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by asmita ahmad

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Antagonist Test of Endophytic Bacteria from Roots of Rice Plant in South Sulawesi Indonesia as a Biological Agent of Bacterial Leaf Blight Disease

Andi Herwati^{1*}, Baharuddin Patandjengi², Muh. Jayadi³ and Masniawati⁴

¹Program of Agriculture, Graduate School, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km.10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

²Department of Plant Pest and Disease, Faculty of Agriculture, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km.10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

³Department of Soil Science, Faculty of Agriculture, Universitas Hasanuddin, Jl. Perintis Kemerdekaan Km.10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

⁴Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Hasanuddin, Jl. Perintis Kemerdekaan Km.10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

*Corresponding author

ABSTRACT

Bacterial leaf blight is a disease in rice plants caused by the bacteria *Xanthomonas oryzae* pv *oryzae* L (Xoo). Biological agents controlling plant diseases that are being developed now are endophytic bacteria that can increase plant resistance to plant pathogens. This study aims to find antagonistic bacteria that are able to provide the highest resistance to the growth of pathogenic Xoo bacteria in rice plants. Antagonistic bacteria were isolated from the leaves and roots of rice plants through surface sterilization and serial dilution. Obtained 53 bacterial isolates consisting of 20 bacterial isolates from leaves and 33 bacterial isolates from roots tested hypersensitive to tobacco plants for filtering potential non-pathogenic bacteria. 29 hypersensitive negative isolates did not occur necrotic on tobacco leaves which were tested antagonist against Xoo in vitro, found 8 isolates of antagonistic bacteria derived from leaves namely AHF9LS, AHF11LS, AHF13LS, AHF3GS, AHF7GS, AHF8GS, AHF5KS, AHF12KS and 7 isolates of antagonistic bacteria from the roots namely AHE1MS, AHE9MS, AHE15MS, AHE1LS, AHE6LS, AHE11LS and AHE23KS which have the potential to inhibit Xoo growth shown by the formation of medium, strong and very strong inhibition zones. In vivo application results of 15 bacterial isolates in rice plants found bacterial isolates with very strong inhibition that is AHE1MS with the smallest average length of the lesion observed 14 days after inoculation is 0.10 cm and 28 days after inoculation is 1.27 cm. For the length of lesion in percentage of diseased leaf area AHE1MS isolate was obtained by 0.22% at 14 days after inoculation and 3.18% at 28 days after inoculation shown by AHF9LS isolates.

Keywords

Antagonist,
Bacterial leaf blight,
Isolates,
Xanthomonas
oryzae pv. *oryzae* L.

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Introduction

One of the main diseases in rice plants is bacterial leaf blight (BLB) caused by the bacterium *Xanthomonas oryzae* pv *oryzae* L. (*Xoo*). BLB can damage rice plants from the nursery to near harvest with two typical symptoms. Crackle is a symptom of disease arising in the seedling phase, marked on the edge of leaf there are wet patches, pale green leaves and curled (Nurkartika *et al.*, 2017). Symptoms of disease in adult plants are marked with orange-yellow on the edge of the leaf, leaf tip, or part of the leaf that has mechanical damage, looks like submerged in hot water and then extends toward the base of the leaf called a blight. This disease can result in yield losses of up to 50-70% (BPTP, 2018). One alternative currently being developed to control bacterial leaf blight disease more wisely and increase production is application of biological agents that can enhance growth and protect plants from disease through several mechanisms (Nurkartika *et al.*, 2017). According to Bhore *et al.*, (2010) biological control agents of plant diseases that are currently being developed are endophytic bacteria. Endophytic bacteria are bacteria that live in the tissues of host plants without causing symptoms of the disease. Endophytic bacteria are reported in addition to increasing plant growth can also induce plant resistance to plant pathogens.

