International Journal of Life Science and Agriculture Research

ISSN (Print): 2833-2091, ISSN (Online): 2833-2105

Volume 03 Issue 09 September 2024

DOI: https://doi.org/10.55677/ijlsar/V03I9Y2024-06

Impact Factor: 6.774 , Page No : 768-773

The Potential Plant Growth-Promoting Bacteria (PGPB) Consortia to Suppress the Growth of *Xanthomonas oryzae* pv. *oryzae* in vitro

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ABSTRACT: Bacterial leaf blight (BLB) is one of the main diseases in rice plants caused by the pathogenic bacteria Xanthomonas oryzae pv. oryzae (Xoo). Plant Growth-Promoting Bacteria (PGBP) are a group of beneficial microorganisms that interact with plants. The interaction of PGPB with plants can act as a biocontrol agent against plant pathogens.This study aims to obtain a PGPB consortium that can potentially suppress the growth of Xanthomonas oryzae pv. oryzae in vitro. The PGPB isolates used were Stenotrophomonas pavanii KJKB 54, Stenotrophomona maltophilia LMTSA 54, Stenotrophomonas maltophilia LMB35, Bacillus cereus AJ34, Serratia marcescens AR1, Ochrobactrum intermedium LMB1, Alcaligenes faecalis AJ14, and bacterial isolate Bacillus thuringiensis LmD13. Seven PGPB consortia were obtained which will be tested for their ability to suppress the growth of Xoo using an experimental method with a completely randomized design consisting of 8 treatments and 4 replications. The antagonist test of PGPB consortia against Xoo bacteria was carried out using the dual culture method. The result showed the consortium of Stenotrophomonas pavanii KJKB54+ Stenotrophomonas maltophilia LMTSA54+ Bacillus cereus AJ34+ Serratia marsescens AR1 cell consortium and supernatant had the highest potential to suppress Xoo. The antagonistic activities of the consortium were 58% and 52%. This consortium shows the ability to produce of protease enzyme, HCN and siderophores and can be developed as a biological agent against Xanthomonas oryzae pv. oryzae.

Published Online: September 20, 2024

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KEYWORDS: antibiosis, leaf blight, HCN, protease enzyme, siderophore.

INTRODUCTION

Bacterial leaf blight (BLB) is one of the main diseases in rice plants caused by the pathogenic bacteria *Xanthomonas oryzae* pv. *oryzae* (Xoo). This disease causes rice crop yield losses of up to 20%-100% during an epidemic (Yang et al., 2020). In Indonesia, yield losses due to this disease reach 21-36% in the rainy season and 18-28% in the dry season. *Xanthomonas oryzae* pv. *oryzae* can damage all phases of rice plant growth from seedling to harvest, with two typical symptoms: kresek and blight. Kresek symptoms appear in the vegetative phase of rice, while blight symptoms appear in the generative phase (Suparyono et al. 2016)

Various strategies have been developed to control bacterial leaf blight, including control using resistant plants and control using synthetic chemical bactericides. However, the continuous use of chemical bactericides negatively impacts environmental and human health (Sopialena et al., 2020). Thus, compatible and sustainable control techniques are needed to reduce the impact of chemical compounds by utilizing beneficial microorganisms around the plants (Wang et al., 2022).

Plant Growth-Promoting Bacteria (PGBP) are a group of beneficial microorganisms that interact with plants; these bacteria inhabit various ecological niches such as the rhizosphere, rhizoplane, colonize plant tissues (as endophytes), the surface of phyllostomid leaves, and even in seeds and flowers (Vuolo et al., 2022). In interacting with plants, bacteria involve various mechanisms, both direct and indirect mechanisms. Direct mechanisms involve nitrogen fixation, mineral solubilization (e.g., phosphorus and iron), siderophore production, and phytohormone production (e.g., auxin, cytokinin, gibberellin, and ethylene) (Katsenios et al., 2022). Meanwhile, indirect mechanisms involve biocontrol activities in response to biotic stress by producing antibiotics (Ngalimat et., 2021) and Induced Systemic Resistance (ISR) (Hata et al., 2021).

