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Research Article

Antagonism Mechanism of Epiphytic Yeast against Anthracnose Pathogen (*Colletotrichum acutatum*) on Chilli

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ABSTRACT

Epiphytic yeasts have the potency as antagonistic agents against various pathogens of post-harvest products. Anthracnose is a major disease of chilli that causes high economic loss. This research was objected to study the antagonism mechanism of epiphytic yeast isolates that have the antagonistic potency against anthracnose pathogen on chilli (*Colletotrichum acutatum*). Twenty-two isolates of epiphytic yeasts, isolated from chilli leaves and fruit, were tested. The characterization of the antagonism was carried out by antibiosis, anti-fungus volatile production, and chitinolytic activity tests. The results showed that all tested isolates did not have antibiosis mechanism against *C. acutatum*. All isolates produced volatile compounds which inhibited the colony growth of *C. acutatum*. Four isolates showed high relative inhibition rate, i.e. isolates B32DEP (35.68%), B30DEP (37.52%), B23DEP (38.52%), and B29DEP (45.42%). Fourteen isolates showed chitinolytic activities. Three of them had high chitinolytic activities, i.e. B12DEP, B2DEP, and G237DEP.

Keywords: antagonistic agents, antibiosis, chitinolytic activity, volatile compound

INTRODUCTION

Anthracnose is an economically important disease of chilli (Than *et al.,* 2008). It is a major disease of chilli both in the tropics and sub-tropics area (Sangdee, 2011). The yield loss may reach 50% (Than *et al*., 2008). Anthracnose is caused by pathogenic fungi from the genera of *Colletotrichum*, i.e. *Colletotrichum acutatum*, *C. gloeosporioides*, and *C. capsici* (AVRDC, 2007). Common control practice to reduce losses due to anthracnose is by using synthetic fungicides. The improper use of synthetic fungicides causes several problems, such as pathogen resistant to the fungicides (Zhang and Huang, 2007), pollution of the environment, and chemical residues on the fruits, hence harmful to be consumed.

Biological control of pre and postharvest diseases is one of the control techniques mostly studied in the past decades. Yeast is an antagonistic microbe that has been known as a biocontrol agent (Irtwange, 2006; Qing & Shiping, 2000). Several studies showed the success of using yeast as a biocontrol agent against pathogens on fruits and vegetables. Yeast species of *Debaryomyces hansenii* had a broad spectrum of biological activity (Sharma *et al.*, 2009). Chanchaichaovivat *et al.* (2007) reported that yeast species of *Pichia guilliermondii*, *Candida musae, Issatchenkia orientalis,* and *Candida quercitrusa* were able to reduce the incidence of anthracnose on chilli caused by *C. capsici*. The mechanisms of the antagonism between biocontrol agents and pathogens can be competition, parasitism, antibiosis, and induced resistance (Sharma *et al.*, 2009). Biological control may involve one or several antagonism mechanisms. According to Nunes (2012), yeast had antagonism mechanisms of nutritional and space competition, hyperparasitism, production of lytic enzyme, and induced resistance in controlling postharvest pathogens.

The ability of yeast to compete in nutrition and space was reported by Liu *et al.* (2013) and Zhang *et al.* (2007). Space and nutritional competition by *Rhodotorula glutinis* were indicated by the speed and the increase of the yeast colonization on strawberries against *Botrytis cinerea* (Zhang *et al.,* 2007). The mechanism of hyperparasitism was

shown by *P. guilliermondii* that attack *B. cinerea* and *Penicillium hyphae* through the degradation of the *B. cinerea* cell wall in the attachment area (Sharma *et al.*, 2009). Antibiosis mechanism can be in the form of production of antibiotics or other toxic compounds such as killer toxin, volatile compounds, and lytic enzymes (Walker *et al.,* 1995; Castoria *et al.*, 2001; Avis & Bélanger, 2002; Huang *et al.,* 2011; Francesco *et al.,* 2014). There are not many studies about the use of yeast as biocontrol agents against anthracnose on chilli in Indonesia. The potency of yeast and its mechanism as biocontrol agent are necessary to be studied. Therefore, this study was objected to determine the antagonism mechanism of epiphytic yeast isolates that may have antagonistic potency against anthracnose pathogen (*C. acutatum*) on chilli.

