Conference Paper

Biocontrol Potential of Endophytic Bacteria Isolated from Healthy Rice Plant against Rice Blast Disease (*Pyricularia oryzae* Cav.)

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Abstract

Isolation was attempted to collect endophytic bacteria as potential biocontrol agents against rice blast disease (*Pyricularia oryzae* Cav.). The disease is one of major threats in rice production as it can cause 100% yield loss. Concern on the environment and human health has led to the searching of alternative controlling method to replace the commonly used pesticide-based method. Endophytic bacteria are bacteria that have intimate relationship with their host without inducing any pathogenic symptom. The use of endophytic microbial as biocontrol agent has its own advantages as the microbes are more easily to adapt to the environment needed by the host plant. We evaluated endophytic bacteria isolated from healthy rice plants and tested for their potential biocontrol activity using dual culture assay. Ten isolates were found to inhibit the growth of *P. oryzae* of more than 50%. Microscopic observation showed that they were able to cause the mycelia malformation of *P. oryzae*. Further work is currently in progress to determine their effectiveness in the pot trial.

Keywords: Endophytic bacteria; Pyricularia oryzae; biocontrol.

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Received: 28 July 2017
Accepted: 14 September 2017
Published: 23 November 2017

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the ICSAFS Conference Committee.

1. Introduction

Providing sufficient food for feeding the growing world population is always a challenge faced by the agricultural communities. Rice feeds more than half of the world population, yet its production is often limited by the invasion of pests, diseases and weeds. It is estimated that the total average of yield losses worldwide caused by these organisms is 36.5%, with 14.1% contributed by plant diseases alone. The rice yield losses caused by plant diseases were worth $220 billion worldwide, with the percentage of the yield lost higher in developing countries. At some countries, the loss due to blast can reach up to 100% [1].

Rice blast, caused by *Pyricularia oryzae* Cav., is one of the most important disease in Indonesia. It is one of the most destructive and widespread disease in rice plantation. The rice yield loss due to the present of this disease can be used to feed approximately 60 million people [2]. Rice blast can infect all stages of rice development in almost all rice plantations [3]. *P. oryzae* causes lesion on leaves, nodes, panicles and grains. Blast attacks on the neck causes more severe losses compared to the leaf blast [4].

The control strategies for blast have been relying on the use of resistant varieties and fungicide application [5]. Neither of the strategies can effectively control the disease. In addition, it is commonly known that the extensive use of fungicide has led to negative impact on human health as well as the environment. Therefore, the need of finding alternative methods to control the disease is an immediate requirement. Using biocontrol agent is one of environmental friendly and saver methods. Endophytic bacteria have an intimate relationship with the plant host without causing any symptom [6]. Although it is located within the plant tissues, its development is also accustomed by both biotic and abiotic factors from within and outside the plant. Consecutively it is able to elicit physiological changes that modify the growth and development of its host [7, 8].

Bacterial endophytes contribute to plant growth promotion, increase the plant yield, supress the pathogen and assimilate nitrogen to plants [9]. Bacterial endophytes also give benefit to its host plant by producing range of natural compounds which can be beneficial to medicine, agriculture and industry. Bacterial endophytes share ecological niche with plant pathogens, which make them become suitable candidate for biocontrol agent [10]. Numerous reports have shown that endophytic bacteria have the ability to control plant pathogens [8, 9, 11, 12]. This present study aimed to isolate endophytic bacteria from rice and determine their antifungal activity against rice blast pathogen *P. oryzae.*

2. Materials and Methods

2.1. Sample collection and endophytic bacteria isolation

Rice plants samples were collected from rice plantation near Bandung, West Java, Indonesia. Healthy 16-weeks old plants were chosen. It was carefully uprooted and packed for transported to the lab to keep the plant fresh. The rice plant samples were cleaned using running water to remove soil thoroughly. Plants were then sectioned into roots, leaf blades and stems followed by air drying at room temperature to remove excess water. The plant tissues were subjected for surface sterilization steps using the following washing series: 60 sec in absolute ethanol, 6 min in 4% sodium hyphochlorite, 30 sec in absolute ethanol and rinse in sterile RO water as the final rinse [13].

The plant tissues were then air dried over night to remove the excess water. It was cut into small fragments before being put into isolation media which were Tryptic Soy Agar (TSA) (tryptic soya broth 17 g, agar 18 g, per litre RO water), Mannitol Soy (MS) (mannitol 20 g, soya flour 20 g, agar 18 g, per litre RO water) agar and Tap Water Yeast Extract (TWYE) agar (yeast extract 0.25 g, K_2HPO_4 0.5 g, agar 18 g, per litre RO water). The plates were observed weekly for the presence of endophytic bacteria which were transferred subsequently into fresh half strength potato dextrose agar (PDA).

