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Evaluation of the Efficacy of *Trichoderma* and *Pseudomonas* Species against Bacterial Wilt (*Ralstonia* Isolates) of Tomato (*Lycopersicum Spp.*)

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ABSTRACT

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

Keywords: Biocontrol; *Pseudomonas*; *R. solanacearum*; *Trichoderma*

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is the second most important vegetable crop in the world next to potato [1]. The center of origin of *Solanum lycopersicum*, (*S.* section *Lycopersicon*) has been localized in the narrow band between the Andes mountain ranges and the Pacific coast of western South and extends from southern Ecuador to northern Chile, including the Galapagos Islands [2]. Tomatoes production accounts for about 4.8 million hectares of harvested land area globally with an estimated production of 162 million tones. China leads world tomato production with about 50 million tones followed by India with 17.5 million tonnes [3]. In Africa, the total tomato production for 2012 was 17.938 million tons with Egypt leading the continent with 8.625 million tones [3]. It is an economically important vegetable in Ethiopia. According to the Central Statistics Authority of Ethiopia, the country produced 27,774.538 tons of tomato in 5235.19 hectares of land in 2018 [4].

R. solanacearum is ranked as the second most important bacterial pathogen among the top ten economically important soil borne pathogens that cause severe yield losses on different solanaceous crops in different parts of the globe [5]. Different studies showed the bacterial wilt pathogen inflict 50-100% loss on potato in Kenya [6], 88% on tomato in Uganda [7], 70% on potato in India [8]. It is one of the most destructive and widespread disease of tomato in Ethiopia and its prevalence is as high as 55% in major tomato producing areas of the country. Different methods, mainly pesticides are employed to control bacterial wilt of tomatoes. Chemical controls with Actigard (e.g., Acibenzolar-S-methyl) and phosphorous acid effective to control bacterial wilt under at greenhouse and to a lesser extent field conditions [9]. The use of excessive agrochemicals is negatively perceived by consumers and

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

However, effective and long term control is possible by using a combination of diverse methods including the use of resistant/tolerance varieties, cultural practices, biological and chemical control as parts of an integrated pest management strategy to control bacterial wilt caused by *R. solanacearum* [11]. The use of biological control agents alone and/or together with other control methods as part of integrated pest management (IPM) practices is widely employed to overcome these problems [12].

Soil bacteria and fungi which flourish in the rhizosphere of plants and, stimulate plant growth are collectively known as plant growth promoting microorganisms (PGPM). The most abundant and useful microorganisms in the rhizosphere are *Pseudomonas*, *Bacillus*, *Burkholderia*, *Agrobacterium*, *Streptomyces*, *Trichoderma*, *Penicillium*, and *Gliocladium*. These microorganisms are used with the aim of improving crop yield by augmenting nutrient availability, enhancing plant growth and protection of plants from diseases and pests [13]. They are capable of secreting hydrolytic enzymes and causing mycoparasitism on pathogens and narrow spectrum antagonistic activity compared to synthetic pesticides, and, thus used singly or in combination with one another and chemicals in integrated pest management (IPM) to suppress plant-pathogens [14].

Trichoderma and *Pseudomonas spp.* are the most frequently isolated fungi and bacteria in all the root ecosystems respectively. *Trichoderma* species effective in controlling phytopathogens due to their ability to grow toward the hyphae of other fungi, coil around them and degrade the cell walls of the pathogen. Morsy, et al. [15] showed that, the dual application of *T. viride* and *B. subtilis* decreased the percentage of pathogen infection and increased survival rate than single inoculation in tomato. The biocontrol potential of two *Trichoderma* species on *sclerotia* rot disease of tomato plants in Chile and Iceland was evaluated and the result showed that, *T. harzianum* and *T. viride* reduced the disease by 74.50%, and 68.75%, respectively [16].