MATERIALS AND METHODS

The tests were conducted in the Laboratory of Plant Mycology, Department of Plant Protection, Faculty of Agriculture, IPB University. The experiment was arranged in the Completely Randomized Design with 22 treatments of epiphytic yeast isolates (B2BEP, B3DEP, B6BEP, B7DEP, B11DEP, B12DEP, B16BEP, B18BEP, B20DEP, B21DEP, B22DEP, B23DEP, B25DEP, B27BEP, B28DEP, B29DEP, B30DEP, B32DEP, G141DEP, G144BEP, G234DEP, and G237DEP.) and 3 replications. The epiphytic yeasts were isolated from the surface of chilli leaves and fruit collected from Bogor and Garut. Those yeast isolates were proven non-pathogenic and had the potency as antagonists against *C. acutatum* with the inhibition rate of more than 40% (Hartati, 2016).

Antibiosis Test

The antibiosis test was carried out using the dual culture method. Both yeast and *C. acutatum* were cultured on PDA. A loopful of 5 days old yeast isolate was harvested and transversely streaked onto the middle of PDA in a petri dish (diameter of 9 cm). After that, 10 days pure culture of *C. acutatum* on PDA was cut by using a cork borrer (6 mm diameter) and placed on the left and right sides of the streaked yeast. The distance between pathogen and yeast was 3 cm. *C. acutatum* isolate was placed in the same position without the yeast as control. Treatments and control were incubated at room temperature with 3 replicates. The clear zone and the relative inhibition rate were assessed daily up to 10 days incubation. The relative inhibition rate was calculated based on the following formula:

$$RIR = \frac{jc - jt}{jc} \times 100\%$$

RIR = relative inhibition rate
jc = radius of the pathogen colony toward the antagonist position on control
jt = radius of the pathogen colony toward the antagonist on treatment

Anti-Fungus Volatile Production Test

Anti-fungus volatile production test was performed by culturing the epiphytic yeast isolates. A loopful of the 5-days-old yeast culture was streaked onto the middle of PDA in a petri dish, and 7-days-old *C. acutatum* (6 mm diameter) culture on PDA was placed in the middle of another petri dish with PDA. Then, the lidless petri dish of yeast isolate was cupped above the lidless petri dish of *C. acutatum*, and sealed with cling plastic wrap (Huang *et al*., 2011). As the control, a petri dish of *C. acutatum* was cupped above a petri dish containing only PDA without yeast. Each treatment of yeast isolates and control were replicated 3 times. The growth of *C. acutatum* in the treatment compared to the control, and the percentage of the relative inhibition rate were assessed daily up to 10 days incubation. The relative inhibition rate (RIRv) was calculated using the similar formula as the antibiosis test, as follow:

$$RIRv = \frac{dc - dt}{dc} \times 100\%$$

RIRv = relative inhibition rate at volatile compound production test
dc = diameter of *C. acutatum* at control
dt = diameter of *C. acutatum* at yeast treatment

Chitinolytic Activity Test

The test of chitinolytic activity of the epiphytic yeast isolates was carried out on 0.2% colloidal chitin agar medium. The colloidal chitin was prepared according to Hsu and Lockwood (1975) as follow: 20 g of chitin $(C_8H_{13}NO_5)n$ derived from shrimp skin (C717O practical grade Sigma) was suspended into 400 ml concentrated HCl. The suspension was incubated for 24 hours in cold condition, and then filtered by using

glass wool. The filtrate was added with 200 ml of cold distilled water, then added with 500 ml of 10 N NaOH to obtain a pH of 7.0 (after 350 ml, the subsequent addition was done drop by drop). Then, the suspension was centrifuged at 7.000 rpm for 10 minutes, resuspended in cold distilled water, and recentrifuged. The colloidal chitin pellet produced was stored at 4ºC, and ready to use.

Chitinolytic activity test was performed by culturing 3 to 5-days-old yeast isolate on colloidal chitin agar medium. Colloidal chitin agar was prepared according to Shurtleff and Averre (1997), by mixing 1−2.5 grams of colloidal chitin and 20 g of agar in 1000 ml of distilled water or mineral salt solution. One liter of mineral salt solution contained 0.7 g K_2HPO_4; 0.5 g KH_2PO_4; 0.5 g crystalline $MgSO_4.7H_2$; 0.001 g $FeSO_4$; and 0.001 g $ZnSO_4$. The test was replicated three times, incubated at room temperature, and observed daily. The chitinolytic activity was shown by the clear zone around the yeast colony.

