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SCHOOL OF GRADUATE STUDIES

Evaluation of the efficacy of *Trichoderma* and *Pseudomonas* species against bacterial wilt (*Ralstonia* isolates*)* of tomato (*Lycopersicum* spp.)

By

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Declaration

I declare that the thesis here submitted by me for the Degree of Master of Science in Biology (Applied Microbiology) to the School of Graduate Studies of Addis Ababa University is my own independent work and has not previously been submitted by me or anybody else where.

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Name Date of Submission Signature

Supervisor statement

I, the undersigned, confirm that this thesis is approved for submission.

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Name Date Signature

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Contents

LIST OF TABLES

LIST OF APPENDIX

Acronyms

AAU	Addis Ababa University
ANOVA	Analysis of variance
BE	Biocntrol efficacy
CFU	Colony forming unit
CRD	Completed randomized design
CSA	Central Statistics Authority of Ethiopia
DIC	Disease incidence of control
EU	European Union
GPE	Growth promotion efficacy
GYA	Glucose yeast extract agar
HCN	Hydrogen cyanide
ISR	Induced Systemic Host Resistance
IPM	Integrated pest management
KB	King's B medium
KOH	Potassium hydro oxide
NA	Nutrient agar
OD	Optical density
P	Phosphorus
PDA	Potato dextrose agar
PGPR	Plant Growth Promoting Rhizobacteria
PSB	Phosphate-solubilizing bacteria
RB	Rhizobacteria
RS	ralstoniasolanacearum
SA	Salicylic acid
SAS	statistical analysis system
TR	trichoderma
TZC	Triphenyltetrazolium chloride agar

ABSTRACT

Ralstonia solanacearum causes bacterial wilt of tomato and limits the crop production, and antagonistic microorganisms such as fungi and bacteria are used to suppress the disease, of which *Trichoderma* spp and *Pseudomonas* spp are the most effective agents to control bacterial wilt of various horticultural and other crops. In the present study, attempt was made to isolate these two microorganisms to evaluate their effectiveness to control *R. solanacearum* the causal agent of bacterial wilt disease of tomato under greenhouse conditions. Thus *R. solanacearum*, *Pseudomonas* and *Trichoderma* spp were isolated from wilted and healthy tomato plants grown from farmer's field in Ziway and Meki. The virulence of the pathogen and the antagonistic effect of the bacteria and fungi were evaluated against *R. solanacearum in vitro* and *in vivo* condition. Based on the *in vitro* results the best two isolates were selected to show their antagonistic effect under greenhouse condition in single and combined designs. The result showed the pathogenicity test of the isolates were evaluated under greenhouse condition, and isolate AAURS1 showed highest virulence (75%) followed by isolate APPRCRS2 with pathogenicity of 50%. With regard to antagonism test, isolates AAURB20 and AAUTR23 showed the highest inhibition against *R. solanacearum* with inhibition zone of 16mm and 15 mm, respectively. Among the treatments co-inoculation (AAURB20+AAUTR23) was more effective and reduced disease incidence by 13.33% and increased the bio-control efficacy by 72.22 % when compared with individual treatment and negative control (un inoculated treatment).The isolates significantly increased the plant height, and dry weight by 72.33 cm, and 12.18 g, respectively. Thus, the combined use of the biocontrol agents significantly reduced the incidence of tomato bacterial wilt disease. However, their performance should be evaluated using other yield parameters under field conditions to produce healthy tomato seedling to minimize the use of chemicals and reduce environmental pollution.

Key words: biocontrol, *Pseudomonas*, *R.solanacearum*, *Trichoderma*

1. Introduction

Tomato (*Lycopersicon esculentum*) is the second most important vegetable crop in the world next to potato (Gebisa *et al*., 2017). The center of origin of *Solanum lycopersicum*,(*S.* section *Lycopersicon*) has been localized in the narrow band between the Andes mountain ranges and the Pacific coast of western South and extends from southern Ecuador to northern Chile, including the Galapagos Islands (Peralta *et al* 2008). Tomatoes production accounts for about 4.8 million hectares of harvested land area globally with an estimated production of 162 million tones. China leads world tomato production with about 50 million tones followed by India with 17.5 million tonnes (FAOSTAT, 2014).In Africa, the total tomato production for 2012 was 17.938 million tons with Egypt leading the continent with 8.625 million tones (FAOSTAT, 2014. It is an economically important vegetable in Ethiopia. According to the Central Statistics Authority of Ethiopia, the country produced 27,774.538 tons of tomato in 5235.19 hectares of land in 2018 (CSA, 2019).

R solanacearum is ranked as the second most important bacterial pathogen among the top ten economically important soil borne pathogens that cause severe yield losses on different solanaceous crops in different parts of the globe (Mansfied *et al*., 2012). Different studies showed the bacterial wilt pathogen inflict 50-100% loss on potato in Kenya (Muthoni *et al*., 2012), 88% on tomato in Uganda (Katafiire *et al*., 2005), 70% on potato in India (APS, 2005). It is one of the most destructive and widespread disease of tomato in Ethiopia and its prevalence is as high as 55% in major tomato producing areas of the country (Biratu *et al*., 2013)

Different methods, mainly pesticides are employed to control bacterial wilt of tomatoes. Chemical controls with Actigard (e.g., acibenzolar-S-methyl) and phosphorous acid effective to control bacterial wilt under at greenhouse and to a lesser extent field conditions (Pradhanang *et al*., 2005). The use of excessive agrochemicals is negatively

perceived by consumers and supermarket chains due to residual chemicals in horticultural products. In addition use of chemical pesticides contaminate groundwater, enter food-chains, and pose hazards to animal health and to the user spraying the chemicals. Consequently, several members of the European Union (EU) (Sweden, Denmark, and Netherlands) decided in the mid–late 1980s to decrease the use of chemicals in agriculture by 50% and ban some of them through time within a 10-year period (Butt *et al.*, 2001).

However, effective and long term control is possible by using a combination of diverse methods including the use of resistant/tolerance varieties, cultural practices, biological and chemical control as parts of an integrated pest management strategy to control bacterial wilt caused by *R. solanacearum* (Persley, 1992). The use of biological control agents alone and/or together with other control methods as part of integrated pest management (IPM) practices is widely employed to overcome these problems (Barari, 2016).

Soil bacteria and fungi which flourish in the rhizosphere of plants and, stimulate plant growth are collectively known as plant growth promoting microorganisms (PGPM). The most abundant and useful microorganisms in the rhizosphere are *Pseudomonas*, *Bacillus*, *Burkholderia*, *Agrobacterium*, *Streptomyces*, *Trichoderma, Penicillium*, and *Gliocladium.* These microorganisms are used with the aim of improving crop yield by augmenting nutrient availability, enhancing plant growth and protection of plants from diseases and pests (Vessey, 2003). They are capable of secreting hydrolytic enzymes and causing mycoparasitism on pathogens and narrow spectrum antagonistic activity compared to synthetic pesticides, and, thus used singly or in combination with one another and chemicals in integrated pest management (IPM) to suppress plant-pathogens (Handima and Kalaivani, 2014) .

Trichoderma and *Pseudomonas* spp are the most frequently isolated fungi and bacteria in all the root ecosystems respectively. *Trichoderma* species effective in controlling phytopathogens due to their ability to grow toward the hyphae of other fungi, coil around

them and degrade the cell walls of the pathogen. Morsy *et al*. (2009) showed that, the dual application of *T. viride* and *B. subtilis* decreased the percentage of pathogen infection and increased survival rate than single inoculation in tomato. The biocontrol potential of two *Trichoderma* species on *sclerotia* rot disease of tomato plants in Chile and Iceland was evaluated and the result showed that, *T. harzianum* and *T. viride* reduced the disease by *74.50*%, and 68.75 %, respectively (Kator *et al*., 2015)

Pseudomonas species are one of the most widely distributed bacteria in the rhizosphere. They play a crucial role in controlling fungal and bacterial plant pathogens. Deshwal (2012) showed that, *Pseudomonas aeruginosa* strains inhibited the growth of *Sclerotina sclerotiorum* by 62-83%. A study conducted by Babu and Paramageetham (2013) also showed that P. *aeruginosa* reduced (inhibited) 73.7% of the radial growth of *S. rolfsii*. Another study showed that P. *aeruginosa* significantly reduced the incidence of fusarium wilt disease of egg plant up to 85 % (Altinok *et al*., 2016), Maurya et al (2014) reported the antagonistic activity of *P. fluorescens* against *Alternaria alternate*, the fungal pathogen of tomato with mycelial growth inhibition of 48.13%. In Ethiopia, Takele et al., (2015) also reported the antagonistic activity of *Trichoderma* against fusarium wilt of tomato with mycilial growth inhibition of 58%-66%.

Several studies also showed that the application of these antagonists have a dramatic effect on bacterial wilt disease (*Ralstonia solanacearum*) of tomato. Narasimha *et al*., (2013) showed that *Trichoderma asperellum* (T4 and T8) isolates delayed wilt development by *R. solanacearum*, effectively decreased the disease incidence (51%) , improved plant growth promotion and increased fruit yield under field conditions. Another study also showed that *Trichoderma spp.* AA2 and *P. fluorescens* PFS were most potent inhibiting the growth of *Ralstonia spp*, and the field study indicated *Trichoderma spp.* and *Pseudomonas fluorescens* alone were able to prevent 92% and 96% of the infection and combination of both were more effective, preventing 97% of infection compared to Chemical control methods that prevented 94% of infection (Yendyo, 2017). This shows the promising potential of native isolates of *Trichoderma spp.* and *Pseudomonas fluorescens* as biocontrol agents against *Ralstonia spp.*

In order to identify successful microorganisms as biocontrol agents, continuous screening of new isolates is needed for effective formulation against specific pathogens. Therefore, this study was initiated with the objective of evaluating the efficacy of *Trichoderma* and Pseudomonas spp individually and in combination against bacterial wilt pathogen, *R. solanacearum* of tomato under *in vitro* and *in vivo* conditions.

