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The Potency of UB Forest Chitinolytic Bacteria to Promote Plant Growth and Inhibit Damping off Disease on Soybean

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KEYWORDS

Chitinase; Plant Growth Promoting (PGP); *Rhizoctonia solani*

Abstract Damping off disease in soybean plants is caused by the fungal *Rhizoctonia solani*. The damping off disease causes a yield loss of up to 85-100%. The purpose of this study was determining the ability chitinolytic bacteria consortium of UB Forest's in suppressing damping off disease in vitro and in vivo as well as its potential to stimulate the growth of soybean plants. The research stages included isolation of the pathogenic fungus *R. solani* and the pathogenicity test. Rejuvenation chitinolytic bacterial isolates of UB Forest, test chitinolytic bacteria antagonist of UB Forest against *R. solani* fungus, test of Plant Growth Promoting (PGP) activities, synergy test of selected chitinolytic bacterial isolates, in vitro test for the inhibition of chitinase crude extract against *R. solani*, and inhibition test of chitinolytic bacteria consortium against damping off disease. The selected chitinolytic bacteria were code bacteria UB12, UB19, and UB52 with plant growth promoting activities with inhibition percentage of the pathogen *R. solani* of 73.9%, 67.4%, and 71.7%. The best chitinolytic bacterial isolates were the genus *Bacillus* sp. and *Pseudomonas* sp. The inhibition test of chitinase crude extract showed an inhibition percentage of 25-55%.

Introduction

Soybean is an important commodity that has a very high, balanced, and complete protein nutritional content. The increase in population and public awareness of food nutrition causes soybean consumption as a home industry product to increase (Aldillah, 2015). However, soybean production during the last 15 years has decreased and is only able to meet domestic needs of less than 15% of the total requirement of 2.45 million tons, so that as much as 86.95% of soybean needs must be met by imports (Kementerian Pertanian, 2017; Mahdi and Suharno, (2019) Therefore, meeting the availability of soybeans is a challenge in increasing soybean production.

One of the inhibiting factors for soybean production is due to disease attack, especially damping off disease by the pathogen *Rhizoctonia solani*. The pathogen infects the root or stem adjacent to the soil. As a result, nutrient and water transportation is clogged so that plants wither and experience rot (Zhang *et al*., 2013). The attack of the pathogenic fungus *R. solani* resulted in yield losses of up to 100% if the attack occurred in the early stages of soybean growth and the loss reached 85% (Khaeruni and Rahman, 2012).

Efforts to control damping off disease generally use synthetic fungicides. Continuous use of synthetic fungicides with excessive doses can pollute the environment, are resistant to plant pathogens and interfere with human health (Arif, 2015). Therefore, biological control that is environmentally friendly and integrated integrated pest control (IPM) needs to be developed by utilizing antagonistic microbes such as chitinolytic bacteria. Chitinolytic

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bacteria are able to produce chitinase enzymes to degrade the cell walls of pathogenic fungi which contain 40% of chitin molecules (Sari and Ira, 2015).

University of Brawijaya Educational Forest or UB Forest is a tropical forest with a high biodiversity category and is known to have complex microbial communities (Hutamy and Yulia, 2019). Previous research has conducted a study on the diversity and abundance of chitinolytic bacteria in UB Forest and obtained 76 chitinolytic bacteria from the genus *Bacillus* sp., *Pseudomonas* sp., *Pantoea* sp., *Erwinia* sp., *Xanthomonas* sp., And *Clostridium* sp. (Karina, 2019). Therefore, in this study, all isolates will be carried out further testing to determine the potential antagonist against the pathogenic fungus R. solani in the form of the best combination of bacteria to control damping off disease and its potential to spur growth of soybean plants.

Materials and methods

Time and Place of Research.

The research was conducted for 3 months at the Plant Disease Laboratory and Glass House, Department of Plant Pests and Diseases Faculty of Agriculture, University of Brawijaya.

Isolation of R. solani Fungi and Pathogenicity Test.

