J. Trop. Plant Pests Dis.
Vol. 22, No. 2, September 2022
Pages: 154–161

ISSN: 1411-7525
E-ISSN: 2461-0399
DOI : 10.23960/j.hptt.222154-161

RESEARCH PAPER

Antagonistic feature displayed by endophytic bacteria consortium for control rice pathogens

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Manuscript received: 9 November 2021. Revision accepted: 4 April 2022. Available online: 12 August 2022.

ABSTRACT

Rhizoctonia solani and *Xanthomonas oryzae* pv. *oryzae* (Xoo) are important pathogens causing severe damage on rice. It makes severe damage and is required for the management of the disease. One of the management of the disease is the use of endophytic bacteria. The purpose of this study was to determine the compatibility of endophytic bacteria as consortium, to examine the potency of the endophytic bacteria consortium against *R. solani* and *Xoo* and to understand the mechanism of inhibition of endophytic bacteria consortium against *R. solani* and *Xoo*. Assessment on the compatibility between endophytic bacteria was performed in a petridish using streak method. The endophytic bacterial consortium antagonism test against rice pathogenic fungi and bacteria was carried out in a completely randomized design with 2 treatments and 15 replications. The variables observed were the compatibility of endophytic bacteria, the percentage of inhibition and the zone of inhibition. The mechanism of inhibition was observed from changes in the form of pathogenic fungi and bacteriostatic or bactericidal. The results showed that among 12 compatible endophytic bacteria as constituents of the consortium, five isolates of endophytic bacteria showed strong and very strong growth with the other isolates. These isolates from Petanahan Kebumen, Karangwangkal Purwokerto and Sumbang Banyumas. The consortium of endophytic bacteria was able to suppress the growth of *R. solani* by 57.14% with antibiosis mechanism causing swelling of the mycelium and formed a red pigment. The endophytic bacteria consortium inhibited the growth of *Xoo* by 15 mm with a bacteriostatic antibiosis mechanism. Consortium endophytic bacteria can be used as an alternative to control plant diseases which also has the opportunity to be formulated and applied to plants.

Key words: antibiosis, bacteriostatic, consortium, endophytic bacteria

INTRODUCTION

Sheath blight (caused by the fungus *Rhizoctonia solani*) and bacterial leaf blight (caused by *Xanthomonas oryzae* pv. *oryzae*) are important plant diseases causing severe damage to rice. Both the diseases have been reported to cause yield loss of 20–60% and 15–70%, respectively (Nuryanto, 2017; Raj et al., 2019). Management of the diseases is strongly needed to avoid further losses. The awareness of the quality of health and environment resulting in the use of eco-friendly management methods now has been widely performed, and one of which is endophytic bacteria (Olanrewaju & Babalola, 2019).

Endophytic bacteria are bacteria that live in plant tissues without causing damage or symptoms to plants, even providing benefits to plants (Melnick et al., 2011). The endophytic bacteria now become one promising biological control agent which has been widely used to control a wide range of plant pathogens, including *R. solani* and *X. oryzae* pv. *oryzae* (Xoo) (Nagendran et al., 2013; Halim et al., 2020). The bacteria have also been reported to be able to promote plant growth and yield (Glick, 2012; Kesaulya et al., 2015; Olanrewaju & Babalola, 2019). Several parts of the plants such as roots, stems, and leaves are subjected to the living environment of endophytic bacteria and the largest population is found in the roots (Harni et al., 2012; Beric et al., 2012).

Application of endophytic bacteria to constrain plant disease development has been widely performed both in single and consortiums. Application of consortium of several endophytic bacteria has been reported ensuring better results than when it was applied singly. Yanti et al. (2020) reported that the application of a consortium of several endophytic bacteria (*Bacillus pseudomycoides* strain SLBE 3.1 AP, *Bacillus thuringiensis* strain SLBE 2.3 BB, *Bacillus toyonensis* strain AGBE 2.1 TL) was able to inhibit development

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anthracnose disease on chili. The effect on the activities of the consortia inoculants a majority of them performed better in all parameters of maize growth component than the single treatment and control (Olanrewaju & Babalola, 2019).

Five isolates of antagonist endophytic bacteria have been preserved in rice, however, their compatibility and potential to be applied as a consortium to inhibit disease development of rice, especially leaf blight has not been fully revealed (Prihatiningsih et al., 2020; Prihatiningsih et al., 2021). This paper reported the potential use of several local endophytic bacteria and the development of bacterial consortium against rice pathogens in vitro as preliminary studies before being applied to plants. The purpose of this study was to determine the compatibility of the five endophytic bacteria, to examine the potential of the application of a consortium of endophytic bacteria to inhibit *R. solani* and Xoo as well as elucidate the inhibition mechanism of the consortium of endophytic bacteria against *R. solani* and Xoo.