Endophytic bacteria can directly increase plant resistance by releasing certain compounds in pathogenic niches, as an antagonism, inducing plant resistance systems, and increasing plant tolerance to biotic environmental pressures (Hallman 2001). In addition endophytic bacteria can support the growth of host plants by producing plant growth regulators, helping fixation of nitrogen, producing antibiotic compounds that induce host plant resistance to pathogens and parasites, and producing

antibiotics (Bhore *et al.*, 2010; Reinhold-Hurek and Hurek 2011; Wiratno *et al.*, 2019). According to Reinhold-Hurek and Hurek (2011), Leiwakabessy and Latupeirissa (2013), Wiratno *et al.*, (2019) endophytic bacteria can protect plants against pathogens through induction mechanism of plant defense, competition in obtaining nutrition and space to conduct colonization and secretion of substances that are antagonistic to pathogens. Endophytic bacteria that colonize the internal tissues of their host plants are protected from competition with other microbes and environmental stress (Hallmann *et al.*, 1997; Balosi *et al.*, 2014). Endophytic bacteria that are used as biological agents and growth stimulants provide better benefits than free-living microorganisms because they do not compete in complex ecosystems (Compant *et al.*, 2005; Yulianti, 2013).

This study aims to find antagonistic bacteria that are able to provide the highest resistance to the growth of pathogenic *Xoo* bacteria in rice plants.

Materials and Methods

Study area

The plant material used is the roots and leaves of healthy rice plants obtained from several regions in Soppeng district, namely Galung Village, Pattojo, Rompegading, and Timusu. Bacteria were isolated by surface sterilization method of Hallmann *et al.*, (2001), roots and leaves of rice were cleaned and cut in 1-2 cm size then immersed in 70% alcohol for 1 minute and in NaOCl 2.5% for 2 minutes then rinsed 3 times with sterile water for 3 minutes. The cuttings of roots and leaves were each applied to Nutrient Agar (NA) medium and incubated for 48 hours. Selected isolates are bacteria that do not grow on NA medium. Furthermore, the plant material is mashed with sterile mortar and mixed with 10

ml of sterile water (Fig. 1). 1 ml of suspension of plant material is homogenized with 9 ml of sterile water in a test tube and diluted in series with concentrations of 10^{-2} , 10^{-3} , and 10^{-4} . Each dilution of 0.1 ml was spread on NA medium and incubated for 48 hours. Bacterial colonies were purified into NA media to obtain pure culture (Ramos, 2004; Risan, 2017; Kristiana *et al.*, 2019).

Hypersensitive reactions of bacterial isolates

Hypersensitive reaction test on tobacco leaves (*Nicotiana tabacum*) based on the method carried out by Schaad *et al.*, (2001) to determine bacterial isolates as plant pathogens. Each isolate was grown on liquid NA medium, incubated for 24 hours in a rotary shaker to a population density of 10^9 cfu / ml (Harni and Ibrahim, 2011). Each inoculum was infiltrated as much as 1 ml using a 1 ml sterile syringe without needle on the underside of a healthy tobacco leaf. Positive control was used *Xanthomonas oryzae*, while sterile distilled water as negative control. Plant response is observed in 24-48 hours. Isolates that do not cause necrosis produce a negative hypersensitive reaction (Abdallah *et al.*, 2016).

Antagonistic test of bacterial isolates against *Xoo* in vitro

This test was carried out to determine the ability of bacterial isolates to inhibit *Xoo* growth and find potential isolates as biological agents. Testing is done by double layer method (Lisboa *et al.*, 2006). The liquid culture of pathogenic bacteria as much as 800 μ L (10^7 cfu/ ml) was inoculated into 80 ml NA semi-solid. 10 ml is poured on a solid NA surface. Paper disk (0.7 cm diameter) is soaked was immersed in a 24-hour-old bacterial solution, then dried and placed in a petri dish containing *Xoo* bacteria on NA

media. The culture was incubated at 37°C for 24 hours and diameter of inhibition zone formed was then observed. Antibacterial activity is positive if an inhibitory zone is formed around the paper disk. Control treatment with sterile distilled water. The antimicrobial activity index is calculated based on the method by Patra *et al.*, (2009). Inhibition abilities are grouped based on inhibitory indexes which are very strong (> 3.0) with symbols (++++), strong (2.0-2.9) with symbols (+++), moderate (1.0-1, 9) with symbols (++) , weak (0.1-0.9) with symbols (+) and do not have antagonistic abilities (0.0) with symbols (-). Inhibition index (IH) is calculated based on the formula:

$$IH = \frac{\text{Clear Zone Diameter} - \text{Blank Discs Diameter}}{\text{Blank Discs Diameter}}$$

