

Paper 2

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APPLICATIONS OF POTASSIUM FERTILIZER AND *Bacillus* sp. BIOPESTICIDE FOR INCREASING TOMATO RESISTANCE TO BACTERIAL WILT DISEASE

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ABSTRACT

Bacterial wilt on tomato caused by *Ralstonia solanacearum* is a crucial disease, because it can reduce yield until 50%. The aims of this research were: 1) to find out biopesticide formula for *Bacillus* sp. growth, 2) to test *Bacillus* sp. against *R. solanacearum* in vitro, 3) to test potassium fertilizer combined with *Bacillus* sp. for enhancing tomato resistance to the bacterial wilt disease. The research was conducted in 2 steps i.e to test the persistence of *Bacillus* sp. in biopesticide formula, and to test the best combination of both potassium and the *Bacillus* sp. biopesticide. The results showed that *Bacillus* B298 was the best isolate in its persistence on the biopesticide formula of organic growth medium+CaCO₃+CMC 1%+mannitol 1%, and in inhibiting *R. solanacearum*. The best biopesticide formula for the *Bacillus* sp. persistence was growth organic media+ CaCO₃+CMC 1%+mannitol 1%. *Bacillus* sp. was able to increase tomato resistance to the bacterial wilt disease from the category of susceptible to be tolerant and becoming resistant.

Keywords: tomato, *Ralstonia solanacearum*, potassium and *Bacillus* sp.

INTRODUCTION

Bacterial wilt disease caused by *Ralstonia solanacearum* is an important disease on tomato and other members of the family Solanaceae. Yield loss by this disease may reach 50%. Biocontrol can be carried out by using *Bacillus* sp., that has been explored from potato rhizosphere in the previous research. Five selected isolates have been tested to control the bacterial wilt disease on potato and tomato. *Bacillus* sp. was capable to suppress either the

disease on potato or tomato, and was able to quicken the maximum harvesting time on tomato (Prihatiningsih and Soedarmono, 2004; Prihatiningsih and Kustantinah, 2005).

Bacillus sp. is also known as stimulant in plant resistance. According to Choudhary and John (2008), it is called as the ISR (*Induced systemic resistance*). Increase in plant resistance may be stimulated by the pathogen infecting limitedly, the avirulen pathogen, and non pathogenic bacterium including that as an antagonist such as *Bacillus* sp. and certain chemical substances (van Loon, 2000; Agrios, 2005). Applications of *Bacillus* sp. combined with potassium fertilizer could enhance the plant resistance, because potassium was required in carbohydrate accumulation and translocation. K (potassium) element together with Cl, F, and Boron would move and be available in the xylem, so they strengthened stem tissues and the plant became more resistant to pathogen attack that its multiplication took place within the stem (Jones *et al.*, 1991). Furthermore, Aziz *et al.* (1992) said that K fertilization could decrease significantly the infection percentage of the bacterial late blight on rice and increased plant resistance from susceptible to be moderate resistant.

Recommendation of K fertilization on horticultural plants that is generally in the form of KCl or K₂O with the dose of 6 g/plant or 20 g/m² may increase growth and yield (Winarso, 2005). Potassium in plant is as organic salt, which is numerous found in plant parts which actively grow and it is useful as catalisator, especially in protein change becoming amino acid. Therefore, when a plant has sufficient protein, the protein will increase. This indicates that K supports in protein synthesis, carbohydrate formation, and breaking it off. Less potassium leads to photosynthesis inhibition and respiration

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increase. Supply of K also strengthen the stem tissues (Jones *et al.*, 1991). Therefore, the plant becomes more resistant to pathogen attack multiplying in the stem xylem tissues like *R. solanacearum*.

The aims of the research were 1) to find out the best biopesticide formula to evaluate the *Bacillus* sp. persistence in the formula, 2) to test the biopesticide formula containing *Bacillus* sp. as the biological agent in suppressing *R. solanacearum* *in vitro*, and 3) to test the combination of potassium fertilizer and *Bacillus* sp. biopesticide for controlling the bacterial wilt disease and to enhance the tomato plant resistance to the disease.

MATERIALS AND METHODS

The research was conducted at Kutasari Village Baturaden Sub District Banyumas Regency, from June to August 2008. Materials used in the research were tomato seeds of Mirah variety, *R. solanacearum* inoculum, medium of YPGA (yeast pepton glucose agar), CPG (Casamino acid, pepton glucose), TTC (triphenyl tetrazolium chloride), phosphate buffer +0,1%pepton, KOH 3%, hydrogen peroxida 3%, alcohol 70%, formula substances such as ultisol soil, organic growth medium, CMC (Carboxy Methyl Cellulose) as sticker and CaCO₃ as rectifier, and mannitol as carbon source, *Bacillus* sp. isolate, B46, B209, B211, B298 dan B315, potassium fertilizer (KCl).

