Plant Growth Promoting Rhizobacteria (PGPR): as a Potential Biocontrol for Curvularia lunata Invitro

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Abstract. Rhizobacteria is an environmentally friendly and sustainable biocontrol agent, used as an alternative in the management of plant pathogens. Furthermore, their suppression mechanism often varies, as some are capable of producing antibiotic compounds, competing with, and increasing plant growth, although some forms possess multiple control. This study, therefore, aims to determine the activity of rhizobacterial antibiosis against Curvularia lunata in vitro. The experiment was conducted at the Laboratory of Biological Control, Department of Pests and Plant Diseases, Faculty of Andalas University. In addition, the procedure required using an experimental method, which was carried out in a Completely Randomized Design. The antagonistic rhizobacterial selection of C. lunata was carried out using a dual culture test, on a total of 7 rhizobacteria, and 4 were selected, encompassing Stenotrophomonas maltophilia KJKB5.4, Stenotrophomonas pavani LMTSA5.4, Bacillus cereus AJ34 and Alcaligenes faealis AJ14 as potential candidates in the control of C. lunata, suppression effectiveness that was above 50%. In addition, it was also established that all four isolates the propensity to produce chitinase enzymes.

1. Introduction

Rice plants (Oryza sativa) are known as one of the main sources of carbohydrates, hence the increased tendency to remain an important part of the strategic agricultural sector. This is practical in both economic and social terms, resulting in prioritization during development. Despite the existence of data on production, harvested area, and the increase in productivity of Indonesian rice, pests, and pathogens attacks have widely been identified, including Curvularia lunata, which originated from the grains of rice, known to cause discoloration [5], and is commonly discovered in the tropics and subtropics, as a facultative parasite. In addition, losses incurred by this infection in rice sometimes reach 20% to 50% [6]. According to Taufik et al., [24] the symptoms tend to appear at the point where the plant has produced panicles, characterized by a change in grain color to a slightly dark or black. Therefore, the surface is covered with fungus mycelium, which is blackish brown and often forms a thick black coating. These properties are easily distinguished from yellowish uninfected rice grains.

C. lunata disease control commonly adopted by farmers involves the use of synthetic fungicides, although the excessive application has been attributed to the incidence of resistance, environmental pollution, and adverse side effects on human health. Hence, controlling with the use of antagonistic...
microorganisms (biological control) is the focus of intense research worldwide \[11\]. This biological technique is considered as a viable alternative substitute for synthetic chemical-based approaches \[12\]. Biological control requires the use of microorganisms that live in the rhizosphere region of plants, a zone in the soil that is directly affected by the root secretion system, and is known to be rich in nutrients (because of the accumulation of exudates containing carbohydrates and amino acids). This function as an energy source for bacteria within the soil, termed rhizobacteria \[26\], and they are also confer some benefits living in the rhizosphere, based on the capacity to increase plant growth (Plant Growth Promoting Rhizobacteria) \[3\]. They also confer some favorable effects on plant growth, which is obtained through direct or indirect mechanisms, better known as Rhizobacteria Promoting Plant Growth (PGPR) \[4\]. This possesses the capacity to increase the host's adaptive potential and also enhance growth, through the presence of hydrogen cyanide (HCN), siderophore, indole acetic acid (IAA), dissolved phosphate, bacteriocin, lytic enzyme production, antibiotics as well as anti-fungal properties \[12\]. Moreover, the production of chitinase has been acknowledged as a part of the lytic system allows bacteria to utilize fungus hyphae that consist of Chitin, as an actual growth substrate, based on the fact that it is an important part of the most fungus cell wall \[7\]. Some rhizobacteria perform the function of inhibiting some phytopathogens by competing for space and nutrients, the enhanced ability to generate bacteriocin, production of lytic enzymes, antibiotics against Bacillus, Burkholderia, Enterobacter, Herbaspirillum, Ochrobactrum, Pseudomonas, Serratia, Staphylococcus, and Stenotrophomonas, possessing the capacity to suppress the growth of phytopathogen \[25\]. Therefore, there is a potential opportunity of developing rhizobacteria as a biological agent for controlling plant pathogens, thus, the study is aimed at determining its activity against Curvularia lunata in vitro.

2. Materials and Methods
This research was conducted at the Laboratory of Biological Control, Department of Pests and Plant Diseases, Faculty of Agriculture, Basic and Central Laboratory, Andalas University, from April to June 2019.

