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Biological Control of *Ralstonia solanacearum*(L.) Using *Trichoderma* species and *Pseudomonas* species Isolates *Invivo* at Varying Dates of Inoculation

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Abstract

Three fungal isolates of trichoderma spp and two bacterial isolates of fluorescent pseudomonas were isolated from ginger rhizosphere soil collected from different ginger growing areas of the country. The isolates were screened in vitro for their antagonistic activity against the pathogen, ralstonia solanacearum (bacterial wilt) on kb agar medium. Out of the tested isolates, only four (type 3, type 4, type 5 and type 6) showed a certain degree of inhibition to the growth of the pathogen. To test their antagonistic effect in a greenhouse, a pot experiment was conducted using trichoderma and pseudomonas spp. Sterilized soil and susceptible ginger rhizome was used for the study. In the experiment, bacterization of ginger rhizomes with the selected isolates i.e., type 7, type 8, type3, type4, type5 and type6 significantly reduced the incidence of bacterial wilt by 59.83%, and increased plant growth: plant height and dry weight by 76.89% and 28.44%, respectively suggesting the importance of the isolates as plant growth promoting rhizobacteria. In this study, isolates of bio-agent have already been found having very effective strong fungicidal effect. In some cases, the reported effect is more than that of chloramphenicol (0.25%) pesticides. Out of the many such reported promising plants, on the simple basis of availability trichoderma isolate type 05, trichoderma isolate type 03, type 04 and trichoderma isolate type 06 have been selected to asses their combat ability against, the highest average rhizome yields however were observed under the treatment t_6 and t_7 and the lowest under the treatment to. As a result low yield was found in control pots and high in the pots where combined treatments were applied. The combined treatment had a highly significant effect on the rhizome yield. The highest average rhizome yields however were observed under the treatment t6 (type sp-s) and t7 (type 2sp) and the lowest under the treatment to. As a result low yield was found in control pots and high in the pots where combined treatments were applied. The combined treatment had a highly significant effect on the rhizome yield. The use of trichoderma isolate type 05 and trichoderma isolate type 03 had shown promising result at 15 dys after innoculation. The result from the benefit cost ratio analysis revealed that highest financial benefit was obtained from the combined treatment t7 where the bcr (3.97) was the highest and the second highest bcr was from t6 (3.96) while the lowest bcr (1.90) was observed in control pot. It was evident from the obtained results that comparatively low yields were responsible for the lower gross return and a lower bcr against each treatment. Hence, trichoderma spp and pseudomonas spp were recommended against bacterial wilt of ginger. Thus, bio-control of ralstnia solanacearum must be strengthened so as to replace the use of toxic chemicals by cheaper, sustainable, eco-friendly antagonists.

Keywords: *Bacterial wilt, biocontrol, ginger, pseudomonas spp., ralstonia solanacearum, trichoderma spp*

Introduction

Ginger (*Zingiber officinale* Roscoe) belongs to a tropical and subtropical spice plant family *Zingiberaceae*. It is thought to be originated in South East Asia and introduced to many parts of the world; and has been cultivated for thousands of years as spice and medicinal purpose in India and China (Rafie and Olczyk, 2003; Shulka and Singh, 2007). 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. In many countries, especially in India and China, fresh ginger is used to prepare vegetable and meat dishes and used in preparation of various foods for seasoning, flavoring and imparting aroma in all over the world (Shukla and Singh, 2007). Ginger is a beneficial cash crop in Ethiopia. In this country, Ginger is a popular species and condiments for its special taste, flavor, color and even long-term storage

ability etc. It 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).