The research was done in two steps, the first step was to test *Bacillus* sp. persistence in the biopesticide formula which was tried to obtain the best biopesticide formula, namely K: Control (*Bacillus* sp. isolate in sterilized water), F1: Formula 1 (ultisol soil+CaCO₃+CMC1%) + *Bacillus* sp., F2: Formula 2 (organic growth medium + CaCO₃ + CMC 1%) + *Bacillus* sp., F3: Formula 3 (ultisol soil+CaCO₃+CMC 1% + mannitol 1%) + *Bacillus* sp., F4: Formula 4 (organic growth medium + CaCO₃ + CMC 1% + mannitol 1%) + *Bacillus* sp. *Bacillus* sp. used comprised five isolates i.e.: B46, B209, B211, B298, dan B315, so there were 25 treatments replicated four times. The comparison among carrier materials (ultisol soil, organic growth medium):CaCO₃:CMC:mannitol was 10:5:1:1 (weight:volume). The volume of *Bacillus* sp. was 5 ml per treatment, prepared from the slant YPGA medium in a tube of two days and was

dissolved in 1,000 ml of sterilized water for each tube. The density was 10¹⁰ CFU/ml. Subsequently, it was conducted an inhibition test of the five *Bacillus* sp. isolates taken from the formula to *R. solanacearum* *in vitro*, by growing them in double layer in a petridish containing the YPGA medium. The second step was a combination test of potassium fertilizer and the biopesticide formula in enhancing the tomato plant resistance to the bacterial wilt disease. The treatment in this step was the best isolate gained from the first step research, namely the isolate B298. This result was seen from the average population during observations at week 2, 4, 8, and 12. Therefore, the treatments were arranged as follows:

- K: Control (without *Bacillus* sp. B298 biopesticide, inoculated with *R. solanacearum*).
- F20: (without *Bacillus* sp. B298 biopesticide and without *R. solanacearum* inoculation)
- F21: Biopesticide formula (ultisol soil + CaCO₃ + CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + *R. Solanacearum* inoculation.
- F22: Biopesticide formula (organic growth medium + CaCO₃ + CMC 1% + mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + *R.solanacearum* inoculation.
- F23: Biopesticide formula (ultisol soil + CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + without *R.solanacearum* inoculation.
- F24: Biopesticide formula (organic growth medium + CaCO₃+CMC 1% + mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) without *R.solanacearum* inoculation.

These six treatments were replicated five times and each used six polybags. The comparison of the formula was similar to the first step, that was a comparison between weight and volume of the arranging materials for the biopesticide formula (10:5:1:1). This implied 10 g of the organic growth medium: 5 g of CaCO₃ : 1 ml of CMC 1%: 1 ml of mannitol 1%, and the *Bacillus* sp.B298 volume used was 5 ml per treatment, with the density of 10¹⁰ CFU/ml. These treatments of the biopesticide formula were on tomato seeds before nursery, by coating them. The concentration of *R. solanacearum* was 10⁹ CFU/ml.

Variables observed were:

1. *Bacillus* sp. persistence in the biopesticide formula calculated basically by populations

in four times of observations with two weeks interval.

2. *Bacillus* sp. inhibition of the biopesticide formula to *R. solanacearum* *in vitro*, measured from inhibition zone formed.
3. Incubation period of the bacterial wilt disease, observed daily since *R. solanacearum* inoculation until the emergence of wilting symptom on tomato.
4. Disease intensity, calculated by using a Ghosh and Mandal (2009) formula:

$$IP = \frac{n}{N + n} \times 100\%$$

Remarks:

- IP = Disease Intensity
 n = Number of infected plants at one plot.
 N = Number of plants that was alive at one plot.

5. Infection rate, counted by using a van der Plank (1963) formula:

$$r = \frac{2.3}{t} \left\{ \log \frac{1}{1 - X_t} - \log \frac{1}{1 - X_0} \right\}$$

Remarks=

- r = Infection rate (unit/day)
 t = observation time ($t_i - t_0$)
 X_t = Infected tissue proportion at t time
 X_0 = Infected tissue proportion at 0 time (initial observation).

