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ABSTRACT

The research objective was to know ability of antagonistic microbes to control bacterial wilt on potato in the field. This research was carried out at Serang Village, Karangreja Subdistrict, Purbalingga Regency from June up to August 2012. The antagonist, originally isolated from potato field, was Bacillus sp. B2 and B4, and Pseudomonas sp. P19 and P20. Based on the research result, Pseudomonas P19 could control the disease on potato with delaying incubation period of 78.95%, suppressing disease intensity of 51.57%, decreasing final pathogenic population of 99.74%, and inducing plant resistance with increasing saponin, tannin, and glycoside content. However, the antagonist could not increase growth and yield of potato.

Keywords: antagonistic microbes, bacterial wilt, potato

INTRODUCTION

Potato is one of the preferred horticultural society of Indonesia, while in some countries potato is consumed as staple food. National potato production in 2010 reached 1,060,805 tons and 66,531 ha (BPS, 2012) in harvest areas. To 2011, Central Java had been one of production centers with the most extensive potato growing areas in Indonesia, which is 16 585 ha with production reaching 250,404 tons (BPS, 2012). Wonosobo district is one of the major centers of the potato crop in Central Java, an area of 3,088 ha and crop production reaching 467,977 tons (BPS Central Java, 2012).

Efforts to increase potato production in Indonesia face many obstacles where plant diseases are present as one of the obstacles. Potato plant disease considered very harmful is bacterial wilt (*Ralstonia solanacearum*) (Champoiseau *et al*., 2009). Plants attacked by this pathogen will languish. In severe attacks, the whole parts of plants get will get rotten. Priou *et al*. (2011) reported that the bacterial wilt could cause yield losses up to 40%.

Control of the disease relies more on synthetic chemical pesticides (Semangun, 2000). The use of such a tactless manner is known to have many negative impacts on environment and humans. Therefore, it is necessary to find other control measures that are effective but environmentally friendly. The use of microbial biopesticides based on antagonists have a high potential because microbes are able to survive in the soil so that their efficacy is sustainable and their multiplication and formulation are easier.

Microbial antagonists that have potential for biological control of plant pathogens have been reported by several researchers, including fluorescent Pseudomonas group, *Gliocladium* sp., *Trichoderma* spp., *Paecilomyces lilacinus*, *Verticilium* spp., *Metarrhizium anisopliae*, *Beauvaria bassiana*, and *Bacillus* sp. (Handayati, 2004; Soesanto, 2004; Santoso *et al*., 2007; Soesanto *et al*., 2005; Hastopo *et al*, 2008). Furthermore, Soesanto *et al*. (2011a) reported the successful isolation of microbial isolates antagonistic *Bacillus* sp 2 and 4, *Pseudomonas fluorescens* isolates 19, 20, and 21, *Gliocladium* sp isolates 1 and 3 and *Penicillium* sp isolates 1 and 2 had been tested *in vitro* for their ability to control the fungus *F. oxysporum*, *R. Solanacearum*, and *Globodera rostochiensis*. Test results show that the *in-planta Bacillus* sp isolates 2 and 4 as well as *Pseudomonas fluorescens* isolates 19 and 20 had a high potential in controlling bacterial wilt of potatoes. This study aims to determine the ability of microbial antagonists to control bacterial wilt disease in potato plants in the field.

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MATERIALS AND METHODS

This research was conducted in the potato field in the Serang Village, Karangreja District, Purbalingga Regency (± 1200 m.asl) for 3 months starting from June to August 2012.

Propagation and Inoculation of *R. Solanacearum*

R. solanacearum pure cultures were aseptically transferred into a erlenmeyer flask containing Nutrient Broth, shaken in a shaker with a speed of 150 rpm for 3 days at room temperature. Inoculation was done by spraying 20 ml (density of 1.18×10^8) suspension of pathogens per planting hole.

Propagation and Treatment of The Antagonists

Pure cultures of *Pseudomonas* P19 and P20 and *Bacillus* sp B4 and B2 were aseptically transferred into Nutrient Broth for *Bacillus* and Kings B liquid for *Pseudomonas*, shaken in a shaker with a speed of 150 rpm for 3 days at room temperature. Density acquired for *Pseudomonas* P19 and P20 and *Bacillus* sp. B4 and B2 was 1.2×10^8, 1.4×10^8, 1.14×10^8, and 1.39×10^8 cfu/ml suspension, respectively. Application of antagonistic microbes was given by spraying 20 ml of suspension per planting hole along with the planting, and repeated 4 times after planting with frequency of 5 days.

