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Yeast metabolites inhibit banana anthracnose fungus *Colletotrichum musae*

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Abstract

The severity of banana anthracnose (*Colletotrichum musae)* was assayed after the application of metabolites from four yeasts; *Debaryomyces hansenii* TISTR 5155, *Candida sake* TISTR 5143, *Aureobasidium pullulans* TISTR 3389 and *Candida utilis*. The yeasts were cultured in either yeast extract malt extract broth (YMB) or potato dextrose broth (PDB). The effects of the addition of non-volatile and volatile metabolites from these yeasts on the *in vitro* growth of *Colletotrichum musae* were also investigated. YMB was a better growing medium for the secretion of metabolites than PDB media. Metabolites from *C. utilis* and *A. pullulans* in YMB gave the highest reduction of disease severity. Non-volatile metabolites from *A. pullulans* and *C. utilis* also significantly inhibited mycelial growth with EC_{50} values of 190 and 158 mg/L. The inhibition of spore germination and germ tube elongation of the non-volatile metabolites from *A. pullulans* and *C. utilis* had EC_{50} values of 149 and 151 mg/L, respectively. Similarly the volatile metabolites of the yeasts greatly reduced hyphal growth, spore germination and germ tube elongation. The secretion of ß-1,3-glucanase and chitinase was observed by the yeast *A. pullulans* in YMB medium, but *C. utilis* only secreted ß-1,3-glucanase. In conclusion, the yeasts studied in this experiment appear to have a high efficacy for reducing the development of banana anthracnose and could provide an alternative control method.

Keywords: culture filtrate, antagonistic yeasts, disease severity, hydrolytic enzymes

Introduction

Anthracnose of banana is caused by the fungus *Colletotrichum musae* (Berk & Curtis) and is the most important disease of banana in tropical areas. *C. musae* is also the causal agent of crown rot, stem-end rot and blossom-end rot in bananas. The worldwide demand for bananas is

increasing and banana for export must be free from exotic diseases, fungal toxins and chemical residues. Biological control agents such as yeasts, have been introduced to manage the diseases on a number of postharvest commodities (Kefialew and Ayalew, 2008; Zhang *et al.,* 2009). Antagonistic yeasts have been selected mainly because they do not damage the host cells and produce toxic metabolites which could have a negative effect on environment, animal nor on human health. Yeasts can survive and multiply on the surface of the fruits but may cause contamination on the commodities for export. Therefore culture filtrates without living cells of yeasts may be used as substances for controlling anthracnose disease in banana. It is therefore the aim of this experiment to assess the severity of banana anthracnose (*Colletotrichum musae)* after the application of metabolites from four yeasts; *Debaryomyces hansenii* TISTR 5155, *Candida sake* TISTR 5143, *Aureobasidium pullulans* TISTR 3389 and *Candida utilis*.

Materials and Methods

1. Preliminary screening of non-volatile metabolites from yeasts against anthracnose disease on banana fruit

Yeast metabolite preparation

Four antagonistic yeasts; *Debaryomyces hansenii* TISTR 5155, *Candida sake* TISTR 5143, *Aureobasidium pullulans* TISTR 3389 and *Candida utilis* were cultured on YMB and PDB media. A liquid culture was made by adding a loop full of yeast cells from an agar plate to a 500 ml Erlenmeyer flask containing a 300 ml YMB or PDB. The samples were then shaken at 120 rpm for 48 h at room temperature (28-30°C). The yeast cells were separated by centrifugation at 9000 rpm for 10 min and the supernatant was filtrated using a 0.45 µm glass filter funnel. Culture filtrates were collected from the supernatant and used as metabolites for inhibition studies.

