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# Antagonist effect of volatile organic compounds produced by *Debaryomyces hansenii* on *Colletotrichum gloeosporoides* as anthracnose reason of tropical apples

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**Key words:** Volatile organic compounds, *Debaryomyces hansenii, Colletotrichum gloeosporoides,* Antrachnose, Apple.

**Abstract**

The usage antagonist yeast for biological control had emerged as one of the most promising alternatives in pre and postharvest protection of apples in Indonesia. The aim of the research was to identify volatile organic compounds (VOCs) produced by *Debaryomyces hansenii* that inhibited the growth of *Colletotrichum gloeosporioides*, pathogenic fungi of tropical apples. *D. hansenii* was isolated from apples in Indonesian. Bioassay using potato-dextrose agar medium in sealed petri dishes showed that the fungi growth inhibition was ultimately due to volatile organic compounds produced by *D. hansenii*. The VOCs were trapped on activated charcoal prior to analyzing by integrated thermal desorption using GC-MS. The VOCs significantly inhibited the mycelia growth of *C. gloeosporioides* by 85.07 %, and induced morphological abnormalities such as mycelia deviations. The identified VOCs include acids, esters, ketones, oximes, heterocyclic, and phthalate compounds. The result indicated that tropical apple yeast is abundant resources of bioactive VOCs and played an important role in reducing apple anthracnose disease of tropical apples in Indonesia.

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## Introduction

Indonesia has tropical climate and the apples are cultivated especially in Batu region, East Java. The main disease of apple plants is anthracnose (bitter rot) which is caused by *C. gleosporioides.* According to Rahman *et al.* (2007) in tropical countries, *C. gleosporioides*. *Penz. Sacc.* is the main causing agent of anthracnose disease and bitter rot, which is eminent postharvest disease.

Anthracnose disease mainly attacked the essential part of plants, such as fruits. Recently, in order to control anthracnose disease of apples was used chemical means using synthetic fungicide. In fact, Chemical method has not given satisfied result. Epidemic disease was continually happened in the garden intensively so that the lost could reach from 40% into harvest failure, especially in the rainy season. The combined disease control using combination some control methods, including biological control, was important effort to achieve the most effective result. Microbe usage, including yeast group as biological control agent of plant disease, rapidly increased and continually got the attention, and was studied and developed.

Some researchers reported that yeast had important role in biological control of plant disease. *D. hansenii* is nonpathogenic yeast of four varieties of tropical apples in Indonesia and it had ability to stand in extremely stress condition because of pesticide compound exposure. Some particular yeast was acted as antagonist to pathogenic infection of postharvest fruits such as apples (Blevea *et al.,* 2006; Janisiewicz *et al.,* 2001).

The killer yeast phenomena had important role in preventing and controlling pathogenic fungi of plants (Starmer & Lachance, 2011). In the previous study, *D. hansenii* showed the ability to inhibit the growth and the breed of *C. gloeosporioides* potentially with antibiotic mechanism such as releasing poisonous volatile organic compounds. The aim of this study is to identify the poisonous volatile organic compounds which were released by *D. hansenii* and its antagonistic effect on *C. gloeosporioides*.

## Materials and methods

*Antagonist yeast strains and anthracnose of fungal pathogen*

The yeast of *D. hansenii* was isolated from two varieties of apples, i.e. Wang Ling and Anna, which was obtained from apple garden in Batu region, East Java, Indonesia. This yeast was identified molecularly using primer ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC -3') and ITS 5 (5'- GGA AGT AAA AGT CGT AAC AAG G -3'). The pathogenic fungi causing anthracnose disease, *C. gloeosporioides*, was isolated from apples of Manalagi variety obtained from Batu region, East Java, Indonesia. This fungi was identified molecularly using specific primer of CgInt species for forward primer with sequence (5'-3') GGCCTCCCG-CCTCCGGGCGG and reverse primer ITS 4 with sequence (5'-3') TCCTCCGCTTATTGATATGC.

*The evaluation of antifungal volatile organic compound production*

The ability of isolated yeast to produce volatile organic compound was evaluated by the modification procedure of Montealegre *et al.* (2003). As many as 100 µl antagonistic yeast suspension ($10^9$ cell/ml) was placed in the center of half part of petri dish contained PDA medium, and as many as 5 mm pure breeding fungi, 7 days of age, was placed in three point in the center of second petri dish. Both petri dishes were placed face to face so that they protect each other and make indirect contact between pathogenic fungi and yeast suspension. The petri dishes were sealed to prevent the loose of produced volatile compound prior to incubate at ambient temperature for 10 days. The evaluation used completely randomized design with treatment of the type of isolated yeast. There are 12 yeast isolated from tropical apples which were detected their ability to produce volatile organic compound, such as *D. hansenii* of A1, W1, W2, W3, W4, and W6 strain. For comparison yeast, this study used *Aureobasidium pullulans* of A5, A6, R7, R9, and M10 strain, and each treatment was replicated for three times.

