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Characterization of Bacterial Wilt Pathogen (*Ralstonia solanacearum L.*) of Ginger (*Zingiber officinale* Roscoe) and Its Controls Using *Trichoderma* and *Pseudomonas* Isolates

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
ANOVA	Analysis of Variance
BV	Biovar
BWG	Bacterial Wilt of Ginger
CABI	Centre for Agricultural Bioscience International
CFU	Colony Forming Unit
DAI	Day after inoculation
DSR%	Percentage of disease severity reduction
EPPO	European and Mediterranean Plant Protection Organization
EPS	Exopolysaccharides
g	Gram
Hr	Hour
KBM	King‟s B medium
KOH	Potassium hydroxide
Ml	Millilitre
RS	*Ralstonia solanacearum*
rRNA	ribosomal ribonucleic acid
AAURS-03	Addis Abab University *Ralosonia solanacearum*
SNNPRS	Southern Nations, Nationalities and Peoples Regional State
TTC	Triphenyl tetrazolium chloride
TZC	Tetrazolium chloride
USA	United States of America
UV	Ultraviolet
W|V	wave length
WD	weight of the diseased rhizome
µg	Micro gram
Spp	Species
w/v	weight by volume

%	Percent
R	correlation coefficient
Rmp	Revolution per minute
s	Seconds
SE	standard error
p	p- value (statistical significance level)
Mm	Millimeter
DLA	Diseased leaf area
CV	coefficient of variation

ABSTRACT

Ralstonia solanacearum (Rs) race 4 causes bacterial wilt of edible ginger (Zingiber officinale) and limits the crop production in the tropics. The present study was undertaken to isolate and characterize ginger bacterial wilt agent (Ralstonia solanacearum) and to evaluate inhibition of Ralstonia solanacearum by Trichoderma and Pseudomonas isolates in a green house and laboratory experiments in 2014/15 season. Totally, 10 isolates of Trichoderma and 10 isolates of Fluorescent pseudomonas were isolated and characterized. Inhibitory effect of the biocontrol agents was evaluated in vitro *following agar well diffusion method for their efficacy against* Ralstonia solanacearum*. The experimental design was complete randomized design (CRD) with three replications. Data on yield and quality parameters, disease reactions, biocontrol efficacy had been collected and analysed. Based on the result of In vitro inhibition test only four isolates that inhibit the growth of the pathogen were selected and used under the green house experiment. In vitro antagonistic activities of Pseudomonas fluorescens1 and Pseudomonas fluorescens2 showed the maximum inhibition of Ralstonia solanacearum with an inhibition zone of 30 and 39.5 mm respectively. Among antagonists tested P. fluorescens, were found to be more effective in the reduction of disease severity and also increased the biocontrol efficiency when compared to untreated control by 44.4 and 92%, respectively. Also, application of antagonists significantly increased plant yield by 1.6 ton/h. ANOVA showed that a wisely designed combined treatment was found to be more effective than single treatment application. Overall, the result showed that the selected antagonistic isolates managed to suppress the disease caused by the pathogen, R. solanacearum effectively. Thus, it can be concluded that the incidence and severity of bacterial wilt of ginger disease can significantly be reduced by the combined use of antagonists in an equal suspension with foliar spray in order to have a higher yield and with minimum health risk as well as environmental pollution. Cultural characteristics of isolates on Tetrazolium Chloride (TZC) Agar selective medium showed light to red color with the characteristic red center which resembles the pathogen Ralstonia solanascearum. Biochemical and physiological characteristic revealed that similarities among isolates and strains were grouped in biovar III and corresponds to race 4 of the pathogen. Hence, further study is necessary to check the ability of the anatagonists to control the disease under field conditions.*

Keywords: bacterial wilt, biocontrol, biovar, ginger, *race 4, Ralstonia solanacearum*

1. INTRODUCTION

Ginger (*Zingiber officinale Rosc*.), belonging to the family Zingiberaceae, is an important commercial crop grown for its aromatic rhizomes which are used as a spice and a medicine (Sharma *et al.,* 2010). Ginger rhizome is typically consumed as a fresh paste, dried powder, slices preserved in syrup, candy (crystallized ginger), as a beverage or as flavoring agent. It is cultivated in almost all the tropical and subtropical parts of India(Tarafdar and Saha, 2007). India is the largest producer of ginger in the world accounting for about one-third of the total world output followed by Thailand and Japan. It is an important crop that earns a sizeable amount of foreign exchange for the country (Tarafdar and Saha, 2007). Ginger is known to have been introduced to Ethiopia as early as in the 13th century (Jansen, 1981). It is cultivated in South, Southwestern and Northwestern parts of Ethiopia as cash crop, and is among the important spices used in every Ethiopian kitchen for the preparation of pepper powder, stew, bread, etc. It has also some use in traditional medicine for the treatment of flu and stomach ache (Jansen, 1981; Girma Hailemichael and Digafie Tilahun, 2004).

Ginger is one of the most important spices, largely for small scale farmer in Ethiopia. The crop is mainly grown in the southern part of the country and it is dominant in the cropping system. In the Southern Nations and Peoples‟ regional states (SNNPRS), 85% of the total arable land often allotted for ginger production and 35% of the farmers in the region produce ginger(Endrias Geta and Asfaw Kifle, 2011). From 2006 to 2008 a total of 0.41 million ton of fresh gingers have been produced from an area of 27,229 hectare in limited woredas in the region with a mean yield of 1.6 ton/ ha. The yield per unit area is low by any standard; however, the land under the crop has increased faster. This shows the important role ginger plays in export market for the country and help to generate income at house hold level(Endrias Geta and Asfaw Kifle, 2011).

The production of ginger, however, is largely affected by diseases caused by bacteria, fungi, viruses, mycoplasma and nematodes, the crop suffers from diseases like bacterial wilt caused by *Ralstonia solanacearum*, rhizome rot caused by *Pythium* species, *Fusarium* species, *Sclerotium* species, *Pseudomonas* species and others (Senapati and Ghose, 2005; Paret *et al*., 2010; Sharma *et al.,* 2010; Kavyashree, 2011).Ginger (*Zingiber officinale* Rosc.), an important horticultural crop in South East Asia, produces pungent, aromatic rhizomes, which are valuable both as a spice and as aherbal medicine. Ginger wilt caused by *R. solanacearum* was first reported in the

1950s and since then it has become increasingly serious in China. Disease incidence in the field usually ranges from 10% to 40% but the disease is also known to destroy the crop completely (Zhang *et al.,* 2001). In addition, the disease could reduce the quantity of the ginger, resulting in huge economic losses. Bacterial wilt disease of edible ginger causes severe economic damage inmany countries, including China, India, Indonesia, Japan, Malaysia, Mauritius, the Philippines, and the United States (Hawaii) (Kumar and Sarma, 2004; Alvarez *et al.,* 2005). Bacterial wilt is the main causal agent of ginger wilt in tropical, subtropical, regions (Agrios, 2005). In the absence of susceptible crops, alternative weed hosts and non-host plants and soil played an important role for the survival of *R. solanacearum* strains (Granada and Sequeria, 1983).
In Ethiopia,bacterial wilt caused by Ralstonia solanascerum (Smith) has been reported on Potato, Tomato, Pepper, Enset, Banana and Ornamentals but on ginger this is the first report(Bekelle Kassa *et al*.,2016). *Ralstonia solanacearum*, the one of causal agent of bacterial wilt disease, is a severe obstacle to the production of solanaceous plants in both tropical and temperate regions. As a diverse species complex, *R. solanacearum* has developed an extremely broad host range throughout the world, including >450 host species representing 54 plant families (Wicker *et al.,* 2007).

Ralstonia solanacearum is classified into five races based on the hosts affected, and five biovars based on the ability to use or oxidize several hexose alcohols and disaccharides (Agrios, 2005). Race1 strains (biovars 1, 3, and 4) are pathogenic to a broad range of hosts, including tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), and peanut (*Arachis hypogaea*); race 2 strains (biovars 1 and 3) infect banana (*Musa acuminata*), plantain (*Musa paradisiaca*), heliconia (Heliconia spp.), and other plants in the Musaceae family; race 3 strains (biovar 2) occur in cool upland areas in the tropics and cause severe wilt in potato (*Solanaum tuberosum*), tomato, and geranium (*Geranium* spp.); race 4 strains (biovars 3 and 4) infect ginger; and race 5 strains infect mulberry (*Morus alba*). According to Stirling (2002), in the years 1997 and 1998, some ginger growers in Queensland (Australia) experienced severe losses of newly planted „seed pieces". Kumar *et al*., (2004) also reported bacterial wilt of ginger caused by *Ralstonia solanacearum* that is widely distributed in tropics, subtropics and temperate regions worldwide. According to Rahaman *et al.,* (2009), over the last few years, rhizome diseases have affected the crop in many states of India resulting in decline of rhizome yield from1:8 ratios (seed rhizome to harvested rhizome) to 1:4.

Fikere Lemessa and Zeller (2007 and Paret *et al*. (2010) have identified sixty two *R. solanacearum* strains from Ethiopia and 19 of them grouped in to biovar1 and the rest of the strains to biovar 2. It was further observed that biovar 2 strains had limited host range (affecting mainly potato) compared to biovar 1 strains (affecting eggplant, tomato and potato) but so far race 4 is not yet reported in association with ginger or other crops in Ethiopia.

A ccording to Habetewold kifelew *et al*. (2015), bacterial wilt of ginger was found widespread in all area surveyed in Ethiopia. Among the different places surveyed, wilt incidence were found ranged from 93.5% sheka zone to 10.7 Gamogofa zone in 2012 survey season. The first bacterial wilt syndrome were reported from Benchmaji zone Bebeka coffee estate farm, then it progress to the neighboring zone Sheka within a short period of time and cause up to 67% yield loss. During 2012 the diseases were less prevalent around Wolayta zone even if the areas have been known for producing ginger as a major crop. Survey results of SNNPRS (Dawro, Wolayta, Kenbatatenbaro, Hadiya, Gomogofa, Konta, Alaba, Sheka, and Bench maji) zone the crop was devastated by the diseases and caused yield loss up to 98%. (Bekelle Kassa *et al.*,2016).

Biological control 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 (Barea and Jeffries, 1995).

Plant beneficial microbes (PBM), a component of extensive microbial biodiversity, affect plant health and development and the importance of these microbes in agriculture is continuously growing. Some PBM like, strains of fluorescent pseudomonads, *Trichoderma* spp. are known antagonists against soil borne plant pathogens including *R. solanacearum* and attempts have been made throughout the world to explore the possibilities of using these saprophytic antagonists for crop disease management(Nautyal,2000; Bora and Bora, 2008; Bora *et al*., 2013).

Hidaka and Murano (1956) studied *in vitro* effect of streptomycin on the behaviour of *R. solanacearum*, and they found that streptomycin at 0.3 µg/ml water inhibited or killed *R. solanacearum.* Singh *et al.* (2000) found that streptomycin and streptopenicillin were superior over other antibiotics against the pathogen under *in- vitro* and *in- vivo* conditions. Traditionally R. solanacearum infections are frequently treated by applying streptomycin (Diogo and Wydra 2007; Chen *et al*., 2008). However, the use of streptomycin is assumed to contribute to the

development of antibiotic resistance in the pathogen and to an increased abundance of antibiotic resistant soil bacteria. An alternative strategy for controlling the bacterial wilt is the use of resistant varieties (Sisson, 1999 and Bhatti *et al.,* 2011). In Ethiopia currently ginger plants are observed to be attacked by pathogen and the intensity of disease in Ethiopia is increasing from time to time, this may be due to introduction of new strains to, Ethiopia from other parts of the world through latently infected planting materials. However, there is no other studiy conducted on the characterizations of bacterial wilt of ginger and its bio control agents using the bacterial and fungal antagonists in South western Ethiopia. Therefore, the present study was undertaken to characterize morphological and biochemical strains of *Ralstonia solanacearum* (*Smith)* and its biocontrols using bacterial and fungal antagonists.

2. OBJECTIVES OF THE STUDY

2.1. General Objective

The general objective of this study is to undertake morphological and biochemical characterizations of *Ralstonia solanacearum* strains (*Smith)* and their biocontrols using bacterial and fungal antagonists

2.2. Specific Objectives

The specific objectives of the study are to:

- examine the morphological, physiological and biochemical differences among some *R. solanacearum* pathogen strains of ginger.
- study pathological variability of the bacterial wilt of ginger strains and conduct pathogenicity test on ginger cultivars.
- identify the biovar (s) of *R. solanacearum* infecting the ginger cultivars in study area
- evaluate potential biocontrol antagonists alone and combined with bactericides against bacterial wilt (*R. solanacearum)* of ginger (*Zingiber officinale* Roscoe) *In vitro* and *In vivo* conditions.

3. LITERATURE REVIEW

3.1. Origin and Production of Ginger

Ginger originated in South-East Asia, probably in India. Today ginger is cultivated in several parts of the world, the most important producing regions being India, China, Nigeria, Sierra Leone, Indonesia, Bangladesh, Australia, Fiji, Jamaica and Nepal. Among them India and China are the dominant suppliers to the world market (Peter, 2001). Ginger is the second most widely cultivated spice in Ethiopia, next to chillies (Biruk Ayenew *et al.*, 2012). Endrias Geta and Asfaw Kifle of Haramaya University who conducted a survey in 2011 on the Production, Processing and Marketing of Ginger in Southern Ethiopia, concluded that ginger had been produced as an important commercial horticultural crop in southern Ethiopia. Farmers produce different varieties and apply traditional management practices (Endrias Geta and Asfaw Kifle 2011).

3.1.1. Botany of Ginger

Ginger is a monocotyledon plant, which consists of more than 300 plant species in 53 genera and belonging to the family *Zingiberaceae* and to the order *Zingiberales*. In the *Zingiberaceae*, it belongs to the subfamily *Zingiberoideae*, which are aromatic with unbranched aerial stems, distichous leaves, open sheaths and hypogeal germination, mainly confined to the old world tropics. Among them, ginger is a slender perennial herb, 30-100 cm tall with branched rhizomes 22 bearing leafy shoots. The leafy shoot is the pseudo-stem formed by leaf sheath and bears 8-12 distichous leaves (Emmanuel *et al.*, 2014).

3.1.2. Dietary Use and Health Benefits of Ginger

Both fresh and dried ginger rhizomes are used worldwide as a spice, and ginger extracts are used extensively in the food, beverage, and confectionary industries for the production of marmalade, pickles, chutney, ginger beer, ginger wine, liquors, biscuits, and other bakery products (Mesomo *et al.*, 2013). In Ethiopia it is among the important spices used in every kitchen to flavor stew, tea, bread and local alcoholic drinks (Nigist Asfaw and Berhanu Abegaz, 2003). Medicinally, it is used mainly to relieve stomach ache, fever, influenza, headache, coughs and tooth ache (NigistAsfaw and Sebsebe Demissew,2009). though it is used also for anorexia, arthritis, atonic dyspepsia, bleeding, cancer, chest congestion, chicken pox, cholera, chronic bronchitis, cold

extremities, colic, colitis, common cold, cough, cystic fibrosis, diarrhoea, difficulty in breathing, dropsy, flatulent, disorders of gall bladder, hyperacidity, hypercholesterolemia, hyperglycemia, morning sickness, prevention of motion sickness, nausea, rheumatism, sore throat, throat ache, stomach ache and vomiting in pregnancy (Haniadka *et al.*, 2013).

3.1.3. History of Ginger in Ethiopia

Ethiopia is one of the fast developing countries in Africa. The small and medium sized production and distribution is generally considered the engine of economic growth, as well as a means for poverty reduction by virtue of their numbers and their significant economic and social contributions. Ginger plays a significant role. Ethiopian agriculture contributing 42% share in the gross domestic product (Vijayalaxmi and Sreepada, 2012) , Ginger is an important commercial crop. A large majority of 95% vegetables, Horticulture products and commercial crops like ginger, produced in the country come from the smallholder(Endrias Geta and Asfaw Kifle, 2011). Ginger is one commonly used ingredient in Ethiopian recipe. In Ethiopia, Ginger locally called Gingibaar. Tea brewed from ginger is a common folk remedy for colds in Ethiopia. It is commonly known as Kasherchai. Ginger ale and ginger beer are also drunk as stomach settlers in countries where the beverages are made. Ginger has pungent taste and interesting aroma. In addition, ginger has been used historically for its medicinal value in a wide variety of diseases, especially in gastrointestinal disorders, such as constipation, diarrhea, anorexia, colic, dyspepsia, nausea, vomiting, and motion sickness and remains an important cooking spice around the world (Endrias Geta and Asfaw Kifle, 2011).

Agriculture is the backbone of the Ethiopian economy. It provides employment opportunities. The production of these commodities should be encouraged in labor abundant countries like Ethiopia. Ginger plays an important role in contributing to local economy. Actually it has Export potential, value added to economic growth, it creates lot of job opportunity locally, linkages with other sectors, supply capacity impact on gender empowerment, accessibility and government priority on small farmers is the benefit in Ethiopia (Vijayalaxmi and Sreepada, 2012). Ginger cultivation in Ethiopia started during 13th century, arabs introduced it from India. Ethiopia is the gate way for many Asian and many East European Countries (Jansen, 1981). Yellow and brown areas of Ethiopia are most suitable. Yellow area is Low rainfall or irregular climate and Brown area is Large grazing areas with irregular climate (Vijayalaxmi and Sreepada, 2012). Ginger

cultivated in many places in Ethiopia, the specific area in Ethiopia has identified as Bahir Dar, Dejen, Debere Markos, Kola Dega Damot, Metekel and Agew Mider, Gamo, Galeb and Hamer Bako, Gofa and Kulo Konta, Wabe, Dolo and Genale, Sidama and Arero Gimbi, Nekemte, Horo Guduru and Arjo and all regions of Ilubabuor and Jima Zone (Vijayalaxmi and Sreepada, 2012).

During 2011, Endrias Geta and Asfaw Kifle conducted a survey on Production, Processing and Marketing of Ginger in Southern Ethiopia, concluded that, Ginger has been produced as an important commercial horticultural crop in southern Ethiopia (Endrias Geta and Asfaw Kifle, 2011).

Farmers produce different varieties and apply traditional management practices. External support in promoting improved varieties and management practices is almost nonexistent. Ginger product markets are very volatile and price fluctuations discouraged farmers to produce the crop. Thus, researchers have to assist farmers in indentifying improved varieties with desirable market traits, appropriate agronomic and post-harvest management practices including drying methods, sorting and grading techniques, as well as processing technologies(Endrias Geta and Asfaw Kifle, 2011). Extension workers and other development practitioners have join hands with the farmers in addressing marketing problems such as: easing barriers to entr into markets by organizing ginger producers into producers and marketing cooperatives (Endrias Geta and Asfaw Kifle, 2011).

3.1.4. Major Ginger-growing Areas in Ethiopia

Ethiopia is a land of diverse climate and soil type that enable prolific growth of several indigenous and exotic spices, herbs, medicinal and other essential oil bearing plants, ginger has been known in Ethiopia since the beginning of 13th century, when the Arabs brought it from India to East Africa (Sileshi Belew, 2010). The major ginger growing areas in Ethiopia include wetter regions at altitude below 2000 m in Kefa, Illuababor, Gamo Gofa, Sidama and Wellega mostly in gardens and around homesteads. However, large scale production and marketing of ginger are reported from Illubabur, Wolaita, Kembata and Tambaro (NigistAsfaw and Sebsebe Demissew,2009). There are also cultivations in Gojam and Gonder regions to cover home consumption. The production of this spice has been expanding in most parts of the country, as it can be grown under varied climatic conditions that do not have frost problem. Ginger thrives well in areas with altitudes from sea level to 1500 m, mean annual temperature of 20-32$^{o}_{C}$ and

with total rainfall greater than 1200 mm. The ideal soil type for the production of ginger is a well-drained, fertile and friable soil and with enough humus, neutral pH (Girma Hailemichael *et al.*, 2008).