1.1. Objectives

1.1.1. General objective

- To isolate and characterize for effective antagonistic bacterial and fungal species from the soil and evaluate their efficacy on *Ralstonia solanacearum* causing bacterial wilt disease of tomato under in *vitro* and in *vivo* (greenhouse) conditions.

1.1.2. Specific objectives

- To isolate and characterize *Ralstonia solanacearum* from diseased tomato plants and evaluate its pathogencity against the host plant
- To isolate antagonistic bacterium (*Pseudomonas*) and fungus (*Trichoderma*) from the rhizosphere and screen for their antagonism against the test pathogen, *Ralstonia solanacearum* under laboratory conditions
- To evaluate the efficacy of selected single and co-inoculants on the test pathogen, *Ralstonia solanacearum* in tomato under greenhouse conditions.

2. Literature review

2.1. Center of origin and diversity of Tomato

The center of origin of Solanum lycopersicum, (S. section Lycopersicon) has been localized in the narrow band between the Andes mountain ranges and the Pacific coast of western South America (WWF and IUCN, 1997). This extends from southern Ecuador to northern Chile, including the Galapagos Islands (Peralta *et al.,* 2008).Mexico is presumed to be the most probable region of domestication, with Peru as the center of diversity for wild relatives (Larry and Joanne, 2007).

The cultivated tomato is a member of the genus Solanum within the family Solanaceae. The Solanaceae, commonly known as the nightshade family, also includes other notable cultivated plants such as tobacco, chilli pepper, potato and eggplant. Tomato classification has been the subject of much discussion and the diversity of the genus has led to reassessment of earlier taxonomic treatments. Tomato was originally named Solanum lycopersicum by Linnaeus in 1753; Lycopersicon lycopersicum (L.) Karsten has also been used (Valdes and Gray, 1998). For a long time tomatoes were known as L. esculentum, but recent research has shown that they are part of the genus Solanum and are now again broadly referred to as Solanum lycopersicum (Peralta et al., 2008).

The genus Solanum consists of approximately 1500 species. The tomato clade (section Lycopersicon, formerly recognized as the genus Lycopersicon) includes the cultivated tomato (Solanum lycopersicum) and 12 wild relatives, all natives to western South America. Tomato (Solanum lycopersicum) is derived from two wild ancestor species, Solanum pimpinellifolium and Solanum cerasiforme. Other wild species are useful for breeding disease resistance, colour improvement and desirable quality traits (Ranc et al., 2008).

2.2. Tomato Production and Constraints

Tomato (*Lycopersicone sculentum* Mill) is an important source of vitamins and antioxidants (Gerrior and Bente, 2002). Among more than 600 carotenoids in plant, only about 14 are found in human tissues (Khachik *et al*. 1995).Tomato and tomato products contribute to 9 of these 14 carotenoids and are the predominant source of lycopene, neuorosporene, gamma-carotene, phytoene, and phytofluene (Sies *et al*., 1995). Frequent consumption of tomato products is associated with a lower risk of prostate cancer (Ambrosini *et al.*, 2008).

Tomato is one of the most important edible and nutritious vegetable crops in the world. About 171 million tons of tomatoes are harvested annually from plantings of 5 million hectares. Almost 60% of world production is from Asia, 11.1% from Africa, 13.3% from Europe, 8.7% from North America, and 6.6% from Central America and South America (FAOSTAT, 2017).The primary tomato producing countries are China, India, United State of America, , Turkey, Egypt, Iran, Italy, Spain, Brazil and Mexico.

Table 1: the top 15 tomato producing countries in the world

Rank	Country	production
1	China	50,000,000
2	India	17,500,000
3	USA	13,206,950
4	Turkey	11,350,000
5	Egypt	8,625,219
6	Iran	6,000,000
7	Italy	5,131,977
8	Spain	4,007,000
9	Brazil	3,873,985
10	Mexico	3,433,567
11	Uzbekistan	2,650,000
12	Russian federation	2,456,100
13	Ukraine	2,274,100
14	Nigeria	1,560,000
15	Portugal	1,392,700

Source: FAOSTAT (2014)

Table 2: The top 15 tomato producing countries in Africa

Rank	Country	Production (tons)
1	Egypt	8 625 219
2	Nigeria	1 560 000
3	Morocco	1 219 071
4	Tunisia	1 100 000
5	Cameroon	880 000
6	Algeria	796 963
7	South Africa	564 740
8	Sudan (former)	529 200
9	Kenya	397 000
10	Ghana	321 000
11	Tanzania	255 000
12	Mozambique	250 000
13	Benin	244 742
14	Libya	225 000
15	Niger	188767

Source: FAOSTAT (2014)

Tomato production is hampered by various soil borne plant pathogens and pests (Foolad *et al*. 2008). Phytopathogens that attack tomato include the fungal pathogens; *Phytophthora infestans, Alternaria solani, Sclerotium sclerotiorum, Rhizoctonia solani*, *Fusarium oxysporum, and bacterial pathogens; Pseudomonas syringae* and *Ralstonia solanacearum* (Yin *et al*. 2013). These pathogens cause a significant reduction in yield and quality of crops.

2.3. Ralstonia solanacearum

Ralstonia solanacearum is formerly known as *P. solanacearum* EF Smith and causal agent of wilt of tomato and other solanaceous crops. The pathogen is also identified as *Burkholderia solanacearum* (Yabuuchi *et al.*, 1992).It widely occurs in tropical,

subtropical and some temperate regions of the world. The disease limits the production of solanaceous crops such as tomato, pepper, eggplant, tobacco and potato as well as other important crops like peanut, banana, ginger and geranium (Swanson *et al*., 2005). It is ranked as the second important bacterial pathogen, causing vascular wilt disease and the most destructive pathogens with rapid and fatal wilting symptoms.

This pathogen infects an extensively wide range of host plants (over 200 species), which distributed worldwide and induces destructive economic impacts (Kelman, 1998). Direct yield losses by the pathogen vary widely according to the host, cultivar, climate, soil type, cropping pattern and strain. For example, yield losses vary from 0 to 91% in tomato, 33 to 90% in potato, 10 to 30% in tobacco, and 80 to 100% in banana and up to 20% in groundnut (Elphinstone, 2005). It is very difficult to control this pathogen due to its abilities to grow endophytically, survive in soil, especially in a deeper layer, travel along water and associate with weeds (Wang and Lin, 2005).

2.4. Morphological and Physiological Characteristics of *R.solanacearum*

Ralstonia solanacearum is a rod-shaped bacterium with an average size varying from 0.5 to 0.7 by 1.5 to 2.5 μm and it is considered as an organism strictly aerobic (Denny and Hayward, 2001). It is catalase positive, oxidase positive, and nitrate reduction positive. The pathogen does not hydrolyze starch and readily degrade gelatin. In broth culture, the organism is inhibited by concentrations of sodium chloride (NaCl) greater than 2%. Liquid and solid (agar) growth media are commonly used for culturing of the bacterium.

On solid agar medium, individual colonies are usually visible after 36 to 48 hours of growth at 28^{o}C, and Kelmans tetrazolium chloride (TZC) agar is regularly used for its isolation (Kelman, 1954). After two days on TZC medium, virulent wild-type colonies are large, elevated, fluidal, and either entirely white or with a pale red center. For most strains, the optimal growth temperature is 28-32^{o}C. However, some strains that are pathogenic on potato have a lower optimal growth temperature of 27^{o}C.

2.5. Epidemiology and survival of the pathogen

Ralstonia solanacearum enters roots through wounds made by transplanting, cultivation, insects, or certain nematodes and through natural wounds where secondary roots emerge .Once inside the host, the bacterium has an affinity for the vascular system, where it multiplies rapidly, filling the xylem with bacterial cells and slime. After infection, it also moves up through the vascular system, the xylem, and finally blocks water transportation, which causes wilting (Wang and Lin, 2005). Typical symptoms of bacterial wilt can be observed few days after infection, such as wilting and later yellowing, dwarfing and finally irreversible, sudden wilting and death of plants.

Death of plant cell is caused by degradation of vessels and adjacent tissues. Further symptoms of bacterial wilt represent discoloration of the vascular system from pale yellow to dark brown and droplets of milky bacterial ooze exuding from affected tissue. Subsequently, *R. solanacearum* cells are set free into the soil from roots or collapsed stems that spread to roots of adjoining plants or to fulfill the saprophytic part of its life cycle (Denny, 2006).

The symptoms of bacterial wilt vary due to the diverse nature of the pathogen in colonizing various host plants. There are many bacterial wilt symptoms; the typical symptom on tomato is a flaccid appearance on the youngest leaves usually at the warmest time of 2 to 5 days after inoculation with *R. solanacearum*. Depending on the environmental conditions, wilting of the whole plant may follow rapidly due to reduced sap flow caused by the presence of large amounts of *R. solanacearum* cells and their exopolysaccharide slime in xylem vessels (Vasse *et al*., 1995). If the conditions are less favorable for the pathogen, the disease develops less rapidly, characterized by stunting and the development of adventitious roots.

The favorable conditions for development of bacterial wilt in tomato are described by (Wang and Lin, 2005). According to these authors, high temperature and moist soils

facilitate the development of bacterial wilt. When the stem is cut, vascular tissues occur in a brown discoloration and white or yellowish bacterial ooze may be visible.

Transmission and dissemination of the pathogen occur through several means. The bacterium can be carried over long distances on vegetative propagating materials surviving about 2-3 years (Coutinho, 2005). Vegetative organs are undoubtedly an important source of inoculum and contribute to short and long distance dispersal of the pathogen. Furthermore, contaminated irrigation water, chicken and cattle manure (2-4 weeks) and waste from the potato processing industry, as well as in latently infected potatoes and tomato seeds and infected potato tubers are potential sources of the pathogen (Hayward, 2000).