MATERIALS AND METHODS

Research Site. This research was carried out at the Laboratory of Plant Protection, Faculty of Agriculture, Universitas Jenderal Soedirman from March to August 2021.

Preparation of Endophytic Bacteria Consortium, *R. solani,* and Xoo**.** Endophytic bacteria consortium prepared by transferring one loopful each of five isolate endophytic bacteria i.e A5, A6 (Petanahan Kebumen Isolates), KR4, KR7 (Karangwangkal Purwokerto isolates), and SB3 (Sumbang Banyumas isolate) into 250 mL flask containing 100 mL NB (nutrient broth) medium (Merck) and it was shaken at 150 rpm in room temperature for 24 h. *R. solani* was isolated from rice sheath blight symptoms, meanwhile the Xoo was obtained from bacterial leaf blight symptoms. The *R. solani* and Xoo were then cultivated on potato dextrose agar (PDA) (Oxoid) and potato sucrose agar (PSA) media (Merck) respectively and were incubated at room temperature. The five days old *R. solani* and 48 h old Xoo were used for further investigation.

Antagonism Assay Endophytic Bacteria Consortium to Fungal and Bacterial Rice Pathogens. The method for inhibition assessment of endophytic bacteria to *R. solani* was carried out using dual culture methods (Wang et al., 2013), in a 9 cm diameter petri dish containing 10 mL PDA. A mycelial plug (diameter 5 mm) of *R. solani* was placed in the middle of the media and incubated at room temperature for 1 day. After incubation, a paper disk (diameter 6 mm) which has been dripped with 10 µL of the endophytic bacteria consortium (10^8 cfu/mL) was put on both sides of the petri dish at a distance of 3 cm from the edges and incubated at room temperature for 5 days. The observation was performed after 5 days by measuring the radius of the colony of *R. solani* which grew opposite the paper disk containing endophytic bacteria consortium (C) and the radius of the colony toward the paper disk containing the endophytic bacteria consortium. The percentage of inhibition was calculated using a formula described by Wang et al. (2013) and Muthukumar & Venkatesh (2013) as follows:

$$I = \frac{C - T}{C} \times 100\%$$

I = inhibition (%);
C = growth of radius colony opposite with the paper disk of endophytic bacteria consortium;
T = growth of radius colony toward the paper disk of endophytic bacteria consortium.

Antagonism assay of endophytic bacteria consortium against Xoo was performed using dual culture method according to Balouiri et al. (2015) which has been modified. The Xoo was inoculated on a NA medium (Resti et al., 2017). The consortium of endophytic bacteria was propagated in NB medium and it was shaken (150 rpm for 24 h) using a shaker (Orbital shaker KBLee 3001 DAIKI) at room temperature. Paper disk with a diameter of 6 mm was dripped into 10 µL of the consortium suspension, then placed on a NA medium that had been poured with Xoo. The paper disk was placed at three different points. Incubation was carried out at room temperature for 24 h. A completely randomized design was used in this experiment with 2 treatments (the Xoo which was grown on a petri dish without endophytic bacteria consortium and with endophytic bacteria consortium) and 15 replications. The bservation was performed on the inhibition zone which was recognized as the clear zone around the paper disk (diameter zone-diameter paper disk). The inhibition zone was obtained by subtracting the diameter zone from the diameter paper disk. The area of inhibition was measured using formula (Balouiri et al., 2015):

$$I = \text{diameter zone} - \text{diameter paper disk}$$

Mechanism Antibiosis Assay of Endophytic Bacteria Consortium. The mechanism of inhibition against pathogenic fungi was observed from the morphological changes of fungi that grew towards bacterial colonies

or paper disks with endophytic consortium bacteria with a microscope. The antibiosis mechanism to Xoo was observed by taking a part of the zone with an Ose needle and then inserted into a test tube containing 0.6% peptone water and shaken for 24 h (150 rpm) at room temperature. Once the peptone water is cloudy, the antibiosis mechanism was bacteriostatic meaning it only inhibited the growth of Xoo, in the other hand, if the peptone water is clear, the antibiosis mechanism is bactericidal, meaning that it is able to kill Xoo (Bernatova et al., 2013). The indicator of antibiosis was detected in the consortium bacteria producing enzymes such as chitinase and protease. Detection of chitinase using Lestari et al. (2017) method and protease using Prihatiningsih et al. (2021) method.

