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Research Article

Effectiveness and Mechanism of Endophytic *Bacillus* in Suppressing Wilt Intensity of Banana Seedling Inoculated by Blood Disease Bacterium

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Abstract

Background and Objective: The main constraint faced by the banana farmers in Indonesia is bacterial wilt caused by Blood Disease Bacterium (BDB). Biological control of the disease using endophytic *Bacillus* is promising to develop. This study aimed at reporting the experimental study of the effectiveness and mechanism of some endophytic *Bacillus* of banana in suppressing wilt intensity of banana seedling artificially inoculated by BDB. **Materials and Methods:** The study was conducted in greenhouse, using 10 isolates of endophytic *Bacillus* of banana and banana seedlings produced by tissue culture acclimated for 3 months. A unit of treatment consisted of three banana seedlings arranged by completely randomized design with three replications. Each seedling was planted on 1 kg of soil media infested by endophytic *Bacillus* and BDB at 10^8 CFU g^{-1} . The pathogen inoculation was done on day 7 after *Bacillus* infestation. Some capability analyses of the isolates of producing growth regulator, enzymatic and chemical compounds were also done *in vitro* for supporting the study of antagonistic mechanism. **Results:** The results showed that some isolates of the endophytic *Bacillus* significantly ($p < 0.05$) suppressed the infection rate and wilt intensity. The *Bacillus* reduced disease intensity by 51.44-24.07%. The antagonistic isolates reduced infection rate from 0.36 to 0.03-0.09 unit day^{-1} . The significance was also showed by the Area Under the Disease Progress Curve (AUDPC) variable. The isolates produced Indole Acetic Acid (IAA) and grown positively on chitin and pectin medium. The volatile and nonvolatile compounds produced by the isolates inhibited growth of the pathogen significantly ($p < 0.05$). **Conclusion:** Some isolates of endophytic *Bacillus* are promising as biological control agents of bacterial wilt of banana caused by BDB as they reduce wilt intensity effectively and they produce IAA, chitinase, pectinase, volatile and nonvolatile compound that are presumably involved in the antagonism mechanism.

Key words: Chitinase, pectinase, volatile compound, nonvolatile compound, antagonism mechanism

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Blood bacterial wilt is the most important disease of banana in Indonesia^{1,3}. The disease is caused by Blood Disease Bacterium (BDB) that formerly was proposed by Gaumann² as *Pseudomonas celebensis*. The pathogen causes yellowing, wilting and eventually death of banana. The pathogen infects all stages of banana growth both vegetative and generative. On the fruiting stage, the diseased banana produces inedible fruits due to the fruit to be browning, broken off growing, maturing and eventually rotting^{1,2,4}. Recently BDB has been categorized as an invasive species because of the rapid spread from Celebes Island where the disease was firstly found by Gaumann² to other islands of Indonesia. It has spread in all of provinces of Indonesia and Malaysia⁴. In the fields, it had been reported that the disease incidence and crop losses could reach 100%^{4,5}.

Blood Disease Bacterium (BDB) is still difficult to control. Therefore, its control development technology needs to be conducted. The development of technology for disease control leading to support sustainable agriculture is a concern of the most experts for the current and futures. Biological control has been considered among the most promising technologies for sustainable agriculture. It reduces the reliance on synthetic pesticides, minimizes the negative impact on the environment and improves workers safety while at the same time maintaining the economic viability of crop production⁶⁻⁸. One of the interesting biological control agents is endophytic *Bacillus*, because it is one of more environmentally friendly alternatives, which is able to colonize an ecological niche similar to that of vascular wilt pathogens such as BDB⁹. Endophytic *Bacillus* lives in associating with various plants. It promotes growth and induces resistance of plants to some pathogens⁹⁻¹⁴. This research aims to study of the effectiveness and mechanism of endophytic *Bacillus* in suppressing wilt intensity on banana seedling artificially inoculated by BDB. The result of evaluation on some antagonism mechanism of some effective isolates to BDB is also presented.

