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The Abilities of Endophytic and Biofertilizing Bacteria and Their Combinations to Suppress Bacterial Wilt Disease (*Ralstonia solanacearum*) of Chili

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Abstract

Bacterial wilt disease (*Ralstonia solanacearum)* is one of the most important diseases in Solanaceae, including chili. Biological control is one of environmentally-friendly method for controlling plant diseases. Microbes that are potential as biological control agents include bacterial endophytes and bacteria that are usually used as biofertilizer. This paper discusses the result of the study that examined the abilities of endophytic and biofertilizing bacteria solely or in combination to suppress bacterial wilt disease (*R. solanacearum*). The endophytic bacteria isolates tested were *Lysinibacillus* sp. and *Bacillus subtilis*, while biofertilizing bacteria used were N-fixing bacteria (*Azotobacter chrococcum*) and P-solubilizing bacteria (*Pseudomonas cepacea*). The results showed that the endophytic bacteria, biofertilizing bacteria and their combination inhibited wilt disease incidence in chili by 46.7-80%. The highest disease suppression (80%) showed by the endophytic bacteria, *B. subtilis.* This endophyte was also able to promote a significant chili growth.

Keywords: *Ralstonia solanacearum*, Endophytic bacteria, Biofertilizer, Biological control, Chili.

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1. Introduction

Bacterial wilt disease caused by *Ralstonia solancearum* is destructive soil-borne disease in Solanaceous plants including chili. The disease results in progressive wilt and finally the death of infected plants [1]. The disease infected all phase of their host plant particularly in early vegetative and generative stages in which the plant is more susceptible to the disease. The disease is difficult to control as the pathogen has wide

range of host plants and can survive in the soil and seeds and other planting materials [2].

The disease can be control by using bactericides. However, the use of bactericide is harmful to the beneficial soil microorganisms and also can lead to soil pollution. Therefore, many researchers have developed environmentally friendly control measures such as biological control.

Microorganisms that are potential as biological control agents for plant diseases include bacterial endophytes and also Plant Growth Promoting Rhizobacteria (PGPR). Many isolates of bacterial endophytes have been reported to control plant diseases [3, 4]. Some isolates of bacterial endophytes from cabbage roots [5] and cogon grass roots [6] suppressed club root disease in cabbage. Some isolates of bacterial endophytes from potato tubers and roots also reduced disease incidence of bacterial wilt in potato [7]. Bacterial endophytes are known to produce various antibiotics that toxic to the pathogen [4] and to induce plant resistance to diseases [8].

Some PGPR can supply certain plant nutrition by solubilizing phosphate or fixing Nitrogen and therefore they can be used as biofertilizers. *Pseudomonas cepacea* is phosphate solubilizing bacteria that produce organic acid and phytohormone [9] and has been shown able to increase the yield of maize by 20.2% [10]. *Azotobacter chroococcum* is Nitrogen fixing bacteria that can produce exopolysaccharide [11] and has been reported to increase the growth of lettuce [12]. Some PGPR that has potential as biofertilizer can also showed suppressive effects on plant diseases [13–16].

Combinations of compatible microbes have been reported to increase their ability to diseases suppression [17, 18]. Endophytic bacteria isolated from cogon grass roots, *Lysinibacillus* sp., and the endophyte isolated from potato tuber, *Bacillus subtilis,* as well as biofertilizing agents Azo*tobacter chroococcum* and *Pseudomonas cepacea* were compatible each other based on their growth in vitro [7]. This paper discusses the effect of these bacteria individually or in combination to control bacterial wilt (*R. solanacearum*) in Chili.

2. Materials and Methods

The endophytic bacteria, *B. subtilis* isolated from cogon grass root, while *Lysinibacillus* sp. isolated from potato tuber. The P-solubilizing bacteria, *P. cepacea* and N-fixing bacteria, *A. chroococcum,* were isolated from corn rhizosphere. The endophytic bacteria were mass cultured on nutrient broth, while *A. chroococcum* and *P. cepacea* were mass cultured in 4% molase broth medium. The culture of endophytic bacteria and *P. cepacea* were incubated on a rotary shaker (150 rpm) for 24 hours at room temperature, while the culture of *A. chroococcum* was incubated on the rotary shaker (150 rpm) for 76 hours at room temperature.

