Mode of action of the endophytic yeast *Rhodotorula mucilaginosa* in controlling basal stem rot caused by *Phytophthora capsici*

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**Abstract.** *Phytophthora capsici* is the cause of basal stem rot (BSR) disease in black pepper plants. The BSR attack rate can reach 67% and can reduce black pepper production in the field by 30–40%. Application of yeast as a BSR control technique has not yet been studied, and this study therefore aims to determine the mechanism of yeast's action in suppressing *P. capsici* mycelium growth *in vitro*. Yeast is obtained from the Piperaceae family, and testing is through the dual culture antibiosis mechanism. There are two *Rhodotorula mucilaginosa* isolates from Piperaceae plants that can significantly inhibit mycelium growth of *P. capsici* through hyperparasitism, production of volatile organic compounds, and the ability to produce β-1,3 glucanase enzyme. Microscopic observations using compound microscopy and scanning of microscopic electrons show that *Rhodotorula mucilaginosa* has the ability to lyse *P. capsici* hyphae development.

1. **Introduction**

In Vietnam, basal stem rot (BSR) caused by *Phytophthora capsica* is a significant disease of black pepper [1]. One of the factors driving the low production of black pepper in Indonesia is BSR, with yield losses reaching 30–40% in Lampung province [2]. *Phytophthora capsici* is a destructive pathogen of black pepper (*Piper nigrum* L.) that attacks all tissues and plant growth stages [3]. Control of BSR is achieved by crop rotation, cultural practices, fungicides, and resistant cultivars [4]. However, the improper use of fungicides has caused problems such as pathogen resistance.

Biological control of BSR is another method available for controlling *P. capsici*. Several studies show that using a biocontrol agent such as *Trichoderma* spp. can inhibit BSR in the field [2]. Endophytic bacterial control of BSR in black pepper has been reported by Aravind et al., [5], using organisms such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Bacillus megaterium*. Yeast is an antagonistic microbe that is known to act as a biocontrol agent. Several yeast antagonists are reported to have antibiotic properties, and most of these properties occur through the production of volatile compounds [6]. Yeast *Aureobasidium pullulans* is reported to produce cell wall degradation in
Monilinia laxa [7]. The yeasts Pichia membranefaciens and Cryptococcus albidus have been reported to have strong hyperparasitic abilities against the hyphae of Monilinia fructicola, Penicillium expansum, and Rhizopus stolonifer [8]. Yeasts can be involved in mechanisms of control such as antagonistic antibiosis, lysis, competition, induction of resistance, and hyperparasitism.

It is important to understand the modes of action of biocontrol agents in controlling BSR. The main modes of action are antibiosis, production of VOCs, hyperparasitism, and the ability to produce β-1,3 glucanase enzymes. Cryptococcus laurentii and Sporobolomyces roseus have high nutritional competitiveness against Botrytis cinerea [9]. This study aims to determine the mode of action of two isolates of Rhodotorula mucilaginosa in controlling BSR caused by Phytophthora capsici.

2. Materials and Methods

Two yeast isolates were obtained from plant endophytes of the Piperaceae family. Sampling was conducted in Dramaga, Bogor, West Java, Indonesia. Both yeast isolates were identified molecularly as Rhodotorula mucilaginosa. P. capsici was isolated from black pepper plantations showing BSR symptoms in Sukamulya, Sukabumi, West Java.

2.1. Preparing yeast isolates

The Rhodotorula mucilaginosa isolates were obtained from the Safitri 2017 collection. The yeast was stored in ampoules and then reisolated on potato-dextrose agar (PDA) medium and incubated for 72 hours. Isolates were stored in a refrigerator at 5°C and rejuvenated when they were ready for use in testing.

2.2. Antibiosis test

2.2.1. Dual culture test. This test refers to the method used by Rosa et al., [10] with some modification. Yeast was scratched onto PDA medium perpendicularly with P. capsici in a petri dish (Ø 8 cm). Pure culture of P. capsici aged ten days was taken with a cork drill (Ø 0.3 cm) and placed next to the yeast scratch (two pieces per petri dish) and then incubated at room temperature in dark conditions. Observations were made by measuring the width of the clear zone formed. For the second culture test, Rhodotorula mucilaginosa yeast was grown on potato-dextrose broth (PDB) medium and incubated for ten days. The yeast suspension was set at a speed of 150 rpm for yeast cell growth. The yeast suspension was inoculated by P. capsici at seven days old using a cork drill (Ø 0.3 cm). The yeast suspension inoculated by P. capsici was observed to reduce the P. capsici mycelium wet weight by three isolate P. capsici per Erlenmeyer.

2.2.2. Formation of volatile organic compounds (VOCs). The medium used was PDA. The production capacity of VOC by R. mucilaginosa was observed using the inverted plate technique. P. capsici aged five days was inoculated onto the bottom of the petri dish, and P. capsici aged ten days (Ø 0.3cm) was inoculated on the top of the plate of poured medium. Observations were made at the end of the incubation period by comparing the diameter of P. capsici with the control [11].