MATERIALS AND METHODS

In vivo test: An experiment to evaluate the capability of endophytic *Bacillus* of suppressing wilt intensity of blood disease was done on banana seedling inoculated artificially. The main materials in this study were BDB isolates. The inoculums of BDB were obtained by culture of the pathogen in CPG broth medium that were incubated for 4 days. The

Bacillus isolates were cultured in nutrient broth and then were incubated for 3 days. A unit treatment consisted of 3 banana seedlings inoculated by BDB suspension with density at 10^8 CFU g^{-1} of soil media. The medium consisted of soil and compost with composition 1:1 in volume. Each seedling was planted on polybag filled by 1 kg of the medium. The treatments included infestation of 10 isolates of endophytic *Bacillus* in the medium with the density at 10^8 CFU g^{-1} of soil media done 7 days before pathogen inoculation. The treatments were arranged by completely randomized design with three replications. The variables observed were wilt intensity, infection rate and area under the disease progress curve (AUDPC)¹⁵⁻¹⁷.

Wilt intensity was assessed by the Eq. 1:

$$IP (\%) = \frac{\sum(n \times v)}{N \times Z} \times 100 \quad (1)$$

where, IP = Wilt intensity, n = Number of seedling with certain score, v = Wilting, Z = Higher score and N = Number of seedling as a treatment unit, where as for scoring used categories: 0 = No wilting leaf, 1 = One-two wilting leaf/leaves, 2 = Three-four wilting leaves, 3 = Five-six wilting leaves and 4 = All of leaves are wilting¹⁵.

Infection rate was assessed by using the Eq. 2¹⁶:

$$r = \frac{2.3}{\Delta t} \left(\log \frac{1}{1 - X_t} + \log \frac{1}{1 - X_0} \right) \quad (2)$$

where, r = Infection rate (unit per week), t = Interval of observation, X₀ = Wilt intensity in proportion and X_t = Wilt intensity in proportion at t.

Assessment of AUDPC was done by the Eq. 3^{16,17}:

$$AUDPC = \sum_{i=1}^n \frac{X_i + 1 + X_{i+1}}{2} (t_{i+1} - t_i) \quad (3)$$

where, AUDPC = Area under the disease progress curve, X_i = Wilt intensity at t_i; t_i = Time of observation and n = Sum of observation.

Indole acetic acid assay: The capability test of *Bacillus* of producing Indole Acetic Acid (IAA) was done as follows: First, standard solution of IAA was made by mixing synthesis IAA with distilled water at 0.0, 0.2 and etc. until 2.0 ppm. After that 3 mL IAA solution was taken and to each of the solution 1 mL Salkowski's solvent was added and then homogenized. The

absorbance value of IAA solutions was assessed by spectrophotometer at $\lambda 530$ nm. The second, pure culture of *Bacillus* isolate was sub-cultured on NA medium and incubated for 48 h. The bacterial suspension was made 10 mL using McFarland standard solution¹⁸ with cell density at 10^8 CFU mL⁻¹. Approximately 3 mL of bacterial suspension was removed into 30 mL LB liquid solution (Luria Bertani) + tryptophan. Each treatment was replicated 3 times and incubated at 28°C in shaker at 1500 rpm for 6 min by which supernatant and pellet were separated. Analysis of IAA content used calorimeter method¹⁹. Approximately 2 mL supernatant and 1 mL Salkowski's reagent were mixed²⁰⁻²¹. The samples were allowed for 60 min before assessing the absorbance value using spectrophotometer at $\lambda 530$ nm. Regression formulation was substituted by absorbance value of sample.

Chitinase and pectinase assay: The capability test of *Bacillus* of producing chitinase and pectinase was based on the activity zone of pectin lyase produced by *Bacillus* on pectin medium. Filter papers of diameter of 0.5 cm dyed into *Bacillus* suspension at 10^8 CFU mL⁻¹ were placed symmetrically on the CPG agar medium containing 0.5% chitin or pectin in Petri dish of diameter of 9 cm. The observation of chitin and pectin lyase activation was based on the clear zone on the pectin medium after incubating for 72 h.