3.2. Taxonomy and Diversity of Ralstonia solanacearum

R. solanacearum has not always been the name of this species. On the contrary, it has been classified in numerous genera since it was first described. Although T.J. Burrill was probably the first to isolate the bacterium in 1890, E.F(Yabuuchi *et al.,*1995). Smith was the first to publish a scientific description and classify it in the genus *Bacillus* as *B. solanacearum* in 1896. However, afterwards it was moved to the genus *Bacterium*, and to the genus *Pseudomonas* with the name of *P. solanacearum* temporary reclassified in the genera *Phytomonas* and *Xanthomonas* and eventually transferred back to the genus *Pseudomonas* in 1948. In 1992 it was placed in the genus *Burkholderia.* But, more recent phylogenetic and polyphasic phenotypic analyses pointed out that it would rather be accommodated in the new established genus of *Ralstonia*, in 1995 (Yabuuchi *et al*., 1995). Following the hypothesis that strains of this species "are the product of long evolution occurring independently in various areas on different hosts". Apart from races *R. solanacearum* strains have been traditionally classified into biovars, on the basis of the pathogen ability to utilize and/or oxidize several hexose alcohols and disaccharides. Five races and biovars have been described so far (Hayward,1991; EPPO, 2004) and Africa is the continent where the highest diversity is found (Elphinstone,2005). Both classification schemes constitute informal groupings at the infrasubspecific level. The relationship between them is only evident with race 3 strains, which generally correlate with biovar 2 phenotype (Hayward, 1991).

Classifications based on molecular techniques have also been reported, as the phylotypes or monophyletic clusters of strains, which are determined after phylogenetic analysis of sequences of particular genes. Thus, the species is divided into four phylotypes corresponding to four broad genetic groups, each of them related to a geographic origin (Fegan and Prior,2005). Phylotype I contain all strains belonging to biovars 3, 4 and 5, isolated primarily from Asia. Phylotype II includes biovar 1 and 2 strains, and 2T (a subgroup of biovar 2 for tropical areas) isolated from America, all race 3 strains pathogenic to potato and the race 2 banana pathogen. Phylotype III comprises strains belonging to biovars 1 and 2T from Africa and surrounding islands. Phylotype IV is more heterogeneous, with biovar 1, 2 and 2T strains from Indonesia, strains isolated in

Australia and Japan, and also *R. syzygii* and the blood disease bacterium (BDB) (Fegan and Prior,2005). The diverse classification schemes proposed for *R. solanacearum* (Fig. 1) reflect the great phenotypic and genotypic variation within the species, which has led to the term "*R. solanacearum* species complex" (Gillings and Fahy, 1993). Defined as "a cluster of closely related isolates whose individual members may represent more than one species"

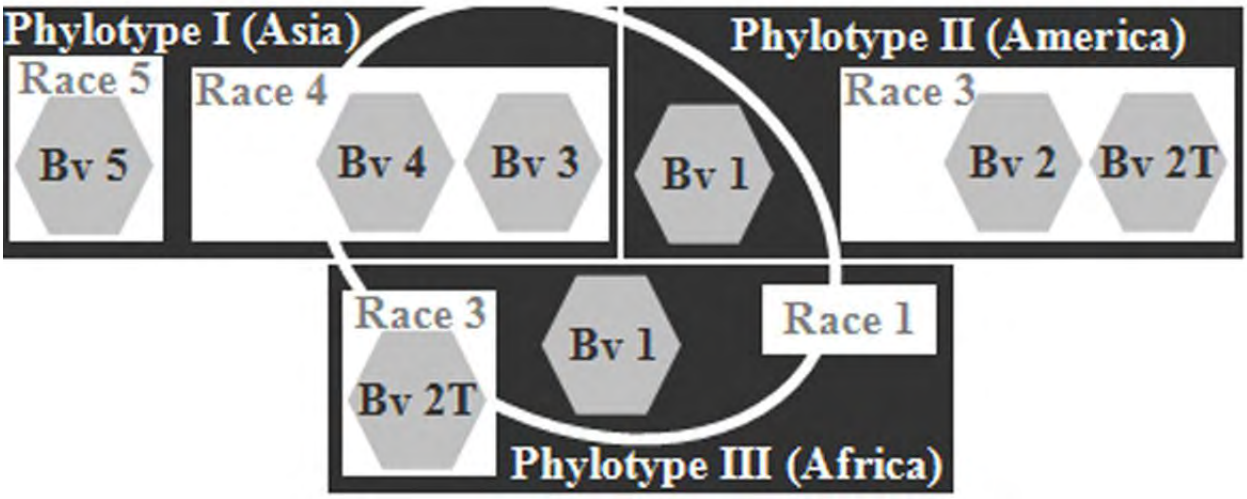

fig 1. Diversity within the *R. solanacearum* species complex.Correspondence among phylotypes, races and biovars of the pathogen. Phylotype IV is not represented because of its high heterogeneity (Fegan and Prior,2005).

3.3. Morphological and physiological characteristics

R. 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). The principal biochemical characteristics are 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°C and Kelman"s 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.

3.4. Symptoms of the bacterial wilt disease

Ralstonia solanacearum, once established in its host, causes the disease, bacterial wilt or bacterial rot which is manifested in a variety of symptoms in the different host plants (Yabuuchi *et al.*, 1995). It is a highly challenging and one of the most destructive diseases of solanaceous crops worldwide (Hayward, 2005). The disease is predominant in warm humid tropical and temperate regions of the world (Hayward, 1991). The following symptoms are based on the observations made by Kelman (1953) and can be externally or internally manifested.

3.4.1. External symptoms

The most common external symptoms of the infected plants are wilting, stunting and yellowing of the leaves, bending of the leaves downwards showing leaf epinasty, growth of adventitious roots in the stems, and the development of narrow dark stripes in the infected vascular bundles beneath the epidermis may also be observed. This results from multiplication of the bacteria in the vascular system to levels that clog the vessels (Boucher *et al*., 2001). Bacterial levels of between 10^{6} and 10^{10} cfu/ml have been reported in highly infected hosts (Clough *et al*., 1997).

Leaf spots, which may coalesce and cause defoliation, may also occur on infected leaves (Saddler, 2005). This may further result in stunting of the whole plant (Saddler, 2005). The degree of the expression of the symptoms and rate of disease development may vary depending on host susceptibility and the aggressiveness of the pathogenic strain(Kelman, 1953). In most of the cases, the disease usually progresses until complete wilting and collapse of the plant occurs. In cases where wilting of the plant does not occur, the plants usually show characteristic dwarfism and stunted growth (Kelman, 1953). Anderson and Gardner (1999) observed interveinal chlorosis which developed 5–10 days after inoculation. The youngest leaves developed chlorosis first which spread to other leaves and finally ending in wilting of the plant within 3-4 weeks following infection.

Another observation they made was the rotting of rhizomes which started in week 5 following inoculation and became advanced at the end of 8-10 weeks.

3.4.2. Internal symptom

The external symptoms result from the pathogenicity of the bacteria once inside the host, the Bacteria possess specific genes that code for hydrolytic enzymes and (*hrp*) genes that control disease development and the hypersensitive reactions (Boucher *et al.,* 2001). The internal symptoms which are a direct reaction to infection include progressive discolouration of the vascular tissue, mainly the xylem, at early stages of infection, and of portions of the pith and cortex, as the disease progresses, until complete necrosis occurs. Slimy viscous ooze typically appears on the vascular bundles of stems that have been cut across, as a result, collapse and death of the plant take place because of the degradation of occluded xylem vessels and the destruction of surrounding tissues(Boucher *et al.,* 2001). These symptoms are generally observed on leaves and stems of all of the different plant species affected by the bacterial wilt caused by the bacterium *R. solanacearum.*

3.4.3. Pathogenicity of *R. solanacearum*

R. solanacearum possesses diverse genes involved in colonization and wilting of host plants, such as those coding for lytic enzymes and EPS, hypersensitive reaction and pathogenicity (*hrp*) genes, structural genes encoding effector proteins injected by a type III secretion system (T3SS) from the bacterium into the plant cell, genes coding for factors implicated in cell adherence, and others (Schell,2000). Phytopathogenic bacteria have often developed enzymes to hydrolyze plant cell wall components to obtain nutrients and energy, which are further involved in early stages of the infection process, favouring the entry and advance of the pathogenic agent in host tissues.
R. solanacearum produces several plant cell wall-degrading enzymes, secreted via the type two secretion system (T2SS) (Boucher *et al*, 2001).

Several phytopathogenic bacterial species produce high amounts of EPSs either in pure culture or during *in planta* multiplication. Although usually related to pathogenicity, it is often difficult to know if the EPSs take active part in symptom production or if they indirectly favour infection (Schell, 2000 and Boucher *et al*., 2001). In *R. solanacearum*, it has been reported that all virulent wild-type strains (mucoid colonies) produce EPS (Poussier et al., 2003) while EPS-deficient mutants (non-mucoid colonies) are avirulent. *R. solanacearum* EPS appears to be highly heterogeneous, since it has a varying composition among strains(Poussier et al., 2003). In *R. solanacearum*, the *hrp* genes control induction of both, disease development and the

hypersensitive reaction (HR) (Boucher *et al*., 2001). Therefore, *hrp* mutants are unable to induce symptoms in susceptible host plants and a HR in resistant plants or non-hosts. HR is a plant defense mechanism that prevent the spread of pathogen infection to other parts of the plant, it is associated with plant resistance and characterized by a rapid and programmed plant cell death localized in the region surrounding the infection (Nimchuk et al., 2003). It can be visualized as necrotic areas in the plant tissues affected. *R. solanacearum hrp* mutants do not seem to be involved in the infection process, since most of them could be isolated from the stems of infected plants but, they showed an impaired ability to multiply *in planta* not observed when cultured on minimum me dia which may indicate a possible role of *hrp* genes in diverting certain plant metabolites from the plant to the bacteria (Boucher *et al.,* 1992*)*.

3.5. Distribution, host range and diversity of *R. solanacearum*

The high economic and social impact of this organism results from its wide geographical distribution around the world. Over 200 plant species, especially tropical and subtropical crops, are susceptible to one or other of the races of *R. solanacearum*. These 'races' were defined more than 50 years ago and mainly correspond to pathovars (*i.e* groups of strains adapted to a single or a group of hosts). Aspects on the biodiversity of the *R. solanacearum* species and strain classification will be given in detail below. Worldwide, the most important plants susceptible to *R. solanacearum* are: tomatoes, Musa spp., tobacco and potatoes. Some minor host crops are: *Anthurium* spp., artichoke, groundnuts, *Capsicum annuum*, cotton, rubber, cassava, castor beans, egglant, ginger, *Eucalyptus, Pelargonium, Piper hispidinervium, Pogostemon patchouli*, pumpkin, sesames, turmeric. In addition, many weeds are also hosts of the pathogen and therefore increase the potential of *R. solanacearum* to build up inoculum (Elphinstone, 2005; Hayward, 1994 and Kelman, 1953). From this list, it is apparent that the *R. solanacearum*'s host range is not restricted to solanaceous plants but encompasses many other botanical families among Dicots and Monocots. Historically, strains of *R. solanacearum* were classified into five races based loosely on host range although this classification does not, with few exceptions, correlate with the taxonomic relationship of the strains (Denny, 2006).

The five races of *R. solanacearum* have different host ranges and geographic distributions. Race 1 is a poorly-defined group with a very wide host range (attacks tobacco, many other

solanaceous crops and many hosts in other plant families) and is endemic to the southern United States as well as Asia, Africa, and South America. Race 2 principally attacks bananas, and is found mainly in Central America and Southeast Asia. Race 3 is distributed worldwide and has primarily been associated with potato, causing the brown rot disease, which ranks among the most destructive diseases of potato in Africa, Asia and Central and South America (CABI, 2003). Race 4 affects ginger in much of Asia and Hawaii, and race 5 affects mulberries in China (Denny, 2006; Champoiseau *et al*., 2010). The ability of the pathogen to utilize and/or oxidize several hexose alcohols and disaccharides also allowed to classify *R. solanacearum* strains into six biovars (Hayward, 1991). Both classification schemes constitute informal groupings at the infrasubspecific level (Hayward, 1991). The relationship between them is only evident with race 3 strains, which generally correlate with biovar 2 phenotype (Hayward, 1991). Race 1 strains have a wide host range contrary to race 2 and 3 strains which infect a number of plants more restricted; therefore, these classification systems show a little correlation. The strain causing bacterial wilt of ginger in India belongs either to biovar 3 or 4; the former being the most virulent in India (Kumar and Sarma, 2004). The pathogen is primarily rhizome-borne and is believed to be transmitted to many ginger-growing areas through latently infected rhizomes. Secondary spread within the field and neighbouring localities is through rain splashes and runoff water (Kumar *et al*., 2004).

3.6. Bacterial Wilt of *Ralstonia solanacearum* Worldwide

Elphinstone (2005) has recently reviewed on bacterial wilt caused by *R. solanacearum,*. is present on all continents throughout wet tropic, sub-tropics and warm temperate regions of the world, this disease is of international concern and is being addressed by collaborative research between national researchers; and by International Agricultural Research Centers such as International Potato Center (CIP) working on potato (Hayward, 2005), International Corp Research Institute for Semi-Arid Tropics (ICRISAT) working on peanut (Hayward, 2005) and Asian Vegetable Research and Development Center (AVRDC) working on tomato, pepper and eggplant (Hayward, 2005). Publications on *R. solanacearum* until 1997, have dealt with finding resistance through breeding and selection (24%), pathogen host range, diversity, and distribution (22%), disease management and control (18%), pathogenicity and host pathogen interactions (17%), biological control (10%), detection and diagnosis (4%) and finally epidemiology and ecology (3%) (Elphinstone, 2005). In terms of host crops studied, 35% of publications have deal

with tomatoes, 24% with potato, followed by eggplant (11%), tobacco (9%), *capsicum* (7%), groundnut (3%), banana (3%), and ginger (2%), the remaining 5% 17 included many other host crops and ornamentals (Elphinstone, 2005).

Hayward, (2005) observed that the developed countries did focus their research on fundamental questions of the genetic basis of virulence, pathogenicity, and regulation of gene expression,while developing countries primarily addressed work on disease resistance, management, many aspects of epidemiology and biology. This situation certainly favors the disparity in the availability of information between the two important Races and 3 biovars present on potato: Race1 biovar3 or biovar 1 and Race 3 biovar 2. More research work has been done on Race 3 biovar 2 since that is the Race present in both tropical highland climates and in warm temperate climates characteristic of most developed countries. Still more attention should be paid to Race 1 that occurs exclusively in tropical or sub tropical regions where soil never freezes. Race 2 biovar 3 is a novel variant of the pathogen adapted to cooler environments where soils freeze (Alvarez *et al*., 2005). While Race identification is based on host crop the biovar is determined through the oxidation test on disaccharides and hexose alcohol. Race 1 is found on diverse crops, and the others Races (2, 3, 4 and 5) are specific on a few crops (Denny and Hayward, 2001). Among the crops infected by *Rs* race 4 strains, bacterial wilt of edible ginger is the most devastating economically, and severe losses have been reported from Australia, China, India, Indonesia, Japan, Malaysia, Mauritius, the Philippines and the United States (Hawaii) (Kumar and Sarma, 2004 and Alvarez *et al*., 2005). In Hawaii, bacterial wilt of edible ginger led to yield losses exceeding 50% in 1998 and 1999 (Yu *et al*. 2003). In 2005, the area harvested was 13% less than the previous season due to the disease (Hudson, 2006).

Table 1. Geographical Distribution of Host Range and Biovars of *Ralstonia solanacearum* (Smith) Worldwide.

Race	Host Range	Geographic distribution	Biovar
1	Wide	Asia,Australia,Americas	1, 3, 4
2	Banana Other *Musa* spp.	Caribbean, Brazil Philippines	1
3	Potato, some other Solanaceae, Geranium; Plus afew other species	Worldwide except US and Canada	2
4	Ginger	Asia	3, 4
5	Mulberry	China	5

Source: Denny and Hayward (2001) modified by Daughtrey (2003).

3.7. Economic impact of *R. solanaceraum*

The high economic and social impact of this organism results from its wide geographical distribution in all warm and tropical countries of the globe but also in more temperate countries from Europe and North America as the result of the dissemination of strains adapted to cooler climates, losses greatly differ in the distinct areas in dependance on (i) local climates, soil types and cropping practices, (ii) the choice of crop and plant cultivar, and (iii) the virulent characteristics of the *R. solanacearum* strains present (Elphinstone, 2005). In areas where the organism has quarantine status, considerable economic losses can result from the destruction of entire infected crops, additional eradication measures and restriction on further production on contaminated land (Elphinstone, 2005).

Disease severity mostly increases if *R. solanacearum* is found in association with root nematodes. In tobacco, nematode infestation changes the physiology of the plants, causing susceptibility to bacterial wilt (Elphinstone, 2005). Experiments in India showed that the combined pathogenic effects of *R. solanacearum* and *Meloidogyne javanica* were greater than the independent effects of either in the case of *R. solanacearum* R3, one of the most damaging pathogens on potato worldwide (Hayward, 1991; Janse, 1996), it has been estimated to affect 3.75 million acres in approximately 80 countries with global damage estimates exceeding $950 million per year (Floyd, 2007). The potential economic impact of potato brown rot caused by R3

is unknown in temperate climates such as where much of US potato production occurs (Champoiseau *et al.,* 2010). The actual potential of this race to cause losses in temperate climates is uncertain because data on yield reductions are limited due to quarantine and eradication efforts in potato fields in the UK, the Netherlands and Sweden. Despite widespread detection of the pathogen in northern Europe, direct losses of potato to the disease have been limited to a few outbreaks during unusually hot summers (Priou *et al*., 2006). In the case of banana, moko disease has affected susceptible bananas and plantains over thousands of square miles in central and South America This disease is one of the most important phytopathological problems of the banana Agri business in tropical countries. In Colombia, the region of Uraba and Magdalena which represent the main exporting region of banana in this country, this disease causes a destruction estimated in 16.5ha/year (Castaneda and Espinosa, 2005). Bacterial wilt disease of edible ginger causes severe economic damage in many countries, including China, India, Indonesia, Japan, Malaysia, Mauritius, the Philippines, and the United States (Hawaii) Kumar and Sarma, 2004; Alvarez *et al.,* 2005). Crop losses of edible ginger resulting from bacterial wilt exceeded 50% in 1998 and 1999 (Yu *et al.,* 2003), and in 2005, the area harvested was reduced by 13% (Hudson, 2006). In India, bacterial wilt is widespread on edible ginger and 100% yield losses have been reported (Sharma and Rana, 1999).