The existence of enormous potential for the production of ginger invaluable crops in the country as a diversification option for coffee especially for lowland spices. Concomitantly, the research findings for highland seed spices were very promising in more of the cereal-based farming system of mid-altitude and highlands of Ethiopia. Therefore, highly promising varieties of ginger had been identified based on their adaptation, yield, extraction quality and chemical composition that could meet the international standards, provided they are produced following appropriate agronomic and postharvest handling techniques. Subsequently, best accessions from each crop species that are highly adaptable and productive under the stated agro ecologies were identified. Potentials of these varieties for high economic return to producers were clearly observed from the results of many year evaluation. Ethiopian export performance (values '000 Ethiopian Birr (ETB); 1 USD = 20 ETB) of dry spices: ginger and chili from 2002/03 to 2012/2013 was 1,586,938.2 and 317,849.08, respectively (EHA, 2015). As a result, their contribution for boosting the national economy through import substitution and/or export, were evident. Similarly, productivity of more than 20 t/ha fresh rhizome yield of ginger and turmeric had been recorded from ginger, which explicitly indicates the potential for the production of these spices in Southwestern Ethiopia. However, the accessions at hand have quality problem that calls for further improvement (Fantahun and Teklu, 1995).

Bacterial wilt of ginger is the most limiting factor in the production of culinary ginger in USA, causing up to 45% loss in ginger production of the country in 1993 (Hepperly, *et al*., 2004). 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. In addition, Rahaman *et al.,* (2009) reported that more than 60% of farmers in India feel that wilt and soft rot of ginger rhizomes were the major limiting factors in ginger cultivation. In traditional agricultural system as well as organic farming, managing rhizome diseases is a great challenge. Consequently, many growers have given up ginger cultivation and others are still struggling to survive because of rhizome diseases in India. Stirling *et al.* (2009) indicated in their survey based observations on the etiology of bacterial wilt of ginger that the disease generally develops during hot and wet conditions, and often causes losses of more than 50% of seed crops. Moreover, a review by Dohroo (2005) indicated that ginger crops can sometimes almost totally destroyed by bacterial wilt. Bacterial wilt is a serious spoilage of rhizome, causing considerable economic loss to growers in different countries (Ghosh and Purkayastha, 2003**).** According to FAO (2004) post-harvest spoilage in ginger is normally due to rough harvesting and handling practices which result in injury to the skin and flesh of the rhizome.

For Kaneshiro and Shintaku (1996), the major factor in dissemination of bacterial wilt of ginger on the island of Hawaii was the unintentional use of plant materials infected by *R. solanacaerum* . Cutting up an infected rhizome for planting divides one in to several infected seed pieces and further disseminates the fungi and/or bacteria via the contaminated cutting instrument. Kumar *et al.* (2004) indicated that primarily rhizome-borne pathogens are believed to be transmitted to many ginger growing areas through latently infected rhizomes and secondary spread within the field and the neighboring localities is through rain splashes and run-off water in the field. Agricultural tools also may contribute in disseminating the pathogens. Reduction of infection can be achieved by split-drying the rhizome to a moisture content of 10- 12%. According to Bernard (2008).Ginger diseases have been minimized further by cultural methods such as the use of clean seeds, crop sanitation, crop rotation, varying time of planting (early planting) to ensure disease escape, and seed treatment.

In southern Ethiopia, ginger is a an indispensible crop and its rhizome quality affects the economic return of the growers, establishment, growth and yield of the crop. At Kembata Tenbaro, and Wolaita Zones, the ginger crop is named as "the crop against poverty" or "the cash in the bank". This multifunctional crop often spoiled, loses quality and deteriorated by microorganisms that caused rhizome product loss. Therefore, these problems can be solved by good management practices based on operational research. Although attempts have been made to conduct research on agronomic aspects and chemical constituents of ginger cultivated in Ethiopia, there has been no research conducted on ginger spoilage microorganisms in Ethiopia. Thus, this bio-control research was initiated with the aim of managing bacterial wilt of Ginger, caused by *Ralstonia solanacearum* through *Trichoderma* spp and *Pseudomonas* spp.

