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Bioprospection of single and a consortium endophytic bacteria of suboptimal field rice isolates as biocontrol of rice bacterial leaf blight pathogens

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Abstract. *Djatmiko HA, Prihatiningsih N, Lestari P. 2023. Bioprospection of single and a consortium endophytic bacteria of suboptimal field rice isolates as biocontrol of rice bacterial leaf blight pathogens. Biodiversitas 24: 2710-2715.* Rice root endophytic bacteria isolated from suboptimal land have the potential to control leaf blight by *Xanthomonas oryzae* pv. *oryzae* (Xoo). The aim of this research was: 1) to test the potential of single and consortium endophytic bacteria single and consortium to control Xoo in vitro, 2) to know the antibiosis mechanism of this endophytic bacteria and 3) to evaluate the biochemical character of the single endophytic bacteria as an antagonist feature. The completely randomized design with 5 single and 1 consortium treatment, 4 replications was used as a research method. The inhibition was carried out using a diffusion method of 10^8 cfu/mL endophytic bacterial suspension. Filter paper 6 mm diameter had been dripped 10 µL suspension of endophytic bacteria, then it placed 3 spots on NA that had been poured with Xoo. The variable observed was the inhibition zone of endophytic bacteria, the inhibition mechanism, and the character as antagonist produces protease; and siderophore. The results showed that both single and consortium endophytic bacteria could potentially suppress Xoo in-vitro, and the best inhibition was the consortium with an inhibitory area of 62.74 mm^2 and the bacteriostatic mechanism. The consortium's antibiosis index was 1.49, it can produce protease and siderophore with clear zone and orange color features. The endophytic bacteria consortium has the potential as a biopesticide to be developed as an alternative to control rice bacterial leaf blight.

Keywords: Antibiosis, bacteriostatic, biopesticide, protease, siderophore

INTRODUCTION

Endophytic microbes, both fungi and bacteria play a role in controlling plant diseases and increasing growth, and inducing plant resistance. Control of plant diseases using endophytic bacteria is one trend that has good prospects, considering the advantages of endophytic bacteria compared to other microbes. Endophytic bacteria are bacteria that live in plant tissues without causing damage or symptoms to plants, even providing benefits to plants (Melnick et al. 2011; Miliute et al. 2015). Lately, endophytic bacteria are also said to be able to promote plant growth and yield and even induce plant resistance to pathogens. This potential is possessed by endophytic bacteria because it is able to produce several compounds as components of plant growth such as Indole Acetic Acid (IAA), siderophores, phosphatases, and HCN, as well as compounds as resistance-inducing components such as phenol, salicylic acid, jasmonic acid, phenylalanine ammonia lyase (Glick 2012; Dawwam et al. 2013; Kesaulya et al. 2015; Olanrewaju and Babalola 2019). Endophytic bacteria are considered as rational and safe pathogen controllers for plants and the environment (Beric et al. 2012; Muthukumar et al. 2017).

Endophytic bacteria consortium is made by mixing several compatible single endophytic bacteria so that they can increase their activity and effectiveness as biocontrol agents and plant growth promoters. Microbial consortia in agriculture have become an area of interest for growth promoters. Positive interactions between rhizobacteria and endophytic bacteria help in colonization and associative means of combatting pathogens. A single rhizobacterium or endophytic bacterium can have more than one mechanism of action, so a combination of two or more endophytic bacteria as a consortium can increase plant growth, combat stress, and control pathogens. To support this claim, studies have shown that the co-inoculation of plant growth-promoting bacteria improves plant growth (Köhl et al. 2019; Prihatiningsih 2020a). The single endophytic bacteria from rice root suboptimal land have some characteristics as plant growth-promoters which produced HCN, siderophore, IAA, and phosphate solubility showed KR4, KR5, KR7, SR5, SR7, SM1, SB1, and SB3 isolate, and the SM1, SB1, and SB3 isolates could suppress the disease intensity with 56.03-60.79% effectiveness. Suboptimal land development for agriculture is generally faced with several problems, including high soil acidity and toxicity of Fe and Al as well as deficiency of nutrients N, P, K, Ca, and Mg. Therefore, improvements need to be done to the condition of soil biology and chemistry, using endophytic bacteria. Suboptimal land spread on the embankment of the river, the lower part of the river terraces, alluvial depression, plain fluvial marine, and peat (Prihatiningsih et al. 2021).

