Control of Postharvest Botrytis Fruit Rot of Strawberry by Volatile Organic Compounds of *Candida intermedia*

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ABSTRACT

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A study was conducted to identify volatile organic compounds or volatiles produced by *Candida intermedia* strain C410 using gas chromatography-mass spectrometry, and to determine efficacy of the volatiles of *C. intermedia* in suppression of conidial germination and mycelial growth of *Botrytis cinerea* and control of Botrytis fruit rot of strawberry. Results showed that, among 49 volatiles (esters, alcohols, alkenes, alkanes, alkynes, organic acids, ketones, and aldehydes) identified from *C. intermedia* cultures on yeast extract peptone dextrose agar, two compounds, 1,3,5,7-cyclooctatetraene and 3-methyl-1-butanol, were

the most abundant. Synthetic chemicals of 1,3,5,7-cyclooctatetraene; 3-methyl-1-butanol; 2-nonanone; pentanoic acid, 4-methyl-, ethyl ester; 3-methyl-1-butanol, acetate; acetic acid, pentyl ester; and hexanoic acid, ethyl ester were highly inhibitory to conidial germination and mycelial growth of *B. cinerea*. Inhibition of conidial germination and mycelial growth of *B. cinerea* by volatiles of *C. intermedia* was also observed. Meanwhile, results showed that incidence and severity of Botrytis fruit rot of strawberry was significantly ($P < 0.01$) reduced by exposure of the strawberry fruit to the volatiles from *C. intermedia* cultures or *C. intermedia*-infested strawberry fruit. These results suggest that the volatiles of *C. intermedia* C410 are promising biofumigants for control of Botrytis fruit rot of strawberry.

Additional keywords: biological control.

Botrytis cinerea Pers. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is a cosmopolitan plant-pathogenic fungus, causing gray mold disease on >200 crop species, including strawberry (*Fragaria* × *ananassa* Duch.) (52). It causes leaf blight, blossom blight, and fruit rot of strawberry (55) and, among these Botrytis diseases, fruit rot is the most important and is usually responsible for severe pre- and postharvest losses for strawberry industry (11,55).

Botrytis cinerea is a necrotrophic plant pathogen. It can secrete cell-wall-degrading enzymes such as polygalacturonases, pectin methylesterases, cellulases, and hemicellulases, and phytotoxic metabolites such as botrydial (7). These chemical factors facilitate infection of strawberry fruit by *B. cinerea* and development of soft rot symptoms. Thus far, there are no strawberry cultivars with high resistance to *B. cinerea* (11). Control of Botrytis fruit rot (BFR) of strawberry depends on application of fungicides at the pre- and postharvest stages (1,35). However, public concerns regarding contamination of strawberry fruit by fungicide residues and formation of fungicide-resistant strains of *B. cinerea* (6,8,19,54,58) suggest a need to explore alternative measures for control of BFR of strawberry.

Fresh postharvest products usually arrive at supermarkets or shops directly, and in consumers' hands for consumption after storage for a certain period of time. Development of measures safe for human beings to control postharvest diseases is of foremost importance for food safety. Biological control using indigenous microorganisms is probably a safe approach for

reducing plant postharvest diseases caused by *B. cinerea*, including BFR of strawberry. Previous reports indicated that selected fungal species such as *Aureobasidium pullulans* (de Bary) G. Arnaud (29), *Candida oleophila* Montrocher (29), *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (= *Gliocladium roseum* Bainer) (45), *Metschnikowia fructicola* Kurtzman & Droby (22), *Pichia guilliermondii* Wick. (53), *Rhodotorula glutinis* (Fresen.) F. C. Harrison (56), *Trichoderma* spp. (47), and *Ulocladium atrum* Preuss (2) are effective agents for suppression of BFR of strawberry under preharvest or postharvest conditions. The proposed mechanisms for control of BFR of strawberry by these biocontrol agents include antibiosis (45), mycoparasitism (26,45), competition for nutrients and space (29), and suppression of sporulation of *B. cinerea* (2,45).