Crop residue left in the fields that were infected by *R. solanacearum* also serves as source of disease inoculum in the field (Wang and Lin, 2005). Insects have been also considered as vectors and a high potential for natural spread of race 2, (Elphinstone, 1998). Hence, extensive host range, wide distribution and long saprophytic survival in the environment make the control of the disease caused by *R. solanacearum* more difficult.

2.6. Economic importance of bacterial wilt disease

Soil-borne diseases are considered to be more limiting than seed-borne or air-borne diseases in the production of many crops and account for 10–20% of yield losses annually (USDA, 2003). Among the top ten economically important soil born bacteria species reported by Mansfied *et al*. (2012), *R. solanacearum* is ranked as the second most important bacterial pathogen that cause severe yield losses on different solanaceous crops in different parts of the globe. Different studies showed that 50-100% on potato in Kenya (Muthoni *et al*., 2012), 88% on tomato in Uganda (Katafiire *et al*., 2005), 70% on potato in India and varying degree losses in many potato growing countries of the world (APS, 2005).

In Ethiopia percent wilt incidence of bacterial wilt is reported as high as 63% (Bekele, 1996) on potato, and 55% on tomato (EIAR, 2002) and 100% on pepper (Assefa *et al*., 2015). In case of potato, since most wilted potato plants do not give marketable tube, crop yield losses from the disease could be very high under Ethiopian conditions. The pathogen has been known to have a high survival and damaging risk to many economic crops and other vegetation in Ethiopia. Hence, there is a need for proper management against this pathogen.

All taken together, the considerable economic losses caused by the pathogen is attributed to its persistence, wide host range and its broad geographical distribution in tropical, subtropical and some warm temperate regions of the world (Elphinstone, 2005). In addition to significant yield losses, bacterial wilt is also responsible for indirect damage on land use and disposal of susceptible crops. The presence of *R. solanacearum* in fields discourages planting of fields with many vegetables on family farms and home gardens, which leads to a considerable reduction in food source (Hayward, 2000).

2.7. Management strategies of bacterial wilt

Bacterial wilt is difficult to control in field production due to the properties as soil-borne pathogen, its broad host range and genetic variation level within the strains (Denny, 2006). Resistance breeding has been effective with tobacco and groundnut, but success with solanaceous crops appears to be limited or linked to climate conditions (French, 1994). However, effective and long term control is possible by using a combination of diverse control methods including the use of resistant/tolerance varieties, cultural practices, biological and chemical control as part of an integrated pest management strategy.

2.6.1. Resistance cultivars

The best strategy to control bacterial wilt caused by *R. solanacearum* is breeding for resistance cultivar (Persley, 1992). (Abdalla and Abdulla ,1998) have found that the degree of susceptibility to bacterial wilt is significantly different among six tomato cultivars which were tested indicating additive genes were more important than the non-additive genes. Thus, in breeding programs, selection for disease resistant plants (genotype) after each generation is recommended. Resistant plants colonized and invaded by *R. solanacearum* showed tolerance to the disease. Nakaho *et al*. (2004) shown that bacterial multiplication in the stems of resistant tomato plants was suppressed due to limited pathogen movement from the proto xylem or primary xylem to other xylem tissues. Furthermore, Dahal *et al*. (2010) elucidated the molecular interactions in the cell walls of resistant and sensitive plants inoculated with *R. solanacearum* using a proteomic approach.

2.6.2. Cultural practices

A number of cultural practices can reduce disease, including crop rotation that affect the ability of the local *R. solanacearum* strains to survive. However, only partial control of the disease can usually be achieved with short-term rotations. Intercropping at different time length with several plant species such as bean, cabbage, cowpea, onion, pea, or several cereals like maize or wheat has proved variable efficiency in bacterial wilt control (Lemaga *et al.*, 2005). Other methods include, planting healthy (tested) seed, using cover crops reduction of weed hosts by herbicides, since there are many asymptomatic infection to *R. solanacearum* infection and avoidance of surface water for irrigation (Champoiseau *et al.,* 2010). An important aspect for successful control of *R. solanacearum* and eradication of bacterial wilt is systematic surveillance on both, imported and home produced plant materials susceptible to the disease (Elphinstone, 2005).

2.6.3. Chemical control

It is established that it is relatively difficult to control bacterial wilt in the field using chemicals for the bacterium localizes inside the plant xylem and is able to survive at depth in soil. Chemical controls with Actigard (e.g., acibenzolar-S-methyl) and phosphorous acid for bacterial wilt have been shown to have effective both in greenhouse and to a lesser extent on field conditions (Pradhanang *et al*., 2005). Other control methods include treatment of irrigation or effluent water with low doses of chlorine or peracetic acid and the soil fumigation with vapam, methyl bromide, or chloropicrin, but it is of limited efficacy and utility (Rai *et al.,* 2008).

The plant-derived volatile compound, thymol was found to effectively reduce bacterial wilt incidence on tomato when used as pre-plant soil fumigation (Ji *et al.,* 2007). Several antibiotics were tested against *R. solanacearum* of which streptomycin and streptopenicillin reduced bacterial infection (Singh *et al*., 2000). Mondal et *al. (*2005) reported the efficacy of different antibiotics on tomato seedlings by root dipping before planting against bacterial wilt disease and found that the incidence of bacterial wilt was reduced to a great extent. Rai *et al.* (2008) also reported minimum wilt incidence and highest yield of bell pepper when seeds were treated with streptomycin.

The increasing use of agrochemicals is negatively perceived by consumers and supermarket chains. In addition use of chemical pesticides contaminate groundwater, enter food-chains, and pose hazards to animal health and to the user spraying the chemicals, for these reasons, it is increasingly banned by governmental policies and several members of the European Union (EU) (Sweden, Denmark, and Netherlands) decided in the mid–late 1980s to decrease the chemical input in agriculture by 50% within a 10-year period (Butt *et al.,* 2001).

2.6.3.1. Rhizobacteria as bio-control agents

Biological control can be defined as management of common components of ecosystem to protect plants against pathogens. It is acceptable as a key practice in sustainable agriculture (Azcon-Augiler and Barea, 1996). Biological control preserves environmental quality by reducing the dependency on chemical input and maintaining sustainable management practices. Biological control agents have potential to shift or antagonize phytopathogenic or deleterious microorganisms in the rhizosphere. Since the method is ecologically friendly, safe and specific for controlling pathogens, it has been developed and given attention and used as supplementary control method for plant pathogens.

The rhizosphere is the narrow zone of soil specifically influenced by the root system (Dobbelaere *et al*., 2003). This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (Gray and Smith, 2005). This situation is reflected by the number of bacteria that are found around the roots of plants, generally 10 to 100 times higher than that in the bulk soil. This zone is populated by a diverse range of microorganisms and the bacteria colonizing this habitat are called rhizobacteria (Schroth and Hancock, 1982).

Plant-associated bacteria can be classified into beneficial, harmful and neutral groups on the basis of their effects on plant growth (Dobbelaere *et al.*, 2003).Beneficial free-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria (Kloepper *et al.*, 1989). Bacteria of diverse genera have been identified as PGPR, of which *Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore, 2006). The production of one or more antibiotics is the mechanism most commonly associated with the ability of plant growth promoting bacteria to act as antagonistic agents against phytopathogens (Glick *et al.*, 2007).

Harmful microorganisms living in the rhizosphere and interacting with the plant roots may cause development of plant diseases. PGPR, which exert a beneficial effect on the

plant they colonize, on the other hand, interact with the plant roots as well as with other microorganisms in the rhizosphere. Some of the PGPR are antagonists to recognized root pathogens and may result in prevention of development of plant diseases (Cook *et al.*, 1995). Therefore the use of disease-suppressive PGPR to keep the level of deleterious microorganisms under control or below a threshold limit can be considered as biocontrol agents. This suggests the introduction of biocontrol agents from outside in the rhizosphere to achieve disease suppression.

Several studies have been made to control bacterial wilt of tomato with exogenous application of PGPR (Nguyen and Ranamukhaarachchi, 2010; Aliye *et al.*, 2008). It is important to evaluate PGPR antagonistic to the pathogen and incorporate them into successful disease management schemes as bio-control agent. A key feature of such organisms is their ability to adjust to the rhizosphere and to aggressively colonize the host roots (Dunne *et al.,* 1997**).** It is recommended that indigenous bio-control agents should be isolated and characterized to achieve greater efficiency for the management of crop diseases (Dubey and Patel, 2001).

Various Bacillus and *Paenibacillus spp* help to promote the health of crops and control diseases by producing antibiotic metabolites, suppressing plant pathogens, others antagonize plant pathogens by competing for nutrients like iron and phosphate. Others indirectly fix nitrogen which they make available to the plants and help stimulate plant nutrient uptake. Among these the bacterial antagonists have the twin advantage of faster multiplication and higher rhizosphere competence hence; *P. fluorescens* have been successfully used for biological control of several plant pathogens (Ramamoorthy *et al*., 2002).

Biological control using PGPR strains especially from the genus *Pseudomonas* is an effective substitute for chemical pesticides to suppress plant diseases Compant *et al*.(2005).Their applicability as bio-control agents has drawn wide attention because of the production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Nagarajkumar *et al*., 2004). Fluorescent

Pseudomonas is uniquely capable of synthesizing many of these antibiotics, not only to enhance its own fitness but also to help in the maintenance of soil health and bio protection of crops from pathogens (Gaur *et al*., 2004)

A number of soil bacteria and plant growth promoting rhizobacteria (PGPR) are currently being investigated for their role in the control of *R. solanacearum*. A list of saprophytic bacteria strains *viz. Corynebacterium sp.* BT6, *Bacillus* spp. FH17 and BB11, *Escherichia* sp. BT4, *Serratia* sp. J2 and *Pseudomonas* sp. J3 have been found most effective against bacterial wilt disease (Guo *et al.*, 2001).Das and Bora (2000) examined the role of biological control agents, *P. fluorescens, B. subtilis, T. harzianum, T. viride, T. koningii, Aspergillus terreus* and *Gliocladium virens,* their inhibitory action and their efficacy in suppression of *R. solanacearum.* Kumar and Sood (2002) reported bacterial wilt management by using *P. fluorescens* in combination with *G. mosseae*. Ramesh and Korikanthimath (2004) reported the use of *P. fluorescens* as a potential biocontrol agent for the management of *R. solanacearum*

Kumar and Sood (2005) observed no bacterial wilt incidence in tomato when biological control agents (*P. fluorescens* and *B. cereus*) were combined with 10 weeks soil solarization. Biswas and Singh (2007) showed the effectiveness of *Pseudomonas fluorescens* as biological control agent against the bacterial wilt disease after soil disinfection with lime one month before transplanting. Bora and Bora (2008) reported that *P. fluorescens* and *T. viride* significantly reduced the bacterial wilt disease incidence in brinjal and also increased the recovery of antagonists from rhizosphere. Ramesh *et al.* (2009) reported that *Pseudomonas* is the major antagonistic endophytic bacterium in eggplant which has potential to be used as a biocontrol agent against *R.solanacearum* in eggplant.