Data Analysis

Data of antibiosis and anti-fungus volatile production tests were analyzed by SPSS software (version 21.0 for Windows). If there were significant differences between treatments, the data were further analysed by using Duncan's Multiple Range Test at 5%.

RESULTS AND DISCUSSIONS

Antibiosis Test

Result of the antibiosis test revealed that the yeast did not cause the formation of a definite clear zone around the colony of the pathogen. Statistical analysis of Relative Inhibition Rate (RIR) showed that the yeast isolates tested did not affect the RIR of *C. acutatum*, hence the epiphytic yeast isolates tested did not produce antibiosis mechanism. The RIR ranged from 6.53−20.85%. There were 7 epiphytic yeast isolates that had RIR more than 15%, i.e. B23DEP, G141DEP, B20DEP, B30DEP, G237DEP, B11DEP, and B29DEP (Table 1).

Table 1. The relative inhibition rate (RIR) of epiphytic yeast against *Colletotrichum acutatum* in the antibiosis test at 10 days after treatment

Isolate code	Average of RIR (%)
B2BEP	8.79
B3DEP	6.78
B6BEP	12.48
B7DEP	8.29
B11DEP	17.09
B12DEP	9.55
B16BEP	11.23
B18BEP	7.88
B20DEP	16.25
B21DEP	9.55
B22DEP	13.32
B23DEP	17.09
B25DEP	9.13
B27BEP	9.55
B28DEP	12.48
B29DEP	19.18
B30DEP	18.76
B32DEP	13.74
G141DEP	20.85
G144BEP	13.74
G234DEP	10.80
G237DEP	15.41

Biocontrol agents have antagonistic activity that involve complex interactions between host, pathogen, antagonist, and environment (Nunes, 2012). According to Sharma *et al.* (2009), antagonistic agents have four antagonism mechanisms, i.e. competition, parasitism, antibiosis, and induced resistance. Antibiosis has an important role in the biological control mechanism by antibiotic producing microbes. Wardhika *et al.* (2014) stated that bacteria unable to produce clear zone in the antagonism test were caused by not producing antibiotic compound to control the pathogen. According to Droby *et al.* (1993), yeasts generally do not produce antibiotics. However, yeasts produce a toxic compound (killer toxin). Killer toxin can be in the form of protein, glycoprotein, or fatty acid. *Williopsis mrakii*, *Saccharomyces cerevisiae*, and *Pichia anomala* had the ability to produce antimycotic killer toxin (Walker *et al.,* 1995). *Pseudozyma flocculosa* also produced extracellular fatty acid that control powdery mildew fungus and other pathogens (Avis & Bélanger, 2002). The fatty acid causes disorganization of cell membranes and cell disintegration. Antagonistic yeast isolates in this study were considered to produce a low concentration of killer toxin.

Anti-Fungus Volatile Production Test

Results of anti-fungus volatile production test, based on RIRv, indicated that the yeast isolates produced volatile compounds. This was indicated by the inhibition of the growth of *C. acutatum* in the

treatments of yeast isolates. The statistical analysis showed that the epiphytic yeast isolates tested significantly affected the RIRv of *C. acutatum*. The RIRv ranged from 8.57-45.42% (Table 2). Some epiphytic yeast isolates had high RIRv at 10 days after treatment (dat), i.e. B11DEP, B18BEP, B23DEP, B29DEP, B30DEP, and B32DEP, with the RIRv of 32.55; 32.76; 38,52; 45.42; 37,52; and 35.68%, respectively (Table 2 and Figure 1). The further analysis showed that there were significant differences between control and the yeast isolates in the ability to inhibit the growth of *C. acutatum*, except B22DEP. The epiphytic yeast isolates generally had the same ability to inhibit the growth of *C. acutatum* by producing volatile compounds. B29DEP isolate caused the highest RIRv. B23DEP and B30DEP isolates caused the same RIRv (Table 2).