The growth of pathogenic fungi was determined and compared to the control (without antagonist yeast presence). The test result was indicated by percentage average of the growth inhibition of *C. gloeosporioides* with or without isolated yeast presence. The percentage of the growth inhibition was calculated with the formula:

$$P = \frac{(a-b)}{a} \times 100\%$$

whereas:

a= the average of diameter of pathogenic fungi mycelia without yeast volatile compound presence

b= the average of diameter of pathogenic fungi mycelia with yeast volatile compound presence

If the test showed the significant inhibition of pathogenic fungi growth, then followed by extraction process of volatile compound produced by *D. hansenii*.

*The extraction of volatile compound from Debary-omyces hansenii*

The volatile organic compounds were extracted using the method of Salgado *et al.* (2013). The yeast of *D. hansenii* was planted using PDA media on the petri dish. The cover of petri dish was replaced by other dish containing 3 g of sterilized activated charcoal. Both petri dish were sealed with transparence tape and incubated at ambient temperature for 5 days. The PDA media without inoculated *D. hansenii* was used as control. Each treatment was replicated for three times. After incubation time, activated charcoal was washed by ethyl acetate 5 ml to extract all captured organic compounds. The next step was to identify volatile organic compounds produced by yeast using gas chromatography – mass spectrometry (GC-MS) technique.

GC-MS test was conducted by GC-MS-QP2010 Plus. As many as 1 µl sample was injected using *split injection* model. The temperature of the oven was set at 50°C for 1 minute. The temperature was elevated with the rate of 3°C per minute until reaching 260°C, and was kept for 2 minutes. The carrying gas was Helium 99% and was conducted in the column with current rate of 1.69 ml/minute. The current control model used pressure control, such as 100 kPa.

The transferred duct from GC to MS was conducted at 250°C. Ionized source temperature was 230°C. The evaluation was done by fully scanned model with ranged from initial m/z (40) until final m/z (350).

*Microscopic observation*

Microscopic observation was conducted to evaluate morphological changes on hypha/mycelium or other structure which was formed by pathogenic fungi due to its interaction with antagonist yeast. The mycelium of fungi was sampled from fungi breed which was exposed by volatile organic compounds released by *D. hansenii*. The control sample was also evaluated from the fungi without volatile organic compounds exposure. Both samples were observed under microscope with zooming at 160 and 640 times.

*Data analysis*

All data were analyzed by descriptive and statistic model, such as analysis of varian, using excel application program. The significance of treatment was determined by F-test. If the F-test is significant, it is followed by average different test, such as Scott-Knott 0.05 test.

**Results and discussion**

*The production of anti-fungal volatile organic compounds from 12 strains of antagonist yeast*

In this research, 12 yeast strains isolated from tropical apples were evaluated for their ability to produce volatile organic compounds (VOCs). From 12 yeast strains, only 6 strains which showed strong inhibition to *C. gloeosporioides*, such as *D. hansenii* strain A1,W1,W2,W3, and W6. Their inhibition effects were range from 71.28% to 85.07%. The strongest growth inhibition to the pathogenic fungi was done by *D. hansenii* strain A1, about 85.07%. *Aureobasidium pullulans* strain A5 and strain R7 and *D. hansenii* strain W4 revealed inhibition effect about 43.69 %, 48.28 %, and 47.14% respectively. While the other yeast strains (strain A6, M13, M10, and R9) indicated weaker inhibition effect, range from 21.83% to 28.72% (Table 1 and Fig. 1). Inhibition effect is classified as weak if inhibition percentage level is less than 10%.

The result showed that anti-fungal VOCs from *D. hansenii* influenced the growth of *C. gloeosporioides*. This fact indicated that VOCs produced by *D. hanseii* had fungistatic and fungicidal activity on *C. gloeosporioides*. The growth inhibition process was seen after 2 day incubation. Not only mycelium growth was influenced by VOCs present, but also the quality of pathogenic mycelium fungi. The evaluation of *C. gloeosporioides* mycelium showed the color change from white (without volatile compound exposure) into brownish white (after volatile compound exposure). Mycelium texture also changed, form thick and cotton-like texture (without volatile compound exposure) into very thin and flat texture (after volatile compound exposure).

