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ENHANCEMENT IN EFFECTIVENESS OF ANTAGONISTIC MICROBES BY MEANS OF MICROBIAL COMBINATION TO CONTROL *Ralstonia solanacearum* ON POTATO PLANTED IN MIDDLE LATITUDE

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ABSTRACT

One of the common problems hampering the cultivation of potatoes in middle latitude is the presence of bacterium *Ralstonia solanacearum* commonly known to cause wilt disease, by which crop failure might be caused when serious attack occurs. The objectives of the research were to obtain the application of antagonistic microbes to inhibit the wilt disease caused by *R.solanacearum* and to increase the growth and yield of potato in middle latitude. The research was conducted from July to October 2012. Antagonistic microbes were used in this research to inhibit the bacterial wilt disease caused by *R.solanacearum* on potato planted in Bumiaji, Batu. *Trichoderma viride, Streptomyces sp.* and *Pseudomonas fluorescens* isolates were selected to be applied as treatments either singly or in combination. Randomized block design was applied on the treatment group with three replications. The results showed that the single application of *Pseudomonas fluorescens* or combined application of *Streptomyces sp*. and *Trichoderma viride* + *Streptomyces sp*. was capable of extending incubation period 4 to 7 days and reducing disease incidence 44.85% – 50.09%, reducing disease intensity up to 61.23 – 72.77%, reducing the population *R.solanacearum* up to 7.28 – 97.88%, increasing the number of leaves and the marketable yield as much as 67.96 – 81.98%.

Keywords: control, *Ralstonia solanacearum*, antagonistic microbes, middle latitude

INTRODUCTION

Potato is one of the crops yields which are potential to be included in food diversification. Potato production in Indonesia was increasing by 5% per annum with the planting area of 60,000 ha in 2006, while it could only cope with 10% national food need or as much as 8.9 million tons per annum (Bisnis Indonesia online, 2008).

The supplies of potatoes mostly come from high lands, while the planting areas in highlands were limited and prone to main environmental problems like landslide, intensive cultivation in highlands which may lead to depleting the natural resources due to low productivity of the land and land degradation. On the other hand, middle land provides more spacious plains giving closer access to market places and consumers. However, potato farming in the middle land is exposed to a serious problem such as bacterial wilt disease caused by *R. solanacearum* causing to 10 – 100% lost when serious attack occurs (Hanudin, 2012; Rosyidah, 2010; Semangun, 2002).

The use of pesticide to control the bacterial wilt disease caused by *R.solanacearum* is believed to be effective but costly and polluting. On the contrary, the use of biological agents against *R.solanacearum* has been reported more effective on tomato, potato, chili and tobacco (Hersanti *et al*., 2009; Heru, 2006; Nurjanani, 2011). *Tricho-derma viride*, *Pseudomonas fluorescens*, and *Streptomyces* are potential antagonistic microbes in hampering *R.solanacearum*.

Trichoderma viride is very competitive in using the space and nutrition so that it experiences faster growth compared to its pathogen (Ernawati, 2000). In addition, it also has capability of inhibiting the intensity of the disease, incubation period

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(Soesanto, 2004) and the *in vitro* growth of *R.solanacearum* on potatoes as much as 36% to 83.33% (Gunawan, 1995; Hersanti *et al*., 2009; Tuju, 2004). *Streptomyces sp*. is capable of producing streptomycin, kanamycin and neomycin antibiotic in great amount. *Streptomyces sp*. also has good antagonistic capability in term of hampering *R. solanacearumin vitro* by means of antibiosis and bacteriostatic mechanism (Djatmiko *et al*., 2007). On chili *Streptomyces sp*. is able to extend the incubation period up to 10.5 days after inoculation and inhibit the percentage of the disease up to 75% (Khairul, 2006).

Pseudomonas fluorescens is capable of controlling *R. solanacearum* due to secondary metabolites bringing together the antimicrobial activity against bacteria and pathogenic fungi. *Pseudomonas fluorescens* also produces siderophore which is capable of inhibiting the growth of pathogens by limiting iron (Fe) uptake from soil (Duijff *et al*., 1993; Dufy and Defago, 1999), gives beneficial influence to the plant growth as Plant Growth Promoting Rhizobacteria (PGPR), and produces cyanide (Landa *et al*., 2002). *Pseudomonas fluorescens* is able to inhibit the growth of *R.solanacearum* on patchouli (Nasrun *et al*., 2005) and tobacco (Heru, 2006).