Volatile organic compounds (VOCs) are a mixture of carbon-based gas-phase compounds, having a small size that can spread through the atmosphere and soil (Morath *et al.*, 2012). Volatile compounds can also be a mixture of simple hydrocarbon compounds, such as aldehyde, ketone, alcohol, phenol, thioalcohol, thioester and their derivatives, benzene derivatives, and cyclohexane (Huang *et al.*, 2011). These volatile compounds have roles in plant pathogen biocontrol. *Candida intermedia* strain C410 was reported to produce 49 types of volatile compounds (ester, alcohol, alkene, alkane, alkyne, organic acid, ketone, and aldehyde) that reduced the incidence and severity of *B. cinerea* rot (Huang *et al.,* 2011). Francesco *et al.* (2015) reported that *Aureobasidium pullulans* strain L1 and L8 produced 2-phenyl, 1-butanol-3-methyl, 1-butanol-2-methyl, and 1-propanol-2-methyl (alcohol group) in the first 96 hours of growth. These volatile compounds had important role in the antagonistic activity against five postharvest pathogens, namely *B. cinerea, C. acutatum*, *Penicillium expansum, P. digitatum,* and *P. italicum.* Volatile compounds and killer toxins are toxic compounds that inhibit growth and even kill pathogens. In addition, they are able to eliminate the signals initiation between pathogen and plant where pathogen elicitors are not able to recognize plant receptors, hence a compatible relationship between pathogen and plant does not occur (Agrios, 2005). It was assumed that this was the mechanism involved in the relationship between the antagonistic yeast isolates and *C. acutatum*.

Table 2. The relative inhibition rate (RIRV) of epiphytic yeast against *Colletotrichum acutatum* in the anti-fungus volatile production test at 10 days after treatment

Isolate Code	Average of RIRV (%)
Control	0e
B22DEP	8.57de
G237DEP	16.08cd
B25DEP	8.79bcd
B20DEP	9.45bcd
B16BEP	10.42abcd
B7DEP	10.51abcd
B3DEP	14.06abcd
G144BEP	12.41abcd
B12DEP	15.61abcd
B27BEP	19.68abcd
B6BEP	21.54abcd
B21DEP	23.56abcd
B2BEP	23.44abcd
G234DEP	26.61abc
B28DEP	28.56abc
G141DEP	27.68abc
B11DEP	32.55ab
B18BEP	32.76ab
B32DEP	35.68ab
B30DEP	37.52ab
B23DEP	38.52ab
B29DEP	45.40a

Remarks: Data followed by the same letters were not significantly different according to Duncan's Multiple Range Test (5%)

Chitinolytic Activity Test

Results of chitinolytic activity test showed that most epiphytic yeast isolates had the potency to generate chitinolytic activities. Fourteen isolates of yeast were found to have the chitinolytic activities at 6 days after treatment (dat) (Table 3). The chitinolytic activity was characterized by the formation of clear zone around the yeast colony on colloidal chitin medium. G237DEP isolate caused the widest clear zone compared to other epiphytic yeast isolates (Figure 2). The clear zone formed in the chitinolytic activity test was an indication that the epiphytic yeast isolates had chitinolytic activities and were capable of producing chitinase. Chitinolytic activities are important for antagonistic agents to control diseases caused by fungal pathogens, because the chitin of the fungi cell wall can be degraded by the enzyme. Chitin degradation will cause lysis of the fungi cell wall.

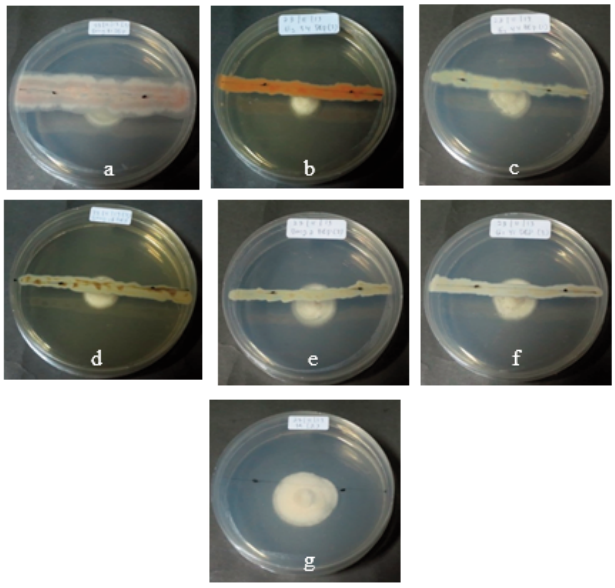

Figure 1. Growth inhibition of *Colletotrichum acutatum* colonies by antagonistic yeast isolates through the production of volatile compounds; (a) B23DEP, (b) B29DEP, (c) B32DEP, (d) B30DEP, (e) B11DEP, (f) B18BEP, (g) control

