Research Journal of Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Microbiology

ISSN 1816-4935
DOI: 10.3923/jm.2016.20.27

Research Article
Synergetic Effect of Rhizosphere Bacteria Isolates and Composted Manure on Fusarium Wilt Disease of Tomato Plants

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Abstract
Composts and bio-fertilizers are the most important elements for agricultural development activities. In agricultural both practices are prominently improves plant growth. Besides, Composts and bio-fertilizers suppress plant disease, presence of a pathogen and a susceptible host in the environment. Plant disease suppression is the direct result activity of consortia antagonistic microorganisms and composted manure. Isolation and identification of the soil borne fungal pathogens and rhizosphere bacteria were done *in vitro* based on their morphological and biochemical examination; to evaluate effects of rhizosphere bacteria isolates and composted manure on Fusarium wilt of tomato/*Lycopersicon esculentum*/plants disease. Additionally, antagonism experiment likewise hydrogen cyanide test and plant growth promotion test (phosphate solubilization, starch hydrolysis and screening of Indole Acetic Acid (IAA)) productions were carrying out. Under greenhouse condition the disease severity, disease incidence and measurement of growth parameters were correlated using two rhizosphere isolates (RhB-7 = Rhizobacteria *Bacillus* spp., RhP-12 = Rhizobacteria *Pseudomonas* spp.) and two different percentages of composted manures (10 and 20%) including their combination. The result revealed 100% disease incidence and severity in control experiment and 29-100% efficacy in combined treatment. Furthermore, the combination of RhB-7, RhB-12 and 20% of composted manure were the most efficient against Fusarium wilt disease of tomato than the rest treatments. Through, rhizosphere bacteria isolates and composted manure it is possible to control tomato/*Lycopersicon esculentum*/plant wilt which is caused by the fungal called *Fusarium* spp. and increase the soil fertility and the product yields.

Key words: Antagonism, biocontrol, composted manure, disease incidence, fungal disease

Received: August 28, 2015 **Accepted:** November 09, 2015 **Published:** December 15, 2015

Citation: Lata Lachisa and Anbessa Dabassa, 2016. Synergetic Effect of Rhizosphere Bacteria Isolates and Composted Manure On Fusarium Wilt Disease of Tomato Plants. Res. J. Microbiol., 11: 20-27.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tomato (*Solanum lycopersicum*) is one of the most important economical crops, which belongs to family "Solanaceae". The economic importance of this crop appears in both local consumption and exportation purposes. Among the numerous plants in the world tomatoes are forms leguminous potch which contains the rhizobacters and other rhizosphere microorganisms. Tomato (*Solanum lycopersicum*, formerly, *Lycopersicon esculentum* Mill.) is the second most important vegetable crop after potato in the world (Panthee and Chen, 2010). However, tomato production is significantly reduced by pathogen because it can destroy roots of tomato at growth stages (Kim and Kim, 2008). As indicated by Van Lenteren and Woets (1988), diseases of tomato were the major limiting factor on their production.

For the duration of seed germination and roots growth through the soil; the loss of organic material provides the driving force for the development of active microbial populations around the root, known as the rhizosphere effect although the stimulation in microbial activity is a general phenomenon largely involving saprotrophs, specific groups of symbionts may be selectively enhanced (Grayston *et al*., 1998). For instance, mutualistic bio trophic symbioses may develop between rhizobia and legumes and mycorrhizal fungi may interact with their plant hosts. Nevertheless, antagonistic symbioses between pathogens and roots can also form resulting in disease. The microbial interactions taking place in the sperm sphere and rhizosphere associated with disease development and especially biocontrol of these diseases form (Beattie, 2006).

Determining of microorganisms very close to epidermis, plants secrete signal molecules for protection against invasion of the heterogeneous microbes in the root zone and at this stage the differentiation takes place between pathogenic, associative, symbiotic, or naturalistic adaptation of microbes with the plant (Mishra *et al*., 2011). The plant rhizosphere includes different group of organisms like (Buee *et al*., 2009) AMF and bacterial groups forming association in the leguminous and non-leguminous plants mostly Tomato (*Solanum lycopersicum*).