The bacterial leaf blight of rice is caused by *Xanthomonas oryzae* pv. *oryzae*, a main disease that constrains the production of the staple crop in many countries. These pathogens were identified based on the disease symptoms, pathogenicity, morphological, physiological, and genetic characteristics of bacterial cultures isolated from the infected plants. The symptoms of bacterial leaf blight showed generally infect leaves, but distribution depends highly on its environmental condition (Resti et al. 2020). To control this disease (Suryadi et al. 2012; Resti et al. 2020) used the endophytic bacterial consortia that have antibiosis abilities and improve the growth of rice seedlings. Introduction of endophytic bacteria consortia is more effective in suppressing disease and increasing the growth of rice plants because more than one type of endophytic bacteria found have different mechanisms of suppressing disease and promoting plant growth. Combining bacteria as a consortium that can produce IAA, siderophore, dissolve phosphate, and active plant defense enzyme will be more effective compared to a single introduction of the endophytic bacteria (Miliute et al. 2015; Oluwambe and Kofoworola 2016). Endophytic bacteria are associated with plants to help the absorption of nutrients because they are able to dissolve phosphate into a form available to plants, and provide Fe through siderophores, and produce phytohormones such as Indole Acetic Acid (IAA), gibberellins, and cytokinins (Glick 2012; Dawwam et al. 2013).

The control of bacterial leaf blight with endophytic bacteria has been carried out according to the consortium (*Bacillus* sp. SJI, *Bacillus* sp. HI) and (*Bacillus* sp. SJI and *Serratia marcescens* isolate JB1E3) is able to control bacterial leaf blight and improve the growth of seedlings and plants (Resti et al. 2020). Control rice bacterial leaf blight was controlled with endophytic bacteria singly with the effectiveness of 64.16% and 49.14 respectively in screen house and field (Prihatiningsih et al. 2021). The control of bacterial leaf blight on rice with endophytic bacteria from suboptimal land in a consortium studied in this study. The combination of endophytic bacteria in the consortium can control various plant pathogens more effectively. Bacteria have more than one beneficial effect on the host, with different disease suppression mechanisms. Combining strains with different disease suppression mechanisms can control pathogens more effectively.

This research was original because started from the exploration of endophytic bacteria from rice roots in suboptimal lands, and the combination of endophytic bacteria as a consortium as an alternative technology for the management of plant disease which is eco-friendly and supported sustainable agriculture. In addition, the application of endophytic bacteria can reduce dependence on the use of synthetic pesticides, thereby supporting food security and safety.

The aim of this research was: (i) to test the potential of endophytic bacteria both single and consortium to control Xoo in vitro, (ii) to know the antibiosis mechanism of this endophytic bacteria and (iii) to evaluate the biochemical character of the single endophytic bacteria as an antagonist feature.

MATERIALS AND METHODS

Research site

This research was conducted from April to July 2021 at the Laboratory of Plant Protection, Faculty of Agriculture, University of Jenderal Soedirman Purwokerto. Rice root endophytic bacteria isolated from sub-optimal land were initially selected as IAA producers and controllers of rice bacterial leaf blight, *Bacillus* sp. Petanahan, Karangwangkal and Sumbang isolates (*Bacillus* sp. A5, A6, KR4, KR7 and SB3) (Prihatiningsih et al. 2020a). Xoo was isolated from a rice plant with leaf blight symptoms in the Karangwangkal area of Purwokerto, Indonesia.