Assay of volatile compound in culture filtrate of *Bacillus*:

This test was done by culturing *Bacillus* on NA and BDB on CPG agar medium in the cover of Petri dish. *Bacillus* cultures incubated for 2 days were covered by BDB cultures in cover Petri dish by positioning upside down than the couple dishes were silted by transparent plastic isolator. Growth of the BDB was observed on the 9th day of incubation. The observation was done by harvesting bacterial culture using 10 mL sterile water. Bacterial cell density was observed by assessing absorbance value of bacterial suspension using spectrophotometer at $\lambda 530$ nm. The absorbance values were converted to colony forming unit using a standard formulation.

Assay nonvolatile compound in culture filtrate of *Bacillus*:

The bacteria were culture in NB incubated for 4 days. Amount of 3 mL *Bacillus* culture filtrate at 10^8 CFU mL⁻¹ was centrifuged at 5 rpm for 20 min. The supernatant resulted from the centrifugation was sterilized by autoclave at 121°C and 1 atm for an hour and then it was placed under UV for 12 h. Approximately 0.1 mL of BDB suspension at 10^8 CFU mL⁻¹

were culture on the CPG agar medium using Petri dish of diameter of 9 cm. Filter papers of diameter of 0.5 cm dyed into *Bacillus* culture filtrate were placed on the agar media in symmetric position. The cultures were incubated for 72 h. The capability observation of inhibiting BDB growth was based on inhibition zone indicated by clear zone around the filter papers.

Statistical analysis: The statistical analysis of quantitative data was conducted using the SPSS 21.0 statistical software (SPSS Inc, Chicago, IL, USA). The data of disease variables were subjected to F-test and the discrimination of the variation among treatments was performed using the Duncan's Multiple Range Test (DMRT) at 5% probability. Where as assays data such as the *Bacillus* ability to produce phytohormone, chitinase, pectinase and inhibitor compounds were subjected to descriptive analysis.

RESULTS

In vivo test: The results showed that all isolates of endophytic *Bacillus* could effectively suppress blood disease intensity on the banana seedlings artificially inoculated by BDB. The isolates could suppress wilt intensity, infection rate and AUDPC (Table 1). The greatest effective one was isolate B18 suppressing wilt intensity at 74.51% (94.44-24.07%), whereas the smallest one was isolate B01 suppressing wilt intensity at 45.00% (94.44-51.85%).

Assay of enzyme and inhibitor compounds: Results of the current researches show that all of *Bacillus* isolates can produce phytohormone of Indole Acetic Acid (IAA), enzyme of chitinase and pectinase. The capability of *Bacillus* to produce IAA was showed by the results of IAA assay from

Table 1: Endophytic *Bacillus* capability of suppressing wilt intensity, infection rate and AUDPC on seedling of banana artificially inoculated by BDB

Isolates of <i>Bacillus</i>	Wilt intensity (%) [*]	Infection rate (unit days ⁻¹)	AUDPC [*]
No treatment	94.44 ± 0.00 ^e	0.36 ^b	1375.00 ± 116.67 ^b
Carrier agent	92.59 ± 3.21 ^e	0.33 ^b	1500.00 ± 63.10 ^b
B01	51.85 ± 3.21 ^d	0.09 ^a	652.78 ± 63.10 ^a
B02	42.59 ± 3.21 ^c	0.07 ^a	533.33 ± 75.00 ^a
B03	40.74 ± 3.21 ^c	0.07 ^a	452.78 ± 04.81 ^a
B04	42.59 ± 6.42 ^c	0.07 ^a	577.77 ± 84.30 ^a
B09	25.93 ± 3.21 ^a	0.04 ^a	327.78 ± 48.83 ^a
B15	29.63 ± 2.62 ^{ab}	0.04 ^a	488.89 ± 48.83 ^a
B17	37.04 ± 11.5 ^{bc}	0.06 ^a	488.89 ± 179.96 ^a
B18	24.07 ± 3.21 ^a	0.03 ^a	300.00 ± 62.55 ^a
B20	35.19 ± 6.42 ^{bc}	0.05 ^a	466.67 ± 76.38 ^a
B22	31.48 ± 3.21 ^{ab}	0.05 ^a	527.78 ± 61.43 ^a