Application of antagonistic bacterial isolates in vivo to rice plants

Inpari 32 rice seedlings that will be sown are cleaned with 95% alcohol for 1 minute then washed with sterile distilled water three times for 3 minutes. Selected seeds are sunk and wrapped in gauze for 4 days to germinate, then planted in a 15x30 cm² pot filled with sterile growing media. 21-day-old rice seeds are transferred to pots with a diameter of 30 cm and each pot is planted with 2 clumps of rice. The selected antagonistic bacteria are bacteria with strong to very strong inhibition patterns in the in vitro test. *Xoo* colonies were grown on liquid NA medium with a density of 10^9 cfu/ ml (Yashitola *et al.*, 2002). Spraying was carried out before inoculation of pathogen *Xoo* (preventive) of 30 ml of bacterial suspension at 7 days, 14 days, 21 days, and 42 days after planting. At the age of 45 days, pathogen *Xoo* was inoculated by leaf clipping method with five rice leaves in each clump. The control treatment was sprayed with sterile aquades, as well as pain control inoculated with *Xoo*. Symptoms of bacterial leaf blight were observed after 14 and 28 days

after inoculation through measurement of the lesion length of bacterial leaf blight using the formula:

$$\% \text{ Disease Leaf Area (\% DLA)} = \frac{\text{Total length of lesion of test sample}}{\text{Total length of leaves of test sample}} \times 100\%$$

Results and Discussion

Isolation of antagonistic bacteria from rice plants

The results of isolation of biological agents from roots and leaves of rice plants are presented in Table 1. Obtained 53 isolates can grow and colonize NA media. 20 isolates originated from leaves and 33 originated from roots (Figure 2). NA medium is a medium used to observe the appearance or morphology of bacterial colonies and contains nutrients for the growth of non-selective microorganisms (Rossita *et al.*, 2016).

Hypersensitive reactions of bacterial isolates

53 bacterial isolates that had been obtained were tested hypersensitive to tobacco leaves (Table 1). 29 hypersensitive negative isolates did not occur necrosis there was no change in tobacco leaves and 24 hypersensitive positive isolates caused tobacco leaves to become necrotic (Figure 3). According to Danaatmadja *et al.*, (2009) hypersensitivity testing in tobacco is a screening of potential non-pathogenic bacteria even though some pathogens give negative results. Hypersensitivity is a defense mechanism that can inhibit the attack of microorganisms. Characteristically, infected cells have increased permeability, deficiency, and death from host cells.

This is consistent with the observation of Wiratno *et al.*, (2019) that hypersensitivity reactions arise in plant tissue are necrotic due to the plant's response to pathogens and are an attempt to inhibit pathogen growth.

Antagonistic bacterial isolate test in vitro

29 bacterial isolates which did not cause necrotic symptoms on tobacco plants were tested antagonist against *Xoo* in vitro (Figure 4), found 8 isolates of antagonistic bacteria derived from leaves and 7 isolates of antagonistic bacteria from roots that have strong and very strong inhibitory potential to inhibit *Xoo* growth is indicated by the formation of inhibitory zones (Table 2).

This is consistent with observations by Octriana (2011), that the growth of pathogens is inhibited due to competition with biological agents causing the pathogen not have room for its place of life. This is because competition occurs when there are two or more microorganisms that directly require the same source of nutrition. The difference in inhibition zones is thought to be due to the condition and nutrient content of media used (Saputra *et al.*, 2015).

In addition to the mechanism of inhibition through nutrition and space competition also has an antibiotic inhibition mechanism. Besides that, according to Vann Loon (2000) and Putro *et al.*, (2014) biological control by antagonistic bacteria can occur through one or several mechanisms such as in biological microbial controllers, namely: antibiosis, competition, hyper-parasite. The difference in inhibition of each isolate is caused by different inhibitory mechanisms. This is in accordance with opinion of Pal and Gardener (2006) and Kurniawati *et al.*, (2015) the production of bioactive compounds such as degrading enzymes, bioactive compounds and other compounds such as carbon dioxide, ammonia and hydrogen cyanide that can inhibit growth and kill other microorganisms which are the mechanism of antibiosis. Antibiotic compounds act as induction agents (elicitors) capable of providing plant resistance to disease. Walters *et al.*, (2013) and Hanudin *et al.*, (2016) states that

resistance induction can be done through application of biological agents (such as nonpathogenic rhizobacteria) and chemical compounds (synthetic and vegetable) can be used as an induction of resistance. The success of inducing compounds ranges from 20-89% in controlling the attack of plant pathogens, depending on physiological conditions, plant species, and abiotic factors such as temperature and humidity.

