



Test of Hypersensitivity and Antagonistic Reaction of Endophytic Bacteria from *Klutuk* Banana (*Musa balbisiana*)

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Abstract

Background: Plants' response and antagonistic reaction against disease-causing organisms are two crucial characteristics of Plant Growth Promoting Bacteria (PGPB). Seventeen endophytic bacteria have been isolated from *Musa balbisiana*. However, the plants' reaction against these isolates and their antagonistic activities against disease-causing organisms remained unknown. This study aims to (1) determine the sensitivity effect of endophytic bacteria isolates to tobacco and (2) test the antagonism of endophytic bacteria isolates against the blast disease actor, namely *Pyricularia oryzae*. **Method:** Sensitivity tests were performed by inoculating bacterial isolates into tobacco leaves with infiltration. Bacterial isolates were prepared with a minimum OD 600: 0.5. Then, a milliliter volume of each isolate was infiltrated into tobacco leaves from the abaxial side using a 3 mL syringe. The lesion on the tobacco leaves was observed seven days after inoculation (DAI). The antagonism test was carried out by growing *Pyricularia oryzae* on a PDA plate for seven days, subsequently transferred to an NA medium for three days then inoculated with endophytic bacteria. The assessed parameter of the antagonistic test was the formation of an inhibitory zone between endophytic bacteria and *Pyricularia oryzae* at 4 DAI. **Results:** No hypersensitive reaction of tobacco leaves against K117, K324, K38, K86, K18, K28, K102 isolates inoculation at 7 DAI. Furthermore, the antagonistic test indicated that all isolates inhibited the growth of *Pyricularia oryzae*, with the range of inhibition from 32.36 to 40.46%. **Implication:** Thus, these results revealed the PGPB characteristics in the newly isolated endophytic bacteria from the banana.

Keywords: Antagonism Reaction; Endophytic Bacteria; Hypersensitivity Reaction Test; *Pyricularia oryzae*



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Introduction

Endophytic bacteria are microorganisms that reside in plant tissue for a specific duration and can form colonies without causing any harm to the host organism. The bacteria thrive within the plant tissue. Each higher plant contains several endophytic microbes capable of producing biological compounds or secondary metabolites, which may result from co-evolution or genetic recombination of the host plant into endophytic microbes (Lestari et al., 2021). Endophytic bacteria can produce compounds that protect plant tissues from attacks by pathogenic microorganisms, while plant tissues provide nutritional needs for endophytic microbes to stay alive (Sadikin et al., 2021). Additionally, endophytic bacteria can produce secondary metabolites similar to their host's secondary metabolite due to genetic transfer from the host plant to endophytic bacteria (Ruslan et al., 2022). Endophytic bacteria can act as biocontrol agents, plant growth boosters, or Plant Growth Promoting Bacteria (PGPB).

The advantages of endophytic bacteria as biological control agents, some of which are also able to increase plant growth, they can increase the availability of nutrients, produce growth hormones, and can induce plant resistance known as induced systemic resistance (ISR) (Wulandari, 2012; Sucipto et al., 2015; Istiyova et al., 2023). It is suspected that endophytic bacteria can suppress the growth of fungi that cause blast disease in rice (*Pyricularia oryzae*). This activity related to endophytic bacteria has the potential as PGPB, although further testing is needed to obtain more accurate information on its capacity.

Several reports showed that some endophytic bacteria improve plant resistance against fungal pathogens. The ability of a compound in bacteria can disturb fungal permeability. This can inhibit the production and excretion of extracellular enzymes from fungi (Zuraidah et al., 2020). Some bacteria are known to be able to produce protease and chitinase enzyme compounds (Oktafiyanto et al., 2018). For example, Chitinolytic enzyme can lyse or degrade chitin into monomers are thought to be able to inhibit the growth of pathogenic fungi in rice by damaging the fungal wall (Suryadi et al., 2013).

One of devastating rice plant diseases is blast disease. Blast disease is caused by the pathogenic fungus *Pyricularia oryzae* that can infect and significantly affect the quality and quantity of production (Zulaika et al., 2018). Blast disease damages rice leaves (leaf blast) or commonly called knot patterns, plant necks in the neck blast or neck blast, collar blast, and rice grains. Symptoms on the leaves can be spots that form rhombus with a pointed tip. The middle of the symptoms appear in the form of gray spots around it, brown to reddish-brown at the edges of the spot. The color of the spots at the beginning of symptoms is white or gray, surrounded by a greenish-brown color (Kusumawati & Istiqomah, 2020).