This result was in accordance with Salgado *et al.* (2013) who stated that *C. gloeosporioides* and *Fusarium culmorum* which were given volatile compound treatment from *B. tropica*, experienced growth inhibition and mycelium quality decreasing. Mycelium texture changed from cotton-like texture into thin and flat texture. According to Chaurasia *et al.* (2005) inhibition effect caused by volatile compounds was bigger than diffusible compound effect. Breuer dan Harms (2006) said that *D. hansenii* was osmotolerance, oleoginus, nonpatogenic yeast with wide spectrum of carbon substrate and resistance in the chemical stress condition. Volatile toxin of *D. hansenii* can be used as therapy agent in medicinal treatment against pathogenic yeast and had function as natural preservatives in the food fermentation in order to control rot yeast.

**Table 1.** The average of colony diameter of *C. gloeosporioides* after VOCs treatment produced by 12 antagonist yeast in day 10 after inoculation.

| Type of yeast | colony diameter of *C. gloeosporioides* (mm)* | The percentage of growth inhibition (%) |
|---|---|---|
| *D. hansenii* strain A1 | 4,33 a | 85,07 |
| *A. pullulans* strain A5 | 16,33 b | 43,69 |
| *A. pullulans* strain A6 | 20,67 c | 28,72 |
| *Rhodotorula* sp. | 21,67 c | 25,28 |
| *A. pullulans* strain M10 | 21,33 c | 26,45 |
| *A. pullulans* strain R7 | 15,00 b | 48,28 |
| *A. pullulans* strain R9 | 22,67 c | 21,83 |
| *D. hansenii* strain W1 | 8,33 a | 71,28 |
| *D. hansenii* strain W2 | 7,67 a | 73,55 |
| *D. hansenii* strain W3 | 5,33 a | 81,62 |
| *D. hansenii* strain W4 | 15,33 b | 47,14 |
| *D. hansenii* strain W6 | 6,00 a | 79,31 |
| Control | 29,00 d | |

*The score followed by the same letter in the same column is insignificant difference according to Scott Knott 0,05

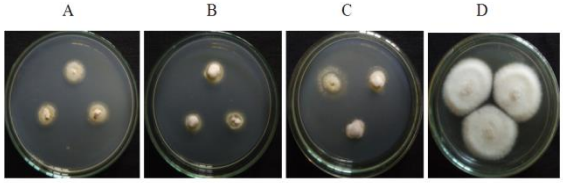


**Fig 1.** The inhibition of *C. gloeosporioides* growth caused by VOCs contact produced by *D. hansenii* strain W6 (A), W3 (B), A1 (C) and Control (D).

*Identification of volatile organic compounds produced by Debaryomyces hansenii*

*D. hansenii* was proofed to produce varying antifungal VOCs and able to produce more than one component of VOCs. One type of yeast could produce 5-19 different VOCs components (Table 2).

VOCs profile of yeast was compared to control profile (without *D. hansenii* inoculation). The GC-MS analysis identified 15 different compounds, including sulfuric compound, dimethyl pyro methane, cyanide compound, and phthalate compound.

**Table 2.** The component of Volatile organic compounds produced by *Debaryomyces hansenii*.

| No | Component | Formula |
|---|---|---|
| 1 | 3-Buten-2-One, 4-(2,2,6-Trimethyl-7-Oxabicyclo[4.1.0] Heptane | C13H20O2 |
| 2 | 7-Hydroxy-7-Phenyl-3,9-Diisopropyl-2,10-Dioxadispiro [3.3.3.1]Dodecan-1,11-Dione | C22H28O5 |
| 3 | Acetonitrile-D3 / Methyl-D3 Cyanide | C2D3N |
| 4 | 5,5'-Dicarboxy-3'-(2-Chloroethyl)-4-(2-Acetoxyethyl)-3,4'-Dimethylpyrromethane | C19H23ClN2O6 |
| 5 | 2-Acetonyl-3-Cyano-2,3-Dimethylcyclobutane-1-Carboxylic Acid | C11H15NO3 |
| 6 | 2-Keto-Butyric-Acid | C4H6O3 |
| 7 | 1,2,6,7-Diepoxy-4-Oxaheptane | C6H10O3 |
| 8 | Sulfurous Acid, Dibutyl Ester / Butyl Sulfite (Bu2so3) | C8H18O3S |
| 9 | 4-T-Butyl-1-Trifluoromethyl Cyclohexanol | C11H19F3O |
| 10 | Acetonyl Decyl Ether | :C13H26O2 |
| 11 | 1,2-Benzenedicarboxylic Acid, Diethyl Ester / Phthalic Acid | C12H14O4 |
| 12 | 3-(Z-Methoxyvinylidene) Phthalide | C11H10O3 |
| 13 | 1,2-Benzenedicarboxylic Acid,Dibutyl Ester/ Benzene-O-Dicarboxylic Acid | C16H22O4 |
| 14 | Di-Isodecyl Phthalate | C28H46O4 |
| 15 | Ditridecyl Ester Of Phthalic Acid | C34H58O4 |

