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Screening for endophytic bacteria from *Ambon Banana* (*Musa paradisiaca*) as biocontrol agent of anthracnose (*Colletotrichum gloeosporioides*) on bananas fruit

ARTINI PANGASTUTI♥, HESTI PRATIWI, RATNA SETYANINGSIH
Microbe Biomaterial Research Group, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: artini_p@staff.uns.ac.id

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Abstract. *Pangastuti A, Pratiwi H, Setyaningsih R. 2023. Screening for endophytic bacteria from Ambon Banana (*Musa paradisiaca *L.) as biocontrol agent of anthracnose (*Colletotrichum gloeosporioides*) on bananas fruit. Nusantara Bioscience 15: 238-244.* Post-harvest *Ambon Banana*s (*Musa paradisiaca* L.) are sensitive to anthracnose disease, caused by *Colletotrichum gloeosporioides*, and causes the fruit to rot quickly. Thus, chemical fungicides are employed, damaging living organisms and the environment. One solution is biocontrol using endophytic microorganisms as antagonistic agents against the anthracnose fungus that causes anthracnose disease. This study aimed to obtain potential endophytic bacteria from the *Ambon Banana* plant that had inhibitory activity against the growth of the pathogenic fungus *C. gloeosporioides* that causes anthracnose in bananas. Endophytic bacteria were recovered from *Ambon Banana* roots by crushing plant components. Therefore, bacterial isolates were tested for antagonistic interactions with pathogenic fungi using the dual culture approach. The 16S rRNA gene sequence analysis was used to identify bacterial isolates with the most significant inhibitory potential. According to the findings of this study, seven isolates of endophytic bacteria, A2-1, A2-2, A5-2, A6-2, A6-3, A8-1, and A9-1, can limit the growth of the pathogenic fungus *C. gloeosporioides*. The strain A6-3, identified as *Pseudomonas pseudoalcaligenes*, exhibited the greatest potential as a biocontrol agent against the pathogenic fungus *C. gloeosporioides*. It achieved the highest inhibition rate of 43.59%, resulting in an extended fruit shelf life and reduced harm susceptibility.

Keywords: Anthracnose, banana, biocontrol agent, *Colletotrichum gloeosporioides*, endophytes

INTRODUCTION

Ambon Banana (*Musa paradisiaca* L.) is an Indonesian tropical horticulture plant commonly used for its fruits, either directly or as a variety of meals. Because of its sweet flavor, this fruit is popular among the general people. Banana fruit farmers, on the other hand, regularly face various challenges, notably during the post-harvest, storage, and transportation procedures. The Ambon fruit banana is affected by pathogenic fungal infestation, which decreases fruit quality and leads it to degrade quickly. One of the pathogens is the fungus *Colletotrichum gloeosporioides*, which causes anthracnose in banana plants (Abd-Elsalam et al. 2010; Gautam 2014a; Gautam 2014b). Anthracnose attacks on various plant organs often start with the appearance of minute black or dark brown spots, which then grow and consolidate to cause damage to these organs (Bele et al. 2018). The pathogenic fungi can infect plants in the field and become inactive; after the banana fruit is harvested, *C. gloeosporioides* begin to grow again as the ripen banana fruit and cause damage. This fungal infection spreads faster in wounded tissue and ripe fruit (Unnithan et al. 2018).

Therefore, to achieve optimal agricultural yields, it is essential to carefully analyze plants' growth and defensive mechanisms throughout their developmental stages. Farmers depend on chemical fertilizers and pesticides to enhance productivity, while infections are treated with antibiotics or antifungals. The long-term consequences of chemical substance overabundance in the cultivation system can lead to detrimental outcomes (Ayesha et al. 2021). Synthetic fungicides, namely thiabendazole, prochloraz, benomyl, and carbendazim, are commonly employed in agriculture for their fungicidal properties. Those substances mentioned above are chemical fungicides that possess the ability to inhibit or mitigate the occurrence of anthracnose in bananas. Spray or dips can be applied to banana fruits before or after harvesting.