The application of combined biological agents in controlling bacterial wilt disease caused by *R. solanacearum* is the better approach in biological control. Antagonistic microbial combination is expected to be able to increase the potential of biological agents to the better protection level (Guetsky *et al*., 2001).

From this research was obtained combination of biological agents which were able to effectively and efficiently control wilt disease by bacterium *R. solanacearum* on potatoes. Consequently, the expectation of optimal growth of potatoes in middle land will be more visible.

MATERIALS AND METHODS

The experiment was conducted from July to October 2012 in endemic wilt disease land in Bumiaji, Batu, Indonesia with the latitude of 670 m above sea leveland the soil texture was clay. The cultivar of potatoes used in this research was DTO-28 whose feature fits the condition of middle land (Wardiyati, 1990). The antagonistic microbes *Trichoderma viride, Streptomyces sp*., and *Pseudomonas fluorescens* were supplied from the collection of Microbiology Laboratory of Mathematics and Science Faculty and Plant Pathology Laboratory of Agriculture Faculty of Brawijaya University, Malang. These microbes were previously selected and tested *in vitro* in Plant Pathology laboratory of Agriculture Faculty of Brawijaya University to find out antagonistic potential against *R. solanacearum* pathogen.

Preparing Antagonistic Microbes

Trichoderma viride was grown on Potato Dextrose Agar (PDA), while *Streptomyces sp*., and *Pseudomonas fluorescens* were separately grown on Kings B medium for 48 hours in the temperature of 30^{o} C. When the pure cultures were obtained, each culture of *Trichoderma viride and Streptomyces sp*. was grown on Potato Dextrose Broth (PDB) media, while the pure culture of *Pseudomonas fluorescens* was grown on Nutrient Broth (NB) by shaking for 24 hours. The available cultures were then suspended to obtain approximately the concentration of 10^{8} cfu.mL^{-1} for *Streptomyces sp.* and *Pseudomonas fluorescens* (Nurbaya *et al*., 2011) and 10^{7} spora.mL^{-1} for *Trichoderma viride*.

Experimental Implementation

The experiment was conducted by applying Randomized Block Design (RBD) with 8 treatments and 3 replications. The treatments tested involved: A0= control (without antagonistic microbes), A1= *Trichoderma viride,* A2= *Strep-tomyces sp.*, A3= *Pseudomonas fluorescens*, A4= *Trichoderma viride* + *Streptomyces sp.*, A5= *Tri-choderma viride* + *Pseudomonas fluorescens,* A6= *Streptomyces sp.*+ *Pseudomonas fluorescens* dan A7= *Trichoderma viride* + *Streptomyces sp.*+ *Pseudomonas fluorescens.* The 25 ml antagonistic microbe was applied by dousing it into the planting media 2 weeks before planting together with organic matter from chicken manure (Rosyidah, 2010), followed by the second application given at 14 days after planting (dap). There were 27 plants per plot with space of 25 x 70 cm between plants.

The organic matter from manure was given at 15 t.ha^{-1}, while urea 300 kg.ha^{-1}, SP-36 200 kg.ha^{-1}and KCl 300 kg.ha^{-1} were given as inorganic fertilizers. The plants were intensively taken care of and watered by using watering pot.

The responses which were observed in this research comprising incubation period, population of antagonistic microbes, pathogenic bacterium population, and disease incidence

were calculated based on the following formula (Sinaga, 2006):

KP = n/N x 100%
KP = disease (%)
n = number of wilting plants
N = number of plants observed

The development of disease intensity was formulated by using Winstead and Kelman (1952):

$$IP = \frac{a_1 n_1 + a_2 n_2 + a_n n_n}{5 \times \text{total number of plants}} \times 100\%$$

Remarks:

IP	=	Disease intensity
a	=	scoring value per plant
n	=	plant number with certain scoring value
5	=	wilt level : 0 : healthy plant 1 : one wilting leaf or two 2 : 3 to 10 wilting leaves 3 : more than 10 wilting leaves 4 : all leaves wilting 5 : dead

The growth component observed was number of leaves, while the potato yield harvested was observed in yield component.

