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Research Article

In Vitro Study of Action Mode of *Rhodotorula minuta* Dmg 16 BEP as Biocontrol Agents on *Alternaria solani*

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ABSTRACT

Rhodotorula sp. is widely known as a biocontrol agent and is reported effective in controlling several diseases on crops. *Rhodotorula minuta* Dmg 16 BEP is an antagonist yeast from Indonesia which was effective in controlling various plant diseases. The objective of this research was to study the *in vitro* mode of action of *R. minuta* Dmg 16 BEP against *Alternaria solani*. The antibiosis ability of *R. minuta* Dmg 16 BEP to *A. solani* might be based on the activity of volatile compounds and not from its metabolites products. The growth of *A. solani* in paper disk assay and dual culture tests were not inhibited nor inhibitory zones developed, whereas the inverse petridish test showed the growth inhibition of *A. solani*. The results of the chitinolytic activity test on chitin agar and proteolytic on skim milk agar showed that no clear zone was formed. *R. minuta* has a strong hyperparasitic ability according to the slide culture test as the yeast cells surrounded *A. solani* hyphae and caused damage to some parts of hyphae.

Keywords: *Alternaria solani*; antibiosis; hyperparasitism; *Rhodotorula minuta*

INTRODUCTION

Rhodotorula sp. is a yeast widely developed as a biocontrol agent. This yeast is from the group of Basidiomycota phylum with the characteristics of the orange colony, oval cell shape with a size of 3× 4 µm, and able to form budding cells. This yeast is found in soil, litter, leaves, stems, and flowers (Sjamsuridzal *et al*., 2010). Several species of *Rhodotorula* were able to suppress the development of post-harvest fruit diseases, such as *R. gutinis* strains HRA4 and YMB4 in pears (Spotts & Cervantes, 2002), *R. gutinis* LS11 in apples (Costaria, 2005), and *R. mucilaginosa* in peaches (Zhang *et al*., 2016).

Rhodotorula sp. mechanism as an antagonistic agent is vary depending on its strain and species. Gholamnejad *et al*. (2010) reported that *R. mucilaginosa* has the antibiotic ability by producing volatile compounds and metabolites that can inhibit the development of *Penicillium expansum* in apples. *R. mucilaginosa* from China was reported to have the ability to induce resistance by increasing the concentrations of PPO and POD enzymes and has strong spatial and nutritional competitiveness (Li *et al*., 2011), while *R. glutinis* LS-11 was reported to have lysis ability by producing Glucanase enzymes (Castoria *et al*., 1997). Li *et al.* (2016) reported that *R. glutinis* also has a strong hyper-parasitic ability against conidia *Botrytis cinerea*.

R. minuta Dmg 16 BEP is a type of antagonistic yeast from Indonesia. In previous studies, this yeast is reported to be able to suppress 90.07% of the anthracnose development in post-harvest chilies (Hartati *et al*., 2014). Yet, this antagonist yeast has not been tried to control brown spot disease in tomatoes. This disease is caused by *A. solani* infection which can result in a yield loss of 86% (Kalay & Sinay, 2015). This study aimed to determine the mode of action of *R. minuta* Dmg 16 BEP in suppressing the development of *A. solani*.

MATERIALS AND METHODS

Isolation and Identification of A. solani

Isolation of *A. solani* was performed using a single spore isolation method. The symptomatic leaves with brown spots were cut to a size of 0.5 × 0.5 cm (1 g) and put into a test tube containing 10 ml sterile distilled water and then shaken with a vortexer to dissolve the conidia. The spore suspension was dropped on 10 µl Potato Dextrose Agar (PDA) media and incubated at room temperature for 24 hours. Hyphae formed from germinating single spores were cut and transferred to new media. Sporulation induction was performed following the method by Shahin & Shepard (1979) and Nasreen *et al.* (2017) with some modifications. *A. solani* isolates were grown on sporulation media with a composition of 10 g of tomato leaves, 15 g of agar, 0.5 g of $CaCo_3$, and 1 L of aquadest. Incubation was performed in the dark condition at 18°C. The pure isolates were stored for further treatment. Molecular identification by Polymerase Chain Reaction (PCR) using a common 18S rDNA primer by the forward primer ITS5 and reverse primer ITS4. Electrophoresis was performed in 1.5% w/v agarose gel (TopVision, Thermo) with GeneRuler 1000 bp marker and colored with ethidium bromide. The PCR products were sent to Macrogen, South Korea for DNA sequencing. The nucleotide sequences were compared with the nucleotide sequences found in the NCBI GenBank database, using the nucleotide basic local alignment search tool (BLAST).