The significance of rhizosphere microorganisms, especially mycorrhizal fungi and PGPR bacteria, in a soils can be enormous, since they can able to increase the tolerance of plants against a biotic stress, stimulate plant growth and contribute in this way to an accelerated remediation of disturbed soils (Gamalero *et al*., 2009; Yang *et al*., 2009). Now a days, biological methods have known as effective and appropriate ways in plant disease control. The application of those microorganisms largely overlooked as biocontrol agents of plant disease including the rhizobacteria that can colonize plant root surfaces and able to suppress plant pathogen microorganisms (Yang *et al*., 2009; Singh *et al*., 2011). Microbial application in bio fertilizer and biocontrol for plant production should be promoted, as the result of its environmental friendly and effect in productivity.

MATERIALS AND METHODS

Study was conducted at Jimma University, which is found in Jimma town, Oromia region, South West Ethiopia, 352 km from Addis Ababa. It is located at latitude of 7°40'N and 36°50'E at altitude of 1,780 m above sea level (Alemu *et al*., 2011). The laboratory study method was conducted at the microbiology and postgraduate research Laboratory of Biology department and in greenhouse from September, 2013 to June, 2014.

Isolation of *Fusarium* spp. and sample preparation: Infected tomato plant tissues (leaf, stem and roots) were surface sterilized in 3% sodium hypochlorite (NaClO) for 3 min, rinsed in three changes of sterile distilled water and then blotted dry with a sterile paper towel pad. Approximately, 2 mm×7 mm tissue sections were cut from the advancing portion of the lesion of surface sterilized tissue using a sterile scalpel blade. The sections were placed on specific *Fusarium* spp. medium [peptone penta-chlorinator-benzene agar (PPA)] containing Diffco agar powder (15 g L^{-1}), peptone (15 g L^{-1}), KH_2PO_4 (1 g L^{-1}), $MgSO_4.7H_2O$ (0·5 g L^{-1}) according to Parry *et al*. (1995).

Isolation of plant growth promoting rhizobacteria (PGPRB): The plant growth promoting Rhizobacteria (PGPRB) was isolated on the agar media. The mechanisms of plant growth promotion the isolate were determined through the Phosphate solubilization and Indole acetic acid production of the identified microorganisms using the method described by Shahab *et al*. (2009).

PGPR bacterial mechanisms of biocontrol

Hydrogen cyanide production: Hydrogen cyanide productions of the isolate were tested. The young culture (48 h) of bacterial isolate (100 µL) were streaked on the surface of medium, then sterilized filter papers were soaked in 2.0% Na_2CO_3 in 5.0% (w/v) picric acid placed in the upper lid of the petri dish. The petri dishes were sealed with parafilm and incubated at 30°C for 4 days. The cyanogenic activities of the isolate were determined.

Experimental design: The experimental designs were done by using (4x3) factorial using randomized block design with three replicas. Four microbial combinations with three different doses (0, 10 and 20%) of composted manure. Each treatment was being replicated three times; which was giving a total of 33.

Disease severity and disease incidence: Fusarium wilts incidence and severity was taken at weekly intervals, starting from 4 Weeks After Transplanting (WAT) to 9 WAT and determined on 0 to 5 scales according to Soonthornpoct *et al*. (2001), where, 0 = no symptoms (neither root discoloration nor leaf yellowing, 1 = 1-25% root discoloration or one leaf yellowed, 2 = 26-50% root discoloration or more than one leaf yellowed, 3 = 51-75% root discoloration or vascular discoloration plus one leaf wilted, 4 = up to 76% root discoloration or more than one leaf wilted and 5 = completely dead plants. Disease Incidence (DI) was determined using the following formula of as follows as the above author stated and result obtained were converted to percentage using the formula: I (%) = (number of infected plants/total number of plants) x100.