Several studies also showed that the application of these antagonists have a dramatic effect on bacterial wilt disease (*Ralstonia solanacearum*) of tomato. Narasimha, et al. [17] showed that *Trichoderma asperellum* (T4 and T8) isolates delayed wilt development by *R. solanacearum*, effectively decreased the disease incidence (51%), improved plant growth promotion and increased fruit yield under field conditions. Another study also showed that *Trichoderma spp.* AA2 and *P. fluorescens* PFS were most potent inhibiting the growth of *Ralstonia spp.* , and the field study indicated *Trichoderma spp.* and *Pseudomonas fluorescens* alone were able to prevent 92% and 96% of the infection and combination of both were more effective, preventing 97% of infection compared to Chemical control methods that prevented 94% of infection [18]. This shows the promising potential of native isolates of *Trichoderma spp.* and *Pseudomonas fluorescens* as biocontrol agents against *Ralstonia spp.*

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

MATERIALS AND METHODS

Sample collection

Soil samples from the rhizosphere of healthy and bacterial wilt infected tomato plants were collected from different fields from Ziway and Meki along the Rift Valley, which is one of the most important vegetable producing areas in the country. Diseased plant samples were selected based on visible characteristic symptom of bacterial wilts [18].

Isolation of *Ralstonia solanacearum* from wilted tomato plants

Isolation of the wilt pathogen was undertaken according to Kelman A [19]. Diseased tomato stem samples were washed with tap water, and surface sterilized with 70% ethanol for 2 minutes and rinsed repeatedly in sterile water for 5 minutes. The samples were then suspended in the five-milliliter sterile distilled water for ten minutes to make them turbid due to oozing of bacterial cells from cut ends of diseased tissue. The bacterial suspensions were prepared to appropriate dilutions from which, 1 ml of the bacterial suspension was spread onto the surface of solidified Triphenyl Tetrazolium Chloride Agar (TZC) medium and incubated at 28 ± 2°C for 48 hrs.

Identification of virulent/avirulent isolates of *Ralstonia solanacearum*

The virulent and a virulent isolates of the pathogen were differentiated by Kelman method [19] on Tetrazolium Chloride (TZC) agar medium and compared with isolates obtained from Ambo Plant Protection Research Center. The virulent isolates were detected based on their pink or light red colored colonies with characteristic red center and whitish margin, whereas the avirulent isolates were differentiated on their colonies characterized by smaller, off-white and non-fluidal or dry texture on TZC medium after 24 hours of incubation.

Pathogenicity test

Virulence of the isolates was carried out by inoculating them on the tomato seedlings according to Margare et al. [20]. Tomato seeds were planted directly in 20 × 18 cm plastic pots containing sand and soil in the ratio of 2:1 (3 kg of soil and 1.5 kg of sand) soil and sand was obtained from AAU. Bacterial isolates were grown on nutrient broth medium for two days at 30°C, suspended in sterile distilled water and adjusted to OD 600 nm = 0.1 (approximately inoculum size of 10^8 CFU/ml) [21]. Inoculation was made at the four true leaf stages by injecting into the stem with a needle. Plants inoculated with sterile water served as control and pots were regularly watered. Tomato plants were observed for development of typical wilt symptoms, and the severity of bacterial wilt was recorded based on the severity scale as follows; (% of shoot wilted, using a scale of 0-5 where, 0=No symptoms, 1=one leaf wilted (1% to 25%), 2= 2 or 3 leaves wilted (26% to 49%), 3=half plant wilted (50% to 74%), 4=

all leaves wilted (75% to 100%), 5=Plant dead).

Biochemical characterization of isolates

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

Isolation of antagonists from tomato rhizosphere soil

Isolation of the bacterial and fungal antagonists was carried out using soil dilution method according to Johnsen and Nielsen [22]. Ten gram of rhizosphere soil sample collected from healthy tomato plants was prepared to appropriate dilutions (10^{-1} to 10^{-5}) and 10^{-3} to 10^{-5} plated on to KB (King's B medium) for rhizobacteria and PDA for *Trichoderma spp.* (fungal antagonists). The Petri plates were incubated at 25°C for 7 days for fungal antagonists and at 28°C for two days for rhizobacteria.