The 4- T- Butyl-1- Trifluoromethylcyclohexanol compound had micro biocide properties which its vapor could kill microorganism, including fungi. Other components, such as sulfuric acid, dibutyl ester or dibutyl phosphate were dangerous substances for living organism. If someone inhaled dibutyl phosphate vapor, it caused breathing organ irritation, such as nose, throat and lungs. The 7–hydroxy-7-phenyl-3, 9-diisopropyl-2,10-dioxadispiro [3.3.3.1] dodecan-1,11-dione was a dangerous compound for living things (Pub Chem, 2015).

Dura *et al.* (2004) stated that *D. hansenii* can produce ammonia compound and some volatile compounds. According to Gori *et al.* (2007) various strain of *D. hansenii* can make ammonia compound in the glycerol medium agar and cheese agar. Acetonitril compound is harmful to living things, including microorganism. Acetonitril was toxic in the low dosage. It can be metabolized to make hydrogen cyanide which had toxic effect.

Korpi (2009) and Vespermann (2007) said that generally VOCs consisted of several simple components, such as ketone, aldehyde, hydrocarbon, alcohol, phenol, thioalcohol, thioester, benzene, cyclohexane, and its derivative compounds; had low molecular weight, low polarity and high vapor pressure. The 1,2-Benzenedicarboxylic acid, diethyl ester and 3-(Z-Methoxyvinylidene) phthalate were phthalate compounds which had toxic and microbiocide properties.

Generally, phthalate compounds were detected in the environment, such as stone, natural water, land, plants and aquatic organism. Although phthalate derivatives were useful for chemical reagent, it needed to consider the risk from its poisonous, carcinogenic, mutagenic properties for environment health. Sultan *et al.* (2010) reported that ester phthalate compound was naturally produced by extracellular of microorganism, such as yeast, bacteria and fungi. One of the bacteria which produced phthalate compound was PGPR *Burkholderia cepacia*. Besides that antifungal active compounds of *B.cepacia* K87 were identified as pyrrolnitrin and two other its derivatives (Sultan *et al.*, 2008).

The 1,2- Benzenedicarboxylic acid dibutyl ester compound was known as Dibutyl phthalate (DBP). In pharmacology, DBP was dangerous compound, had aromatic smell. DBP was used as ectoparacyte killer.

The component of VOCs had important role in biological control of pathogenic plants. According to Bruce (2005) yeast and bacteria produced keton, dimethyl disulfide and dimethyl trisulfide which controlled wood fungi. The 1,2-Benzenedicarboxylic acid, diethyl ester or phthalic acid was known as Diethyl phthalate (DEP). DEP was abundant in the environment. The biodegradation of DEP by mediated-microbe process potentially produced harmful product for microorganism.

Yeast was resulted volatile organic compounds by extracellular on the cell wall surface (Jiménez-Moreno, 2009). The *Tilletiopsis pallescens* produced fatty acid ester, antifungal compound which can inhibit mildew fungi and other competitor growth (Urquhart, 2002). Arfi *et al.* (2002) found that *D. hansenii* can deliver volatile sulfur compound, especially a great quantity of methylthiopropanal. *D. hansenii* can also synthesize volatile acid, alcohol and carbonyl compound.

*Candida intermedia* strain C410 produced 49 volatile organic compounds, such as ester, alcohol, alkena, alkane, alkyna, organic acid, ketone, dan aldehyde. The1,3,5,7-cyclooctatetraene and 3-methyl-1-butanol compounds can decline disease incident and rot fruit intensity caused by *Botrytis cinerea* (Huang *et al.*, 2011). According to Medina-Cordova *et al.* (2016), *D. hansenii* was antagonist yeast which indicated high effective against various phytopathogenic fungi in the various surroundings. The result showed that the inhibition of mycelium growth of *Mucor circinelloides, Aspergillus* sp., *Fusarium proliferatum* and *Fusarium subglutinans* was around 97.2-98.3 % on corn dent. The growth of *F. proliferatum* and *F. subglutinans* were inhibited by volatile organic compounds produced by

*D. hansenii,* as many as 54.2% and 43.5%, respectively, compared to the control one. According to Francesco *et al.* (2014) VOCs resulted from *A. pullulans* strain L1 and L8 had important role on postharvest pathogenic antagonist activity, for example *C. acutatum.* and *A. pullulans* produced alcohol on the first 96 hours of their growth. The 1-propanol-2-methyl was active to the test pathogenic fungi. The VOCs production by *A. pullulans* significantly can inhibit pathogenic infention. The high inhibition power of yeast to the pathogenic plants was probably due to VOCs which it produced and chitinolitic activity (Hartati *et al.*, 2015).

