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Induced resistance of Potato *(Solanum tuberosum* L.*)* toward *Ralstonia solanacearum* disease with combination of several bio-control microbes

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Abstract

The research aims to study the influence of bio-control microbes of Trichoderma viride., Streptomyces sp. and *Pseudomonas fluorescens* on the resistence of potato crop toward *Ralstonia solanacearum* disease and to get a combination of the best bio-control microbes to increase potato growth and yield. The research was conducted on February to June 2013 at the screen house of School of Agriculture of Unisma. As the research treatment, the selected isolates of *Trichoderma viride, Streptomyces sp*., and *Pseudomonas fluorescens* were applied as a single treatment or in combination. The tested treatments were arranged in completely randomized block design (RAK) with nine treatments and three repetitions. Research result showed that treatment of application with *Pseudomonas fluorescens,* either as single treatment or in combination with *Streptomyces sp.* and *Trichoderma viride* + *Streptomyces sp.*, has potential to induce resistance toward *Ralstonia solanacearum* disease on potato. It is showed by its ability to extend the incubation period of 11 – 13 days, reduce the disease incidence of 72.01 – 76.55%, reduce population density of *R. solanacearum* of 50.79% - 57.81%, increase the total phenol of crop's leaves, increase crop's length 7.09 – 12.49% and increase tuber fresh weight of 149.29 – 150.27%.

Keywords: induced resistance, *Solanum tuberosum* L., *Ralstonia solanacearum,* bio-control microbes

1. Introduction

Indonesia still has relatively low and unstable potato productivity. The productivity ranges around 13-17 to ha^{-1} of the potential of 30 ton ha^{-1} (Ridwan, 2010). One of the causes of the low potato productivity in Indonesia is bacterial wilt caused by *Ralstonia solanacearum*. The disease has caused production loss of 43 – 80% (Nurbaya, 2011; Sulistyo *et al*., 2012; Rosyidah *et al*., 2013).

Bactericidal spray to overcome the disease is very dangerous for the health of potato tuber product, the environment and farmer's wealth. It is reported that the use of pesticide to overcome the disease has been effective; however, besides expensive, it raises problems related to farmer's wealth level, product health and environmental pollution. *Trichoderma viride, Streptomyces sp.* and *Pseudomonas fluorescens* are antagonistic microbes potential in controlling *Ralstonia solanacearum* as reported by Heru, 2006; Hersanti *et al.,* 2009; Nurjajani, 2011; 2013; Rosyidah *et al*., 2013.

Resistance induce mechanism, generally, is characterized by the increase of chemical compound formation able to prevent pathogen's growth and development. The compound can be *Pathogenesis-Related Proteins*, secondary metabolites in form of alkaloid, phenol, flavonida , glycoside, phytoalexin compounds and so on (Chairul, 2003). A resistant plant has higher concentration on those chemical compounds (Agrios, 2005). Applying antagonistic fungus and bacteria in combination is one of strategies to increase plant' resistance through ISR (*Induced Systemic Resistance*) and there are not many researches on this strategy.

The research aims to study the influence of bio-control microbes *T. viride., Streptomyces sp.* and *P. fluorescens* on the resistance of potato crop toward *Ralstonia solanacearum* disease and to get a combination of the best bio-control microbes to increase potato growth and yield.

2. Material and Method

The research was conducted at the Screen House of School of Agriculture, Malang Islamic University on February to June 2013. The site is located on 460 m above sea level and having clay soil texture. In this study, the variety of the potato used was DTO-28, a variety that resistance to be planted in a medium land (Wardiyati,

1990). The bio-control microbes of *T.viride, Streptomyces sp.,* and *P. fluorescens* obtained from the collection of School of Natural Sciences and Mathematic (MIPA) and School of Agriculture of Brawijaya University, Malang. The microbes have been selected and in-vitro tested on their antagonistic ability using pathogen *R. solanacearum* at the bacteria laboratory Department of Pest and Disease, Brawijaya University.

2.1 Preparation of Antagonistic Microbes and R. *solanacearum* Pathogen

T. viride isolate was growth in PDA (Potato Dextrose Agar) medium, whereas *Streptomyces sp.,* and *P. fluorescens* were growth in Kings B medium with temperature of 30^0C for 48 hours. Once the pure culture is obtained, each was propagated in PDB (Potato Dextrose Broth) medium for *T. viride* and *Streptomyces sp.,* and in NB (Nutrient Broth) medium for *P. fluorescens* and put into shaker for 24 hours. The available culture was suspended to reach concentration of 10^8 cfu.mL^{-1} for *Streptomyces sp.* and *P. fluorescens* (Nurbaya *et al*., 2011) and 10^7 spora.mL^{-1} for *T. viride*.