Isolation of the pathogenic fungus *R. solani* using soybean plant parts that are symptomatic of damping off disease and then they are planted on PDA media. The pathogenicity test of *R. solani* refers to the method of Rustam *et al*., (2011) by preparing a 48-hour-old pure *R. solani* fungal culture, diluting it into 10 mL of sterile distilled water and calculating the concentration with a fungal density of 106 cfu mL^{-1}. After that, the fungus inoculation of *R. solani* at the base of the stem of healthy soybean plants aged 2 weeks and incubated for 7 days. The symptoms that arise must have the same initial symptoms.

Rejuvenation of chitinolytic bacteria isolates.

The rejuvenation of isolates consisted of 62 UB Forest chitinolytic bacteria isolates with bacterial genera including *Bacillus* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Pantoea* sp., *Erwinia* sp., dan *Clostridium* sp. The rejuvenation was carried out by using the streak plate method on Nutrient Agar (NA) media and incubated at room temperature for 48 hours. Furthermore, bacterial isolates were made into culture stock on NB media and stored at 6-8°C in a refrigerator

UB Forest Chitinolytic Bacteria Antagonist Test against R. solani Fungi.

The antagonist test between *R. solani* and chitinolytic bacterial isolates was carried out in vitro which refers to the method of Herliyana *et al.* (2013) namely a modified multiple culture method in one confrontation plate. The filter paper was immersed in chitinolytic bacterial suspension and placed on PDA media at 4 points with a distance of 3 cm from the center of the petri dish. After 24 hours, *R. solani* fungals were inoculated in the center of the petri dishes and incubated at room temperature. Observations were made 6 days after isolates were paired. The mycelium growth of *R. solani* fungi measured the radius R1 (radius of fungal mycelium growth towards the edge of the petri) and R2 (radius of mycelium growth towards antagonistic bacteria), then calculated the percentage of inhibition of chitinolytic bacterial isolates against *R. solani* fungi with using the formula (Padmaja *et al*., 2013).

$$I = \frac{R1-R2}{R1} \times 100\% \quad \text{..... (1)}$$

Note: I: Percentage of inhibitory power, R1: pathogen radius of *R. solani* in control treatment, R2: radius of non-control *R. solani* pathogen.

Plant Growth Promoting (PGP) Activity Test.

(a) Phosphate Solvent Activity, testing bacteria as a phosphate solvent using Pikovskaya selective media refers to Silitonga *et*

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al. (2013) modified. A total of 1 ose of bacterial isolate was diluted in 10 mL of sterile distilled water and added with a biocide (fungicide for bacterial isolates). A total of 1 mL of bacterial suspension and aseptically poured into a petri dish containing Pikovskaya agar medium. Shake the petri dish until the bacterial suspension is evenly distributed on the surface of the media. Furthermore, the bacterial culture is incubated for 24-48 hours at room temperature. Phosphate dissolution indicators are indicated by a clear zone around the growing bacterial colony. (b) Nitrogen Fixing Activity, the nitrogen fixation test was carried out by pouring 1 mL of the bacterial suspension on Burk media. The bacterial cultures were then incubated for 24-48 hours at room temperature. Bacteria that can grow on Burk media are bacteria that can bind nitrogen. The composition of Burk media is composed of 1.3 g Burk salt, 15 g agar, 1 mL Fe-Mo, 20 g sucrose and 1 L distilled water. Burk salt is composed of MgSO4 20 g, K2HPO4 80 g, KH2PO4 20 g, CaSO4 13 g, whereas Fe-Mo consists of 1.45 g FeCl3, NaMoO4 0.253 g, 1 L distilled water.

Synergy Test of UB Forest Chitinolytic Bacteria Isolates.

The selected chitinolytic bacterial isolates were tested for synergy between the bacterial isolates obtained. The method used refers to Asri and Zulaika (2016) research by growing chitinolytic bacterial isolates intersecting on the same NA media. Bacterial isolates were scratched and rubbed against each other so that the isolates would meet. Bacterial cultures were incubated for 24 hours and observed whether a clear zone was formed indicating inhibition of the two isolates. Isolates are said to be compatible if an inhibition zone is not formed in the area where the two isolates meet and vice versa is said to be incompatible when an inhibition zone is formed in the area where the two isolates meet (Silitonga *et al*., 2013).