Antibiosis assay of endophytic bacterial both single and consortium against Xoo

The antibiosis assay was carried out by following the method used by Balouiri et al. (2016) with modifications. It was carried out by growing Xoo by pour plate on NA medium, then the endophytic bacterial consortium suspension was grown in NB medium shaking at 150 rpm for 24 hours, room temperature. Filter paper Whatman no. 4 with a diameter of 6 mm was dripped with 10 µL of the suspension, then placed on a NA medium that had been poured with a Xoo pour plate in three parts. Incubation was carried out at room temperature for 48 hours. A completely randomized design was used in this experiment with 6 treatments (5 single endophytic bacteria and 1 consortium from 5 isolates) and 3 replications. The observed variables were the zone of inhibition, the area of the inhibition, the mechanism of inhibition, and the antibiosis index. The inhibition zone was measured based on the clear zone formed, the diameter of the zone, the diameter of the filter paper, and the radius of the clear zone was measured to calculate the area of inhibition.

Mechanism antibiosis assay

The antibiosis mechanism was observed by taking part of the zone with an Ose needle then inserted into a test tube containing 0.6% peptone water and shaken it for 24 hours at 150 rpm at room temperature, then observed if the peptone water was cloudy, which the antibiosis mechanism was bacteriostatic meaning it only inhibited the growth of Xoo, but if the peptone water was clearly which the antibiosis mechanism is bactericidal, meaning that it is able to kill Xoo (Köhl et al. 2019).

Antibiosis index

The antibiosis index is measured to determine the antibiosis activity which is usually indicated by the ability of bacteria to produce enzymes or compounds that play a role in antagonism with the antibiosis mechanism. Measurement of the antibiosis index using the formula proposed by (Halimahtussadiyah et al. 2017):

$$AI = (A-B) : B$$

Where:
AI : Antibiosis Index
A : Diameter of the zone
B : Diameter of colony antagonist or diameter paper disc

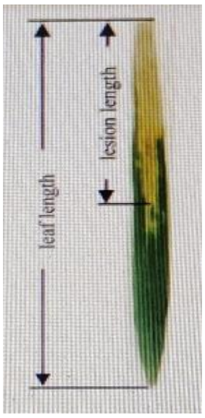

Figure 1. The disease was scored by measuring the lesion length. The lesion length is the length from the inoculation site to the edge of thoroughly blighted leaf middle veins (Ke et al. 2017)

The results of the calculation of the antibiosis index are included in the inhibition category according to (Pan et al. 2009) as follows, very strong if AI > 2.0 with the symbol (+++), strong if AI (1-1.9) with the symbol (++), weak if AI (0.1-0.9) with the symbol (+) and does not have antibiosis ability (0,0) with the symbol (-).

Biochemical characteristic of endophytic bacteria as an antagonist

The protease was produced by endophytic bacteria, it was assayed on skim milk agar medium. The bacterial culture was grown on skim milk agar medium (casein 5 g, yeast extract 2.5 g, glucose 1g, agar 15 g, distilled water 1000 mL, skim milk 7% was added as an inducer (Prihatiningsih et al. 2021). The qualitative method for protease assay of endophytic bacteria was carried out through protease screening using a skim milk agar medium with the scratched inoculated method of one loop full (Vijayaraghavan and Vincent 2013). The clear zone that appeared the around scratch was protease secreted by endophytic bacteria.

The siderophore produced by endophytic bacteria was carried out on SD-CASA medium, dark blue, 2% agar w/v in order to be added. As much as 10 mL of CAS-blue agar was the basis of the plate. After being solidified, it was coated with 6 mL of YPGA (yeast extract peptone glucose agar) as the second layer medium. Then, the plate was incubated overnight at 32°C, ten μL of supernatant endophytic bacteria was dropped on a paper disc, forming an orange or pink zone around the paper disc was found (Hu and Xu 2011).

Bacterial leaf blight control by endophytic bacteria

The Xoo was inoculated to the leaf with clipping methods 1-1.5 cm from the tip of the leaf (Naqvi et al. 2015) at 34 days after planting. The density of the Xoo was 10^9 cfu/mL. The scissor was sterilized by immersing 70% alcohol before use to cut the tip of the leaf and then dipping it into Xoo suspension. The application of the endophytic bacteria both single and a consortium by spraying at a foliar leaf of rice 2 days before inoculation the Xoo and 10, 20, 30 days after inoculation. The endophytic bacteria both single and a consortium arranged in wet formula 1 week before application. The variables observed were incubation period, disease intensity, and infection rate. The measuring of disease intensity uses the formula by Ke et al. (2017):

$$DI = a/b \times 100\%$$

Where:
DI : Disease Intensity
a : Length of bacterial leaf blight (cm)
b : Overall leaf length (cm) (Figure 1).