The *R. solanacearum* isolate used was the result of potato crop isolation in Ngantang attacked by *R. solanacearum*. Purification isolation and propagation of *R. solanacearum* was conducted using TZC (2,3,5-triphenyl tetrazolium chloride) medium. Population density for the inoculation is $2.91x10^8$ cfu.mL^{-1} measured with spectrophotometer at OD 600.

2.2 Experimental Implementation

The research was an experiment research using completely randomized block design (RAK) with nine treatments and three repetitions. The treatments were: A0= control positive (without pathogen *R.solanacearum* and bio-control microbes), A1= control negative (*R.solanacearum* without bio-control microbes), A2= *T. viride,* A3= *Streptomyces sp.*, A4= *P.fluorescens*, A5= *T. viride* + *Streptomyces sp.*, A6= *T. viride* + *P. fluorescens,* A7= *Streptomyces sp.* + *P. fluorescens* and A8= *T. viride* + *Streptomyces sp.*+ *P.fluorescens.*

The planting medium was soil: sand: organic matter of chicken feces with comparison of 2:1:1 and has been sterilized using thermal steam at 50-70^{0}C for five hours. Planting medium of 4 kg was filled into a polybag and added with NPK (Nitrogen, Phosphor and Potassium) inorganic fertilizer (1:1:1) of 6 gram per polybag. At two weeks before planting, a 20 ml antagonistic microbe was sprayed on the planting medium (Sulistyo *et al.,* 2012). The second application of antagonistic microbes was conducted at 14 days after planting. Pathogen *R. solanacearum* was infected to the crop's root when the age of the crop is 25 days by wounding the root using sterile needle. *R. solanacearum* suspension of 20 ml was applied to each plant. The crop was nurtured intensively and spraying is conducted using watering can.

Observation was conducted on the following response: incubation period, total phenol (Folin-Denis), population of final pathogenic bacteria, antagonistic effectiveness, and disease incidence calculated using the following formulation based on Sinaga (2006):

$$KP = n/N \times 100\% \quad (1)$$

KP = disease incidence (%)

n = number of wilt plant

N = number of crop observed

In order to find out the level of crop's resistance toward the application of tested antagonistic microbes, the percentage value is converted into the degree of resistance according to Thaveecha et al (1989) in Table 1.

Table 1. The Degree of Resistance according to Thaveecha *et al.* (1989)

Percentage of Disease Incidence (%)	Level of Resistance
0 - 20	Resistance
21 – 40	Fairly Resistance
41 – 60	Fairly Susceptible
61 – 100	Susceptible

Growth component observed was crop's height and yield component observed was fresh weight of potato tuber at harvest time.

2.3 Data Analysis

The data was analyzed by using analysys 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.

3. Result and Discussion

3.1 The Content of Total Phenol Compound

Total phenol compound test shows varied results. The increase of phenol is observed in treatment with bio-control microbes and pathogen *R. solanacearum*. Potato crops treated with bio-control microbe of *T. viride, Streptomyces sp.,* and *P. fluorescens*, either as single treatment or in combination with pathogen *R. solanacearum,* shows significant difference ($p < 0.05$) on the number of total phenol compare to treatment of A0 control positive, without pathogen *R. solanacearum* and antagonistic microbe (Figure 1). This increase is assumed to be related to the application of bio-control microbes. The application of bio-control microbe is able to increase biochemical resistance of potato crop toward bacterial wilt disease of *R. solanacearum*. Phenol compound is one of secondary metabolites with biological function as antioxidant and anti-bacterial activities. This compound functions as a protection mechanism for plant toward predation by insect, herbivore and microorganism (Williams and Harborne, 1989; Cowan, 1999; Beckman, 2000).

The formation of chemical compound in form of secondary metabolite such as alkaloid, flavonoid, glycoside, phytoalexin, and phenol is one of plant's mechanisms to avoid pathogen attack. Phenol compound is able to prevent pathogen's growth and development (Hammerschmidt and Dann, 2000; Chairul, 2003; Vallad and Goodman, 2004). Further, it is stated that secondary metabolic compound is toxic in nature.