Materials and methods

Description of the study Area

The study was carried out in Kembata Tenbaro, and Wolaita Zones of south west of Ethiopia (Specifically, Durame and Hadaro Tunto in kembata 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 "WoniaDega" Agro –ecological zone. Consequently, it experiences mean annual temperature between 14^{0}c and 26^{0}c (EARI, 2009; Teferra and Leikun., 1999; CSA, SNNPR, 2007; 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) where as 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)

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 fluorescens*

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. Re-isolation and selection of bio-antagonistic *Trichoderma* species: *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 re-identification of the strains was done by the Addis Ababa University, College of Natural and computational Sciences, Department of Molecular, Cellular and Microbial Biology, mycology laboratory. Antagonistic activity of *Trichoderma* spp.which was tested against ten highly virulent strains of *R. solanacearum* by *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 were 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

Effects of bio-agents on disease reaction, yield, and growth of different ginger varieties had been evaluated. Data on different parameters, with all of its organization, conversion and analysis are presented in the Tables (7-13) in the subsequent subsections. The effect differences of the treatments significantly varied from one another and gave a clear picture about the effects on plant growth and yield promoting factors and yield and thus eventually on the financial return and cost benefit ratio.

Plant Infection

Symptoms of plant infection were observed and compared in 15, 22 and 30 DAI on two medium age leaves in two treated and non-treated plants (To). The very next day the designated treatments were given to the plants. The results are presented in Table 7. 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 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 85.5 in To (Control) and it was the lowest (21.43) in T8 (Chloramphenicol (0.25%)). Percent plant infection in T2 (*Trichoderma* spp isolate 1), and T3 (*Trichoderma* spp Isolate 02) were statistically similar level of significance at 15 DAI though numerically different. At 22 DAI, the

highest percent plant infection was found in To (control) which was 82.2 and the lowest percent plant infection was found in T8 (Chloramphenicol (0.25%)) which was 30.6.

Fig 1. Antagonistic activity of *Trichoderma viride* against R. *solanacearum*.

The combination treatments T7 (combination of antagonists and the chemical) was found to be most effective and had 32.31% leaf infection. At 30 DAI, the highest percent plant infection was found in To (control) which was 80.4 and the lowest percent plant infection was found in T8 (Chloramphenicol (0.25%)) which was 37.2. 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. At 30 DAI the % LAD under T7 was only 42.2.

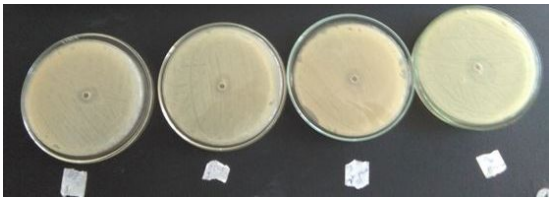

Fig 1. Antagonistic activity of Pseudomonas fluorescens against *R. solanacearum*.

Table 1. Percent plant infection per plant due to at Bacterial wilt of ginger at different days after inoculation as in influenced by some management practices

Treat. No.	Treatments	15 DAI	22 DAI	30 DAI
To	No treatment	81.1a	81.2a	81.4a
T1	*Trichoderma* spp 1	71.8b	75.33b	79.6a
T2	*Trichoderma* spp 2	72.66b	73.7b	78.66a
T3	*Pseudomonas* spp 1	39.62c	44.54d	54.2b
T4	*Pseudomonas* spp 2	44.44c	49c	53.25b
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 +chlora.	24.66e	32.31ef	42.2c
T8	Chloramphenicol (0.25%)	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(s) in a column did not differ at 5% level by LSD.*