3.8. Status of Bacterial Wilt of Ginger (*Ralstonia solanacearum*) in Ethiopia

In Ethiopia, the bacterial disease has been reported on potato, tomato, pepper, enset, banana and ornamentals but ginger bacterial wilt is not yet reported and new to Ethiopia (Bekell kassa,2016). However, ginger is now well known in Ethiopia as fresh or dry rhizomes for sale on most markets. It is mainly grown in the south and Southwestern part of the country with high land coverage and farmers percentage in the cultivation. In different parts of the world, like most cultivated crops, ginger is affected by biotic and abiotic factors. In most ginger producing countries like India, ginger is affected by different pathogens. Bacterial wilt (*Ralstonia solanacearum* soft rot/rhizome rot, leaf spot (*Phyllostica zingiberi,* Nematodes were commonly known to cause disease on ginger crop. Ginger bacterial wilt disease caused by R. *solanacearum* is known as most limiting factor in Hawaii. Ethiopia, in the history of spice research achievements, no disease prevalence‟s have been recorded recently, in 2011/12 and 2012/13 cropping season regional plant health clinics have reported that ginger crop was infected by

unusual disease. (Endrias Geta and Asfaw Kifle 2011). Based on such regional reports, survey was conducted during the 2011/12, 2012/13 and 2013/ cropping seasons in the Southern Nations, Nationalities and People and Gambela Regional States to investigate the status and causal agent of the recently occurred ginger disease (Habetewold kifelew *et al*., 2015). Bacterial wilt of ginger was found widespread in all area surveyed, among the different areas surveyed, wilt incidence were found ranged from 93.5% Sheka zone to 10.7% Gamogofa zone in 2012 survey season. The first bacterial wilt syndrome were reported from Benchmaji zone Bebeka coffee estate farm, then after it found in the neighboring zone Sheka within a short period of time and cause up to 67% yield loss. During 2012 the diseases were less prevalent around Wolayta zone even if the area have been known for producing ginger as a major crop; at the same year even if the diseases is less prevalent at Wolayta area which is different in geography and agro-ecology to that of Benchmaji and Sheka zones, (Habetewold kifelew *et al*., 2015), collected and checked the seed rhizomes from Wolayta and surrounding for latent infection, from the laboratory result indicated that the seed rhizomes were found 100% latently infected(Habetewold kifelew *et al*., 2015). In subsequent year 2014 survey result, SNNPRS (Dawro, Wolayta, Kenbata tenbaro, Hadiya, Gomogofa, Konta, Alaba, Sheka, and Bench maji) and Gambella region (Majang) zone ginger were found devastated by the bacterial wilt and caused yield loss up to 98%. (Habetewold kifelew *et al*., 2015).

In many tropical and subtropical regions the pathogen has been widely distributed and associated with a wide range of hosts (Agrios, 2005). *Ralstonia solanacearum*(RS) grouped in to five races based on the different in host ranges and geographic distributions; those races principally attack Bananas (race 1), ornamental planes (race 2) , potato (race 3), Ginger (race 4) and mulberries (race 5) (Kelman, 1997). Race 4, of the pathogen limits the production of ginger in the tropics (Paret, et al, 2010) and has a narrow host range, and restricted to ginger (Kelman, 1997). Fikre Lemessa and Zeller (2007) identified sixty two RS strains from Ethiopia out of this 19 were grouped as biovar I and rest of the strains as biovar II. It was further observed that biovar II strains had limited host range (affecting mainly potato) as compared to biovar I strains (affecting eggplant, tomato and potato) but so far race 4 is not yet reported in association with ginger or other crops in Ethiopia**.**

3.9. Management Strategies of Bacterial Wilt of Ginger

Bacterial wilt caused by *R. solanacearum* is a disease difficult to control in field production on crops, since pathogen‟s properties as a soilborne bacterium, their broad host range and the genetic variation level within the strains; they have not allowed implementing a universal control measure for this disease (Saddler, 2005 and Denny, 2006). Disease management requires a thorough knowledge on three main components such as host, pathogen and environment. The disease development and progress depends on the interaction of all three factors and the reaction must be positive when host become susceptible, pathogen develops virulence and environmental conditions are favorable. Resistance breeding has been effective with tobacco and groundnut, but success with solanaceous crops appears to be limited or linked to climate conditions (Hayward, 1991; French, 1994). In other cases, the resistance or more accurately tolerance as asymptomatic infections are common place with solanaceous hosts can be neutralized by the actions of other pests as nematodes (Saddler, 2005). 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 parts of an integrated pest management strategy.

3.9.1. Resistance cultivars

The best strategy to control bacterial wilt caused by *R. solanacearum* is breeding for resistance cultivar (Persley, 1992). A virulent mutant of *R. solanacearum* has been used as a bio-control agent for the virulent pathogen (Trigalet and Trigalet-Demery, 1990). Abdalla and Abdulla (1998) have found that the degree of susceptibility to bacterial wilt is significantly different among six tomato cultivars which were tested and this indicated that the 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. The infection by *R. solanacearum* can be controlled by the use of hot pepper accessions (*Capsicum annuum* L.), sweet pepper (*Capsicum annuum* L.) in Japan. Bacterial multiplication in stems of resistant tomato plants was suppressed owing to the limitation of pathogen movement from the proto xylem or the primary xylem to other xylem tissues(Nakaho *et al*., 2004). The limitation was most conspicuous in Hawaii 7996. Grafting experiments indicated that the percentage of wilting of

Ponderosa scions was less on Hawaii 7996 rootstocks than that on the most resistant rootstock (LS-89) used in Japan. Hawaii 7996 could be an alternative genetic source for breeding for resistance to bacterial wilt (Nakaho *et al*., 2004).

3.9.2. Cultural practices

In regions where the disease is endemic cultural control methods and phytosanitation have the potential to ameliorate its worst excesses (Saddler, 2005). In regions where bacterial wilt of potato is endemic or in locations where *R. solanacearum* is present but not yet established, these methods can be effective under some conditions (Champoiseau *et al.,* 2010). A number of cultural practices can reduce disease, including crop rotation which dependent on factors such as the ability of the local *R. solanacearum* strains to survive and maintain a remaining infective population in the absence of a host (Akiew and Trevorrow, 1994). Thus, only partial control of the disease can usually be achieved with short-term rotations. Intercropping of 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 (Devaux *et al.,* 1987; Hayward, 1991; Akiew and Trevorrow, 1994; French, 1994; Dhital *et al*., 1996; Terblanche, 2002; Katafiire *et al.,* 2005 and Lemaga *et al.,* 2005). Other methods include, planting healthy (tested) seed, using of cover crops and other measures to reduce the impact of weed hosts as herbicides, since there are many considered as asymptomatic to *R. solanacearum* infection and avoidance or testing and treatment of surface water for irrigation (Champoiseau *et al.,* 2010). An important aspect for successful control of *R. solanacearum* dissemination and bacterial wilt eradication is the realization of systematic surveillance on both, imported and home produced plant material susceptible to the disease (Elphinstone, 2005).

3.9.3. Biological control

The biological control is one of the control means most investigated today, however the promising results generated under controlled conditions have failed to be replicated in the field. A number of soil bacteria and plant growth promoting rhizobacteria (PGPR) are currently being investigated for their role in the control of *R. solanacearum*, alist of saprophytic bacteria that have been studied as biological control agents against include six bacterial strains *viz*. *Corynebacterium sp.* BT6, *Bacillus* spp. FH17 and BB11, *Escherichia* sp. BT4, *Serratia* sp. J2 and *Pseudomonas* sp. J3. were found most effective against bacterial wilt disease (Guo *et al*., 2001). Jagadeesh *et al.* (2001) evaluated the role of a fluorescent siderophore using Tn5 mutants

of bacterial wilt in tomato. 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 use of *P. fluorescens* in combination of *G. mosseae*. Ramesh and Korikanthimath (2004) reported the use of *P. fluorescens* as a potential biocontrol agent for the management of *R. solanacearum* in aubergine. 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) have proved that soil disinfection with lime one month before transplanting and the use of *Pseudomonas fluorescens* as biological control agent were effective to minimize the bacterial wilt incidence in brinjal field. 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. Other studies have focused on the use of derivates from the *R. solanacearum* wild type itself, for example spontaneous avirulent mutants deficient in exopolysaccharide production capable of colonize the host but survive asymptomatically. Saddler (2005) has indicated that the use of *hrp*O mutants which showed a significant reduction in the onset of disease and disease severity when plants where subsequently challenged with a pathogenic strain of fungi or bacteria. However, the use of all these biological organisms are currently being investigated in small scale experiments and none are currently available commercially(Saddler, 2005). Biological control can be defined as the direct and accurate 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 displace or antagonise phytopathogenic or deleterious microorganisms in the rhizosphere. Biocontrol bacteria produce chemicals, but these are degradable and only produced in low amounts at targeted locations. The biocontrol approach fits well in the worldwide strategy to grow healthier plants in a sustainable way and, therefore produce high quality food. Biological control has the potential to control crop diseases while causing no or minimal detrimental

environmental impact (Haggag *et al.,* 2007). Biological control of plant pathogens has been developed and given attention and used as alternative control method due to some chemical pesticides pose harmful effects to human health and the environment. There is also a need to control various diseases for which there is currently no control or only partial control because there is little or no genetic resistance in the host, crop rotation is impractical or not economically feasible, or reliable, economical chemical controls are not available (Cook, 1993).

3.9.4. Limitation of Potential Biological Control Agents

Although an array of microorganisms has been shown to protect crop plants from disease under experimental conditions, commercial development of many antagonists has been hampered due to inconsistent performance between field locations and seasons(Ownley and Windham, 2006). Biocontrol is less consistent than chemical control, variation in consistence and performance of biological control agents has been attributed to many factors like abiotic, biotic, and ecological factors (Kamilova *et al*., 2005). In addition, survival and efficacy of biocontrol affected by host plant genotype, agricultural practices, mutation of the biocontrol organism and resistance of the pathogen to biocontrol mechanisms (Ownley and Windham, 2006). Biological control may also competitively displace and pathogens to non-target organisms (Cook *et al.*, 1996). Unintended effects would occur if this competitive displacement seriously affect a non target organism; perhaps even leading to its extinction or in some other way detrimentally affecting a component of the ecosystem(Goettel *et al*., 2001).Furthermore, some fungi that intended for biological control could infect a wide variety of hosts, which sometimes include mammals. Thus, evaluation of potential microbial control agents must include an evaluation of their virulence towards non-target organisms (Goettel *et al*., 2001).

3.9.5. Chemical control

Direct control of bacterial wilt diseases caused by *R. solanacearum* in the field has showed to be difficult due to the fact that 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 efficacy both in greenhouse and to a lesser extent field (Anith *et al.,* 2004; Pradhanang *et al*., 2005 and Ji *et al.,* 2007). Other control ways include the 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.,* 2005). Several antibiotics were tested against *R. solanacearum* in which streptomycin and streptopenicillin recorded less bacterial infection (Singh *et al*., 2000). Mondal *et al.* (2005) reported the efficacy of different antibiotics *viz*., streptomycin, chloramphenicol, oxytetracycline hydrochloride, norfloxacin, amoxicillin + cloxacillin, chloroquine phosphate and dichlorophen 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) reported minimum wilt incidence and highest yield of bell pepper when seeds were treated with streptomycin.

3.9.6. Disadvantage of Chemical Control

Although chemical compounds have been applied to control plant diseases, they result in negative impact on wide range of organisms. The increasing use of agrochemicals is negatively perceived by consumers and supermarket chains. Chemical pesticides contaminate groundwater, enter food-chains, 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). Frequent applications of copper-based bactericides amended with an ethylenebisdithiocarbamate fungicide (maneb or mancozeb, class B2 carcinogens) provide some disease suppression of xanthomonas leaf blight and other bacterial diseases of onion in Colorado, but they must be applied regularly eight or more times to be effective and suppress disease per season (Schwartz and Otto, 1998).

3.9.7. Effects of Trichoderma , Pseudomonas and Streptomycin sulfate as biocontrol agents (BCA) against Ralstonia wilt on ginger

Common control measures against bacterial wilt include the use of resistant varieties, healthy seeds, crop rotation, agronomic practices, biological control, and integrated management. Potential biological control agents (BCAs) explored so far to control the wilt include avirulent mutants of R. solanacearum on bean (He and Kang, 1990; Dong et al., 1999), genetically engineered antagonistic bacteria on banana (Kang et al., 1995), the arbuscular mycorrhizal(AM) fungus Glomus versiforme (Zhu et al., 2004) on pepper, and antagonistic rhizobacteria such as Bacillus spp. (Fikere Lemessa and Zeller, 2007), Pseudomonas spp. (Guo et al., 2001; Götz et

al., 2006), and Streptomyces spp. (El Albyad et al., 1996) on tomato. Currently, Bacillus spp. and Pseudomonas spp. are the most popular BCAs. Several living microbial products have been commercialized as BCAs, such as a wettable powder of Bacillus subtilis (Cohn) Y1336, water

suspension of Pseudomonas fluorescens (Migula), a mixture of wet- table powder and granule of Paenibacillus polymyxa (Ash, Priest and Collins) (Sun et al., 2004), and a wettable powder of Bacillus mega- terium (LS 20031261) for ginger and tomato against R. solanacearum. However, ginger wilt continues to be a serious problem in China and other countries, which has spurred research on the specificity of the pathogen to ginger. Ram *et al*. (1999) conducted an experiment to evaluate fungal and bacterial biocontrol agents (BCA) individually in combination and finally both BCA‟s in integration with fungicidal rhizome treatment. Soil application of *T. harzianum* and rhizome treatment with *Pseudomonas sp*. and fungicides was the most effective among all the tested treatments (Ram *et al*., 1999). Seed were treated with three biocontrol agents before planting and these biocontrol agents already multiplied along with neem cake (200g/plot) were applied to soil prior to planting (Jayasekhar *et al*., 2000). In integrated management of ginger against *Pythium*, *Fusarium* and *Ralstonia*, the results indicated that mancozeb, seed solarization and hot water treatments of ginger rhizomes were effective in increasing the emergence and yield of ginger (Anon., 2005). Soil rectification with lime one month before transplantation and use of *Pseudomonas flourescens* as biocontrol agent were effective to minimize the bacterial wilt incidence in field (Biswas *et al*., 2008). Dagostin *et al.* (2008) reported that *Trichoderma sp.* have ability to produce a series of antibiotics and fungal cell wall- degrading enzymes proteinase (Prb1), which play important role in mycoparasitism and mycelial lysis of the target pathogenic microbes. Fluorescent pseudomonads could show diverse mechanisms of biocontrol which include antibiosis, cyanide production, siderophore production, competition for space and nutrient and induced systemic resistance, Fluorescent pseudomonads produce yellow green fluorescent siderophores (pyoverdine type) under low iron condition, which is an important mechanism for the biocontrol activity of PGPR (Bakker *et al.,*1993)

Hidaka and Murano (1956) studied *in vitro* effect of streptomycin on the behaviour of *R. solanacearum*, and they found that streptomycin at 0.3 µg/ml water inhibited and killed or *R. solanacearum.* Singh *et al.* (2000) found that streptomycin and streptopenicillin were superior over other antibiotics against the pathogen under *in- vitro* and *in- vivo* conditions. Traditionally

R. solanacearum infections are frequently treated by applying streptomycin (Diogo and Wydra 2007; Chen *et al.* 2008). However, the use of streptomycin is assumed to contribute to the development of antibiotic resistance in the pathogen and to an increased abundance of antibiotic resistant soil bacteria. An alternative strategy for controlling the bacterial wilt is the use of resistant varieties (Sisson, 1999 and Bhatti *et al.,* 2011). Chemical control such as bactericides, in addition to being potentially harmful to the environment, has not proved to be efficient in controlling *R. solanacearum*. Alternatively, biological control has been proposed to be an effective, safe and eco-friendly approach in plant disease management (Almoneafy *et al.,* 2014; Dey *et al.,* 2014). The public demand for sustainable agriculture has helped to drive research on the biological control of plant diseases and the practical use of antagonistic microorganisms. Recent publications have indicated that biological control of bacterial wilt disease could be achieved by using antagonistic microbes (Guo *et al.,* 2004; Kurabachew and Wydra, 2013; Maji and Chakrabartty, 2014; Ramesh and Phadke, 2012). When compared with treatments using a BCA alone, mixtures are expected to provide greater efficacy. However, a number of factors, including type of bacterial and/or fungal strains, and the existing pathogen pathovar, influence the effectiveness of single or co-inoculation of BCAs. For example, some earlier studies demonstrated that a co-inoculation was more effective than single inoculation of either bacteria or fungi (Abo-Elyousr *et al.,* 2012; Liu *et al*., 2009; Zhou *et al.,* 2014).

4. MATERIALS AND METHODS

4.1 Description of the sampling site

The sampling site were Durame and Hadaro Tunto in(Kembata Tenbaro) and Areka in (Welayita) Zones of south west of Ethiopia. Durame is a town in southeastern Ethiopia the administrative center of the Kembata Tembaro Zone in the Southern Nations, Nationalities and Peoples Region (SNNPR).The town has a latitude and longitude of 7°14′N and 37°53′E with an elevation of 2101 meters above sea level. Durame has an estimated total population of 12,849 of whom 6,461 are men and 6,388 are women (Teferra and Leikun., 1999).

. Durame is about 219Km from Addis Ababa, two roads connect the town from Addis Ababa through Shashamene and from Addis Ababa through Hossana. The records of National Meteorological Service Agency from Durame Observatory substation show that the mean annual rainfall for twenty seven years i.e. from 1983 to 2010, at an altitude of 2101m above mean sea level, latitude of 7°14′N and longitude of 37°53′E was 1260mm. The rainy season is from the months of April to September. Around 75% of the rainfall arrives in these months. In a mountainous tropical country like Ethiopia altitude is by far the most important factor in controlling climate. It affects distribution of both temperature and rainfall. Generally, regions between 1500 - 2300 meters above mean sea level. (categorized as 'woina dega' or sub tropical climate) have temperatures that range between 15 – 20°C, areas between 500 – 1500 meters above mean sea level. (i.e. 'Kola' or tropical climate) have 20 -30^{o}C and areas below 500 meters above mean sea level (i.e. 'Bereha' or desert climate) have a temperature of 30°C and above.The highest temperatures occur during months of Feb., March, April whereas Oct. Nov .and December have low temperature (FitsumMarkos, 2013).

Areka (also known as Areka Ancheto) is a twon in southern part of Ethiopia, located in the wolaita zone of the southern Nations Nationalities and peoples region , about 300 km southwest of the capital, Addis Ababa. This town has altitude and longitude of 7°4′N 37°42′E and in elevation of 1774m above sea level. The rainfall pattern is bimodal, short rainy season which runs from March to May, followed by long rainy season which runs from June to September.

Mean annual rainfall of Areka,is about1000-1300mm and an annual temperature range of 25-35°C. This town has an estimated total population of 31,408 of whom 15,795 are men and 15,613 are women (EARI, 2009).

Hadero Tunto is one of the woredas in the Southern Nations, Nationalities, and Peoples' Region of Ethiopia. Part of the Kembata Tembaro Zone, Hadero Tunto is bordered on the south by the Wolayita Zone, on the west by Tembaro, on the north by the Hadiya Zone, and on the east by Kacha Bira. the town lies at the coordinate of 37^{o} 7^{0} north latitude and 7^{0} 37^{0} East longitudes. Towns in Hadero Tunto include Hadero and Tunto. Hadero Tunto was part of former Omo Sheleko woreda. Based on the 2007 Census conducted by the CSA, this woreda has a total population of 98,375, of whom 48,548 are men and 49,827 women (CSA, 2007).

