Utilizing Bacillus to inhibit the growth and infection by sheath blight pathogen, *Rhizoctonia solani* in rice

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**Abstract.** *Rhizoctonia solani* Kuhn is a common pathogen of rice. The pathogen causes sheath blight of rice. The pathogen can cause loss in the production of rice up to 45%. So far, the disease however is still poorly taken care of by the farmers and researchers, so the control measures is nearly never practiced by the farmers in the fields. It due to the unavailability of effective control method of the disease. Therefore, development to control the disease is important. *Bacillus* is one of popular bacteria which is effective as biological control agent of a lot of pathogens in plants, but it has not been used for control sheath blight in rice yet. The current researches were aimed to study the potential of *Bacillus* collected from healthy rice as candidates of biological control agent of the disease. The results showed that some isolates showed indications to inhibit significantly the growth and infection of the pathogen. We obtained at least five isolates of *Bacillus* collected from leaves, sheath, and stem of healthy rice fields. All of the isolates could effectively inhibit the growth of *R. solani* in vitro on potato dextrose medium at range 30.33-58.00%, whereas in vivo B05 isolate was the most effective in inhibiting the infection of pathogen at 30.43%. It was not significantly different (*P*≥0.05) to application of hexaconazol with dosage of 2 ml L⁻¹.

1. **Introduction**

Rice is one of the main commodities in Indonesia. The demand of rice will continue to increase along with the increase in population [1]. Based on data from [2], Indonesia rice production is 44.72 million tons in 2013, and was decreased to 44.45 million tons in 2014. Efforts to supply the demand for rice production must be continued, but the challenge in increasing production is also increasing due to climate change, threat of pests and plant pathogens [3].

*Rhizoctonia solani* Kuhn is one of the pathogens that attacks rice. *R. solani* is a pathogen that causes rice sheath blight disease that becomes one of the main problems in the attempt to increase rice production, especially in intensive cultivation system [4]. The pathogen can cause yield loss up to 45% [5]. This pathogen can survive in soil, plant debris, and has a wide range of hosts [6]. *R. solani* can survive as sclerotia and sclerotia produced by *R. solani* can survive in soil for more than one year [7].

The control measures were nearly never practiced by the farmers in the fields. Therefore, the development to control the disease is needed. Some examples of biological agents that have been developed as biological pesticides were *Bacillus, Pseudomonas fluorescent* group, *Actinomycetes*, and *Trichoderma* [8]. Antagonistic bacteria, particularly of the genus *Bacillus*, are most widely used as biological agents to control many plant pathogens [9] including *Rhizoctonia* [10]. This study aims to
evaluate the inhibitory capacity of *Bacillus* obtained from the isolation of healthy rice plants from the field to *R. solani* in vitro and in vivo.

2. Methods

The experiment was conducted in laboratory and green house of the Laboratory of Plant Pests and Diseases Control, Faculty of Agriculture, Universitas Sebelas Maret from August 2016 to April 2017. Materials used in the lab includes alcohol 70%, NA medium, PDA medium, LB medium, soil, rice seeds, *Bacillus* isolates and *R. solani* isolates. The tools used in this research are Petri’s dish, test tube, measuring cylinder, plastic wrap, vortex, ose needle, Bunsen’s lamp, loop, microscope, Erlenmeyer’s tube, autoclave, shaker, camera, blight plant of blight, healthy rice plant and Polybags.

The treatments were arranged by Completely Randomized Design (CRD) consisted of 8 treatments and repeated three times. The treatments were control (no biological agents and no pathogen (K-)), negative control in the presence of pathogens (K+), fungicides chemicals (P0), *Bacillus* 01 (B01) isolates, *Bacillus* B02 (B02) isolates, *Bacillus* B03 (B03) isolates, *Bacillus* B04 (B04) isolates and *Bacillus* B05 (B05) isolates. Observated data were analyzed using F test followed by Duncan’s Multiple Ranged Test (DMRT) with 5% confidence level.

Variables observed in this study were growth inhibition, onset of symptoms, disease incidence, and spotting area. The inhibition of pathogenic fungi was performed during in vitro antagonism test in the laboratory. Inhibition measurements were performed when the inhibition zone was formed between *Bacillus* and *R. solani* isolates. The inhibition measurement of the fungus with the formula of the colony radius away from the antagonist is reduced by the radius of the colony approaching the antagonist divided by the colony radius away from the antagonist multiplied by 100%. Observation of *R. solani* symptoms was performed on treated rice plants, which were observed daily with direct observation when there was appearance of spots on the rice stem. The observation of disease incidence was done by the number of affected plant samples divided by the total number of plants multiplied by one hundred percent. The area of spots was observed using millimeters of grid. Observations were performed on all spots that appeared from the treatment.