Statistically identical results were recorded in T2 (*Trichoderma* spp isolate 1), T3 (*Trichoderma* spp isolate 2) with values of 71.8, and 72.66, respectively, in 15 DAI. Besides, 22 DAI, the same result was observed in these treatments with values of 75.33 and 73.7 % of DLA. On T4 (*Pseudomonas* spp isolate 1), T5 (*Pseudomonas* spp isolate 2), and their combination (*Pseudomonas* spp isolate 1+ *Pseudomonas* spp isolate 2), on the other hand, lesser infections were observed with values of 39.62, 44.44, and 40.66, respectively, at 15 DAI. At 22 DAI, *Pseudomonas* spp isolate 1, T5 (*Pseudomonas* spp isolate 2), and their combination (*Pseudomonas* spp isolate 1+ *Pseudomonas* spp isolate 2), had 44.54, 49, and 46.22 percent of plant disease infection. Combination treatment (*Trichoderma* Isolate 1 + *Trichoderma* Isolate 2) or combination of pseudomonas spp (*Pseudomonas* spp isolate 1+ *Pseudomonas* spp isolate 2) had shown superior results as compared to *Trichoderma* spp or pseudomonas spp applied alone. But treatment chloramphenicol (0.25%) , despite having the highest performance, was not environment friendly though significantly differed from To (Control), T_1, T2 T3, T4 T5 and T6. Besides, statistically similar percent plant infection showed *Trichoderma* spp Isolate 01, and *Trichoderma* spp Isolate type 02 in all 15, 22 and 30 DAI regimes. According to the last observation at 30 DAI on the percent plant infection per pot. the least effective treatments compared to the control were T1 (*Trichoderma* spp 1), T2 (*Trichoderma* Isolate 02), T3 (*Pseudomonas* spp Isolate 01). T4 (*Pseudomonas* spp Isolate type 02) and T5 (Combination of *Trichoderma* Isolate 1 and 2). However, all these treatments had significant efficacy. On the contrary, the highly effective treatments under which the pots had the lowest or nearly lowest percent infected plants were under T5 (Combination of *Trichoderma* Isolate 1 and 2) and T6 (*Pseudomonas* spp 1 and 2). T6 (*Pseudomonas* spp isolates 1 and 2) and T7 (Combination of antagonists and chemical) were the most effective integrated treatments.

Treatment T8 was chloramphenicol (0.25%) fungicide which reduced the disease at lateral spread of the lowest condition was but it must be considered that chloramphenicol (0.25%) is a very toxic chemical which is injurious to human health and not an environment-friendly product it will directly destroy the beneficial microorganism too.

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 or so. Affected leaves have been observed to wither and fall off. The results obtained on the parameter % leaf infection are presented in Table 2. Effect of different treatments on percent leaf infection are presented in Table 2. At 15 DAI, the highest percentage of leaf infection 24.98% was observed in the treatment To (control).The result clearly indicated that the treatments had significant effect on percent leaf infection. The lowest (5.56%) percent leaf infection was observed in pots received chloramphenicol (0.25%) treatment. Percent leaf infection was statistically similar in control (no treatment) and T1 (*Trichoderma* Isolate 01) in 22 DAI and 30DAI with value of 27.4% and 29.9% and 25.33 29.6%, respectively. The rest of treatments had shown statistically significant effect (Table 2).

Table 2. Percent leaf infection per plant due to bacterial wilt of ginger at different days after inoculation

Treat. No.	Treatments	15 DAI	22 DAI	30 DAI
To	No treatment	25.2a*	27.4b	29.9a
T1	*Trichoderma* spp 1	22.8bc	25.33b	29.6a
T2	*Trichoderma* spp 2	23.66b	23.7c	28.66ab
T3	*Pseudomonas* spp 1	20.62d	24.54b	26.2b
T4	*Pseudomonas* spp 2	24.44a	29a	29.25a
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 +chlora.	24.66a	22.31c	22.2d
T8	Chloramphenicol (0.25%)	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(s) in a column did not differ at 5% level by LSD*

At 30 DAI, the highest percentage of leaf infection 28.86% was observed in the treatment To (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 (6.760%) percent leaf infection was observed in treatment chloramphenicol (0.25%) (Chemical) .Percent leaf infection was statistically similar in T2 (Trichoderma Isolate type 03) and T3 (Trichoderma Isolate type 04) having of 20.20% and 20.32% respectively .The rest the of treatments had shown statistically significant effect (Table 2).

The result clearly indicated that the treatments had significant effect on percent leaf infection. At 30 DAI. the highest leaf infection 32.32% was observed in the treatment To (control). The lowest (8.13%) percent leaf infection was observed in treatment T l (Chloramphenicol (0.25%)). Percent leaf infection was statistically similar in T2 (Trichoderma Isolate type 03) and T3 (Trichoderma Isolate type 04) having of 21.50% and 22.00%, respectively as well as T6 (Neern + Trichoderma Isolate type 04 + Trichoderma Isolate type 06) and T7 (Trichoderma Isolate type 03+ Trichoderma Isolate type 04 + Trichoderma Isolate type 05) were statistically similar having 17.09% and 14.40%, respectively. The rest of treatments had shown statistically significant effect (Table 8). The progress in the % leaf infection from 15 DAI 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.