2.6.3.2. *Pseudomonas fluorescence*, a potential bacterial antagonist to control plant diseases

Pseudomonas fluorescens encompasses a group of common, nonpathogenic saprophytes that colonize soil, water and plant surface environments. It is a common gram negative, rod-shaped bacterium. As its name implies, it secretes a soluble greenish fluorescent pigment called fluorescein, particularly under conditions of low iron availability (Palleroni, 1984). Because they are well adapted in soil, *P. fluorescens* strains are being investigated extensively for use in applications.

Certain members of the *P. fluorescens* have been shown to be potential agents for the biocontrol which suppress plant diseases by protecting the seeds and roots from fungal infection. They are known to enhance plant growth promotion and reduce severity of many diseases (Hoffland *et al*., 1996). This is due to the production of a number of secondary metabolites including antibiotics, siderophores and hydrogen cyanide Competitive exclusion of pathogens as the result of rapid colonization of the rhizosphere by *P. fluorescens* may also be an important factor in disease control (Hass and Defago, 2005).

2.6.3.3. Trichoderma as biocontrol agent

Trichoderma, a genus of asexually reproducing fungi, that is widely distributed in nearly all temperate and tropical soils. They are strong opportunistic attackers, fast growing, prolific producers of spores and powerful antibiotic producers. These properties make these fungi ecologically very successful (Kubicek *et al.,* 2002). They show a high level of genetic diversity and can be used to produce a wide range of commercial products of industrial and horticultural importance much of the known biology and many of the uses of these fungi have been documented recently (Druzhinina, 2006).

Trichoderma species produce different volatile and non-volatile compounds which it inhibit the growth of phytopathogens. The mechanism of antibiosis constitutes a much

more complex system, leading to the phenomenon of biological control**.** Among these antibiotics, the production of gliovirin, gliotoxin, viridin, pyrones, peptabiols and others have been described extensively (Vey *et al*., 2001). Besides, the production of a large variety of volatile secondary metabolites by *Trichoderma* (e.g. ethylene, hydrogen cyanide, alcohols, aldehydes and ketones up to C4 chain-length) also plays an important role in biocontrol. Another antibiotic compound, i.e. peptabiols exhibit antibacterial and antifungal properties, represented by alamethicin (Landreau *et al*., 2002).

With the growing interest in the biological properties of *Trichoderma* species, several bioactive metabolites have been isolated and identified from different strains. These compounds possess diverse structures which belong to different classes of molecule and accordingly possess a range of bioactivities.

2.7. Biocontrol Agents and Their Mechanism of Action in the Management of Plant Pathogens

Bio control agents involve a puzzling array of mechanisms in achieving disease control. The mechanisms employed by bio control agents in controlling plant diseases are broadly classified into: direct and indirect antagonisms.

2.7.1. Direct Antagonism

Direct antagonism results from the physical contact and / or high degree of selectivity for the pathogens by bio control agent. This includes:

2.7.1.1. Production of antibiotics

Many PGPR have the ability to produce peptide antibiotics. These are oligopeptides that inhibit synthesis of pathogens cell walls, influence membrane structures of cells, and inhibit the formation of initiation complex on small subunit of ribosomes (Kundan *et al.,*

2015). A variety of antibiotics have been identified, including compounds such as amphisin, 2,4 diacetylphloroglucinol, hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin are produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp (Avinash *et al.*, 2016).

More than 12 antibiotics are synthesized by *B. subtilis* strains: bacillomicin, mycobacillin, fungistatin, iturin, phengicin, plipastatin, surfactin, bacilizin, etc. The antibiotics produced by the majority of *Bacillus* sp is active against both Gram positive and Gram negative bacteria (for example, polymyxin, circulin, and colistin) and pathogenic fungi *Alternaria solani, Aspergillus flavus, Botryosphaeriaribis, Colletotrichum gloeosporioides, Fusarium oxysporum, Helminthosporium maydis, Phomopsis gossypii*. Some studies showed an active influence of bacterial antibiotics in the regulation of defense system of the plant. It was revealed that *B. subtilis* surfacine is able to stimulate induced systemic resistance by activation of components like lipoxygenases, lipid peroxidases and the formation of reactive oxygen species (Satyavir *et al*., 2009).

2.7.1.2. Secretion of lytic enzymes

The production of lytic enzymes by rhizospheric bacteria involved in the control mechanisms against plant root pathogens including *Fusarium oxysporium* and *Rhizoctonia solani* (Nico *et al.*, 2010). Hydrolytic enzymes act as agents for prevention of plant diseases by causing lysis of deleterious microbes in the close vicinity of the plant as they secrete increased level of cell wall lytic enzymes (chitinases, glucanases and proteases) (Satyavir *et al*., 2009).

Some PGPR strains have been found to produce enzymes that can lyse fungal cell walls. Cell walls of most of the phytopathogenic fungi (except Oomycetes) are made up of chitin, which is a homo polymer of N-acetyl 21 glucosamine residues linked in P-1, 4 linkages. Chitinase which hydrolyse this polymer are produced by various organisms and

have been implicated in the control of fungal diseases. Inactivation of genes involved in their biosynthesis has been used to provide evidence for their contribution in biocontrol in planta (Kundan *et al.,* 2015).

The soil borne fluorescent *Pseudomonas* has received particular attention because of their capacity to produce a wide range of enzymes and metabolites. Antagonistic or biocontrol activity of fluorescent *Pseudomonas* is due to the production of different types of cell wall degrading enzymes like chitinase, protease/elastase and β-1, 3 glucanase. These enzymes are supposed to degrade the cell wall of various bacterial and fungal plant pathogens. Interestingly, some allelochemicals produced by PGPB are used as experimental pharmaceuticals, and this group can serve as additional resources to deal with the alarming ascent of multidrug-resistant human pathogenic bacteria (Premachandra *et al.,* 2016).

2.7.1.3. Hydrogen cyanide (HCN) production

Hydrogen cyanide (HCN) is secondary metabolite produced by many rhizosphere bacteria and plays a significant role in biological control of the pathogens. It is likely to inhibit electron transport chain and energy supply to cell, leading to death of cells. It also seems that PGPR inhibit proper functioning of enzymes and natural receptors reversible mechanism of inhibition and also known to inhibit the action of cytochrome oxidase (Behargavi *et al*., 2016). To date many different bacterial genera have shown to be capable of producing HCN, including species of *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* (Jan *et al*., 2013). Generally there is no negative effect on the host plants by inoculation with cyanide-producing bacterial strains. Various studies attribute a disease protective effect to HCN, in the suppression of "root-knot" and black rot in tomato and tobacco root caused by the nematodes *Meloidogyne javanica* and *Thielaviopsis basicota*, respectively (Satyavir *et al*., 2009).

2.7.2. Indirect antagonism

Biocontrol agents can reduce the disease incidence of crops by increasing their growth at least during the early stages of the life cycle. The best example of this is the resistance of damping off of Solanaceous crops with advance of age Chaube *et al*.(2003) reported that, bio agents both fungal and bacterial help in managing the plant diseases by promoting the growth of plants through increased solubilization of nutrients, increased nutrient uptake through enhanced root growth and sequestration of nutrients.

2.7.2.1. Phosphate solubilization

Phosphorus (P) is the second most important plant growth-limiting nutrient after nitrogen. The majority of phosphorus is in its insoluble form, whilst the plants can only absorb phosphorus when it is bonded with oxygen as in monobasic ($H2PO^{4-}$) and dibasic forms (HPO^{4-}) $_2$. A low abundance of phosphorus is typical in many agricultural soils, where phosphates make complexes with soil constituents, making them unavailable to many organisms (Lambers *et al*., 2013). To overcome phosphate deficiency, phosphate dense fertilizers are applied to crops regularly. However, plants can only absorb limited amount of phosphates and the rest is rapidly converted into insoluble P. There is also an extensive loss of phosphates in agricultural lands via run off and much of the phosphate ends up in water reservoirs.

There are microorganisms that are associated with hydrolyzing organic through phosphatase enzymes and solubilize and inorganic phosphates through organic acid production. The latter are known as phosphate-solubilizing microorganisms. Since more than 90% of them are bacteria, they are known as Phosphate solubilizing bacteria (PSB). These organisms are known to solubilize P from substrates and make it available to plants, and hence are a possible alternative to phosphate rich fertilizers. Bacteria of the genera *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia,*

Flavobacterium, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia*are reported as the most effective PSB (Bhattacharyya *et al*., 2012).

2.7.2.2. Induced Systemic Host Resistance (ISR)

Induced systemic resistance (ISR) is the most indirect form of antagonism. Biocontrol agents induce a sustained change in the plant, increasing its tolerance to infection by a pathogen, a phenomenon known as induce systemic resistance (ISR) (Satyavir *et al*., 2009). Salicylic acid (SA) and non-expresser of pathogenesis-related genes1 (NPR1) are key players in systemic acquired resistance *Trichoderma harzianum* when inoculated on to roots or on to leaves of grapes provides control of diseases caused by *Botrytis cineria* on leaves spatially separated from the site of application of the bio control agent (Desmukh *et al.,* 2006). Many classes of compounds are released by the *Trichoderma* spp. In to the zone of interaction and induce resistance in plants. The first class is proteins with enzymatic or other activity. Fungal proteins such as xylanase, and cellulases are secreted by T*richoderma* species (Martine *et al.,* 2001). *Trichoderma* endochitinase can also enhance defense, probably through induction of plant defense related proteins.