Experimental Design

The trial used Randomized Block Design (RBD) with 6 treatments and 4 replications. The treatments tested were: control, *Pseudomonas* P19 and P20, *Bacillus* sp B4 and B2, and bactericide (a.i. streptomycin, 2 ml/l).

Planting and Maintenance of Crop

Land was cultivated by using hoes and cleared from weeds. Before planting, the land of organic fertilizers involved 5,000 kg/ha, Urea 200 kg/ha, SP36 200 kg/ha, and KCl 75 kg/ha. The spacing was 60x25 cm and 50 cm distance beds with one tuber per planting hole. Supplementary fertilizer was given 45 days after planting with a dose of 150 kg urea/ha, SP-36 250 kg/ha, and KCl 75 kg/ha. Plant maintenance was done by watering the plants when rain was not present, and weeding was done 2 times at 3 and 6 weeks.

Observed Variables and Observations

The variables observed in this study include: (1) incubation period, (2) the disease intensity, where the observations were done every day by calculating the intensity of pathogen attack occurring, (3) the density of the pathogen and final antagonist, where the density calculation was done by taking a sample of 1 g of soil diluted and grown in CPG medium-TTC for measurements of *R. solanacearum*, while *P. flourescens* was grown in medium Kings B, the density of *Bacillus* sp. was done in a soil sample oven for 10 minutes at 80^0C, diluted and then grown in a medium Nutrient Broth. The density of the pathogen and antagonists performed colony forming units (cfu) per gram of soil. (4) Components of plant growth (height, number of leaves, plant dry weight), (5) potato yield (tuber number and weight), (6) phenol content in plants (saponins, tannins and glycosides), content of phenolic compounds were analyzed qualitatively using Chairul (2003) modified as followed.

Glycosides were tested by Keller Kiliani reagent. Testing was done by extracting 10 g of plant material (roots and stems) with 80% ethanol, then filtered using filter paper and dried in a water bath. Fat was removed by washing using hexane until the pigment was lost or colorless hexane solution. Further residue heated over a water bath to remove residual hexane. After the rest of hexane was removed, 3 ml of $FeCl_3$ reagent was added, stirred and transferred into a test tube. One ml of concentrated sulfuric acid was dripped through the tube wall. The mixture was allowed a few moments until it changed in color. The color change indicated a positive reaction to 2-deoxy-sugar.

Tannin was identified with $FeCl_3$ reagent. Testing was done by extracting 10 g of plant material (roots and stems) with 80% ethanol, filtered and dried over a fire bath. The residue was dissolved in 20 ml of hot water. Further extracts were coupled with 5 drops of 1% NaCl solution. Reagent test with ferric chloride ($FeCl_3$) was done by adding 3 drops of reagent into the $FeCl_3$ extract. Hydrolyzed tannins would give you blue black, while the tannin condensation gave blue green, then compared with the controls.

Saponin test was performed using a test foam or froth (froth the test). *Sapindus rarak* was prepared as a control; approximately 1 g of

Sapindus rarak was diluted in 10 ml of 80% ethanol, 2 ml of the extract and then inserted into a test tube. Testing was done by extracting 2 g of plant material (roots and stems) with 80% ethanol and put into a test tube. Each tube was added with 10 ml of water, covered, shaken strongly for 30 seconds, and allowed to stand for 30 minutes. In the event of froth or foam on the surface of the solution, a positive means of plant material containing saponins. Plant material that produced little foam or froth remained stable and showed the presence of hard acid - free fatty acids, and then compared with the controls.

