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In Vitro Evaluation of Phosphate-Solubilizing Bacteria as Antagonists for Bacterial Leaf Blight Disease (*Xanthomonas oryzae* pv. oryzae)

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ABSTRACT

Rice is a crucial staple food crop in Indonesia, with over 50% of the Indonesian population relying on it for sustenance. However, the productivity of rice has been significantly reduced by 50-80% due to the prevalence of bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The extensive use of pesticides, which not only harm the environment but also facilitate the rapid spread of these bacteria, poses a major challenge in combating this issue. As an alternative approach, the utilization of biological agents, such as phosphate-solubilizing bacteria (PSB), has been explored. PSBs not only promote plant growth but also possess biocontrol capabilities. This study aims to investigate the inhibitory capacity of PSB derived from acidic soil against bacterial growth in vitro. The test was performed using the dual culture technique with the zone-of-inhibition method. The results showed that ten isolates of PSB inhibited *Xoo* with various diameters. The four isolates with the highest inhibitory activities were *Pseudomonas* sp. EF.NAP 3 (5.267 ± 0.189 mm), *Pseudomonas* sp. GSP 6 (4533 ± 0.772 mm), *Pseudomonas* sp. EF.NAP 4 (3,424 ± 0.161 mm), and *Pseudomonas* sp. EF.NAP 9 (3.167±0.136 mm). This indicates that the PSB isolate acts as a bio stimulant for the dissolution of phosphate in the soil and as a bioprotectant. The ability of PSB to inhibit *Xoo* can be used as an alternative to control bacterial leaf blight in rice plants.

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INTRODUCTION

Rice is an important agricultural crop in Indonesia, with over 50% of the population relying on it as a staple food (Hastuti et al., 2012). Rice consumption in Indonesia is increasing every year, in line with the growing population. In 2021, national rice production is expected to reach 54,415 million tons, with a harvested area of 10,411 million hectares, and rice productivity will reach 52.26 kg/ha (BPS, 2021). Indonesian people's dependence on rice makes them vulnerable to food insecurity if there are problems with rice production, such as crop failure due to pests and pathogens. *Xanthomonas* is a gram-negative bacterium that commonly infects several crops, including rice, citrus, cassava, tomatoes, sugarcane, passion fruit, and brassica (Xie et al., 2018). Rice leaf blight is caused by bacteria of this specific genus. The presence of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) bacteria have been reported to damage and destroy rice production worldwide, leading to the inability to meet the basic food needs of almost half of the global population (Jin et al., 2020). *Xoo* can reduce rice production by 10-12% in cases of mild infection and by 50-80% in cases of severe infection (BPS, 2018; Mew et al., 1993). In 2012, the land area in Indonesia affected by bacterial leaf blight was

81,119 ha (Direktorat Perlindungan Tanaman Pangan, 2012).

The highland areas affected by *Xoo* experienced significant losses. Symptoms caused by *Xoo*-infected rice leaf surfaces appear as yellow to green spots at the beginning of the leaf, followed by gray to white lesions along the leaf veins (Qian et al., 2013; Zhang & Wang, 2013). These symptoms lead to suboptimal photosynthesis, resulting in the formation of numerous empty grains and a decrease in rice productivity. Additionally, rice attacked by *Xoo* undergoes incomplete grain filling, causing the grains to break easily during milling (Saylendra et al., 2017). Consequently, the value of grain forselling decreases. *Xoo* bacteria can spread rapidly through improper handling, posing a threat to farmers worldwide (Xie et al. 2018). Chemical control of *Xoo* has been attempted using copper-based chemicals; however, these chemicals have negative impacts on human health, wildlife, and the environment (Bulgari et al., 2019). A more sustainable alternative for treating the disease is the use of biological control agents directly (bacterial isolates) or indirectly through secondary metabolites involved in biocontrol (Marin et al., 2019).