Diseased Leaf Area

The results obtained on the parameters percent LAD has been presented in Table 4.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 in To (control) having value 35.00 % and the lowest leaf area disease was in T8 (Chloramphenicol (0.25%)) having value 8.32 %. Treatment T2 (Trichoderma Isolate type 03), T3 (Trichoderma Isolate type 04) and T4 (Trichoderma Isolate type 06) showed statistically similar significant effect having values 25.22 %, 24.44 % and 26.18 % , respectively. The rest of the treatments also showed significant effect (Table 3). A combined treatment T7 which include bioagents of Trichoderma Isolate type 03, Trichoderma Isolate type 04 and Trichoderma Isolate type 05 as mixture spray was found as efficient as Chloramphenicol (0.25%). The relative efficiencies of treatments suffered only a slight change at 30 DAI probably due to environmental factors and the growth stage physiology of the plants. The disease development in the infected leaves at this period (15-30 DAI) was rather low compared to the next period of observation (30-20). Only treatment with Chloramphenicol (0.25%) (T8) has shown a significant difference higher efficacy from T7. At 30 DAI, the highest percent leaf area disease was observed in To (control) having value 15.62 % and lowest leaf area disease was in T8 (Chloramphenicol (0.25%)) having value of 10.08 %. Treatment T2 (Trichoderma Isolate type 03), T3 (Trichoderma Isolate type 04) and T4 (Trichoderma Isolate type 06) showed statistically similar effect having values 27.40 %,

28.21 % and 28.48 %, respectively. The rest of the treatment showed significant effect (Table 4 . 4). Engulfment of leaf area by the disease progress was faster in this period of observation (22-30 DAI). This was probably because the plants grew older and the approach of warm weather with longer day length.

Table 3. Percent diseased leaf area (%DLA) due to bacterial wilt of ginger at different days after inoculation

Treat. No.	Treatments	15 DAI	22 DAI	30 DAI
To	No treatment	35.2a*	37.4a	39.9a
T1	*Trichoderma* spp 1	32.8b	35.33b	39.6a
T2	*Trichoderma* spp 2	33.66b	33.7c	38.66a
T3	*Pseudomonas* spp 1	30.62c	34.54c	36.2bc
T4	*Pseudomonas* spp 2	34.44ab	39a	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 +chlora.	34.66ab	32.31c	32.2d
T8	Chloramphenicol (0.25%)	31.43d	30.6d	37.2bc
	$LSD_{0.05}$	1.5	2.3	2.4
	CV (%)	16.7	19.5	13.5

*** Means with the same letter(s) in a column did not differ at 5% level by LSD*

At 30 DAI. the highest percent leaf area disease was observed in T0 (control) having value 50.12 % and the lowest leaf area disease was in T8 (Chloramphenicol (0.25%)) having value 12.16 %. Treatment T7 (Trichoderma Isolate type 03+ Trichoderma Isolate type 04+Trichoderma Isolate type 05) and T5 (Chemical) statistically similar effect having values 10.12 %, and 8.32 % .respectively. The rest of the treatment also showed significant effect (Table 3).

"Stem" Infection

'Stem', in the context of this research, was considered the above ground part from petiole to the surface of the soil. Stems of ginger plants are often observed to be affected and even produce visible symptoms. The symptoms on the stem were very similar to the bacterial wilt of solanaceous crops. The bark of the stem develops cankerous lesions which were sunken or crateriform spitting the stem longitudinally. The stem became weak at such points and easily broken down. Stem infection was not considered as a parameter because, to provide information about disease severity two other variables (% leaf infection and % LAD are already included).