RESULTS AND DISCUSSION

The results of testing the use of microbial antagonists for the control of wilt disease in potato caused by *R. solanacearum* can be seen in Table 1. Based on the statistical analysis, the treatment had a significant effect on the incubation period (the time shown symptoms) of bacterial wilt disease on potato. The incubation period after inoculation pathogen was calculated until the emergence of the early symptoms of the disease. Early symptoms of bacterial wilt was characterized by the wilting petiole especially on a hot day, and the subsequent development of symptoms experienced wilting leaves and stems, which starts from the top of the plant. This is in accordance with the opinion shared by Semangun (2000) stating that wilt occurs in young leaves or yellowing of older leaves. If the trunk, branches, or petiole vascular bundle are cut, they will look brown and from the cut location will come out the mass of bacteria like milky white mucus.

Based on the observations (Table 1), the control treatment showed the most rapid incubation period was 28.25 days after inoculation (dai). The lack of control on the control effort resulted in rapid pathogen into the plant and caused disease symptoms. Treatment in the control treatment was not significantly different from the bacteria *Bacillus* sp B2 and B4 and *P. fluorescens* P20 with an incubation period of between 34.75 and 35.75 dai.

Pseudomonas fluorescens P19 was the best antagonistic microbes capable of delaying the incubation period (50.75 dai); they have delayed incubation period by 22.50 days (78.95%) when compared with controls. This result is also better when compared with the use of bactericide which was only able to delay the incubation period by 14.97 days (52.99%). Delays expected incubation period associated with the presence of bacterial antagonists that could be a contender for the bacterium *R. solanacearum* in attacking the plant.

Table 1. The incubation period, the intensity of wilt disease, the final density of *R. solanacearum* and the final density of bacterial antagonists on potato

Treatments	Incubation period (dai)	Disease intensity (%)	Final density of *R. solanacearum* (cfu/g soil)	Final density of antagonist bacteria (cfu/g soil)
Control	28.25 a	36.61 b	$7.50x10^{21}$	$1.72x10^{18}$
Bacillus sp B2	34.75 ab	42.23 b	$3.59x10^{20}$	$1.10x10^{20}$
Bacillus sp B4	35.75 ab	35.61 b	$1.05x10^{20}$	$2.77x10^{20}$
P. fluorescens P19	50.75 c	17.73 a	$1.90x10^{19}$	$8.30x10^{21}$
P. fluorescens P20	35.00 ab	36.47 b	$5.70x10^{19}$	$7.40x10^{21}$
Bactericide	43.25 bc	29.43 ab	$8.60x10^{19}$	$2.40x10^{17}$

Remarks: Numbers followed by the same letter in a column indicate no significant difference at 5% Duncan Multiple Range Test (DMRT)

Based on the intensity of the disease, the use of bacterial antagonists to suppress the intensity of the disease was compared with controls with no control. Control by *P. fluorescens* P19 is the best, which is not significantly different from control bactericide, with emphasis on the intensity of the disease, respectively, 51.57% and 19.61%. These results are consistent with research of Soesanto *et al.* (2011a), on the inhibition of bacterial antagonists against *R. solanacearum* that bacterial antagonist *P. fluorescens* P19 is able to inhibit bacterial growth *in vitro* and in the greenhouse test results suppressed wilt disease intensity bacterial wilt in potato crops by 79.6%.

Giving microbial antagonists with watering can also reduce late pathogen population of *R. solanacearum* by 98.60 to 99.74%. The lowest density in the treatment of bacterial antagonists was *P. fluorescens* P19 (1.90 X 10^{19} cfu/g soil or decreasing by 99.74%). These results are in line with the length of the incubation period and the low intensity of the disease in the treatment of bacterial antagonists. The low pathogenic bacteria were also allegedly associated with high density of bacterial antagonists end of treatment *P. fluorescens* P19 reaching 8.30 X 10^{21} cfu/g soil. This is presumably related to the ability of the bacteria to colonize potato plant roots and defend themselves from changes in environmental conditions. The high population of antagonistic bacteria in the soil will increase competition and increase the amount of antibiotics that can be generated, thereby increasing the ability of antagonists in controlling the disease.

Testing the phenol content qualitatively in plants (Table 2) note that the content of glycosides, saponins and tannins in the plants increased after treated with bacterial antagonists. This shows that bacterial antagonists, given around the potato plant roots able to induce plant resistance. Increasing the highest phenol content was shown in the treatment of *P. fluorescens* P19. Similar results were also presented by Soesanto *et al.* (2010, 2011a, 2011b), which states that the provision of treatment of bacterial antagonist *P. fluorescens* P60 was able to induce plant resistance.