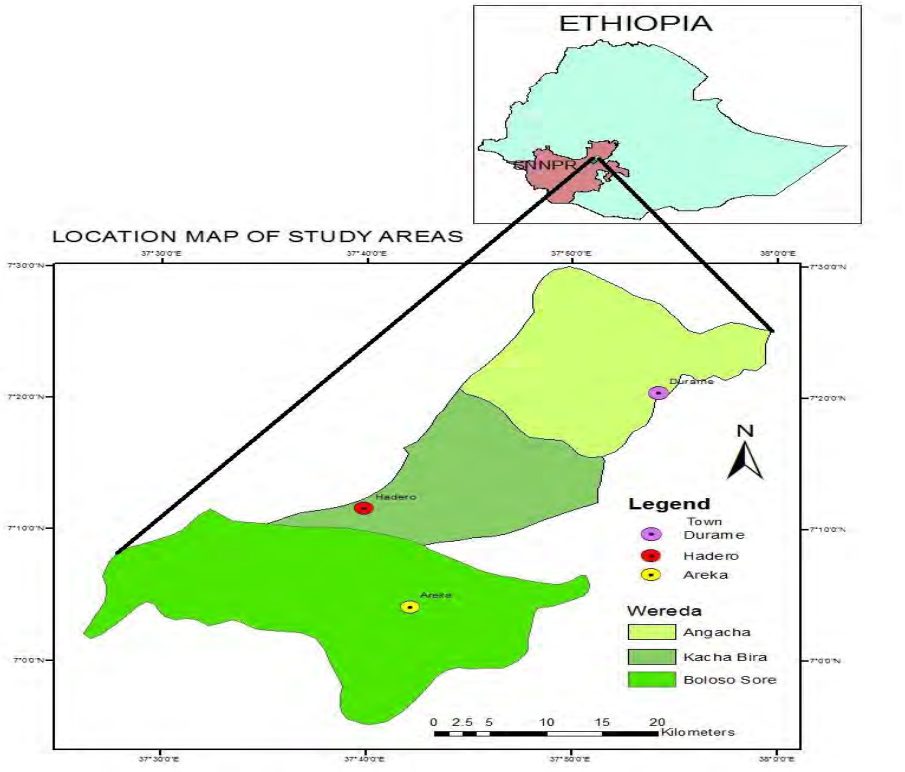

Fig 2. Map of Ethiopia with SNNP Region highlighted in purple color and Sampling site highlighted in different color of yellow, brown and Green in South western Ethiopia.

4.2 Sample Collections

Samples were collected from three ginger growing areas of the Kembat Tenbaro and Welayita zone of SNNP region (Durame and Hadaro Tunto in Kembata Tenbaro and Areka in woliata zone) South western parts of Ethiopia. Total 50 samples of infected ginger rhizomes and Rhizosphere soil were collected from study site(14 from Durame,17 from Hadaro,19 from Areka), and brought to the laboratory for the isolation of *R. solanacearum.* Diseased organs of the plants were taken from the rhizome of all the present cultivars in the study sites. The isolates were collected from the farmer‟s field of all the agro climatic regions in three different altitudinal ranges (high, medium and low). Roots with adhering soils of healthy ginger plants were collected sub-sampled and transfered into sterile plastic bags. The method that was used for the collection of ginger pathogens from the plant materials was based on the examination and sampling of observed diseased sample. Standard sampling methods was used in the process developed by Aneja (2005). Samples were collected in 8 to 12 km intervals from farmer‟s field samples were kept in clean plastic bags (envelopes) and were brought to the mycology laboratory and then was stored at 4°C for further study. The *in vitro* study was carried out in the Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University (AAU) where as the *in vivo* study was conducted in the Green house College of Natural and Computational Sciences, at AAU.

Fig 3. Collected diseased rhizome sample form study site of Durame, Hadero and Areka.

4.3. Isolation of the test pathogens

The bacteria were isolated from the plant rhizomes within 2 days of collecting the samples, as follows. For isolating the bacteria from the plant rhizomes samples (3 g each) of rhizome were surface-disinfected with 1% sodium hypochlorite for 2 min washed three times with sterile water, and 0.1 mL from the last washing is plated onto Nutrient Agar (NA) to confirm that the pieces were completely sterile from outside: if no growth is seen after incubation at 28 ^{0}C for 48 hr, samples were assumed to be sterile. The rhizome samples were directly plated on Nutrient Agar medium (NA), incubated at 28 ^{0}C for 48 hr, and pure cultures obtained from single colonies were selected and purified. Pure culture of *R. solanacearum* were transferred to nutrient agar media slants and stored at 4 ^{0}C for further investigation (Aneja (2005) and Dingera and Sinclair (1993).

Designation of bacterial wilt disease of ginger was carried out using eight isolates, Addis Ababa University *R. solanacearum* isolates 03 (AAURS-03), AAURS-04, AAURS- 05,AAURS- 07, AAURS -08, AAURS-018, AAURS-026 and AAURS- 034.

The bacterial wilt disease of ginger and biocontrols of bacterial and fungal antagonists were designated as followed:

1. Bacterial wilt disease of ginger (BWDG)
2. bacterial and fungal antagonists
3. Streptomycin Sulfate (Bactricide)

4.4. Isolation and identification of Ralstonia solanacearum

Diseased ginger plant parts (rhizomes) and soil samples were collected from the ginger growing areas of Ethiopia. Field diagnosis of diseased plant samples were done by critically observing the bacterial wilt symptoms. *Ralstonia solanacearum* was isolated on Nutrient Agar (NA) medium.

The vascular portion of infected rhizome sections were cut into small pieces, washed and sterilized by the same procedure as described above and were directly placed on Nutrient Agar medium,incubated at 28°C for 48 hr . After isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Tetrazolium Chloride (TZC) medium as described

by Kelman (1954). The pathogenicity test was performed on three month old healthy ginger seedlings by stem inoculation method. A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the center was selected and adjusted to 3.2 x 10^8 cfu ml-1 for inoculation. Pure culture of *R. solanacearum* were maintained on nutrient agar medium slants and maintained at 4°C for further studies.

4.4.1 Identification of Virulent/Avirulent isolates of *R. solanacearum*

The virulent and avirulent isolates of *R. solanacearum* were differentiated by Kelman (1954). Tetrazolium Chloride (TZC) agar medium containing 0.005% 2, 3, 5-tripenyl tetrazolium chloride (TTC). In this test, virulent isolates produce pink or light red colour colonies or colonies with characteristic red centre and whitish margin and avirulent isolates produce smaller, off-white and non-fluidal or dry on TZC medium after 24 hours of incubation (Kelman ,1954, Champoiseau,2008, Rahman *et al*., 2010).

4.4.2 Culture based Identification

The culture based identification was found the bacteria is fluidal, presents irregular shape and white with pink centered colonies on tetrazolium chloride (TZC) media, which is similar with the description of *R. solanascearum* by Kelman (1954) and Hayward, (1964)

4.5. Morphological Characterization

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

4.5.1. Motility Test

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

4.5.2. Gram reaction

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 30 seconds and then washed with running tape water for one minute. It was then flooded with iodine for one minute, rinsed in tape water and decolorized with 95% ethanol until colorless runoff. After washing the specimen was counter-stained with safranin for approximately 10 seconds, washed with water, dried and observed microscopically at 10X, 40X and 100X using oil (Schaad,1980).

4.6. Physiological factors affecting on the grwoth bacterial wilt of ginger (R. solanacearum)

4.6.1.. Effect of hydrogen ion concentration (pH)

The ability of isolates to grow on acidic and alkaline media was checked by inoculating the bacterial culture on 100 ml of Nutrient Broth Medium in 250 ml flasks and adjusted to pH levels of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0,7.5, 8.0 and 8.5 at 28± 1 °C by using pH meter with addition of diluted HCL and NaoH., the Growth (OD at 600 nm) of all the isolates of *R. solanacearum* was recorded at 24, 48 and 72 hr by using spectrophotometer (Kai *et al.,*2014).

4.6.2. Effect of temperature on the growth of *R. solanacearum*

The effect of temperature on the growth of egiht isolates of *R. solanacearum* was determined at different temperatures. Bacterial isolates were streaked on Nutrient agar for 48 hr to obtain single colonies, which were transferred into Nutrient broth and allowed to grow at 28 ± 1 °C with shaking at 150 rpm for 20 hr to obtain exponential growth phase (OD at 600 nm: 0.1). 100 µl cultures of these isolates of *R. solanacearum* were inoculated into 25 ml of Nutrient broth in 100 ml of conical flask and incubated at 4, 10, 15, 20, 25, 30, 35 and 40 °C up to 72 hr with three replications. Growth (OD at 600 nm) of all the isolates of *R. solanacearum* was recorded, at 24, 48 and 72 hr by using spectrophotometer (Dinesh Singh, *et al.*,2014).

4.6.3. Salt Tolerance

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

4.7. Biochemical Characterization

4.7.1. Catalase Test

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

4.7. 2. Oxidation fermentation test

The oxidation and fermentation of a carbohydrate by isolates were determined on oxidation - fermentation medium using the method described by Aneja (2005). The medium contains: 2.0 g of peptone, 1.0 g of yeast extract, 5.0 g of NaCl, 0.2 g of K_2HPO_4, 0.08 g of bromothymole blue per 1 liter of distilled water and pH was adjusted to 7.1. The medium was freshly prepared and 5 ml of broth medium was dispensed in each test tube. After sterilization, the medium was cooled to about 35^0C with tap water to avoid dissolution of oxygen in the medium to which 10% (w/v) of sterilized glucose was added. Two test tubes inoculated by stabbing with straight wire loop. One of the tubes were sealed the surface with a thin layer of paraffin oil to create anaerobic condition whereas the other tube was incubated aerobically. Acid production associated with color change from green to yellow in the aerobic tube was indicative of oxidation whereas the color change from green to yellow in the both tubes showed fermentation (Aneja, 2005).).

4.7.3. Fluorescent Pigment Production

The ability of bacterial isolates to produce yellow- green fluorescent pigments or flourescein siderophore was tested by culturing the bacterial isolates on KB medium. Twenty four hours old culture was inoculated into the medium and incubated at 28^0C for 48 hr. The fluorescent pigments were observed for the fluorescence under 366 nm UV light after 48hr incubation (Stieglitz and Weimer, 1985).

4.7. 4. Indole Production Test

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

4.7. 5. Starch Hydrolysis

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

4.7.6. Levan production from sucrose

Single colonies of bacteria were streaked on nutrient agar plates supplemented with 5% sucrose. The plates were then incubated at 28°C for 48 hr. Separate, discrete colonies were observed for the Levan production with naked eye (Schaad, 1980).

4.7.7. Lipase activity on Tween 80 agar

The constituents of the medium, 10.0g peptone, 5.0 g NaCl, 0.1g $CaCl_2 \cdot 2H_2O$, 10.0gTween-80 (Sigma), 20.0g Agar perlitre,were dissolved in distilled water and heated, at 100°C with periodic swirling to dissolve the agar. The pH was adjusted to 6.0. The molten medium was dispensed in 100 ml quantities in 200 ml screw-capped bottles. Using a wire loop bacterial inoculums were radially streaked across separate halves of Petri plates and incubated at 28°C for 7 days. The plates were examined daily for the presence of a dense precipitate around the bacterial growth (Aneja, 2005).

4.8. Grouping of *R. solanacearum* into biovars

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize disaccharides (lactose, maltose) and sugar alcohols (manitol, sorbitol) as described previously by Hayward (1964) and He et al. (1983). Ten ml of each 10% lactose, maltose, mannitol, and sorbitol was added in to mineral medium $NH_4H_2PO_4$ 1.0 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, Difco bacto peptone 1.0 g, Agar 3.0 g and Bromothymol blue10-20 ml per litre) containing 1% sugar. The disaccharides was sterilized by filtration using 0.22 mm micron millipore membrane and the hexose alcohol was 121^0C for 15 minutes. Bottles of semi-solid basal medium were melted in water bath and cooled to70°C. Media was then allowed to solidify at room temperature. Control was kept by adding 10ml sterilized distilled water instead of sugar solution into the basal medium. Tewenty (20) ml of the melted medium was dispensed into the wells of microtitre plate. Inoculums for each group of isolate was prepared by several loop full of bacteria from 24 hours old cultures to sterilized distilled water to make suspension containing about 10^8

CFU/ml. Then 100 µl of bacterial suspension was added to the wells of microtitre plate incubated at 28°C. The plates were then examined after 2,7 and 14 days of inoculation for the presence of indicator change from olivaceous green to orange colour on the surface of medium (Schaad *et al.,* 2001).

4.8.1. Preparation of inoculum

Selected *R. solanacearum* isolate was cultured in nutrient broth at 28±2^{0}c on orbital shaker. Twenty four hours old culture was taken and transferred into 100ml of the medium and incubated on orbital shaker at 120 rpm for 48hrs. Then the cultures were diluted with sterilized distilled water to 10^8 cells/ml. Plants were inoculated at eighth leaf stage by stem puncture and leaf infection pin pricks. The bacterial suspension was inoculated in to the two leaves of each test plant **(**Stromberg *et al.,* 2004).

4.8.2.1 Pathogenicity test

Virulence character of the *R. solanacearum* isolates was carried out on a set of three ginger cultivars, Durame, Hadero tunto in Kambata Tanbaro and Areka in Walita zone which are susceptible to bacterial wilt of ginger. Nine to twelve (9-12) months old healthy rhizomes obtained from Areka Agricultural Research Center were selected and used for further pathogenicity assay. Rhizomes were transplanted in plastic pots (9 cm in diameter) containing soil and sand ratio(1:1) and then grown under the greenhouse. The bacterial inoculums were grown on TZC agar medium for three days at 28°C, suspended in sterile distilled water, and spectrophotometrically adjusted to OD 600nm = 0.1 (approximately 10^8 CFU per ml (Ran *et al*., 2005). 1 ml of inoculum per plant was inoculated in to ginger sedling by stem puncture and leaf infection pin pricks. Plants at the eighth leaf stage were inoculated by puncturing the basal part of the stem with a needle dipped in inoculum. Each experiment was repeated three times. The day and night temperatures varied between 25-35^{0}c with 12hr light and 12h dark (Klement *et al.*, 1990). Symptoms of disease were observed for one week after inoculation (Takamitsu, *et al.,* 2013). If the plant showed typical wilt symptoms, the interaction was considered as pathogenic. The *R. solanacearum* isolates were categorized into 4 group‟s viz., highly pathogenic, moderately pathogenic, weakly pathogenic and non pathogenic based on the symptomatological variations on the test ginger plants, whereas non inoculated ginger seedlings showed no symptoms and hence served as control (Tans *et al.,* 2001). The severity of bacterial wilt of ginger

was recorded based on the severity scale as described by Horita and Tsuchiya (2001). Briefly, 1= No symptom, 2 = Top young leaves wilted, 3 = Two leaves wilted, 4 = Four or more leaves wilted and 5 = Plant died

4.8.3. In vitro evaluation of Pseudomonas fluorescens and Trichoderma isolates against R. solanacearum

The effects of different isolates of *Pseudomonas* and *Trichoderma* isolates against the bacterial wilt of ginger was indicated in the following sub-sections.

4.8.3.1. Isolation and evaluation of antagonistic effect of Pseudomonas fluorescens

Isolation was carried out according to the methods described by Labeda (1990). The potential bioantagonistic bacteria were isolated from rhizoplane soil of healthy ginger plants by soil dilution method using King‟s B medium and incubated at 28 ± 2 ^{0}C for two days and maintained on Kings B slants by regular sub culturing. The identity of the *P. fluorescens* isolates were confirmed by morphological, cultural and biochemical tests (Rekha *et al.*, 2010). Among ten *P. fluorescens* isolates, only two were selected for further study. The *R. solanacearum* suspensions were adjusted to 10^8 cfu per ml and swabbed on nutrient agar medium (NA) . The swabbed plates were spot inoculated with 100 µl *P. fluorescens* isolates and incubated at 28 ± 2 ^{0}C for three days. After incubation, the zone of inhibition was observed and recorded. There were three replicates for each treatment.

Trichoderma isolates were isolated from rhizoplane soil of healthy ginger plants by standard soil dilution agar plate method on Potato Dextrose Agar (PDA) at 25± 2°C (Aneja, 2005). Pure cultures of the *Trichoderma* isolates were maintained on PDA and identified using cultural and morphological characters (Watts *et al*., 1988). Antagonistic activity of *Trichoderma* isolate which was tested against eight virulent isolates of *R. solanacearum* by *in vitro* techniques using nutrient agar medium (Ran *et al*., 2005). Hundered (100) µl supernatants from one week old culture broths of *Trichoderma* grown in Potato Dextrose Broth (PDB) were tested by well diffusion method (Kamal *et al*., 2008). After incubation of inoculated plates for seven days, the zone of inhibition was observed and recorded. There were three replicates for each treatment.

4.8.4. In Vivo/ Pot Experiment

A pot experiment was designed under greenhouse conditions using plastic pots containing reasonable weight of sterilized soil that was brought from the sampling site. Disease free suckers

of ginger plants from Areka Agricultural Research Center were planted in the pots and kept to grow in green house. Thirty days after pathogen inoculation, disease severity based on the disease index (DI) 1–5 was recorded as follows: DI 1 wilt symptoms on 1- 25% of the leaves, DI 2 wilt symptoms on 26-50% of the leaves, DI 3 wilt symptoms on 51-75% of the leaves, DI 4 wilt symptoms on more than 76% of the leaves and DI 5 the whole plants dies (Kempe and Sequeira 1983). Disease severity and biocontrol efficacy were calculated according to the following formula.

$$\text{Disease severity (\%)} = \frac{\sum (\text{The number of diseased plants} \times \text{DI})}{\text{Total number of plants investigated} \times \text{the highest disease index}} \times 100$$

$$\text{Biocontrol/ Chemical-control efficacy} = \frac{\text{Disease severity of control plants} - \text{Disease severity of antagonist treated plant}}{\text{Disease severity of control}} \times 100\%$$

4.8.5. Biological Treatments

The pot experiment was conducted to evaluate the biocontrol potential of each selected isolates solely and integrated with the bactericide. Surfaces of pots was sterilized with 70% of ethanol and filled with 3Kg of sterilized soil from the sampling site. Ginger seedlings were brought from Areka Agricultural Research Center, Planted ginger were infected eighth leaf stages and one (1)ml of inoculums of R. solanacearum per plant was prepared and inoculated into the stem, leaf and wounded collar root areas with concentration 10^8 cells/ml. And simultaneously, 1 ml of each bacterial and fungal antagonists were applied eight selected isolates of *R. solanacearum* and equal suspension of bacterial and fungal antagonist and Streptomycin Sulfate were also applied in to virulent isolate alone (AAURS-03) of *R.solanacearum* according to the following treatments:(i)Control(not-treatment)(ii)Trichoderma1+AAURS03,(iii)Trichoderma2+AAURS03, (iv)pseudomonas1+AAURS03,(v)pseudomonas2+AAURS03,(vi)combinationTricoderma1,2+AAURS03, (vii) combination pseudomonas1,2+AAURS03, (viii) (combination of antagonists and bactericide+AAURS-03) and (ix) Streptomycin Sulfate were applied.Three replicate pots were specified for each treatment in completely randomized experimental design (Morsy et al., 2009).

4.8.6. Disease developments

Observation on ginger wilt disease development was taken at 15 days interval from the time of inoculation and continued till harvest of the crop. Data on Plant infection, leaf infection, leaf area diseased, rhizome infection were Collected and analyzed. The pathogenicity index was determined by using the expression (Salami and Akintokun, 2008):

Pathogenicity index (PI) = $100-\frac{[WD \times 100\%]}{WH}$

Where: WD=weight of the diseased rhizome and WH= weight of healthy rhizome.