The experiment was arranged in a randomized complete block design with 10 treatments and 3 replications. Each replication consisted of eight plants. The treatments were bacterial isolates individually, the biofertilizing bacteria (*P. cepacea* and *A. chroococcum*), combination between each or both isolates of endophytic bacteria (*B. subtilis, Lysinibacillus* sp.) and the biofertilizing bacteria, bactericide and check. The bacterial isolates were applied as seed treatments. The chili seed were soaked in bacterial cell suspension (10^7 cfu/ml) for 30 minutes. For combination treatments, the bacterial suspension was mixture of the individual cell suspension (concentration) in equal volume. The seeds were then planted in medium consisted of pasteurized soil and compost (2:1, v/v) with rice husk charcoal 10% (v/v).

The bacterial isolates individually or in combination were applied at the time of transplanting the seedlings into a tray (40 × 30 × 15 cm in size) that would contain 8 chili seedlings. The bacterial suspension (10^7 cfu/ml) with the dosage of 30 ml per plant were applied in planting hole. As it was difficult to obtain highly virulent *R. solanacearum* isolate as pure culture (the pathogenicity tests were always failed), the inoculum of the pathogen was prepared by collecting oose bacteria from the infected plants in the field. The bacterial pathogen suspension was checked its density using *Tetrazolium chloride* (TZC) agar medium. The pathogen suspension (10^7cfu/ml) with the dosage of 30 ml suspension per plant was inoculated in planting hole after application of tested bacteria (just before transplanting the seedlings. The chili seedlings (3 week old) were then transplanted into the infested planting holes in the tray, in which 8 seedlings were planted in each tray.

Variables observed were the height and numbers of leaves of chili seedlings before pathogen inoculation, incubation period of the disease (the appearance of the symptom at the first time), the percentage of infected plants that was observed every day until there was no new infected plants anymore. As in this experiment not all plants in the positive check (inoculated with the pathogen) were dead, so the fresh and dry weight of the survived chili plants in each treatments were also measured at the end of the experiment. Data were analyzed statistically using SPPS 20. The significance differences between treatments were further analyzed using Tukey HSD 5%.

3. Results and Discussion

3.1. The abilities of endophytic and biofertilizing bacteria to suppress wilt disease incidence of chili

The results showed that the abilities of the bacteria to suppress bacterial wilt were varied depending on the isolates and their combination. The endophytic bacteria (*Lysinibacillus* sp. and *B. subtilis*) were able to inhibit the development of *R.*

Table 1: The abilities of endophytic bacteria and PGPR individually or in combination to suppress bacterial wilt disease (*R. solanacearum*) in chili.

Treatments	Incubation period (days after pathogen inoculation)	Disease incidence (%) at 30 days after pathogen inoculation		Percentage of inhibition (%)
A: *Lysinibacillus* sp	16	16.7	ab	73.3
B: *B. subtilis*	23	12.5	ab	80.0
C: *A. chroococcum*	8	37.5	bc	40.0
D: *P. cepacea*	12	20.8	ab	66.7
E: *A.chrococcum* + *P.cepaceae*	9	25.0	ab	60.0
F: *Lysinibacillus* sp + *A. chroococcum* + *P. cepacea*	14	16.7	ab	73.3
G: *B. subtilis* + *A. chroococcum* + *P. cepacea*	9	33.3	b	46.7
H: *Lysinibacillus* sp + *B. subtilis* + *A. chroococcum* + *P. cepacea*	10	33.3	b	46.7
I: Bactericide	16	4.2	a	93.3
J: Check	7	62.5	c	0.0

Note: Data in the same column followed by different letters were significantly different ($P < 0.05$) based on Tuckey HSD test

solanacearum infection. This was showed by longer incubation period of the disease compared to the check. In these treatments, the symptom of wilt disease appeared at 16 and 23 days after pathogen inoculation respectively. These incubation periods were the same or even longer than that of bactericide treatment. The earliest disease symptom was appeared in the check plants at 7 days after pathogen inoculation (Table 1). No additional of the infected plant was detected at 26 days after pathogen inoculation.