Various non-pathogenic *Pseudomonas* rhizobacteria have the ability to induce a state of systemic resistance in plants, which provides protection against a broad spectrum of phytopathogenic organisms including fungi, bacteria, and viruses (Satyavir *et al*., 2009).

2.7.2.3. Competition

From the microbial perspective, soils and living plant surfaces are nutrient limited environments. So to colonize the phytosphere, a microbe must effectively compete for the available nutrients. Both the bio control agents and the pathogens compete with one another for the nutrients and space to get established in the environment. This process of competition is considered to be an indirect interaction between the pathogen and the bio control agent whereby the pathogens are excluded by the depletion of food base and by physical occupation of site (Pal *et al.,* 2006)

Thus, bio control agents compete for the rare but essential micronutrients, such as iron and manganese especially in highly oxidized and aerated soils for they have more efficient nutrient uptake system for the substances than the pathogens (Nelson, 1990).This property can be attributed to the production of iron binding ligands called siderophores as in *Erwinia caratovora* (Kloepper *et al.,* 1980). Siderophores chealate the Fe (II) ions and the membrane bind protein receptors specifically recognize and take up the Siderophore-Fe-complex (Mukhopadhyay and Mukherjee, 1998).Bio controls agents also compete with the pathogen for physical occupation of site and thereby reduce or delay the root colonization by the pathogen. This is because the pathogen cannot gain a foothold for establishment on host; bio control can thus reduce the severity of root rot of pine (Maloy, 1993).

3 Materials and methods

3.1. Sample Collection

Soil samples from the rhizosphere of healthy and bacterial wilt infected tomato plants were collected from different fields from Ziway and Meki along the Rift Valley, which is one of the most important vegetable producing areas in the country .Diseased plant samples were selected based on visible characteristic symptom of bacterial wilts (Yendyo and Pandey, 2017).

3.2. Isolation of *Ralstonia solanacearum* from wilted tomato plants

Isolation of the wilt pathogen was undertaken according to Kelman (1954). Diseased tomato stem samples were washed with tap water, and surface sterilized with 70 % ethanol for 2 minutes and rinsed repeatedly in sterile water for 5 minutes. The samples were then suspended in the five-milliliter sterile distilled water for ten minutes to make them turbid due to oozing of bacterial cells from cut ends of diseased tissue. The bacterial suspensions were prepared to appropriate dilutions from which, 1 ml of the bacterial suspension was spread onto the surface of solidified Triphenyltetrazolium chloride agar (TZC) medium and incubated at 28±2˚C for 48 hrs.

3.3. Identification of virulent/avirulent isolates of *Ralstonia solanacearum*

The virulent and a virulent isolates of the pathogen were differentiated by Kelman method (1954) on Tetrazolium Chloride (TZC) agar medium and compared with isolates obtained from Ambo Plant Protection Research Center. The virulent isolates were detected based on their pink or light red colored colonies with characteristic red center and whitish margin, whereas the avirulent isolates were differentiated on their colonies

characterized by smaller, off-white and non-fluidal or dry texture on TZC medium after 24 hours of incubation.

3.4. Designation of isolates

Bacterial and fungal isolates were found to be biocontrol agent designated as by the prefix AAU (Addis Ababa University), letters RB (Rhizobacteria), TR (*Trichoderma*), RS *Ralstonia solanacearum* followed by specific numbers to separate different isolates.

3.5. Pathogenicity Test

Virulence of the isolates was carried out by inoculating them on the tomato seedlings according to Margare *et al*., (2011). Tomato seeds were planted directly in 20 x18 cm plastic pots containing sand and soil in the ratio of 2:1(3kg of soil and 1.5kg of sand) soil and sand was obtained from AAU. Bacterial isolates were grown on nutrient broth medium for two days at 30°C, suspended in sterile distilled water and adjusted to OD 600nm =0.1 (approximately inoculum size of 10^8CFU/ml) (Ran et al., 2005).Inoculation was made at the four true leaf stages by injecting into the stem with a needle. Plants inoculated with sterile water served as control and pots were regularly watered. Tomato plants were observed for development of typical wilt symptoms, and the severity of bacterial wilt was recorded based on the severity scale as follows; (% of shoot wilted, using a scale of 0-5 where, 0=No symptoms, 1=one leaf wilted (1%-25%), 2= 2 or 3 leaves wilted (26%-49%), 3=half plant wilted (50%-74%), 4= all leaves wilted (75%-100%), 5=Plant dead).

3.6. Biochemical Characterization of isolates

The selected virulent isolates were also inoculated on nutrient agar plates and incubated at 28^0C for 24 hrs for biochemical characteristics including Gram reaction, catalase test and oxidase test.

3.6.1. Gram staining

The isolates were gram stained using standard methods (Schaad, 1980). A loop full of the bacterium was spread on a glass slide and fixed by heating on a very low flame. Aqueous crystal violet solution (0.5%) was spread over the smear for one minute and then washed with running tape water. It was then flooded with iodine for one minute, rinsed in tape water and decolorized with 95% ethanol until colorless runoff is formed. After washing the specimen was counter-stained with safranin for 45seconds, washed with water, dried and observed microscopically at 100X using oil immersion objective.

3.6.2. Catalase test

A loop-full of bacterial culture (24 hrs old of each isolate was placed on a clean glass slide. A drop of H_2O_2 solution (3%) was added and mixed with the culture. Production of gas bubbles indicated positive reaction for the presence of catalase (Collins and Lyne, 1976).

3.6.3. Oxidase test

Oxidase test was performed by taking a loopful bacterial culture and rubbed on a disc of Whatmann filter paper No. 1 impregnated with a freshly prepared 3% (w/v) solution of tetra methyl-p-phenylenediaminedihydro chloride in distilled water. The change of the disc to purple color within 10 sec indicated positive result (Collins and Lyne, 1976)

3.6.4. Motility Test

Motility of isolates was determined by stabbing isolates in test tubes containing motility agar medium with straight wire loop .Motile isolates showed diffused growth whereas non motile isolates showed non-diffused/ straight growth (Collins and Lyne, 1976).

3.6.5. Indole production test

Indole production from isolates was determined by using 1% tryptone broth and incubated at 28^0C or 48 hrs. One ml of Kovac‟s reagent (diethylamide benzaldehyde) was added to each test tube. The development of bright- pink color on the top layer indicated indole production (Aneja, 2005).

3.7. Isolation of antagonists from tomato rhizosphere soil

Isolation of the bacterial and fungal antagonists was carried out using soil dilution method according to Johnsen and Nielsen(1999)Ten gram of rhizosphere soil sample collected from healthy tomato plants was prepared to appropriate dilutions (10^{-1} to 10^{-5}) and 10^{-3} to 10^{-5} plated on to KB (King's B medium) for rhizobacteria and PDA for *Trichoderma spp* (fungal antagonists). The Petri plates were incubated at 25 °C for 7 days for fungal antagonists and at 28°C for two days for rhizobacteria.

3.8. *In vitro* antagonism test against the Pathogen

The antagonism tests were carried out on the fungal and bacterial isolates against the bacterial wilt pathogen *in vitro* used disk diffusion method (Nguyen and Ranamukhaarachchi, 2010**)**. The bacterial wilt pathogen was grown on nutrient broth for 48 hr from which, 100µml was swapped onto Petri plates with nutrient agar. And the bacterial antagonist grow on nutrient broth for 48 hrs and *Trichoderm*a were grown in Potato Dextrose Broth (PDB) (20g/l dextrose, 4g/l potato extract and 15g/l agar) for 7 days and sterilized Paper disc (5 mm) was immersed in each test antagonist solution and was spotted at the center of the pathogen-inoculated-plate. Paper disc immersed in sterile distilled water and spotted at the center of the plates with the pathogen was used as control. Plates were incubated at 28°C for 48hrs to measure inhibition zone. Based on the antagonistic potential (percentage inhibition) on the test pathogen, twelve isolates were

studied in detail for cultural and growth, biochemical and Plant growth promoting characteristics of the rhizobacteria.

3.9. Morphological characterization of fungi antagonists

Morphological characterizations of the fungal antagonists were performed by growing them on PDA at 25 °C for 7 days. They were characterized by observing their cultural characteristics (colony color on the front and reverse side of the plate, growth rate, conidiophore branching, conidial shape and compared with the culture collection from AAU.

3.10. Biochemical characterization of bacterial antagonists

The selected bacterial antagonistic isolates were characterized by the following biochemical tests including Gram differentiation and gram reaction, growth at 41^{o}c, catalase test, oxidase test, pigment production, gelatin liquefaction using standard methods.

3.10.1. Fluorescent pigment production

The ability of bacterial isolates to produce yellow- green fluorescent pigments was tested by culturing the bacterial isolates on KB medium. The fluorescent pigment was observed for the fluorescence under 366 nm UV light after 48hr incubation (Stieglitz and Weimer, 1985).

3.10.2. Gelatin liquefaction

The overnight cultures of the isolates were inoculated in to nutrient gelatin deep tubes and incubated at 28 ± 2^{o}C for 24 h. Then the tubes were kept in the refrigerator at 4^{o}C for 30 minutes. The isolates showing liquefied gelatin were taken as positive and those which resulted in solidification of gelatin on refrigeration were recorded as negative (Blazevic and Ederer, 1975).

3.10.3. Carbohydrate fermentation Test for bacterial antagonist

Carbohydrate fermentation test of the bacterial isolates was under taken following the method of (Shaw and Clarke, 1995) various carbohydrates viz., glucose, maltose, fructose and lactose were incorporated in the peptone water. One percent of each sugar and phenol red as an indicator (0.5%) was added to peptone water tubes. About 10 ml of broth transferred into each test tube. Test tubes were autoclaved and inoculated with loopful of 24 h old culture of test organisms then incubated for 2-7 days at 28 ^{0}C. Change in color from pink to yellow is taken as positive.