2.2. Validation of surface sterilization method

The effectiveness of the surface sterilization method was conducted at every isolation process. This was done to verify that the isolated microbes were truly endophyte. Surface sterilization method was validated by imprint the sterilized plant tissues into TSA and MS agar media. The plates were incubated and observed for the presence of microbes on the imprinted path. When no microbes present at the imprinting path, then the bacteria isolated from the particular isolation process considered as endophytes [14].

2.3. Screening for potential antifungal activity of the endophytic bacteria

The endophytic bacteria were screened for their antifungal activity against *P. oryzae*, the pathogen of rice blast disease. *P. oryzae* was isolated from infected paddy leaf showing typical blast symptom. *P. oryzae* was grown and maintained on PDA. The antifungal screening was conducted using dual culture assay. The bacteria were pre-inoculated by streaking the bacteria at 2 cm near the periphery of the plate. A six mm in diameter of actively growing fungi was positioned at 6 cm apart from the pre-inoculated endophytic bacteria 7 days before. As control, the same diameter of fungal pathogen was inoculated on PDA without any bacteria isolates at 2 cm near the side of the plate. The plates were then incubated in the dark in a constant temperature room at 27°C for 7 days or until the control plates (*P. oryzae* on PDA without bacteria) were full.

The antagonistic activity was checked by measuring the growth radius of *P. oryzae* toward the direction of the bacterial antagonist colony (R2) and the growth radius of *P. oryzae* in the control plate (R1). The potential ability of the isolated endophytic bacteria was determined by calculating the percentage of inhibition in radial growth (PIRG) using the following formula [15]:

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

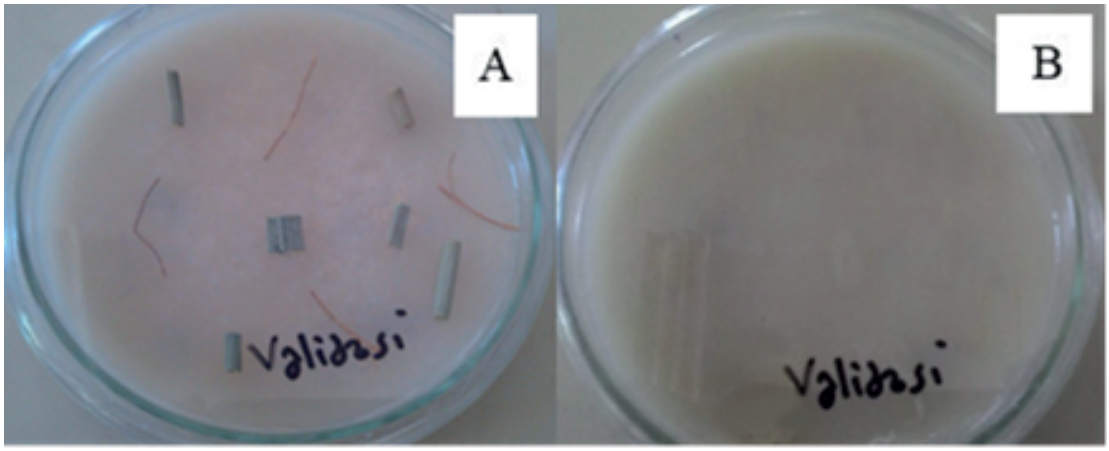

Figure 1: Validation of surface sterilization method. The plant tissues were imprinted on the agar media (A). No microbes grew on the imprinted path of the plant tissues (B) following plant tissues removal and incubation. This demonstrated that the surface sterilization method was able to sterilise the plant surface from microbial epiphytes.

2.4. Statistical analysis

The data obtained from the observation on the fungal colony radial was subjected to analysis of variance. The means were separated by Duncan's Multiple Range -Test (DMRT) at $P = 0.05$ with SPSS statistical software version 17.

3. Results and Discussion

Surface sterilization is a mandatory step in isolation of endophytic microbes. This aims to remove the microbes that present on the plant surface. The sterilization agent needs to be effective to kill the microbes on the plant surface but also cause no damage to the plant. Thus, the validation of the surface sterilization method is very important to confirm the success of the surface sterilization method and that the isolated microbes are truly endophytes. Sodium hypochlorite and ethanol have known as an effective sterilization agent [16, 17]. No microbes present on the imprint path of the rice plant tissues which were surface sterilized earlier (Fig 1). Therefore, bacteria isolated from this study were confirmed to be endophytes.