In addition to plants, PGPB also interacts with other microorganisms that will later form colonies that will have a beneficial or neutral impact on plants (Morales-Cedeno et al., 2021). Several PGPB bacteria combined into a consortium can become more effective biological agents than when used singly (Sarma et al., 2015). Duncker et al., (2021) reported that the combination of microorganisms in a consortium can control various plant pathogens more effectively. The consortium's PGPB can also offer better protection against plant pathogens. With various protection mechanisms provided by various PGPB species, the risk of pathogen attack is lower than using a single isolate. This study aims to obtain a PGPB consortium that can potentially suppress the growth of *Xanthomonas oryzae* pv. *oryzae* in vitro.

MATERIAL AND METHODS

The research was conducted at the Microbiology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Universitas Andalas, Indonesia, from June - November 2023. Seven PGPB consortia were obtained which will be tested for their ability to suppress the growth of *Xanthomonas oryzae* pv. *oryzae* (Xoo) using an experimental method with a completely randomized design consisting of 8 treatments and 4 replications. The treatment consists of the following PGPB consortia:

A. *Stenotrophomonas pavanii* KJKB54+ *Stenotrophomonas maltophilia* LMTSA54+ *Bacillus cereus* AJ34+ *Serratia marsescens* AR1

B. *Stenotrophomonas pavanii* KJKB54 + *Ochrobactrum intermedium* LMB1+ *Stenotrophomonas maltophilia* LMB35+ *Bacillus cereus* AJ34

C. *Bacillus cereus* AJ34+ *Stenotrophomonas pavanii* KJKB54+ *Serratia marsescens* AR1+ *Alcaligenes faecalis* AJ14

D. *Stenotrophomonas maltophilia* LMTSA54+ *Alcaligenes faecalis* AJ14+LMD13+ *Stenotrophomonas maltophilia* LMB35

E. *Stenotrophomonas maltophilia* LMB35+ *Stenotrophomonas pavanii* KJKB54+ *Alcaligenes faecalis* AJ14+ *Serratia marsescens* AR1

F. *Alcaligenes faecalis* AJ14 + *Bacillus thuringiensis* LMD13+ *Ochrobactrum intermedium* LMB1+ *Stenotrophomonas maltophilia* LMB35

G. *Ochrobactrum intermedium* LMB1+LMD1+ *Stenotrophomonas maltophilia* LMB35+ *Stenotrophomonas marsescens* AR1

H. Control

Rejuvenation of PGPB

The PGPB used in this study were rejuvenated from culture stocks in 20% glycerol. The rejuvenation process, a crucial step in our research methodology, involved the quadrant streak method on nutrient agar (NA) medium in a 9-cm-diameter petri dish, then incubated the rejuvenated isolates for 24-hour periods at room temperature to obtain single colonies and subsequently multiplied the bacteria for further testing.

Rejuvenation of Pathogenic Bacteria *Xanthomonas oryzae* pv. *oryzae* (Xoo)

The Xoo isolate used in this study is a collection from the Microbiology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Universitas Andalas. The Xoo from 20% glycerol media was rejuvenated using the quadrant streak method, a technique that allows for the isolation of a single colony, on Wakimoto Agar media and incubated for 2 x 24 hours.

Antagonist Test of PGPB Consortium Against *Xanthomonas oryzae* pv. *oryzae* in Vitro

The antagonist test of PGPB consortia against Xoo bacteria was carried out using the dual culture method. 100 µl of Xoo suspension (population density of 10^6 cells/mL) was spread evenly on Wakimoto media with glass beads. Then, sterile disc paper pieces (diameter 5 mm) were dipped into the PGPB consortium suspension (population density of 10^8 cells/mL) and dried for 1 minute. As a control, one paper disc was dipped into sterile distilled water. Furthermore, four disc paper that had been dipped with the PGPB consortium was arranged on a Wakimoto medium containing Xoo and one disc containing distilled water positioned in the center of the dish, and then incubated at room temperature for 2 × 24 hours. Antibiotic production was indicated by a clear zone around the paper disc pieces containing PGPB consortium cells. The antagonistic activity of the PGPB consortium was calculated using the following equation:

Inhibitory (%) = (clear zone diameter-disc paper diameter)/disc paper diameter

Bioactive Compound Activity of Supernatant of PGPB Consortia.