In treatment A1 (control negative, application of pathogen *R. solanacearum* without bio-control microbe) an increase in phenol compound is also observed compare to treatment A0 (control positive, without the application of pathogen *R. solanacearum* and without bio-control microbe). It is happened as crop reaction toward infection of pathogen *R. solanacearum* and root wounding before pathogen inoculation. Pieterse *et. al*. (2009) states that an increase in plant resistance through SAR (Systemic Acquired Resistance) is happened after local pathogen infection on plant; this infected plant then activate gens play role in resistance by producing chemical compound to protect the plant, such as salicylic acid. Once the plant has the resistance, it will be able to defend itself when another pathogen attacks; therefore, the pathogen cannot develop. According to Goodman et. al., (1986) plant's tissue infected by pathogen shows change on metabolic pattern, among others is the activation of peroxide enzyme and other phenol oxidase. The similar is stated by Marten *et al.,* (1995) and De Ascensao et.al. (2003) that a big phenol synthesis will occur if plant is attacked by pathogen. Agrios (2005) states that pathogenic microorganism causing mechanical and chemical damage will stimulate plant to produce toxic compound toward pathogen (phytoalexin).

The mechanism of bio-control microbe in inducing resistance is by colonizing plant's tissue; thus, stimulate the plant to increase the production of metabolite compounds play role in plant's resistance, such as peroxide enzyme, polyphenol oxidase, increase chitinase, β - 1,3 glucanase, pathogenesis related protein and phytoalexin activities (Press *et al*., 1977). Plants need peroxidase enzyme to produce defensive compounds such as lignin, chitin and various cell wall constitutive compounds (Hallman, 2001). Moreover, Bruce et.al (1989) states that peroxidase is another component of plant's initial response toward pathogen attack and plays key role in lignin biosynthesis that limit pathogen spread. This enzyme product has anti-microbe and anti-virus activities (Van Loon and Callow, 1983).

The result of this research is similar to Harni *et al*., (2011) testing on the mechanism of bacteria *P. putida* on patchouli that able to induce plant resistance by increasing the level of salicylic acid, peroxidase and phenol. The same result also proved by Soesanto (2008) using *P. fluorescens* P 60 and *P. fluorescens* P 32 potential in inducing resistance toward fusarium wilt disease on banana seedling indicated by the increase of phenol compounds content, which are glycoside, saponin and tannin. Moreover, M. Karthikeyan *et al*., (2006) states that the application of bio-control agent combined with *P. flueorescens, T. viride* and chitin is able to increase phenol accumulation and PAL (phenilalanin ammonia liase), PO (peroxidase), PPO (polyphenol oxidase), chitinase and β-1,3 glukanase activities on Cocos *nicifera* L compare to other treatments and control.

3.2 Pathosystem Component

Based on research result, it is known that a test by applying bio-control microbe gives significant influence ($p < 0.05$) on pathosystem component consists of incubation period, population density of final *R. solanacearum*, antagonistic effectiveness, disease incidence and resistance criteria.

Table 2 shows that the application of tested bio-control microbe indicates a longer incubation period compare to treatment without application of bio-control microbe. The symptom of *R. solanacearum* attack appears between 12 – 25 days after pathogen inoculation. The application of bio-control microbe is able to slow down the time needed by the pathogen to infect the plant since penetration until the occurrence of wilt symptom on leaves of young potato crop. According to Samanhudi (2009), this incubation period is influenced by several factors, host plant, environment and pathogen. The incubation period of pathogenic bacteria *R. solanacearum* is depend on virulence power, applied nutrient and plant resistance.

Research result shows that treatment by applying bio-control microbe *Streptomyces sp.* and *P. fluorescens* tends to have longer incubation period even though it is not significantly different to treatment by applying *P. fluorescens* , *T. viride* + *Streptomyces sp.*+ *P.fluorescens* and *Streptomyces sp.* It is assumed that it is related to phenol compound produced by the plant. There is a relationship between incubation period and total phenol with R^2 of 0.814 (Figure 2). The research also obtains relationship pattern between incubation period and disease incidence with R^2 of 0.909 (Figure 3). The application of bio-control microbe *Streptomyces sp.* and *P. fluorescens*, either as a single treatment or in combination, is better in controlling pathogen *R. sonacearum* attack than treatment without application of bio-control microbe; therefore, the incidence of wilt disease is low. The application of *Sreptomyces sp.* and *P. fluorescens*, single or in combination, is able to decrease disease incidence of 72.01% – 76.55%.