Leaf infection = $\frac{\text{Number of infected leaves} \times 100\%}{\text{Total number of leaves}}$

4.8.7. Yield of ginger

Rhizomes yield per pot was recorded by taking the weight of the entire produce harvested from the pot and the data were expressed in gram (g) (Singh, 2002).

$$\text{Yield/ha} = \frac{\text{Yield per pot} \times 10{,}000}{\text{Area of the pot}}$$

4.9. Data Analysis

The experimental data was analyzed by using one way analysis of variance and comparison of means, at 0.05 level was made by LSD t- test. Mean and standard deviation and standard error of the mean were analyzed by using SPSS (version 20.0). ANOVA was performed for means comparison at ($p<0.05$).

5.RESULTS

5.1. Characterization and identification of aggressive isolates of *R. solanacearum* from ginger

Among 50 bacterial isolates isolated from ginger rhizome, only eight isolates were selected based on the morphological, physiological, cultural, biochemical and pathogenicity studies that were carried out in the laboratory All the isolates were grown on TZC plates at 28 $\pm 2^0$C for 48hr The virulent (colonies with pink or light red colour or characteristic red center and whitish margin) and avirulent (smaller, off-white and non-fluidal colonies) isolates of *R. solanacearum* were identified in TZC medium containing 0.005% TTC. All isolated 50 isolates from ginger rhizomes had relatively close colony appearance on TZC medium resembling to *R. solanacearum*. Eight isolates were showed typical symptoms of wilting, where as the remaining 42 were non pathogenic and could be rated as saprophytic although they had similar colony appearance with *R.solanacearum*. Based on the morphological studies it was found that all the isolates were gram-negative, motility, rod shaped, pink color, non-capsulated and non-spore forming (Table 3).

Table 2. Collections and Isolation of *Ralstonia solanacearum* isolates from ginger rhizomes

Isolates of *R.solanacearm*	Source of ino.	Geographical Area/zone/Region				Pathogeniciy to ginger
		Zone	Town	Woreda	Region	
AAURS-03	Soil	Welyita,	Areka,	Boloso Sore,	SNNPR	Highly Pathogenic
AAURS-04	Ginger	Welyita,	Areka,	Boloso Sore,	SNNPR	Highly Pathogenic
AAURS-05	Soil	kambata,	Hadero,	Kacha Bira,	SNNPR	Highly pathogenic
AAURS-07	Ginger	kambata,	Durame,	Angacha ,	SNNPR	moderatly Pathogenic
AAURS-08	Ginger	kambata,	Hadero.kacha Bira,		SNNPR	moderately pathogenic
AAURS-018	Ginger	Kambata,	Hadero,	Kacha Bira,	SNNPR	moderatly Pathogenic
AAURS-026	Ginger	Kambata,	Hadero,	kacha Bira,	SNNPR	non pathogenic
AAURS-034	Ginger		Hadero,	kacha Bira,	SNNPR	weakly pathogenic

The threads of bacterial ooze exuding from the infected xylem vascular bundles were observed from wilted rhizome. (Fig. 4).

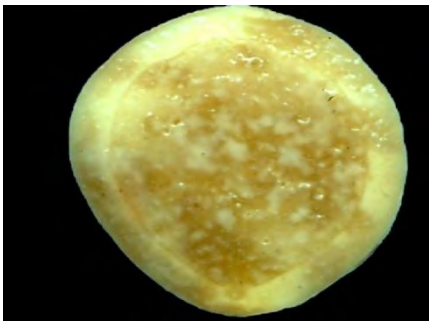

Fig 4. Bacterial ooze from an infected ginger rhizome.

5.2 Culture media based identification of *R. solanascearum*.

The culture identification was based on the bacterial is fluidal, presents irregular shape and white with pink centered colonies on tetrazolium chloride (TZC) media, which is similar with the description of *R. solanascearum* (Fig.5).

(a) onTZC medium (b) on nutrient agar medium

Fig 5. Morphology of *R. solanascearum* onTZC and nutrient agar medium

5.3 Identification of Virulent/Avirulent isolates of *R. solanacearum*

In this test, virulent isolates produce pink or light red colour colonies or colonies with characteristic red centre and whitish margin and avirulent isolates produce smaller, off-white and non-fluidal or dry on TZC medium after 24 hours of incubation.

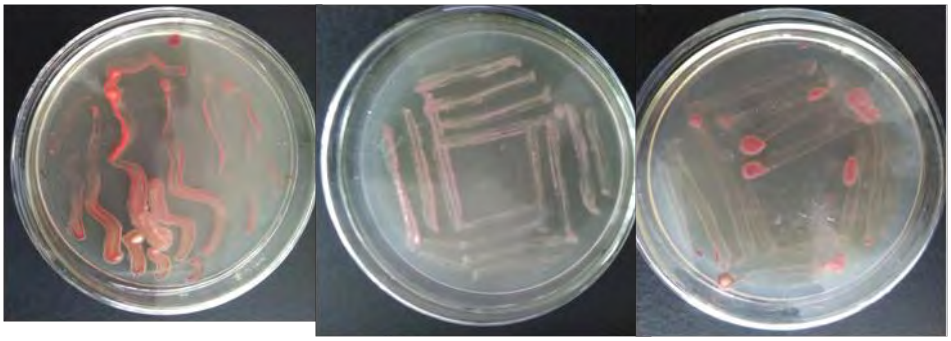

Fig 6. Pink centered virulent colonies of *R. solanacearum* on TZC agar medium

5.5 Biochemical Characterization of *R. solanacearum*

On the basis of all tests performed, data in the Table 3 shows morphological and biochemical characteristics of bacterial isolates. The isolates which were positive for catalase test, indol production test and oxidation of glucose, Hydrolysis of Tween 80, while they gave negative response in levan production from sucrose and remained non-fluorescent,were assumed as *R.solanacearum*. However, some isolates tested in this study showed differential behavior for starch hydrolysis.

5.5.1. Catalase

All eight isolates were catalase positive, release gas upon addition of hydrogen peroxide (Table 3).

5.5.3 Levan production from sucrose:

The tested isolates were negative for levan production, and levan formation results from the enzymatic activity of levan sucrase on sucrose (Table 3) .

5.5.4 Starch hydrolysis:

The tested isolates give different response for starch hydrolysis,seven isolates were negative for starch hydrolysis, but the only one isolates (AAURS -026) showed that clear zone surrounding the bacterial growth was observed when the plates were flooded with lugol‟s iodine solution for starch hydrolysis (Table 3).

5.5.5 Production of Fluorescent Pigment:

All tested isolates were found nonfluorescent. None of fluorescent cultures produced brown diffusible pigments which are the characteristic of *Ralstonia solanacearum* isolates , whereas presence of green, diffusible fluorescent pigment was evident in fluorescent isolates but did not produce fluorescent pigment on Kings B medium (Table 3)

5.5.6 Indole Production Test

The tested isolates showed the development of bright- pink color on the top layer mass of bacterial growth so all isolates were positive for Indole Production Test (Table 3)

5.5.7. Oxidation-fermentation of glucose:

All tested isolates were found positive for oxidation of glucose there was a gradual pH change at the surface of the open tubes resulting in color change from green to yellow, no pH change was observed in sealed tubes (Table 3)

5.5.8. Growth at 35 and 40^{o}C:

It was seen that all isolates grow at 35^{o}c and all of them failed to grow at 40 ^{o}C (Table 3)

5.5.9 NaCl tolerance:

In all the isolates, heave growth appeared in 1 % Nacl, medium with weak growth in 2 % Nacl. As the characteristic of *R. solanacearum*, none grow at 3,4,5,6,7,8,9,% NaCl medium (Table 4).

Table 3 . Biochemical and physiological characteristics of virulent bacterial isolates from wilted ginger for confirmation of *Ralstonia solanacearum.*

Isolates	Gram stain	Motility	Shape	Color	Catalase	Oxidationd glucose	Hydrolysis of Tween 80	Levan producti on	Indole producti on test	Starch hydrolysi s	Fluorescen pigment n KBM	Growth 40^0c
AAURS-03	Gm-	+	R	Pink	+	+	+	_	+	–	_	_
AAURS-04	Gm-	+	R	Pink	+	+	+	_	+	–	_	_
AAURS - 05	Gm-	+	R	Pink	+	+	+	_	+	–	_	_
AAURS-07	Gm-	+	R	Pink	+	+	+	_	+	_	_	_
AAURS-08	Gm-	+	R	Pink	+	+	+	_	+	–	_	_
AAURS-018	Gm-	+	R	Pink	+	+	+	_	+	–	_	_
AAURS-026	Gm-	+	R	Pink	+	+	+	_	+	+	_	_
AAURS-034	Gm-	+	R	Pink	+	+	+	_	+	–	_	_

Note: All tests were conducted in three replicates; „+" indicates positive reaction; „-" indicates negative reaction and Gm- =Gram negative, Gm+=Gram Positive, R=rod.

5.6 Ecophysiological Characteristics of Effective Isolates

The influences of different hydrogen ion concentrations, incubation time and temperature regimes on optical densities of *R. solanacearum* has been observed.

5.6.1 Effect of hydrogen ion concentration (pH) on R. solanacearum

There was a significant variation among the isolates in terms of their optical densities at different pH regimes and incubation times. The optical density of all isolates was high at pH of 6.5, the suitable pH for the growth of *R. solanacearum* was 5.0,5.5 and 7.0, and the optical density with pH ranging from 4.0 to 4.5 or 7.5 to 8.5 inhibited the growth of *R. solanacearum* and the high optical density was observed at 72 hours of incubation. The low optical density was observed at 24 and 96 hours of incubation in all tested pH conconcentrations (Fig.7).

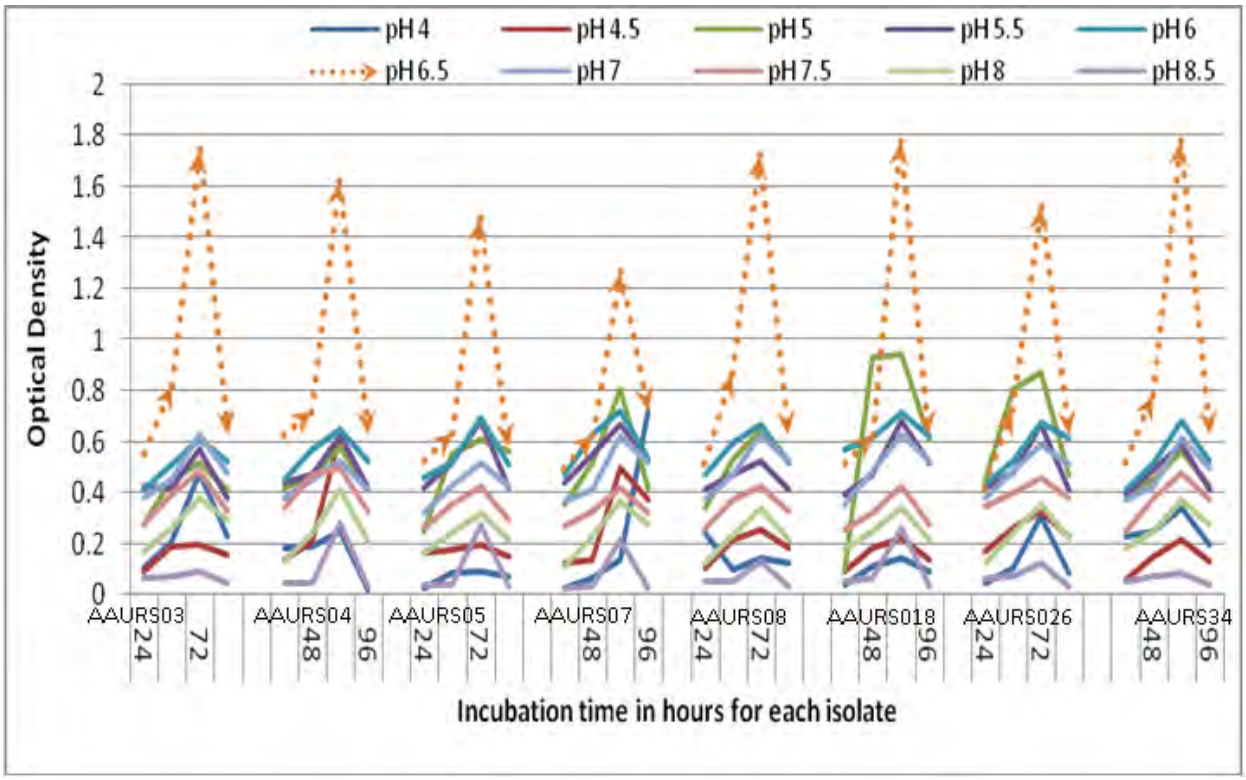

Fig 7. Effect of pH and incubation time on optical density of *R. solanacearum* isolates

5.6.2 Effect of incubation time and temperature on the growth of *R. solanacearum*

The data in the Figure 8 showed that variations with different ecophysiological conditions among the bacterial isolates with regard to temperature of their growth, all of them were found to grow with the temperature range of 10^0C to 35^0C at 24, 48 and 72 hrs. All isolates showed slow growth at 10, 15 where as all managed to moderate growth between temperatures at 20, 25 and 30^OC. All isolates showed best growth at 35^0C. All isolates showed growth neither at 40^0C nor at 4^0C (Figure 8).

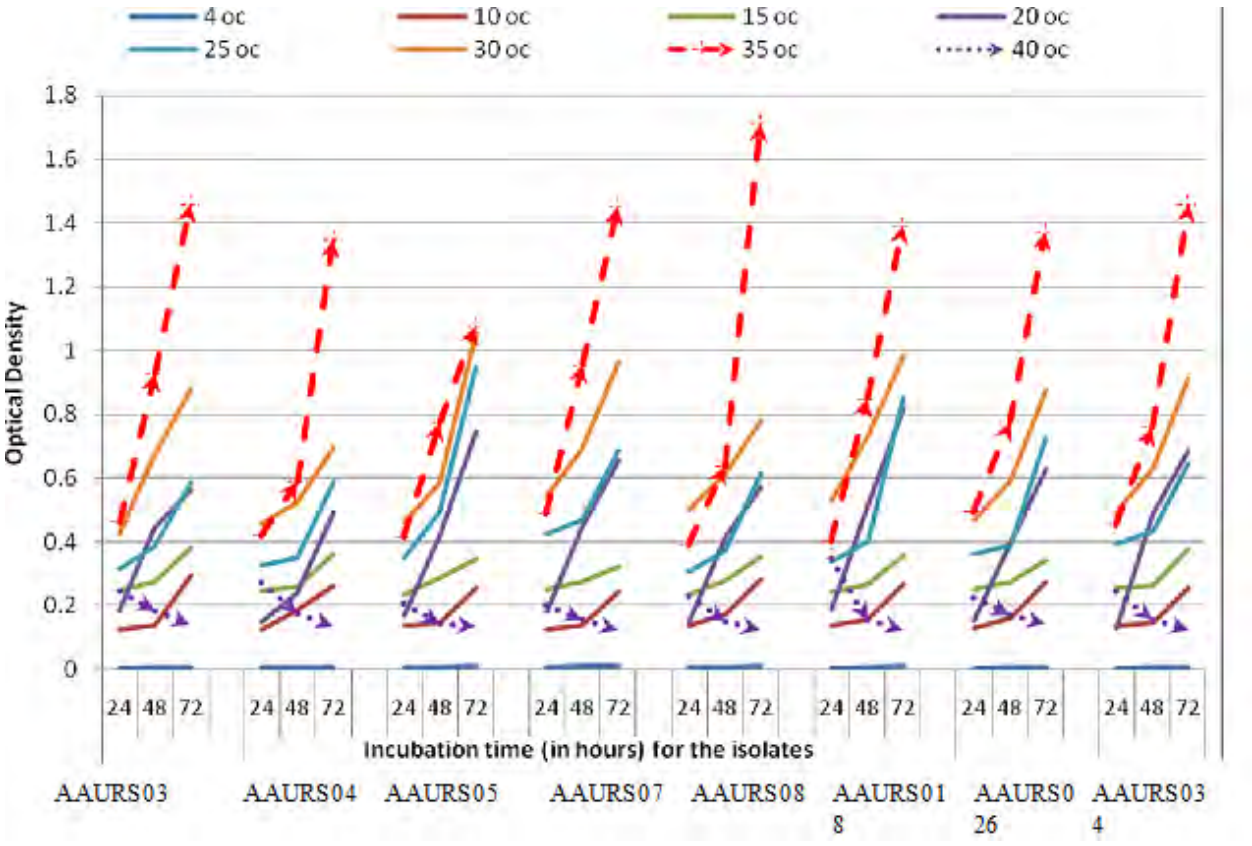

Fig 8. Effect of incubation time and temperature on optical density of R. solanacearum isolates.

5.6.3 Salt Tolerance on *R. solanacearum*

It has been observed that *R. solanacearum* isolates showed variation growth of different concentration of sodium chloride. They showed abundant growth at 1%, moderate growth at 2%, scant growth, at 3% and 4%, no growth at 5%, 6%, 7%, 8% and 9% in nutrient broth (Table 4).

Table 4. Salt Tolerance

Isolates	Salt concentration (%)								
	1%	2 %	3%	4 %	5 %	6 %	7 %	8 %	9 %
AAURS- 03	+++	++	+	+	–	–	–	–	–
AAURS- 04	+++	+	+	+	–	–	–	–	–
AAURS-05	+++	++	+	+	–	–	–	–	–
AAURS-07	+++	++	+	+	–	–	–	–	–
AAURS- 08	+++	++	+	+	–	–	–	–	–
AAURS-018	+++	++	+	+	–	–	–	–	–
AAURS- 026	+++	++	+	+	–	–	–	–	–
AAURS- 034	+++	++	+	+	–	–	–	–	–

Key: + = Scant growth, ++ = Moderate growth, +++ = Abundant growth, - = No growth

5.7. Biovar differentiation

The biovar of *R. solanacearum* isolates was identified by utiliztion of disaccharides and hexose alcohols. The result of the biovar test showed that all eight *R. solanacearum* isolates oxidized disaccharides (lactose, maltose) and sugar alcohols (manitol and sorbitol) within 7 days. The oxidation reaction was indicated by the change of color. The results revealed a change of color green to yellow color indicating the oxidization of sugars by bacterial isolates. Therefore, all *R. solanacearum* isolates belong to biovar III as shown in (Table 5 and Fig.9). On the other hand all the control plates of different sugars and sugar alcohols remain unchanged.