Previous studies have documented that fungicides have a greater propensity to elicit adverse impacts on non-target soil microbial communities when compared to insecticides and herbicides, potentially compromising soil fertility (Muñoz-Leoz et al. 2013). In addition, fungicides have a significant influence on several non-target beneficial organisms, such as antagonistic organisms, predators, and parasites that play a crucial role in controlling harmful insects. Certain fungicides can undergo leaching, thereby infiltrating the deeper soil layers and contaminating the groundwater table. This contamination poses a significant risk to human health (Pathak et al. 2022). In addition, pathogens can acquire resistance due to the indiscriminate application of fungicides, leading to more aggressive strains (Goswami et al. 2018).

Moreover, with the many losses connected with chemical fungicides mentioned above, biocontrol is considered a more environmentally sustainable and less

hazardous approach for mitigating anthracnose infestation in banana crops (Fu et al. 2017; Vieira et al. 2017). Endophytic bacteria are found in plants and can be used to treat pathogenic fungi because they produce compounds that inhibit or kill pathogenic germs. Endophytic bacteria live in the same ecological niche as plant pathogens. As a result, they are more effective at treating plant infections (Hong and Park 2016). Moreover, it can be argued that endophyte bacteria exhibit more excellent ecological friendliness and sustainability than chemical fungicides. These entities do not contribute to environmental pollution, soil degradation, or residue-related issues (Xu et al. 2021). In addition, these substances do not harm advantageous creatures, including pollinators, predators, and decomposers. Endophytes can produce bioactive compounds with antimicrobial, antifungal, or antiviral activity, such as siderophores, antibiotics, hydrolytic enzymes, organic acids, and volatile organics (Alam et al. 2021). Endophytic bacteria, such as *Bacillus* CA8 and *Pseudomonas* PK5, have been widely reported to suppress various plant diseases, including blood disease in banana plants (Nawangsih 2007). The presence of endophytic bacteria within the roots of plants leads to the production of chitinase and protease enzymes, which possess the ability to impede the growth and development of pathogenic fungus (Pradana et al. 2016). This study aimed to obtain potential endophytic bacteria from the *Ambon Banana* root that have inhibitory activity against the pathogenic fungus *C. gloeosporioides* that causes anthracnose in bananas. Endophytic bacteria derived from the same host are expected to have more potential to be applied as biocontrol agents for anthracnose disease on bananas.

MATERIALS AND METHODS

Isolation of endophyte bacteria

The roots of the *Ambon Banana* plant from Sukoharjo District in Central Java, Indonesia were utilized to obtain endophytic bacteria. The banana roots from 5 individual plants were thoroughly cleaned with running water, dried with a paper towel, and weighed up to 1 gram. The roots were surface sterilized by immersing them in 70% alcohol for 30 seconds, followed by 2 minutes in a 2% NaOCl solution. Next, using sterile distilled water, the roots were cleaned four times. The surface-sterilized roots were mashed in a mortar with 9 mL of distilled water. The filter paper (Whatman grade 1 qualitative filter papers) filters the root solution. To each petri dish containing Luria Agar (LA) medium, 100 µL of root solution was spread with dry galski rods. The inoculated media were incubated for 72 hours. The formed single colonies were then isolated and purified.

Fungus culture

The *C. gloeosporioides* isolates were collected from the Indonesian Culture Collection (InaCC) LIPI. The fungus was rejuvenated using a Potato Dextrose Agar (PDA) medium. PDA medium was prepared by dissolving 3.9 grams of PDA powder (Oxoid, England) in 100 mL of distilled water and autoclaved for 15 minutes at 121ºC. Petri dishes were filled with PDA medium and left to cool. PDA medium was inoculated with the fungus *C. gloeosporioides* using a culture needle at the center. The mixture was then cultured for 5-7 days to allow the fungus to grow (Mayadianti et al. 2020).