The bioactive compound activity test of PGPB supernatant was carried out using the (Monjezi et al., 2023): the PGPB consortium was taken as much as 1 ml and put into 100 ml sterile nutrient broth (NB) medium in a 250 ml Erlenmeyer flask. It was then incubated on a rotary shaker (100 rpm, at room temperature) for 24 hours. The suspension of the PGPB consortium was centrifuged at 14,000 rpm for 15 minutes to separate the bacterial cells from the supernatant containing secondary metabolites from the bacteria. Antibacterial activity from the supernatant was carried out using the Disc Diffusion method. The suspension of Xoo was spread on Wakimoto media in a 10 cm diameter petri dish. There was four pieces of sterile paper discs with a diameter of 5 mm were dipped into the PGPB consortium supernatant and then arranged in a petri dish containing Xoo; as a control, one paper disc was dipped in sterile distilled water positioned in the center and incubated at room temperature for 2x24 hours. Antibiotic production was indicated

by a clear zone around the pieces of a paper disc containing the PGPB consortium supernatant, and its inhibitory ability against Xoo was measured.

Detection of Antibacterial Properties of PGPB Consortium.

Hydrogen Cyanide (HCN) Production. HCN production by PGPB consortia was detected using the Abd El-Rahman et al., (2019) method. Consortia of PGPB were cultured on NA media. The filter paper was dipped in cyanide detection solution (CDS) (2 g picric acid and 8 g sodium carbonate in 200 ml sterile water) and then dried for 5 minutes. The filter paper was placed on the lid of a petri dish. As a control, the disc paper was placed in the same way but without endophytic bacteria culture, then incubated at room temperature for 4x24 hours.

Production of Protease Enzyme. Production of protease enzyme by the PGPB consortium was carried out according to Denizci et al. (2004); protease synthesis by PGPB was carried out on skim milk agar media supplemented with 0.1% glucose, 0.2% peptone, 0.5% yeast extract, 0.1% K2HPO4, 0.02% MgSO4.7H2O, and 0.5% skim milk. The pH of the media was adjusted to 10 using sterilized 10% Na2CO3. The skim milk agar media was inoculated with PGPB isolates and consortium at a temperature of 30±2 C, and the formation of a clear zone around the bacterial colony was observed.

Siderophore Production. The siderophore production by PGPB consortia was carried out using Chrome Azurol Sulfonate (CAS) Agar media based on Sultana et al., (2021). Each 1 liter of CAS Agar media consists of four solutions with each composition as follows: Solution (1) is a Fe-CAS indicator solution (composition: 10 ml of FeCl3.6H2O 1 mM dissolved in 10 mM HCL; 50 ml of CAS solution (1.21 mg/ml) and 40 ml of hexadecyl-trimethylammonium bromide (HDTMA) solution 1.82 mg/ml). Solution (2) is a buffer solution made by dissolving 30.40 g of piperazine-N, N-bis[2-ethanesulfolfonic acid] (PIPES) into 750 ml of salt solution (3 g KH2PO4, 5 g NaCl, 10 g NH4Cl, 20 mM MgSO4, 1 mM CaCl2). Aquadest is added until the solution volume reaches 800 ml; the pH of the solution is then measured and calibrated with 50% KOH until it reaches pH 6.8. Furthermore, 20 g of agar-agar is added to the solution before sterilization. Solution (3) contains 2 g of glucose, 2 g of mannitol and microelements consisting of 493 mg of MgSO4.7H2O, 11 mg of MnSO4.H2O, 1.4 mg of H3BO3, 0.04 mg of CuSO4.5H2O, 1.2 mg of ZnSO4.7H2O, and 1 mg of NaMoO4.2H2O, all components of solution 3 are dissolved in 70 ml of distilled water. Solution (4) consists of 30 ml of 10% (w/v) cassamino acid, sterilized using a 0.45 µm membrane filter. CAS medium is made by mixing solutions 2 and 4 at a temperature of 50 oC after sterilization, then solutions 3 and 1 are added slowly and then homogenized using a magnetic bar. CAS medium has a dark green color. The siderophore production test is carried out by scratching PGPB consortia aged 2 x 24 hours on CAS medium. The orange color around the PGPB consortium indicates that the bacteria can produce siderophores.

Data Analysis.

The data obtained from the experimental results were analyzed using Analysis of Variance (ANOVA). Data that were significantly different continued with the Least Significant Difference (LSD) test at a 5% significance level.