^{*}Averages followed by the same letter are non-significantly different based on DMRT at 5% level and the values following the averages are standard error of mean

Table 2: Capability of endophytic *Bacillus* of producing phytohormone, enzyme, volatile and nonvolatile compounds

<i>Bacillus</i> isolates	IAA (ppm)	Enzymes		Inhibitor compounds	
		Chitinase	Pectinase	Volatile × 10 ⁸ CFU mL ⁻¹	Nonvolatile* diameter of clear zone (cm)
B01	9.66	+	+	1.50	0.91 ^{ef}
B02	6.85	+	+	1.65	0.85 ^{ef}
B03	9.58	+	+	1.37	0.90 ^{ef}
B04	6.15	+	+	1.48	0.99 ^d
B09	9.62	+	+	1.92	0.85 ^{ef}
B15	6.59	+	+	1.98	1.29 ^c
B17	6.76	+	+	1.80	0.89 ^e
B18	5.59	+	+	1.25	1.84 ^a
B20	8.59	+	+	2.02	0.80 ^f
B22	6.87	+	+	1.84	1.45 ^b
No treatment	0.00	-	-	2.89	0.00 ^g
Sterile water	0.00	-	-	2.95	0.00 ^g

+ = Produce pectinase activity zone, - = No activity zone, *Averages followed by the same letter are non-significantly different based on DMRT at 5% level

5.59-9.66 ppm where no IAA was in the media with no treatment and treated with sterilized water. All of isolates showed the growth well on both chitin and pectin medium. It means that the *Bacillus* could use the chitin and pectin as carbon source due to the ability to produce the enzyme of chitinase and pectinase. All of the isolates also produced volatile and nonvolatile inhibitor compounds showed by decreasing growth of BDB on the treated medium (Table 2).

DISCUSSION

The current research results showed that the endophytic *Bacillus* had effective capability of suppressing wilt intensity of banana artificially inoculated by blood disease bacteria. The results supported the previous research indicating that endophytic *Bacillus* were effectively reduced wilt intensity of blood disease on plantlets and *in vitro* test of the bacteria that could inhibit the growth of banana wilt pathogens^{10,11}. The result was linear to different pathogens according to previous researches of Ji *et al.*¹³, Nagendran *et al.*²² and Nawangsih *et al.*²³, Ji *et al.*¹³ did a screening on antagonistic activities against *Ralstonia solanacearum in vitro* and four isolates showed a remarkable inhibitory effect. The evaluation of the antagonistic strains against bacterial wilt of mulberry indicated that the strain Lu144 effectively reduced disease incidence. Nagendran *et al.*²² reported that rice plots treated with endophytic *Bacillus* had a significantly ($p < 0.05$) lower intensity of bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* compared to untreated control plots by which also recorded a higher grain and straw yield. Whereas, Nawangsih *et al.*²³ did a greenhouse test on six selected of endophytic bacteria to *R. solanacearum* by which they obtained BL10 isolates (similar *Bacillus amyloliquefaciens* strain JK-SD002) as promising bio-control agents. At six weeks

after transplanting, plants treated with BL10 isolate showed a significantly ($p < 0.05$) lower disease incidence (43.33%) than those of control (83%).