Data Analysis

The data was analyzed by using analysis of variance (ANOVA), where LSD test at 5% followed when significant influence was present. Statistical analysis (regression and correlation) was done by using Microsoft Excel.

RESULTS AND DISCUSION

Wilt Disease Incidence by *R. solanacearum*

The emergence of wilt disease by *R. solanacearum* on potato in the research land brought various results. The selected isolates *Trichoderma viride, Streptomyces sp.* and *Pseudomonas fluorescens* given either singly or in combination were proven effective in inhibiting the development of wilt disease by *R. solanacearum*.

Generally, the application of *Pseudomonas fuorescens* given singly or combined with *Streptomyces sp*., or combined with *Trichoderma viride* + *Streptomyces sp*. was capable of extending the incubation period up to 4 – 7 days compared with the treatment without the presence of antagonistic microbes (Figure 1).

It can be concluded that the symptoms caused by *R. solanacearum* were observable from 45 to 53 days after planting (Figure 1). Hersanti (2009) stated that the incubation period of this disease would depend on its virulence capacity to the host plants with the incubation period ranging from 36 to 49 days. Such an incubation period was affected by several factors: host plant, environment and pathogen. The immunity of plants plays a role in determining the period required by the symptom of bacterial wilt disease caused by *R. solanacearum* to emergence (Samanhudi, 2009).

The incubation period is positively correlated with the occurring disease incidence (Sastra, 2009). The research results indicated that the single application of antagonistic microbe *Streptomyces sp*. and *Pseudomonas fluorescens* was capable of effectively controlling pathogenic attack of *R. solanacearum* so that the disease incidence was hampered to low (Figure 2). This low disease intensity occurred due to the application of *Streptomyces sp.* and combination of *Streptomyces sp. + Pseudomonas fluorescens* (Figure 3).

The application of *Streptomyces sp*. given either singly or in combination with *Pseudomonas fluorescens* was capable of reducing disease incidence by 62.38% and 50.09%, disease intensity by 85.73% and 72.77% compared with the treatment without the presence of antagonistic microbes. Table 1 reveals that the ability of *Streptomyces sp*. in colonizing potato plant roots was lower when applied in combination than thatapplied singly. It showed that the inhibition on *R. solanacearum* did not result from the colonisation, but it was more because of the antibiosis. This was in line with the report by Shimizu (2002) that *Streptomyces sp*. was capable of producing antibiotics of bleomycin, erythro-mycin, josamycin, kanamycin, neomycin and tetracycline and geldamycin.

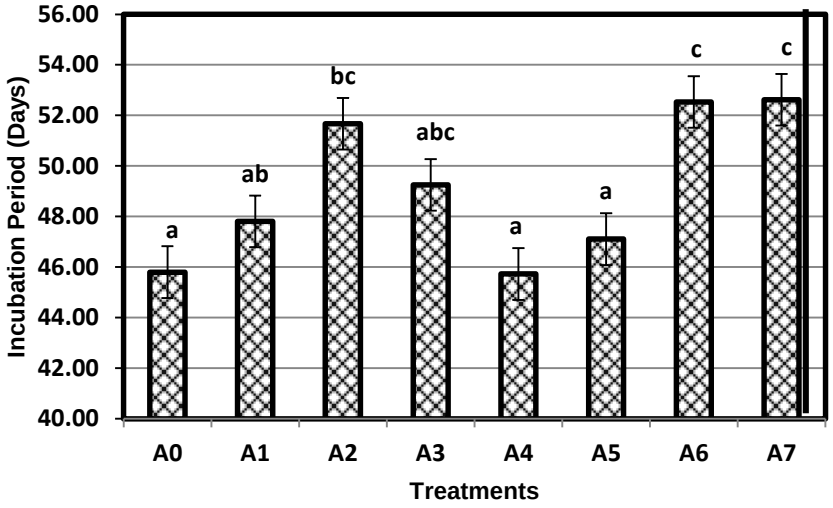

Figure 1. Incubation period of bacterial wilt disease in various treatments with application of antagonistic microbes. A0= control A1= *Trichoderma viride,* A2= *Streptomyces sp.*, A3= *Pseudomonas fluorescens*, A4= *Trichoderma viride* + *Streptomyces sp.*, A5= *Trichoderma viride* + *Pseudomonas fluorescens,* A6= *Streptomyces sp.*+ *Pseudomonas fluorescens* dan A7= *Trichoderma viride* + *Streptomyces sp.*+ *Pseudomonas fluorescens*