Table 5 . Differentiation of *Ralstonia solanacearum* into biovars and races

Isolate group	Utilization of Carbohydrates				Biovars	Races
	Maltose	Lactose	Sorbitol	Manitol		
AAURS- 03	+	+	+	+	III	4
AAURS- 04	+	+	+	+	III	4
AAURS- 05	+	+	+	+	III	4
AAURS- 07	+	+	+	+	III	4
AAURS- 08	+	+	+	+	III	4
AAURS- 018	+	+	+	+	III	4
AAURS- 026	+	+	+	+	III	4
AAURS- 034	+	+	+	+	III	4

Note: + Utilization of sugars, - No utilization of sugars

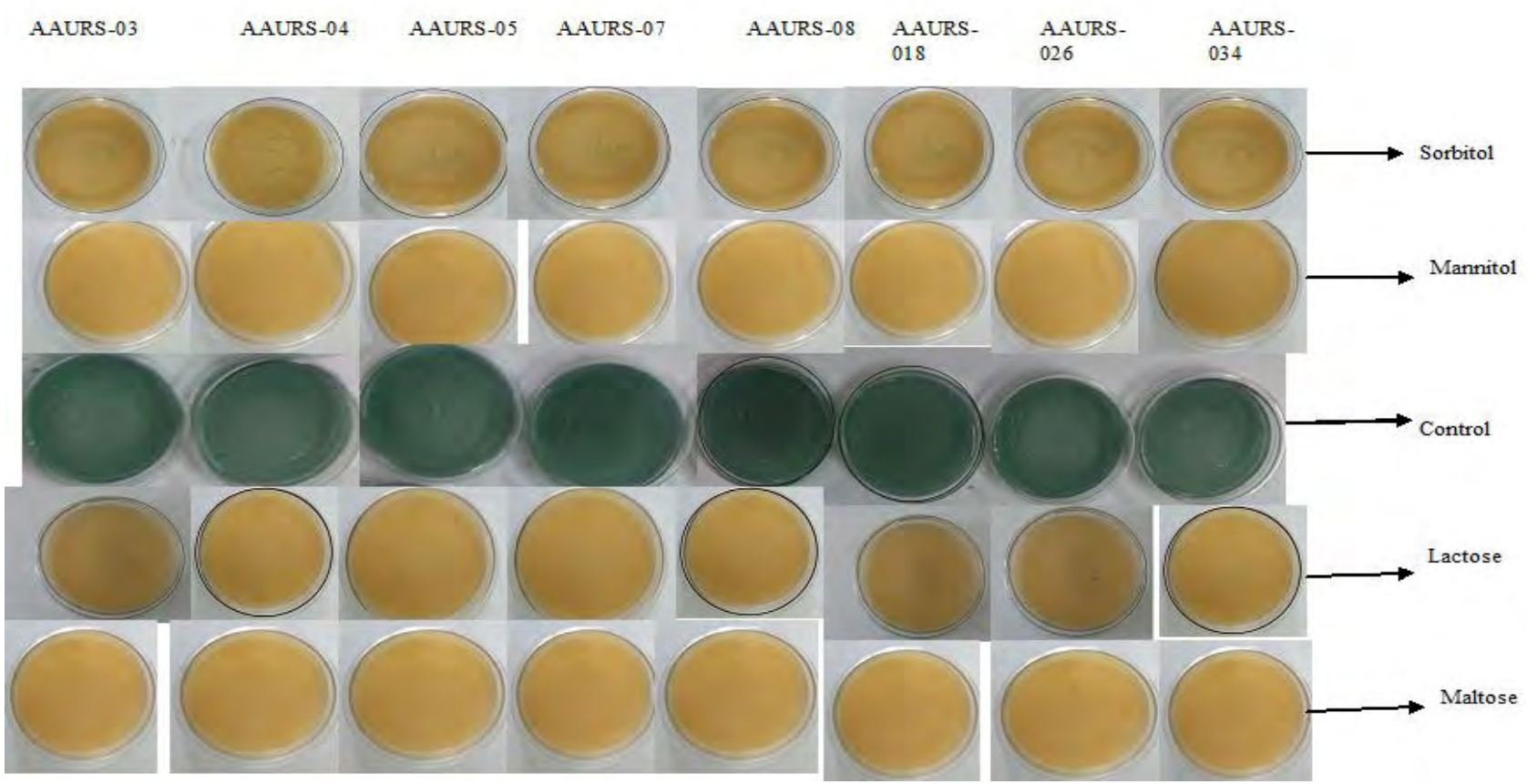

Fig 9. Biovar test showing positive (+ve) yellow color and negative (-ve) green color reaction indicating the utilization of sugar and alcohol by *Ralstonia solanacearum* isolates in microtitre plate.

5.8. Pathogenicity Test

It is clear evident from the result of pathogenicity test of *R. solanacearun* isolate under artificial stem inoculation method revealed that bacterial wilt of ginger occur within 7 to 15 days after inoculation (Fig.10).

Fig 10. The symptomatical variation on ginger plants of 1-5 scale studied under green house.

Table 6.Grouping of *R. solanacearum* isolates based on the variation of pathogenicity test .

Isolates	pathogen city	Scale	Percentage (%)
AAURS-03	Highly pathogenic	3	75
AAURS -04	Highly pathogenic	3	75
AAURS-05	Highly pathogenic	3	75
AAURS-07	moderately pathogenic	2	50
AAURS-08	moderately pathogenic	2	50
AAURS-018	moderately pathogenic	2	50
AAURS-026	Non pathogenic	0	0
AAURS-034	weakly pathogenic	1	25

Fig 11. Illustrations of the treatments for comparisons of sympotoms development(a) before inoculation (b) Application time of inoculation of biocontrols and test pathogen (c) Inoculation with treatment (d) inoculation only with pathogen

5.9.. Inhibition zone of *Trichoderma* and *Pseudomonas* isolates against *R. solanacearum*

The antagonists could produce varying level of inhibition zones against *R. solanacearum* in agar well diffusion method *in vitro* (Table 6, and Figure,14 and 15). The inhibition zone due to *P. fluorescens 1,2,* was relatively higher (30 and 39.5 mm) followed by *Trichoderma1,2, isolates* (20 and 22.5 mm).

Table 7. Inhibition zone (mean and standard deviation) of *Pseudomonas isolates*1,2,and *Trichoderma* isolates 1 and 2 using agar diffusion methods.

	Inhibition zone (mm)			
Isolates	***Pesudomonas* 1**	***Pesudomonas* 2**	***Trichoderma*1**	***Trichoderma* 2**
AAURS- 03	22.5±4.91	29±9.41	12.5±2.95	17.5±3.39
AAURS- 04	17.7±4.77	16.5±8.96	20±2.64	13.5±3.30
AAURS- 05	16.5±5.11	39.5±9.48	15.5±2.32	12±3.47
AAURS- 07	16.5±5.42	14.5±5.72	15.5±2.54	12.5±3.37
AAURS- 08	16±5.76	19±5.99	16.5±2.84	22.5±3.15
AAURS- 018	21.5±5.94	27±6.92	20±3.24	16.5±0.85
AAURS- 026	30±7.27	10.5±5.82	12.5±1.66	16±0.78
AAURS- 034	16±2.54	18.5±2.34	14±1.28	14.5±0.80
Mean	**19.59**	**21.81**	**15.81**	**15.63**

Fig 12. Antagonistic activity of *Pseudomonas fluorescens* against *R. solanacearum*.

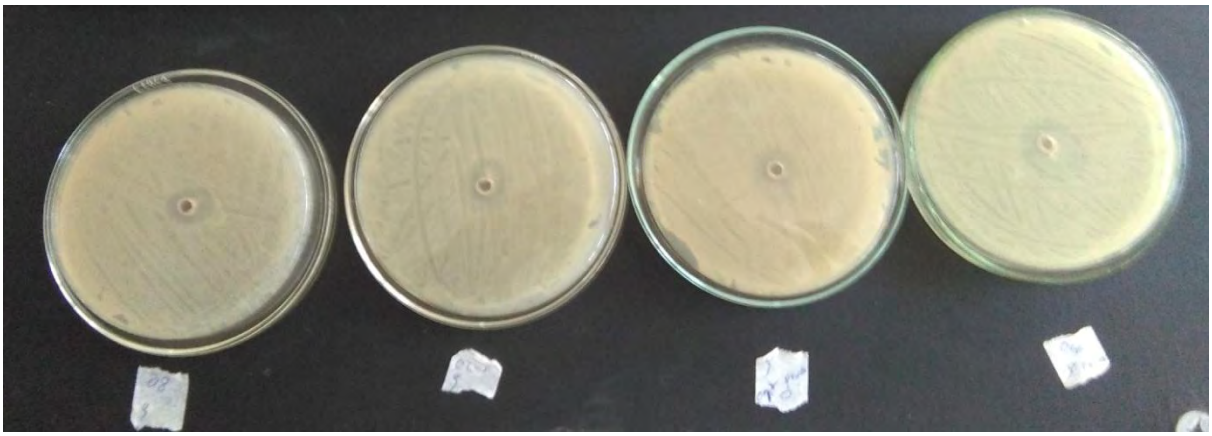

Fig 13. Antagonistic activity of *Trichoderma* isolate against *R. solanacearum*

5.9. 1. Effects of Bioagents on Disease Reaction, Biocontrol Efficacy , Yield and Growth of Ginger.

All treatments controlled the infection extensively giving higher yield compared to no treatment (control) (Tables 8-16). Through the Streptomycin sulfate which gave the best result both in terms of disease control and yield of ginger. The combined application of bactericide+antagonists performed as effective as chemicals and claimed as the most effective to reduce % severity, leaf and rhizome infection as well as to limit lesion area on rhizome. combination of bactericide + antagonistic resulted significant impact to reduce disease incidence and increased yield of ginger. this indicating that a combination of management practice is environment friendly and healthy . There is differences among the treatments and they are relatively varied from one another and gave a clear picture about the effects on plant growth and yield promoting factors and eventually on Yield.

5.9.2. Infection of bacterial wilt of ginger (R. *solanacearum*) on ginger cultivars

Symptoms of plant infection were observed and compared in 15, 22 and 30 DAI on treated and non treated plants (To).On day one the designated treatments were given to the plants. The results infection of bacterial wilt of ginger (R. *solanacearum*) on ginger cultivars are indicated in Table 8.The treatments (T1-T8) reduced the incidence of bacterial wilt of ginger disease relatively compared to the control treatment throughout the observation period, i.e., 15, 22 and 30 DAI. At all the observation dates, the control pots had the highest percent of plant infection. The highest percentage of plants showing bacterial wilt of ginger symptoms at 15 DAI

was 79.1% over control and it was the lowest (21.43%) in T8(Streptomycin sulfate). Percent plant infection in T1 (*Trichoderma* isolate1), and T2 (*Trichoderma* 2 isolate) were similar relatively at 15 DAI though numerically different. At 22 DAI, the highest percent plant infection was found in To (control) which was 80.3% and the lowest percent plant infection was found in T8(Streptomycin sulfate) which was 30.6%. The combination treatments T7(combination of antagonists and bactericide) was found to be most effective and had 32.31% plant infection. At 30 DAI, the highest percent plant infection was found over control which was 81.1% and the lowest percent plant infection was found in T8 (Streptomycin sulfate) which was 37.2%. The combination treatments T7(combination of antagonists and bactericide) was found to be most effective and only inferior to the pots treated with Streptomycin sulfate treatment. At 30 DAI the percentage plant infection under T7 was only 40.2%.

Table 8. Percent of ginger infection per plant with bacterial wilt of ginger after different days of inoculation.

Treat. No.	Treatments	15 DAI*	22 DAI*	30 DAI*
To	No treatment	79.1a	80.3a	81.1a
T1	*Trichoderma* isolate 1	72.8b	76.33b	78.6a
T2	*Trichoderma* isolate 2	72.66b	74.7b	77.66a
T3	*Pseudomonas* isolate 1	38.62c	43.54d	53.2b
T4	*Pseudomonas* isolate 2	45.44c	49c	54.25b
T5	Tri 1 + Tri 2	40.66c	46.22cd	51.23b
T6	Pseud. 1 + Pseud. 2	32.14d	35.88e	41.56c
T7	Tri +Pseu +Strepto.	24.66e	32.31ef	40.2c
T8	Streptomycin Sulfate	21.43e	30.6f	37.2d
	$LSD_{0.05}$	6.5	5.4	4.1
	CV(%)	16.6	12.1	14.0

* Means followed by the same letter within a column are not significantly different ($P<0.05$)

Similar results were recorded in T1(*Trichoderma* 1 isolate), T2 (*Trichoderma* 2 isolate) with values of 72.8, and 72.66%, respectively, in 15 DAI. Besides this, 22 DAI, the same result was observed in these treatments with values of 76.33 and 74.7 % of plant disease infections. On T3 (*Pseudomonas* 1 isolate), T4 (*Pseudomonas* 2 isolate), and their combination (*Pseudomonas*1+ *Pseudomonas* 2 isolates), on the other hand, lesser infections were observed with values of

38.62, 43.44, and 32.14%, respectively, at 15 DAI. At 22 DAI, T3(*Pseudomonas* 1 isolate), T4 (*Pseudomonas 2* isolate), and their combination (*Pseudomonas* 1+ *Pseudomonas* 2 isolates), had 45.54, 49, and 35.88 percentof plant infection. Combination treatment (*Trichoderma* 1 +*Trichoderma* 2 isolates) or combination of *Pseudomonas* isolate (*Pseudomonas*1+ *Pseudomonas* 2 isolate) had shown superior results as compared to *Trichoderma* or *Pseudomonas* isolate applied alone. But treatment Streptomycin sulfate, despite having the highest performance, was not environment friendly though significantly differed from control and T1, T2 T3, T4 T5 and T6. Besides, statistically similar percent of plant infection shown*Trichoderma*1 and *Trichoderma* 2 isolates in all 15, 22 and 30 DAI regimes. According to the observation at 30 DAI on the percent of plant infection per pot, the least effective treatments compared to the +ve control were T1 (*Trichoderma*1 isolate), T2 (*Trichoderma* 2 isolate), T3(*Pseudomonas*1 isolate) T4 (*Pseudomonas* 2 isolate) and T5 (Combination of *Trichoderma* 1 and 2). However, all these treatments had considerable efficacy. On the contrary, the relativly effective treatments under which the pots had the lowest or nearly lowest percent infected plants were under T5 (Combination of *Trichoderma* 1 and 2 isolate) and T6 (combination of *Pseudomonas* 1 and 2 isolates) and T7 (Combination of antagonists and bactericide) were the most effective integrated treatments. Streptomycin sulfate bactericide which reduced the infection of pathogen but it must be considered that Streptomycin sulfate toxic chemical and not an environment-friendly product.

5.9.3 Leaf Infection

Extended nectrotic wilting symptoms appeared on mature leaves and grow fast. Large spots have been found to kill the leaf within three weeks. Affected leaves have been observed to wither and fall off. The results obtained on the parameter % of leaf infection and effect of different treatments on percent leaf infection are presented in Table 8. At 15 DAI, the highest percentage of leaf infection 24.2% was observed in the treatment to (control). The result clearly indicated that the treatments had relativly effect on percent leaf infection. The lowest (21.43%) percent leaf infection was observed in pots received Streptomycin sulfate treatment. Percent leaf infection was relatively different with in control (no treatment) and T1 (*Trichoderma* 1 isolate) in 22 DAI and 30DAI with value of 28.4% , 25.3% and 30.9, 28.6%, respectively. The rest of the treatments had shown relatively high effect o n percent of leaf infection(Table 9).

Table 9. Percent leaf infection per plant with bacterial wilt of ginger after different days of inoculation.

Treat. No.	Treatments	15 DAI*	22 DAI*	30 DAI*
To	No treatment	24.2a	28.4b	30.9a
T1	*Trichoderma* isolate 1	21.8bc	25.33b	28.6a
T2	*Trichoderma* isolate 2	22.66b	23.7c	27.66ab
T3	*Pseudomonas* isolate 1	20.62d	23.54b	25.2b
T4	*Pseudomonas* isolate 2	23.44a	29a	28.25a
T5	Tri 1 + Tri 2	20.66d	26.22b	28.23ab
T6	Pseud. 1 + Pseud. 2	22.14b	25.88b	26.56b
T7	Tri +Pseu +Strepto.	24.66a	22.31c	22.2d
T8	Streptomycin Sulfate	21.43c	20.6d	27.2a
	$LSD_{0.05}$	2	2.1	3.3
	CV (%)	8.9	10.4	11.5

*Means followed by the same letter within a column are not significantly different ($P<0.05$)

At 2 2 DAI, the highest percentage of leaf infection 28.4% was observed in the treatment over the control and this was significantly higher than under any other treatments. The result clearly indicated that the treatments had significant effect on percent leaf infection. The lowest (21.4%) percent leaf infection was observed in treatment Streptomycin sulfate (Chemical) .Percent leaf infection was statistically different in T1(*Trichoderma,*1isolate) and T2 (*Trichoderma* 2) having of 25.3% and 23.7%, respectively.The rest the of treatments had shown statistically significant effect on control of the test pathogen (Table 9).

At 30 DAI, the highest leaf infection 30.9% was observed in the treatment To (control). The lowest (22.2%) percent leaf infection was observed in treatment T7 (Combination of antagonists and chemical). Percent leaf infection was statistically similar in T1 (*Trichoderma* 1) and T2 (*Trichoderma* 2) having of 28.6% and 27.6%, respectively as well as T5 (Tri 1 + Tri 2) and T6 (Pseud.1 + Pseud.2) were having relatively the same effect of 28.23% and 26.66%, respectively. The rest of treatments had shown relatively high effect (Table 8). The progress in the % leaf infection from 15 to 30 DAI and then through the next period 22-30 DAI appeared to have same speed. This scenario was more or less same under all the treatments including control.

5.9.4 Leaf area diseased (LAD)

The results obtained on the parameters percent LAD has been presented in Table 9. ln case of percent leaf area diseased, the result clearly indicated that the treatment had significant effect o n percent leaf area disease. At 15 DAI, the highest percent leaf area disease was observed int o (control) having value 36.2 % and the lowest leaf area disease was in T5 (*Trichoderma1*+ *Trichoderma2*) having value 30.6 %. Treatment T1(*Trichoderma*1), T2(*Trichoderma* 2), T3(*Pseudomonas* 1) and T4 (*Pseudomonas* 2) showed relatively similar effect having values of 31.8 %, 33.66 % and 30.62 %,34.4%, respectively. The rest of the treatments also showed significant effect (Table 9).A combined treatmentT7 which include bioagents of *Trichoderma*1 + *Pseudomonas*1 and Streptomycin sulfate as mixture spray was found as efficient as Streptomycin sulfate. The disease development in the infected leaves at this period (15-30 DAI) was rather low compared to the next period of observation (20-30). Only treatment with Streptomycin sulfate (T8) has shown a significant difference higher efficacy from T7.

At 22 DAI, the highest percent of leaf area disease was observed into (control) having value 38.4 % and lowest leaf area disease was in T8 (Streptomycin sulfate) having value of 30.6%. Treatment T1(*Trichoderma*1 isolate), T2(*Trichoderma* 2 isolate) and T3 and 4 (*Pseudomonas* isolates 1,2) showed statistically similar effect having values 3 4 . 3 %, 34.7 % and 35.54 %, 38.%, respectively. The rest of the treatment showed significant effect (Table 1 0). Engulfment of leaf area by the disease progress was faster in this period of observation (22-30 DAI).

Table 10. Percent diseased leaf area (% DLA) with bacterial wilt of ginger after different days of inoculation.

Treat. No.	Treatments	15 DAI*	22 DAI*	30 DAI*
To	No treatment	36.2a	38.4a	39.9a
T1	*Trichoderma* isolate 1	31.8b	34.33b	39.6a
T2	*Trichoderma* isolate 2	33.66b	34.7c	38.66a
T3	*Pseudomonas* isolate 1	30.62c	35.54c	37.2bc
T4	*Pseudomonas* isolate 2	34.44ab	38a	39.25a
T5	Tri 1 + Tri 2	30.66c	36.22ab	38.23ab
T6	Pseud. 1 + Pseud. 2	32.14b	35.88b	36.56bc
T7	Tri +Pseu +Strepto.	34.66ab	32.31c	32.2d
T8	Streptomycin Sulfate	31.43d	30.6d	37.2bc
	$LSD_{0.05}$	1.5	2.3	2.4
	CV (%)	16.7	19.5	13.5

* Means followed by the same letter within a column are not significantly different ($P<0.05$)

At 30 DAI the highest percent leaf area disease was observed into (control) having value 39.9 % and the lowest leaf area disease was in T7 (Combination of antagonists and chemical) having value 32.2 %. Treatment T5(*Trichoderma*1+Trichoderm2, T6(*Pseudomonas* 1 + *Pseudomonas*2 isolates) and T8 (bactericide) statistically similar effect having values 38.23 %, 36.56% and 37.2 % .respectively. The rest of the treatment also showed significant effect (Table 1 0).

