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Biovar Differentiation and Pathogenicity of *Ralstonia solanacearum* (L.) Isolates on Ginger *(Zingiber officinale* Rosc.*)*

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Abstract

Ralstonia solanacearum (L.) 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 greenhouse and laboratory experiments in 2014/15 season. Totally, 10 isolates of Ralstonia solanacearum 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 biovar and pathogenecity of Ralstonia spp had been collected and analysed. Based on the result of pathogenecity test, only four isolates that had the highest virulence and growth of the pathogen were selected and used under the greenhouse experiment. Cultural characteristics of isolates on Tetrazolium Chloride (TZC) Agar selective medium showed light to red color with the characteristic red centre 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 antagonists to control the disease under field conditions.

Keywords: *Bacterial Wilt, Biocontrol, Biovar, Ginger, Race 4, Ralstonia solanacearum*

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Introduction

Ginger (Zingiber officinale Rosc.) 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 a herbal 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 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). 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 more than 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.

According 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, Kenbata Tenbaro, 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 behavior of *R. solanacearum*, and they found that streptomycin at 0.3 µg/ml water inhibited *R. solanacearum*. Singh et al. (2000) found that streptomycin and penicillin 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 study 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.

Materials and methods

Description of the Study Area

The study was carried out in Kembata Tenbaro, and Welayita Zones of south west of Ethiopia.(Durame and Hadaro tunto in kembat Tenbaro and Areka in Welayita Zone). Durame town is found in Kembata Tembaro zone in SNNPRS at a distance of 125km west of the regional capital, Hawassa, 350 km south of Addis Ababa via Shashemene and 298 Kms via Hossana. It also located at a distance of

80 Km south of Hossana town and 12km form the Shashemene-Wolata Sodo road form Mazoria which is new part of shone town. Astronomically, the town lies at the coordinate of 7^{o} 14^{o} north latitude and 37^{0} 35^{0} east longitudes. As a result of its topography, within the Ethiopian context, the town of Durame experiences cool temperate climate. According to the traditional temperature zone classification of Ethiopia, (Which is based on altitude) the town is found within the "Wonia Dega" Agro–ecological zone. Consequently, it experiences mean annual temperature between 14^{0}c and 26^{0}c (EIAR, 2009, Municipality of Durame Town, 2008).

Sample Collection

Samples were collected from three ginger growing areas of the Wolaita zone of SNNP region (Durame and Hadaro Tunto in kembat Tenbaro and Areka in Wolaita zone) south west of Ethiopia. Rhizosphere soil samples and rhizome of ginger (*Zingiber officinale* Roscoe) were collected from ginger growing areas. 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 transfer into sterile plastic bags. The collected sample was brought to College of Natural sciences, Addis Ababa University for isolation, characterization and evaluation of environmental factors on the growth, distribution, incidence and severity of the pathogens to the plant. The methods 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) and Dingera and Sinclair (1993). Samples were collected in 8 to 12 km intervals from farmer's field. Sample contained in clean plastic bags (envelopes) was brought to the laboratory and then was stored at 4°C for farther study. The *in vitro* study was carried out in the Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, College of Natural Sciences, Addis Ababa University (AAU) whereas the *in vivo* study was conducted in the Green house, at AAU.

Isolation of the Test Pathogens

The bacteria were isolated from the plant rhizomes within 2 d of collecting the samples, as follows. The samples were re-suspended in sterile saline (0.85% NaCl) and stirred briefly. The resulting cell suspension is serially diluted, plated on Nutrient Agar (NA), incubated at 28 ^{0}C for 48 h, and pure cultures obtained from single colonies were selected and purified. For isolating the bacteria from the plant rhizomes samples (3 g each) of roots were surface-disinfected with 1% sodium hypochlorite for 5 min and 70% ethanol 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 was completely sterile from outside: if no growth is seen after incubating at 28 ^{0}C for 48 h, the samples were assumed to be sterile. These samples were then ground using a sterilized mortar and pestle, mixed with 3 mL sterile water, and filtered through sterilized gauze. The filtrate is diluted 10-fold and plated onto Nutrient Agar (NA) maintaining 3 replications. After incubation at 28 ^{0}C for 48 h, a single colony was picked and inoculated into Nutrient broth, after shaking at 28 ^{0}C for 20 h, 0.5 mL of the suspension was blend with 0.5 mL 80 % Glycerin and stored at 4 ^{0}C for further investigation.