2.2.3. Glucan activity. Testing β-1,3 glucan production activity was carried out using glucan medium. The antagonistic yeast isolate was streaked onto the glucan medium. Incubation was carried out at room temperature for 3–5 days. The production of the glucanase enzyme was indicating by formation of a clear zone around the yeast [12].

2.2.4. Hyperparasitism test. This test was carried out using water agar and the agar block method. 48-hour-old yeast was inoculating onto a water agar block which had been overgrown with P. capsici hyphae. This was then dripped with sterile water (10 μL) and covered with a glass lid. The sample was incubated for 4–5 days and then observed under a compound microscope with a 100x magnification and a scanning electron microscope with JSM-IT200.
2.2.5. Statistical analysis. The in-vitro test was carrying out using a completely randomized design with three replications and two sub-units. Tables and graphs were processed by MS Office Excel 2010. The in-vitro test treatment which was significantly impacted was further tested by the Student-Newman-Keul (SNK) test at $\alpha = 5\%$ using SAS 9.1 software.

3. Results and Discussion

3.1. Antibiosis test and lysis of Rhodotorula mucilaginosa

One of the advantages of biological control is the variety of control mechanisms offered, making the control effectiveness of the antagonistic agent both high and sustainable. The results showed that yeast isolates exhibited more than one pathogen inhibition mechanism. One study reported that fifteen epiphytic yeast isolates did not have the antibiotic capacity to inhibit $P. capsica$ [13]. However, epiphytic yeast from chilies have been reported as exhibiting hyperparasitism, producing volatile compounds, and producing chitin. The second culture test results showed minimal inhibition (13.09 mm) between the yeast colonies of $R. mucilaginosa$ and $P. capsici$ (Figure 1A). The results of the metabolite test showed that there was an effect of $R. mucilaginosa$ yeast suspension treatment on growth of $P. capsici$, which was characterized by inhibition of the wet weight of $P. capsici$ mycelium in the PDB media of 0.13 grams compared to control of $P. capsici$ isolates on PDB (without treatment with yeast antagonists) (Table 1). In reduction in wet weight of $P. capsici$ mycelium treated with $R. mucilaginosa$, yeast suspension treatment with a density of $10^7$ had a role in inhibiting the growth of $P. capsici$ mycelium, formation of VOCs, the ability to hyperparasitize, and the potential to produce $\beta$-1,3 glucanase enzymes that reflected the size of the yeast population. Schisler et al., [14] reported that the agent's antagonistic ability is strongly influenced by the density of cells. The biocontrol activity of the antagonistic agent will increase when the cell density is increased. $Candida saitoana$ was effective at a density of $10^7$ mL$^{-1}$ cells in controlling $P. expansum$ in apples [15]. $A. pullulans$ at a density of $10^7$ and $108$ mL$^{-1}$ cells can control $Penicillium digitatum$ in grapefruit, $Botrytis cinerea$, $Rhizopus stolonifer$, and $Aspergillus niger$ in grapes, and $B. cinerea$ and $R. stolonifer$ in tomatoes [16].

![Figure 1. a) Growth of $P. capsici$ on PDA medium; b) Dual culture test between $R. mucilaginosa$ and $P. capsici$ on PDA medium; c) Growth of $P. capsici$ on PDB medium; d) Effect of $R. mucilaginosa$ suspension $P. capsici$ on PDB medium; e) test of $R. mucilaginosa$ glucanase activity in glucan agar medium.](image-url)
a significant role in the mechanism of *R. mucilaginosa* as a biocontrol agent of *P. capsici*. Plant pathogenic activity of biocontrol agents is based on the extracellular secretion of lysis enzymes [17]. Glucanase activity is reported to be involved in the yeast mechanism that acts as a biocontrol agent [18].

### Table 1. Physiological characterization of antagonistic yeast in inhibiting *P. capsica*

| Isolate code         | Antibiosis inhibition zone on PDA medium (mm) | Decrease of biomass mycelium of *P. capsici* on PDB medium (gram) | Relative inhibition rate of volatile compounds (%)* | Hyper-parasitism (The number of affinities of yeast to *P. capsici* [colonies of yeast]) | Production of β-glucanase on glucan medium (mm)* |
|----------------------|-----------------------------------------------|---------------------------------------------------------------|---------------------------------------------------|---------------------------------------------------------------------------------|-----------------------------------------------|
| Control              | 0.00b                                         | 6.34a                                                         | 0.00b                                             | 0                                                                              | 0b                                            |
| *R. mucilaginosa* (END6) | 13.09a                                        | 0.13b                                                         | 31.62a                                            | > 50                                                                            | 18.5a                                         |
| *R. mucilaginosa* (EPT69) | 0.00b                                         | 0.13b                                                         | 23.71a                                            | >10                                                                             | 0b                                            |