Data were analyzed using Analysis of Variance (ANOVA) and the significant difference was continued using Duncan Multiple Range Test (DMRT) at P = 0.05.

RESULTS AND DISCUSSION

Antibiosis mechanism of endophytic bacterial both single and consortium against Xoo

The results of the observation of the inhibition zone of endophytic bacteria against Xoo both single and consortium are shown in Table 1. The highest inhibitory power was the endophytic bacteria consortium, which was 4.47 mm. This showed that the consortium of 5 endophytic bacteria has stronger antibiosis activity than the single effect. One endophytic bacteria can produce more than one antibiotic compounds, so they become more effective in inhibiting pathogens when combined. Similar results were also shown observed from research conducted by (Resti et al. 2020) which revealed that the consortium endophytic bacteria treatment was more effective than the control. Further Oluwambe and Kofoworola (2016) also showed that the consortium of several effective strains for growth enhancement performed better than their individual culture of rhizobacteria. The single of endophytic bacteria has various control mechanisms against pathogens, so combining them in a consortium can increase the effectiveness of control. Compatibility tests have been carried out in the preparation of the consortium, so that the secondary metabolites produced will be able to synergize effect in increasing their effectiveness. One of the advantages of applying endophytic bacteria is because they live in associations in plant tissues so that they are more resistant to unfavorable biotic and abiotic factors.

The area of inhibition was calculated to detect inhibition which showed antibiosis activity around the colony or extracellular metabolites. The consortium of endophytic bacteria showed 2-7 fold activity different compare to the single endophytic bacterium. This research showed that the area of inhibition reach 62.74 mm^2 (Figure 2). The consortium endophytic bacteria can produce several secondary metabolites for plant growth promoters and disease controllers. All of the endophytic bacteria showed a bacteriostatic mechanism of their antibiosis effect (Table 1).

Antibiosis index of endophytic bacteria

The results of the Antibiosis Index (AI) calculation showed that a consortium endophytic bacteria was the strong character of inhibition, with an antibiosis index of 1.49 (Table 2). The strong antibiosis index showed that the bacteria can produce several secondary metabolites. The evaluation of the bacterial consortium in reducing Xoo under a limited control environment in the screen house showed various effectiveness (Suryadi et al. 2012).

Evaluation of endophytic bacteria as antagonist feature

Production of protease was shown by endophytic bacteria as a qualitative assay, which was observed asa clear zone around the scratch (Figure 3). In a qualitative plate assay, the clear zone in the protease-producing test of endophytic bacteria means that this bacteria is capable of producing proteases by hydrolysis of protein in skim milk agar.

The orange zone expression around a colony of endophytic bacteria indicated the production of siderophore by endophytic bacteria (Prihatiningsih et al. 2021). The bacterial siderophore test formed an orange zone indicating that the type of siderophore was hydroxamate (Ahmed and Holmström 2014; Prihatiningsih et al. 2017, 2021). Siderophores are low molecular weight compounds produced by bacteria and are capable of chelating iron into a form of siderophore-Fe^{3+} bonds available to plants. This siderophore is also one of the biocontrol mechanisms by binding to Fe^{3+} so the pathogen is limited in the need for Fe^{3+}. Therefore, the pathogen is inhibited from reproducing to reach the rhizosphere due to iron deficiency (Glick 2012; Prihatiningsih et al. 2017).