The ability of bacterial antagonist *P. fluorescens* P19 in the press of incubation, bacterial wilt disease intensity, and the amount of the final pathogen was thought to be caused by several mechanisms that had antibiosis, siderophore, resilience affected, and nutrient competition. This is consistent with the results of the study Kloepper *et al.* (1980) and Soesanto *et al.* (2010, 2011b), indicating that bacterial antagonist *P. fluorescens* is capable of producing antibiotics, siderophore, and inducing plant resistance. *P. fluorescens* reported to produce antibiotics such as phenazine-1-carbocylic acid (P1C), HCN, and 2.4 diacethyl-phloroglucinol. Other antibiotic types shown to suppress fungal pathogens are pyoluteorin and pyrrolnitrin.

In the iron-deficient medium, siderophore is capable of binding the iron making it unavailable for pathogenic organisms (Alabouvette *et al.* 1996). According to Soesanto (2008), it has a role as fungistatic siderophore and bacteriostatic in low iron conditions. Some isolates of *P. fluorescens* reportedly produces chitinase enzymes, which may play a role in the control of plant pathogens (Kumar *et al.*, 2007; Shan-lang *et al.*, 2008; Soesanto *et al.*, 2011b). Further said by Soesanto *et al.* (2010), the bacterial antagonist *P. fluorescens* P60 is able to improve the content of phenolic compounds in the test plants in addition to suppressing Fusarium wilt disease components, inducing plant resistance and supporting plant growth.

Table 2. The content of phenolic compounds in the treatment of potato bacterial antagonists for control of bacterial wilt disease

Treatments	Glycoside	Saponin	Tannin
Control	+	++	+
Bacillus sp B2	++	++	+++
Bacillus sp B4	++	+++	+
P. fluorescens P19	+++	+++	+++
P. fluorescens P20	++	++	+++
Bactericide	++	++	++

Remarks : - = not contain phenol, containing phenol + = slight, + + = fairly containing phenol, + + + = many contain phenol

Table 3. Components of growth and yield of potato crop in the treatment of bacterial antagonists for control of bacterial wilt disease

Treatments	Crop height	Dry weight of crop	Dry weight of root	Root length	Tuber weight	Number of tubers
Control	34.48	9.35	0.84	15.35	186.78	11.57
Bacillus sp B2	37.80	9.78	1.00	16.38	192.55	13.05
Bacillus sp B4	36.69	10.71	0.85	15.61	226.20	12.22
P. fluorescens P19	36.25	9.89	1.12	15.88	230.19	13.28
P. fluorescens P20	33.96	10.50	1.24	16.62	220.09	13.35
Bactericide	34.55	9.88	0.99	16.45	231.52	11.73

The effect of treatment on the growth and yield components of potato plant height, plant dry weight, root dry weight, number and weight of potato tubers of potato tubers, showed no statistically significant difference (Table 3). All treatments were not able to increase the growth and yield of potato plants. This indicates that bacterial antagonists as Plant Growth Promoting Rhizo-bacteria (PGPR) were not optimal in spurring growth and yield. According to Soesanto (2008), PGPR applied in the field were not always optimal. The population density was high enough so that the bacterial antagonists could not be built in a short period of time to enhance the growth and yield of crops.

Nevertheless, several parameters such as the weight of tuber and treatment of bacterial antagonists tended to increase from 3 to 23.95% yield. Presence of other diseases such as late blight disease caused *Phythophthora infestans* reaching 58.69 to 83.08%, was also suspected to be the factor in fact not different antagonistic bacterial treatment effect on the growth and yield of potatoes. The existence of this disease caused the potato plant leaves to dry out, so it would disrupt the photosynthesis process in plants. The existence of this disease also caused the plants to be harvested before the time, at the age of 64 days after planting (dap), faster than it should (90 dap).

CONCLUSIONS

Pseudomonas fluorescens P19 to control bacterial wilt disease in potato plants was capable of delaying incubation with 78.95%, 51.57% suppressing disease intensity, lowering end of the pathogen population 99.74%, and inducing plant resistance by increasing the content of saponins, tannins, and glycosides. However, it was not able to increase the growth and yield of potato plants.

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