Volatile organic compounds (VOCs) are substances of low molecular weight (usually <300 Da), low polarity, and high vapor pressure (50). Production of VOCs with antimicrobial activity has been reported in bacteria (5,15,17,21), *Streptomyces* spp. (27,51), filamentous fungi (23,43,44), yeasts (3,16), and higher plants (24,25). Antimicrobial VOCs from the plant-endophytic fungus *Muscador albus* Worapng, Strobel & W. M. Hess were found to have great potential for control of postharvest fruit diseases (31,33,42) and soilborne plant diseases (32,38). Previous reports also indicated that VOCs of *Streptomyces platensis* F-1 were effective for suppression of BFR of strawberry (51), and VOCs of 'Isabella' grape (*Vitis labrusca* L.) were effective for suppression of *B. cinerea* on table grape (*V. vinifera* L.) (24) and kiwi fruit (*Actinidia deliciosa* Liang and Ferguson) (25). Fialho et al. (16) reported that *Saccharomyces cerevisiae* Meyen ex E. C. Hansen can produce VOCs inhibitory to *Guignardia citricarpa* Kiely, the causal agent of citrus black spot (16). However, information on the potential of the VOCs produced by *S. cerevisiae* and other

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yeast species in biological control of plant diseases, including BFR of strawberry, is not available in literatures.

The yeast strain C410 of *Candida intermedia* (Cif. & Ashford) Langeron & Guerra was isolated from a healthy leaf of a strawberry plant grown in a greenhouse in Wuhan, China (18). Our previous study showed that application of strain C410 of *C. intermedia* to freshly harvested fruit of strawberry was effective in reducing incidence and severity of BFR of strawberry (18). This result suggests that strain C410 of *C. intermedia* is a potential agent for control of BFR of strawberry. Meanwhile, our preliminary observation indicates that cultures of strain C410 grown on yeast extract peptone dextrose agar (YEPDA) have a strong aromatic smell, suggesting release of VOCs. The objectives of this study were to (i) analyze the composition of the VOCs produced by strain C410 of *C. intermedia* and (ii) determine efficacy of the VOCs of *C. intermedia* in suppression of conidial germination and mycelial growth of *B. cinerea* and control of BFR of strawberry.

MATERIALS AND METHODS

Fungal strains and culture media. The yeast used in this study, strain C410 of *C. intermedia*, was identified on the basis of morphological and physiological characteristics, and the DNA sequence of the internal transcribed spacer (ITS) region (ITS1-5.8S rDNA-ITS2) of ribosomal gene (GenBank accession number GQ913345). Strain SB-1 of *B. cinerea* was isolated from a diseased fruit of strawberry collected from a greenhouse in Wuhan, China. Stock cultures of the two fungal strains were maintained on potato dextrose agar (PDA) and stored at 4°C. Working cultures were prepared by transferring yeast cells of *C. intermedia* or mycelia of *B. cinerea* on PDA in petri dishes, and the cultures were incubated at 20°C for 3 to 5 days. Conidia of *B. cinerea* were harvested from 14-day-old PDA cultures (26). Yeast cells were harvested by washing the PDA cultures of *C. intermedia* with sterile distilled water. The resulting yeast cell suspensions ($\approx 1 \times 10^9$ yeast cells/ml) of *C. intermedia* and the conidial suspensions of *B. cinerea* (1×10^7 conidia/ml) were used as inoculum for the bioassays of this study. The percentage of germinated conidia of *B. cinerea* was >90%.

Three culture media (PDA, water agar (WA), and YEPDA) were used in this study. PDA was made of fresh potato (200 g of peeled potato, 20 g of glucose, 20 g of agar, and 1,000 ml water). WA contained 20 g of agar in 1,000 ml of water. YEPDA contained 10 g of yeast extract, 20 g of peptone, 20 g of glucose, 20 g of agar, and 1,000 ml of sterile distilled water. All these media were autoclaved at 121°C for 20 min.