In vitro antagonistic assay

The dual culture approach using PDA media was used to observe bacterial isolates' ability to inhibit the growth of *C. gloeosporioides*. The test was conducted for 10 pure bacterial isolates from the previous process. Fungal mycelium was taken as an agar plug using the cork borer. A fungal agar plug was placed in the center of each Petri dish. Bacteria were scratched along a 2 cm line beside the plug; a 3 cm distance separated bacteria and fungus. In addition, as a control, a petri dish containing only the fungus *C. gloeosporioides* was placed in the center of the petri dish. The inoculated Petri dishes were incubated for 7 days at 25ºC (Hamaoka et al. 2021). Inhibitory measurement data is calculated to determine the percentage of inhibition using the formula:

$$P = \frac{R1-R2}{R1} \times 100\%$$

Note: P= inhibition percentage; R1 = average diameter of the pathogenic fungal colonies in the control; R2 = average diameter of pathogenic fungal colonies in the treatment

Chitinase assay

Colloidal chitin was prepared by dissolving 2 grams of powdered chitin into 20 mL of 12 M HCL. The mixture was added to 400 mL of cold distilled water and incubated at 4ºC for 24 hours. Colloidal chitin was washed with water until the pH was 7. Solid chitin media was made with the composition of colloidal chitin 3 g, K_2HPO_4 0.105 g, $MgSO_4$ 0.075 g, KH_2PO_4 0.045 g, $FeSO_4$ 0.0015, agar 2.25 g, and 150 mL distilled water. All ingredients were stirred using a magnetic stirrer until dissolved and put into an Erlenmeyer flask. The chitin media solution was then sterilized. The chitin medium was poured into a petri dish and allowed to harden, and the bottom of the petri dish was divided into four regions. Bacterial isolates were inoculated on the specified area. The petri dish was incubated at 30ºC for 16-48 hours. A clear zone around the bacteria was observed (Lau et al. 2020). The chitinolytic index of each isolate was calculated. A chitinolytic index is a ratio between the clear zone's and bacterial colonies' diameter. According to Suryadi et al. (2013), the formula for determining the Chitinolytic Index (CI) is:

$$CI = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

Morphological characterization of selected bacterial isolates

The bacteria that demonstrated the ability to inhibit the growth of *C. gloeosporioides* were selected for further characterization. Luria Agar (LA) media was used

to characterize bacterial isolates morphology. The LA media composition consisted of 3.75 g of Luria-Bertani broth (HiMedia, India) and 2.25 g of Bacto agar (Oxoid, England) mixed with 150 mL of distilled water. The media was sterilized with autoclaving at a temperature of 121°C for 15 minutes, transferred into petri dishes, and subsequently allowed to solidify. The bacterial isolate was aseptically streaked in a quadrant on an agar plate. The sample was subjected to incubation for 24-48 hours until the emergence of a solitary colony. The single bacterial colonies formed were observed for various characteristics, including color, shape (round or spread), fringe (regular or irregular), and elevation (rising, convex, or flat) (Sanjaya et al. 2019).

Gram stain

A single loop of bacterial culture was deposited onto the surface of a glass slide and afterward submerged in sterile distilled water. Subsequently, the isolate was fixed on the surface of the slide by passing it over an open flame. The isolate was subjected to a 1-minute exposure to crystal violet, followed by a thorough washing and subsequent drainage. The isolate was subjected to a 2-minute iodine treatment followed by a subsequent washing process for the next step. The isolate was then subjected to a treatment involving the application of 96% ethanol, followed by a subsequent washing and draining process. Last, the bacterial isolates were subjected to a 30-second safranin treatment followed by a next washing step.

Bacteria identification

Identification of selected bacterial isolates was carried out at PT. Genetika Science Indonesia, based on the gene sequences encoding 16S rRNA (Stackebrandt and Goebel 1994). The genomic DNA of the isolates was extracted with Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005) and then amplified using a 16S RNA PCR primer (27F/1492R) with MyTaq HS Red Mix (2X) (Bioline, BIO-25048). The PCR products were sequenced, and then the sequences were compared with the database using BLAST.