Infected Rhizomes per Pot

The most devastating symptoms of bacterial wilt of ginger were also detected in the rhizomes. The symptoms of this disease appeared on matured rhizomes and therefore sometimes the disease is called "matured-rhizome rot. Circular and sunken lesions with black margins appeared on the matured rhizomes. A pinkish mass of fungal spores covered the sunken-spot. In the advanced stage of the disease, the concentric markings with dark acervuli appeared on the affected parts. The spotted rhizomes dropped down prematurely and heavy losses resulted.

Effect of different treatments on percent rhizome infection is summarized in Table 4. At 30 DAI, all the treatments showed statistically significant effect on reducing percent rhizome infection compared to control. The highest percent of rhizome infection was observed in control pot To (35.12%) and the lowest percent of rhizomes infection was recorded in chloramphenicol (0.25%) (4.72%).The treatments T3 (24.06%) and T1 (23.69%) had shown statistically similar significant effect. Treatment T1 (22.06%) and T4 (21.38) were statistically similar as well as T6 (16.38%) and T7 (14.65%) were statistically similar. The rest of the treatments showed significant effect. (Table 4).

Rhizome infection at 30 DAI, the treatments showed statistically significant effect on percent rhizome infection. The highest percent of rhizome infection was observed in control pot To (52.12%) and the lowest percent of rhizome infection was recorded in chloramphenicol (0.25%) (12.25%).The treatments T3 (25.50%) and T, (25.53%) had shown statistically similar effect. At 30 DAI the Ginger plants are quite old. Especially, the test cultivar plants almost reached their full maturity. Thus the plants became weak with hardly any new tissue development. These reasons coupled with the environmental and other factors some interesting findings appeared within Table 4.5. Thus, control where no treatment was given the % rhizome infection at 30 DAI became more than double compared to the situation observed at 30 DAI. But the plants which received treatment, whichever it may be, the rhizome disease spread was considered low. The highest rhizome disease was observed in T_0 (Control) and significantly small different in other treatments.

Among the botanical bioagents, the strongest anti- *R. solanacearum* reaction has been shown in terms of percent rhizome infection by Trichoderma Isolate type 04 leaf extract (1.44), which is lower than Trichoderma Isolate type 05extract (1 .84). This individual antifungal strength has also been reflected in T6 and T7. Chemical's effect in reducing % rhizome infection was as anticipated, the strongest. That is at 60 DAI only 12.25% infected rhizome per plant was observed and the increase from the 30 DAI was only 1.53%.

Throughout the experiment in all the parameters taken into account 0.25% application of chloramphenicol as foliar spray performed excellent. It was stated that chloramphenicol treatment was a positive control treatment as no treatment was considered as a control treatment. The aim was to compare the effects of the bio-control treatments with both. The results and their analyses revealed that the test treatments, bioagents, are capable of reducing bacterial wilt of Ginger was higher quite significantly even when applied singly. However, combination biological agents were more strong and effective even to a level that with minimum risk such treatment can replace a highly effective chloramphenicol (0.25%) fungicidal treatment.

Table 4. Percent infected rhizomes per pot due to Bacterial wilt of ginger at different days after inoculation

Treat. No.	Treatments	30 DAI	15 DAI
To	No treatment	37.4b	49.9a
T1	*Trichoderma* spp 1	36.33c	49.6a
T2	*Trichoderma* spp 2	35.7d	48.66b
T3	*Pseudomonas* spp 1	35.54d	46.2c
T4	*Pseudomonas* spp 2	39.11a	49.25a
T5	Tri 1 + Tri 2	37.22b	48.23b
T6	Pseud. 1 + Pseud. 2	37.88b	46.56d
T7	Tri +Pseu + Chlora.	36.31c	42.2e
T8	Chloramphenicol (0.25%)	36.6c	47.2c
	$LSD_{0.05}$	1.1	1.2
	CV (%)	14.7	16.5