In addition, endophytic bacteria acquire nutrition from plants and protect plants from pathogens throughout their life (Eris et al., 2018). Endophytic bacteria can inhibit colony growth due to competition for growth space and secretion of antibiotic compounds in the media, giving rise to a clear zone around the colony (Marwan et al., 2021). The inhibition of endophyte fungi against *Pyricularia oryzae* can be seen from the non-proliferation of the pathogen colony through the endophyte fungi (Sunariasih et al., 2014a). Some soil microbes can produce plant hormones that can stimulate plant growth. Plants will absorb the hormones produced to grow faster or larger (Selangga & Listihani, 2021). Hypersensitivity reactions can provide a plant response to pathogens that appear in plant tissues, which is an attempt to inhibit the growth of pathogens (Wiratno et al., 2019).

According to previous reports, 82 endophytic bacteria from banana *klutuk* plants have been isolated. These isolates were sampled from bananas, which were grown in loamy sand (LM) and silt loam (SL) soils at Universitas Gadjah Mada (UGM), Indonesia. Among all isolates, 17 showed inhibition activity against the growth of *Fusarium oxysporum* f. sp. cubense (Foc) (Rahayu et al., 2021). However, the 17 selected isolates were not tested toward plant hypersensitivity and antagonism reaction against pathogenic fungi, *Pyricularia oryzae*. The hypersensitivity test is crucial for advantageous endophytic bacteria before crop application. Also, the antagonistic test against pathogens will give additional information on the benefits of isolated endophytic bacteria. Therefore, this study examined the prospective 17 isolates toward plant hypersensitive using tobacco plants and fungal pathogen antagonism reaction against the blast-disease-causing organism *Pyricularia oryzae*.

Method

Experimental design and materials

This study was conducted with an experimental design and three repetitions for each treatment. The used materials in this study were isolates of endophytic bacteria isolated from the roots, corm, and petiole of Klutuk banana with codes K1, K2, K7, K8, K9, K115, K117, K118, K324, K33, K38, K40, K86, K18, K28, K58A, K102 which grows in loamy sand (LM) and silt loam (SL) soils, is a collection of the Microbiology Laboratory, Department of Microbiology, Faculty of Agriculture, UGM Indonesia. The selected bacterial isolates were

grown in Nutrient Agar (NA) and Nutrient Borth (NB), while the pathogenic fungus was grown on Potato Dextrose Agar (PDA). *Pyricularia oryzae* isolate is the collection of the biology education laboratory, Universitas Muhammadiyah Surakarta. It was used to test the antagonistic ability of endophytic bacterial isolates.

Regeneration of bacterial isolate

Each isolate of endophytic bacteria was precultured on NB media with a 24-hour shaker, after which it was inoculated on NA media by a scratch method. Subsequently, the isolates were incubated for 2 x 24 hours.

Hypersensitivity reaction test

Hypersensitivity reaction test on tobacco leaves uses qualitative approach which observes the appearing symptoms after isolates inoculation. This treatment was carried out to determine the pathogenesis of 17 isolates of endophytic bacteria selected from the Klutuk banana. The observable response was a hypersensitivity reaction of tobacco leaves inoculated by 17 isolates of endophytic bacteria from the banana plant. Prepared 17 isolates of endophytic bacteria from Klutuk banana rejuvenated in oblique order. Then each isolate of endophytic bacteria from the Klutuk banana plant was inoculated into 5 ml of NB media at 48 hours. Then, each of bacterial isolate was inoculated using a 3ml syringe into tobacco leaf tissue, injecting a sterile syringe between the leaf epidermis on the abaxial side of leaf, between the leaf bones without damaging the leaf epidermis until parts appear wet. Furthermore, tobacco leaves were labeled according to endophytic bacterial isolates that had been inoculated and observed for seven days. Hypersensitivity reactions are positive when necrosis forms in tobacco leaf tissue.