Observation on the final population density of pathogen *R. solanacearum* indicates that potato crop treated with *P. fluorescens*, as a single treatment or in combination, is able to reduce the number of final population of pathogen *R. solanacearum* of 98.43% compare to other treatments. It is an indication that the application of *P. fluorescens* is able to reduce final population of pathogen. This result is similar to Rosyidah (2013). Oka (1993) states that developmental epidemiology of a disease, including *R. solanacearum*, is determined by the number of initial inoculum and infection rate in a time unit. Initial density of inoculated pathogen *R. solanacearum* is 2.91 x 10^8 cfu mL^{-1}, whereas the average final population of pathogen *R. solanacearum* in treatment with application of *Pseudomonas fluorescens is* 4.57 x 10^6 cfu mL^{-1}. The difference in pathogen population is due to the competition with antagonist; therefore, pathogen unable to maximize control to the existence space and nutrient. Pathogenic bacteria *R. solanacearum* lives side by side with antagonistic bacteria in soil causing poor nutrient availability in the environment and the existence of toxic metabolite. The result is that pathogenic bacteria are unable to breed well (Agrios, 2005). Furthermore, it is states that when the population of bio-control microbes decrease during harvest time means that their role to control disease is effective.

P. fluorescens ability to suppress pathogen population can be associated with its ability to protect root from pathogen *R. solanacearum* infection by colonizing root surface, producing chemical compounds such as anti-fungus, antibiotic, and competing with pathogen in absorbing Fe cation. According to Goto (1992), *P. fluorescens* produce secondary metabolic of *pyroverdin* or *pseudobactin* functions as siderophore. The siderophore develops quickly covering plant root and move Fe into plant root zone causing ion Fe deficiency for pathogen and impede its development. This fact has caused reduce in *R. solancearum* population and increase in *P. fluorescens* population.

The application of bio-control microbe *Streptmyces sp*. and *P. fluorescens* as a singe treatment or in combination tends to show high effectiveness compare to other treatments, which is 68.92% - 79.99%. This *Streptmyces sp*. and *P. fluorescens* ability is assumed to be related to their ability to produce defensive compound such as lignin, chitin, suberin and various cell wall constitutive compounds (Hallman, 2001; Maryani and Kasiamdari, 2004).

3.3 Growth and Yield Components

Result from the observation of plant growth at 61 days after planting and tuber fresh weight per plant at harvest time is presented in Table 3. The table shows that treatment with application of bio-control microbe *T. viride, Streptomyces sp.* and *P. flurescens* as a single treatment or in combination tends to increase plant height. It is due to the application of bio-control microbe that able to increase organic matter decomposition and mineralization activities. Fungus, bacteria and actinomycetes are some microorganism play role in organic matter decomposition as well as some soil fauna. Soil microflora and microfauna interacts each other with their

requirement on organic matter because organic matter provides carbon as energy source. The result of organic matter decomposition process by microorganism is nutrient discharge contained within the organic matter. Metabolism process in a plant occurs normally if there is sufficient nitrogen available for plant growth. This, in turn, will influence photosynthesis and respiration processes (Evans and Farquar, 1991). The application of bio-control microbe contributes to the increase of essential nutrient element absorption to help plant growth. In addition, the increase of plant growth occurs due to the increase of available nutrient and smooth nutrient absorption.

T. viride is able to decompose lignin, selulose and chitin from organic matter into nutrient available for plant absorption (Suryanti et. al., 2003). *T. viride* capable to increase plant growth as stated by Bentez *et al*., (2004) that *Trichoderma sp*. is able to colonize root; thus, stimulate plant growth and increase resistance toward pathogen infection. As bio-fertilizer, *Trichoderma sp.* capable to live in plant roots; thus, it is able to increase root growth, yield, resistance toward environmental stress and nutrient absorption.

It is assumed that the role of *Streptomyces sp.* in increasing plant growth is due to its ability to produce auxin *indole-3-acetid acid* (IAA) functioned in stimulating plant growth. IAA is auxin resulted by microbe. It is useful in the soil and is estimated as one of mechanism in plant growth promoting rhizobacteria (Soesanto, 2008).