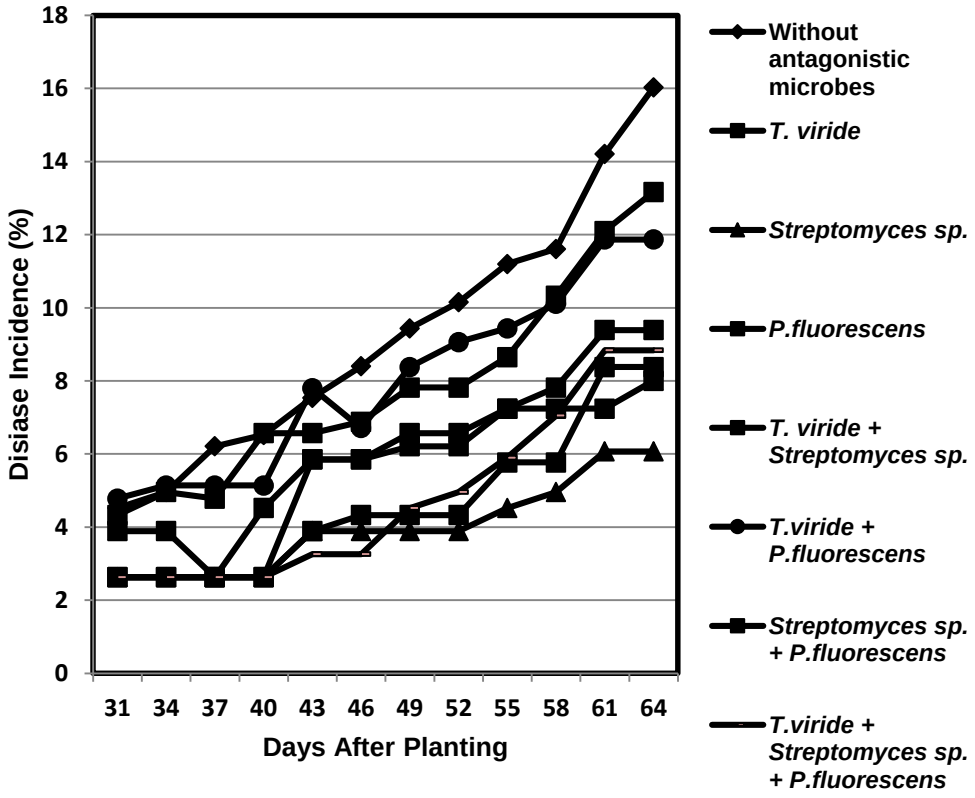

Figure 2. The disease incidence of wilt disease *R. solanacearum* at certain ages of plant