Microbes are effective and safe biocontrol agents for humans and other non-target organisms (Usta, 2013). Soil bacteria or rhizospheres can be beneficial because they induce resistance to plant pathogens (Berendsen et al., 2012; Jain & Pandey, 2016). Rhizosphere microbiota can enhance plant defense responses against disease threats by activating the innate immunity of the host plant (Jain & Pandey, 2016). After *Xoo* infection, the induction and accumulation of defense-related enzymes, such as glucanase, protease, chitinase, and siderophores, can suppress disease at the site of infection, preventing colonization and disease progression (Bardin et al., 2015). Rhizosphere microbes can produce various secondary metabolites that benefit plants with functions such as inhibitors, antimicrobials, and enzymes (Emmert & Handelsman, 1999; Krishanti et al., 2015).

Pseudomonas spp. are recognized as one of the genera capable of suppressing various plant diseases caused by soil-borne pathogenic microbes. This capability stems from its capacity to biosynthesize antimicrobial metabolites, including antibiotics, cyclic lipopeptides, siderophores, and hydrogen cyanide (Gross & Loper, 2009). Furthermore, *Pseudomonas* spp. can solubilize phosphate, thereby enhancing the availability of phosphate ions absorbed by plants (Rasul et al., 2019). During the phosphate solubilization process, phosphate-solubilizing bacteria (PSB) can generate organic acids, such as gluconic acid, through the glucose dehydrogenation process (De Werra et al., 2009; Gross & Loper, 2009). Gluconic acid is predominantly produced by endophytic and diazotrophic bacteria, such as *Gluconacetobacter diazotrophicus* Pal5. This organic acid, derived from glucose oxidation by glucose dehydrogenase associated with pyrroloquinoline-quinone in plant growth-promoting bacteria, serves as an antimicrobial agent that safeguards plants against pathogenic bacteria (Nieto-Peñalver et al., 2014), such as *Paenibacillus larvae* (Sagona et al., 2015).

The presence of phosphate-solubilizing bacteria is advantageous for plants, as they not only furnish nutrients but also confer protection against pathogenic bacteria. Thus, the exploration and isolation of PSB is imperative to identify potential bacterial candidates. In 2020, PSB were isolated from acidic soils in Lampung, with *Pseudomonas* spp. dominating the diversity of all obtained isolates (Asril et al., 2021; Asril & Lisafitri, 2020). However, further investigation into the potential of these PSB isolates as biocontrol agents against pathogenic bacteria, particularly *Xoo*, remains lacking. Considering PSB's classification of PSB as plant growth-promoting bacteria (PGPB), wherein another notable ability is biocontrol of plant diseases, PSB is hypothesized to possess antimicrobial activity against *Xoo* pathogenic bacteria. Therefore, this study aimed to assess the capacity of PSB from acidic soil to inhibit *Xoo* growth in vitro.

MATERIALS AND METHODS

This research was conducted at the Microbiology Laboratory of Institut Teknologi Sumatera in October 2021. Ten phosphate-solubilizing bacteria (PSB) isolates were obtained from acidic soil samples collected at the Institut Teknologi Sumatera. The isolate codes used were *Burkholderia territorii* EF.NAP 1 (Asril et al., 2023), *Pseudomonas* sp. EF.NAP 2, *Pseudomonas* sp. EF.NAP 4, *Pseudomonas* sp. EF.NAP 5, *Pseudomonas* sp. EF.NAP 9, *Pseudomonas* sp. EF.NAP 10 (Asril et al., 2021), *Pseudomonas* sp. GSP 1, *Pseudomonas* sp. GSP 6, and *Pseudomonas* sp. GSP 13 (Asril & Lisafitri, 2020). These isolates were cultured on Nutrient Agar (NA) supplemented with 5% $Ca_3(PO_4)_2$ and incubated at 30 ^oC for 48 h. *Xanthomonas oryzae* pv. *oryzae* strains were obtained from the Microbiology Laboratory collection at IPB University. *Xoo* was cultured on Nutrien Agar (NA) medium containing 5 g peptone, 1 g yeast powder, 3 g beef extract, 10 g sucrose, and 16 g agar powder per 1000 ml of ditilled water. The *Xoo* cultures were incubated at 30 ^{0}C for 24 h.