In vitro antagonism test against the pathogen

The antagonism tests were carried out on the fungal and bacterial isolates against the bacterial wilt pathogen *in vitro* used disk diffusion method [23]. The bacterial wilt pathogen was grown on nutrient broth for 48 hr from which, 100 µml was swapped onto Petri plates with nutrient agar. And the bacterial antagonist grow on nutrient broth for 48 hrs and *Trichoderma* were grown in Potato Dextrose Broth (PDB) (20 g/l dextrose, 4 g/l potato extract and 15g/l agar) for 7 days and sterilized Paper disc (5 mm) was immersed in each test antagonist solution and was spotted at the center of the pathogen-inoculated-plate. Paper disc immersed in sterile distilled water and spotted at the center of the plates with the pathogen was used as control. Plates were incubated at 28°C for 48hrs to measure inhibition zone.

Morphological characterization of fungi antagonists

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

Biochemical characterization of bacterial antagonists

The selected bacterial antagonistic isolates were characterized by the following biochemical tests including Gram differentiation and gram reaction, growth at 41°c, catalase test, oxidase test, pigment production, gelatin liquefaction, hydrogen cyanide production, ammonia production, phosphate solubilization, and carbohydrate fermentation test by using standard methods.

Compatibility test

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

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

Tomato seeds from local Gelelima variety and Galilea variety that were obtained from Melkasa Agricultural Research Center were sown in seedling bed. After 25 days, the seedlings were transplanted in pots filled with potting mixture (soil: sand at 2:1 w/w/) at the rate of three seedlings per pot. Inoculum of the pathogen and the selected biocontrol agents; *Pseudomonas* and *Trichoderma* were prepared at 10^8cfu $ml^{-1}$and conidial suspension of (10^8 spores ml^{-1}) respectively as described by Sivan, et al. [24]. Fifty ml of the mixed inoculum of the pathogen and antagonists were inoculated into the pots at the same time using soil drench method [25]. Each treatment was replicated thrice in completed randomized design (CRD). The treatments were;

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

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

T3 Ralstonia solanacearum+ Trichoderma spp. + pseudomonas (AAURS+AAURB20+AAUTR23)

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

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

According to Song, et al. [26], wilt incidence was calculated by the following formula:

Where

BE = Biocontrol efficacy

DIC = Disease incidence of control

GPE=Growth promotion efficacy

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

Data analysis

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

RESULTS AND DISCUSSION

Cultural and biochemical tests for identification of *Ralstonia solanacearum*

A total of fifteen bacterial isolates were collected from infected tomato plants with bacteria wilt, of which four isolates that showed the typical cultural characteristics of virulent *R. solanacerum* were selected for *in vivo* pathogenicity studies (Table 1). These isolates exhibited pink or light red colonies or red center with whitish margin. All of them were rod shaped, gram negative, non-spore forming, motile, and catalase and oxidase positive and indole negative bacteria (data not shown). These results conformed to the characteristics of virulent strains of *R solanacearum* on TZC medium after 24 hours of incubation reported elsewhere [19].

Pathogenicity tests

The result showed that bacterial wilt of tomato occurred within 15 to 21 days after inoculation. All isolates were pathogenic on tomato plants and produced typical symptoms of wilt. Isolate AAURS1 exhibited the highest disease incidence (75% wilting) followed by 50% of wilting with APPRCRS2, whereas isolates AAURS3 and AAURS4 induced weak infection on the host (Table 1). Other reports also showed 50-71% wilting on different tomato varieties [27]. El-Ariqi, et al. [28] also reported that different isolates of *R. solanacearum* caused 52%-97% of wilting. Selim, et al. [29] have also reported that different isolates of *R. solanacearum* showed different wilt incidence ranging from 40%-96%.