Antibiosis Index. The antibiosis index was measured to determine the antibiosis activity which is usually indicated by the ability of bacteria to produce enzymes or compounds that play a role in antagonism with the antibiosis mechanism. Measurement of the antibiosis index was analyzed using the formula proposed by Halimahtussadiyah et al. (2017).

$$AI = \frac{(A - B)}{B}$$

A = diameter of zone;

B = diameter of colony antagonist or diameter filter paper.

The results of the calculation of the antibiosis index are included in the inhibition category according to Pan et al. (2009) as follow:

Very strong, if AI > 2.0 with symbol (+++); Strong, if AI (1–1.9) with symbol (++); Weak, if AI (0.1–0.9) with the symbol (+); Does not have antibiosis ability (0,0) with symbol (-).

RESULT AND DISCUSSION

The compatibility test of endophytic bacteria as the basis for consortium composition was evaluated based on intact scratches, no lysis or rupture occurred, the two test isolates were compatible, indicated by isolates A5, A6, KR4, KR7, and SB3 consistently, whereas if there was lysis at the meeting of the scratches the two isolates showed less compatible (Table 1, Figure 1). Based on the characteristics shown in Table 1 and Figure 1, five isolates that showed potential to be used as a consortium (Table 2) were further investigated on their antagonistic capability to inhibit the growth of *R. solani* and Xoo (Figures 2, 3, and 4). The results of the inhibition capability of the consortium of endophytic bacteria to *R. solani* and Xoo are shown in Table 3, which was 57.14% for *R. solani* and 15 mm for Xoo (Table 3). The antibiosis index for Xoo was 4, which is in the group of a very strong inhibition level (Pan et al. (2009). Inhibition of antagonistic bacteria against *R. solani* can be recognized in the occurrence of lysis or changes in shape at the tips of the hyphae (Abbas et al., 2019). Margani et al. (2018) reported that *Bacillus*

Table 1. Selection of 12 isolates rice root endophytic bacteria form marginal lands as a consortium

Number isolate of rice root endophytic bacteria	The origin of isolate (Central Java)	Compatibility between isolates	Respon of colony growth
A1	Petanahan Kebumen	+	a little lysis
A4	Petanahan Kebumen	+	a little lysis
A5	Petanahan Kebumen	+++	strong growth
A6	Petanahan Kebumen	++	growth
SR5	Patikraja Banyumas	+	a little lysis
SR7	Patikraja Banyumas	+	a little lysis
SM1	Somagede Banyumas	+	a little lysis
KR4	Karangwangkal, Banyumas	+++	strong growth
KR5	Karangwangkal, Banyumas	+	a little lysis
KR7	Karangwangkal, Banyumas	+++	strong growth
SB1	Sumbang Banyumas	+	a little lysis
SB3	Sumbang Banyumas	+++	strong growth

(+) a little lysis (sometime strong, anytime lose with the other isolates, lysis, rupture); (++) growth (good growth with the other isolates); (+++) growth and strong (very strong growth with the other isolates).

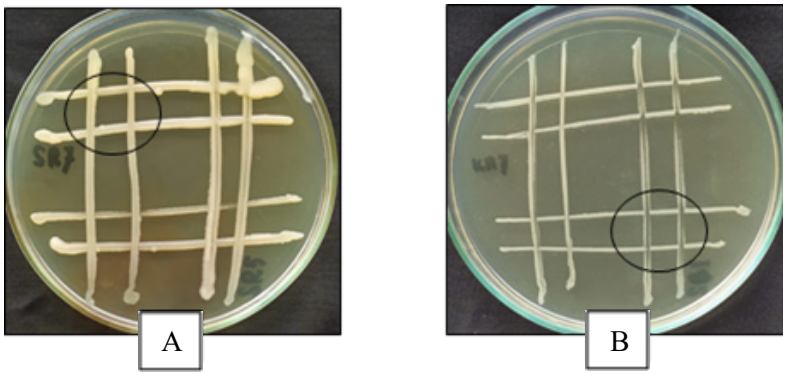

Figure 1. The compatibility test of two isolates endophytic bacteria in each petridish (A) compatible and (B) less compatible.