2.1. Materials
The Rizobacteria used were stock culture obtained from the laboratory of Biological Control, Department of Pests and Plant Diseases, Faculty of Agriculture, Andalas University. These include Alcaligenes faecalis AJ14, A. faecalis ANO6, Bacillus cereus AJ34, Serratia marsecens AR1, Ochrobactrum ciceri RK12; Stenotrophomonas pavanii LMTSA5.4 and Stenotrophomonas maltophilia KJKB5.4, Curvularia lunata, Luria Bertani Agar (LB), and Media Potato Dextrose Agar (PDA).

2.2. Methods
Rhizobacteria Rejuvenation. The Rizobacteria was cultured on Luria Bertani Agar (LB) medium (Tripton 10 g, NaCl 5 g, yeast extract 5 g, and 1000 ml distilled water), at pH 7.3), followed by incubation at 30°C for 48 hours. Then a single colony of each bacterium was grown on an LB Broth media. Besides, they were incubated at 30°C and continuously shaken using a shaker (150 rpm) for 24 hours at a population of 108 CFU/ml.

2.2.1. Rejuvenation of Curvularia lunata. The C. lunata used stock culture obtained from the Microbiology Laboratory, Department of Pests and Plant Diseases, Faculty of Agriculture, Andalas University. These fungi were bred on Potato Dextrose Agar (PDA) media (200 g potatoes, 20 g dextrose, 17.5 g agar and 1000 ml distilled water, pH 7), and the pure culture was grown for 7 days for use in further testing.
2.2.2. *Rhizobacteria Inhibition against C. lunata*. This experiment was carried out on seven rhizobacterial species using dual culture test methods. C. lunata on PDA media were cut using a cork borer with a diameter of 0.5 cm, then placed in the middle of a petri dish containing the new PDA media. The rhizobacterial suspension (10⁸ CFU / ml) was dropped on sterile filter paper with a diameter of 0.5 cm and placed on four sides of a petri dish with four repetitions. As a control, the pieces of C. lunata were put in a petri dish without adding rhizobacteria. The ability of rhizobacteria to inhibit C. lunata was observed after the control had grown to fill the plates. The rhizobacteria can to inhibit C. lunata indicated by the presence of a clear zone around the rhizobacterial colony. Inhibition calculated using the recommended formula (Simarmata et al., 2007):

\[ L_z = L_{av} - L_d \]  

Explaination:
IZ = Inhibitory zone diameter (mm)  
Lav = Inhibition zone diameter with filter paper (mm)  
Ld = Filter paper diameter (mm)  

Effectiveness of pathogenic fungus suppression:

\[ \text{Effectiveness} = \frac{\text{Diameter of Control Colony} - \text{Diameter of treatment colony}}{\text{Diameter of Control Colony}} \]  

2.2.3. Potential Test of Rhizobacteria Bioactive Compounds. The potential of bioactive compounds produced by rhizobacteria tested using the Rustam method (2012). One rhizobacterial colony was put into 100 ml liquid LB media in a 250 ml Erlenmeyer flask and then incubated on a rotary shaker (100 rpm at room temperature for 2 x 24 hours (population 10⁸ cells/ml). The bioactive compounds produced by bacteria were obtained by separating bacteria from the solvent by centrifuging for 5 minutes at 14,000 rpm (25 oC).

The resulting supernatant sterilized using a millipore filter (0.22 µm). The activity test of bioactive compounds carried out using the disc diffusion method based on Guechi et al., 2012. Sterile disc paper with a diameter of 0.5 cm was dipped into the supernatant then dried for about 1 minute. And then, put the disc paper at a distance of 3 cm from the tip of a petri dish containing PDA media. Furthermore, the C. lunata were collected using a 5 mm diameter cork borer and placed at a distance of 3 cm from the edge of the petri dish and opposite to the rhizobacterial supernatant. As a control, the sterile distilled water droplet on the paper discs. To see the bioactive compounds was done by measuring the colony diameter of C. lunata then compared with the control. The ability of rhizobacterial bioactive compounds was calculated using a formula (Simarmata et al., 2007).

The activity of inhibiting C. lunata growth was measured based on the weight of the fungus mycelia formed, using Riyadi et al. (2008). A total of 10 ml of 2.5% HCl was added to the Petri dishes covered with fungi. Then it is heated on an electric stove so that the PDA media melts, swallowing mycelium on the Whatman No. paper 40 which has known its weight, then the mushroom mycelium is weighed to measure its wet weight and then dried to determine the dry value.