3.11. Characterization of bacterial antagonists for their PGPR characters

3.11.1. HCN production

The ability of the isolates to produce HCN was conducted according to Rhitu *et al*, (2017).The isolates were streaked on King's B medium amended with 4.4 g/l glycine. Whatman No.1 filter paper discs dipped in 0.5% picric acid in 2% sodium carbonate solution were placed under the lid of each petriplate and sealed with paraffin. Plates were incubated at 28±2^{o}C for 4 days. Color change of the filter paper from deep yellow to orange and orange to brown indicated the production of HCN.

3.11.2. Production of NH_3

Bacterial isolates were tested for the production of ammonia in peptone water (Amjad *et al*., 2015). Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated at 30°C for 48–72 h Nessler's reagent (0.5 ml) was added in each tube.

Development of faint yellow to dark brown color was a positive test for ammonia production.

3.11.3. Phosphate solubilization

Capacity of isolates to solubilize inorganic calcium phosphate was checked by using glucose yeast extract agar (GYA) medium containing per 1 L distilled water; 10 g glucose, 2 g yeast extract and 15 g agar. In addition, two other solutions were prepared separately; first 5 g K_2HPO4 was dissolved in 50 ml distilled water and second 10 g $CaCl_2$ in 100 ml distilled water. These two solutions were added to 1 L GYA just before pouring medium to plates (Beneduzi et al., 2008). Each isolate was grown in GY broth for 24 h, and then 10 µL of bacterial culture was spot-inoculated on the medium and incubated for 7 days at 28ºC. Isolates which showed clear halos around their colonies were considered as phosphate solubilizers.

3.12. Compatibility test

In vitro compatibility test between the selected bacterial and fungus isolate was conducted using dual culture method in order to determine whether they can be used in combination. Thus, an overnight culture of the bacterium grown in King's B broth was streaked on one side of a petri-dish containing NA containing 2% sucrose. The other side of the petri-dish was inoculated with 1 cm disc of 7 days old *Trichoderma* sp. The plates were then incubated at 25^0C to test the presence of inhibition between the two isolates.

3.13. Antagonistic test of the isolates against the test pathogen on tomato under greenhouse condition

Tomato seeds from local Gelelima variety and Galilea variety that were obtained from Melkasa Agricultural Research Center were sown in seedling bed. After 25 days, the seedlings were transplanted in pots filled with potting mixture (soil: sand at 2:1 w/w/) at the rate of three seedlings per pot. Inoculum of the pathogen and the selected biocontrol agents; Pseudomonas and *Trichoderma* were prepared at 10^8cfu $ml^{-1}$and conidial suspension of (10^8 spores ml^{-1}) respectively as described by Sivan *et al*. (1984).Fifty ml of the mixed inoculum of the pathogen and antagonists were inoculated into the pots at the same time using soil drench method (Algam *et al*., 2010) .Each treatment was replicated thrice in completed randomized design (CRD). The treatments were;

T1 *Ralstonia solanacearum*+ *Trichoderma*. (AAURS+AAURB20)

T2 *Ralstonia solanacearum*+ *pseudomonas* (AAURS+AAUTR23)

T3*Ralstoniasolanacearum*+ *Trichoderma* spp. + *pseudomonas* (AAURS+AAURB20+AAUTR23)

T4 Inoculated control with *Ralstonia solanacearum* (diseased control)(AAURS) and

T5 un-inoculated control (healthy control) (DW)

According to Song *et al*., 2004, wilt incidence was calculated by the following formula:

$$\textbf{Wilt incidence}\ (\%) = \frac{\textbf{scale X number plants infected}}{\textbf{highest scale X total number of plants}}\textbf{X100}$$

$$\textbf{BE}\ (\%) = \frac{\textbf{DIC} - \textbf{Disease incidence of antagonist treated group}}{\textbf{DIC}}\textbf{X100}$$

$$\textbf{GPE} = \left(\frac{\textbf{Antagonist treated group} - \textbf{Control}}{\textbf{Control}}\right)\textbf{X100}$$

Where

BE = Biocntrol efficacy

DIC = Disease incidence of control

GPE=growth promotion efficacy

Plant growth was measured in terms of shoot height and shoot dry weight 2 months after sowing. For dry weight measurement, plants were dried in an oven at 70°C for 3 days to constant weights.

3.14. Data Analysis

All variables measured were subjected to one-way ANOVA. Duncan's multiple range tests was applied when one-way ANOVA revealed significant differences ($P < 0.05$). All statistical analysis was performed with SAS software.

4. Results and discussion

4.1. Cultural and biochemical tests for identification of *Ralstonia solanacearum*

A total of fifteen bacterial isolates were collected from infected tomato plants with bacteria wilt, of which four isolates that showed the typical cultural characteristics of virulent *R.solanacerum* were selected for *in vivo* pathogenicity studies (Table 1). These isolates exhibited pink or light red colonies or red center with whitish margin. All of them were rod shaped, gram negative, non-spore forming, motile, and catalase and oxidase positive and indole negative bacteria (data not shown).These results conformed to the characteristics of virulent strains of *R solanacearum* on TZC medium after 24 hours of incubation reported elsewhere (Kelman,1954;(Narasimha et a1.,2013).

4.2. Pathogenicity tests

The result showed that bacterial wilt of tomato occurred within 15 to 21 days after inoculation. All isolates were pathogenic on tomato plants and produced typical symptoms of wilt. Isolate AAURS1 exhibited the highest disease incidence (75% wilting) followed by 50% of wilting with APPRCRS2, whereas isolates AAURS3 and AAURS4 induced weak infection on the host (Table 3). Other reports also showed 50-71% wilting on different tomato varieties (Abo-Elyousr and Asran, 2009). El-Ariqi *et al.* (2005) also reported that different isolates of *R.solanacearum* caused 52%-97% of wilting.

Selim *et al*. (2011) have also reported that different isolates of *R.solanacearum* showed different wilt incidence ranging from 40%-96%.

Table 3: Variations in Pathogenicity of R. *Solanacearum* isolates on the Host Tomato Variety (Gelilema)

Isolate	Percentage infection	Scale	Pathogenicity
AAURS1	75	4	Highly pathogenic
APPRCRS2	50	3	Moderately pathogenic
AAURS3	25	1	Weekly pathogenic
AAURS4	25	1	Weekly pathogenic

4.3. Isolation and Screening of Plant growth promoting antagonist

A total of twenty rhizobacterial and six fungal isolates were collected and preliminarily screened for their antagonistic property on the test pathogen. They were evaluated against two isolates of *Ralstonia solanacerum* using paper disc diffusion method under *in vitro* conditions.

The data showed that the bacterial isolate, AAURB20 showed the highest mean inhibition diameter of 15mm and 16 mm followed by the fungus, AAUTR23, isolate with inhibition diameters of 14 mm against the two test pathogens AAURS1and APPRCRS2 respectively (Table 4). This implies that the antagonists have potential to be used in the greenhouse for in vivo bio protection of tomato plant .The in vitro antagonistic activity of *P. fluorescens* was also reported by Alyie *et a1*. (2008) where *P. fluorescens* isolates (PF20) had the greatest inhibition zone in vitro *against R. solanacearum* with the inhibition diameter of 14.15 mm and other two isolates (PR-3-I-x , PR-4-I-x) 3.2 showed and 3.5 mm respectively. This suggests that the mode of action or the type of antibacterial metabolite production may vary among the isolates tested (Williams and Asher, 1996).The inhibitory activity of *P. fluorescens* against the pathogen in the study is in line with that of Henok *et al*. (2007), Alyie *et a1*. (2008) and Yendyo *et al*. (2017) where they reported that isolates of *P. fluorescens* had significantly inhibited under the bacterial growth of *R. solanacearum* under *in vitro* condition.

The in vitro antagonistic activity of *Trichoderma sperellum* was reported by Narasimha *et a1*. (2013) that inhibit the growth of *Ralstonia solanacerum* with inhibition zone ranging from 11mm-27mm diameter.

Table 4: Antagonistic activity of antagonists against *R. Solanacerum* under *in vitro* condition grown on NA medium and incubated at 28°c for 2 days

Isolates	Group	Inhibition zone in mm(mean±SD)	
		AAURS1	APPARCRS2
AAURB1	Rhizobacteria	9.0 ± 0.00^{cdef}	7.5 ± 0.70^{d}
AAURB2	"	6.5 ± 0.71^{fg}	$7.\pm1.41^{de}$
AAURB3	"	9 ± 0.00^{cdef}	$10.\pm0.00^{bcd}$
AAURB4	"	7.5 ± 2.12^{defg}	7.5 ± 0.70^{d}
AAURB5	"	0	
AAURB6	"	9.5 ± 1.41^{cdef}	8.00 ± 1.41^{d}
AAURB7	"	8.5 ± 0.71^{cdef}	10.5 ± 0.70^{bcd}
AAURB8	"	10 ± 0.00^{cdef}	10 ± 0.00^{bcd}
AAURB9	"	7 ± 1.41^{efg}	8.5 ± 2.12^{cd}
AAURB10	"	0	
AAURB11	"	9.5 ± 0.62^{cdef}	7.5 ± 2.12^{d}
AAURB12	"	8 ± 0.00^{cdef}	9.0 ± 0.00^{bcd}
AAURB13	"	7.5 ± 0.70^{defg}	9.0 ± 1.41^{bcd}
AAURB14	"	4.5 ± 0.71^{g}	7 ± 0.00^{de}
AAURB15	"	0	2.5 ± 0.71^{ef}
AAURB16	"	9.5 ± 0.66^{cdef}	10 ± 0.00^{bcd}
AAURB17	"	8 ± 1.41^{cdef}	8 ± 2.83^{d}
AAURB18	"	9.0 ± 0.04^{dce}	7.5 ± 0.71^{d}
AAURB19	"	11 ± 0.30^{bc}	13 ± 1.41^{abc}
AAURB20	"	$15.\pm0.71^{a}$	16 ± 0.70^{a}
AAUTR21	Fungi	9.5 ± 0.71^{cdef}	10 ± 0.00^{bcd}
AAUTR22	"	9 ± 0.00^{cdef}	10 ± 1.41^{bcd}
AAUTR23	"	14 ± 1.41^{ab}	13.5 ± 0.70^{ab}
AAUTR24	"	10 ± 1.41^{cde}	8 ± 1.41^{d}
AAUTR25	"	10.5 ± 0.70^{cd}	9.5 ± 0.70^{bcd}
AAUTR26	"	10.5 ± 0.70^{cd}	9.5 ± 0.14^{bcd}

Data are presented as mean value ±standard division of three replicates. Values with different letters within each column indicate significant difference at $p < 0.05$.