Most of the treatments with the tested bacteria was significantly reduced the wilt disease incidence by 46.7-80%. The treatment, that was not significantly different to the check, was only the treatment with *A. chroococcum*. In this experiment, this N-fixing bacteria was not effective in inhibiting *R. solanacearum* infection. However in previous experiment, this isolate was demonstrated a significant ability to reduce the incidence of damping off (*Rhizoctonia solani*) and fusarium wilt (*Fusarium oxysporum*) diseases in chili [7]. Another biofertilizing agent, the P-solubilizing bacteria *P. cepacea* was able to reduce the bacteria wilt disease incidence significantly at 66.7% reduction compared to the check. This bacteria was also able to suppress damping off disease and fusarium wilt disease in the previous experiment [7]. The ability of P-solubilizing bacteria *Burkholderia* or *Pseudomonas cepacea* to control plant diseases was also reported in other experiments. They were able to suppress *Phytophthora* disease in chili [16], damping off disease and Fusarium diseases in French bean [15].

In this study, combination of bacterial isolates did not showed any significant increase in their disease suppressive effect. In case of *B. subtilis,* its individual effect to suppress the disease was relatively higher (80%) compared to its combination (46.7%). This is contrasted to the previous experiments in which the disease suppressive effects of microbial consortia were better than the individual effects [17–19]. In other experiment using the same isolates, the combination between *B. subtilis*, *Trichoderma harzianum* and biofertilizing bacteria *(A. chroococcum* and *P. cepacea*) tended to result in better disease suppression on damping off and Fusarium diseases in chili [7]. The difference on those effects may be due to the difference in the patho-system. Roberts *et al*. [17] also reported the different effect of combinations of some antagonistic isolates in suppressing different plant diseases over their individual isolates.

3.2. The effects of endophytic bacteria and PGPR on chili growth

The effects of endophytic bacteria and PGPR were observed before and after pathogen inoculation. Observation on the effects of the bacteria before pathogen inoculation was intended to know the abilities of the bacteria to promote the plant growth. The results showed that most of the treatments did not significantly increase the plant growth. The treatments that significantly increased the plant height were only two treatments which were *Lysinibacillus* sp. and *P. cepacea* individually (Table 2). *P. cepacea* used in this study was found to produce phytohormone [9].

In this experiment, the ability of N-fixing bacteria, *A. chroococcum,* to promote plant growth was not obvious. This was probably due to the presence of the nutrition in the growth media that was still sufficient to support chili seedlings, so that the effect of N-fixing bacteria was not significant. Soleimanzadeh and Gooshchi [20] found that the positive effect of *Azotobacter* on plant growth was relatively decreased if N levels increased.

The effects of bacterial treatments on plant growth were also observed after pathogen inoculation. In this experiment, most of the treatments did not increase the shoot and root fresh and dry weight, compared to the pathogen inoculated check. The significant effect on shoot was only observed on the treatment with *B. subtilis,* whilst the significant effect on roots was found in the treatments with *P. cepacea* and combination of *B. subtilis, A. chroococcum* and *P. cepacea*. Based on total shoot and root weight, it was also found that most of the treatments did not increase the chili growth significantly. The significant increase was only observed in the treatment with the endophytic bacteria, *B. subtilis* (Table 3). The significant better growth of the plants, treated with *B. subtilis* and inoculated with *R. solanacearum,* were likely related to the

Table 2: The effect of biofertilizing agent or endophytic bacteria individually or in combination on the growth of chili (before pathogen inoculation).