Disease intensity of bacterial leaf blight on rice by endophytic bacteria treatment was not significantly different in both single and consortium, but the treatment of KR4 and KR7 isolates was different from the control. The consortium treatment has an infection rate as same as the KR7 isolates of endophytic bacteria were 0.032 units/day. This is because that isolate KR7 comes from Karangwangkal, a marginal area because it is always planted with rice continuously, and because of its ability to produce siderophores. The endophytic bacterial consortium was composed of 5 isolates including KR7, thus showing the same bacterial leaf blight suppression activity. The evaluation of the bacterial consortium in reducing Xoo under a limited control environment in the screen house showed various effectiveness (Suryadi et al. 2012). Munif et al. (2019) mentioned that the application of a combined antagonistic formulation could decrease the disease intensity compared to the single antagonistic formulation in low-tide land rice plants.

The symptoms of rice blight are shown in Figure 4 that initially the symptoms only develop around the cut scar with scissors and then develop elongated white straw and dry. Characteristic symptoms of yellow to white water soaked stripes with wavy margins at the edges and on the leaf blade. The development of symptoms required a relatively short time during the study in the screen house, only within 10 days from the time the symptoms appeared, the infected leaves experienced quite severe symptoms. This suggests a virulent Xoo pathogen and environmental conditions favor disease development.

The development of Xoo pathogen infection can be suppressed by the application of antagonists such as endophytic bacteria with various control activities. Endophytic bacteria isolated from roots and leaves from a paddy field showed 6 isolates of positive antagonistic activity against Xoo on the nutrient agar plate, and successfully identified at molecular level by 16S rRNA amplification as *Enterobacter* sp., *Geobacillus thermoparaffinivorans*, *Gamma proteobacterium*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *B. cereus*. Subsequent tests at the experimental screen house showed that endophytic bacteria had the potential to reduce disease severity with suppressive efficacy of more than 80% demonstrated by endophytic bacteria isolates *Bacillus subtilis* and *Geobacillus thermoparaffinivorans* (Halim et al. 2020). Similarly, *Bacillus* isolates from potato rhizosphere have antibiosis activity in vitro with bacteriostatic mechanism against *Ralstonia solanacearum* and in the screen house, it can suppress bacterial wilt disease in potatoes with 64.8% effectiveness (Prihatiningsih et al. 2020b).

Table 1. Zone inhibition and antibiosis mechanism of endophytic bacteria against Xoo

Treatment of endophytic bacteria	Zone inhibition (mm)	Antibiosis mechanism
A5	2.20 ± 0.20 bc	Bacteriostatic
A6	1.67 ± 0.12 c	Bacteriostatic
KR4	2.40 ± 0.20 bc	Bacteriostatic
KR7	2.57 ± 0.15 bc	Bacteriostatic
SB3	2.83 ± 0.15 b	Bacteriostatic
Consortium	4.47 ± 0.15 a	Bacteriostatic

Note: Numbers followed by the same letter in the same column show no significant difference at 5% DMRT. A5, A6: Isolates from Petanahan Kebumen, KR4 and KR7: Isolates from Karangwangkal Banyumas, SB3: Isolate from Sumbang Banyumas, Indonesia

Table 2. The antibiosis index of endophytic bacteria against Xoo

Treatment of endophytic bacteria	Antibiosis index	The inhibition category (symbol and character)	
A5	0.73 ± 0	+	Weak
A6	0.56 ± 0.02	+	Weak
KR4	0.80 ± 0.03	+	Weak
KR7	0.87 ± 0.01	+	Weak
SB3	0.94 ± 0	+	Weak
Consortium	1.49 ± 0.02	++	Strong

Note: A5, A6: Isolates from Petanahan Kebumen, KR4 and KR7: Isolates from Karangwangkal Banyumas, SB3: Isolate from Sumbang Banyumas, Indonesia