RESULTS AND DISCUSSION

Results

A total of ten isolates of endophytic bacteria were acquired from the roots of *Ambon Banana* plants. The isolates were assigned alphanumeric identifiers A1-1, A2-1, A2-2, A4-1, A5- 2, A6-1, A6-2, A6-3, A8-1, A9-1. Furthermore, 7 isolates can prevent the growth of the pathogenic fungus *C. gloeosporioides* (Figure 1). The isolate exhibiting the highest percentage of inhibition was A6-3, which had an inhibition index of 43.59% (Table 1). In comparison to earlier experiments, the isolate exhibited low levels of antifungal activity. None of the isolates showed chitinolytic activity, which suggested that the mechanism of inhibition of fungi from these bacterial isolates was not through the production of chitinase enzymes that damage the cell walls of fungi.

Endophytic bacteria selected had different morphological characteristics in color, shape, periphery, and colony elevation (Table 2). Root endophytes encompass a wide array of taxonomic groups, each contributing distinct functional attributes to the plant-microbe association. This diversity manifests in various forms, from taxonomic richness to functional versatility, impacting nutrient acquisition, pathogen suppression, and overall plant fitness. Most of the bacterial isolates belong to the group of gram-negative bacteria. Based on the results of identification using the gene marker encoding 16S rRNA, isolate A6-3, which had the highest inhibition against the growth of the fungus *C. gloeosporioides*, was *Pseudomonas pseudoalcaligenes*.

Table 1. Inhibition index of fungal growth of *C. gloeosporioides* by endophytic bacteria from *Ambon Banana* roots with two replicates

Bacterial Isolate	Inhibition Index Against Fungus C. gloeosporioides (%)	Chitinolytic Activity
A1-1	0	-
A2-1	17.95	-
A2-2	16.67	-
A4-1	0	-
A5-2	30.77	-
A6-1	0	-
A6-2	7.69	-
A6-3	43.59	-
A8-1	21.79	-
A9-1	11.54	-
Control	0	-

Table 2. Characteristics of selected endophytic bacteria isolates from *Ambon Banana* roots

Isolate Code	Gram Stain	Colony Color	Form	Periphery	Elevation	Identification (based on 16S rRNA gene sequence)
A2-1	+	White	Round	Regular	Convex	na
A2-2	+	Cloudy white	Round	Regular	Rising	na
A5-2	-	Clear white	Round	Regular	Flat	na
A6-2	+	White	Round	Regular	Flat	na
A6-3	-	White	Spread	Irregular	Flat	*Pseudomonas pseudoalcaligenes*
A8-1	-	White	Round	Irregular	Flat	na
A9-1	-	Yellowish	Round	Regular	Flat	na