In Vitro Test for the inhibition of chitinase crude extract against R. solani.

Testing of chitinase crude extract was carried out using agar well diffusion method (Wibowo *et al*., 2017). Chitinolytic bacteria were grown on 150 mL NB medium and incubated in a room temperature orbital shaker at a speed of 120 rpm for 72 hours, then 10 mL of culture were centrifuged at 4000 rpm for 20 minutes. One mL of the supernatant was filtered using a 0.45 µm Millex-HV Sterile Filter Durapore PVDF Membrane bacteria filter and put in an eppendorf tube. A total of 30 µL of chitinase crude extract was inserted into the well on PDA media which was 3 cm from the 3 day old R. solani colony. The in vitro experimental design used a completely randomized design (CRD) with 9 treatments and 3 replications. The percentage of fungal inhibition was measured for 7 days using the formula (Elfina *et al*., 2015).

$$\% \text{ Inhibition} = \frac{a-b}{a} \times 100\ \% \quad \text{.....(2)}$$

Note: a: r mycelium to the edge of the petri dish, b: r mycelium to the well

Result and discussion

Isolation of R. solani Fungi and Pathogenicity Test.

The pathogenic fungus *R. solani* was isolated from the base of the soybean plant that showed symptoms of damping off. The pure culture from the isolation was grown on PDA media. The result of pure culture is a straight white hyphae isolated from the cortex of the plant stem. The results of the isolation of the pathogenic fungus *R. solani* on PDA media were originally white, the longer the age of the fungus will cause a brownish color change and so on the longer it is stored, the media will be dark brown. It is shown in Figure 1.

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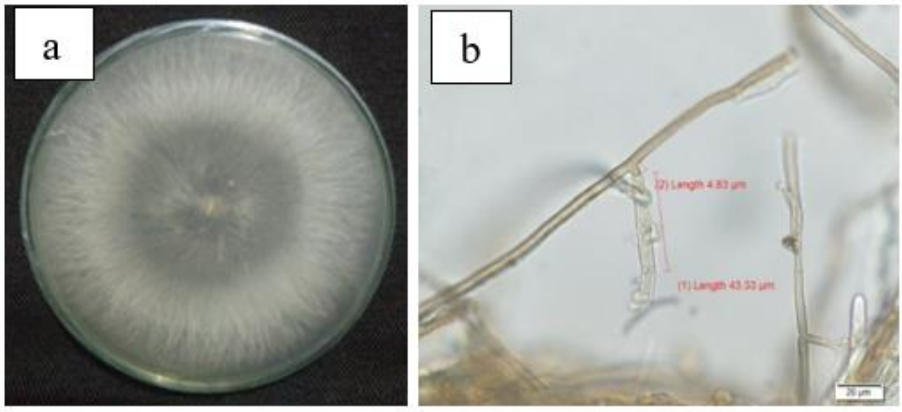

Figure 1. Pathogen isolation results (a) Macroscopic appearance of the pathogen *R. solani* (b) Microscopic appearance of the pathogen R. solani at 400x magnification

Fungal growth is relatively fast and reaches a colony diameter of 90 mm within 4 days after inoculation, with distinctive white, dark brown mycelia and irregularly shaped sclerotia structures. The observation results of the pathogenic fungus basidiospora *R. solani* at a microscope magnification of 400x were insulated and branched unicellular hyphae measuring 4.83 µm and a branch that formed a right angle. This is in accordance with Novina *et al*. (2015) who stated that the characteristics of the fungus are colorless young hyphae, adult hyphae are white to blackish brown, hyphae length 4 - 10 µm, septa, forming a branch with an angle of 90°.

UB Forest Chitinolytic Bacteria Antagonist Test against R. solani Fungi.