In addition to its ability to suppress plant disease, *P. flurescens* also plays role in accelerating plant growth by dissolving phosphate, capable in stimulating root system growth and impeding harmful fungus and bacteria (Weller, 1988). The statement is similar to Azizah (2009) stating that *P. fluorescens* gives positive influence to tested plant growth by increasing plant height. Another ability of *P. fluorescens* is the ability to produce growth hormone IAA (3-indol acetic acid). IAA influences plant cell division, differentiation, stimulates seed germination and tuber; increases root development, controls plant's vegetative growth and increase resistance toward stress condition (Tsavkelova *et al*., 2006; Spaepen *et al*., 2011).

Treatment with application of antagonistic microbe *P. flurescens*, either as a single treatment or in combination with *Streptomyces sp*. and with *T. viride* + *Streptomyces sp*., resulting in the biggest tuber fresh weight at harvest time and it is significantly different ($p < 0.05$) to other treatments (Table 3). The higher the level of wilt disease incidence, the lower the weight of the resulted tuber is. This is similar to Rosydah *et.al.* (2013) reporting that marketable potato tuber formed at harvest time is increase at 67.96-81.98% compare to the one without antagonistic microbe application.

The increase of this potato tuber yield in those treatments can be related to the indirect influence of *P. fluorescens* activities to produce growth hormone that stimulate root growth (Cambell, 1989) and its ability to synthesize hydrogen cyanide and anti-biotic. This result also similar to the one reported by Weller (1988) that the application of *P. fluorescens* on potato seedling is able to increase yield of 5-33% and 60-144% on radish. Arwiyanto (1998) also reports that the application of *P. fluorescens* on tobacco is able to increase production of 88-92%. *Streptomyces* sp capable in increasing plant growth and yield with its ability to dissolve phosphate, nitrogen fixation (Thakuria *et. al.,* 2004), stimulate lateral roots growth and produce growth hormone IAA (Vasudevan *et al*., 2002; Vanderwell *et al*., 2001). *Trichoderma sp.* applied to potato crop is proven to reduce wilt plant of 100% and increase marketable tuber yield of 52.54% (Sulistyo *et.al*., 2012).

4. Conclusion

1. The application of *P. fluorescens* as a single treatment or in combination with *Streptomyces sp.* and *T. viride* + *Streptomyces sp.* potential to induce resistance toward *R.* disease on potato crops indicated by the increase of total phenol.
2. The application of *P. fluroscens* as a single treatment or in combination with *Streptomyces sp.* and *T. viride* + *Streptomyces sp.* has a tendency to increase plant height of 7.09 – 12.49% and tuber fresh weight of 149.29 – 150.27%.

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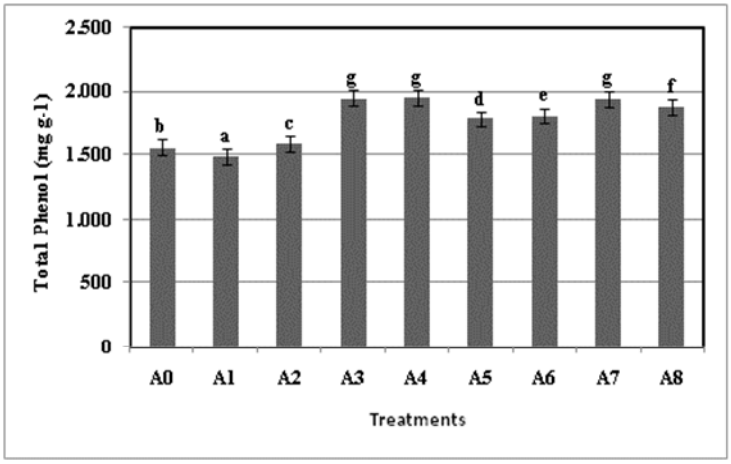

Figure 1. Total phenol in microbial testing various biological control treatments. A0 = positive control (without *R.solanacearum* and microbial biological control), A1 = negative control (*R.solanacearum* without microbial biological control), A2 = *T.viride*, A3 = *Streptomyces sp*., A4 = *P.fluorescens*, A5 = *T . viride* + *Streptomyces sp.*, A6 = *T. viride* + *P. fluorescens*, A7 = *Streptomyces sp*. + *P.fluorescens* and A8 = *T. viride* + *Streptomyces sp.* + *P. fluorescens.*