Antagonistic bacterial isolate test *in vivo* on rice plants

In vivo application results showed that bacterial isolates were able to inhibit the growth of diverse *Xoo* on rice leaves. This shows that all endophytic bacterial isolates tested have the potential to be antagonistic to *Xoo*. Bacterial isolates with very strong inhibition were AHE1MS with the smallest average length of the lesion that is 0.10 cm at 14 days after inoculation and 1.27 cm at 21 days after inoculation. These results indicate that ability of antagonistic bacterial isolates in suppressing the growth of *Xoo* pathogenic bacteria. This is consistent with observations made by Zheng *et al.*, (2012), inhibition of pathogen growth by antagonistic bacteria is indicated by a delay in development of symptoms. The use of antagonistic microbes can produce antibiotic compounds that can inhibit the growth of pathogens so that their growth is faster than pathogen. Shehata *et al.*, (2008) states that antagonistic bacteria produce different amounts of antibiotics so that they have different inhibiting abilities. Antagonistic bacteria as bio-control agents must have the ability to survive in order to suppress the transmitted infection. Because only antagonistic bacteria can survive on plant parts that are efficient and effective in managing transmitted infections.

The lowest percentage observation of leaf area was found in AHE1MS isolates of 0.22% at 14 days after inoculation and 3.18% at 21 days after inoculation shown by AHF9LS isolates. Obtained isolates that have a strong inhibitory to the growth of *Xoo* at *e in vitro* level differed at *in vivo* level. This is consistent with the results of Mew *et al.*, (2004) and Rustam *et al.*, (2011), when antagonistic bacteria are applied at different places and times, the ability to inhibit antagonistic bacterial isolates *in vitro* is often inconsistent with *in vivo* applications in the field. This indicates the influence of environmental conditions on the growth and development of bacterial isolates that are applied so that it is less adaptive at *in vivo* level which causes the antagonistic potential it has to play less role as at *in vitro* level (Rusatam *et al.*, 2011).

Based on observations obtained isolates that have a strong inhibition against *Xoo*. Quadth-Hallmann *et al.*, (1997) and Zuraidah (2013) stated that isolates which have strong inhibition on *Xoo* growth. One important aspect for efficacy of biocontrol agents is internal colonization of rice leaves by bacteria that can determine antagonistic activity in protecting stomata area of rice leaves and ability of bacterial cells to enter plant tissue while competing with other bacteria associated with plants.

Based on observations of the application of biological bacteria that are antagonistic, it is more effective in suppressing *Xoo* growth when the rice plants are in generative growing stage, namely age 7, 14, 21, and 42 days after planting. This is consistent with observations by Utkhede (2005) and Zuraidah (2013) biocontrol agents are generally more effective when applied before disease develops as a preventive treatment (Table 3).

Table.1 Results of isolation and hypersensitive reactions of antagonistic bacteria from rice plants

Bacterial isolate	Antagonistic bacterial origin	Hypersensitive reaction
AHF3MS	Leaf	+
AHF4MS	Leaf	+
AHF7MS	Leaf	-
AHF9MS	Leaf	+
AHF15MS	Leaf	+
AHF8LS	Leaf	+
AHF9LS	Leaf	-
AHF11LS	Leaf	-
AHF13LS	Leaf	-
AHF16LS	Leaf	-
AHF3GS	Leaf	-
AHF5GS	Leaf	-
AHF7GS	Leaf	-
AHF8GS	Leaf	-
AHF3KS	Leaf	-
AHF4KS	Leaf	+
AHF5KS	Leaf	-
AHF8KS	Leaf	-
AHF12KS	Leaf	-
AHF13KS	Leaf	+
AHE1MS	Root	-
AHE2MS	Root	+
AHE6MS	Root	+
AHE8MS	Root	-
AHE9MS	Root	-
AHE13MS	Root	+
AHE14MS	Root	+
AHE15MS	Root	-
AHE1LS	Root	-
AHE4LS	Root	+
AHE5LS	Root	+
AHE6LS	Root	+
AHE9LS	Root	-
AHE11LS	Root	-
AHE12LS	Root	-
AHE13LS	Root	+
AHE15LS	Root	-
AHE20LS	Root	-
AHE2GS	Root	-
AHE4GS	Root	-
AHE5GS	Root	+
AHE6GS	Root	-
AHE8GS	Root	+
AHE10GS	Root	+
AHE5KS	Root	+
AHE14KS	Root	-
AHE15KS	Root	+
AHE16KS	Root	+
AHE17KS	Root	+
AHE20KS	Root	+
AHE21KS	Root	+
AHE22KS	Root	-
AHE23KS	Root	-