R. minuta *Isolates Preparation*

Isolate *R. minuta* Dmg 16 BEP was obtained from the results of previous studies (Hartati *et al*., 2014). The yeast stored in ampoules was rejuvenated on PDA media and incubated for 72 hours. Isolates were stored in a refrigerator at 5°C and rejuvenated when it was used for the test.

Dual Culture Test

This test was according to Gholamnejad *et al.* (2010). Yeast was scratched on PDA media perpendicularly in the middle of the petri dish (Ø 9 cm). Pure culture of *A. solani* was taken with a cork drill (Ø 0.6 cm) and placed next to the yeast streak, then incubated at room temperature in the dark condition. Observations were made by measuring the width of the formed inhibition zone.

Rough Metabolite Test for R. minuta

This test was performed using the paper disc assay method with several modifications (De Beer & Sherwood, 1945). *R. minuta* was grown on potato dextrose broth (PDB) media and incubated for 10 days. The *R. minuta* suspension was centrifuged at 14000 rpm to separate the supernatant from *R. minuta* cells. The supernatant was taken and filtered using a 0.22 µm filter. The 10 µl of the crude metabolite from the filtering results was dropped on a paper disc that was placed around the colonies of *A. solani* which was four pieces per petri dish.

Volatile Organic Compounds (VOC) Production Ability

The production capability of VOC by yeast was performed using the inverted plate technique. *R. minuta* aged 5 days was inoculated on the bottom of the petri dish and *A. solani* (Ø 0.6 cm) aged 10 days was inoculated on the top of the plate that had been poured with media. Observations were made at the end of the incubation period by comparing *A.solani* diameter with the control.

Chitinolytic Activity Test

R. minuta aged 5 days was streaked on 0.5% chitin medium perpendicularly. Incubation was conducted at room temperature for 3–5 days. Daily observations were performed during the incubation period of the clear zone formed on the edge of the yeast colony (Murthy & Bleakley, 2012).

Proteolytic Activity Test

This test used a medium of Skim Milk Agar (SMA). *R. minuta* was streaked upright on SMA media. Incubation was performed at room temperature for 3–5 days. Proteolytic activity was indicated by the formation of a clear zone around the yeast colony (Jones *et al*., 2007).

Hyperparasitism Ability Test

This test was conducted using water agar with the agar block/slide culture method (Wijedasa & Liyanapathirana, 2012). *R. minuta* aged 48 hours was inoculated on Water Agar (WA) block that had been overgrown with *A. solani* hyphae, then dropped with sterile water (10 µl) and covered with a cover glass. The incubation was performed for 4–5 days and then observed the affinity of the yeast in colonizing the mycelium under a microscope.