Table 2. The five selected isolates as consortium constituens

Compatible isolates	Non compatible isolates	Non stabil compatible
KR4	KR5	A1
KR7	SB1	A4
SB3	SR5	
A5	SR7	
A6	SM1	

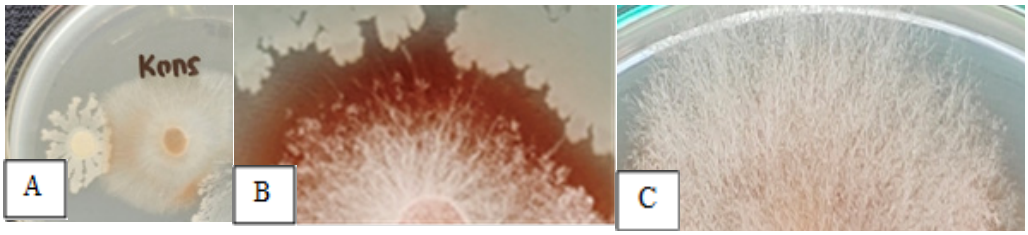

Figure 2. Inhibition of endophytic bacteria consortium against *R. solani.* (A) The growth of colony *R. solani* toward endophytic bacteria consortium and the opposite one; (B) The malformation of the hyphae tip; (C) *R. solani* at the normal growth.

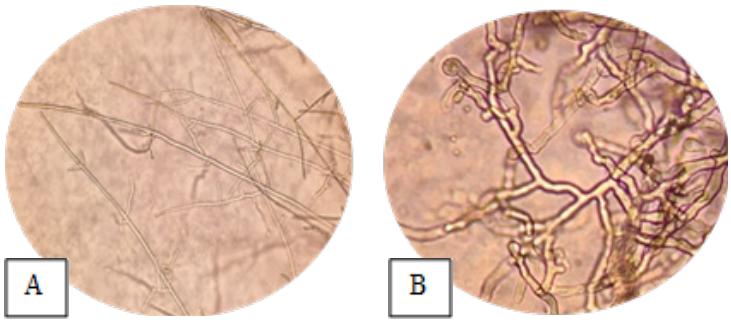

Figure 3. Inhibition mechanism of endophytic bacteria consortium against R. solani, malformation of the hyphae tip swelling, lysis and rupture. (A) Normal of hyphae; (B) Swelling of the hyphae tip.

Single Consortium

Figure 4. Inhibition growth of Xoo by single endophytic bacteria and consortium.

sp. B05 can inhibit the growth of *R. solani* in vitro by 30.33–58.00%.

The antagonistic *Bacillus* spp. controls the mycelial growth of fungi, preventing plant fungal disease (Khan et al., 2018). The bacteria attach to the mycelial cell walls, and the chitosanase (EC 3.2.1.123 enzyme), protease (EC 3.4.21.112 enzyme), cellulase (EC 3.2.1.4 enzyme), glucanase (EC 3.2.1.21 enzyme), siderophores, and cyanide acid of the bacteria crack and deform the hyphae, which leads to altered cell structure and functions due to vacuolation and protoplast leakage and mycelial crack (Abbas et al., 2019). The bacteria synthesize antifungal lipopeptides, such as iturin, fengycin, mixirin, pumilacidin, and surfactin, that are involved in the destruction of the pathogenic fungi in rhizospheres (Kulimushi et al., 2017; Toral et al., 2018).

The endophytic bacteria possess direct and indirect mechanisms for controlling plant pathogens. The direct mechanism is conducted by producing antimicrobial, siderophore, and chitinase enzymes, and other hydrolytic enzymes while the indirect mechanism is through the induction of systemic resistance in plants (Wang et al., 2019; Jacob et al., 2020). Biofilm formation of *Bacillus* spp. around the root surface and their secretion of toxins (surfactin, iturin, macrolactin, bacillomycin, and fengycin) destroy the pathogenic bacterial populations and reduce disease incidence in plants (Rais et al., 2017; Wang et al., 2019). The consortium of endophytic bacteria investigated in this study showed the capability to inhibit Xoo by antibiosis mechanism (Figure 5).

The inhibition of endophytic bacteria against pathogens has an antibiosis mechanism as indicated by the formation of a zone around the paper disk which was dripped in 10 µL suspension of endophytic bacteria consortium. The zone shows the effect of inhibiting the growth or killing of pathogenic fungi and bacteria. The antagonistic bacterial suspension (cell+extracellular) produces secondary metabolites, or only extracellular capable of producing several compounds such as chitinase, and protease that acts as inhibiting factors for fungi and bacterial pathogens. Chitinase from extracellular *Bacillus subtilis* B298 was produced as an inhibiting factor for the growth of *Colletotrichum* sp. the cause of chili anthracnose with the activity of 6.937 U/mL at 15 hours incubation. The effect of various temperatures on chitinase activity showed that optimum activity was achieved at 40 ºC with an

Table 3. Inhibition growth of *R. solani* and Xoo by endophytic bacteria consortium