6. Components of plant resistance (total phenol compound on the plant), the resistance was determined by using the disease intensity category such as follows:

Resistance = DI of 0 – 25%
 Moderate resistance = DI of 26 – 50%

Moderate susceptible = DI of 51 – 75%
 Susceptible = DI of 76 – 100%

Remarks : DI = Disease Intensity

Two steps experiment were conducted in RCBD (Randomly Completely Block Design). Data obtained were analyzed by using analysis of variance and when significant then continued by using DMRT of 5%.

RESULTS AND DISCUSSION

Bacillus sp. Persistence in the Biopesticide Formula

The result of observations at the step 1 was *Bacillus* sp. persistence obtained from tested biopesticide formula, based on the observation of *Bacillus* sp. population within 12 weeks.

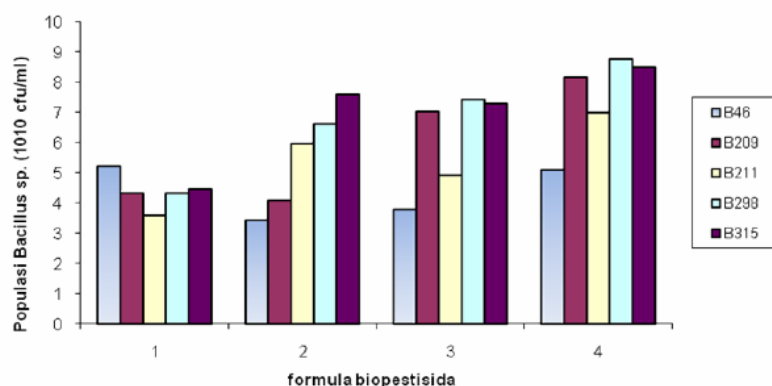
Figure 1 performs the average population of five isolates of *Bacillus* sp. in four formula tested. The highest population of *Bacillus* sp. was at the formula 4 (organic growth medium+ CaCO₃+CMC1%+mannitol 1%). The highest mean population was 7.01×10^{10} CFU/ml at the *Bacillus* sp. B298, followed by B315, B209, B211, and B46 reaching 6.98×10^{10} CFU/ml, 5.89×10^{10} CFU/ml, 5.37×10^{10} CFU/ml, and 4.38×10^{10} CFU/ml. The best biopesticide formula for the growth of five *Bacillus* sp. isolates was F4, followed by F2, F3 and F1 that were indicated by mean population of five *Bacillus* sp. isolates at those formulas reaching 7.51×10^{10} CFU/ml, 6.20×10^{10} CFU/ml, 6.09×10^{10} CFU/ml, and 4.39×10^{10} CFU/ml respectively. The F4 (organic growth medium+ CaCO₃+CMC1%+manitol 1%) was the best one for *Bacillus* sp. persistence because the formula consisted of the organic growth medium as a carrier substance. The organic growth medium components were goat manure, bokasi, cocopit, and burnt husk. These substances consist of organic matters namely organic C content of 16.103% and total N of 0.901% with C/N ratio of 17.872 that are still high when compared to the carrier substance of ultisol soil. This condition was suitable for *Bacillus* sp. viability, so the population was high and persistent up to 12 weeks. This was related to the research conducted by Kilian *et al.* (2000) stating that the microbial population density as *B. subtilis* was affected by organic compounds, soil humidity, soil type, and oxygen supply in soil. *Bacillus* sp. required enough nutritions such as C and N needed for growing and surviving in soil. They also stated that the bacteria can be abundant within soil reaching mean number of 6×10^8 cells/g of soil. Phae *et al.* (1992) stated that addition of organic matters into soil treated with an antagonistic bacterium such as *B. subtilis* NB22 could cause suppressive effect to *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FoR) causing crown and root rot, and to *R.*

solanacearum causing the bacterial wilt disease on tomato plants.

R. solanacearum* Inhibition by *Bacillus* Sp. Isolated From the Formula *In Vitro

These inhibition observations were carried out by growing *R. Solanacearum* and *Bacillus* sp. in double layer on the YPGA medium in the Petridish. Suppression of *R. solanacearum* by five isolates of *Bacillus* sp. that were persistent in the biopesticide formula was shown by the presence of the inhibition zone as seen in Table 1. Not all isolates of *Bacillus* sp. grown at the biopesticide formula that have been isolated, were capable to perform the inhibition zone to *R. solanacearum*, although in the previous observations taken from the pure isolate grown at the YPGA medium, the isolates were able to show the inhibition zone to *R. solanacearum* (Prihatiningsih and Soedarmono, 2004). This