According to Carstens *et al.* (2003), antagonistic activity in biological control of plant pathogens is based on the extracellular secretion of lytic enzymes. Chitinase hydrolyzes β-1,4 bonds in chitin which is a component of the fungi cell wall. *P. guilliermondii* produced chitinolytic activities to control mango rot pathogen, *Botryodiplodia theobromae* (Sugiprihatini *et al.,* 2011). Castoria *et al.* (2001) reported that extracellular exocytinase and β-1,3-glucanase of *A. pullulans* were involved in antagonistic activity against postharvest pathogens, such as *B. cinerea, P. expansum, Rhizopus stolonifera*, and *Aspergillus niger*. In this study, the epiphytic yeast isolates tested were able to produce chitinase to hydrolyze the colloidal chitin from shrimp skin. The hydrolysis process converts chitin to N-acetyl glucosamine monomer as a carbon source. Carbon needed by microorganisms is usually obtained by hydrolyzing various forms of chitin by the chitinase (Yanai *et al*., 1992). *C. acutatum* is a fungus that has chitin cell wall, thus the yeast isolates tested were able to degrade chitin on the cell wall of *C. acutatum* by their chitinolytic enzyme.

Table 3. Clear zone formation in the chitinolytic activity test at 6 days after treatment

Isolate Code	Clear Zone
Control	Not formed
G234DEP	Formed
B22DEP	Not formed
B6BEP	Formed
B23DEP	Not formed
G141DEP	Not formed
B2BEP	Not formed
B20DEP	Not formed
B25DEP	Not formed
B30DEP	Formed
B7DEP	Formed
G144BEP	Formed
B21DEP	Formed
B16BEP	Not formed
B12DEP	Formed
B27BEP	Not formed
B28DEP	Formed
B3DEP	Formed
G237DEP	Formed
B18BEP	Formed
B11DEP	Formed
B29DEP	Formed
B32DEP	Formed

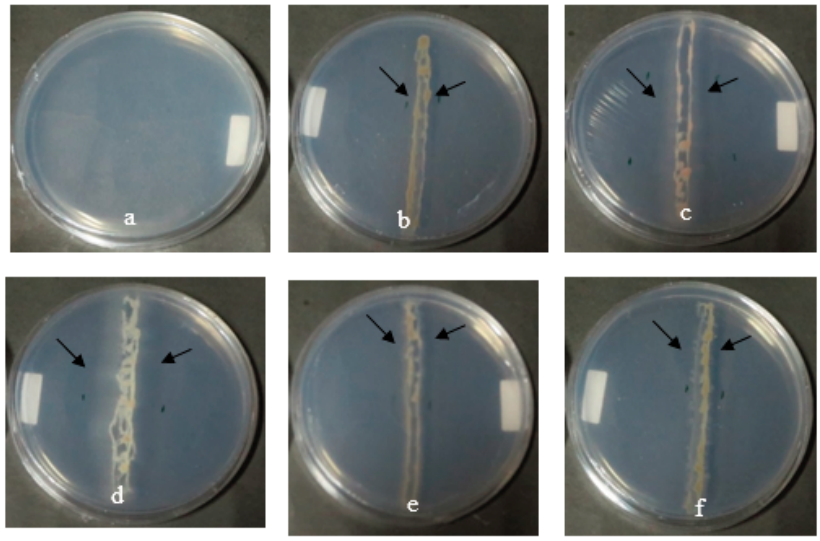

Figure 2. Chitinolytic activities of several epiphytic yeasts at 6 days after treatment; (a) control, (b) B29DEP, (c) B12DEP, (d) G237DEP, (e) B32DEP, and (f) B28DEP

CONCLUSION

Results of the dual culture test showed that the epiphytic yeast isolates tested did not have antibiosis mechanism through antibiotic production. However, all epiphytic yeasts had antibiosis mechanism through the production of volatile compounds and chitinolytic activities. The value of the relative inhibition rate in the production of volatile compounds test ranges from 8.57− 45.42% at 10 dat. Fourteen epiphytic yeast isolates had chitinolytic activities at 6 dat.

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