Yeast metabolite treatments on C. musae development in banana fruit

Hands of green bananas were cut into the single fruit. Fruits were surface-disinfected with 5% sodium hypochlorite solution for 5 min, washed with tap water and dried in air. Ten fruit per treatment were individually sprayed with the culture filtrate from each yeast using an airbrush (BADGER AIR-BRUSH™, U.S.A.). After spraying, the banana fruits were stored on plastic trays and enclosed with plastic bag to maintain high humidity at room temperature for 24 h. Banana fruits were then inoculated with spore suspension of *C. musae* (10^6 spores/ml) using an airbrush. The source of the *C. musae* was obtained from diseased fruit. After inoculation, the treated fruit were returned to the incubator in the same condition for 24 h. After 7 days storage the severity (%) of the anthracnose on the banana fruits was recorded. The metabolites from the yeasts which showed the best control of the anthracnose disease on the banana fruits were selected for further studies.

2. Study of metabolites from yeasts on the in vitro growth of C. musae

Non-volatile metabolites from yeasts on the growth of C. musae

The growing mycelium obtained from 7-day-old culture of *C. musae* grown on PDA was cut with a 5 mm-diameter cork borer at the edge of colony and transferred to an empty 9 cm-diameter Petri dish. The agar plug with mycelium was placed at the middle of Petri dish. An agar plug in YMB medium was served as control. Twenty mL of the culture filtrate from each yeast was added to a Petri dish and incubated at room temperature for 5 days. Following this the filtrate of the culture was removed by filtration using a Whatman No.1 filter paper. The mycelial mat was subsequently dried in the oven at 80˚C for 6 h and weighed.

A spore suspension of *C. musae* was obtained from a 7-day-old culture on PDA by flooding the dishes with sterile 0.85% sodium chloride solution then centrifuged at 3000 rpm for 5 min. A loop full of spore masses was then transferred to vial containing 10 ml of each of the four yeast culture filtrates. All vials were stored at 4°C for 12 h. After this time, a 100 µl of spore suspension was spread on the surface of the water agar and incubated at room temperature. The numbers of germinated spores were observed at 6 h after incubation. Germ tube elongation was also observed using micrometer. 400 spores per replication were randomly selected.

The EC_{50} values of the metabolites from each of the yeasts on the mycelial growth and spore germination of pathogen were determined. Yeast metabolites were diluted with sterile YMB medium at various concentrations. After incubation for 6h for spore germination and 5 days for mycelial growth, the mycelial mat was dried in hot air oven (80°C for 6 h) and weighed. In addition the number of germinated spores were determined.

Volatile metabolites from yeasts on the growth of C. musae

A 5 cm-diameter Petri dish containing PDA media was placed inside of a 9 cm-diameter Petri dish containing YMA medium. The antagonistic yeasts were streaked onto the YMA and incubated for 48 h. Subsequently a 5 mm-diameter agar plug of a 7-day-old culture of *C. musae* was transferred into the middle of PDA Petri dish. The Petri dish was then sealed with paraffin and incubated for 4 days. The diameter of the pathogen was measured and compared to the control which did not contain the antagonistic yeasts.

A spore germination test on the volatile metabolites was conducted using the method as described above. The PDA medium was replaced by water agar in a 5 cm-diameter Petri dish. A spore suspension of *C. musae* (10^5 spores/ml) was spread on the water agar then the Petri dish was sealed with paraffin and incubated for 6 h. Total spore numbers were randomly selected with 400 spores per replication measured. The germinated spores were counted under the microscope compared to the control.

3. Determination of cell wall degrading enzymes against C. musae in yeast culture filtrates

ß-1,3-glucanase and chitinase activities were assayed from the culture filtrates of each of the four yeast cultures**.** ß-1,3-glucanase was determined according to the method of Chanchaichaovivat *et al.* (2008) with some modifications. 260uL of 2 mg/ml larminarin in 0.05 M sodium acetate buffer (pH 5.0) was added to 1 ml of each yeast culture filtrates. The reaction mixtures were incubated at 35˚C in a water bath for 30 min, and the reaction was terminated by placing the mixture in boiling water for 10 min. 580 µl of 3,5-dinitrosalicylic acid solution and 2.16 ml of 0.05 M sodium acetate buffer (pH 5.0) were added and subsequently boiled for a further 5 min. After boiling, the reaction tubes were left at room temperature to cool, then 1.58 ml of distilled water was added prior to reading the absorbance at 540 nm. The enzyme activity was determined by quantifying the glucose released from laminarin by comparing with the standard curve of glucose. Enzyme activity was expressed as microgram per mL of culture filtrate.