*Microscopy Observation*

The microscopy of observation of hypha of *C. gloeosporioides* contacted to VOCs from *D. hansenii* showed it experienced morphological changes.

The hypha was distortion, swollen and many big structures like bubbles. Hypha which was contacted VOCs did not have conidia at all. The abnormality of hypha was many helix structures like coil (Fig. 2).

All abnormal hypha group exhibited thin, flat, and curly mycelia. The longer exposure of Hypha can cause death. It is obvious from the decrease of *C. gloeosporioides* mycelia diameter as the time passed. On the contrary, the control one, without VOCs contact, the hypha of *C. gloeosporioides* could grow normally. The hypha grew straight, tube-like, and no helix or swollen structure. In the mycelia growth was seen conidia and dense mycelia. This fact indicated that VOCs produced by *D. hansenii* had strong fungistatic activity.

Rahman *et al.* (2007) reported the inhibition of *C. gloeosporioides* mycelia growth as many as 26.6% after 7 day incubation because of volatile antibiotic produced by antagonist bacteria of *Burkholderia cepacia* and *Pseudomonas aeruginosa*, isolated from papaya. The research of Salgado *et al.* (2013) indicated that *C. gloeosporioides* grew together with *Burkolderia tropica* experienced mycelia morphology changes as a result of bacteria volatile compound synthesized by *B. tropika*.

The hypha morphological changes, including hypha swelling, abnormal form, bubble cell, and the aggregation of cytoplasm and protoplasm. It is observed the vacuolization and granulation of mycelia structure and coil-like form.

The damage of fungal hypha due to volatile compound was reported Chaurasia *et al.*, (2005) who stated that structural damage of six pathogenic under in-vitro culture condition. The destruction was related with antijamur volatile compound of *B. substilis*. This bacteria strain was success to limit all sample fungi growth in the dual culture test and inducting morphology abnormality like deviation of mycelia and conidia. The inhibition effect caused by volatile compound was bigger than diffusible compound.

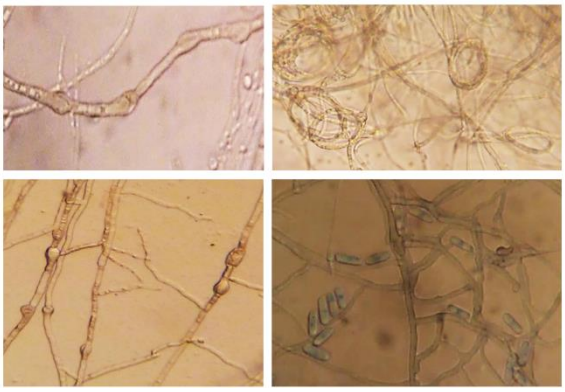

**Fig. 2.** The morphology of hypha of *C. gloeosporioides* exposed by VOCs produced *D. hansenii*. (A) bubbled, vacuolized and swollen hypha;(B) coil-like structure hypha; (C) thickened and swollen hypha, (D) Normal hypha.

According to Medina-Cordova *et al.* (2016) *D. hansenii* was antagonist yeast and highly effective against phytopatogenic fungi in various surrounding. The research result showed the the inhibition level of mycelia growth of *Mucor circinelloides, Aspergillus* sp., *Fusarium proliferatum* and *Fusarium subglutinans* were about 97.2-98.3% in the corn seed. The potential biological agent of *D. hansenii* against toxigenic fungi on corn seed due to synergetic effect of some factors, such as nutrition and space competition, volatile compound and soluble extracellular compound production. Furthermore, the growth of isolated fungi, such as *F. proliferatum* and *F. subglutinans* was inhibited by volatile compound resulted from *D. hansenii*, as many as 54.2% and 43.5% respectively, compared to the control one.

## Conclusion

The result showed that *D. hansenii* is strong antagonist to *C. gloeosporioides* which was pathogenic anthracnose fungi of tropical apples. The poisonous volatile organic compound had important role in the growth and breed inhibition of pathogenic fungi. *D. hansenii* was nonpathogenic yeast in four varieties of tropical apples in Indonesia, had ability to resist in the extremely stress condition because of chemical compound of pesticide. It produced poisonous volatile compound, so it was potentially developed as biological control agent to pathogenic fungi especially anthracnose disease of fruits.

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