In vitro Evaluation of Phosphate-Solubilizing Bacteria (PSB) Inhibition Against Xanthomonas oryzae pv. oryzae Using Dual Culture Assay

Ten phosphate-solubilizing bacterial isolates were assessed for their ability to inhibit *X. oryzae* pv. *oryzae* (*Xoo*) using a dual-culture method. *Xoo* suspensions were prepared in sterile distilled water at a concentration of 10^8 cfu/ml. Subsequently, 0.1 ml of the *Xoo* suspension was evenly spread onto the surface of a petri dish containing solid sterile Muller Hinton Agar (MHA) medium. Additionally, a single loop of PSB bacteria was inoculated onto the surface of the MHA medium spread with *Xoo*. Each experimental procedure was replicated three times, and sterile distilled water was used as a control.

The test petri dishes were then incubated at 28±1 ^{0}C, and the inhibition of bacterial growth was assessed after 48 h of treatment (Yasmin et al., 2016). Inhibition diameter was determined using the following formula (Asril et al., 2020; Yasmin et al., 2016). The antimicrobial inhibition zone activity was categorized into four groups: weak (<5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (>20 mm) (Ouchari et al., 2019).

Inhibition diameter (mm) = Clear zone diameter (mm) – Colony diameter (mm)…. (1)

RESULTS AND DISCUSSION

A dual-culture antagonist test revealed that ten isolates of phosphate-solubilizing bacteria exhibited inhibitory activity against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The presence of a clear zone surrounding the bacterial colonies indicated this inhibitory activity (Figure 1). The inhibition diameters of the isolates ranged from 1.136±0.192 to 5.267±0.189 mm. Among the ten PSB isolates, four were identified as potential candidates due to their significant inhibitory powers, namely *Pseudomonas* sp. EF.NAP 3, *Pseudomonas* sp. GSP 6, *Pseudomonas* sp. EF.NAP 4, and *Pseudomonas* sp. EF.NAP 9, which exhibited inhibitory diameters exceeding 3.0 mm (Figure 2). The inhibitory activities of the PSB isolates against *Xoo* were categorized as either moderate or weak. Among the ten isolates, only one fell into the moderate category, while the others were categorized as weak. However, this level of inhibitory activity surpassed that of the *Pseudomonas* isolates obtained from the rice rhizosphere of the Kasemen District, India, which exhibited inhibition diameters ranging from 0.36-0.78 mm (Saylendra et al., 2017).

These findings underscore the high potential of PSB as bioinoculant for managing *Xoo* leaf blight. Additionally, PSBs aid in soil phosphorus acquisition and provide protection against *Xoo*, thereby enhancing the crop yield. Application of phosphate-solubilizing antagonistic bacteria has been demonstrated to effectively suppress bacterial leaf blight while promoting root growth (Rasul et al. 2019; Yasmin et al., 2016). Compared to screening the total bacterial population isolated from soil, phosphate-solubilizing bacteria present promising candidates in the selection process for potential antagonistic bacteria against target pathogens (Jahangir et al., 2016).

The ability of PSB isolates from soil to inhibit *Xoo* growth is attributed to various pathogen control mechanisms, including the production of antibiotic compounds, competition exclusion, bioactive compounds, extracellular enzyme secretion, and the synthesis of secondary metabolites, as well as the induction of plant resistance and promotion of plant growth. Bacteria inhabiting soil or the rhizosphere exhibit a multitude of inhibitory mechanisms against pathogens (Rahma et al., 2019). For instance, *Pseudomonas* sp. strain E227 harbors a gene responsible for the synthesis of the antibiotic 2,4-diacetylohloroglucinol (DAPG). The antagonistic activity of bacterial isolates is also due to their capacity to produce lytic enzymes and hydrogen cyanide (HCN), or from synergistic interactions between different metabolites (Santoyo et al., 2012; Velusamy et al., 2013).