** *Means followed by the same letter(s) in a column did not differ at 5% level by LSD*

Rhizome Yield per Pot

Effect of different treatments on yield of Ginger in terms of dry weight was determined and presented in Table 4. The highest yield was recorded in treatment T6 pot (330 g/pot) and the lowest yield was recorded in control pot To (l50 g/pot). Yield of Ginger greatly varied from treatment to treatment which ranged from 150 – 330 g/pot. The second highest yield was observed in T7 (328 g/pot). The treatments T3 and T5 as well as T6 and T1 showed statistically significant effect. However compared to control the highest yield was observed in the T6 (Trichoderma Isolate type 03+ Trichoderma Isolate type 04+ Trichoderma Isolate type 06) treatment (Table 5). From above, it is revealed that rhizome production is inversely proportional to rhizome infection. It is interesting to find in the Table 4 which contains the findings about rhizome yield per pot that the weakest proved treatment T1 (*Trichoderma* spp 1) increased dry rhizome yield upto 33.2%. While spraying with Trichoderma Isolate type 03 extract 70.6%. Trichoderma Isolate type 04 bioagents 93.3%, Trichoderma Isolate type 06 bioagents 66.6%, Trichoderma Isolate type 05extract 106.6%, Trichoderma Isolate type 03, Trichoderma Isolate type 04 and Trichoderma Isolate type 06 combines extract and Trichoderma Isolate type 03, Trichoderma Isolate type 04 and Trichoderma Isolate type 05extract 118.6%. The chloramphenicol (0.25%) increased dry rhizome production per pot by 108%.

Table 5. 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* spp 1	200f
T2	*Trichoderma* spp 2	265c
T3	*Pseudomonas* spp 1	284b
T4	*Pseudomonas* spp 2	245d
T5	Tri 1 + Tri 2	299a
T6	Pseud. 1 + Pseud. 2	224e
T7	Tri +Pseu +chlora.	296ab
T8	Chloramphenicol (0.25%)	301a
	$LSD_{0.05}$	12.5
	CV (%)	11.3

** *Means followed by the same letter(s) in a column did not differ at 5% level by LSD*

Further interesting matter was that the plants who received Chloramphenicol (0.25%) treatment produced lesser quantity of rhizome than those under T6 and T1 had Trichoderma Isolate type 06 extract in combination which probably promoted production physiology. The possible reason may be chloramphenicol might have adversely influenced the reproduction physiology of the ginger plants. This adds to the already existing reasons why environment friendly community would like to find a safe replacement of chloramphenicol.

Discussion

Extended nectrotic wilting symptoms were appeared on mature leaves within 15 days after inoculation and grow fast. Large spots have been found to kill the leaf within three weeks or so. Affected leaves have been observed to wither and fall off. The results obtained on the parameter % leaf infection were recorded. Effect of integrated antagonists had shown inferior percent leaf infection at the earliest of times. At 15 DAI, the highest percentage of leaf infection 24.98% was observed in the treatment To (control).The result clearly indicated that the treatments had significant effect on percent leaf infection. The lowest (5.56%) percent leaf infection was observed in pots received chloramphenicol (0.25%) treatment. Percent leaf infection was statistically similar in control (no treatment) and T1 (*Trichoderma* Isolate 01) in 22 DAI and 30 DAI with value of 27.4% and 29.9% and 25.33 29.6%, respectively. The rest of treatments had shown statistically significant effect. Antagonistic activity of *Trichoderma* spp and *Pseudomanas* spp against the pathogen, *Ralstonia solanacearum* (Bacterial wilt) on KB agar medium had been . Out of the tested isolates, only four (type 3, type 4, type 5 and type 6) showed a certain degree of inhibition to the growth of the pathogen. 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. In the experiment, bacterization of Ginger rhizomes with the selected isolates i.e., type 7, type 8, type3, type4, type5 and type6 significantly reduced the incidence of bacterial wilt by 59.83%, and increased plant growth: plant height and dry weight by 76.89% and 28.44%, respectively suggesting the importance of the isolates as plant growth promoting rhizobacteria.