The antagonist selection results showed that there were 59 bacteria from 62 UB Forest chitinolytic bacteria which were antagonistic to the pathogen *R. solani.* A total of 40 bacteria had an inhibitory percentage of 51-76% and 19 bacteria had an inhibitory percentage of 37-50% and 3 bacteria had no antagonistic activity. UB Forest chitinolytic bacteria which are classified as having high antagonistic activity reach 66% of the total bacteria. This is because the isolates were obtained from the UB Forest environment with high biomass and decomposition activity by decomposers and detritivores composed of chitin. Terahara *et al*. (2009) reported that chitinolytic bacteria from natural ecosystems with high soil fertility levels are more potential, diverse, and distinctive. According to Suryadi *et al*. (2013) a high chitinolytic index indicates that bacteria produce large amounts of chitinase which can affect the antagonistic properties of bacteria.

Plant Growth Promoting (PGP) Activity Test.

The activity of Plant Growth Promoting (PGP) is known from the ability of chitinolytic bacteria to fix nitrogen and dissolve phosphate. The test results showed that there were 11 bacteria with the potential to fix nitrogen, 4 bacteria had the potential to dissolve phosphate, 39 bacteria had both activity, and 8 bacteria did not have PGP activity. The potential is indicated by the presence of a clear zone on the pikovskaya media. The clear zone that is formed around the bacterial colony is due to the production of the enzyme phosphatase to dissolve phosphate (Goenadi *et al*., 2000). Meanwhile, chitinolytic bacteria that are able to grow on Burk media are bacteria that have the potential to fix nitrogen. Chitinolytic bacteria can fix nitrogen because

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they have a nitrogenase enzyme that will help bind free N2 in the air (Ramamoorthy and Samiyapn, 2001). It can be seen in Figure 2.

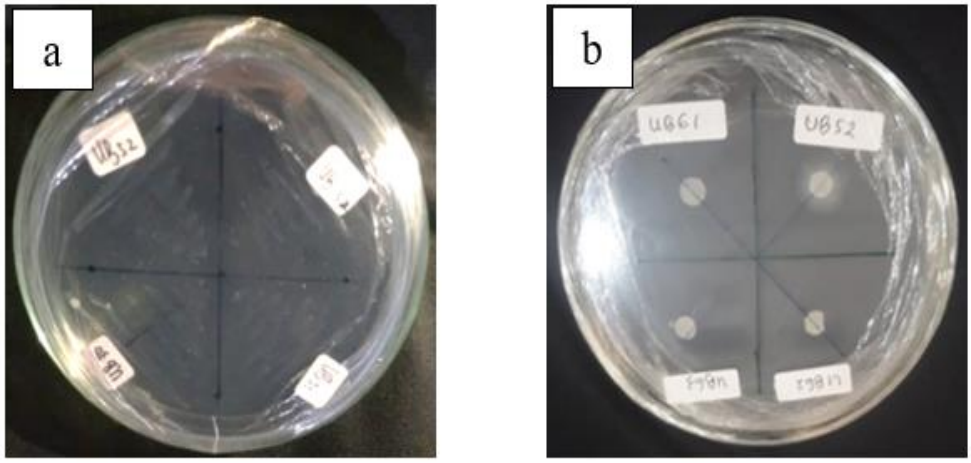

Figure 2. Activity test results *Plant Growth Promoting* (PGP) (a) nitrogen anchors on Burk media (b) phosphate solvent on Pikovskaya media

Based on the selection results of the Plant Growth Promoting Activity (PGP) test and the in vitro antagonist test, then three bacteria with the best abilities were selected for further testing.

Table 1. Selection of selected chitinolytic bacteria

Chitinolytic Bacteria Isolates	Phosphate Solvent	Nitrogen binders	Percentage of Inhibition
UB12 (*Bacillus* sp.)	+	+	73.9%
UB19 (*Pseudomonas* sp.)	+	+	67.4%
UB52 (*Pseudomonas* sp.)	+	+	71.7%

Three chitinolytic bacteria were selected, namely the bacterial code UB12, UB19, and UB52 which have plant growth promoting activities with inhibition percentage against pathogenic fungi R. solani of 73.9%, 67.4%, and 71.7%. The three best chitinolytic bacterial isolates were the genus Bacillus sp. and Pseudomonas sp. which will then be carried out further testing with a consortium combination.

Synergy Test of UB Forest Chitinolytic Bacteria Isolates.