4.4 Morphological and biochemical characterization *P.fluorescens*

Based on the antagonistic potential characteristics, twelve isolates of *P. fluorescens* were studied in detail for colony, colour, growth type, cell shape, and fluorescens of the isolates. Those all the isolates showed similar results with regard to round yellow colony texture on King,s B medium with production of fluorescent pigment gelatin liquefaction positive, catalase, oxidase, gram stain negative, positive KOH and lack of growth at

41^{o}c.This, together with rod shape cell morphology and fast growth further confirmed the isolates to be *Pseudomonas fluorescens* as reported by earlier workers Meera and Balabaskar (2012).

4.4. Carbohydrate fermentation test for bacterial isolates

The isolates utilized the tested carbohydrates and produced yellow color on the medium, which was an indication of the utilization of each carbohydrate. All isolates were capable of utilizing glucose followed by maltose, fructose and lactose (Table 5). The utilization of different carbohydrate sources by the isolates was similar with *P. fluorescens* reported by Henok et al., (2007).

Table 5: Carbohydrate fermentation test results of different indigenous bio-control agents.

Isolates	Fructose	Glucose	Lactose	Maltose
AAURB 1	+	+	±	+
AAURB 3	+	+	+	+
AAURB6	+	+	+	+
AAURB 7	+	+	+	+
AAURB 8	+	+	+	+
AAURB 11	±	+	±	±
AAURB 12	±	+	+	+
AAURB16	±	+	±	±
AAURB 17	+	+	+	+
AAURB 18	±	+	±	±
AAURB19	+	+	+	+
AAURB 20	+	+	+	+

Note: +=positive,-=negative± intermediate reaction

4.6. Morphological characterization of fungi

The fungal isolates were characterized by fast growth with dark green mycelia colony on PDA. Microscopic study revealed that it produced globes to ellipsoidal conidial shape, which was much branched.

Table 6: Morphological Characterization of Fungi

Isolate characters	AAUTR21	AAUTR22	AAUTr23	AAUTR24	AAUTR25	AAUTR26
Colony growth rate(cm)	8-9cm in 6 days	8-9cm in 6 days	8-9cm in 3 days	8-9cm in 5 days	8-9cm in 4days	8-9cm in 4 days
Colony colour	green	green	gark green	dark green	dark green	dark green
Reverse colony colour	colorless	colorless	colorless	colorless	colorless	colorless
Conidiopore	branched	branched	Branched	Branched	Branched	Branched
Conidial shape	globes to ellipsoidal	globes to ellipsoidal	globes to ellipsoidal	ellipsoidal	globes to ellipsoidal	globes to ellipsoidal

4.7. PGPR characterization of rhizbacteria

Among isolates that were screened for their plant growth promoting activities viz., HCN production, ammonia production, phosphate solubilization. Isolate AAURB20 and AAURB19 exhibited strong HCN production followed by isolates AAURB8 and AAURB16 (Table 7). Among test isolates, AAURB 7 and AAURB 20 displayed three PGP characters; whereas most of the isolates exhibited only one of the PGP characters (Table 5). The strains of *P. fluorescence* isolated from rice fields are found to produce HCN against *S. oryzae* Meera and Balabaskar (2012).

Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia. In this study, isolate AAURB7 and AAURB20 produced ammonia. Another study showed that 95% of the isolates from the rhizosphere of rice crops produced ammonia (Joseph *et al*., 2007).

Phosphorous is a major essential macronutrient for biological growth and development. With regard to solubilization of inorganic phosphate four isolates 4 (33%) (AAURB7, AAURB16, AAURB19, and AAURB20) of were able to solubilize phosphate in the plate-based assay, by showing a clear halo zone around the colony. Several species of

Pseudomonas such as *P. fluorescens*, *P. aeruginosa* and *Bacillus* species have been reported as good phosphate solubilizers in agricultural soils (Jha *et al*., 2013).

Table 7: Characterization of rhizobacteria for their PGPR characters

Isolates	HCN production	NH3 production	Inhibition zone(mm)	Phosphate solubilization	Multiple PGP characters
AAURB 1	+	+	9	-	2
AAURB 3	+	+	9	-	2
AAURB6	+	-	9.5	-	1
AAURB 7	+	+++	8.5	+	3
AAURB 8	++	-	10		1
AAURB 11	+	-	9.5	-	1
AAURB 12	+	-	8	-	1
AAURB16	++	+	9.5	+	3
AAURB 17	-	+	8	-	1
AAURB 18	-	+	9	-	1
AAURB19	+++	++	11	+	3
AAURB 20	+++	+++	15	++	3

Note +: low production: ++: medium production: +++: strong production: -: no production

4.8. Compatibility test

The compatibility test between the selected isolate, AAURB20 and selected fungal isolate AAUTR23 indicated that, the colonies of the fungus and the bacterium met on the 7th day without showing inhibitory activity with one another. This observation was the basis for testing a combination of the two antagonists as "mixed culture" in the greenhouse trial. Similary, under *in vitro* compatibility between *T.viride* and *P fluorescens* was reported by Ephrem *et al*. (2011) there was no inhibition between them.

4.9. Effects of isolates on disease incidence and biocontrol Efficacy

The biocontrol efficacy and antagonistic effect of the treatments on disease incidence was highly significant ($p \leq 0.05$) when compared to the control treatments. The highest disease incidence of 80% and 60 % was recorded from the control (Pathogen infection only) on

Galilea and Gelelima varieties, respectively. All treatments reduced disease incidence ranging from 13%-35%; and biocontrol efficacy of 48%-72% (Table 8). Similar results also reported by Selim *et al*. (2011) plants treated with PGPR isolates significantly disease reduced ranging from (15%-57%) compared to infected control, as well as greater amount of biomass compared to the control.

The combined treatments exhibited the lowest value (13.33%) of disease incidence as well as the highest value (72.22%) of biocontrol efficacy against *R.solanacearum*, on Gelelima variety. While isolate AAURB20 exhibited the highest (31.11%) disease incidence and lowest value (61.11%) of biocontrol efficacy on Galilea variety, and 35% and 48% on Gelelima variety, respectively (Table 8). The results could be attributed to the synergistic effect between the combinations of the two microorganisms in this treatment. These results were in harmony with those reported by (Yendyo *et al*., 2017) that *Trichoderma* spp and *P. fluorescence* seems to be more effective than treatment using each individual biocontrol agent that has been achieved 97% of biocontrol efficacy. The dual application of *T. viride* and *B. subtilis* decreased the percentage of pathogen infection and increased survival rate than single inoculation in tomato (Morsy *et al*., 2009). Another study showed that the number of wilted chickpea infected with *Fusarium oxysporium* plants was reduced by 67.93% due to inoculation/suppression by *T. harzianum* (Subhani *et al*., 2013). The highest percentage of disease incidence was found on galilee variety, which may be due to variety resistance. These results were in harmony with those reported by (Chatterjee *et al.,* 1997) which stated that differences of wilt incidence and severity were due to diversity of host plants, the virulence of the pathogen, and other environmental factors.

Table 8: Effect of AAURB20, AAUTR23, and their combination (AAURB20+AAUTR23) on disease incidence

Treatment	Disease incidence (%)		Biocontrol efficacy (%)	
	Galilea variety	Gelelima variety	Galilea Variety	Gelelima Variety
AAURs1+AAURB20	31.11[c]	35.56[c]	61.11[b]	48.1[c]
AAURs1+AAUTR23	26.67[cd]	22.22[d]	66.67[ab]	63.0[b]
AAURs1+AAURB20+AAUTR23	22.22[d]	13.33[de]	72.22[a]	70.37[a]
AAURs1(control)	80[a]	60.00[b]	-	-

Data are presented as mean value of three replicates. Values with different letters within each column indicate significant difference at $p < 0.05$.

4.10. Plant growth promotion efficacy of antagonists in greenhouse condition

Results of this experiment showed that antagonists (bioagents) stimulated plant growth promotion under greenhouse conditions and indicated that tomato plants treated with rhizobacteria and Trichoderma strains significantly grew better than control biomass increase of tomato plants treated with rhizobacteria and Trichoderma strains are shown in (Table 9).

Table 9: Effect of plant growth promotion of antagonists on tomato

Treatments(pathogen s+ bioagents+variety)	Plant height(cm)		Plant dry weight(g)	
	Mean	GPE (%)	Mean	GPE (%)
AAURs1+AAURB20+V1	54±2.65[bcd]	26.5	9.46±0.73[abc]	52.21
AAURs1+AAUTR23+V1	54±2.65[bcd]	40.35	9.54±0.65[abc]	51
AAURs1+AAURB20+AAUTR23+V1	67±3.81[a]	55.4	11.25±1.23[ab]	47.6
AAURs1+V1	43±3.61[c]		6.27±1.20[c]	
Distil water+V1	55.67±3.21[abcd]		10.22±3.25[ab]	
AAURs+AAURB20+V2	58±3.46[ab]	30.36	10.79±1.24[ab]	42.4
AAURs+AAUTR23+V2	64.67±4.16[abc]	45.27	12.18±1.82[ab]	66.2
AAURS1+AAURB20+AAUTR23+V2	72.33±3.23[a]	61.66	12.73±0.48[a]	81.5
AAURs1+V2	44.67±2.31[cd]		7.81±1.42[bcd]	
Distil water+V2	57.33±4.13[bcd]		11.65±2.61[ab]	

Data are presented as mean value ± standard division of three replicates, and each replicate contains three plants. Values with different letters within each column indicate significant difference at $p < 0.05$.GPE= plant promotion efficacy.V1=Galilea variety, V2=Gelelima variety.