Isolation and Screening of Plant growth promoting antagonist

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

The data showed that the bacterial isolate, AAURB20 showed the highest mean inhibition diameter of 15mm and 16 mm followed by the fungus, AAUTR23, isolate with inhibition diameters of 14 mm against the two test pathogens AAURS1and APPRCRS2 respectively (Table 2). This implies that the antagonists have potential to be used in the greenhouse for *in vivo* bio protection of tomato plant. The *in vitro* antagonistic activity of *P. fluorescens* was also reported by Aliye, et al. [30] where *P. fluorescens* isolates (PF20) had the greatest inhibition zone *in vitro against R. solanacearum* with the inhibition diameter of 14.15 mm and other two isolates (PR-3-I-x, PR-4-I-x) 3.2 showed and 3.5 mm respectively. This suggests that the mode of action or the type of antibacterial metabolite production may vary among the isolates tested [31]. The inhibitory

Table 1: Variations in pathogenicity of R. *solanacearum* isolates on the host tomato variety (Gelilema).

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

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

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

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

activity of *P. fluorescens* against the pathogen in the study is in line with that of Henok, et al. [32], Aliye, et al. [30] and Yendyo, et al. [18] where they reported that isolates of *P. fluorescens* had significantly inhibited under the bacterial growth of *R. solanacearum* under *in vitro* condition.

The *in vitro* antagonistic activity of *Trichoderma sperellum* was reported by Narasimha, et al. [17] that inhibit the growth of *Ralstonia solanacerum* with inhibition zone ranging from 11mm-27mm diameter.

Morphological and biochemical characterization *P. fluorescens*

Based on the antagonistic potential characteristics, twelve isolates of *P. fluorescens* were studied in detail for colony, colour, growth type, cell shape, and fluorescens of the isolates. Those all the isolates showed similar results with regard to round yellow colony texture on King,s B medium with production of fluorescent pigment gelatin liquefaction positive, catalase, oxidase, gram stain negative, positive KOH and lack of growth at 41°C. This, together with rod shape cell morphology and fast growth further confirmed the isolates to be *Pseudomonas fluorescens* as reported by earlier workers Meera and Balabaskar [33].

Carbohydrate fermentation test for bacterial isolates

The isolates utilized the tested carbohydrates and produced yellow color on the medium, which was an indication of the utilization of each carbohydrate. All isolates were capable of utilizing glucose followed by maltose, fructose and lactose (Table 3). The utilization of different carbohydrate sources by the isolates was similar with *P. fluorescens* reported by Henok et al., [32].

Morphological characterization of fungi

The fungal isolates were characterized by fast growth with dark green mycelia colony on PDA. Microscopic study revealed that it produced globes to ellipsoidal conidial shape, which was much branched (Table 4).

PGPR characterization of rhizbacteria

Among isolates that were screened for their plant growth promoting activities viz., HCN production, ammonia production, phosphate solubilization. Isolate AAURB20 and AAURB19 exhibited strong HCN production followed by isolates AAURB8 and AAURB16. Among test isolates, AAURB 7 and AAURB 20 displayed three PGP characters; whereas most of the isolates exhibited only one of the PGP characters (Table 5). The strains of *P. fluorescence* isolated from rice fields are found to produce HCN against *S. oryzae* [33].

Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia. In this study, isolate AAURB7 and AAURB20 produced ammonia. Another study showed that 95% of the isolates from the rhizosphere of rice crops produced ammonia [34].

Phosphorous is a major essential macronutrient for biological growth and development. With regard to solubilization of inorganic phosphate four isolates 4 (33%) (AAURB7, AAURB16, AAURB19, and AAURB20) of were able to solubilize phosphate in the plate-based assay, by showing a clear halo zone around the colony. Several species of *Pseudomonas* such as *P. fluorescens*, *P. aeruginosa* and *Bacillus* species have been reported as good phosphate solubilizers in agricultural soils [35].

Table 3: Carbohydrate fermentation test results of different indigenous bio-control agents.

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

Note: +=Positive, -=Negative ± Intermediate reaction

Table 4: Morphological characterization of fungi.