UB Forest's chitinolytic bacteria consortium can be successful by showing the synergistic activity or compatibility between bacterial isolates that are grown intersectively. The test results showed that the three selected chitinolytic bacteria were UB12 (*Bacillus* sp.), UB19 (*Pseudomonas* sp.), and UB52 (*Pseudomonas* sp.) Synergistic or compatible. This is evidenced by the absence of the formation of an inhibition zone in the area where the isolates were grown in petri dishes. Bacterial isolates that are synergistic or compatible are characterized by not forming a clear zone caused by the compatibility between two or more bacteria that associate with each other and form a mutualistic symbiont, resulting in significant abilities (Hartanti, 2020). It is shown in Figure 3.

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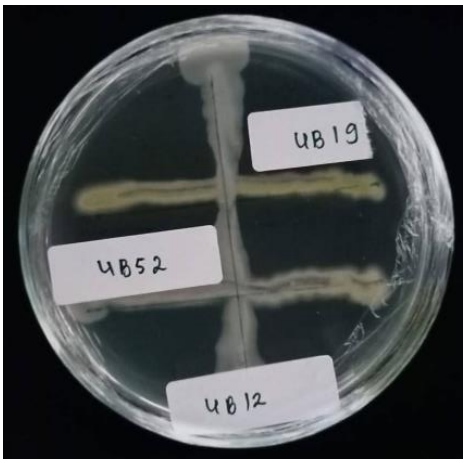

Figure 3. Synergy test results for selected chitinolytic bacteria

Inhibition of Chitinase Crude Extract against R. solani.

Selected UB Forest chitinolytic bacteria chitinase crude extract (UB12, UB19, and UB52) had 25-55% greater inhibition power than the 80% mancozeb fungicide treatment. The treatment of UB Forest chitinolytic bacteria chitinase crude extract had a higher inhibitory power than the fungicide control treatment in inhibiting the pathogen *R. solani.* The highest inhibition was found in UB12 (*Bacillus* sp.) Treatment with an inhibition percentage of 55%, so that it was 4 times more effective than the 80% Mancozeb fungicide.

Table 2. In vitro test results of bacterial chitinase crude extract against *R. solani*

Treatment	Percentage inhibition (%)
Akuades	0.00^{a}
Fungisida	12.67^{ab}
UB12 (*Bacillus* sp.)	55^{c}
UB19 (*Pseudomonas* sp.)	25.33^{abc}
UB52 (*Pseudomonas* sp.)	40.67^{bc}

Chitinase crude extract of bacterial can lyse the structure of chitin as the main compound making up the fungal cell wall (Suryadi *et al.*, 2013). The potential of chitinase crude extract in suppressing damping off disease had been proven by (Nurdin *et al.,* 2013) who reported that the application of bacterial and supernatant formulations containing chitinase crude extract was able to reduce the severity of damping off disease. This is supported by (Alshehri *et al.*, 2016) who stated that chitinase from bacterial activity plays an important role in controlling fungal pathogens and increasing disease tolerance in plants with the production of citioligosaccharides which are further degraded to N-acetylglucosamine by chitobiose. The chitinase enzyme can be produced by the bacteria Bacillus sp. (Gomaa, 2012) and Pseudomonas sp. (Haedar *et al*., 2017). Crude bacterial chitinase extract is known to be able to lyse the chitin structure as the main compound for the cell wall of pathogenic fungi (Suryadi *et al.,* 2013).

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Conclusion

UB Forest's chitinolytic bacteria with the best ability, namely UB12 (*Bacillus* sp.), UB19 (*Pseudomonas* sp.), and UB52 (*Pseudomonas* sp.) have Plant Growth Promoting (PGP) activity and high percentage of inhibition against pathogen *R. solani*. The best consortium of three chitinolytic bacteria showed synergistic or compatible results. Bacteria that have synergistic activity were tested for inhibition of chitinase crude extract against *R. solani* pathogens in vitro with the inhibition percentage of 25-55%. The UB12 treatment had the highest inhibitory activity, namely 55% so that it was 4 times more effective than the Mancozeb 80% fungicide.

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