The inhibitory activity against *Xoo*, characterized by the formation of a clear zone, has been attributed to isolates of *Stenotrophomonas malthopilia* strain LMG 958, *S. pavanii* strain LMG 25348, *Ochrobactrum ciceri* strain ca-34, *B. cereus* AJ34, and *A. faecalis* AJ14, which are capable of producing compounds such as antibiotics and extracellular enzymes (Rahma et al., 2019). Bacillus species, for instance, produce volatile compounds such as decyl alcohol and 3,5,5-trimethylhexanol, which exhibit antibiosis against *Xoo*. In addition to inhibiting *Xoo* growth in vitro, these compounds also suppress the transcription of *Xoo* virulence genes (Xie et al., 2018).

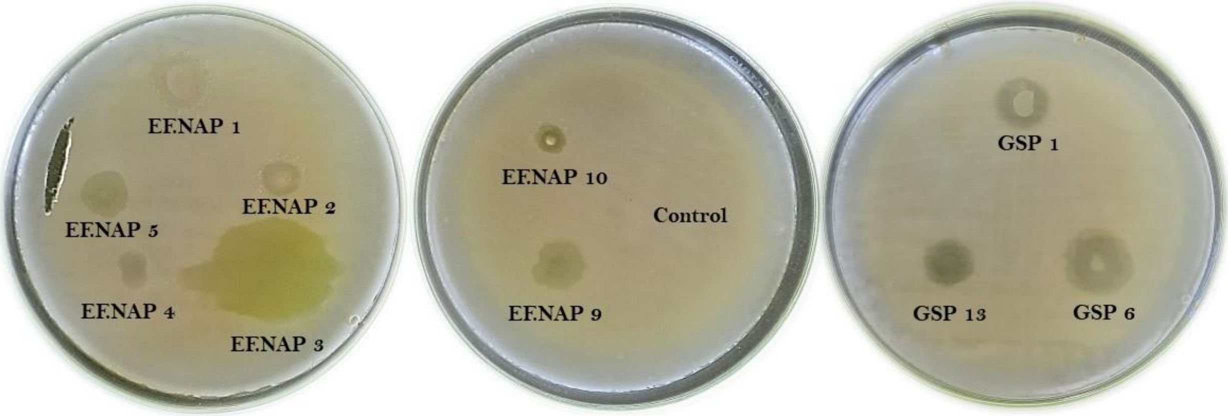

Figure 1. Inhibitory activity of PSB isolates against Xoo after 48 h of incubation