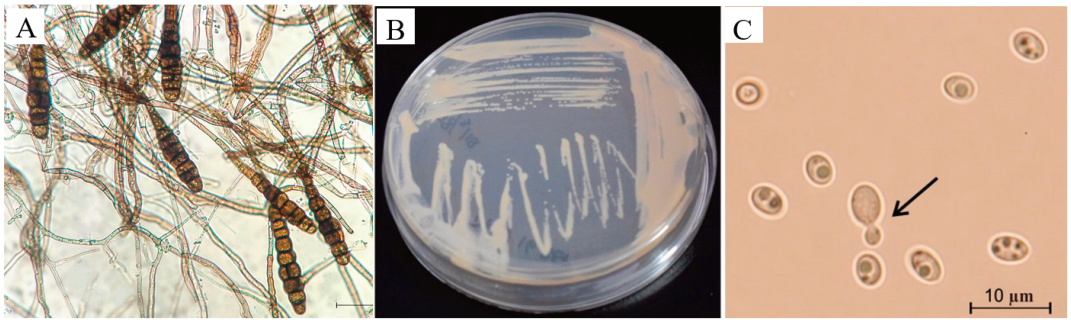

Figure 1. Morphological characteristics of pathogenic isolates and antagonistic yeast of (A) *Alternaria solani* and (B) *Rhodotorula minuta* on PDA medium; (C) microscopic morphological appearance of cell and budding *R. minuta* Dmg 16 BEP

RESULTS AND DISCUSSION

Pathogenic Isolates and Antagonistic Agents

A. solani isolation was taken from the leaves and stems of symptomatic tomatoes of brown spot disease. The results of the isolation were grown on PDA media characterized by the blackish-brown bottom surface of the colony, the white edges of the colony, and the gray colony surface. In the sporulation medium, the shape of conidia was like a club, brown, and has 6–9 horizontal bulkheads. The hyphae were hyaline and septate (Figure 1). The PCR products visualized by electrophoresis showed amplified DNA bands at 540 bp. The results of the BLAST nucleotide analysis showed that isolate B02 was 99% similar to *A. solani* F10 based on GenBank data. *R. minuta* Dmg 16 BEP yeast was grown on PDA media and the colonies formed were orange, convex, shiny, and had a flat surface. Yeast cells were oval, hyaline, and formed budding in some of the cells. Yeast cells had a size of 3.32 × 4.44 µm (Figure 1).

Antibiosis Ability

The results of the dual culture test and paper disk assay were negative. *A. solani* growth was not inhibited or no inhibition zone was formed (Table 1). This showed that *R. minuta* did not produce dissolved antibiotic compounds. In contrast to the results of the inverted plate test, *A. solani* growth was inhibited when compared to the control (Figure 2). This finding showed that *R. minuta* Dmg 16 DEP could produce volatile compounds that inhibited the development of *A. solani*. The previous studies also stated that most of the ability of yeast antibiosis was based on the produced volatile compounds (Hartati *et al*., 2015). Other yeast groups such as *Candida saitoana*, *Debaryomyces hansenii*, *Cryptococcus* sp., and *R. mucilaginosa* were also reported to have the ability to produce VOC (Buzzini *et al*., 2005). Di Francesco *et al*., (2015) reported that antagonistic yeasts could produce volatile compounds that play a role in inhibiting the development of pathogens, such as *Botrytis cinerea*, *Colletotrichum acutatum*, *P. expansum*, *P. digitatum,* and *P. italicum*.

Table 1. Inhibition mechanism of *Rhodotorula minuta* Dmg 16 BEP against the development of *Alternaria solani*

Mechanism		Activity
Antibiosis	Dual culture	-
	Paper dish assay	-
	Inverted petridish	+
Lysis	Agar chitin	-
	Skim milk agar	-
Hiperparasitism	Slide culture	+

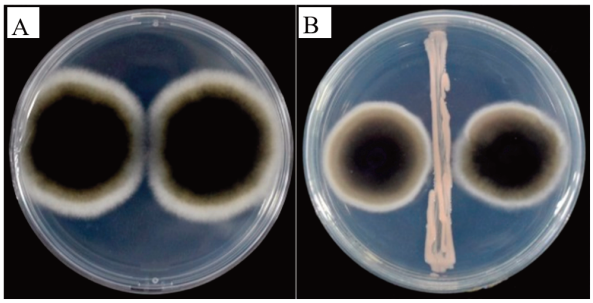

Figure 2. Effect of volatile compounds of *Rhodotorula minuta* Dmg 16 BEP on development of *Alternaria solani*; (A) control for inverted plate test, (B) invers plate test with test yeast treatment, *Alternaria solani* diameter was smaller than the control