Antagonism test

Pyricularia oryzae antagonism test was performed in vitro in a petri dish. *Pyricularia oryzae* culture was taken using a spatula and then placed in the middle of Petri PDA media, then *Pyricularia oryzae* was incubated for seven days. Furthermore, *Pyricularia oryzae* was taken using a spatula and grown on NA media incubated for three days, then inoculated bacterial isolate as much as 4 points per Petri. Observations were made on *Pyricularia oryzae* by measuring the percentage of inhibitory power of endophytic bacteria against endophytic bacteria according to the formula (Lamsal et al., 2012):

$$\text{Inhibition (\%)} = [(R-r)/R \times 100]$$

r = radius of the fungal colony opposite to the bacterial colony

R = maximum radius of the fungal colony

Result

Hypersensitivity reaction test

The results of the hypersensitivity test at 7 DAI are shown in [Figure 1](#) and [Table 1](#).

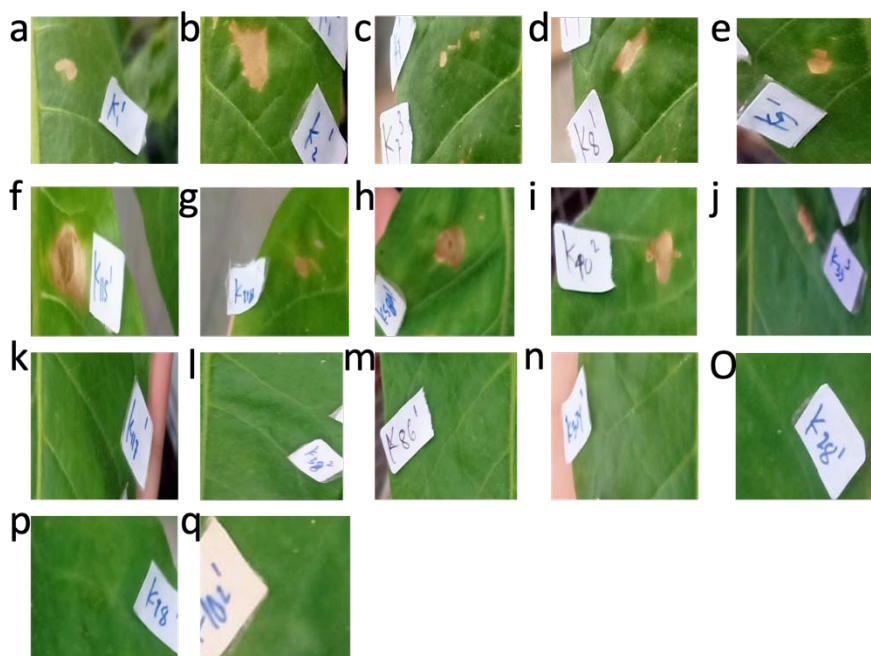


Figure 1. Hypersensitivity Reaction on tobacco leaves that have been inoculated by 17 Isolates of endophytic bacteria with three repetitions and for 7 DAI. (a-j) After Inoculation with Characteristics Showing Symptoms of Necrosis. Isolates K1, K2, K7, K8, K9, K115, K118, K33, K40, K58A, (k-q) did not show symptoms of necrosis Isolates K117, K324, K38, K86, K18, K28, K102.

Table 1. Results of hypersensitivity test on tobacco leaves inoculated by endophytic bacteria at 7 DAI.

Isolate Code	Hypersensitivity reactions
K1	+
K2	+
K7	+
K8	+++
K9	++
K115	+
K117	-
K118	++
K324	-
K33	++
K38	-
K40	++
K86	-
K18	-
K28	-
K58A	+++
K102	-
Control (aquades)	-

Information: (+) positive necrosis slightly; (++) positive moderate necrosis; (+++) positive multiple necrosis; (-) negative necrosis

Antagonistic test

The tested isolates can inhibit the *Pyricularia oryzae* with an average percentage that is not much different can be seen in Figure 2 and Table 2.

