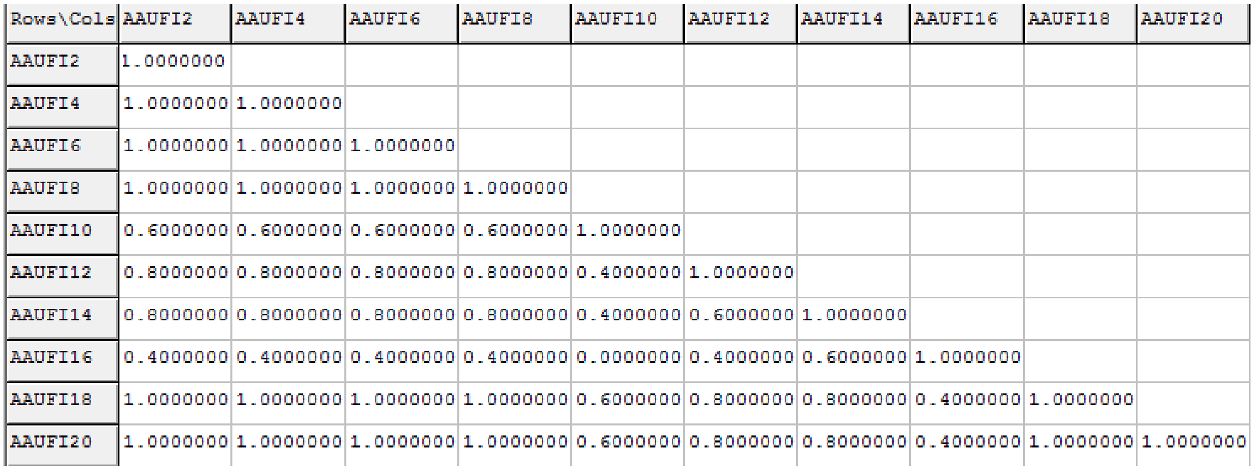
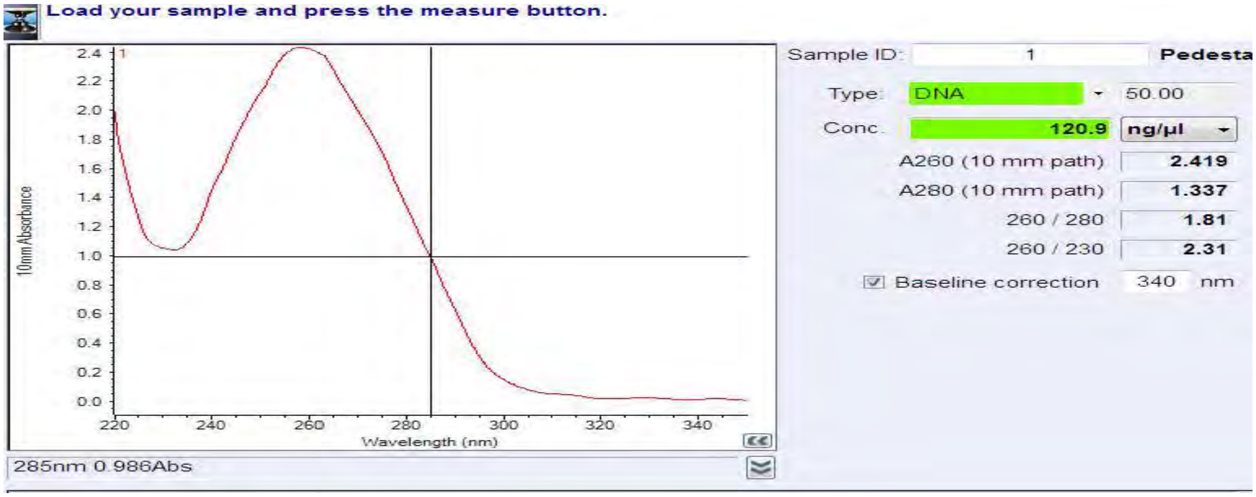
10. APPENDIXS

Appendix 1.Genetic similarity coefficient matrix among 10 isolates of *Fusarium* calculated from RFLP patterns digested with two enzymes.