The percentage of wet weight effectiveness was calculated by the formula:

\[ E_{BB} = \frac{(BB \text{ Control} - BB \text{ Treatment})}{BB \text{ Control}} \times 100\% . \]  

The percentage of effectiveness of dry weight is calculated by the formula:

\[ E_{BK} = \frac{(BK \text{ Control} - BK \text{ Treatment})}{BK \text{ Control}} \times 100\% . \]  

Description:
E = Effectiveness  
BB = Wet weight  
BK = Dry weight

2.2.4. Production of Chitinase Enzyme. Detecting the chitinase enzyme producing activity of rhizobacterial isolates required adopting the method recommended by Dukare et al. [7]. This involved
transferring the 48 hours old suspensions (108 cfu/ml) using a micropipette into a petri dish that contains filter paper to a minimum salt medium with a composition of 0.5 g MgSO4.7H2O, 0.7 g K2HP04, 0.3 g KH2PO4, 0.01 g FeSO4.7H2O, 0.001 g ZnSO4, 0.001 g MnCl2, 1% colloidal chitin, and 1000 ml aquades. Furthermore, the bacterial cultures were incubated for 1-3 days at room temperature, and the clear zones formed were indicators of chitinolytic activity, followed by the index measurement (Syahfitri et al., 2018): 

\[ \text{SI} = \frac{\text{Clear zone diameter} - \text{Colony diameter}}{\text{Colony Diameter}} \]  

(5)

2.2.5. **Statistical analysis.** Data were assessed using analysis of variance (ANOVA), and least significant difference (LSD) tests at a 5% probability to compare the differences among treatments.

3. **Results**

3.1. **Macroscopic and Microscopic Forms of C. lunata**

Observing the macroscopic and microscopic forms of *C. lunata* obtained its fungus colony, with morphological characteristics of a dark brown, brown to the black pigmented colony, velvety appearance, branched mycelium, having septa, subhyaline to brown. Furthermore, the conidiophores were brown to black, with conidia arranged in spirals or verticillate, symmetrical, curved, or unequilateral, involving three or four septa, with one of the middle cells being bigger and darker than those on the tip, as shown in Figure 1.

![Figure 1. Fungi C. lunata (A) Colonies of C. lunata on PDA (B) Microscopic of C. lunata 40x magnification (1 = conidia, 2 = bulkhead in conidia, 3 = insulated hyphae)](image)

3.1.1. **Rhizobacteria Inhibition against C. lunata.** Each rhizobacteria showed significantly different abilities in inhibiting *C. lunata*, as all isolates were capable of constraining growth, and the highest was shown by *S. maltophilia* KJKB5.4, with suppression effectiveness of 60.12%. However, the lowest value was exhibited by *O. ciceri* RK12, to be 29.03%, as shown in Table 1.

| Rhizobacterial Isolates         | Inhibitory Zone (mm) | Effectiveness (%) |
|---------------------------------|----------------------|-------------------|
| *Stenotrophomonas maltophilia*  |                      |                   |
| KJKB5.4                         | 34.00 a               | 60.12             |
| *Stenotrophomonas pavani*       |                      |                   |
| LMTSA5.4                        | 35.00 a               | 58.94             |
| *Bacillus cereus*               |                      |                   |
| AJ34                            | 41.00 b               | 51.91             |
| *Alcaligenes faecalis*          |                      |                   |
| AJ14                            | 41.75 b               | 51.03             |
| *Serratia marsecens*            |                      |                   |
| AR1                             | 54.25 c               | 36.36             |
| *Alcaligenes faecalis*          |                      |                   |
| ANO6                            | 57.00 c               | 33.14             |
| *Ochrobactrum ciceri*           |                      |                   |
| RK12                            | 60.50 d               | 29.03             |
| Control                         | 85.25 e               | 00.00             |

The numbers followed by the same letters in the same column are not significantly different at the 5% level (LSD).
A total of 7 rhizobacterial isolates were tested, and 4 isolates showed the potential ability, encompassing *S. maltophilia* KJKB5.4, *S. pavanii* LMTSA5.4, *B. cereus* AJ34, and *A. faecalis* AJ14. These tend to portray significantly different abilities from other isolates with effectiveness suppression levels above 51.03%, thus indicating the potential to control *C. lunata*.

The ability of rhizobacterial inhibition against the growth of *C. lunata* is shown from the zone of inhibition formed around the rhizobacterial isolate colony (Figure 2). Therefore, the ability to inhibit isolates growth is assumed to be related to the antibiotic compound and extracellular enzyme production capacity.