Emergence of endophytic bacteria from various rice plant tissues are shown on Fig 2. A total of 38 isolates were recovered from various rice plant parts. The small number of the isolated endophytic bacteria was due to the isolation media that being used in this study. To be able to get wide range of bacteria specific isolation media needs to be employed. Especially media with low nutrients that is accessible to the bacteria in the plant [18].

TWYE is low nutrient medium, however the use of TWYE did not able to boost the number of endophytic bacteria isolated from the rice plant. This medium had been

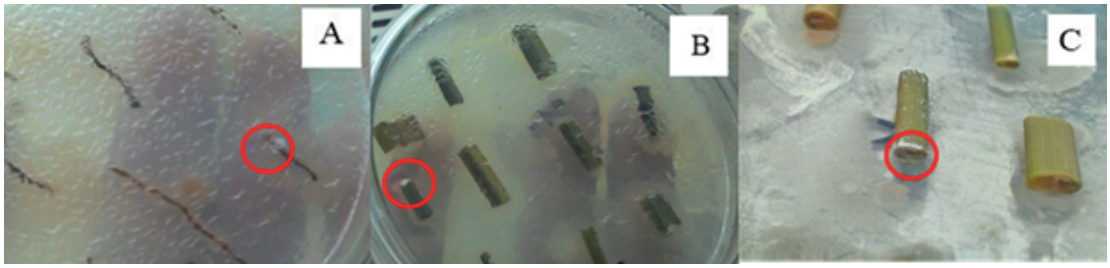

Figure 2: Endophytic actinobacteria (red circle) emerged from rice plant tissues; root (A), leaf blade (B) and stem (C).

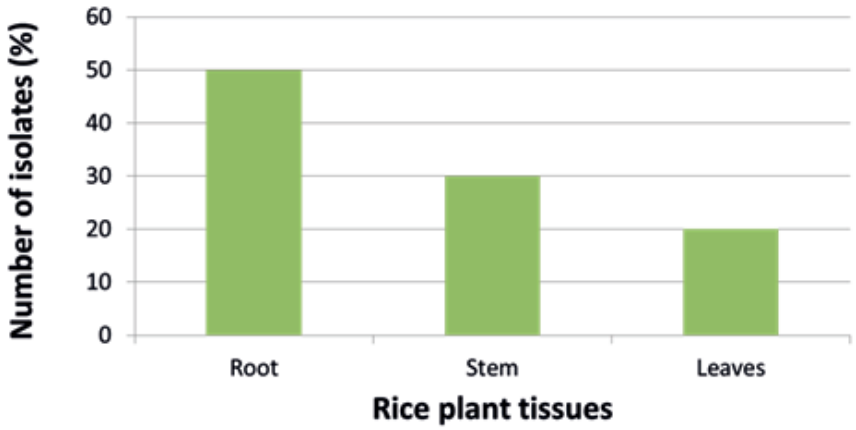

Figure 3: The number of endophytic bacteria isolated from the respected rice plant tissues.

successfully use to isolate actinobacterial endophyte from wheat which later demonstrated highly potential biocontrol agent [13, 19] Furthermore, the incubation time also participate in the finding of the small number of endophyte bacteria. Endophytic microbes take time to be able to emerge from inside of the plant tissue. Only microbial endophytes that present at the meristematic tissues that were able to be isolated [20]. Kaewkla & Franco [18] reported that to be able to get wide range of bacteria from inside the plant, longer incubation period up to 16 weeks is needed. The incubation time in the present study could not be extended beyond 8 weeks. This was due to the presence of common bacteria which were covered the presence of rare bacteria.

Most endophytic bacteria isolated in this study were obtained from roots. As much as 50% bacterial endophytes were isolated from root (Fig 3). Whereas the endophytic bacteria isolated from stem and leaf were 30% and 20% of the total number isolated bacteria, respectively. Endophytic bacteria are distributes heterogeneously within their host plants part. However, most endophytic bacteria can be found more in root and stem rather than in leaf. Soil is the major source for bacteria that become endophytic inhabitant within the plant. Bacteria are able to invade the plant tissue through the roots opening before started to colonize the root. It is then move upward toward stem, leaf sheath and finally leaf blade [16]. Therefore, it was not surprising to find out that the isolated endophytic bacteria mostly came from root.

Table 1: Antifungal activity of the selected 10 endophytic bacteria isolates against *P. oryzae*, the rice blast pathogen.