3. Result and Discussion

3.1 Characteristics of *Bacillus* isolates

*Bacillus* isolates were obtained from healthy rice plants. The isolates obtained were 9 with the origin of different parts of the rice plant (leaf, stem and sheath). The characterization of the isolates obtained was done by observing the colony morphology directly on the Nutrient Agar (NA) medium on Petri’s dish (Table 1). Observation was done to morphological types of bacterial colonies include shape, edges, elevation, color and surface [11].

| No | Source | Character Morphology | Gram |
|----|--------|----------------------|------|
|    |        | Shape | Edge | Elevation | Surface | Color |     |
| 1  | Leaf   | Circular | Entire | Flat | Smooth | White | +   |
| 2  | Leaf   | Irregular | Entire | Flat | Smooth | Cream | -   |
| 3  | Leaf   | Irregular | Serrate | Flat | Smooth | White | -   |
| 4  | Sheath | Irregular | Lobate | Flat | Smooth | White | -   |
| 5  | Sheath | Irregular | Lobate | Flat | Smooth | White | -   |
| 6  | Sheath | Irregular | Entire | Flat | Smooth | White | +   |
| 7  | Stem   | Circular | Undulate | Flat | Smooth | White | +   |
| 8  | Stem   | Irregular | Entire | Flat | Smooth | White | +   |
| 9  | Stem   | Irregular | Undulate | Flat | Smooth | White | +   |
Based on the observations, the character of colonies of bacterial isolates obtained, were varied with circular (rounded), irregular (rounded irregular) colonies, with flat elevation, with edge variation of the entire (smooth), undulate (curvy), and lobate (Wavy) and smooth surface (smooth). The colonies of bacterial isolates obtained varied, some were white and cream. Colonies of bacteria were dominated by the irregular edge shape with flat elevation, smooth surface and white color. Bacterial isolates showed colony morphological characteristics which tend to vary in culture medium [12].

Bacterial morphological characters obtained from observations in nutrient media to conform to a statement from Tay et al. [13] that Bacillus cereus can grow well on NA medium incubated at an average temperature of 28.8 °C. The most common form of bacterial colonies morphology is irregular, this is in accordance with the results of research [14] that bacteria class B. cereus has an irregular shape. Similarly, the colony elevations are flat in all bacterial colonies observed. The observations indicated that the morphology were quite diverse; one of the causes is the bacterial density. Several factors affecting the population density of bacteria are plant species, plant age, tissue type, habitat and resilience formed by plants and environmental factors [15-16]. Isolates of purified bacteria, then characterized by morphology, Gram testing using KOH 3% [17]. According to [18] Bacillus is a Gram positive and rod-shaped bacterium. Nine bacterial isolates obtained, showed 5 Gram positive and 4 gram negative isolates.

3.2 Characteristics of R. solani isolate
The isolate of R. solani was identified visually and microscopically. The pure cultures and hypha of R. solani used, are presented in Figure A. The microscopic appearance of R. solani is shown in Fig. B where there are arrows. Without the arrows it shows the R. solani hypha that has almost angle branching at 90 degree, 6-10 μm wide, and hyaline color [19].

![Figure 1. Characteristics of R. Solani: (A) R. Solani culture on PDA medium, (B) Hypha of R. Solani that makes angle of 90°](image)

3.3 Bacillus antagonism against R. solani in vitro
The results of this study showed that the growth of pathogenic fungi could be inhibited by Bacillus isolates with varying inhibitory power (Figure 2). The result of observation of antagonism tested, showed that the treatment of isolate B05 has the highest inhibitory, which is 58.00%. Bacillus B02 isolate was ranked second in inhibiting R. solani at 48.67%. The next Bacillus isolate inhibiting R. solani was B01 at 47.67%. Bacillus B04 isolate was at 38.67% inhibitory capacity and Bacillus isolate having the lowest inhibition to R. solani was B03 at 30.33%. This is in line with statements from [20] that the use of B. cereus isolate can suppress R. solani and S. rolfsii by 68.9% and 33% and were able to slow down the growth rate of R. solanion PDA media. According to [21] strong inhibition of pathogenic fungi caused by the presence of fungicidal compounds produced bacterial cultures.
Figure 2. Growth inhibition of *R. solani* by *Bacillus* on PDA medium

*Bacillus* isolates having ability to inhibit growth of *R. solani* on PDA medium indicates that the isolates produce some antibiotic compounds involved in antagonistic mechanism. The result supports previous research done by [22] that *Bacillus* sp. has ability to produce antibiotic compounds. The formation of inhibitory zones indicated a mechanism of antibiosis by *Bacillus* isolates [23]. This is in accordance with the research of [24] where endophytic bacteria have antibiotic ability to form highly variable inhibitory zones. Inhibition of *R. solani* is indicated by the distance between pathogenic fungi and *Bacillus* bacteria (Figure 3).