Note: +: positive reaction, -: negative reaction

Table.2 Inhibition of *Xoo* by antagonistic bacterial isolates in the double layer test

Bacterial isolate	Colony diameter			Inhibition index	Ability to inhibit
	1	2	3		
AHF3MS	1.4	1.0	1.2	0.7	+
AHF4MS	0.8	0.7	0.6	0.0	-
AHF7MS	1.2	1.0	1.3	0.7	+
AHF9MS	0.7	0.7	0.8	0.0	-
AHF15MS	1.3	1.3	1.1	0.8	+
AHF8LS	1.4	1.3	1.3	0.9	+
AHF9LS	2.5	2.7	2.7	2.8	+++
AHF11LS	1.8	1.5	1.6	1.3	++
AHF13LS	2.0	2.0	2.1	1.9	++
AHF16LS	1.3	1.1	1.2	0.7	+
AHF3GS	1.8	1.6	1.6	1.4	++
AHF5GS	0.8	0.6	0.8	0.0	-
AHF7GS	1.6	1.5	1.9	1.4	++
AHF8GS	1.4	1.3	1.3	0.9	+
AHF3KS	2.7	2.8	2.8	3.0	++++
AHF4KS	1.3	1.3	1.2	0.8	+
AHF5KS	1.7	1.6	1.5	1.3	++
AHF8KS	1.3	1.4	1.3	0.9	+
AHF12KS	1.8	1.5	1.7	1.4	++
AHF13KS	1.3	1.0	1.3	0.7	+
AHE1MS	2.5	2.2	2.3	2.3	+++
AHE2MS	1.3	1.2	1.4	0.9	+
AHE6MS	0.6	0.8	0.7	0.0	-
AHE8MS	1.1	1.1	1.0	0.5	+
AHE9MS	1.7	1.4	1.5	1.2	++
AHE13MS	1.3	1.3	1.1	0.8	+
AHE14MS	1.3	1.3	1.3	0.9	+
AHE15MS	1.6	1.6	1.9	1.4	++
AHE1LS	2.0	2.0	2.0	1.9	++
AHE4LS	0.8	0.7	0.7	0.0	-
AHE5LS	1.3	1.4	1.2	0.9	+
AHE6LS	1.1	1.3	1.4	0.8	+
AHE9LS	1.2	1.4	1.6	1.0	++
AHE11LS	1.6	1.4	1.8	1.3	++
AHE12LS	1.4	1.2	1.3	0.9	+
AHE13LS	1.2	1.3	1.2	0.8	+
AHE15LS	0.7	0.7	0.7	0.0	-
AHE20LS	1.2	1.1	1.3	0.7	+
AHE2GS	1.2	1.0	1.4	0.7	+
AHE4GS	1.2	1.3	1.2	0.8	+
AHE5GS	1.2	1.3	1.4	0.9	+
AHE6GS	1.2	0.9	0.8	0.4	+
AHE8GS	0.8	0.7	0.7	0.0	-
AHE10GS	0.6	0.6	0.8	0.0	-
AHE5KS	1.2	1.4	1.3	0.9	+
AHE14KS	0.8	1.1	0.9	0.3	+
AHE15KS	1.3	1.3	1.3	0.9	+
AHE16KS	1.3	1.1	1.2	0.7	+
AHE17KS	0.9	0.7	0.5	0.0	-
AHE20KS	1.4	1.3	1.1	0.8	+
AHE21KS	1.3	1.3	1.2	0.8	+
AHE22KS	0.7	0.8	0.7	0.0	-
AHE23KS	2.7	2.3	2.5	2.6	+++