Measurement of growth parameters: The growth parameters of tomato plants measured were: shoot height (cm), root height (cm), number of leaves/plant, petiole length (cm), blade/lamina length (cm) and total plant length (cm).

Statistical analyses: All experiments were set up through a complete randomized block design. The One-way ANOVA was used to analyze differences between treatments. Duncan's multiple range tests at $p< 0.05$ was used for mean separation.

RESULTS AND DISCUSSION

The summary statistics of the measured parameters among study Tomato plant growth were shown in Table 1. In the treatment combination assessed Leaf number was varied from five with control+tomato+Fusarium, RhB-7+ RhB-12+COMP-10%+Fusarium+tomato and RhB-1+COMP-20%+Fusarium+tomato treatment combination. The mean length of petiole was relatively low ranging from 2-4 cm. Shoot length varied from small length (20 cm) to highly length (50 cm) and Leaf length ranged from 6-20 cm, while Root length varied from 9-15 cm and the total plant length was relatively normal ranging from 34-65 cm.

Furthermore, the results from the Table 1 indicate the shoot length, leaf length and total length have greater variability between the group with 51.018, 19.255 and 78.218 and law variability within the group with STDV 2.844, 0.809, 7.143, 4.388, 1.753, respectively. The variability in leaf number, root length and petiole length were the data points closer to the mean with the variability 8.091, 0.655 and 3.073.

Morphological and biochemical examination of the rhizosphere bacteria isolates: Morphological and biochemical examination of the rhizosphere bacteria isolates were done and summarized as the following Table 2. Based up on the morphological and biochemical characteristics the

Table 1: Summary statistics measurement of minimum (min), maximum (max), average and standard deviation values in the grow parameter for tomato plants

Parameters	N	R	Min	Max	S	X	SE	STDV	Var
Leaf No	11	9	5	14	111	10.09	0.858	2.844	8.091
Petiole L (cm)	11	2	2	4	37	3.36	0.244	0.809	0.655
Shoot L (cm)	11	25	25	50	481	43.73	2.154	7.143	51.018
Leaf L (cm)	11	14	6	20	150	13.64	1.323	4.388	19.255
Root L (cm)	11	6	9	15	149	13.55	0.529	1.753	3.073
Total	11	31	34	65	630	57.27	2.667	8.844	78.218

N: Total number, R: Range, Min: Minimum, Max: Maximum, X: Mean, STDV: Standard deviation of vector, Var: Variability, S: Sum, L: Length, SE: Standard error

Table 2: Morphological and biochemical examination of the rhizosphere bacteria isolates

Parameters	RhB-07	RhB-12	RhB-13	RhB-24	RhB-25
Shape	Rod	Rod	Rod	Rod	Rod
Endospore staining	+	-	+	-	-
Gram reaction	+	-	+	-	-
Growth at 80°C	+	-	+	-	
Oxidase test	-	+	-	+	-
Catalase test	+	+	+	+	+
Phosphate-Solubilization	+	+	+	+	-
Cyanide test	+	+	+	+	-
IAA Production	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-

+: Positive reaction; -: Negative reaction, IAA: Indole acetic acid

plant growth promoter microorganisms were identified. The best candidates Rhb-7 and RhB-12 were identified for their phosphate solublization. The rest isolates Rhb-13, Rhb-24 and Rhb-25 were intermediate solubilizer's microorganisms. Though the biocontrol activities of the best candidates RhB-7 and RhB-12 were evaluated *in vitro* and their activities were evaluated in green house.

Biochemically and morphological tests were the common mechanisms of microorganisms. Gram staining of bacterial cultures was done as described previously (HPA., 2007). Oxidase test was performed according to Jurtshuk and McQuitty (1976), endospore staining, growth at 80°C, catalase test, phosphate solubilization, cyanide test, IAA production, starch hydrolysis were used of the identification of rhizosphere bacteria.