Rows\Cols	AAUFI2	AAUFI4	AAUFI6	AAUFI8	AAUFI10	AAUFI12	AAUFI14	AAUFI16	AAUFI18	AAUFI20
AAUFI2	1.0000000									
AAUFI4	1.0000000	1.0000000								
AAUFI6	1.0000000	1.0000000	1.0000000							
AAUFI8	1.0000000	1.0000000	1.0000000	1.0000000						
AAUFI10	0.6000000	0.6000000	0.6000000	0.6000000	1.0000000					
AAUFI12	0.8000000	0.8000000	0.8000000	0.8000000	0.4000000	1.0000000				
AAUFI14	0.8000000	0.8000000	0.8000000	0.8000000	0.4000000	0.6000000	1.0000000			
AAUFI16	0.4000000	0.4000000	0.4000000	0.4000000	0.0000000	0.4000000	0.6000000	1.0000000		
AAUFI18	1.0000000	1.0000000	1.0000000	1.0000000	0.6000000	0.8000000	0.8000000	0.4000000	1.0000000	
AAUFI20	1.0000000	1.0000000	1.0000000	1.0000000	0.6000000	0.8000000	0.8000000	0.4000000	1.0000000	1.0000000

Appendix 2: Result from genomic DNA quality and quantity test using nanodrop.



Load your sample and press the measure button.

Sample ID: 1 Pedesta

Type: DNA 50.00

Conc. 120.9 ng/µl

A260 (10 mm path) 2.419

A280 (10 mm path) 1.337

260 / 280 1.81

260 / 230 2.31

☑ Baseline correction 340 nm

285nm 0.986Abs

Appendix 3: ANOVA table from experiment, effects of *Fusarium* isolates on growth parameters of onion plants.

Growth Parameter	Source of variations	Sum of Squares	Df	Mean Square	F	Sig.
Root length	Between Groups	880.095	10	88.010	5.951	.000
	Within Groups	325.335	22	14.788		
	Total	1205.430	32			
Shoot length	Between Groups	21561.741	10	2156.174	4.653	.001
	Within Groups	10195.086	22	463.413		
	Total	31756.827	32			
Dry weight	Between Groups	42.949	10	4.295	18.948	.000
	Within Groups	4.987	22	.227		
	Total	47.935	32			

Appendix 4: ANOVA table from experiment, effects of *Bacillus* isolate on growth parameters of onion seedlings infected with pathogenic *Fusarium* isolates.

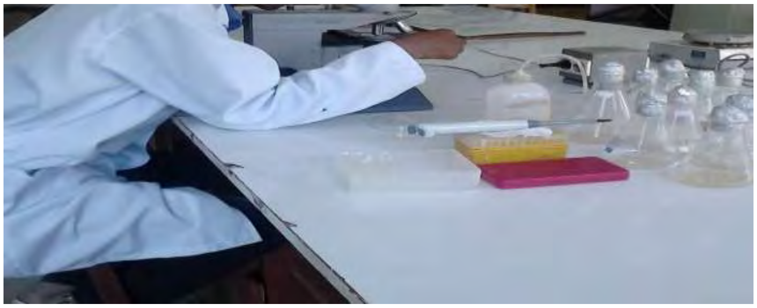
Growth parameters	Source of variations	Sum of Squares	Df	Mean Square	F	Sig.
Root length	Between Groups	2427.412	21	115.591	4.04	.000
	Within Groups	1257.155	44	28.572		
	Total	3684.567	65			
Shoot length	Between Groups	56647.677	21	2697.508	3.96	.000
	Within Groups	29972.882	44	681.202		
	Total	86620.559	65			
Dry weight	Between Groups	84.667	21	4.032	19.298	.000
	Within Groups	9.193	44	.209		
	Total	93.859	65			

Appendix 5: Micro conidia of ten pathogenic isolates of *Fusarium*.

Appendix 6: Onion bulb preparation and surface sterilization using 1% sodium hypochlorite for *in vitro* pathogenicity test.

Appendix 7: Onion seed preparation and surface sterilization using 70% alcohol for *in vivo* pathogenicity test

Appendix 8: *Fusarium* inoculums preparation and adjustment using hemacytometer.



Appendix 9: Glasshouse experiments

Declaration

I, the undersigned hereby declare that, this thesis is my original work and has not been presented nor is being currently submitted for a degree in other Universities or publication. All sources of materials used have been duly acknowledged. It is free for use as far as proper citation and acknowledgment is made.

Signature	Date
....	....
Abreham Chebte	

This thesis has been submitted for examination with the approval of University advisor.

	Signature	Date
Dr, Tesfaye Alemu (Advisor)	....	....