Isolate characters	AAUTR21	AAUTR22	AAUTr23	AAUTR24	AAUTR25	AAUTR26
Colony growth rate (cm)	8-9 cm in 6 days	8-9 cm in 6 days	8-9 cm in 3 days	8-9 cm in 5 days	8-9 cm in 4 days	8-9 cm in 4 days
Colony Colour	Green	Green	Gark Green	Dark Green	Dark Green	Dark Green
Reverse Colony Colour	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
Conidiospore	Branched	Branched	Branched	Branched	Branched	Branched
Conidial shape	Globes to ellipsoidal	Globes to ellipsoidal	Globes to ellipsoidal	ellipsoidal	Globes to ellipsoidal	Globes to ellipsoidal

Compatibility test

The compatibility test between the selected isolate, AAURB20 and selected fungal isolate AAUTR23 indicated that, the colonies of the fungus and the bacterium met on the 7th day without showing inhibitory activity with one another. This observation was the basis for testing a combination of the two antagonists as "mixed culture" in the greenhouse trial. Similary, under *in vitro* compatibility between *T. viride* and *P. fluorescens* was reported by Ephrem, et al. [36] there was no inhibition between them.

Effects of isolates on disease incidence and biocontrol efficacy

The bio control efficacy and antagonistic effect of the treatments on disease incidence was highly significant ($p \leq 0.05$) when compared to the control treatments. The highest disease incidence of 80% and 60% was recorded from the control (Pathogen infection only) on Galilea and Gelelima varieties, respectively. All treatments reduced disease incidence ranging from 13%-35%; and bio control efficacy of 48%-72% (Table 6). Similar results also reported by Selim et al. [29] plants treated with PGPR isolates significantly disease reduced ranging from (15%-57%) compared to infected control, as well as greater amount of biomass compared to the control.

The combined treatments exhibited the lowest value (13.33%) of disease incidence as well as the highest value (72.22%) of bio control efficacy against *R. solanacearum*, on Gelelima variety. While isolate AAURB20 exhibited the highest (31.11%) disease incidence and lowest value (61.11%) of bio control efficacy on Galilea variety, and 35% and 48% on Gelelima variety, respectively (Table 6). The results could be attributed to the synergistic effect between the combinations of the two microorganisms in this treatment. These results were in harmony with those reported by Yendyo, et al. [18] that *Trichoderma spp.* and *P. fluorescence* seems to be more effective than treatment using each individual biocontrol agent that has been achieved 97% of bio control efficacy.

The dual application of *T. viride* and *B. subtilis* decreased the percentage of pathogen infection and increased survival rate than single inoculation in tomato [15]. Another study showed that the number of wilted chickpea infected with *Fusarium oxysporium* plants was reduced by 67.93% due to inoculation/suppression by *T. harzianum* [37]. The highest percentage of disease incidence was found on galilee variety, which may be due to variety resistance. These results were in harmony with those reported by Chatterjee, et al. [38] which stated that differences of wilt incidence and severity were due to diversity of host plants, the virulence of the pathogen, and other environmental factors.

Plant growth promotion efficacy of antagonists in greenhouse condition

Results of this experiment showed that antagonists (bioagents) stimulated plant growth promotion under greenhouse conditions and indicated that tomato plants treated with rhizobacteria and Trichoderma strains significantly grew better than control biomass increase of tomato plants treated with rhizobacteria and Trichoderma strains are shown in (Table 7).

Significant differences ($P \leq 0.05$) among treatments regarding plant height and biomass were observed. Plants treated with combined isolates of AAURB20+AAUTR23 showed the highest values of

Table 5: Characterization of rhizobacteria for their PGPR characters.

Isolates	HCN production	NH_3 production	Inhibition zone (mm)	Phosphate solubilization	Multiple PGP characters
AAURB 1	+	+	9	-	2
AAURB 3	+	+	9	-	2
AAURB6	+	-	9.5	-	1
AAURB 7	+	+++	8.5	+	3
AAURB 8	++	-	10		1
AAURB 11	+	-	9.5	-	1
AAURB 12	+	-	8	-	1
AAURB16	++	+	9.5	+	3
AAURB 17	-	+	8	-	1
AAURB 18	-	+	9	-	1
AAURB19	+++	++	11	+	3
AAURB 20	+++	+++	15	++	3

Note: +: Low production: ++: Medium production: +++: Strong production: -: No production

Table 6: Effect of AAURB20, AAUTR23, and their combination (AAURB20+AAUTR23) on disease incidence.