* means results with the same letter are not significantly different on SNK (Student-Newman-Keul) α=5%; †RIR: relative resistance levels

#### 3.2. Formation of volatile organic compounds (VOCs)

The VOCs effect test on the development of *P. capsici* showed that there was a difference in the relative inhibitory ability of the yeast *R. mucilaginosa* on the colony diameter of *P. capsici* in the control treatment of 0% (Table 1). The colony diameter of *P. capsici* produced in the treatment of *R. mucilaginosa* antagonist yeast with the relative inhibition rate of volatile compound has value of 31.62%. Several other yeast groups, such as *Candida saitoana*, *Debaryomyces hansenii*, *Cryptococcus* sp., and *Rhodotorula mucilaginosa*, have also been reported to have the ability to produce VOCs [19].

![Figure 2](image1.png)

**Figure 2.** a) Growth of *P. capsici* on PDA medium; b) Growth of *P. capsici* and *R. mucilaginosa* isolate END 6 on PDA medium; c) Growth of *P. capsici* and *R. mucilaginosa* isolate END 69 in PDA medium.

Two isolates from *R. mucilaginosa* can produce volatile compounds. This indicates that *P. capsici* was stunted due to the presence of volatile compounds produced by *R. mucilaginosa* (Figure 2). This study showed that growth of the *P. capsici* mycelium’s hyphae was inhibited by *R. mucilaginosa* compared to controls in which hyphal growth of *P. capsici* was not inhibited (without *R. mucilaginosa* treatment) with no physical contact occurring between *P. capsici* and *R. mucilaginosa*. From this
finding, it can be concluded that volatile compounds are produced by *R. mucilaginosa*. Limtong et al., [20] reported that *R. mucilaginosa* was able to produce volatile compounds that inhibited the growth of *Curvularia lunata* and *Helminthosporium oryzae*, while Di Francesco, Ugolini, Lazzeri, and Mari [6] reported that *A. pullulans* produce volatile compounds that have an essential role in biocontrol activity against postharvest pathogens such as *B. cinerea*, *C. acutatum*, *Penicillium expansum*, *Penicillium digitatum*, and *Penicillium italicum*.

3.3. Hyperparasitism

The results of observations with electron microscopy (SEM) showed that the hyperparasitism process of *R. mucilaginosa* was able to lyse the hyphae of *P. capsici* (Figure 3b) enabling hyphae cell walls to be damaged. Presumably, *R. mucilaginosa* produces enzymes that can damage the cell walls of *P. capsici*. Based on the research results for *R. mucilaginosa*, it can be concluded that it has antibiotic properties, is volatile, and can produce β-1,3 glucanase enzymes. In addition to lysis of the cell walls of *P. capsici* by *R. mucilaginosa*, SEM results showed that for the *R. mucilaginosa* isolates, the fungus was unable to develop correctly when *R. mucilaginosa* was present near the *P. capsici* hyphae. This property is due to the uptake of nutrients by yeast antagonists against *P. capsici* hyphae.

Contact between *R. mucilaginosa* and *P. capsici* cells occurred as early as 24 hours after application, and hyphal damage began to occur at 120 hours of incubation (Figure 3a). The ability to hyperparasitize has been reported in several other antagonistic yeast types, such as *P. membranefaciens*, *C. albidas* [8], and *A. pullulans* [13]. The attachment of yeast to the pathogenic structure is closely related to its biocontrol ability. The destruction of hyphae cells (Figure 3b) is strongly suspected to be related to the lysis mechanism. When contact occurs between the yeast cells of *R. mucilaginosa* and *P. capsici* at points where the cell walls of *P. capsici* consist of cell glucans, wall-degrading enzymes from the yeast *R. mucilaginosa* will attach to the cell walls of *P. capsici*. Also, the damage to the cell walls of *P. capsici* was possibly due to anti-fungal activity (VOCs) or a killer toxin from *R. mucilaginosa*.

![Figure 3](image_url). The interaction between *R. mucilaginosa* and *P. capsici* cells on water agar medium, under a light microscope b) View under 5000 x electron microscope, magnification Pc: *P. capsici* hyphae; Rm: *R. mucilaginosa*.

4. Conclusion

In summary, this study provides information indicating that the mode of action of the yeast *R. mucilaginosa* (isolate END6) in controlling BSR disease (*P. capsici*) is achieved through several mechanisms, such as antibiosis, VOCs, glucanase activity, and hyperparasitism. Furthermore, *R. mucilaginosa* (END6) may involve other mechanisms which inhibit BSR in black pepper, such as enzyme hydrolysis, resistance induction, and space and nutrition competition, and so further research needs to be carried out into these potential properties.
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