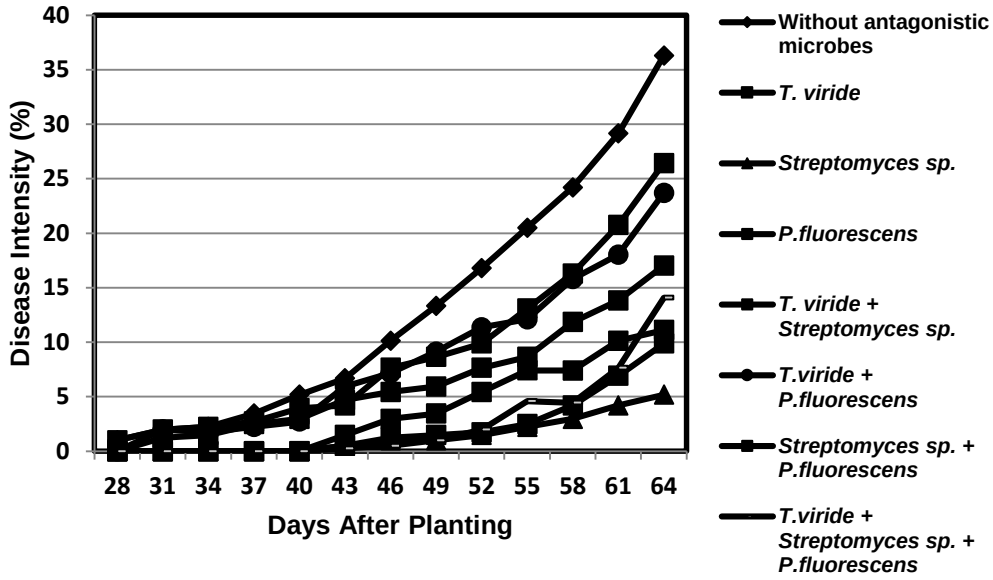

Figure 2. The disease intensity of wilt disease *R. solanacearum* at certain ages of plant

The ability of *Pseudomonas fluorescens* to reduce the attack level and intensity was believed to be correlated with its various mechanisms. *Pseudomonas fluorescens* was said to be able to produce secondary metabolites such as siderophore, pterin, phyrole and phenazine. Siderophore can serve as fungistatic and bacteriostatic agents (Soesanto *et al.*, 2008) and improve the systematic immunity of the plant by increasing the phenol (Park *et al*., 2009). The ability of *Pseudomonas fluorescens* to inhibit the population of pathogen was associated with its ability to protect the roots from the infection caused by pathogenic *R.solanacearum* by colonising the surface of the roots and competing with pathogen in infiltration of cation Fe.

Pseudomonas fluorescens provides secondary metabolites such as pyoverdin and pseudobactin functioning as siderophore (Baharudin *et al.,* 2005). The siderophore developed rapidly covering the roots of plants and translocated Fe in the root zone.

Consequently, this condition supported the root growth, while, at the same time, the absence of ion Fe would hamper the growth of pathogen. The absence of pathogen caused the pathogenic infection on the plant to decrease, which also led to the lack of *R. solanacearum* population (Table 1).

The close correlation between *R. solanacearum* population and the attack level of wilt disease indicated that there was polynomial increase of *R. solanacearum* population with the increase of wilt disease attack level resulting from the increasing population of *R. solanacearum* (Figure 4), which is in line with the research conducted by Soesanto *et al*. (2011) and Hasanah *et al*. (2013).

Potato Yield and Growth

The potato plant treated with the application of *Trichoderma viride*, *Streptomyces sp*. and *Pseudomonas fluorescens* given either singly or in combination gave the various height of plants (41.16-47.39 cm) (Table 2). The application of antagonistic microbe *Trichoderma viride* caused the plant to grow taller due to the ability of *T. viride* to stimulate the plant growth by excreting growth hormone such as auxin and cytokine (Glick, 1995).

The plant treated with the application of antagonistic microbes given either singly or in combination was capable of giving a lot more of leaves than that treated with no antagonistic microbes (Table 2). Fewer leaves were caused by the attack of *R. solanacearum*.

Tabel 1. Population of antagonistic microbes and *R. solanacearum* during harvest time

Antagonistic microbe application	Antagonistic microbes	Antagonistic microbial population ($x10^7$)	*R. solanacearum* population
Without antagonistic microbes		2.27 a	$2.02x10^9$ c
Trichoderma viride		5.10 c	$5.40x10^8$ b
Streptomyces sp.		4.50 bc	$3.45x10^8$ b
Pseudomonas fluorescens		3.80 b	$4.30x10^7$ a
T. viride+Streptomyces sp.	*T. viride*	2.10 c	$3.61x10^8$ b
	Streptomyces sp.	2.84	
T. viride+P. Fluorescens	*T. viride*	1.55 c	$4.71x10^8$ b
	P. fluorescens	3.75	
Streptomyces sp.+P. Fluorescens	*Streptomyces sp.*	3.67 d	$5.50x10^7$ a
	P. fluorescens	3.01	
T. viride+Streptomyces sp.+Pf	*T. viride*	1.67 d	$5.10 x10^7$ a
	Streptomyces sp.	2.43	
	P. fluorescens	3.35	
LSD 5%		**0.81**	**2.16**