Isolate	Mean of *P. oryzae* colony radial (mm)	PIRG (%)	Antagonism Category
TD1	6.33 a*	89.92	Strong
TD4	12.33 b	80.5	Strong
MB3	16.33 bc	74.09	Moderate
MA1	19.00 cd	69.87	Moderate
MB1	19.00 cd	69.74	Moderate
MD5	20.33 cd	67.77	Moderate
TA2	23.00 de	63.42	Moderate
TA2	26.00 ef	58.65	Moderate
TA5	28.67 fg	54.5	Moderate
TA3	30.67 g	51.25	Moderate
Control	63.00 h	-	-

*Means with the same letter are not significantly different from each other following DMRT test at $P = 0.05$.

Among the 38 isolates, based on the preliminary antifungal testing, 10 isolates were chosen for further determination of their antifungal activity. The mean of the radial *P. oryzae* colony and the respected PIRG are shown on Table 1. All the 10 isolates demonstrated good ability in inhibiting the growth of *P. oryzae*. The percentage of inhibition ranged from the lowest of 51.25% to the highest of 89.92%. Following the antagonism criteria by Zivkovic *et al.* [21], two isolates (TD1 and TD4) were categorized to have strong antagonism activity as both isolates demonstrated PIRG values of 80.5% and 89.92%, respectively. To be categorized as having strong antagonism activity, the PIRG value should be between 76-100% [21]. The other isolates were categorized as having moderate antagonism activity as they had PIRG value of below 76%.

The dual culture plates were observed further for the presence of defect to the *P. oryzae* mycelia. Comparison of *P. oryzae* colony between control plate and the treated plate are shown at Fig 4A and 4B. Whereas the microscope observation of the effect of endophytic bacteria against *P. oryzae* (represented by isolate TD1) were shown in Fig 4C. All of the 10 bacterial isolates were able to produce clearing zone. This indicated that the isolates might produce secondary metabolites that able to inhibit the growth of *P. oryzae*. Endopytic bacteria are reported able to produce enzymes and secondary metabolites which can inhibit the growth of pathogenic fungi. Those compounds affect the pathogen through the mycelia malformation, swelling, lysis, and fragmentation [22].

Mycelial malformation by the presence of the endophytic bacteria at current study showed that it was able to produce active secondary metabolite. This secondary metabolite was able to disintegrate the mycelia of *P. oryzae*. Rahman *et al.* [23] reported secondary metabolite produced by bacteria penetrates the fungal pathogen

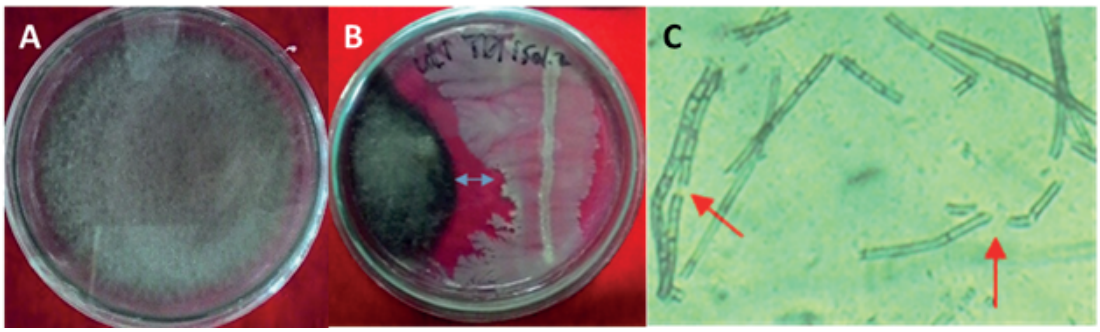

Figure 4: Comparison of *P. oryzae* growth without the presence of endophytic bacteria on control plate (A) and *P. oryzae* with the presence of endophytic bacteria TD1 (B). The visualization of mycelia of *P. oryzae* from the treated plate observed under microscope showing fragmentation and lysis (arrow) (C).

mycelia. The metabolite was further causing protoplasmic disruption and disintegration.

This study demonstrated the potential ability of endophytic bacteria isolated from healthy rice plant as biocontrol agent. Although the in vitro test does not necessarily provide the overall interaction between endophytic bacteria, plant and pathogen, nevertheless, the result of this study provided likely candidate for biocontrol agent. These bacteria need to be subjected for further studies including the isolates identification, secondary metabolites production and its effectiveness as biocontrol agent against other important pathogens of rice as well as its efficacy in the pot trial.

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