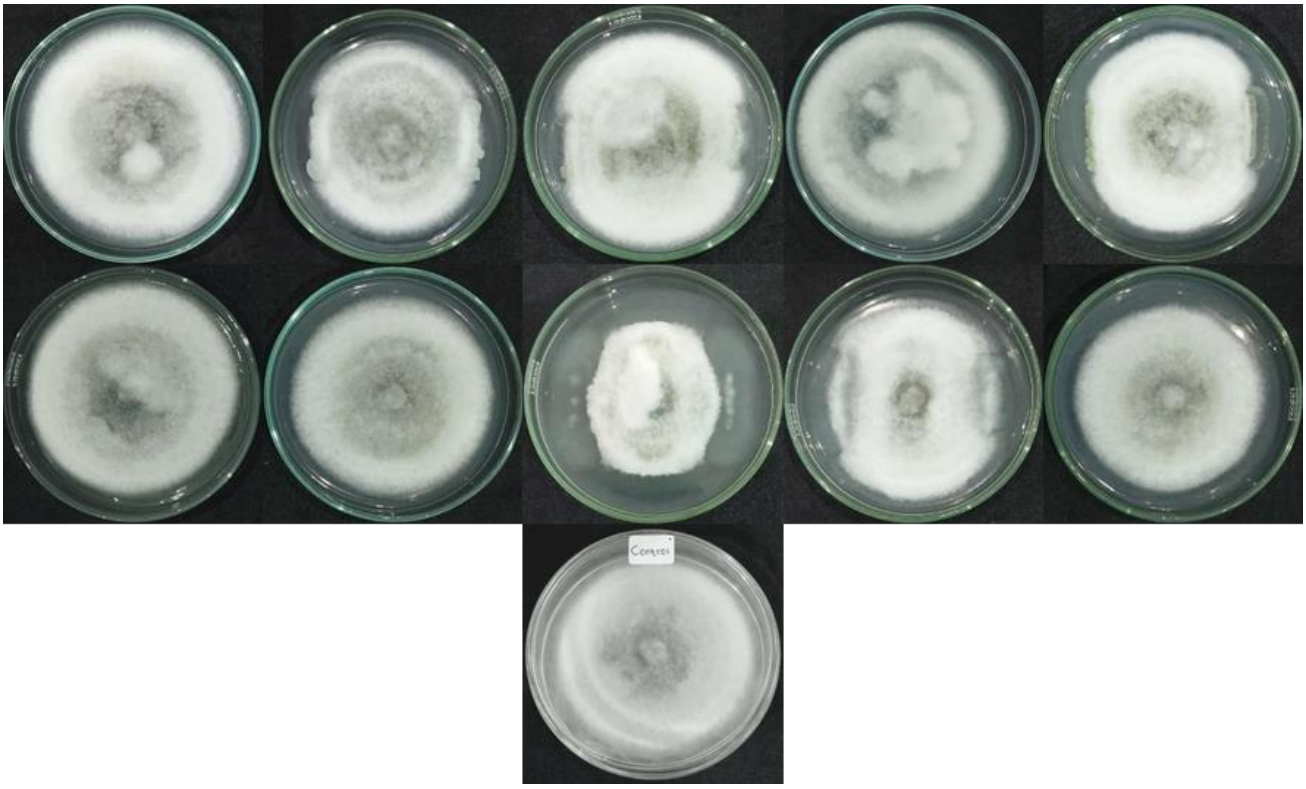

Figure 1. Inhibition of the growth of pathogenic fungi *C. gloeosporioides* by endophytic bacteria of *Ambon Banana* roots *in vitro*: from left to right, top: A1-1, A2-1, A2-2, A4-1, A5- 2; middle: A6-1, A6-2, A6-3, A8-1, A9-1, below; control *C. gloeosporioides*

Discussion

This study successfully obtained several endophytic bacterial isolates from the roots of banana ambon that showed inhibitory activity of the fungus *C. gloeosporioides*. However, the fungal inhibitory activity of these bacterial isolates was still relatively low to be applied as a biocontrol agent. The effectiveness of endophytic bacteria in inhibiting the pathogenic fungus is considered adequate if it exceeds 60%. Nevertheless, it is advisable to employ endophytic bacteria with an inhibitory activity surpassing 90% to suppress pathogenic fungi in agricultural settings successfully (Sanjaya et al. 2019). Isolate A6-3 with an inhibition index of less than 60% was classified as less effective if it was to be applied as a biocontrol agent. Suboptimal growth conditions may contribute to diminished antifungal effectiveness by impeding the production of bioactive antifungal compounds. In order to increase this isolate's antifungal activity, it is necessary to characterize the ideal conditions for growth and metabolite production. The symbiotic relationship between endophyte and host plants affects the production of secondary metabolites, such as antifungal compounds (Alam et al. 2021), so obtaining antifungal activity outside the host requires specialized strategies. Pathogenic infections cause the host to produce certain compounds, such as salicylic acid, jasmonic acid, and ethylene, that can trigger the production of antifungal compounds in bacterial endophytes (Ryan et al. 2008). This may be fine if the endophyte is applied to banana plants because it comes from the same host, so it can produce an antifungal compound when the pathogenic fungi infect the host. Therefore, further tests are needed to apply this bacterium on banana plants to prevent *C. gloeosporioides* infections.