**Figure 2.** Test results of rhizobacterial inhibiting the *C. lunata*. (A) Control (B) *S. pavanii* LMTSA5.4 (C) *A. faecalis* AJ14 (D) *S. maltophilia* KJKB5.4 and E. *B. cereus* AJ34. Arrows: (a) inhibitory zone (b) *C. lunata* and (c) Isolate rhizobacteria (12 days after treatment)

### 3.1.2. Wet and Dry Weight of *C. lunata*.

The analysis of variance results of the wet and dry weight of *C. lunata* fungi colonies showed a significantly different effects produced by rhizobacteria isolates. In addition, it is established that all were capable of suppressing fungi growth, with *B. cereus* AJ34 being the most effective, based on the ability to diminish the wet and dry weight of the fungi colonies at 74.55% and 95.69%, as shown in Table 2. Observations on the dry weight of *C. lunata* colonies were made after drying, and the shape formed is shown in Figure 3.

**Table 2.** The effectiveness of rhizobacterial suppression on the wet and dry weight of *C. lunata* fungi colonies

| Rhizobacteria Isolates | Wet weight of *C. lunata* (g) | Effectiveness (%) | Dry Weight of *C. lunata* (g) | Effectiveness (%) |
|------------------------|-------------------------------|------------------|-----------------------------|------------------|
| *B. cereus* AJ34       | 0.85 a                        | 74.55            | 0.04 a                      | 95.69            |
| *A. faecalis* AJ14     | 1.21 b                        | 63.77            | 0.09 b                      | 90.32            |
| *S. pavanii* LMTSA5.4  | 1.47 c                        | 55.99            | 0.13 c                      | 86.02            |
| *S. maltophilia* KJKB5.4 | 1.63 d                      | 51.19            | 0.16 d                      | 82.79            |
| *A. faecalis* ANO6     | 1.87 e                        | 43.86            | 0.20 e                      | 78.49            |
| *S. marsecens* AR1     | 2.05 f                        | 38.62            | 0.20 e                      | 78.49            |
| *O. ciceri* RK12       | 2.05 f                        | 38.62            | 0.21 e                      | 77.41            |
| Kontrol                | 3.34 g                        | 0.000            | 0.93 f                      | 0.000            |

The numbers followed by the same letters in the same column are not significantly different at the 5% level (LSD).
3.1.3. The Ability of Rhizobacteria Isolates to Produce Chitinase Enzymes. All rhizobacterial isolates were able to produce chitinase enzymes. The highest ability was shown by *S. pavanii* LMTSA5.4 with a chitinolytic index of 4.10, while *A. faecalis* AJ14 showed the lowest index value, as shown in Table 3.

| Rhizobacteria                  | Chitinolytic Index |
|-------------------------------|--------------------|
| *S. pavanii* LMTSA5.4         | 4.10.              |
| *S. maltophilia* KJKB5.4      | 3.50               |
| *B. cereus* AJ34              | 3.00               |
| *A. faecalis* AJ14            | 2.50               |
| *A. faecalis* ANO6            | 1.50               |
| *S. marsescens* AR1           | 1.50               |
| *O. ciceri* RK12              | 1.00               |

Rhizobacteria that are able to produce chitinase enzymes are characterized by the clear zone around the colony as shown in Figure 4.

Figure 3. Dry form of *C. lunata*; (A). control, (B). *S. maltophilia* KJKB5.4, (C). *S. pavanii* LMTSA5.4, (D). *A. faecalis* AJ14, (E). *B. cereus* AJ34.

Figure 4. Chitinolytic activity of rhizobacteria (a) *S. maltophilia* KJKB5.4, (b) *B. cereus* AJ34, (c) *A. faecalis* AJ14 and, (d) *S. pavanii* LMTSA5.4. Arrows indicate clear zone formation by rhizobacteria (8 days after treatment).
4. Discussion

A total of seven rhizobacteria were used in the dual culture test, and 4 with the potential to suppress *C. lunata* growth were identified, indicating the propensity of adoption as antagonistic agents for inhibition. This ability is seen from comparing the pathogenic fungus diameter of treated *C. lunata* with that of the control.