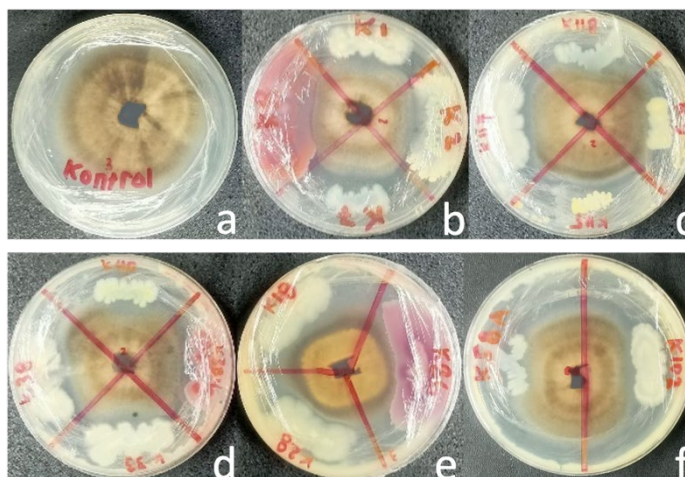


Figure 2. Microscopyisolate: Test results of antagonism of endophyte bacteria against the growth of *Pyricularia oryzae*. (a) control (no bacterial isolate given), (b-f) inability of colonization of *Pyricularia oryzae* beyond endophytic bacterial colonies

Table 2. Average of antagonistic test of endophytic bacteria against *Pyricularia oryzae*

Isolate Code	Average Large Percentage of Inhibition (%)
K1	40.13
K2	36.56
K7	34.50
K8	40.13
K9	34.46
K115	36.53
K117	37.73
K118	37.60
K324	36.90
K33	35.53
K38	37.76
K40	32.36
K86	40.00
K18	38.96
K28	40.46
K58A	35.70
K102	40.00

Discussion

Based on the hypersensitivity reaction test carried out for 7 DAI. Table 1 showed that among 17 tested bacterial isolates in this study, ten bacterial isolates showed positive reactions (showing symptom after inoculation) on tobacco leaves, namely K1, K2, K7, K8, K9, K115, K118, K33, K40, K 58A. Seven other bacterial isolates showed negative or nonpathogenic responses, namely K117, K324, K38, K86, K18, K28, and K102. Ten bacterial isolates show positive necrosis. With a slightly positive reaction given (+), namely in isolate with codes K1, K2, K7, K115, then bacterial isolate that shows a moderate positive reaction is being given (++), namely in isolate with codes K9, K118, K33, K40. Bacterial isolates that show positive necrosis are most given the isolated code (+++), namely in isolates with codes k8 and k58A. In this study, some isolates show negative necrosis results, or it can be said that these isolates are not positive hypersensitivity reaction tests. Seven isolates of endophytic bacteria show negative necrosis results with isolate codes K117, K324, K38, K86, K18, K28, K102. Hypersensitivity reaction is a program of rapid and localized cell death (Oktafiyanto et al., 2018). The results of the hypersensitivity reaction test can be seen from isolates that do not cause necrosis symptoms will show a negative or nonpathogenic response and vice versa if isolates that cause necrosis symptoms show a

positive or pathogenic response in plants (Fajarfika et al., 2022). A hypersensitivity test on isolating SA-1 tobacco leaves and isolating SA-2 and SA-3 showed a negative or asymptomatic reaction. Test hypersensitivity with a positive reaction will cause necrosis symptoms on tobacco leaves. Hypersensitivity testing is crucial for identifying most potential pathogens (Anasari et al., 2022).

Tobacco leaves are commonly employed as indicator plants to determine bacteria's safety against plants as they can clearly respond to pathogenic bacteria. Hypersensitivity reactions are characterized by the drying and death of host cells around the invasion site. According to Wibowo et al. (2020), Hypersensitivity response tests are performed on chitinolytic isolates to see the sensitive response of bacteria to tobacco plants. A hypersensitivity test is performed to determine the pathogenicity of pathogens (the ability of pathogens to cause disease). According to Manalu et al. (2023)'s research, necrosis reactions arise when pathogens interact with plants after injury (Ramdan et al., 2021). Hypersensitivity in plants is a rapid defense reaction of plants against pathogens accompanied by rapid cell death or tissue necrosis around the area injected with bacterial suppression (Yatni et al., 2018).