None of the isolates had chitinolytic activity on the chitin agar media. Chitinase is one of the antifungal mechanisms of bacteria. Endophytes employ various enzymes that facilitate the sequential hydrolysis of the plant cell wall to establish colonization on plants' surfaces. These enzymes play a role in the indirect reduction of phytopathogens and contribute to the destruction of fungal cell walls. However, the expression of endophytic bacterial chitinase enzymes also requires induction from the host. Some bacterial endophytes may produce this enzyme constitutively, while others may produce them only under certain conditions, such as nutrient availability, environmental stress, or signal molecules from the host (Mushtaq et al. 2023). Thus, if the bacteria are outside the host, the chitinase enzyme will not be produced due to the absence of signals from the host. There is also the possibility that these bacterial isolates inhibit fungal growth by other enzymes or metabolites. Besides chitin, glucans are also found in the cell walls of fungi and some bacteria, and glucanases can inhibit fungi by breaking down their cell wall structure and integrity (Kulshreshtha and Sharma 2022). Several enzymes produced by bacteria can also degrade the cell walls of pathogenic fungi, such as proteases and cellulases (Boiu-Sicuia et al. 2023). Other non-enzymatic antibacterial and antifungal compounds, such as siderophores, antibiotics, organic acids, and volatile organics, can be produced by bacterial endophytes (Digra

and Nonsom 2023). These compounds inhibit fungal pathogens' growth, metabolism, or cell integrity.

Based on the sequence of the 16S rRNA gene, isolate A6-3, which had the highest inhibition against the growth of the fungus *C. gloeosporioides*, was identified as *P. pseudoalcaligenes*, a species of bacteria that belongs to the genus *Pseudomonas*, which is known for producing various secondary metabolites with antimicrobial activities. One study reported that an isolate of *P. pseudoalcaligenes* (FP-2) from the root nodules of *Vicia faba* L. showed antifungal activity against *Fusarium* spp. These fungal pathogens cause plant wilt disease (Eshetu and Tesfaye 2020). According to the research of Pliego et al. (2007), *P. pseudoalcaligenes* strain AVO110 was able to control white root rot disease in avocados caused by the pathogen *Rosellinia necatrix* by reducing disease progression to 45% compared to controls. The mechanism of inhibition by *P. pseudoalcaligenes* strain AVO110 against the pathogen *R. necatrix* is a competition for nutrients and niches (Pliego et al. 2019). The *P. pseudoalcaligenes* also produce siderophores, which mediate iron competition with pathogenic fungi (Gamit and Tank 2019).

Some species of *Pseudomonas* were also reported to be able to produce volatile compounds. The Volatile Organic Compounds (VOCs) emitted by *Pseudomonas* sp., such as acetophenone, 1,3-butadiene, 2-undecanone, benzaldehyde, 1,2-benzisothiazol-3(2H)-one, dimethyl trisulfide, dimethyl disulfide, benzothiazole, nonanal, N,N-dimethyldodecylamine, 3,5,5-trimethyl-1-hexanol, isovaleric acid, cyclohexanol, 2-ethyl 1-hexanol, n-decanal, decyl alcohol, have been documented for their antagonistic properties (Naz et al. 2022). These compounds have been found to induce resistance in host plants against various bacterial and fungal pathogens. Dimethyl disulfide is one of the volatile compounds produced by *Pseudomonas fluorescens* that can inhibit the growth of the fungus *Penicillium italicum*, which causes green mold disease in citrus, which is characterized by changes in spore morphology, spore collapse, hyphae rupture, and shrinks. Mycelium is broken without any attached conidia (Wang et al. 2021). Schulz-Bohm et al. (2017) investigated the VOCs produced by *Pseudomonas putida* KT2440 and their effects on *Fusarium oxysporum* f. sp. *lycopersici*, a fungus that causes wilt disease in tomato plants. They found that 2-phenylethanol was the most effective VOC in inhibiting fungal growth and spore germination and that it induced plant defense responses and enhanced plant resistance to the fungus.