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

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

Table 7: Effect of plant growth promotion of antagonists on tomato.

Treatments (pathogen s+ Bioagents+Variety)	Plant height (cm)		Plant dry weight (g)	
	Mean	GPE (%)	Mean	GPE (%)
AAURs1+AAURB20+V1	54 ± 2.65^{bcd}	26.5	9.46 ± 0.73^{abc}	52.21
AAURs1+AAUTR23+V1	54 ± 2.65^{bcd}	40.35	9.54 ± 0.65^{abc}	51
AAURs1+AAURB20+AAUTR23+V1	67 ± 3.81^{a}	55.4	11.25 ± 1.23^{ab}	47.6
AAURs1+V1	43 ± 3.61^{c}		6.27 ±1 .20^{c}	
Distil water+V1	55.67±3.21^{abcd}		10.22±3.25^{ab}	
AAURs+AAURB20+V2	58 ± 3.46^{ab}	30.36	10.79 ± 1.24^{ab}	42.4
AAURs+AAUTR23+V2	64.67 ± 4.16^{abc}	45.27	12.18 ± 1.82^{ab}	66.2
AAURS1+AAURB20+AAUTR23+V2	72.33 ± 3.23^{a}	61.66	12.73 ± 0.48^{a}	81.5
AAURs1+V2	44.67 ± 2.31^{cd}		7.81 ± 1.42^{bcd}	
Distil water+V2	57.33 ± 4.13^{bcd}		11.65 ± 2.61^{ab}	

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

plant height, and dry weight (72.33 cm, and 12.73 g) respectively, when compared with the control (AAURS1) and plants treated by individual isolates AAURB20 and AAUTR23 in variety two (Table 7). Likewise plants treated with isolates AAURB20+AAUTR23 showed high GPE (%) (62%, and 81.5%) for height, and dry weight respectively in variety two (Table 7).

Significant differences ($P \leq 0.05$) among treatments regarding plant height and biomass were also noted on variety one (Table 7). Plants treated with combined isolates of AAURB20+AAUTR23 presented the highest values of plant height, and dry weight (67 cm, and 11.25 g) respectively, when compared with the control (AAURS1) and plants treated by individual isolates AAURB20 and AAUTR23 in variety one (Table 7). Generally combined treatments showed best performance compared to individual treatments. Significant differences were observed in the vegetative growth parameters due to the inoculation of isolated bio-inoculants. This result was in harmony with that of Nguyen and Ranamukhaarachchi [23] on tomato the use of beneficial microorganisms as biocontrol agents led to enhance plant growth parameters (70.4 cm plant height and 19.5 g of dry weight). Such enhancement may be due to induce plant resistance [39], production of extracellular enzymes and antifungal or antibiotics, which reduce the negative effect of biotic stress on plant and produce growth promoting substances [40]. Similar results also reported by Selim, et al. [29] plants treated with PGPR isolates significantly reduced disease compared to infected control, as well as caused greater amount of biomass compared to the control.

CONCLUSION

The combined use of the biocontrol agents significantly reduced the incidence of tomato bacterial wilt disease. Therefore the use of this bioagent would be important for to manage bacterial wilt at greenhouse conditions.