Significant differences ($P \leq 0.05$) among treatments regarding plant height and biomass were observed. Plants treated with combined isolates of AAURB20+AAUTR23 showed the highest values of plant height, and dry weight (72.33 cm, and 12.73 g) respectively, when compared with the control (AAURS1) and plants treated by individual isolates AAURB20 and AAUTR23 in variety two (Table 7). Likewise plants treated with isolates AAURB20+AAUTR23 showed high GPE (%)(62%, and 81.5%) for height, and dry weight respectively in variety two (Table 9).

Significant differences ($P \leq 0.05$) among treatments regarding plant height and biomass were also noted on variety one (Table 7). Plants treated with combined isolates of AAURB20+AAUTR23 presented the highest values of plant height, and dry weight (67 cm, and 11.25 g) respectively, when compared with the control (AAURS1) and plants treated by individual isolates AAURB20 and AAUTR23 in variety one (Table 9). Generally combined treatments showed best performance compared to individual treatments. Significant differences were observed in the vegetative growth parameters due to the inoculation of isolated bio-inoculants. This result was in harmony with that of (Nguyen and Ranamukhaarachchi, 2010**)** on tomato the use of beneficial microorganisms as biocontrol agents led to enhance plant growth parameters (70.4cm plant height and 19.5g of dry weight). Such enhancement may be due to induce plant resistance (De Meyer et al., 1998), production of extracellular enzymes and antifungal or antibiotics, which reduce the negative effect of biotic stress on plant and produce growth promoting substances (Szczech and Shoda, 2004).Similar results also reported by (Selim et al.,2011)plants treated with PGPR isolates significantly reduced disease compared to infected control, as well as caused greater amount of biomass compared to the control.

5. Conclusion and recommendations

In this study a total of fifteen bacterial isolates were collected from infected tomato plants with bacteria wilt, of which four isolates that showed the typical cultural characteristics of virulent *R.solanacerum.* Isolates exhibited pink or light red colonies or red center with whitish margin. All of them were rod shaped, gram negative, non-spore forming, motile, and catalase and oxidase positive and indole negative bacteria.

The pathogenicity test results showed that bacterial wilt of tomato occurred within 15 to 21 days after inoculation. All isolates were pathogenic on tomato plants and produced typical symptoms of wilt. Isolate AAURS1 exhibited the highest disease incidence (75% wilting) followed by 50% of wilting with APPRCRS2.A total of twenty *pseudomonas* and six *Trichoderma* isolates were preliminarily screened for their antagonistic property on *Ralstonia solanacearum*, of which isolate, *pseudomonas* (AAURB20) and *Trichoderma* (AAUTR23) gave the highest mean inhibition diameter of 15mm and 16 mm against the test pathogen.

The isolates utilized the tested carbohydrates and produced yellow color on the medium, which was an indication of the utilization of each carbohydrate. Among isolates that were screened for their plant growth promoting activities viz., HCN production, ammonia production, phosphate solubilization. Isolate AAURB20 and AAURB19 exhibited strong HCN production. Four isolates 4 (33%) (AAURB7, AAURB16, AAURB19, and AAURB20) of were able to solubilize phosphate. In green house study combined treatments of Trichoderma and Pseudomonas spp (AAURB20+AAUTR23) showed best performance and reduced disease incidence by 13.33 % and increased the bio-control efficacy by 72.22 % when compared with individual treatment and negative control (un inoculated treatment). The isolates significantly increased the plant height and dry weight by 72.33 cm, and 12.18 g, respectively. Thus, the combined use of the biocontrol agents significantly reduced the incidence of tomato bacterial wilt disease .Therefore the use of this bioagent would be important for to manage bacterial wilt at greenhouse conditions. However, further study will be required to uses the bio-agent in the field condition for bio-control development program.

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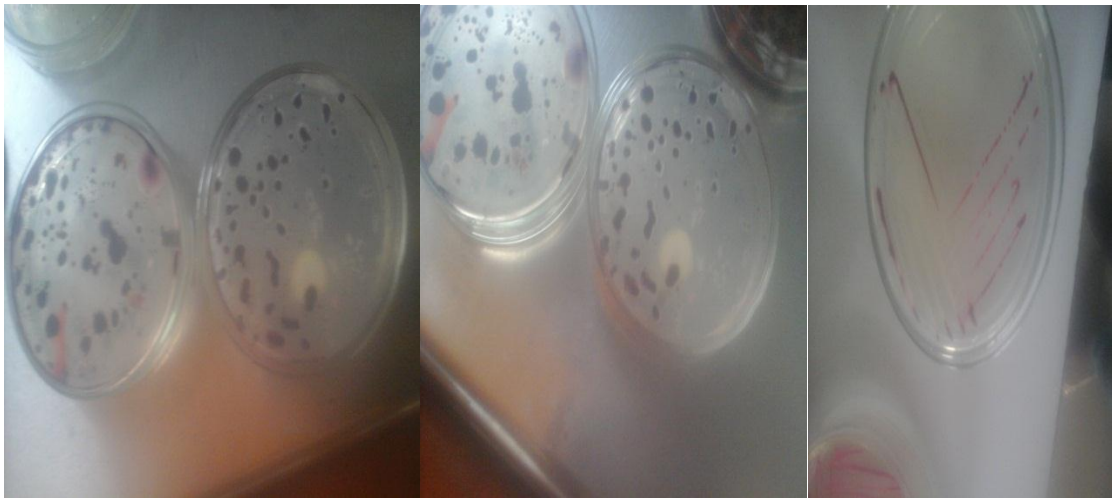
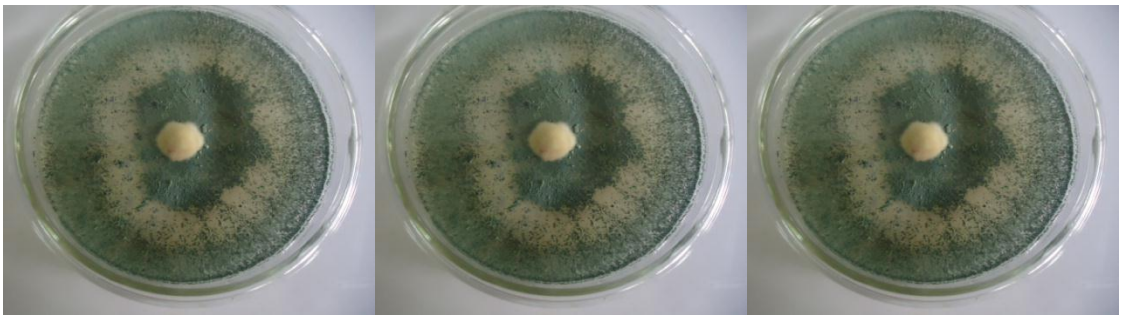
7. APPENDICES

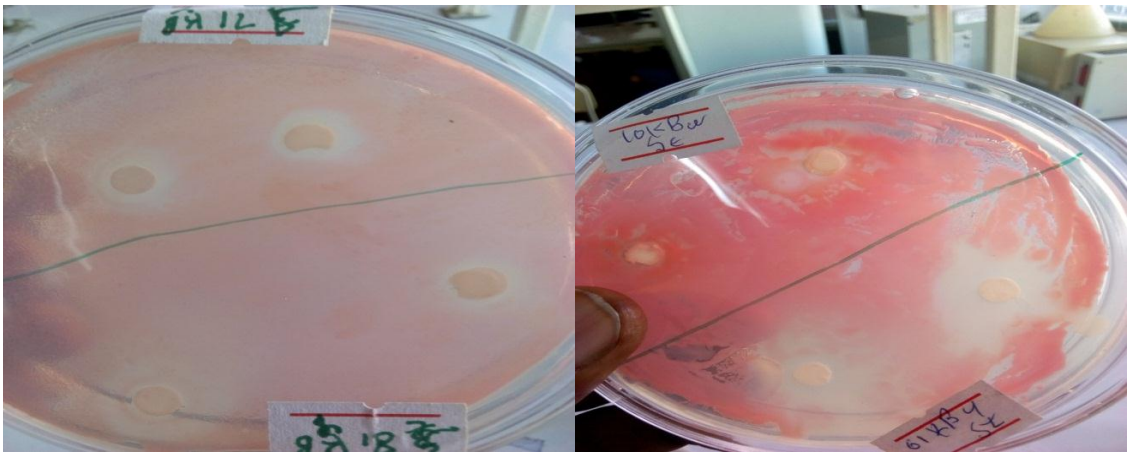
Appendix 1: Sample collection



Appendix 2: Virulent isolates of *R.solanacearum*



Appendix 3: Trichoderma isolates



Appendix 4: Inhibition ability of antagonists against *R.solanacearum*

Appendix 5: PGPR character of antagonists

Appendix 6: Carbohydrate utilization test of antagonists

Appendix 7: Other laboratory works

Appendix 8: Effect of antagonists to suppress *R.solanacearum*

Isolates	Colony type	Colony colour	Type of growth	Growth at $41^{O}C$	Flourecent pigment	Cell shape	KOH	Gm stain	Catalase	Oxidase	Motility	Gelatin liquification
AAURB 1	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 3	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB6	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 7	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 8	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 11	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 12	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB16	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 17	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 18	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB19	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+
AAURB 20	Round	Yellowish	Fast	-	+	Rod	+	-	+	+	+	+

Appendix 9: Morphological and biochemical tests of Pseudomonas