VOCs produced by bacteria can inhibit the fungus *C. gloeosporioides* and overcome anthracnose disease on fruit. According to research by Huang et al. (2014) and Reyes-Perez et al. (2019), *Bacillus atrophaeus* and *Stenotrophomonas rhizophila* produced volatile compounds that interfered with hyphae development and spore germination of the pathogenic fungus *C. gloeosporioides*. This happens because volatile compounds can destroy the primary function consistency and degrade fungal cell walls, causing cell damage and leakage of intracellular substances, resulting in bending and morphological changes in fungal hyphae and spores (Tenorio-Salgado et al. 2013). Further research is needed on whether VOCs are the compounds responsible for inhibiting *C. gloeosporioides* by *P. pseudoalcaligenes* A6-3 isolates.

Previous studies have reported using endophytic bacteria as biocontrol agents for anthracnose disease on bananas or other fruits. Damasceno et al. (2019) reported the isolation and identification of endophytic and rhizosphere bacteria from sisal plants (*Agave sisalana*) that showed antagonistic activity against *C. musae*, the causal agent of anthracnose in bananas. The most effective strains belonged to the genera *Pseudomonas*, *Bacillus*, and *Paenibacillus*. They also showed plant growth promotion and induced systemic resistance in banana plants. The utilization of endophytic bacteria as biocontrol agents has been demonstrated to exhibit greater efficacy compared to chemical fungicides. The efficacy of *S. rhizophila* in treating mangoes for anthracnose was found to surpass that of synthetic fungicides, establishing the bacteria as a superior, cost-effective, and environmentally benign alternative for managing *C. gloeosporioides* (Reyes-Perez et al. 2019).

Several advantages are associated with using endophytes as biocontrol agents for suppressing host diseases. Endophytic microorganisms can establish residence within the internal tissues of plants and engage in competitive interactions with phytopathogens, impeding their growth and development (Fadiji and Babalola 2020). In addition to producing various antimicrobial compounds, endophytes can augment plants' resilience by initiating and preparing the host's defensive responses, including synthesizing phytoalexins, phenolics, and enzymes (Tewari et al. 2019). Endophytic microorganisms enhance plant development and improve stress tolerance by many mechanisms, such as hormone production, mineral solubilization, nitrogen fixation, and pollutant detoxification. *Pseudomonas* spp. produce plant hormones, such as auxins, cytokinins, gibberellins, and ethylene, that regulate plant growth and development. For example, *P. fluorescens* can produce Indole-3-Acetic Acid (IAA), an auxin type that stimulates root elongation and branching in maize and wheat plants (dos Santos et al. 2020). *Pseudomonas* spp. is also known as a producer of organic acids, siderophores, and chelating agents that dissolve insoluble forms of these minerals and make them accessible to plants (de Andrade et al. 2023). The *P. stutzeri* form nodules on the roots of legumes and non-legumes and fix atmospheric nitrogen into ammonia, a form that plants can assimilate. Therefore, the *P. pseudoalcaligenes* A6-3 that we successfully isolated can be used as a biocontrol agent for anthracnose disease on banana fruit while assisting the growth of the host. The advantage of this bacterium is that it was isolated from banana plants, so it is expected to colonize banana fruit and inhibit the growth of pathogens that cause anthracnose disease that damages post-harvest banana fruit.

The occurrence of anthracnose disease on bananas is classified as a post-harvest disease. Consequently, applying biocontrol strategies to mitigate this disease typically involves post-harvest protection techniques. These strategies aim to ensure the safety of the fruit from transit

and storage (Morales-Cedeño et al. 2021). Most practices for applying bacteria as a biocontrol agent during the post-harvest phase involve spraying a bacterial suspension onto the specific fruit or vegetable being targeted. Numerous research studies have investigated using post-harvest biocontrol techniques on fruit, employing diverse application means. Damasceno et al. (2019) applied the endophytic bacterial suspension onto bananas post-harvest using spraying, as the bacteria obtained were determined to be non-pathogenic to humans. So far, *P. pseudoalcaligenes* is not considered pathogenic to humans or plants, making it safe to apply as a biocontrol agent. In the future, *P. pseudoalcaligenes* A6-3 may be used by applying a bacterial suspension to the root of banana plants or onto post-harvest *Ambon Banana*s. Further research is still needed to apply this bacterium in the field.

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