Many factors may affect the capability of bio-control agent of decreasing disease intensity. The capability of biological control agent of producing some compounds may be involved in antagonistic mechanism. Seemingly, the inhibitor compounds are involved in antagonism mechanism and reducing wilt intensity of blood disease of banana. Hadiwiyono and Widono¹⁰ showed that some isolates of *Bacillus* formed a clear zone inhibition on the dual culture with BDB and *Fusarium oxysporum* f. sp. *ubense* by then it was suspected that the bacteria could produce inhibitor compounds. Based on the current evident it was proved that endophytic *Bacillus* could produce volatile and nonvolatile compounds inhibiting growth of BDB. Toxic compounds produced by biological control agent have also been reported as an important mechanism in biological control of plant diseases. Arrebola *et al.*²⁴, Islam *et al.*²⁵ and Yuan *et al.*²⁶ reported that some isolates of *Bacillus* could produce some volatile compounds inhibiting pathogen growth. Ryu *et al.*^{27,28} discovered that bacterial-produced Volatile Organic Compounds (VOCs) triggered plant growth enhancement and induced systemic resistance constitutes a novel mechanism for rhizobacteria-plant interaction. Of the PGPR tested, two of seven strains (*B. subtilis* GB03 and *B. amyloliquefaciens* IN937a) elicited constitutively growth promotion and induced systemic resistance of Arabidopsis seedlings, suggesting that synthesis of bioactive VOCs is a strain-specific phenomenon.

According to Reddy¹⁴ *Bacillus* can produce some secondary metabolites such mycobactin, bacitracin and zwittermicin being useful in inhibiting bacterial pathogen. Other researchers reported that inhibiting capability of *Bacillus*

was caused by the existence of toxic chemical compound of acetoin²⁹, bacilycin³⁰, fengycin and bacillomycin³¹. Some biosynthetic genes of antimicrobial compounds produced by *Bacillus* have been identified; *srfAA* (surfactin), *bacA* (bacylisin), *fenD* (fengycin), *bmyB* (bacillomycin), *spaS* (subtilin) and *ituC* (iturin)³².

Possibly, phytohormone could also be involved in suppressing disease intensity of blood disease due to the ability to produce Indole Acetic Acid (IAA). The compound has been also reported that it could promote growth³³⁻³⁵ and induce resistance to plant pathogens^{27,36-38}. The IAA is important for vigor of the plant and most frequently it is used to explain the mechanism of plant growth promoting microbes. The IAA will take role in regulating the plant growth to be healthy plant. By IAA the healthy plant will be more resistant or tolerant to pathogen infection because of the role in cell proliferation, elongation and enlargement^{39,40}. Some researchers however reported that IAA could promote plant disease development or induced susceptibility^{40,41}. Wang *et al.*⁴¹ added that signaling IAA pathway could be repressed using salicylic acid.

This research proves that all of *Bacillus* isolates produce chitinase and pectinase enzyme. The results are the same as those of the previous researches of Huang *et al.*⁴², Saha *et al.*⁴³, Zhao *et al.*⁴⁴ and Kim and Chung⁴⁵. The role of chitinase in antagonism to pathogenic fungi has been explained well. Chitinase can cause enzymatic dissolution of cell walls leading to loss of fungal protoplasm. It is one of the main antagonistic mechanisms involved in the activity of bio control agents^{46,47}. All of the isolates can produce chitinase, a degrader of the fungal cell wall. It is an indication that cell wall degradation may be one of the mechanisms of antagonism exhibited by the endophytic *Bacillus* isolates. To pathogenic bacteria however both chitinase and pectinase are still poorly understood. Possibly, the role is indirect that the capability of the endophytic *Bacillus* of producing chitinase and pectinase together with IAA is helpful to pathogen in proliferation and colonization of the antagonistic bacteria. It has been reported that pectinase is one of the determinants of induced systemic resistant^{48,49}.

CONCLUSION

All isolates of endophytic *Bacillus* tested, were capable of suppressing wilt intensity, infection rate and AUDPC. The isolate of B18 was the most effective in suppressing wilt intensity from 24.07 to 94.40%. All of isolates could produce IAA, chitinase, pectinase, volatile compound and non volatile compound that were presumably involved in the antagonism mechanism of *Bacillus*.

SIGNIFICANCE STATEMENTS

- From this study it was proved that endophytic *Bacillus* could produce some compounds that were possibly involved in biological control mechanism
- This study discovers that endogenous endophytic *Bacillus* is promising as a biological control agent for control of bacterial wilt

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