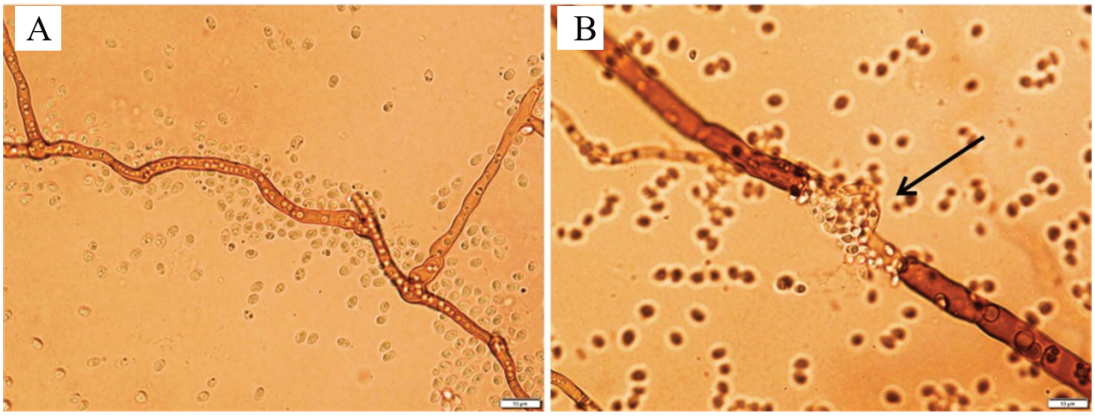

Figure 3. Hyperparasitism test; (A) yeast cell *Rhodotorula minuta* Dmg 16 BEP approaching *Alternaria solani* hypha, (B) hypha damage of *Alternaria solani*

Lysis Ability

Lysis ability test aimed to determine the potential of *R. minuta* in degrading pathogenic cell walls. The composition of the fungal cell wall in the Ascomycota group is generally chitin (39%), glucan (29%–60%), protein (7%–13%), and lipid (6%–8%) (Wabster & Waber 2007). However, the cell wall composition of *A. solani* is still unknown. The results of the protease and chitinase lysis ability test showed that *R. minuta* was unable to produce both enzymes characterized by the absence of clear zones on the edge of the colony (Table 1).

Hyperparasitism Ability

Contact between *R. minuta* and *A. solani* cells occurred 24 hours after application. *R. minuta* colonized the hyphae and caused lysis (Figure 3). The attachment of yeast to the pathogenic structure was closely related to its biocontrol ability (Chan & Tian, 2005). Antagonistic yeast which had a high colonization ability would help the performance of other modes of action such as antibiosis and lysis. When there was contact between yeast and pathogenic cells, the enzymes that degrade the cell wall of the yeast would meet the substrate, so the hydrolysis process occurred. The chitinolytic and proteolytic activities in this study showed negative results, hence the damage to hyphae might be due to antifungal activity (VOC) or other toxins (Liu *et al.*, 2013; Chen & Chou, 2017).

El-Ghaouth *et al.* (2003) reported that *Candida saitoana* could attach to the *B. cinerea* hyphae and inhibit its development. *Pichia guilliermondii* was reported to have a strong adherence to *B. cinerea* hyphae (Wisniewski *et al.*, 1991). Raharjo *et al.* (2006) reported that yeast antagonists could parasitize *C. lagenarium*. Chan and Tian (2005) reported that *P. membranefaciens* and *Cryptococcus albidus* cells were able to attach to the hyphae of *Monilinia fructicola*, *P. expansum,* and *Rhizopus stolonifer*. The attachment of these yeast cells was reported to be largely determined by the role of extracellular polysaccharide (Andrews *et al.*, 1993), uronic acid (Pouliot *et al.*, 2005), and the concentration of growth media (Chan & Tian, 2005).

CONCLUSION

The mechanism of *R. minuta* Dmg 16 BEP which was involved in the inhibition of *A. solani* includes the production of volatile compounds and hyperparasitism. *R. minuta* Dmg 16 BEP did not produce protease enzymes, chitinase enzymes, and antifungal metabolites.

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