RESULT AND DISCUSSION

Antagonist Test of PGPB Consortium Against *Xanthomonas oryzae* pv.*oryzae*

The antagonist test of the PGPB consortia against pathogenic bacteria Xoo showed that all consortia could suppress the growth of Xoo colonies. All PGPB consortia showed the ability to suppress the growth of Xoo and were significantly different from the control in PGPB cells (PGPB pellets) and in supernatants from PGPB (Data in Table 2). The results of this study indicate that PGPB strain included in the consortium can jointly produce metabolite compounds that can suppress the growth of Xoo bacteria, which is indicated by the appearance of a clear zone around the PGPB consortium colony (Figure 2). The PGPB KJKB + LMTSA + AJ34 + AR1 cell consortium had the highest potential to suppress Xoo. This consortia was similar to the LMB1 + LMD1 + LMD35 + ARI and AJ34 + KJKB + AR1 + AJ14 consortia. The antagonistic activities of the three consortia were 58%, 56.00, and 42%, respectively. Meanwhile, the supernatant from the PGPB KJKB + LMB1 + LMB35 + AJ34 consortium showed a significant difference with other consortia with an antibiosis potential of 52.60%. In general, this consortium looks better compared to other consortia because both the cells and the supernatant can potentially suppress the growth of *Xoo* which is higher than other consortia. Danquah et al. (2022) reported that antibiotics are secondary metabolite compounds that kill other microorganisms at low concentrations (<10 ppm). Caulier et al., (2019) reported that antibiotics consist of a heterogeneous group of low molecular weight organic compounds that interfere with the synthesis of pathogen cell walls, cell membrane structures, and biogenesis of initiation complexes in smaller ribosomal subunits.

Table 1. The ability of PGPB consortia to suppress the growth of *Xanthomonas oryzae* pv. *oryzae*

PGPB consortia)**	Inhibition index of the PGPB consortia (%)	Inhibition index of PGPB consortia supernatant (%^
A. KJKB+ LMTSA+AJ34+AR1	58,0 a)*	52 a
B.KJKB+LMB1+LMB35+AJ34	34,0 bc	28 b
C.AJ34+KJKB+AR1+AJ14	42,0 abc	31 b

D.LMTSA+AJ14+LMD13+LMB35	22,0	c	19	b
E.LMB35+KJKB+AJ14+AR1	28,0	c	22	b
F.AJ14+LMD13+LMB1+LMB35	30,0	c	23	b
G.LMB1+LMD13+LMD35+ ARI	56,0	ab	16	b
Control	0,0	d	0.0	c

Description:

*)Numbers in the same column and followed by the same lowercase letter were not significantly different based on the LSD significance level of 5%

**)

A. *Stenotrophomonas pavanii* KJKB54+ *Stenotrophomonas maltophilia* LMTSA54+ *Bacillus cereus* AJ34+ *Serratia marsescens* AR1

B. *Stenotrophomonas pavanii* KJKB54 + *Ochrobactrum intermedium* LMB1+ *Stenotrophomonas maltophilia* LMB35+ *Bacillus cereus* AJ34

C. *Bacillus cereus* AJ34+ *Stenotrophomonas pavanii* KJKB54+ *Serratia marsescens* AR1+ *Alcaligenes faecalis* AJ14

D. *Stenotrophomonas maltophilia* LMTSA54+ *Alcaligenes faecalis* AJ14+LMD13+ *Stenotrophomonas maltophilia* LMB35

E. *Stenotrophomonas maltophilia* LMB35+ *Stenotrophomonas pavanii* KJKB54+ *Alcaligenes faecalis* AJ14+ *Serratia marsescens* AR1

F. *Alcaligenes faecalis* AJ14 + *Bacillus thuringiensis* LMD13+ *Ochrobactrum intermedium* LMB1+ *Stenotrophomonas maltophilia* LMB35

G. *Ochrobactrum intermedium* LMB1+LMD13+ *Stenotrophomonas maltophilia* LMB35+ *Serratia marsescens* AR1

H. Control

Detection of Antibacterial Properties of PGPB Consortium.