Biocontrol activities of the isolate

Screening of IAA production: For the study on the effect of IAA producing rhizosphere bacterial isolates on plant growth, pot assay was executed. The Tomato plant seeds were used for seed coating. The Tomato seeds were surface sterilized by immersing in 75% ethanol for 1 min and mercury chloride (0.2%) for 5 min. Then further to remove traces of mercury chloride, the disinfected seeds were washed 5 times by sterile distilled water according to Etesami *et al.* (2015). The 0.1 mL overnight grown culture (0.5 OD) was applied on seed surface for seed coating. Seeds were dried and sowed into sterile soil as carrier. Six seeds were sown in each pot used per pot at equal distance and experiment was performed in triplicates for each isolates. The uncoated seeds were used as control.

After appearing seedlings of soil 0.1 g of Trp- per kg soil after being solving in water was added to every pot. Pots were irrigated with sterile distilled water every day and kept in sunlight. At the interval of every 5th day, plant was uprooted and seedlings were measured for shoot and root length and other parameter content upto 15th day according to Etesami *et al.* (2015).

The colonies obtained from rhizosphere soil were screened for their ability to produce IAA. Colour reaction of various isolates of rhizosphere bacteria with Salkowaski reagent resulted in the appearance of red color (Fig. 1).

Among the five isolates only two isolates of rhizosphere bacteria showed red colour reaction with salkowaski reagent in nutrient broth in the presence of tryptophan indicating their ability to produce IAA organisms. The production of indole acetic acid was shown through all isolates in the presence of tryptophan. The RhB-12 isolate had the most activity in IAA production. As indicated by Patten and Glick (2002) that compared to other strains Pseudomonas strains had higher level of indole acetic acid (IAA) production. Our result is consistence with the study conducted by Leveau and Lindow (2005) that adding tryptophan increased the production of indole acetic acid. It is possible to suggest that tryptophan acted as a precursor and therefore led to presence of this material in rhizosphere plant exudates. Authoritative rhizobacteria can convert tryptophan to indole acetic acid resulting in increasing plant growth.

The result demonstrated that the change from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively (Fig. 2). Cyanide hydrogen is up setter of perspiration and chelating agent of metals and has been reported as effective in control of Fusarium wilt caused by *Fusarium* spp. (Karimi *et al.*, 2012). As study conducted by Raupach and Kloepper (1998) the results suggest that, greater emphasis on the development of mixtures of biocontrol agents is needed, because they may better adapt to environmental changes that occur throughout the growing season and protect against a broader range of pathogens.

Multiple organisms may more stable rhizosphere community and increase the effectiveness in a wide range of environmental conditions. Mixtures of biocontrol agents with taxonomically different organisms that require different optimum temperatures, free of Particularly Hazardous Substance (PHS) in the environment and moisture conditions may colonize roots more aggressively, improving plant growth and the efficacy of biocontrol agents via different disease suppression mechanisms.

Many types of compost were reported to have suppressed Fusarium wilts in different crops. *Fusarium oxysporum*, Radicis-lycopersici with the use composted poultry manure was suppressed Fusarium wilts (Youssef, 2007). Organic wastes, primarily by-products of many industrial activities,

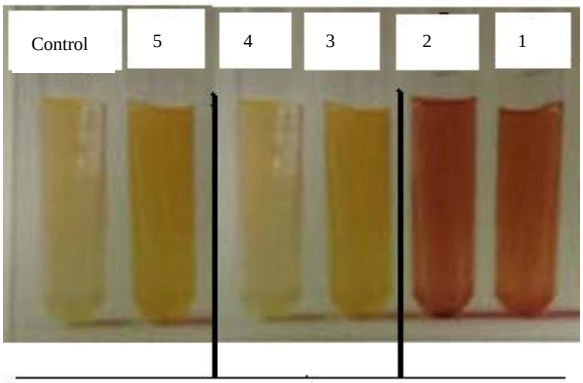

Fig. 1: Qualitative test of rhizosphere bacteria isolates ability of indole acetic acid production