Remarks: the numbers with the same letters of the same column indicate not significant difference according to LSD at α = 5%

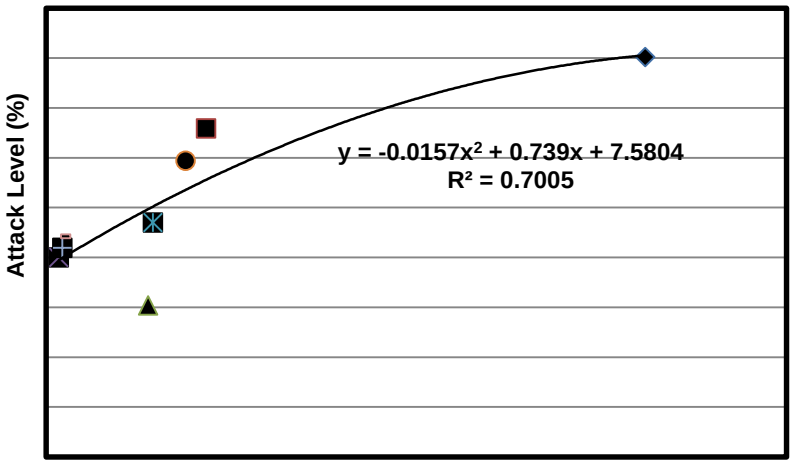

Figure 4. Correlation between *R. solanacearum* population and attack level

The application of *Pseudomonas fluorescens* given either singly or combined with *Streptomyces sp*. and in combination of *Trichoderma viride* + *Streptomyces sp*. was capable of giving more total and marketable weight of tuber than those treated with no antagonistic microbes (Figure 5). More number of leaves indicated the low attack level of wilt disease. More number of leaves in a certain volume would increase the weight of tubers per plant (Figure 6). The higher the attack of wilt disease means the lower the total weight of marketable yields (Figure 7). This result is linear with the research reported by Rosyidah (2010), where it was stated that the insignificant weight of the yield resulted from the water translocation which was hampered due to the disturbed xylem tissues.