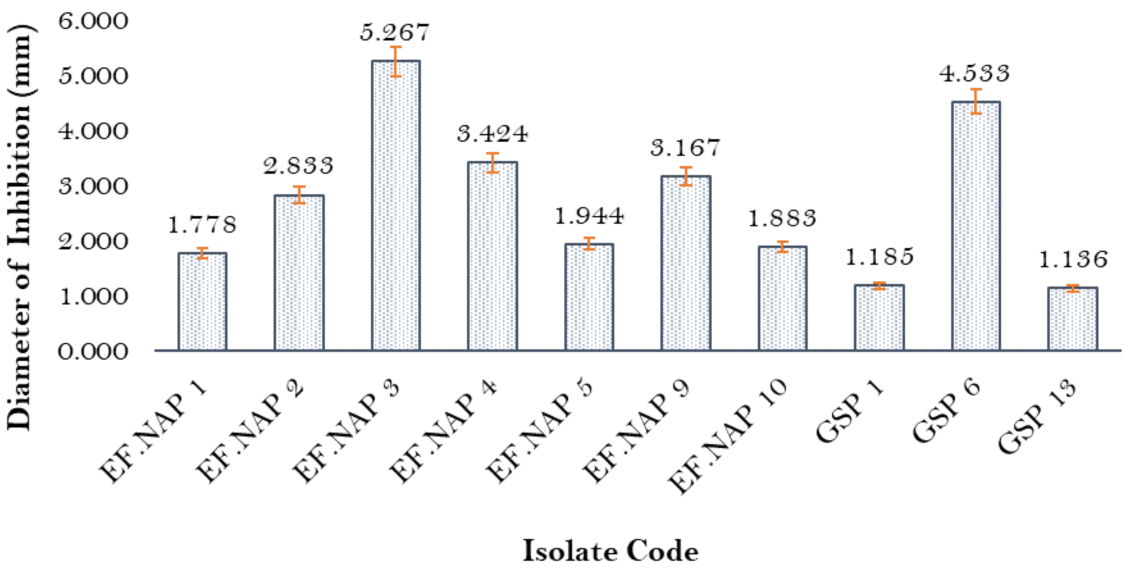

Figure 2. Diameter of inhibition of *Xoo* by PSB isolates

Furthermore, siderophore production serves as a determining factor for bacterial isolates to exert general biocontrol, as evidenced by isolates of *Pseudomonas* spp. E227, E233, Rh323 (Yasmin et al., 2016). Siderophore production is crucial for suppressing bacterial leaf blight (Naureen et al. 2009). *Pseudomonas* bacteria possess the ability to hydrolyze starch, produce cyanide, and exhibit glucanase activity. Bacteria capable of producing HCN can function as biocontrol agents to inhibit the growth of plant pathogens (Ramette et al., 2003). The Secondary metabolites of HCN produced by *Pseudomonas* have been demonstrated to suppress disease growth (Reetha et al., 2014). Moreover, glucanase activity contributes to direct antagonism (Santoyo et al., 2012).

In this study, three potential PSB isolates, *Pseudomonas* GSP 1, *Pseudomonas* GSP 6, and *Pseudomonas* GSP 13, belonging to the genus *Pseudomonas*, were used as biocontrol agents. *Pseudomonas* spp. are recognized for their potential as broad-spectrum biocontrol agents capable of enhancing plant growth (Santoyo et al., 2012; Velusamy et al., 2013). These PSB isolates from *Pseudomonas* sp. with diverse activities are expected to serve as bio controls against bacterial leaf blight (*Xoo*), bio stimulants, and bio-fertilizers, concurrently increasing phosphate absorption in diseased plants that lack nutrients. Phosphate-solubilizing bacteria have the ability to absorb and produce nutrients (such as nitrogen, phosphorus, and zinc) and phytohormones (such as indole-3-acetic acid (IAA)), thereby promoting plant growth (Naureen et al., 2009).

During the process of phosphate dissolution, PSB releases organic acids, which play a crucial role in phosphate solubilization and the biocontrol of *Xoo*. The presence and enrichment of organic acids contribute to the suppression of pathogen growth. Notably, the quantity of organic acids, particularly gluconic acid derived from glucose metabolism via dehydrogenation, significantly influences antibacterial activity and phosphate solubilization (Blok et al., 2007; Suleman et al., 2018). Several studies have underscored the integral role of glucose metabolism in the regulation of microbial metabolites that are essential for pathogen suppression (De Werra et al., 2009; Gross & Loper, 2009). Glucose metabolism in bacteria is mediated by genes such as pqq and gcd (glucose dehydrogenase). The presence of the gcd gene in *Pseudomonas* sp. confirms the contribution of glucose (in the form of gluconic acid) to phosphate solubilization and the biocontrol of plant pathogens (Chen et al., 2016). Further investigations into the diverse abilities of PSB, particularly those belonging to the genus *Pseudomonas* spp., to suppress *Xoo* growth warrant exploration. Specifically, elucidating the mechanism employed by isolates to suppress *Xoo*, whether through direct or indirect means, is imperative for a comprehensive understanding.

CONCLUSION

Ten phosphate-solubilizing bacterial isolates obtained from acidic soils inhibited the growth of *X. oryzae* pv. *oryzae*. Among these isolates, four exhibited the highest inhibitory activity, namely *Pseudomonas* sp. EF.NAP 3 (5.267±0.189 mm), *Pseudomonas* GSP 6 (4533±0.772 mm), *Pseudomonas* sp. EF.NAP 4 (3,424 ± 0.161 mm), and *Pseudomonas* sp. EF. NAP 9 (3.167±0.136 mm). The observed inhibitory activity against *Xoo* underscores the potential of PSB isolates as candidate agents for promoting plant growth. Nevertheless, as the ability of PSB bacteria to inhibit *Xoo* is primarily assessed in vitro, further investigation is warranted to ascertain their significant effect in reducing *Xoo* infection in planta, particularly in rice plants.

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