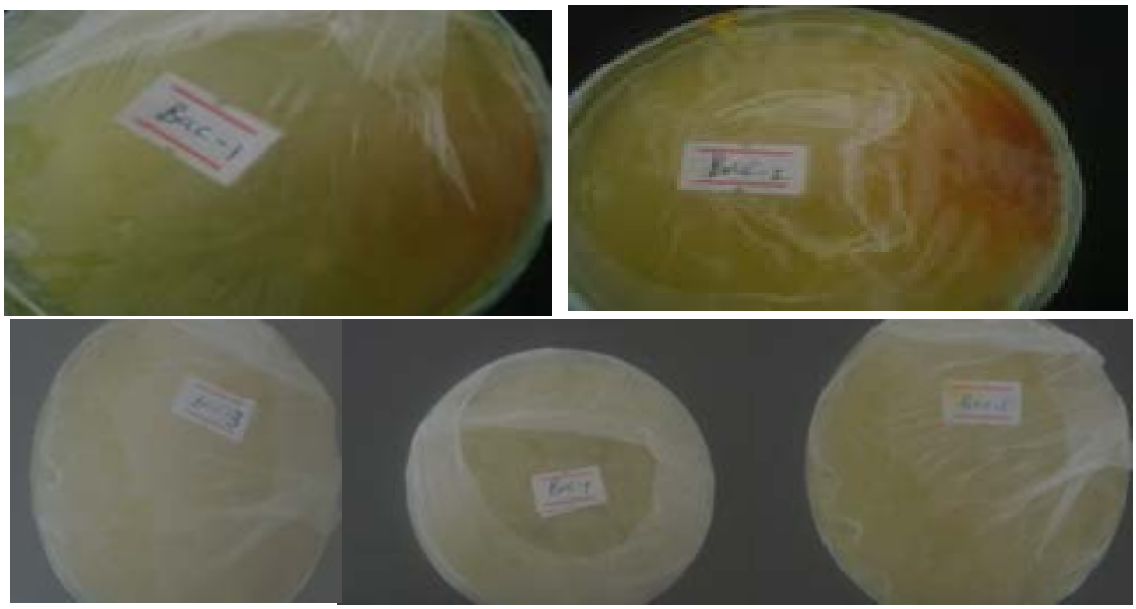

Fig. 2: Qualitative test of rhizosphere bacteria isolates ability of hydrogen cyanide production

Table 3: Measurements of tomato plants growth parameters

Treatments	No. of leaves	Petiole length (cm)	Shoot length (cm)	Leaf length (cm)	Root length (cm)	Total length (cm)
Control+ tomato+Fusarium	5	1.6	24.6	6	9.3	33.9
RhB-7+Fusarium+tomato	10	4.3	42	11.2	13.4	55.4
RhB-12+Fusarium+tomato	9	4	49	12.2	14.7	63.7
COMP-10%+Fusarium+tomato	7	2.1	44.6	11.2	14.2	58.8
RhB-7+COMP-10%+Fusarium+tomato	8	3	41	14.2	13	54
RhB-12+COMP-10%+Fusarium+tomato	11	2.7	48.9	11.1	14.9	63.8
RhB-7+RhB-12+COMP-10%+Fusarium+tomato	14	3.5	50	17	15.4	65.4
COMP-20%+Fusarium+tomato	12	3	40	19.5	12.6	52.6
RhB-7+COMP-20%+Fusarium+tomato	14	4.3	44.2	11.4	14.2	58.4
RhB-12+COMP-20%+Fusarium+tomato	9	4.2	49	17	15	64
RhB-7+RhB-2+COMP-20%+Fusarium+tomato	12	4	47	20	13	60

RhB-7: Rhizibacteria, *Bacillus* spp., RhP-12: Rhizobacterial *Pseudomonas* spp., COMP-10%: Ten percent of composted manure, COMP-20%: Twenty percent of composted manure

include animal manures, crop residues and municipal bio-solids. To reduce the environmental strain, it is essential to make organic wastes a resource rather than a by-product. Naturally, farmers have used animal manures as a crop fertilizer to improve soil physical and chemical properties (Li *et al*., 2011).