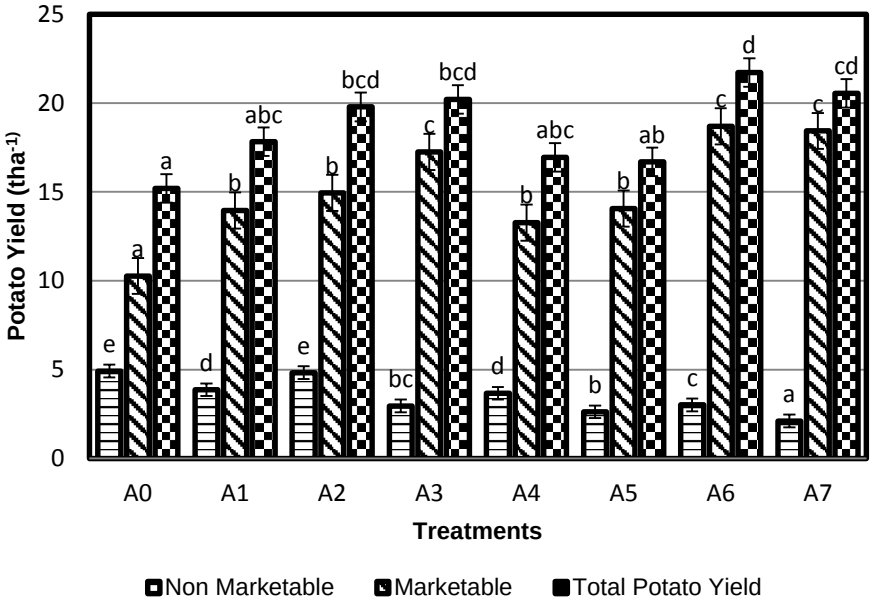

Figure 5. Potato yields in harvesting time

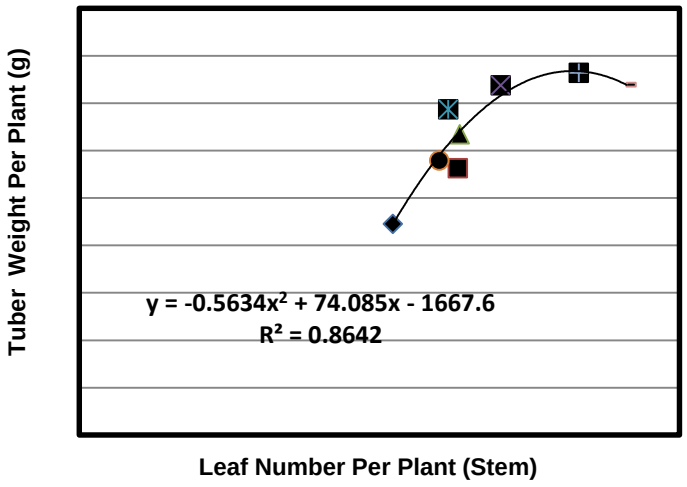

Figure 6. Correlation between leaf number per plant and tuber weight per plant

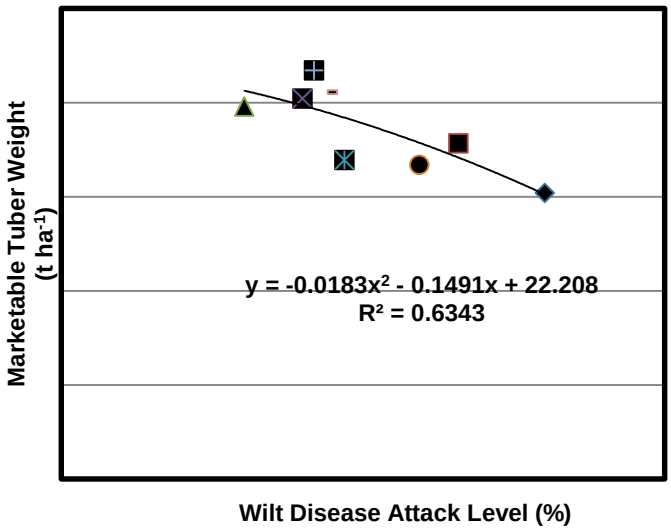

Figure 7. Correlation between wilt disease attack level and marketable tuber weight

Table 2. Height of plant and numbers of leaves in potato 56 days after planting

Antagonistic microbe application	Plant height (cm)	Leaf number (stem)
Without antagonistic microbes	40.72 a	41.80 a
T. viride	47.39 c	50.48 ab
Streptomyces sp.	41.99 ab	50.52 ab
P.fluorescens	42.62 ab	49.20 a
T. viride+Streptomyces sp.	41.61 a	66.9 c
T. viride+P.fluorescens	41.16 a	47.98 a
Streptomyces sp.+P.fluorescens	42.09 ab	68.40 c
T.viride+Streptomyces sp..+Pf	45.27 bc	72.97 c
LSD 5%	4.16	16.39

Remarks: the numbers with the same letters of the same column indicate not significant difference based on LSD at α = 5%

The increasing yields of potato tuber treated with *Pseudomonas fluorescens* given either singly or combined with *Stretomyces sp*. and in combination of *Trichoderma viride* + *Pseudomonas fluorescens* could be correlated with the indirect influence of *Pseudomonas fluorescens* activity in term of producing growth hormone capable of stimulating root growth (Campbell, 1989).

This result is also linear with that reported by Weller (1988) stating that *Pseudomonas fluorescents* applied on potato seeds was able to increase the potato and redish yields by 5-33% and 60-144%, respectively. Moreover, Arwiyanto (1998) confirmed that *Pseudomonas fluorescens* given in tobacco increased the production as much as 88-92%. *Streptomyces sp*. is capable of enhancing the plant growth and production by dissolving phosphat, nitrogen fixation (Thakuria *et al*., 2004), stimulating lateral growth of root and producing IAA growth hormone (Vasudevan *et al*., 2002 ; Vonderwell *et al*., 2001). *Trichoderma sp*. applied in potato was proven to be able to hamper the wilt disease by 100% and increase marketable tuber yield by 52.54% (Rosyidah, 2010).

CONCLUSIONS

All antagonistic microbes applied in this research were capable of inhibiting the attack level and the intensity of wilt disease by bacterium *R. solanacearum* on potato. Antagonistic microbes given singly were not always more effective than those given in combination.

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