The results showed that the tested consortium produced antibacterial secondary metabolite compounds from bacterial cells and supernatants from the PGPB consortium. All of PGPB consortium able produced several secondary metabolite compounds bacteria include antibiotics, protease enzymes, cyanide acid, and siderophores, which act as antimicrobial compounds (Table 2). Previous study reported by Rahma et al. (2019) the single PGPB characterization showed that bacteria *Serratia marcescens* AR1, *Bacillus cereus* AJ34, and *Alcaligenes faecalis* AJ14 able to produce phosphatase enzymes, siderophores, and phytohormones Indole Acetic Acid. These compounds are used to interact with other microorganisms in nature. Santoyo et al. (2021) reported that the study of PGPB interactions individually have a beneficial effect on plants. Combined, it will have more beneficial potential because each microorganism has a different role, such as increasing plant growth, biocontrol agents, phytohormone modulation, and nutrient availability for plants. Montaño et al. (2013) reported that the consortium of *Serratia liquefaciens* and *Serratia phymuthica* can to stimulate root development and plant growth and biomass, while *Sinorhizobium fredii* and *Pantoea ananatis* can to stimulate biofilm formation on the roots of rice and legume plants.

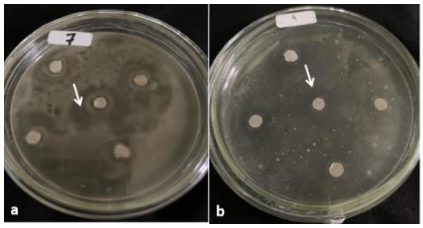

Figure 1. The ability of the consortium of *Ochrobactrum intermedium* LMB1+ *Bacillus thuringiensis* LMD13 + *Stenotrophomonas maltophilia* LMB35+ *Serratia marsescens* AR1 to suppress the growth of *Xanthomonas oryzae* pv. *oryzae.* a). Bacteria consortium, b). Supernatan of consortium

Table 2. Characterization of PGPB Consortia as a biocontrol agent against Xoo

PGPB Consortia	Production of Protease Enzyme	HCN Production	Siderophore Production
A. KJKB+ LMTSA+AJ34+AR1	+	+	+
B. KJKB+LMB1+LMB35+AJ34	+	+	+
C. AJ34+KJKB+AR1+AJ14	+	+	+
D. LMTSA+KJKB+LMB1+LMB35	+	+	+
E. LMTSA+KJKB+LMB1+LMB35	+	+	+
F. AJ14+LMD13+LMB1+LMB35	+	+	+
G. LMB1+LMD1+LMD35+ ARI	+	+	+

Description:

A. *Stenotrophomonas pavanii* KJKB54+ *Stenotrophomonas maltophilia* LMTSA54+ *Bacillus cereus* AJ34+ *Serratia marsescens* AR1

B. *Stenotrophomonas pavanii* KJKB54 + *Ochrobactrum intermedium* LMB1+ *Stenotrophomonas maltophilia* LMB35+ *Bacillus cereus* AJ34

C. *Bacillus cereus* AJ34+ *Stenotrophomonas pavanii* KJKB54+ *Serratia marsescens* AR1+ *Alcaligenes faecalis* AJ14

D. *Stenotrophomonas maltophilia* LMTSA54+ *Alcaligenes faecalis* AJ14+LMD13+ *Stenotrophomonas maltophilia* LMB35

E. *Stenotrophomonas maltophilia* LMB35+ *Stenotrophomonas pavanii* KJKB54+ *Alcaligenes faecalis* AJ14+ *Serratia marsescens* AR1

F. *Alcaligenes faecalis* AJ14 + *Bacillus thuringiensis* LMD13+ *Ochrobactrum intermedium* LMB1+ *Stenotrophomonas maltophilia* LMB35

G. *Ochrobactrum intermedium* LMB1+LMD13+ *Stenotrophomonas maltophilia* LMB35+ *Serratia marsescens* AR1

CONCLUSSION AND IMPLICATION

The result showed the consortium of *Stenotrophomonas pavanii* KJKB54+ *Stenotrophomonas maltophilia* LMTSA54+ *Bacillus cereus* AJ34+ *Serratia marsescens* AR1 cell consortium and supernatant had the highest potential to suppress Xoo. The antagonistic activities of the consortium were 58% and 52%. This consortium shows the ability to produce of protease enzyme, HCN and siderophores and it can be developed as a biological agent against *Xanthomonas oryzae* pv. *oryzae*.

ACKNOWLEDGEMENTS

Thanks' to the Directorate of Research, Technology, and Community Service. Directorate of Higher Education, Research and Technology of the Ministry of Education, Culture, Research and Technology, by Research Contract No. 115/E5/PG.02.00.PL/2023/Fiscal Year 2023.

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