Fusarium spp. is economically significant disease on tomatoes. Due to the soil-borne nature of the disease use of chemical methods for the control of disease is rarely successful. In consistencies in biocontrol under varying environmental conditions has been a common limitation of soil borne pathogens (Ahemad and Kibret, 2014). The use of rhizobacteria for plant disease control is more effective when rhizobacteria is isolated from rhizosphere of the same host plant. In this study, all bacterial isolates were selected from rhizosphere of healthy tomatoes in fields. Some bacterial isolates showed high inhibition activity on pathogen, whereas others showed only mild. According to previous study of Ortiz-Castro *et al*. (2009) showed that many microorganisms such as bacteria can had positive influence on plant growth and plant health as PGPR. The role of rhizobacteria in promoting root and shoot growth of different crops has been demonstrated. The use of rhizobacteria against Fusarium wilts of tomatoes plant caused by the *Fusarium* spp. (Van Lenteren and Woets, 1988) often the yellowing is restricted to one side of the plant or to leaflets on one side of the petiole. The affected leaves soon wilt and dry up, but they remain attached to the plant. The wilting continues on successively younger foliage and eventually results in the death of the plant (Table 4). The stem remains firm and green on the outside, but

Table 4: Effect of treating tomato seeds with rhizobacteria of antagonist Fusarium wilt

Treatments	Disease Incidence (DI)%	Disease sever (%)	Efficacy (%)
Control+tomato+Fusarium	100	100	0
RhB-7+Fusarium+tomato	20	20	80
RhB-12+Fusarium+tomato	0	0	100
COMP-10%+Fusarium+tomato	71.43	71.43	29.67
RhB-7+COMP-10%+Fusarium+tomato	50	50	50
RhB-12+COMP-10%+Fusarium+tomato	18.18	18.18	78.92
RhB-7+RhB-12+COMP-10%+Fusarium+tomato	0	0	100
COMP-20%+Fusarium+tomato	16.67	16.67	86.47
RhB-7+COMP-20%+Fusarium+tomato	0	0	100
RhB-12+COMP-20%+Fusarium+tomato	0	0	100
RhB-7+RhB-12+COMP-20%+Fusarium+tomato	0	0	100

RhB-7: Rhizibacteria *Bacillus* spp., RhP-12: Rhizobacterial *Pseudomonas* spp., COMP-10%: Ten percent of composted manure, COMP-20%: Twenty percent of composted manure

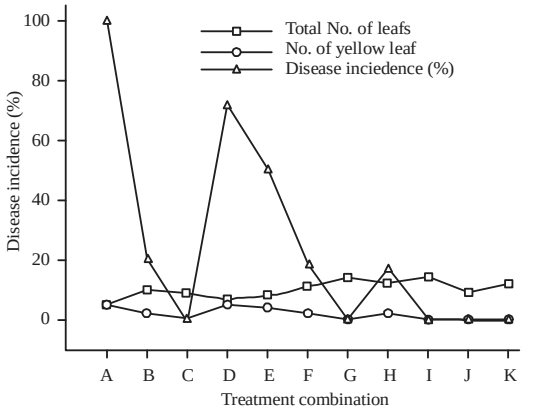

Fig. 3: Evaluation of different treatment combinations of plant growth promoting rhizobacteria and Fusarium wilt disease treatments under greenhouse conditions at Jimma University Natural science college, Department of Biology, 20014. Where, A: Control+tomato+ Fusarium, B: RhB-7+Fusarium+ tomato, C: RhB-12 +Fusarium+ tomato, D: COMP-10%+Fusarium+tomato, E: RhB-7+COMP-10%+Fusarium+tomato, F: RhB-12+COMP-10%+Fusarium+tomato, G: RhB-7+RhB-12+COMP-10%+Fusarium+tomato, H: COMP-20%+Fusarium+tomato, I: RhB-7+COMP-20%+Fusarium+tomato, J: RhB-12+COMP-20%+Fusarium+tomato, K: RhB-7+RhB-12+COMP-20%+Fusarium+tomato