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REFERENCES

1. Benti G, Degefa G, Biri A, Tadesse F. Performance evaluation of Tomato (*Lycopersicon esculentum Mill.*) varieties under supplemental Irrigation at Erer Valley, Babile District, Ethiopia. J Plant Sci. 2017:5;1-5.
2. Peralta IE, Spooner DM, Knapp S. Taxonomy of wild tomatoes and their relatives (Solanum sect. Lycopersicoides, sect. *Juglandifolia*, sect. Lycopersicon; Solanaceae). Systematic botany monographs. ASPT. 2008;84:1-186.
3. FAOSTAT. Global tomato production in 2012. Rome, FAO. 2014.
4. Central Statistic Authority. Report of Federal Democratic Republic of Ethiopia, Statistical Report on Socio-Economic Characteristics of the Population in Agricultural Households, Land Use, Area and Production of Crops. Addis Ababa, Ethiopia. 2018.
5. Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, et al. Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular plant pathology. 2012 ;13(6):614-629.
6. Muthoni J, Shimelis H, Melis R. Management of bacterial wilt [*Rhalstonia solanacearum* Yabuuchi et al., 1995] of Potatoes: Opportunity for host resistance in Kenya. J Agric Sci. 2012;4(9):64-78.
7. Katafiire M, Adipala E, Lemaga B, Olanya M, El-Bedewy R, Ewell P. Management of bacterial wilt of potato using one-season rotation crops in Southwestern Uganda. Bacterial wilt disease and the *Ralstonia solanacearum* species complex. 2005:197-203.
8. APS (American Phytopathological Society). Bacterial wilt diseases and the *Ralstonia solanacearum* species complex. APS Press. 2005.
9. Pradhanang PM, Ji P, Momol MT, Olson SM, Mayfield JL, Jones JB. Application of acibenzolar-S-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. Plant Dis. 2005;89(9):989-993.
10. Butt TM, Jackson C, Magan N. Fungi as biocontrol agents: Progress problems and potential. CABI. 2001.
11. Persley GJ. Ecology of *Pseudomonas solanacearum*, the causal agent of bacterial wilt. ACIAR Proc. 1992;45:71-75.
12. Barari H. Biocontrol of tomato Fusarium wilt by Trichoderma species under *in vitro* and *in vivo* conditions. Cercetari Agronomice in Moldova. 2016;1(165):91-98.
13. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil. 2003;255(2):571-586.
14. Ali H, Nadarajah K. Evaluating the efficacy of' *Trichoderma*' spp and *'Bacillus substilis'* as biocontrol agents against' *Magnaporthe grisea'* in rice. Aust J Crop Sci. 2014;8(9):1324-1335.

15. Morsy EM, Abdel-Kawi KA, Khalil MN. Efficiency of *Trichoderma viride* and Bacillus subtilis as biocontrol agents against *Fusarium solani* on tomato plants. EJP. 2009;37(1):47-57.

16. Kator L, Hosea ZY, Oche OD. *Sclerotium rolfsii*: Causative organism of southern blight, stem rot, white mold and sclerotia rot disease. Ann Biol Res. 2015;6(11):78-89.

17. Narasimha Murthy K, Nirmala Devi D, Srinivas C. Efficacy of *Trichoderma asperellum* against *Ralstonia solanacearum* under greenhouse conditions. Ann Plant Sci. 2013;2:342-350.

18. Yendyo S, Ramesh GC, Pandey BR. Evaluation of Trichoderma spp., Pseudomonas fluorescens and Bacillus subtilis for biological control of *Ralstonia* wilt of tomato. F1000Research. 2017;6.

19. Kelman A. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance in a tetrazolium medium. Phytopathology. 1954;44(12):693-695.

20. Mwangi MW, Monda EO, Okoth SA, Jefwa JM. Inoculation of tomato seedlings with Trichoderma harzianum and arbuscular mycorrhizal fungi and their effect on growth and control of wilt in tomato seedlings. Braz J Microbiol. 2011;42:508-513.

21. Ran LX, Liu CY, Wu GJ, Van Loon LC, Bakker PA. Suppression of bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. in China. Biol Control. 2005;32(1):111-120.

22. Johnsen K, Nielsen P. Diversity of *Pseudomonas* strains isolated with King's B and Gould's S1 agar determined by repetitive extragenic palindromic-polymerase chain reaction, 16S rDNA sequencing and Fourier transform infrared spectroscopy characterization. FEMS Microbiol. 1999;173(1):155-62.