5.9.5 Infected Rhizomes per pot

Among the bioagents, the strongest antagonists against *R. solanacearum* reaction has been shown in terms of percent rhizome infection by Tri +Pseu +Strepto (42.2%), which is lower than *Trichoderma1,Trichoderma12,Pseudomonas*1,*Pseudomonas*2,(49.6,48.66,46.2,49.25) respectively.This individual antibacterial strength has also been reflected in T6 and T7. The effect of bactericide in reducing % rhizome infection was as likely. Throughout the experiment in all the parameters taken into account application of Streptomycin sulfate as foliar spray performed excellent. It was stated that Streptomycin sulfate treatment was a positive control treatment as no treatment was considered as a control treatment. The results and their analyses revealed that the test treatments, bioagents, are capable of reducing bacterial wilt of ginger was relatively effective

even when applied singly. However, co noculation had greater infection control performance than single treatment application

Table 11. Percent infected rhizomes per pot with bacterial wilt of ginger after different days of inoculation.

Treat. No.	Treatments	30 DAI*
To	No treatment	49.9a
T1	*Trichoderma* isolates 1	49.6a
T2	*Trichoderma* isolates 2	48.66b
T3	*Pseudomonas* isolates 1	46.2c
T4	*Pseudomonas* isolates 2	49.25a
T5	Tri 1 + Tri 2	48.23b
T6	Pseud. 1 + Pseud. 2	46.56d
T7	Tri +Pseu +Strepto.	42.2e
T8	Streptomycin Sulfate	47.2c
	$LSD_{0.05}$	1.2
	CV (%)	16.5

* Means followed by the same letter within a column are not significantly different ($P<0.05$)

5.9.6 Biocontrol Efficacy

The biocontrol efficacy of the *Trichoderma* isolate 1,*Trichoderma* isolate 2, P*seudomonas* isolate 1 and *Pseudomonas* isolate 2 had been compared with the control and determined against the eight isolates of the *R. solanacearum*. The respective percent of disease severity and biocontol efficacy were indicated in Table 12-15.

Table 12 . Effect of *Trichoderma* isolate 1 mediated biocontrol on different bacterial wilt of ginger (*Ralstonia solanacearum*) isolates.

R. solanacearum **isolates**	**Disease severity of control (%)(T0) (Mean±SD)**	**Disease severity of treatment (%) (Mean±SD)**	**Biocontrol efficacy (%) (Mean±SD)**
AAURS-03	81.1±30.66	71.8±24.38	11.47±5.81
AAURS-04	81.2±30.76	75.33±27.91	7.23±1.57
AAURS-05	81.4±30.96	79.6±32.18	2.21±3.45
AAURS-07	25.2±25.24	22.8±24.62	9.52±3.86
AAURS-08	27.4±23.04	25.33±22.09	7.55±1.89
AAURS-018	29.9±20.54	29.6±17.82	1.00±4.66
AAURS-026	37.4±13.04	35.33±12.09	5.53±0.13
AAURS-034	39.9±10.54	39.6±7.82	0.75±4.91

As indicated in Table 12 *Trichoderma* isolates 2 mediated biocontrol of ginger wilt caused by eight different *Ralstonia solanacearum* isolates,have a variation among the isolate to developed pathogencity on ginger host. The highest percentage of disease severity was found in +ve control (AAURS -03, AAURS-04, AAURS-05) which was 81.1%, 81.2%, 81.3% respectively. The lowest percentage of disease severity was found in +ve control (AAURS-07, AAURS-08, AAURS-018, AAURS-026, AAURS-034 which was (25.5%, 27.4%, 29.9%, 37.4%, 39.9% respectively). Disease severity is reduced from (81.1%, 81.2%, 81.4 to 71.8%,75.33%,79.6%) respectively.

The highest percentage of biocontrol efficacy was observed in treatment AAURS-03, AAURS-07, AAURS-08, and AAURS-04, which was 11.47%, 9.52%, 7.55%, 7.23% respectively. The lowest percentage of biocontrol efficacy was observed in treatment AAURS-026.AAURS-05, AAURS-018, AAURS-034 which was (5.53%, 2.21%, 1.00%, and 0.75% respectively.

Table 13. Effect of *Trichoderma* isolate 2 mediated biocontrol on different bacterial wilt of ginger (*Ralstonia solanacearum*) isolates.

***R. solanacearum* isolates**	**Disease severity of control (%)(T0) (Mean±SD)**	**Disease severity of treatment (%) (Mean±SD)**	**Biocontrol efficacy (%) (Mean±SD)**
AAURS-03	81.1±30.66	72.66±25.98	11.75±6.09
AAURS-04	81.2±30.76	73.7±27.02	9.96±4.3
AAURS-05	81.4±30.96	78.66±31.98	3.44±2.22
AAURS-07	25.2±25.24	23.66±23.02	6.75±1.09
AAURS-08	27.4±23.04	23.7±22.98	14.61±8.95
AAURS-018	29.9±20.54	28.66±18.02	4.19±1.47
AAURS-026	37.4±13.04	33.7±12.98	10.47±4.81
AAURS-034	39.9±10.54	38.66±8.02	3.13±2.53

As indicated in Table 13, *Trichoderma* isolates 2 mediated biocontrol of ginger wilt caused by eight different *Ralstonia solanacearum* isolates have a variation among the isolate to developed pathogencity on ginger host. The highest percentage of disease severity was found in +ve control (AAURS -03, AAURS-04, AAURS-05) which was 81.1%, 81.2%, 81.3% respectively. The lowest percentage of disease severity was found in +ve control (AAURS-07, AAURS-08, AAURS-018, AAURS-026, AAURS-034which was (25.5%, 27.4%, 29.9%, 37.4%, 39.9% respectively). Disease severity is reduced from (81.1%,81.2%,81.4 to 72.66%,73.7%,78.66%) respectively.

The highest percentage of biocontrol efficacy was observed in treatment AAURS-08, AAURS-03, AAURS-026, and AAURS-04, which was 14.61%, 11.75%, 10.47%, 9.96% respectively. The lowest percentage of biocontrol efficacy was observed in treatment AAURS-07.AAURS-018, AAURS-05, AAURS-034 which was (6.75%, 4.19%, 3.44%, and 3.13% respectively.

Table 14. Effect of *Pseudomonas* isolate 1 mediated biocontrol on different bacterial wilt of ginger (*Ralstonia solanacearum*) isolates.

***R. solanacearum* isolates**	**Disease severity of control (%)(T0) (Mean±SD)**	**Disease severity of treatment (%) (Mean±SD)**	**Biocontrol efficacy (%) (Mean±SD)**
AAURS-03	81.1±30.66	39.62±4.56	57.09±31.61
AAURS-04	81.2±30.76	44.54± 9.48	49.74±24.26
AAURS-05	81.4±30.96	54.2± 19.14	34.58±9.1
AAURS-07	25.2±25.24	20.62 ±14.44	19.36±6.12
AAURS-08	27.4±23.04	24.54± 10.52	12.07±13.41
AAURS-018	29.9±20.54	26.2± 8.86	12.91±12.57
AAURS-026	37.4±13.04	34.54± 0.52	8.49±16.99
AAURS-034	39.9±10.54	36.2 ±1.14	9.57±15.91

As indicated in Table 14 *pseudomonas* isolate 1 mediated biocontrol of ginger wilt caused by eight different *Ralstonia solanacearum* isolates, have a variation among the isolate to developed pathogencity on ginger host. The highest percentage of disease severity was found in +ve control (AAURS -03, AAURS-04, AAURS-05) which was 81.1%, 81.2%, 81.3% respectively. The lowest percentage of disease severity was found in +ve control (AAURS-07, AAURS-08, AAURS-018, AAURS-026, AAURS-034 which was (25.5%, 27.4%, 29.9%, 37.4%, 39.9% respectively). Disease severity is reduced from (81.1%, 81.2%, 81.4 to 39.62%, 44. 54.2%) respectively.

The highest percentage of biocontrol efficacy was observed in treatment AAURS-03, AAURS-04, AAURS-05, and AAURS-07, which was 57.09%, 49.74%, 34.58%, 19.36% respectively. The lowest percentage of biocontrol efficacy was observed in treatment AAURS-018, AAURS-08, AAURS-034, AAURS-026 which was (12.91%, 12.07%, 9.57%, and 8.49% respectively.

Table 15. Effect of *Pseudomonas* isolate 2 mediated biocontrol on different bacterial wilt of ginger (*Ralstonia solanacearum*) isolates.

***R. solanacearum* isolates**	**Disease severity of control (%)(T0). (Mean±SD)**	**Disease severity of treatment (%) *P.fluorescens (2)* (Mean±SD)**	**Biocontrol efficacy (%) *(*Mean±SD)**
AAURS-03	81.1±30.66	44.44±5.99	92.53±65.84
AAU5RS-04	81.2±30.76	49±10.55	72.29±66.63
AAURS-05	81.4±30.96	53.25±14.8	51.94±46.28
AAURS-07	25.2±25.24	24.44±14.01	3.69±1.97
AAURS-08	27.4±23.04	29±9.45	-6.52±12.18
AAURS-018	29.9±20.54	29.25±9.2	2.48±3.18
AAURS-026	37.4±13.04	39±0.55	-4.63±10.29
AAURS-034	39.9±10.54	39.25±0.8	1.8±3.86

As indicated in Table15 *pseudomonas* isolates 2 mediated biocontrol of ginger wilt caused by eight different *Ralstonia solanacearum* isolates, have a variation among the isolate to developed pathogen city on ginger host. The highest percentage of disease severity was found in +ve control (AAURS -03, AAURS-04, AAURS-05) which was 81.1%, 81.2%, 81.3% respectively. The lowest percentage of disease severity was found in +ve control (AAURS-07, AAURS-08, AAURS-018, AAURS-026, AAURS-034 which was (25.5%, 27.4%, 29.9%, 37.4%, 39.9% respectively). Disease severity is reduced from (81.1%, 81.2%, 81.4 to 44.44%, 49. 53.25%) respectively.

The highest percentage of biocontrol efficacy was observed in treatment AAURS-03, AAURS-04, AAURS-05, and AAURS-07, which was 92.53%, 72.29%,51.94%, respectively. The lowest percentage of biocontrol efficacy was observed in treatment AAURS-07, AAURS-018, AAURS-034, AAURS-026,AAURS-08which was (3.69%,2.48%, 1.8%, and 8.49%,-6.52% respectively(Table 14).

5.9.7 Rhizome Yield per Pot

Effect of different treatments on yield of ginger in terms of Fresh Yield per Pot (g) was determined and presented in Table 15. The highest yield was recorded in treatment T5 pot (299 g/pot) and the lowest yield was recorded in control pot To (l52 g/pot). Yield of ginger greatly varied from treatment to treatment which ranged from 152 – 299 g/pot. The second highest yield was observed in T7 (296 g/pot). The treatments T3 and T4 as well as T6 and T1 showed relatively high effect. However, compared to control the highest yield was observed in the T5 (*Trichoderma* 1 + *Trichoderma* 2) treatment (Table 16). From above, clearly revealed that rhizome production is inversely proportional to rhizome infection. Effect different treatment on yiled of Ginger in terms of dry weight was recorded and yiled per pot converted in to yiled per hectare land. It is interesting to find in the Table 16, which contains the findings about rhizome yield per pot that the weakest proved treatment T1 (*Trichoderma* 1) increased dry rhizome yield upto 33.2%. While spraying with *Trichoderma* Isolate 1 has increased yield with 70.6%, *Trichoderma* 2 bioagents has increased by 93.3%,*Pseudomonas* 1 bioagents 66.6%, *Pseudomonas* 2 has increased by 106.6%, combination of *Trichoderma* 1 and *Trichoderma* 2 and Pseudomonas 1 and 2 and combination of antagonists and chemical had shown an increase of 118.6%. The Streptomycin sulfate increased dry rhizome production per pot by 108%. Further interesting matter was that the plants that received Streptomycin sulfate treatment produced lesser quantity of rhizome than those under T5 and T7 had extract in combination which probably promoted plant growth property. The possible reason may be Streptomycin sulfate might have adversely influenced the plant growth property of the ginger plants. This adds to the already existing reasons why environment friendly community would like to find a safe replacement of Streptomycin sulfate .

Table 16. Effect of different treatments on the yield of ginger per pot as affected by bacterial wilt of ginger.

Treat No.	Treatments	Fresh Yield per Pot(g) *
To	No treatment	152g
T1	*Trichoderma* isolate 1	200f
T2	*Trichoderma* isolate 2	265c
T3	*Pseudomonas* isolate 1	284b
T4	*Pseudomonas* isolate 2	245d
T5	Tri 1 + Tri 2	299a
T6	Pseud. 1 + Pseud. 2	224e
T7	Tri +Pseu +Strepto.	296ab
T8	Streptomycin Sulfate	301a
	$LSD_{0.05}$	12.5
	CV (%)	11.3

*Means followed by the same letter within a column are not significantly different (P<0.05)

5.9.8 Yield ton/ha

Effect of different treatments on yield of ginger per hectare was determined and presented in Table 17. It was found that the highest yield was recorded in treatment T7 pot (1.6 ton/ha) and the lowest yield was recorded in control pot To (0.75 ton/ha). Yield of ginger profoundly varied from one treatment to another ranging from 0.75 ton/ha to 1.6 ton/ha. The second highest yield was observed in T8 (1.52 ton/ha). The treatment T5, To, T_7, Streptomycin sulfate showed statistically similar effect. But the highest yield was observed in T7 (antagonists and the chemical) treatment (Table 16). From above, it is revealed that when rhizome infection decreased then yield of ginger increased, conversely when increased rhizome infection then decreased yield of ginger.

Table 17. Effect of different treatments on the yield of ginger (ton/ha) as affected by bacterial wilt of ginger.

Treat. No	Treatments	Yield (Ton/Ha) *
To	No treatment	0.75e
T1	*Trichoderma* isolate 1	1.0d
T2	*Trichoderma* isolate 2	1.0d
T3	*Pseudomonas* isolate 1	1.33bc
T4	*Pseudomonas* isolate 2	1.1cd
T5	Tri 1 + Tri 2	1.28cd
T6	Pseud. 1 + Pseud. 2	1.45ab
T7	Tri +Pseu +Strepto.	1.6a
T8	Streptomycin Sulfate	1.52ab
	$LSD_{0.05}$	0.2
	CV (%)	17.4

* Means followed by the same letter within a column are not significantly different ($P<0.05$)

6. DISCUSSION

The virulent isolates of *R. solanacearum* were observed in the present study eight selected isolates of *R. solanacearum* ioslated from different ginger growing areas produced pink or light red colour colonies or colonies with characteristic red centre and whitish margin on TZC medium (Table 2). *Ralostonia solanacearum* produced fluidal colonies with pink or light red colour on TZC media after 24 hours of incubation (Rahman *et al.,* 2010). These results are in agreement with the findings of Kelman (1954),Schaad (1980), Suslow *et al.* (1982), French *et al.* (1995), Garcia *et al.* (1999) and Melo *et al.* (1999) have reported that the virulent isolates produce pink or light red colour colonies or colonies with characteristic red centre and whitish margin and avirulent isolates produce smaller, off-white and non-fluidal or dry on TZC medium after 24 hours of incubation. Many diagnostic and identification tests are based upon structural and chemical properties of bacteria (Lelliott and Stead, 1987). The chemical compositions of certain substances in bacterial cells can be detected with specific staining techniques. Information about the presence or absence of such substances is used for identification of bacteria (Agrios, 2005).

Microscopic observations clearly indicated that the organism was gram negative and thus the preliminary identification of *R. solanacearum* was confirmed that all of the isolates of *R. solanacearum* did not retain violet colour i.e. the isolates retained counter stain (pink colour). These results are similar with the findings of Suslow *et al*. (1982) they observed that the isolates of *R. solanacearum* did not retain violet colour i.e. the isolates retained counter stain (pink colour).

The present study showed that all the isolates tested produced gas bubbles during these test indicating that these might be *Ralstonia solanacearum.* This finding agrees with the work of Schaad, (1980) who reported that all the isolates tested produced gas bubbles during these tests when mixed with a drop of H_2O_2 on glass slide. All gram negative bacteria produced gas bubbles when these were mixed with a drop of H_2O_2 on glass slide. H_2O_2 is a by product of aerobic respiratory metabolism in aerobic bacteria. Production of gas bubbles gives a clue for presence of aerobic bacteria. The result showed that all group of *R. solanacearum* isolates were not able to produce distinctive domed shaped or round colonies due to production of levan in

sucrose containing nutrient agar (NA) medium. Similar findings were reported by (Zubeda and Hamid, 2011), the tested isolates were negative for levan production, and Levan formation results from the enzymatic activity of levan sucrase on sucrose. Levan formation is responsible for the production of mucoid colonies by some *Pseudomonas.* Production of levan as an extra cellular capsule or slime layer results in colonies which are characteristically raised, convex and dome shaped in appearance (Schaad, 1980).

The current finding showed that positive isolates of this test formed dense precipitate around the bacterial growth, evident mostly after 3-4 days of incubation. According to Sierra (1957) hydrolysis of Tween 80 is a sign of lipolytic activity or, more correctly, esterase activity, because the substrate is freely soluble in water. After hydrolysis of Tween 80 by *R. solanacearum* isolates, the precipated hydrolysed Tween 80 accumulated around colonies indicating positive lipase/esterase activity.The present study showed that tested isolates were found non fluorescent. Similar, finding was reported by (King *et al.,* 1954) Presence of green, diffusible,fluorescent pigment was evident in fluorescent strains, whereas non fluorescent cultures produced a brown diffusible pigment, which is the characteristic of *R. solanacearum.*

The present study showed that the response of *R. salanacearum* to different temperature is variable. All isolates of *R. salanacearum* showed slow growth at 10^0C, 15^0C where as all managed to moderate growth between temperatures at 20^0C, 25^0C and 30^0C. All isolates showed best growth at 35^0C. Thus, it could be shown that biovar III strains prefer high temperature. This is supported the assumption that biovar II and biovar I and III are more adapted to cool and warm tropical conditions (Horita *et al*., 2005 and Swanson *et al*., 2005). According to EPPO (2004) the optimum growth temperature of *R. solanacearum* in the tropics was (35°C) whereas strains occurring at higher altitudes in the tropics and in subtropical and temperate areas is lower (27°C); no growth has been observed at 40°C or 4°C. According to Kelman (1953) approximate minimal and maximal growth temperature values would be 8-10°C and 37-39°C respectively.
The result of hydrogen ion concentration on the growth Ralstonia solanacearum was high at pH 6.5, medium at pH 5,5.5 and 7.0. The pH ranging from 4.0 to 4.5 or 7.5 to 8.5 inhibited the growth of pathogen significantly. This result is an arrement with finding (Kai *et al.,*2014) the growth of R. solanacearum was found greatest in the medium with pH 6.5, the suitable pH for

the growth of R. solanacearum was shown as 6.0~7.0, and the pH ranging from 4.0 to 5.5 or 7.5 to 8.5 inhibited the growth of *R. solanacearum* significantly.