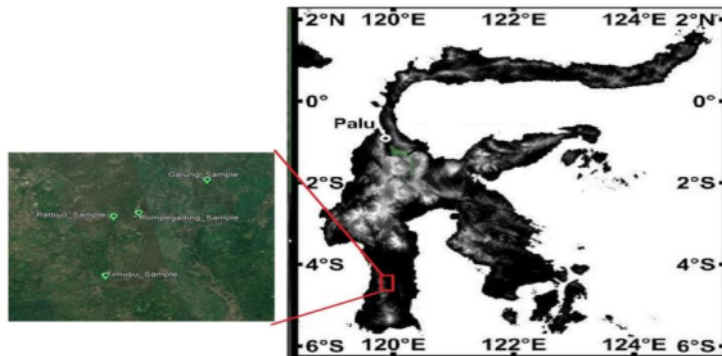
Note: +: Inhibition is weak, ++: Medium inhibitory, +++: Strong inhibition, ++++: Inhibition is very strong

Table.3 Average length of lesions and diseased leaf area in rice plants

Antagonistic bacteria	Average length of the lesion (cm)		Diseased leaf area (%)	
	14 dai	21 dai	14 dai	21 dai
Sterile water	0.27 d	3.67 c	0.62	7.19
AHF9LS	0.13 ab	1.60 ab	0.31	3.18
AHF11LS	0.20 bcd	1.83 ab	0.38	3.82
AHF13LS	0.17 abc	1.80 ab	0.34	3.54
AHF3GS	0.17 abc	1.90 ab	0.37	3.83
AHF7GS	0.13 ab	1.87 ab	0.34	3.74
AHF8GS	0.17 abc	1.83 ab	0.34	3.55
AHF5KS	0.23 cd	1.70 ab	0.50	3.44
AHF12KS	0.20 bcd	2.03 b	0.46	4.10
AHE1MS	0.10 a	1.27 a	0.22	3.65
AHE9MS	0.17 abc	1.97 b	0.43	3.96
AHE15MS	0.20 bcd	2.03 b	0.45	4.04
AHE1LS	0.17 abc	1.90 ab	0.36	3.69
AHE6LS	0.23 cd	2.00 ab	0.56	4.03
AHE11LS	0.20 bcd	2.17 bc	0.48	4.36
AHE23KS	0.13 ab	1.70 ab	0.32	3.33

7 Note: Numbers followed by the same letter in the same column are not significantly different in Duncan range test at the level of 5% Duncan Multiple Range Test (DMRT)

Figure.1 Location of sampling sites in Soppeng District, South Sulawesi, Indonesia



3 **Figure.2** Isolation of endophytic bacteria from leaves and roots of rice plants. A. AHF3GS, B. AHE1MS

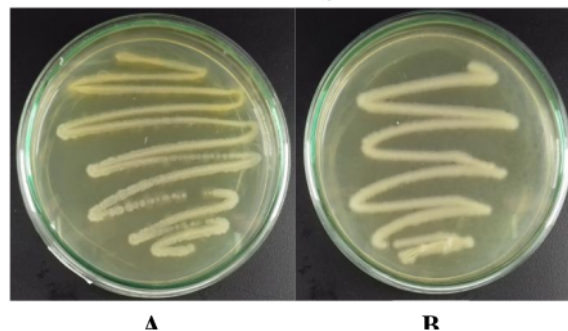
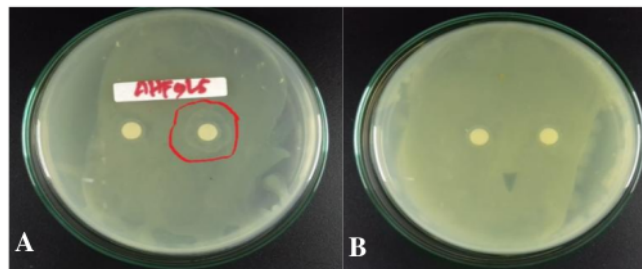


Figure.3 Hypersensitive reaction in tobacco plants 72 hours after inoculation of endophytic bacteria. A. Bacterial isolates were hypersensitive negative; B. Bacterial isolates were hypersensitive positive



Figure.4 Antagonist test of bacterial isolates in vitro on the third day 3; A. Inhibition zone is formed, B. Without inhibition zone



This study presents the isolation of endophytic bacteria derived from leaves and roots of rice tested for pathogenicity in healthy tobacco plants to obtain non-pathogenic bacteria and to inhibit the growth of pathogenic bacteria in rice plants. All endophytic bacterial isolates tested had the potential to be antagonistic to *Xoo* pathogen shown by the formation of a strong inhibitory zone. Endophytic bacteria can suppress infections that are transmitted and can survive on the plant, colonization of rice leaves can protect the stomata of rice leaves so that the potential as antagonistic bacteria as a biocontrol agent.

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