![Figure 2: Growth inhibition of *R. solani* by *Bacillus* on PDA medium](image)

Figure 3. *Bacillus* antagonism against *R. solani* on PDA media: (A) Isolate B01 to *R. solani*; (B) Isolate B02 to *R. solani*; (C) Isolate B03 to *R. solani*; (D) Isolate B04 to *R. solani*; (E) Isolate B05 to *R. solani*; R: isolate *R. solani*; B: isolate *Bacillus*

The inhibition of pathogen growth is characterized by the formation of clear zones in PDA medium, indicating that *B. cereus* produces certain metabolite compounds that can inhibit *R. solani* [20]. Metabolite compounds were suspected as antifungal; thereby inhibiting the growth of pathogenic fungi. The results of [25] showed inhibition by class *B. subtilis* up to 50% of *R. solani* in vitro and suspected *B. subtilis* produced antibiotic compounds such as inturin A and surfactin.
3.4 Bacillus antagonism against R. solani in vivo

Results of Bacillus test showed that the appearance of symptoms between the treatments was not significantly different from each other. The appearance of symptoms was emerged at 3.39 to 10.00 days after inoculation. The disease incidences were at the ranges 33.33% to 100%. The effectiveness in inhibiting of disease was at 0% to 54.35% (Table 2).

Table 2. Bacillus isolate test on R. solani infection at green house

| Bacillus Isolates | Appearance of symptoms | Disease Incidence (%) | Effectiveness in Inhibiting of Disease (%) |
|-------------------|------------------------|-----------------------|------------------------------------------|
| B01               | 8.33 ± 1.53 c          | 55.57 ± 19.24 bc      | 14.49 ± 18.10 abc                        |
| B02               | 10.00 ± 0.00 c         | 66.67 ± 00.00 cd      | 09.42 ± 16.31 ab                         |
| B03               | 8.67 ± 0.58 c          | 100.00 ± 00.00 d      | 00.00 ± 00.00 a                          |
| B04               | 8.67 ± 1.53 c          | 66.67 ± 33.34 cd      | 11.59 ± 20.08 ab                         |
| B05               | 8.67 ± 1.15 c          | 55.57 ± 19.24 bc      | 30.43 ± 13.57 bcd                        |
| Heksaconazol**    | 8.00 ± 1.73 c          | 33.33 ± 00.00 b       | 54.35 ± 28.42 d                          |
| No Bacillus       | 3.39 ± 0.35 b          | 88.89 ± 19.24 cd      | -                                        |

The value followed by the same letters in the same column are non-significantly different based on Duncan’s test at 5%. * Based on data transformed to $\sqrt{x + 1}$; ** Dosage 2 mL L$^{-1}$.

Tests at the in vivo level of 5 isolated Bacillus in isolate B01 showed the earliest symptoms when compared with isolate B02. When the symptoms appear more slowly, Bacillus isolates slow down the growth rate of R. solani, according to [20] statements that the B. cereus treatment allows to slow or delay the timing of symptoms in plants. However, B01 isolate showed a lower incidence of disease and spotted area compared to B02 isolate. For the highest incidence of disease occurs in the treatment of B03 that is 100%, where rice plants inoculated R. solani all show symptoms. We suspected that B03 isolates cannot colonize the plants. This is in accordance with the statement of [26] that emphasis on the intensity of disease attacks is related to the ability of bacteria to colonize the plants and produce the same metabolism as secondary to protect the plants from pathogenic infection.

Bacillus isolates based on the results of the study showed the effectiveness of various disease suppressors. The highest Bacillus isolate in suppressing R. solani pathogen was B05 treatment with 30.43%. Isolates B01 has 14.49% of disease suppression efficacy, B02 isolate is 9.42%. Isolate B03 effectiveness of disease suppression is 0%, this is due to incident disease that happened 100%, so all plants infected by R. solani. The use of hexaconazole fungicide has the highest disease suppression efficacy of 54.35%. Isolate B03 shows that it is in line with in vitro test. Isolate B03 in in vitro test showed inhibitory resistance to R. solani but in vivo test could not inhibit. According to [27] it is possible that the isolate has no or less ability to produce antifungal compounds, or less adaptive so the antagonism role disappears.

Isolate B04 shows a somewhat weak resistor to R. solani growth at the in vitro level, but has a strong inhibitory at the in vivo level. The isolate has a weak antibiotic mechanism to inhibit R. solani growth directly, but indirectly has other mechanisms that are strong enough to suppress symptoms when applied to seeds or plants. In other words, B04 isolates do not directly suppress the growth or development of pathogens, but rather increase plant inhibition to pathogens. The mechanisms of plant inhibition that are affected by antagonistical bacteria are known as the induced systemic resistance [28].

4. Conclusion

The conclusions can be drawn from this study is that all of the isolates could effectively inhibit the growth of R. solani in vitro on PDA at 30.33-58.00%, and isolate B05 was the most effective. It also occurred in vivo that the isolate was most effective inhibiting the infection of pathogen at 30.43% that was non-significantly different different (P≥0.05) from the application of hexaconazol with dosage 2 mL L$^{-1}$. 


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