Isolation and identification of Ralstonia solanacearum

Diseased ginger plant parts (rhizome) 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. *R. solanacearum* was isolated in Nutrient Agar (NA) plate by streaking the bacterial ooze streamed out into the water from the Rhizome. The plates were then incubated at 280C for at 24 hrs. After isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Triphenyl Tetrazolium Chloride (TTC) medium as described by Kelman (1954). The pathogenicity test was performed in three month old healthy ginger seedlings by steam 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 transferred to Nutrient agar media slants and maintained at 4°C for further studies.

Identification of Virulent/Avirulent Strains of R. solanacearum

The virulent and avirulent isolates of *R. solanacearum* were differentiated by Kelman Tetrazolium Chloride (TZC) agar medium containing 0.005% 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).

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)

Grouping into biovars

Ten ml of each 10% lactose, maltose, cellulose, mannitol, sorbitol and dulcitol was added in sterilized screw-capped test tubes and then heated at 100°C for 30 min to sterilize these solutions. Bottles of semi-solid basal medium were melted in water bath and cooled to70°C. Carbohydrate solution (10 ml) was added and mixed in basal media ($NH_4H_2PO_4$ 1.0 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, Difco bacto peptone 1.0 g, Agar 3.0g and Bromothymol blue 80.0 mg per litre). Five ml solution was poured into labelled tubes. 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. Bacterial suspensions from individuals isolates (with concentration of 10^8 cfu/ml) were prepared from 48 hrs old cultures on 523 plates. Inoculation of the medium was carried out by adding 1-2 drops of bacterial suspension to each tube containing sugar solutions, three replicates of sugar solution and control were maintained for each isolate. Test tubes were incubated at 30°C and examined after 2,7 and 14 days for the presence of indicator change from olivaceous green to orange colour on the surface of medium(Hayward, 1964).

Pathogenicity testing of isolates

Virulence analysis of the isolates was carried out on a set of three ginger cultivars, Durame, Hadero Tunto in Kambat Tanbaro and Areka in Wolaita zone which are susceptible to bacterial wilt. The 20 days old healthy seedlings were selected and used for further pathogenicity assay. Bacterial inoculum was prepared in sucrose peptone broth (Hendrick *et al.* 1984, Mitsuo *et al.* 2004) and pelleted by centrifuging at 12,000 rpm for 10 min, suspensions were prepared in sterile distilled water and spectrophotometrically adjusted to OD600nm = 0.1 (approximately 108 CFU per ml) (Ran *et al*. 2005). Root dip method: the roots were trimmed with a sterile scissor and submerged in the bacterial suspension for 30 mins (Prior and Steva, 1990). The inoculated seedlings were transplanted to mini pots containing soil and sand in 1:1 ratio and incubated in greenhouse. The day and night temperatures varied between 25-35^0 C with 12h light and 12h dark (Klement *et al.* 1990). Soil drenching method: the root system of each plant was wounded with a scalpel; 5 ml of inoculum per plant was poured on the wounded root system. Plants were placed in a greenhouse and observed daily (Williamson *et al.* 2002). Each experiment was repeated twice, with two replications of 10 plants for each treatment. Observations were made from one week after inoculation. If the plant showed typical wilt symptoms, the interaction was considered as pathogenic. The isolates were categorized into 4 group's viz., highly pathogenic, moderately pathogenic, weakly pathogenic and non-pathogenic based on the symptomatological variations in the test ginger varieties, whereas non inoculated ginger seedlings showed no symptoms and hence served as control (Veerapaneni *et al*.1997; Tans *et al.* 2001).

Inoculants Preparation

Selected bacterial isolate was cultured in Nutrient Broth at 28±2^{0}c on orbital shaker. 24hr old culture will be taken and transferred to 100ml of the medium and incubated on orbital shaker at 120 rpm for 48hrs. Then the cultures were diluted in sterilized distilled water to 10^8 cells/ml. Plants were inoculated at three leaf stage by stem puncture and leaf infection pin pricks. The bacterial suspension was inoculated in to the leaves of each test plant **(**Stromberg*, et al.,* 2004).