could be caused by the reduction in the antagonistic effectiveness, due to the pure isolates collected for four years and incubated at the YPGA medium treated with sterilized liquid parafin at the temperature of $\pm 18^{\circ}\text{C}$. To maintain the antagonistic effectiveness in soil, it could be added nutrition substances and organic matters in soil at the antagonist application, as using rice husk and other organic matters (Phae *et al.*, 1992; Muhibuddin, 2008). This research also used the carrier of the organic growth medium as the arranging substance for the biopesticide formula in order to maintain the *Bacillus* sp. effectiveness at soil application. This was shown in the glasshouse observations, the antagonistic effectiveness in the F4 formula was capable to control the potato bacterial wilt disease reaching 84.21%, whereas at the high land it only controlled 51.20%, and 46.40% at the medium land (Prihatiningsih *et al.*, 2008).



Remarks = F1:Formula 1(ultisol soil+ CaCO_3 +CMC1%)+*Bacillus* sp., F2: Formula 2 (organic growth medium+ CaCO_3 +CMC1%)+*Bacillus* sp., F3: Formula 3 (ultisol soil+ CaCO_3 +CMC1%+mannitol 1%)+*Bacillus* sp., F4: Formula 4 (organic growth medium+ CaCO_3 +CMC1%+mannitol 1%)+*Bacillus* sp.

Figure 1. The population of five *Bacillus* sp. isolates in four biopesticide formula.

Control of the Bacterial Wilt Disease and Increase of Plant Resistance

The treatments of *Bacillus* sp. B298 combined with potassium fertilizer at the *in planta* research could control the bacterial wilt disease and increase the tomato plant resistance to the disease. This can be seen from the vulnerable-tolerant variety of Mirah to the bacterial wilt disease which performed the presence of increasing resistance. Biopesticide treatments which was combined with the potassium fertilizer, as seen in Table 2, decreased the disease intensity and increased the tomato plant resistance to the bacterial wilt disease from being susceptible-tolerant to be resistant. According to Aziz *et al.* (1992), the potassium fertilizer increased rice plant

resistance to the blast disease by *P. oryzae*, reduced the percentage of leaf blight invasion, increased plant height, and quickened flowering period until 75%, and added milled dry weight. Moreover, Mengel and Kirby (1979) stated that the increase in resistance caused by potassium fertilization was due to formation of the outer epidemic wall that was thicker.

Plant resistance can be measured based on the data of disease intensity and total phenolic compound of plants at each treatment. Symptom of the bacterial wilt disease only emerged at the treatment of F20 without *Bacillus* sp. This performed that the biopesticide treatments could suppress the disease.

Table 1. Inhibition of *R. solanacearum* by *Bacillus* sp.

Pure isolate of <i>Bacillus</i> sp. a)	Inhibition zone, mm a)	The best <i>Bacillus</i> sp. isolate from the formula b)	Biopesticide formula b)	Inhibition zone, mm b)
B46	16.0	B46	K	1.0
B209	15.0	B209	F1	1.0
B211	15.0	-	F2	-
B298	15.0	B209	F3	2.0
B315	15.0	B298	F4	2.0

Remarks= a) Inhibition of *R. solanacearum* by *Bacillus* sp. pure isolate . b) Inhibition of *R. solanacearum* by *Bacillus* sp. isolates from formula. K: control; F1: ultisol soil + CaCO₃+ 1 ml CMC 1%; F2: organic growth medium + CaCO₃ + 1 ml CMC 1% ; F3 ultisol soil + CaCO₃ + 1 ml CMC+ 1 ml mannitol 1%; F4: organic growth medium + CaCO₃ + 1 ml CMC 1% + 1 ml mannitol 1%.

Table 2. Incubation period, disease intensity, infection rate and resistance level of tomato to bacterial wilt disease

Treatments	Incubation period dai	Disease intensity %	Infection rate, unit/day	Plant response
K	0	0	0	Resistant
F20	21	10	0.015	Resistant
F21	0	0	0	Resistant
F22	0	0	0	Resistant
F23	0	0	0	Resistant
F24	0	0	0	Resistant

Remarks= dai: day after inoculation, K: Control (without *Bacillus* sp. B298 biopesticide, inoculated with *R. solanacearum*). F20:(without *Bacillus* sp. B298 biopesticide and without *R. solanacearum* inoculation), F21: Biopesticide formula (ultisol soil+ CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + *R.solanacearum* inoculation, F22: Biopesticide formula (organic growth medium+ CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + *R.solanacearum* inoculation, F23: Biopesticide formula (ultisol soil+ CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + without *R.solanacearum* inoculation, F24: Biopesticide formula (organic growth medium + CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) without *R.solanacearum* inoculation.