The inhibition zones formed (clear zones) were assumed to be due to the release of antibiotic substances from the rhizobacterial isolate. Also, the fungus hyphae of *C. lunata* became thin as a result of the secretion of antifungal compounds, leading to the occurrence of damage during cell wall formation, as well as on the pathogenic fungus hyphal structure. This was in accordance with the report by Rahma et al. [17], which stipulated the enhanced propensity for *S. maltophilis* KJKB5.4 and *S. pavanii* LMTSA5.4 to inhibit the growth of *Diplodia maydis* with efficacy above 50%. Furthermore, Rahma et al. [14] also stated that *B. cereus* AJ34, *A. faecalis* AJ14, *A. faecalis* ANO6, and *S. marsescens* AR1 possess the ability to synthesize IAA, dissolve phosphate, and produce siderophore. (Rahma, Nurballis & Kristina [18] added that the *S. maltophilis* KJKB5.4 and *S. pavanii* LMTSA5.4 have the capacity to produce siderophore, and were also able to dissolve phosphate, and stated the tendency for two isolates, encompassing *S. malthopilia* KJKB5.4 and *S. pavanii* LMTSA5.4 to synthesize IAA phytohormones.

According to Hernandez et al. [16], Rhizobacterial strain B23 has the capacity to inhibit the growth of four pathogenic fungal lines, up to 55.56%, leading to abnormality in the pathogenic fungus hyphae. This occurred due to its ability to produce secondary metabolite compounds and IAA. Furthermore, the results of the study conducted by Abidin et al. [1] indicated that the rhizobacteria of the Bacillus sp. possess an antagonistic mechanism in the form of antibiosis by producing antifungal compounds with the capacity to cause abnormal hyphal growth (malformation). Furthermore, Wang et al., [27] also ascertained the propensity of antagonist rhizobacteria Paenibacillus jamilae HS-26 to produce anti-endorinal and antibiotic compounds, siderophore, and also stimulate plant growth on application.

The antibiosis ability of rhizobacteria in the current study is also supported by the chitinase enzyme and siderophore production capacity [14, 16]. This effect was observed in all four potential isolates, evidenced by the varying clear zone. Thus, the formation of greater areas around the rhizobacterial isolate was indicative of enhanced chitinase enzyme production ability. Meanwhile, the rhizobacteria tend to secrete secondary metabolites in an unfavorable condition, in order to maintain survival, which includes antibiotics and extracellular enzymes, e.g., chitinase. Conversely, chitin as one of the constituent compounds of the fungus cell walls is decomposed due to the action of the enzyme produced by rhizobacteria, subsequently leading to destruction (lysis). Thus, abnormal growth of the pathogenic fungus is observed, based on the thinning of the fungus hyphae, and the pathogenic fungus comparably thickens upwards.

According to El-Sayed et al. [8], the forms of interaction between PGPR and plants occur both indirectly and directly, characterized by interactions in the form of an increase in plant growth through N2 fixation mechanisms; promotion of phosphate minerals and zinc dissolution ability; iron absorption with siderophore production; generation of phytohormones, including auxins, cytokines and gibberellins; and the manufacture of 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme, which hydrolyzes ACC as a precursor to the formation of ethylene in plants. Furthermore, there is also a marked reduction in ethylene concentration, and the stimulation of root length in seedlings [2]. According to Saraf et al. [21], the indirect interaction of PGPR with plants occurs through the mechanism of antibiotics production; decreased iron from the rhizosphere; generation of fungal cell wall lysis enzymes, e.g., β- (1,3) - glucanase and chitinase; synthesis of antifungal metabolites, encompassing cyanide, and systemic resistance induction.

Rhizobacteria possesses the capacity to antagonize *C. lunata*, as about 7 rhizobacteria were tested, to obtain the best 4, encompassing *S. malthopilia* KJKB5.4, *S. pavanii* LMTSA5.4, *B. cereus* AJ34 and *A. faecalis* AJ14. These were selected as potential candidates for the control of C. oryzae, with
suppression effectiveness above 50%. Furthermore, additional tests showed the propensity for all isolates to produce the chitinase enzyme.

5. Conclusions
Rhizobacteria have the capacity to antagonize the fungus C. lunata, because about 7 isolates were tested, to get the best 4, including S. malthophilia KJKB5.4, S. pavanii LMTSA5.4, B. cereus AJ34 and A. faecalis AJ14. This was chosen as a potential candidate for C. lunata control, with an emphasis above 50% effective. Furthermore, additional tests showed a tendency for all isolates to produce the chitinase enzyme. Further testing is needed in the form of a rhizobacteria consortium to optimize its ability to suppress pathogenic fungi.

Acknowledgements
This research funded through research grant of The Directorate of Research and Community Service, Directorate General of Research and Technology Strengthening Ministry of Research, as well as the Technology and Higher Education, in accordance with Research Contract Number: 163/SP2H/AMD/LT/DRPM/2020 and T/26/UN.16.17/PT.01.03/AMD/PD-Pangan/2020

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