Chitinase activity was determined according to the method of Reissig *et al.* (1955) with some modifications. 1% Swollen chitin in 0.05 M citrate-phosphate buffer (pH 6.6), was incubated with 1 ml of each yeast culture filtrate for 1 h at 50˚C. The reaction was terminated by boiling the solution for 5 min. The reaction mixture was left to precipitate at room temperature. The supernatant was collected for N-acetylglucosamine (NAG) analysis; where 0.5 mL of supernatant was mixed with 100 µl of 0.8 M $K_2B_4O_7$, then subsequently boiled for 3 min. After cooling down, 3 ml of dimethylaminobenzaldehyde solution was added, then allowed to stand

in water bath at 37˚C for 20 min. After the mixture has cooled to room temperature (10 min) the absorbance was measured at 585 nm. Chitinase activity was assayed by measuring the concentration of NAG and calculated by comparing with the standard curve of NAG. Enzyme activity was expressed as microgram per mL of culture filtrate.

Results and Discussion

1. Preliminary screening of non-volatile metabolites from yeasts against anthracnose disease on banana fruits

The metabolites from all four yeasts in YMB medium showed a reduction in the severity of the disease on banana fruits than metabolites in PDB (Figure 1). Metabolites from *C. utilis* and *A. pullulans* in YMB showed the greatest reduction of disease severity, reducing the incidence of the disease by 72 and 62%, respectively, compared to that of the control (Figure 1A). The addition, the YMB metabolites of *C. utilis* resulted in the a greater reduction of the disease than metabolites in PDB media (25 and 49% disease severity, respectively) suggesting that the yeast *C. utilis* may produce higher yields of antifungal substances in YMB medium than in PDB. Calvente et al. (1999) showed that components in different liquid cultures resulted in antagonistic yeasts secreting different antifungal substances at different levels. They also showed that the yeast *Rhodotorula glutinis* which was cultured in a medium containing iron could produce a high concentration of rhodotorulic acid and siderophores against *Penicillium expansum.* In addition, it has been reported that different carbon sources in liquid cultures result in the secretion in different amounts of hydrolytic enzymes by the yeast *Candida guilliermondii* (Saligkarias *et al.,* 2002).

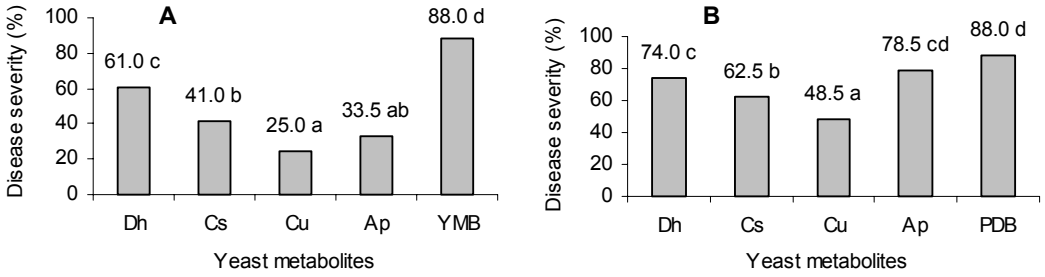

Figure 1: Disease severity on banana fruit by application of culture filtrates in yeast extract malt extract broth; YMB (A) and potato dextrose broth; PDB (B) from four yeasts including *Debaryomyces hansenii* TISTR 5155 (Dh), *Candida sake* TISTR 5143 (Cs), *Candida utilis* (Cu) and *Aureobasidium pullulans* TISTR 3389 (Ap) before inoculation with *Colletotrichum musae* for 24 h. Banana fruits were then incubated for 7 days at room temperature (28-30°C) and compared with YMB or PDB media used as no metabolites. Mean values followed by a different letter indicated significant different (P=0.05), according to Duncan's multiple range test.