In vitro evaluation of *Pseudomonas fluorescens* and *Trichoderma* Spp against *R. solanacearum*

The effects of different strains of pseudomonas and trichoderma species against the bacterial wilt of ginger were discussed in the following sub-sections.

***Isolation and selection of bio-antagonistic of* Pseudomonas fluorescence**

Isolation was carried out according to the methods described by Labeda (1990). The potential bio-antagonistic 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* strains were confirmed by morphological, cultural and biochemical tests (Rekha *et al.* 2010). Among ten *P. fluorescens* strains, only two strains were selected for further study. The *R. solanacearum* suspensions were adjusted to 10^8 cfu per ml and swabbed on NA (Nutrient Agar). The swabbed plates were spot inoculated with *P. fluorescens* strains and incubated. Following incubation, the zone of inhibition was observed. There were four replicates for each treatment. Isolation and selection of bioantagonistic *Trichoderma* species were also undertaken under laboratory conditions. *Trichoderma* strains were isolated from rhizoplane soil of healthy ginger plants by standard soil dilution method on PDA (Potato Dextrose Agar) at 25± 2°C. Pure cultures of the *Trichoderma* isolates were maintained on PDA and identified using cultural and morphological characters (Watts *et al*. 1988). The reidentification of the strains was done by National Fungal Culture collection of India (NFCCI), Agharkar Research Institute, Pune. Antagonistic activities of *Trichoderma* spp, which were tested against ten highly virulent strains of *R. solanacearum* through, *in vitro* techniques using PDA (Ran *et al*. 2005). 100µl 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). Following incubation, the zone of inhibition was observed. There were four replicates for each treatment.

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 Center were planted in the pots and kept to grow in green house. Planted ginger was infected after three leaves were produced according to the following treatments: 1) plant infected with sterilized water 2) plant infected with pathogen only 3) plant infected with pathogen and antagonist and 4) plant infected with pathogen, chloramphenicol (0.25%) and antagonists. Three replicate pots were specified for each treatment in completely randomized experimental design (Morsy*et al*. 2009).

Experimental Design and 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 ginger National Center. The percentage of disease severity (DS %) was evaluated 15 days after inoculation by estimating the percentage of leafs with lesions areas. The percentage of disease severity reduction (DSR%) was calculated according to Edginton *et al*., (1971): DSR (%) = [(DSc - DSt)/DSc)] x 100, where DSc = leaf area with lesions on the control plants that treated with only pathogen and DSt = leaf area with lesions on the treated with antagonist and pathogen or antagonist, bactericide and pathogen.

Data Analysis

The experimental data was analyzed by using one way analysis of variance and comparison of means, at 5% level was made by LSD t-test. Mean and standard deviation and standard error of the mean were analyzed by using SPSS (version 16.0, SPSS Inc, Chicago, IL, USA, 2007). ANOVA was performed for means comparison at ($p<0.05$) using the same program.), Wilcoxon- Mann-Whitney tests, (Proc GLM, SAS Institute Inc., 2002) were also carried out.

Results

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 reactions 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 3 and Fig. 4). On the other hand all the control plates of different sugars and sugar alcohols remain unchanged.

Table 1 . Differentiation of *Ralstonia solanacearum* into biovars and races

Isolate group	Utilization of Carbohydrates				Biovars	Races
	Maltose	Lactose	Sorbitol	Manitol		
type 03	+	+	+	+	III	4
type 04	+	+	+	+	III	4
type 05	+	+	+	+	III	4
type 2-s	+	+	+	+	III	4
type sp- s	+	+	+	+	III	4
type m-t	+	+	+	+	III	4
type 07	+	+	+	+	III	4
type 08	+	+	+	+	III	4