The result of the biovar test showed that all *R. solanacearum* isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol) with in 5-7 days.
Classification of biovars was done based on utilization of carbon sources which was disaccharide and sugar alcohol. If the biovar is biovar IV it is excepted to utilize sugar alcohol while the biovar is biovar III. It is excepted to utilize both disaccharides and sugar alcohol. The oxidation reaction was indicating by the change of colour. The results revealed the change of colour green to yellow indicating the oxidization of sugars by bacterial isolates. Therefore, all *R. solanacearum* isolates belong to biovar III. On the other hand, all the control plates of different sugar and sugar alcohol remain unchanged. The *R. solanacearum* isolate from SNNP region of Ethiopia confirm that all the isolates are biovar III. This could be due to the increasing of latently infected seed rhizome exchange from one location to another, which indicates the population is clonally propagated and transmitted. Large-scale cultivation of ginger in Ethiopia is mainly through the seed rhizome material brought from the predominant ginger growing locations, particularly Wolayta and Kembata. This is indicating that the pathogen is spread throughout ginger growing regions and within the locality. Similar findings were reported by (Bekelle,*et al*., 2016 and Habetewold kifelew,*et al*.,2015) that the disease that threat ginger in Ethiopia is caused by *Ralstoniasolanacearum* biovar III race 4 and the diseases were distributed in major ginger growing areas of the region.

This strains causing bacterial wilt of ginger in Ethiopia is also similar to that of Indian strain which is belong to biovar 3 that causes wilt in 5–7 days (Kumar and Sarma, 2004). The differentiation of biovars of *R. solanacearum* based on the utilization of carbohydrates was reported by Kumar *et al*. (1993). They observed that biovar III oxidizes both disaccharides and hexose alcohols whereas biovar I oxidize hexose alcohols but not disaccharides, biovar II oxidizes only disaccharides and biovar IV oxidizes only hexose alcohols Kumar *et al*. (1993).

The members of family *Psudomonadaceae* such as genus *Pseudomonas* and *Ralstonia* are oxidative in nature as compared to members of family *Enterobacteriaceae* like *Erwinia,* which are fermentative bacteria (Henok, 2014). There was a gradual pH change at the surface of the

open tubes resulting in color change from green to yellow, no pH change was observed in sealed tubes indicating that *R. solancearum,* which was aerobic bacterium, is responsible for such type of change in pH. All the hypersensitive positive isolates were found positive for oxidation of glucose. *Ralstonia solanacearum* isolates varied in utilization pattern of different sugars.

The result of pathogenicity test of *R. solanacearun* isolate under artificial stem inoculation method reviled that it has able to produce wilt symptom in ginger plants with in 7 to 15 days after inoculation. The result were found in range of pathogenecity study in India which is wilt of ginger occur within 5-7 days after inoculation **(**Kumar and Sarma, 2004). *Ralstonia solanacearum* have different host ranges and geographic distributions; race 1, 2, 3, 4 and 5 principally attacks bananas, ornamental plants, potato, ginger and mulberries respectively (Kelman, 1997). *Ralstonia solanacearum* race 4 was found restricted to ginger (Paret *et al*., 2010). According to Kumar and Sarma, (2004) race 4 didn"t attack banana, potato, tomato, pepper and curcuma and cause wilt on ginger within 5 to 7 days. The causal pathogens were resembling as race 4 based on their specific pathogenicity to *Zingiberaceae* crops (Tsuchiya *et al.*, 2005; Yano *et al.*, 2005 and Yano *et al.*, 2011).

Biological control using plant growth promoting microorganisms is an efficient approach to wilt management and is also considered environment-friendly. Recently, several studies have focused on biological control of *Ralstonia* wilt (Ji *et al*., 2008; Takenaka *et al*., 2008; Vanitha *et al*., 2009; Singh and Siddiqui, 2015). Several beneficial microorganisms have been used as biocontrol agent for suppressing *R. solanacearum*. In this study, root associated bacteria were isolated from the rhizosphere of mainly ginger in order to select potential and effective antagonists against bacterial wilt of ginger (*Ralstonia solanacearum*), the causal agent of bacterial wilt. This is likely because of the development of the pathogen and the bio-control agents is influenced by environmental factors as well by the presence of other microbial species. In an *in vitro* screening on nutrient agar (NA) medium by agar diffusion methods, four strains Fluorescent pseudomonads 1, 2 and *Trichoderma* 1and 2 with the highest inhibitory effect were observed under the greenhouse conditions. The results indicated that the antagonistic microorganism *Pseudomonas fluorescens,1* and, *Pseudomonas fluorescens* 2 resulted in maximum inhibition of the *Ralstonia solanacearum* with an inhibition zone of 30, 39.5 mm,

respectively which was found relatively superior over other treatments followed by *Trichoderma,1 and Trichoderma,2 20* and *22.5 mm* respectively.

Similarly laha *et al.* (1992) have stated that the fluorescent pigments produced by *Pseudomonas fluorescens* were appropriate Fe3+ and were considered as siderophores which inhibits the large number of phytopathogenic bacteria and fungi. In this study it was found that biological controls, *(Trichoderma,*1 isolate and *Pseudomonas fluorescens*) were found relatively higher antagonisze pathogen under *in vitro* conditions on the growth of the pathogen. Thus the main increase in inhibitory activity of pseudomonas strains in nutrient agar medium might be siderophore production that inhibited *R. solanacearum* growth. This is in agreement with other observations that the inhibition zone due to *P. fluorescens* was significantly higher (38.7 mm) followed by combination of *P. fluorescens* and *T. harzianum* (31.8mm) (Bora *et al.*,2016). Ciampi-Panno *et al.* (1996) reported *P. fluorescens* to be the primary candidates for biological control of bacterial wilt pathogen because of their own ability to synthesize a wide range of secondary metabolites, many of which have antibacterial and fungal activity. The fluorescent pseudomonads could inhibit the growth of plant pathogens by producing a range of metabolites like antibiotics, siderophores and other substances such as cyanide (Loper and Buyer, 1991). The general mechanism of antagonistic activity of *Trichoderma* spp. has been reported to be antibiosis, lysis, competition and mycoparasitism (Papavizas and Lumsden, 1980).

The isolates were screened *in vitro* for their antagonistic activity against the bacterial wilt pathogen of ginger (*Ralstonia solanacearum*) on PDA and NA medium. Out of the tested isolates, only two fungal and two bacterial isolates showed a certain degree of inhibition to the growth of the pathogen. Testing *in vitro* antagonistic activity is often used as a first straightforward step in selecting potential biocontrol agents. A good ability to colonize therhizosphere has been identified as another major factor for efficient biocontrol (Raaijmakers *et al*., 1999). To test their antagonistic effect in a greenhouse, a pot experiment was conducted using sterilized soil and ginger rhizome that was susceptible to the pathogen.

The treatments (T1-T8) reduced the incidence of bacterial wilt of ginger disease significantly compared to the control treatment throughout the observation period, i.e., 15, 22 and 30 day after inoulation. Similarly, reports of decline of pathogen in the soil due to antagonists and disease reduction were presented recently (Szczech and Shoda, 2004; Pieterse *et al*., 2001;). A

general question raised over the technique employed in conventional biocontrol programs has concerned coinoculation introductions against a single antagonist. It is perceived that co-inoculation of microbial species can result in synergistic, additive, or inhibitory effects on target performance compared to the effect of each antagonist alone. In this study, it has also found that coinoculation had greater disease suppression than those of single inoculation. It might be due to similar type of antagonistic mechanisms or other growth enhancing mechanisms. Stiling and Cornelissen, (2005) also have supported that multi-species application of BCAs were consistently more effective than single species application. Bora *et al.* (2015) suggested utility of *T. parareesei* combined with other saprophytic antagonists for management of bacterial wilt of bhootjolokia caused by *R. solanacearum.*

The combination treatments T7 (combination of antagonists and the chemical) was found to be most effective and only inferior to the pots treated with chloramphenicol (0.25%) treatment. But, treatment Streptomycin sulfate, despite having the highest performance, was not environment friendly though significantly differed from control and T1, T2, T3, T4, T5 and T6 experimental condition. This might be due to the fact that higher population of bioagents resulted either in suppression of the pathogen population or segregation of the pathogen from rhizosphere niches by their enhanced antagonists activity (Mulya *et al.,*1996*).*

Deuri (2013), reported positive compatibility amongst saprophytic antagonists like *P. fluorescens*, *T.viride* and *M.anisopliae*. Similarly, compatible observations amongst bioagents like *P.fluorescens, T. viride, T.harzianum, M.anisopliae* and *Beauveria bassiana* was made by Bora *et al.* (2013). In this experiment, bacterization of ginger rhizomes with the selected antagonistic isolates significantly reduced the incidence of bacterial wilt by 59.83%, and increased plant height and dry weight by 76.89% and 28.44%, respectively.This suggested that the importance of the isolates as plant growth promoting rhizobacteria. This finding is inagreement with Besides Papavizas (1985) reported that *T. harzianum* could inhibit the growth of *R. solanacearum* by production of diffusible substances and over crowding the pathogen. Sutunya (1984) reported that *P. fluorescens* could effectively reduce the growth of *R.solanacearum* in rhizosphere of tomato and ginger plants. In this study, isolates of biocontrol agents have already been found very effective strong antigonistic in controling *R. solanacearum*.

In some cases, the result was found more effective than that of Streptomycin sulfate (bactericide).

Plant infection, leaf infection, leaf area diseased, rhizome infection, yield per pot (g) and yield ton/ha were the parameters for data collection. To observe the effect on pathogenesis, three observations: on (15, 22, 30 DAI) were made, rhizome disease was observed on the last two observation data and the yield data was a cumulative of all the rhizome collection from the respective pots. The ginger test plants which have received chemicals, had the lowest disease intensity as well as severity parameters.

The severity of bacterial wilt and the biocontrol efficacy of the *Trichoderma* isolate 1, 2, and *Pseudomonas* isolate 1, 2 had been compared with the control and determined against the eight isolates of the *R. solanacearum*. The disease severity and biocontol efficacy (%) were compared. Among the antagonists tested, fluorescent pseudomonas showed the most beneficial characteristics, as it consistently suppressed the *R. solanacearum* wilt and also reduced severity and increase biocontrol efficiency compared to untreated control by 44.4 and 92%, respectively (Table 14). The plant growth promotion by rhizosphere bacteria might be associated with secretion of auxins, gibberellins and cytokinins (Viswanathan and Samiyappan, 2001) and suppression of deleterious microorganisms in the rhizosphere (Gamliel and Katan, 1993). The use of rhizosphere bacteria for increasing the yield and crop protection is an attractive approach in the modern system of sustainable agriculture (Gamliel and Katan,1993). Differences of wilt incidence and severity were also reported due to the great diversity of host plants affected by this pathogen, phenotype and genotype of *R. solanacearum,* its wide geographical distribution, and the range of environmental conditions conducive to bacterial wilt (Chatterjee *et al.,* 1997). The disease incidence varied from treatment to treatment, because of cropping pattern, environmental conditions and build up of inoculum load. The higher disease incidence may be attributed to heavy rainfall leading to improper drainage, which favoured the rhizome rot disease, further, monocropping system also aggravated the disease situation. This was clearly indicated by the disease intensity depending on factors like location, cultural practices followed by use of infected rhizomes and meteorological factors like temperature, relative humidity and rainfall (Chatterjee *et al.,* 1997). The highest average rhizome yields were observed under the treatment T_6 and T_7 and the lowest under the treatment of control (To). As a result low yield was found in control pots and high in the pots where combined treatments were applied. This might be due

to direct effect of the antagonists on reduction of the pathogen population present in the plant rhizosphere. Bora, *et al.*, (2016) reported, *P. fluorescens* and *T. harzianum* formulated in vermicompost (VC) and mustard oil cake (MOC) was significantly effective showing minimum diseases incidence (15.63%) as well enhancement of ginger yield (265.5q/ha).

The combined treatment had a highly significant effect on the rhizome yield. The results obtained during this experiment indicated that an intelligently designed combined treatment even may be better than a Streptomycin sulfate bactericide treatment of all the rhizome collection from the respective pots. The ginger test plants which have received bactericide had the lowest disease intensity as well as severity parameters. The treatment that applied alone has shown the weakest disease control potential but significantly superior to the control (To). The combine treatments T5, T6 and T7 have shown very strong response though lesser than the T8 (chemical) in controlling bacterial wilt of ginger. This was finding in agrrement with Liza Barua *et al*. (2009) study on the compatibility of *Trichoderma harzianum* and *Pseudomonas flourescens* against *Meloidogyne incognita* and *Ralstonia slanacearum* complex on egg plant (brinjal). They studied the population dynamics of *Trichoderma harzianum* and *Pseudomonas flourescens* from two different substrates viz, vermicompost and wheat bran after different days of storage related to both the substrates, the population density of *Pseudomonas flourescens* significantly increased up to 45 days of storage and the highest reduction of *Meloidogyne incognita* and *Ralstonia slanacearum* observed in combined application of *Trichoderma harzianum* and *Pseudomonas flourescens.*

Generally, the combination of antagonists with bactericide has proved to be consistently efficient in the control of *R. solanacearum* wilt disease at an in vivo test under greenhouse conditions. Inaddtion to the in *vitro* studies have shown that *Pseudomonas fluorescens* have potential antibacterial activity against the bacterial wilt of ginger (*R. solanacearum*).

7. CONCLUSION AND RECOMMENDATIONS

7.1. Conclusion

The biocmecial characterization revealed that the disease that threat ginger in SNNP region is caused by *Ralstonia solanacearum biovar III race 4* and the diseases was found distributed in major ginger growing areas of region. The syptomology and pathogenicity test confirmed that R. solanacearum causing bacterial wilt of ginger. All isolates of R. solanacearum were found virulent producing pink or light red color or characteristic red center and whitish margin on TZC medium after 48 hours of incubation.

Based on the result of *In vitro* inhibition test two isolates of *Pseudomonas fluorescent* and two isolates of *Trichoderma* had shown inhibetary effects on eight test isolates were selected and used under glasshouse experiment. The *in vitro* studies have shown that *Pseudomonas fluorescens* have potential antibacterial activity against the test pathogen (*R. solanacearum*).

Using of the *Tric+Tric*,*Pseud+pseudo* isolates and their combination with a Streptomycin sulfate is attracting a lot of interest in the biological control of soil borne phytopathogen than individual treatments. But *Pseudomonas fluorescens* have showed decrease severity and increase biocontrol efficience than *Trichoderma* under glasshouse condtion.

The results obtained through this experiment indicated that a wisely designed combined treatment (combination of antagonists and bactericide) was found to be most effective and only inferior to the pots treated with Streptomycin sulfate treatment. But, treatment Streptomycin sulfate, despite having the highest performance, was not environmental friendly. Thus, it can be concluded that the incidence and severity of bacterial wilt of ginger can significantly be reduced by the combined application of antagonists.

7.2 Recommendation

Looking at the seriousness of the disease the following recommendations were made:

The findings of the present study will step forward in determination of the bacterial isolates (*R. solanacearum*) to design an effective methods of detection through molecular based analysis of the test pathogen with special emphasis in order to develop integrated disease management.

Further study is necessary to check the ability of the antagonists to control the disease under field conditions and develop environmental friendly, integrated pest management methods.

Future research should be directed towards detailing the mode of action of these strains.

An urgent need also required on establishing diseases free ginger seed rhizome production system using greenhouse culturing system.

The triangular farmer extension and research link has to be strengthened and evenly applied to entire ginger producing areas in order to reduce *R. solanacearum.*

8. REFERENCES

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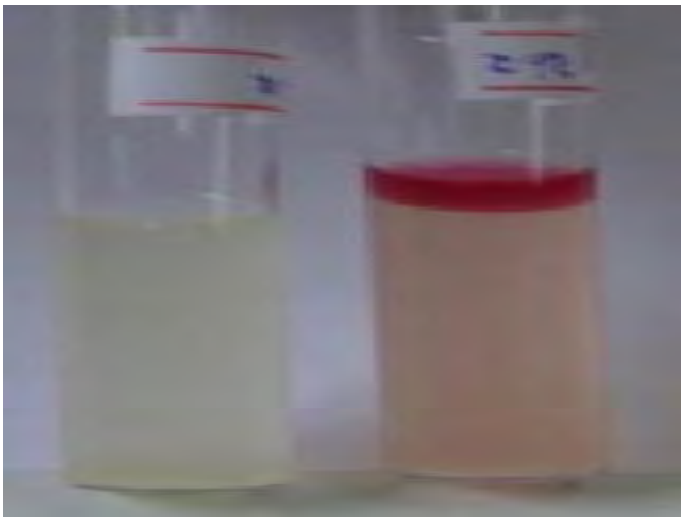
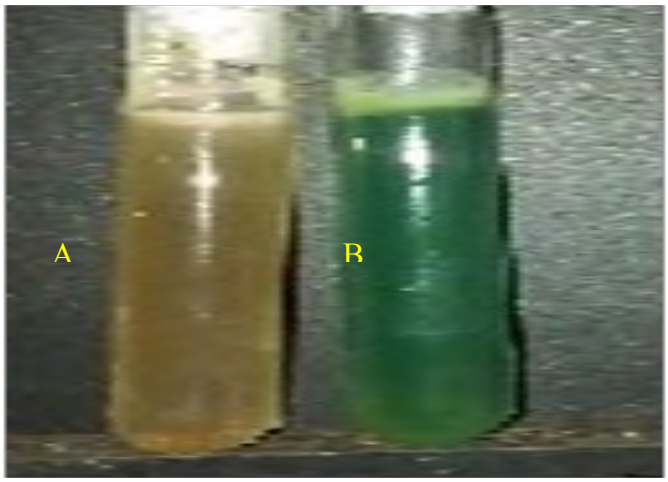
APPENDICES



Appendix 1.The development of bright- pink color on the top layer mass of bacterial growth



Appendix 2. Oxidation of glucose (a) unsealed test tube (+), the color change from green to yellow, (b) sealed test tube (-), no pH change was observed in sealed tubes.

Appendix 3. Starch hydrolysis clear zone with black back ground

Appendix 4. comparation of Trichoderma alone treated Ginger by best and poor performance

Appendix 5. comparation of psuedomonas alone treated Ginger by best and poor performance comparing with control

Appendix 6. Comparation of combination of antagonistic alone and with bactericide

Appendix 7. Different Basal Medium For Compositions.

Basal Medium of TZC or tetrazolium chloride	Amount gm/l	**Basal medium of carbohydrate for biovar determination**	Amount gm/l	**King B Agar for Compositions**	Amount gm/l
Dextrose	10 gm	Ammoniumdihydrogen phosphate (NH4H2PO4)	1 gm	Peptone.	20.0 gm
Peptone	10 gm	Potassium chloride (kcl)	0. 2gm	Glycerol .	10.0 mL
Casamino acids(caseinhydrolysate)	1 gm	Magnesium sulphate (Mgso4.7HO2)	0. 2gm	Dipotassium phosphate	1.5 gm
Agar	18 gm	Peptone	1 gm	Magnesium sulfate . 7 H2O	1.5 gm
Distilled water	1000 ml	Bromothymol blue	0.o3gm	Agar	15.0 gm
		Agar	3.0gm	Distilled Water	For 1 liter of medium
		Distilled water	1000ml		

DECLARATION

I declare that this thesis manuscript is my original work, prepared by my effort with the guidance and close supervision of my advisor. The thesis has been submitted in partial fulfillment of the requirements for M.Sc. degree at Department of Microbial, Cellular and Molecular Biology, Addis Ababa University. Sources of information other than my own have been fully acknowledged and a reference list has been appended. This work has not been previously submitted to any other university for award of any type of academic degree.

Abdallah Ibrahim

Signature _____

Date _____

This thesis has been submitted for examination with my approval as University advisor.

Tesfaye Alemu (Ph.D)

Signature _____

Date _____