The plant used was Mirah variety that owned susceptible-tolerant trait. However, after planted in this research, they showed to be resistant to the bacterial wilt disease. The plant resistance could also be looked at the total phenolic compound in the stem (Table 3). The total phenolic compound in the plants has been normally formed, but if there are pathogen attack and another stimulation of non-pathogenic microbes, the phenolic compound is enhanced. The result of the research indicated that the treatments of biopesticide and potassium fertilizer could increase the total phenolic compound up to 31.80%.

Table 3. Content of total phenolic compound in tomato plants after having treatments with *Bacillus* sp. B298 biopesticide and potassium fertilizer

Treatments	Total phenolic compound, ppm
K	1264.61
F20	1854.22
F21	1426.95
F22	1305.52
F23	992.53
F24	1177.59

Notes: K:Control (without *Bacillus* sp. B298 biopesticide, inoculated with *R. solanacearum*). F20:(without *Bacillus* sp. B298 biopesticide and without *R. solanacearum* inoculation), F21:Biopesticide formula (ultisol soil+ CaCO₃+ CMC 1% +mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + *R.solanacearum* inoculation, F22: Biopesticide formula (organic growth medium+ CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + *R.solanacearum* inoculation, F23: Biopesticide formula (ultisol soil+ CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) + without *R.solanacearum* inoculation, F24:Biopesticide formula (organic growth medium + CaCO₃+CMC 1%+mannitol 1%), *Bacillus* sp. B298 + Kalium (KCl 6 g/plant) without *R.solanacearum* inoculation.

The total phenolic compound on tomato plants which had the best treatment of *Bacillus* sp. B298 biopesticide and the potassium fertilizer was the F21 treatment by the increase of the phenolic compound up to 11.38%. This result performs that the treatment of the *Bacillus*

sp. B298 and potassium fertilizer treatments could enhance the tomato resistance to the bacterial wilt disease. The potassium is capable to increase the resistance by strengthening stem tissues and as a catalisator of some chemical reactions in plants. Potassium is useful as catalisator, particularly in protein alteration to be amino acid. If the plant has sufficient K, then its protein will increase (Jones *et al.*, 1991). According to Aziz *et al.*, (1992), provision of potassium may decrease the percentage of the leaf blight invasion and makes the plant become moderate resistant.

Plant resistance can be seen from the phenolic compound in the plant tissues. The content of the total phenolic compound in stem parts of tomato plants tested performed that *Bacillus* sp. biopesticide application was capable of increasing the total phenolic compound at F21 (ultisol soil+ CaCO₃+CMC 1% 1 ml) that was higher than K (control, without biopesticide). F23 and F24 treatments had lower total phenolic compound than K. This indicates that the total phenolic compound in the experimental plants has not been able to be used as the criterion of increase in the plant resistance because the compound has not been specific yet. According to Agrios (2005), the phenolic compounds functioning to increase the plant resistance were more specific, such as groups of chlorogenic acid, jasmonic acid etc. This research has not analyzed the content of specifically phenolic compounds for resistance. Kapoor (2008) stated that the treatment of *Glomus macrocarpum* or *G. fasciculosum* mycorrhiza 20 days after getting infection with *Fusarium oxysporum* f.sp. *lycopersici* could induce the systemic resistance on tomato indicated by the increasing activity of Phenylalanine Ammonia Lyase (PAL), phenolic concentration, leaf trichome density, and jasmonic acid concentration until nine fold.

CONCLUSIONS AND SUGGESTION

CONCLUSIONS

The best biopesticide formula for the persistence of five *Bacillus* sp. isolates was with the materials of organic growth media + CaCO₃+CMC 1%+ mannitol 1%. The best inhibition ability of *Bacillus* sp. to *R. solanacearum* was shown by the B298 isolate with the inhibition zone reaching 2 mm. The potassium fertilizer combined with the *Bacillus*

sp. biopesticide was able to enhance the tomato plant resistance from the category of susceptible to be tolerant to resistant.

SUGGESTION

It is suggested that further field research is required to test the capability of *Bacillus* sp. B298 in enhancing the tomato plant resistance to the bacterial wilt disease combined with the potassium fertilizer in order to achieve the *Bacillus* sp. B298 biopesticide having stabil character.

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