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

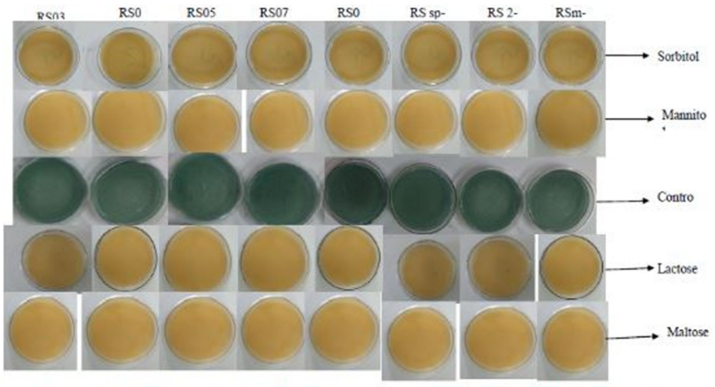

Fig 1. Biovar test showing positive (+ve) yellow color and negative (-ve) green color reaction indicating the utilization of sugar and alcohol by *Ralstonia solanacearum* isolates in microtitre plate.

Pathogenicity Test

Result of pathogenicity test of *R. solanacearun* isolate under artificial stem inoculation method reviled that wilt of ginger occur within 7 to 15 days after inoculation (Fig 2). The severity of bacterial wilt was recorded based on the severity scale as described previously by Horita and Tsuchiya (2001).

Discussion

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* (King *et al.,* 1954). All the hypersensitive positive (HR +ve) and Levan tested, 48 h old *R. solanacearum* cultures on Kings B Medium were examined under short and long wavelength UV

radiation (UV Lamp) and intensity of fluorescence was compared. All tested isolates were found non fluorescent . Comparable results were revealed by all the strains throughout the biochemical tests (Table 2), similar to those reported in identification studies of *R. solanacearum* strains made by Hayward (1994) and Nouri *et al*. (2009).

The result of the biovar test showed that all *R. solanacearum* isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5 days. The oxidation reaction was indicating by the change of colour. The results revealed the change of color green to yellow indicating the oxidization of sugars by bacterial isolates.

Fig 2. The symptomatical variation on tomato plants of 1-5 scale studied under green house (Key Briefly, 1= No symptom, 2 = Top young leaves wilted, 3 = Two leaves wilted, 4 = Four or more leaves wilted and 5 = whole Plant dead)

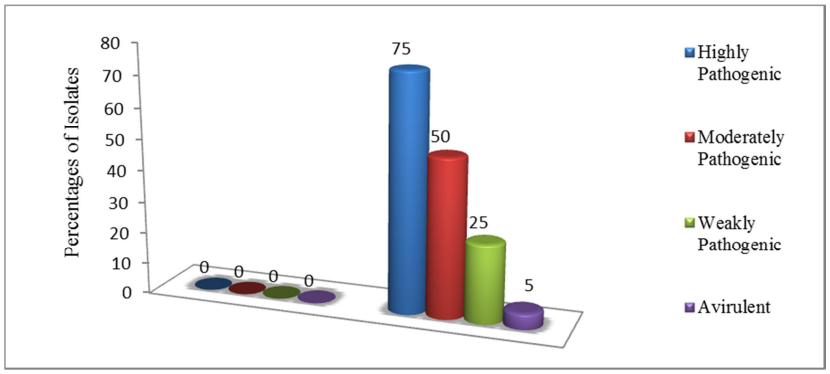

Fig 3. Grouping of R. *solanacearum* isolates based on pathogenicity variation.

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 different geographic origins 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. This strains causing bacterial wilt of ginger in Ethiopia is 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 previously 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 alcohols.

The result of the biovar test showed that all *R. solanacearum* isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol) within 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 found 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 *Ralstonia solanacearum* 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).

Conclusion

Based on the result of In vitro inhibition test two isolates of Pseudomonas fluorescent and two isolates of Trichoderma had shown inhibitory effects on eight test isolates were selected and used under glasshouse experiment. The in vitro studies have shown that *Pseudomonas fluorescent* 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 phyto-pathogen than individual treatments. But *Pseudomonas fluorescent* has showed decrease severity and increase bio-control efficiency than Trichoderma under glasshouse condition. 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.

RECOMMENDATION

From the findings of the present study, it is concluded to step forward in the determination of the bacterial isolates (*R. solanacearum*) to design 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.*

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