23. Nguyen MT, Ranamu Khaarachchi SL. Soil-borne antagonists for biological control of bacterial wilt disease caused by *Ralstonia solanacearum* in tomato and pepper. J Plant Pathol. 2010:395-406.

24. Sivan A, Elad Y, Chet I. Biological control effects of a new isolate of Trichoderma harzianum on *Pythium aphanidermatum*. Phytopathology. 1984;74(4):498-501.

25. Algam SA, Xie G, Li B, Yu S, Su T, Larsen J. Effects of *Paenibacillus* strains and chitosan on plant growth promotion and control of *Ralstonia* wilt in tomato. J Plant Pathol. 2010:593-600.

26. Song W, Zhou L, Yang C, Cao X, Zhang L, Liu X. Tomato Fusarium wilt and its chemical control strategies in a hydroponic system. J Crop Prot. 2004;23(3):243-247.

27. Abo-Elyousr KA, Asran MR. Antibacterial activity of certain plant extracts against bacterial wilt of tomato. Arch Phytopathol Plant Prot. 2009;42(6):573-578.

28. El-Ariqi SN, El-Moflehi M, El-Arbara K, El-Kobati A, El-Shaari A. Antibacterial activity of extracts from *Withania somnifera* and Aloe vera aginst *Ralstonia solanacearum* in potato. Arab J Pl Prot. 2005;23:95-99.

29. Seleim MA, Saead FA, Abd-El-Moneem KM, Abo-Elyousr KA. Biological control of bacterial wilt of tomato by plant growth promoting rhizobacteria. Plant Pathol J. 2011;10(4):146-153.

30. Aliye N, Fininsa C, Hiskias Y. Evaluation of rhizosphere bacterial antagonists for their potential to bioprotect potato (*Solanum tuberosum*) against bacterial wilt (*Ralstonia solanacearum*). Biol Control. 2008;47(3):282-288.

31. Williams GE, Asher MJ. Selection of rhizobacteria for the control of *Pythium ultimum* and *Aphanomyces cochlioides* on sugar-beet seedlings. J Crop Prot. 199;15(5):479-486.

32. Kuarabachew H, Assefa F, Hiskias Y. Evaluation of Ethiopian isolates of Pseudomonas fluorescens as biocontrol agent against potato bacterial wilt caused by *Ralstonia (Pseudomonas) solanacearum*. Acta Agri Solvenica. 2007;90(2):125-135.

33. Meera T, Balabaskar P. Isolation and characterization of *Pseudomonas fluorescens* from rice fields. JFAV. 2012;2(1):113-120.

34. Joseph B, Ranjan Patra R, Lawrence R. Characterization of plant growth promoting rhizobacteria associated with chickpea (Cicer arietinum L.). Int J Plant Prod. 2012;1(2):141-152.

35. Jha PN, Gupta G, Jha P, Mehrotra R. Association of rhizospheric/ endophytic bacteria with plants: a potential gateway to sustainable agriculture. Greener J Agric Sci. 2013;3(2):73-84.

36. Zegeye ED, Santhanam A, Gorfu D, Tessera M, Kassa B. Biocontrol activity of Trichoderma viride and *Pseudomonas fluorescens* against *Phytophthora infestans* under greenhouse conditions. J Agric Sci Technol. 2011;7(6):1589-1602.

37. Subhani MN, Talib S, Sahi AL, Hussain S, Iqbal J, Hussain N. Management of chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* through antagonistic microorganisms. Can J Plant Pathol. 2013;1(1):1-6.

38. Chatterjee S, Mukherjee N, Khatun DC. Status of bacterial wilt in West Bengal J interacademicia. Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, India. 1997;1(1):97-99.

39. De Meyer G, Bigirimana J, Elad Y, Höfte M. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. Eur J Plant Pathol. 1998;104(3):279-286.

40. Szczech M, Shoda M. Biocontrol of *Rhizoctonia* Damping-off of Tomato by *Bacillus subtilis* Combined with *Burkholderia cepacia*. J Phytopathol. 2004;152(10):549-556.