2. Study of metabolites from yeasts on the in vitro growth of C. musae

Non-volatile metabolites from yeasts on the growth of pathogen

The non-volatile metabolites from two yeasts, *C. utilis* and *A. pullulans* significantly reduced the mycelial growth of *C. musae* by 86 and 84%, respectively (Figure 2A). Similarly, spore germination and germ tube growth were also significantly reduced in both metabolites. The metabolites from *A. pullulans* showed higher reduction on spore germination and germ tube elongation than metabolites from *C. utilis* (Figure 2B, 2C).

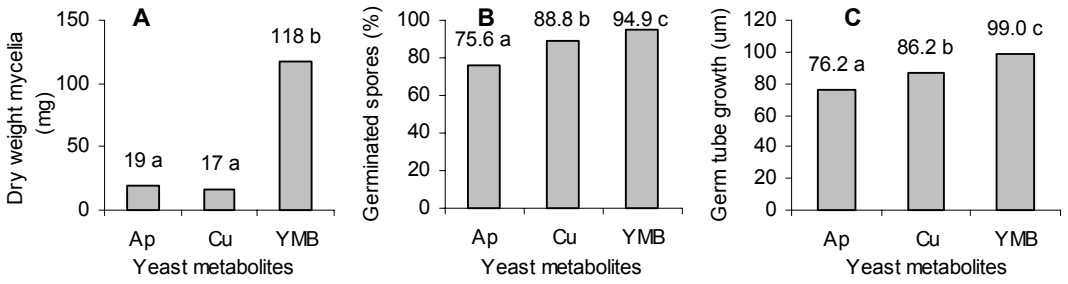

Figure 2: Effect of metabolites from yeasts *Aureobasidiu pullulans* TISTR 3389 (Ap) and *Candida utilis* (Cu) on mycelial growth (A), germinated spores (B) and germ tube elongation (C) of *Colletotrichum musae* incubated for 5 days and 6 h, respectively, at room temperature (28-30°C), compared with yeast extract malt extract broth (YMB) used as the control. Mean values followed by a different letter indicated significant different (P=0.05), according to Duncan's multiple range test.

The EC_{50} values for mycelial growth and spore germination inhibition of *A. pullulans* metabolites calculated from the regression equation ($y = 0.236x + 5.058$, $R^2 = 0.942$ and $y = 0.389x - 8.04$, $R^2 = 0.914$) were 190 and 149 mg/L, respectively. Whereas, the metabolites from yeast *C. utilis* showed the highest growth inhibition as it had the lowest EC_{50} on mycelial growth at 158 mg/L ($y = 0.3415x - 3.972$, $R^2 = 0.952$), while spore germination inhibition had the EC_{50} at 151 mg/L ($y = 0.4068x - 11.214$, $R^2 = 0.91$).

Volatile metabolites from yeasts on the growth of pathogen

The mycelial growth of *C. musae* was significantly reduced in the presence of the volatile metabolites from both yeasts (Figure 3A). However the number of germinated spores and germ tube elongation were only reduced in the presence of volatile metabolites from *A. pullulans*, and not but *C. utilis* (Figure 3B, 3C). Volatile compounds from other microorganisms such as bacterium *Bacillus subtilis*, have been shown to inhibit the growth of *Penicillium digitatum* up to 70% (Leelasuphakul *et al.,* 2008). In addition, the volatile metabolites from *Bacillus mycoides* have been found to inhibit the growth of *Botrytis cinerea* and also activate the defense system in strawberry fruit (Guetsky *et al.,* 2002). However volatile metabolites from the strains of yeasts, *Debaryomyces melissophilus, Rhodotorula glutinis, Cryptococcus laurentii* have not been reported to inhibit fungal growth (Ragaert *et al.,* 2006).