Isolates of bio-agent have already been found having very effective strong fungicidal effect. In some cases, the reported effect is more than that of chloramphenicol (0.25%) pesticides. Out of the many such reported promising plants, on the simple basis of availability Trichoderma Isolate type 05, Trichoderma Isolate type 03, type 04 and Trichoderma Isolate type 06 have been selected to asses their combat ability against, the pathogen , that is the ability against bacterial wilt of Ginger. Plant infection, leaf infection, diseased leaf area, rhizome infection, yield per pot (g) and yield ton/ha were found to be better in combination treatments. The effect on pathogenesis, three observations: one on 15 DAI, the second on 30 DAI and the third and final on 22 DAI were made, rhizomes treated early yield the highest cumulative means of all disease reaction and yield parameters on the rizomes from the respective pots. The pots that received chemicals had the lowest disease intensity as well as severity parameters. The type m-t(T1) has shown the weakest disease control potential but significantly superior to the control (To). The isolates of bio-agent treatments have shown significantly better control than the T1, though they varied widely amongst themselves. The combine treatments T6 (Trichoderma Isolate type 03 + Trichoderma Isolate type 04 + Trichoderma Isolate type 06) and T7 (Trichoderma Isolate type 03+Trichoderma Isolate type 04 +Trichoderma Isolate type 05) have shown very strong response though lesser than the T8 (Chemical) in controlling bacterial wilt of Ginger. The highest average rhizome yields however were observed under the treatment T_6 and T_7 and the lowest under the treatment *To*. As a result low yield was found in control pots and high in the pots where combined treatments were applied. The combined treatment had a highly significant effect on the rhizome yield. The results obtained by Laksman (1990) with the use of Trichoderma Isolate type 05and Achium and Schloesser (1992) with Trichoderma Isolate type 03 and Trichoderma Isolate type 03 seed bioagents nicely corroborate with the present findings. The result from the benefit cost ratio analysis revealed that highest financial benefit was obtained from the combined treatment T_7 where the BCR (3.97) was the highest and the second highest BCR was from T_6 (3.96) while the lowest BCR (1.90) was observed in control pot. It was evident from the obtained results that comparatively low yields were responsible for the lower gross return and a lower BCR against each treatment.

Conclusion

The results obtained through this experiment indicated that a judicially designed combined organic treatment even may be profitable than a chloramphenicol (0.25%) fungicide treatment. 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 (Trichoderma Isolate type 03+ Trichoderma Isolate type 04+Trichoderma Isolate type 05) in equal suspension with foliar spray in order to have a higher profitable yield and eventual higher economic return with minimum health risk as well as environmental pollution. However, meticulous with higher potency or efficacy must go on as the pathogenecity of the causal agent is quite dynamic. Therefore, the farmers may be advised to take an integrated approach which should to raise a profitable production without polluting the environment and adding toxins in the food chain. Overall, the result showed that the selected antagonistic isolates managed to suppress or control the disease caused by the pathogen, *R. solanacearum* effectively. In addition they promoted the growth of the Ginger plant indicating the bacteria may be member of the plant growth promoting rhizobacteria. Hence, *P .fluorescens* can be taken as good candidate of antagonistic bacteria to be used in the biological control of strategy of the economically important pathogen *R. solanacearum* which cause significant yield loss in the country. Such kind of disease control is complicated because of the many variable factors such as host, strains of the pathogen, antagonistic bacteria and environment. So the impact of each factor must be further studied in the field so that the beneficial bacteria can control bacterial wilt effectively.

Recommendation

From the findings of this study, biological control through trichoderma spp and pseudomonas spp are recommended against *Ralstonia solanacearum* in Ginger. Though, plant protection rendered this way is very effective, the data showed that it is difficult to control bacterial wilt completely. Therefore, combining different methods such as resistant variety and biocontrol together can give better protection than a single method. Moreover, addition or use of specific substrate that enhance selective growth and multiplication of the antagonist, use of multiple microbial inoculant rather than a single species alone, genetic manipulation of the desired isolate (the

promising one), improving delivery of the formulation of the biocontrol agent etc, can be considered as untapped potential of *Trichoderma* spp and *P. fluorescens* in the biological control of Ginger bacterial wilt in the SNNP region of country.

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