exhibits a narrow band of brown discoloration (streaking) in the vascular tissue. This discoloration can be viewed by slicing vertically through the stem near the soil line and looking for a narrow column of browning between the central pith region and the outer portion of the stem (Raupach and Kloepper, 1998). The management of Fusarium wilt pathogen is particularly complex because it lives in or near the dynamic environment of rhizosphere and can frequently survive long periods in soil through the formation of certain resistant structures of the pathogen and increasing the yield of several crops through biocontrol of plant pathogens.

According to results presented in this study, suppression of disease was better in soil treatment as shown in greenhouse experiments. Similar results reported by Panthee and Chen (2010). This may be related to the rate and extent of colonization of roots by isolates and percentage of composted manures in soil treatment, since soil treatment pots were completely saturated by bacterial isolates and composted manures. In both methods, RhB-7 and RhB-12 isolates and composted manures were the most effective in reduction of disease under greenhouse conditions (Fig. 3). The result revealed that the efficacy of RhB-12 on Fusarium wilt better than RhB-7 as compared by their disease incidence reduction as well as the yellowing leaf numbers. Moreover, the combination of RhB-7, RhB-2 and 20% of composted manure and RhB-7, RhB-12, 10% of composted manure were the most efficient against Fusarium wilt disease than the rest treatments (Table 3, 4 and Fig. 3).

The control (tomato+Fusarium) and composted manure +Fusarium were the highest incidence in Fusarium disease. On the contrary the treatments with the combination of composted manure and bacteria of different dose have the lowest incidence in Fusarium disease of tomato (Fig. 2) and also supported by Al-Amri (2013).

CONCLUSION

Using a resistant cultivar is the best method in controlling disease. However, due to the possible appearance of new races in pathogen fungi and breaking of resistance in host defense is the reason for infection tomato plant by fungal disease. Therefore, the application of rhizobacteria and composted manure, support plant growth and suppress potato infecting microbes. Tomato wilt disease, caused by fungal pathogen (*Fusarium* spp.) is common in all tomato growing areas in the country. Though, the present study of

Tomato wilt disease was evaluated for his posing considerable tomato loss at green house.

The results of antagonism study clearly demonstrated that rhizobacteria isolates exhibited inhibition of the radial growth of Fusarium isolate under *in vitro* and green house conditions. The fungal mycelium growth reduction was occurred via several modes of actions (HCN, IAA). The antagonistic bacterial biocontrol agents were effective through producing at least a single bio control mechanism.

In general, the present rhizobacteria antagonists were found to reduce tomato wilt disease severity and incidence indicating that the rhizobacteria biological control has considerable promise for reducing Fusarium wilt disease. To increase the productivity of tomato yield farmers should use rhizosphere bacteria isolates and composted manures as well. The government and research institution should works hard on new and powerful technologies for studying synergetic microbial interactions in the rhizosphere and compost, their successful applications in biotechnology and expansion of application of biofertilizer to replace the widely use of the hazardous chemical fertilizer to the natural environment.

ACKNOWLEDGMENTS

I would like to express my deepest appreciation and special thanks to Department of Biology, Jimma University and Natural Science College for the material and facility support to the accomplishment of this research work. My sincere appreciation and thanks goes to my advisor Mr Anbessa Dabassa who stands behind me and provide unlimited personal support and professional assistance throughout this study.

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