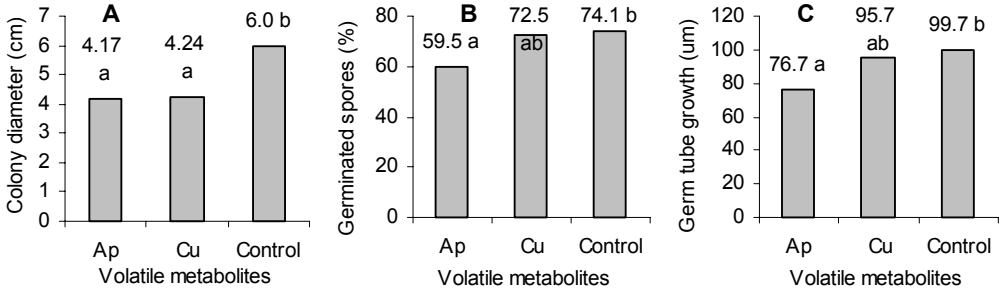

Figure 3: Effect of volatile metabolites from yeasts *Aureobasidiu pullulans* TISTR 3389 (Ap) and *Candida utilis* (Cu) on colonial diameter (A), germinated spores (B) and germ tube elongation (C) of *Colletotrichum musae* incubated for 4 days and 6 h, respectively, at room temperature (28-30°C) as compared with no volatile metabolites (Control). Mean values followed by a different letter indicated significant different (P=0.05), according to Duncan's multiple range test.

3. Determination of cell wall degrading enzymes against C. musae in yeast culture filtrates

Both the hydrolytic enzymes, ß-1,3-glucanase and chitinase, were produced by *A. pullulans* in a 5-day-old culture in YMB media. However the culture filtrate of *C. utilis* only contained ß-1,3-glucanase (Figure 4). These results suggest that the hydrolytic enzymes within the culture filtrates had the potential to inhibit the growth of *C. musae*. Yan *et al.* (2008) also have found that crude chitinase obtained from recombinant rice chitinase, significantly inhibited the spread of green mold in loquat fruit caused by *Botrytis cinerea.*

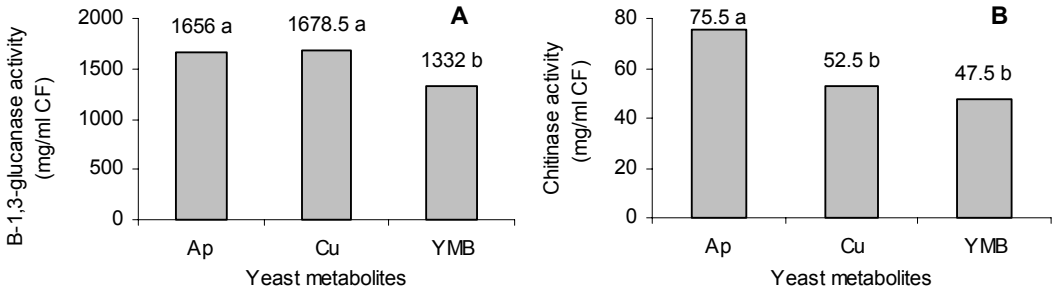

Figure 4: Cell wall degrading enzymes, ß-1,3-glucanase (A) and chitinase (B) produced by yeasts *Aureobasidium pullulans* TISTR 3389 (Ap) and *Candida utilis* (Cu) in 5-day-old liquid culture of yeast extract malt extract broth (YMB), incubated at room temperature (28-30°C). Mean values followed by a different letter indicated significant different (P=0.05), according to Duncan's multiple range test.

Conclusions

Metabolites from the yeasts *C. utilis* and *A. pullulans* cultured on YMB media resulted in the greatest reduction in anthracnose severity. Both the non-volatile and volatile metabolites from the two yeasts also significantly reduced the *in vitro* mycelial growth, spore germination and germ tube elongation of *C. musae. A. pullulans* secreted both the wall degrading enzyme, ß-1,3-glucanase and chitinase, whereas *C. utilis* only secreted ß-1,3-glucanase.

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