The results showed that the tested bacterial isolate was able to inhibit the growth of the pathogenic fungus *Pyricularia oryzae*. The percentage of inhibitory power produced by endophytic bacterial isolate is in average shown in Table 2. K28 bacterial isolate produced the highest percentage of inhibitory power against the growth of colonies of *Pyricularia oryzae* pathogenic fungi of 40,46%. And bacterial isolate that produces the smallest average percentage, namely K40 isolate of 32,36%. The difference in the diameter of the inhibitory power in each treatment is thought to be caused by the ability of bacterial isolates to produce different inhibitory compounds (Flori, 2020). screening of randomly selected 1152 bacterial species from *Musa paradisiaca* indicated that the highest proportion of bacterial strains with antagonistic properties had been noticed in the endo-sphere (9.4%), followed by the rhizosphere (6.5%) and soil (4.4%) (Nakkeeran et al., 2021). The formation of inhibitory forces in isolates of endophytic bacteria grown on media can produce metabolite compounds that cause the pecking of clear zones that show stunted *Pyricularia grisea* growth (Asmoro & Munif, 2020). Endophytes occur in different parts of plants, but more diversity has been reported in roots followed by stem and leaf (Savani et al., 2021), isolates of bacteria with codes k28, k1, k8, k86, k102 resulting in a percentage of inhibitory power of $\geq 40\%$ where three of the isolates are derived from roots, on several physiochemical and biological factors, such as easy entry sites such as cracks near the emergence of lateral roots, intercellular spaces, as well as blood vessels; however, roots are also the preferred place for the entry of bacteria on plants (Ramses, 2021).

Endophytic bacteria inoculated on NA medium which had been previously inoculated by *Pyricularia oryzae*. Subsequently, the NA medium was incubated for three days in room temperature (Indaryaningsih et al., 2021). The antagonists reaction against the fungus began to be seen at three DAI, and this inhibition continued to increase to 9 DAI. Inhibition of the growth of *Pyricularia oryzae* is suspected due to the mechanism of antibiosis of endophytic bacteria against *Pyricularia oryzae*. The research of Wijianto et al., (2018) showed that there was a nutritional competition between endophytic bacteria and *F. oxysporum* fungi in vitro experiments.

After the treatment, endophytic bacterial isolate with the code K40 showed low inhibitory power allegedly because the bacterial isolate produces toxic antibiotic compounds. According to Pitasari & Ali's (2018) research, antibiotic compounds in excessive amounts can be toxic so that they can inhibit and even kill the bacteria themselves, which has resulted in a low bacterial population and the inhibitory force going down. Adding the obtain, according to Marsaoli et al. (2020), the ability of each endophytic strain to occupy its niche or habitat so that the endophytic population between plant parts is different. That can determine the difference in the extent of resistance in each isolate.

The greatest inhibitory ability can be seen in the bacterial isolate code K28. This is because the bacterial isolate can produce high antifungal substances. As said by

(Sunariasih et al., 2014b), the higher concentration of antifungal substances produced the inhibitory power shown by the small growth of the colony. In each treatment, results were not obtained that were too different because endophytic bacteria can increase plant growth supported by (Parida et al., 2017). Endophytic research has been reported to increase plant growth, decompose pathogenic cell walls, and inhibit pathogen growth by producing antimicrobial pests such as siderophores.

Several papers reported similar results of pathogen inhibition by endophytic bacteria from banana plants. Two endophytic bacteria from the banana stem, namely CA8 and PK5 isolates, showed inhibition against the blood disease actor, *Ralstonia solanacearum* (Nawangsih, 2007). In addition, Marwan et al. (2021) also stated that two isolates, EAL15 and EKK22, from banana can significantly suppress blood disease in planta. Moreover, among 60 isolates from banana roots, 30 isolates indicated inhibition against blood disease agents, and three isolates were selected as of their massive inhibition (Hastuti et al., 2014). Some researchers reported the beneficial potencies of endophytic bacteria from banana against the disease agent through both in vitro and in-planta testing.

Conclusions

In-vitro analysis showed the inhibition action of all isolates against *Pyricularia oryzae*, with the inhibition ranges from 32.36 – 40.00%. At the same time, the hypersensitivity test indicated that only K117, K324, K38, K86, K18, K28, and K102 isolates exhibited nonpathogenic bacteria on tobacco.

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Declaration statement

The authors reported no potential conflict of interest

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