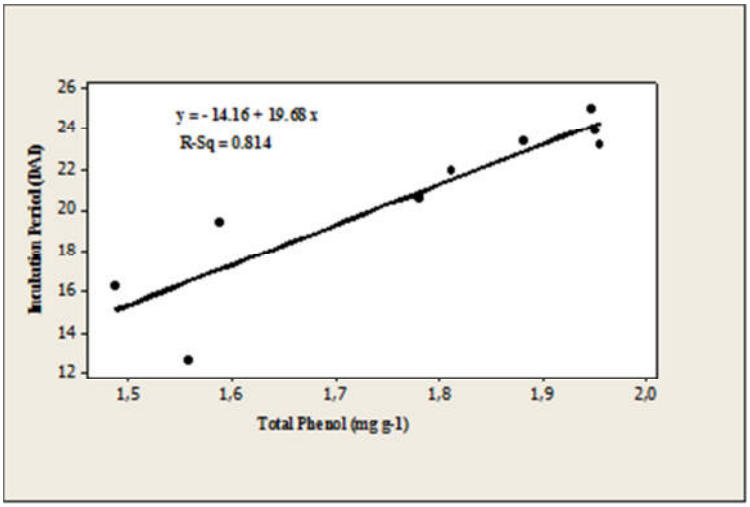

Figure 2. Correlation between total phenol and incubation period

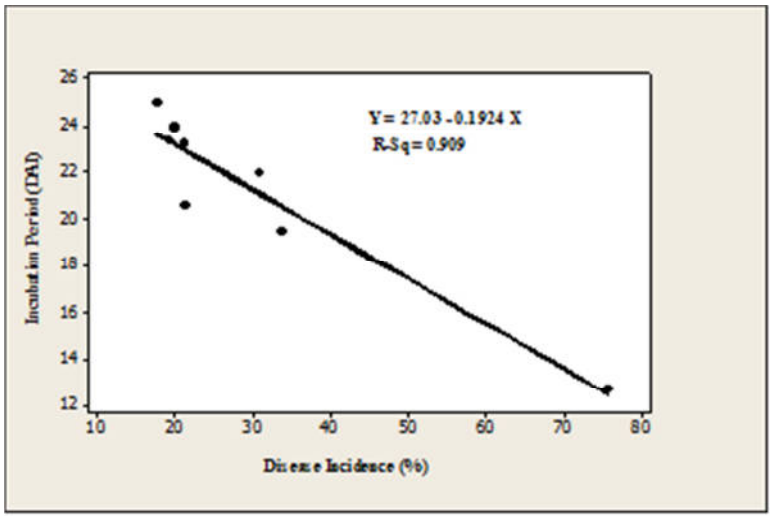

Figure 3. Correlation between disease incidence and incubation period

Table 2. Effect of microbial biological control application of the components pathosystem

Microbial biological control application	Incubation period (days)	*R.solanacearum* population ($x10^6$)	The effectiveness of antagonist (%)	Disease incidence (%)	Level of resistance
without microbial biological control	12.69 a	10.127 e		75.67 c	S
T.viride	19.45 b	8.277 d	56.79 a	33.75 b	FR
Streptomyces sp.	23.92 de	5.080 a	75.99 bc	19.87 ab	R
P.fluorescens	23.17 cde	4.983 a	68.92 abc	21.18 ab	FR
T.viride + Streptomyces sp.	20.61 bc	6.080 b	70.39 abc	21.32 ab	FR
T. viride + P. fluorescens	21.94 bcd	7.077 c	57.73 ab	30.86 ab	FR
Streptomyces sp. + P.fluorescens	25.00 e	4.273 a	79.99 c	17.67 a	R
T. viride + Streptomyces sp.+ Pf	23.39 de	4.470 a	77.59 c	19.23 ab	R
LSD 5%	2.70	0.898	19.16	13.99	

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

R = resistance, FR = Fairly Resistance, S = Susceptible

Table 3. Effect of microbial biological control application of height of plant and tuber fresh weight per plant

Microbial biological control application	Plant height (cm)	Tuber fresh weight per plant (g tan^{-1})
Positive control	44.27 ab	100.30 ab
Negative control	42.53 a	91.10 a
T.viride	55.83 e	126.10 cd
Streptomyces sp.	46.23 abc	118.83 bc
P.fluorescens	53.70 de	216.37 e
T.viride + Streptomyces sp.	49.40 bcd	141.57 d
T. viride + P. fluorescens	47.53 abcd	140.40 d
Streptomyces sp. + P.fluorescens	49.40 bcde	227.10 e
T. viride + Streptomyces sp.+ P. fluorescens	51.47 cde	230.73 e
LSD 5%	6.23	20.61

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