Treatments	Inhibition against *R. solani* (%)	Inhibition mechanism	Inhibition against Xoo (mm)	Antibiosis index	Inhibition mechanism
Endophytic consortium >< *R. solani*	57.14	Swelling, red pigmen	-	-	-
Endophytic consortium >< Xoo	-	-	15	4 (very strong)	Bacterio-static (cloudy)

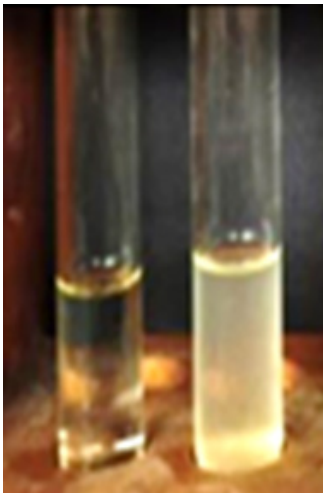

Figure 5. Bacteriostatic mechanism of endophytic bacteria consortium against Xoo.

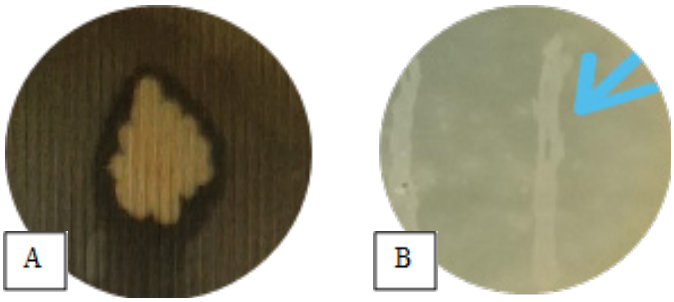

Figure 6. The qualitative chitinase (A) and protease (B) produsedby consortium endophytic bacteria as a indicator antibiosis effect.

activity of 5.764 U/mL. Meanwhile, the optimum pH for chitinase activity was achieved at pH of 5.0 by 6.813 U/mL (Lestari et al., 2017). Extracellular protease secreted by *B. subtilis* B315 affects the virulence of bacterial pathogens by combating biofilm formation and induces stress tolerance in plants. *Ralstonia solanacearum* of chili was inhibited by *B. subtilis* B315 with extracellular protease (Prihatiningsih et al., 2021).

The consortium bacteria produced chitinase and protease as an indicator of the antibiosis effect (Figure 6). Chitinase produced by the consortium acts as a degrader of fungal cell walls composed of chitin (Lestari et al., 2017). Protease as a degrading component of extracellular polymeric substances (EPS) and eradicates pathogenic bacterial biofilms (Prihatiningsih et al., 2021). A microbial consortium is a group of species of microorganisms that work together as a community. In a consortium, the organisms perform mutual activities in a complex and synergistic way. Each endophytic bacterial species has different antagonistic mechanisms, here the endophytic bacterial consortium can provide various control mechanisms simultaneously, thereby it is more effective in controlling plant pathogens (Kavya et al., 2020). It has been reported that consortium of plant growth promoting rhizobacteria (PGPR) showed better options as biological control of plant pathogens such as *Phytophthora capsici* on chili pepper (Zhang et al., 2019), *Pythium* sp. causing damping-off disease on cabbage, and *Fusarium* sp. causing wilt disease on cabbage (Sudharani et al., 2014).

CONCLUSION

The consortium composed of five isolates of endophytic bacteria that were compatible growth were A5, A6, KR4, KR7, and SB3. The inhibition of the endophytic bacteria consortium in suppressing the growth of *R. solani* was 57.14%, with the mechanism of mycelium malformation into swelling of the tip of hyphae and lysis with chitinase as an indicator of antibiosis effect. Antibiotic index resulted by Xoo was 4 which is categorized as a very strong level with a bacteriostatic inhibition mechanism with protease as an indicator antibiosis effect. The consortium of endophytic bacteria are potential to be applied for suppressing sheath blight and bacterial leaf blight disease on paddy fields.

FUNDING

This research was supported by Kementerian Pendidikan, Kebudayaan, Riset dan Teknologi through DRPM as funding research at “Penelitian Terapan” Scheme with contract number: 273/SP2H/LT/DRPM/2021.

AUTHORS’ CONTRIBUTIONS

NP was in charge of planning and responsible for the implementation of the research stages. HAD carried out the endophytic bacteria test for antagonistic to Rhizoctonia solani and Xanthomonas oryzae pv. oryzae. PL contributed for prepare the manuscript. Author’s agree with the final manuscript.

COMPETING INTEREST

We are declare that we have no competing interest for this article.

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