Treatments	Plant height before pathogen inoculation (cm)		Numbers of leaves	
A: *Lysinibacillus* sp	5.8	bc	6.0	a
B: *B. subtilis*	5.1	abc	5.6	a
C: *A. chroococcum*	4.3	ab	5.0	a
D: *P. cepacea*	6.1	c	4.5	a
E: *A.chrococcum* + *P.cepaceae*	5.0	abc	4.0	a
F: *Lysinibacillus* sp + *A. chroococcum* + *P. cepacea*	4.6	abc	4.5	a
G: *B. subtilis* + *A. chroococcum* + *P. cepacea*	4.0	a	4.0	a
H: *Lysinibacillus* sp + *B. subtilis* + *A. chroococcum* + *P. cepacea*	4.7	abc	4.1	a
I: Bactericide	4.2	a	5.0	a
J: Check	4.1	a	4.9	a

Note: Data in a column followed by different letters were significantly different ($P < 0.05$) based on Tuckey HSD test

inhibition of plant infection as the growth of the plants were not significantly different to the uninoculated check (negative check).

The overall results suggested that the endophytic bacteria, *B. subtilis,* can be used to control bacterial wilt disease (*R. solancearum*) in chili. This bacteria can suppressed bacterial wilt by 80% and also increased the plant growth up to 6.5 times compared to the positive check (the pathogen inoculated plant) and 1.9 times compared to the negative check (uninoculated plant).

4. Conclusion

Based on the results of this study, it can be concluded that:

- The endophytic bacteria *Lysinibacillus* sp., *B. subtilis* and the P-solubilizing bacteria, *P. cepacea* suppressed bacterial wilt (*R. solanacearum*) in chili by (66.7–80.0%).
- Combination of the bacteria did not increase the disease suppressive effect. The wilt disease suppression in the treatments combining endophytic and biofertilizing bacteria were 46.7-73.3%.
- Most of the treatments did not significantly increase the growth of the young chilli plants (7 week old). The significant increase in plant fresh and dry weight

Table 3: The effect of biofertilizing agent and endophytic bacteria individually or in combination on the growth of chili, inoculated with pathogen (30 days after pathogen inoculation).

Treatments	Fresh weight (g)			Dry weight (g)		
	Shoot	Root	Total	Shoot	Root	Total
A: *Lysinibacillus* sp	0.983 ab	1.060 abc	2.043 ab	0.123 ab	0.177 ab	0.300 ab
B: *B. subtilis*	1.610 b	1.433 bc	3.043 b	0.213 b	0.223 ab	0.437 b
C: *A. chroococcum*	0.387 a	0.510 ab	0.897 a	0.043 a	0.077 ab	0.120 a
D: *P. cepacea*	0.560 a	1.577 bc	2.137 ab	0.077 a	0.240 b	0.317 ab
E: *A. chrococcum* + *P.cepaceae*	0.630 ab	0.500 ab	1.130 ab	0.070 a	0.080 ab	0.150 a
F: *Lysinibacillus* sp + *A. chroococcum* + *P. cepacea*	0.530 a	0.483 ab	1.013 a	0.063 a	0.077 ab	0.140 a
G: *B. subtilis* + *A. chroococcum* + *P. cepacea*	0.483 a	1.673 c	2.157 ab	0.063 a	0.267 b	0.330 ab
H: *Lysinibacillus* sp + *B. subtilis* + *A. chroococcum* + *P.cepacea*	0.337 a	0.613 abc	0.950 a	0.037 a	0.093 ab	0.130 a
I: Bactericide	0.573 a	0.910 abc	1.483 ab	0.063 a	0.140 ab	0.203 ab
J: Positive check (with pathogen inoculation)	0.247 a	0.253 a	0.500 a	0.030 a	0.037 a	0.067 a
K: Negative check (without any treatment)	0.570 a	1.060 abc	1.630 ab	0.067 a	0.163 ab	0.230 ab

Note: Data in a column followed by different letters were significantly different ($P < 0.05$) based on Tuckey HSD test

(6.1- 6.5 times compared to the pathogen-inoculated check) was found in the treatment using *B. subtilis*.

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