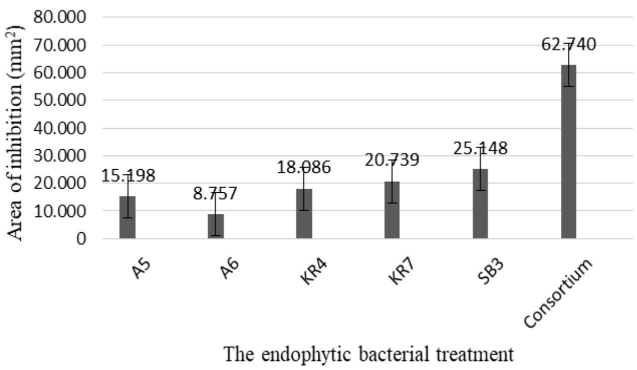

Figure 2. The area of inhibition between Xoo and endophytic bacteria. Note: A5, A6: Isolates from Petanahan Kebumen, KR4 and KR7: Isolates from Karangwangkal Banyumas, SB3: Isolate from Sumbang Banyumas, Indonesia

Table 3. The endophytic bacteria both single and a consortium to suppress bacterial leaf blight on rice

Treatment	The incubation period (days)	Disease Intensity (%)	Infection rate (r) (unit/days)
A	2.55 a	62.25 b	0.040
B	3.10 a	54.59 ab	0.030
C	3.25 a	46.75 ab	0.034
D	3.00 a	44.41 a	0.036
E	3.60 a	42.97 a	0.032
F	3.50 a	48.76 ab	0.034
G	4.10 a	46.65 ab	0.032

Note: Numbers followed by the same letters in the same column show no significantly different effect on the DMRT test with an error rate of 5%. A: Control, B: Endophytic bacteria A5, C: Endophytic bacteria A6, D: Endophytic bacteria KR4, E: Endophytic bacteria KR7, F: Endophytic bacteria SB3, and G: A consortium of 5 endophytic bacteria

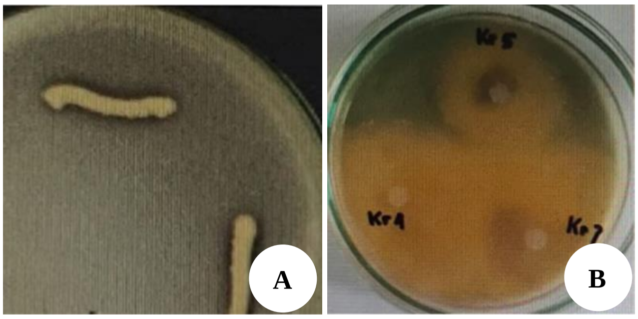

Figure 3. Protease and siderophore production by endophytic bacteria. A. the clear zone as protease test, B. the orange zone around a colony of endophytic bacteria or paper disk with 10 uL suspension of endophytic bacteria as siderophore test

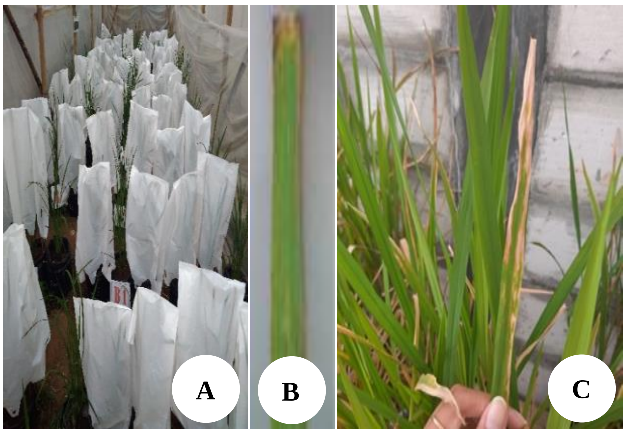

Figure 4. Inoculation treatment and symptoms of bacterial leaf blight. A. Cover the plants with polythene bag after inoculation, B. The symptom after 10 days of inoculation Xoo with endophytic bacteria treatment, C. Blight symptoms after 10 days of inoculation Xoo without endophytic bacteria treatment

From the present research it can be concluded that both the single endophytic bacteria and a consortium had the potential to suppress Xoo growth in vitro, and the highest was the consortium with an inhibitory area of 62.74 mm^2 and the mechanism of inhibition was bacteriostatic. The endophytic bacteria consortiums showed a strong antibiosis mechanism by 1.49 of the antibiosis index value. The endophytic bacteria consortium has the prospective as a biopesticide to be developed as an alternative to control rice bacterial leaf blight.

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