Gas chromatography–mass spectrometry analysis of the VOCs of *C. intermedia*. Yeast cells of strain C410 of *C. intermedia* were inoculated on YEPDA in petri dishes (9 cm in diam-

eter), 20 ml/dish, at $\approx 1 \times 10^8$ yeast cells/dish. Fresh YEPDA without inoculation of *C. intermedia* (uncolonized) was used as control. The yeast cultures were incubated at 20°C for 24 h and the yeast biomass in each dish reached 10^9 yeast cells. Then, a dish of the *C. intermedia* culture or uncolonized YEPDA was transferred to a 100-ml extraction glass vial. The vial was sealed with a silicon rubber mat and incubated at 40°C for 15 min to equilibrate the solid phase and the phase of VOCs in the vial. A 50/30- μ m solid-phase microextraction fiber containing divinylbenzene/carboxen/polydimethylsiloxane (Supelco, Bellefonte, PA) was inserted into the vial for extraction of the VOCs from the yeast culture or the uncolonized YEPDA at 40°C for 40 min. Then, the fiber was pulled out from the vial and inserted into the injection port of a gas chromatography–mass spectrometry (GC-MS) (6890N-5975B; Agilent Technologies Inc., CA) equipped with a J & WHP-5MS fused-silica capillary column (30 m by 0.25 mm i.d., 0.25- μ m-thickness film) (Agilent Technologies Inc.) for separation of the VOCs. GC was operated in splitless mode with the injector temperature set at 250°C. The column temperature was programmed as follows: 40°C for 3 min, and then increased at 3°C/min to 160°C, held at 160°C for 2 min, increased further at 8°C/min to 220°C, and finally held at 220°C for 3 min. Helium (ultra-high purity) was used as the carrier gas, which was set at the flow rate of 1.2 ml/min. MS was operated in electron ionization mode at 70 eV and 230°C. Mass spectra were obtained using a scan modulus with the total ion counts within the range of 50 to 550 m/z. Identification of the VOCs was done by comparing the mass spectra and retention times of the individual VOC with those for the standard compounds deposited in the database of the National Institute of Standards and Technology (NIST)/EPA/NIH library (NIST05) and the Wiley Registry of Mass Spectral Database (Wiley 7.0) in the MS. Analysis of the VOCs in the sample of the *C. intermedia* culture or the uncolonized control was repeated twice. The VOC components appearing in both the culture of *C. intermedia* and the uncolonized YEPDA were not considered to be produced by *C. intermedia*.

Antifungal activity of the selected VOCs from *C. intermedia* (bioassay 1). Thirteen VOC components identified in YEPDA cultures of strain C410 of *C. intermedia* were selected (Table 1). These components were artificially synthesized by three chemical companies, including Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), Aladdin Reagent Database Inc. (Shanghai, China), and Sigma–Aldrich (St. Louis) (Table 1). The synthetic VOCs were individually tested for inhibition of mycelial growth of *B. cinerea* on PDA and for inhibition of conidial germination of *B. cinerea* on WA. The bioassay was done in closed petri dishes (9 cm in diameter) in the presence of *B. cinerea* and a given synthetic VOC. In the test for inhibition of mycelial growth, a mycelial agar plug removed from the colony margin of a 2-day-old PDA culture of *B. cinerea* was inoculated in a petri dish

TABLE 1. Synthetic volatile organic compounds used for testing antifungal activity against *Botrytis cinerea* in this study

Compound	Source ^a	Purity (%) (grade) ^b	Storage conditions
Acetic acid, pentyl ester	A	≥ 99.5 (GC)	4°C, in darkness
1-Butanol, 3-methyl-	A	≥ 98.5 (AR)	4°C, in darkness
1-Butanol, 3-methyl-, acetate	A	≥ 99.5 (AR)	4°C, in darkness
Butanoic acid, ethyl ester	B	≥ 99.0 (GC)	4°C, in darkness
1,3,5,7-Cyclooctatetraene	C	98 (GC)	–20°C, in darkness
Ethyl acetate	A	≥ 99.0 (AR)	4°C, in darkness
Hexanoic acid, ethyl ester	A	≥ 99.5 (GC)	4°C, in darkness
2-Nonanone	B	> 99.0 (GC)	4°C, in darkness
Pentanoic acid, 4-methyl-, ethyl ester	C	≥ 97.0 (GC)	4°C, in darkness
Phenylethyl alcohol	B	≥ 99.5 (GC)	4°C, in darkness
Propanoic acid, ethyl ester	B	≥ 99.7 (GC)	4°C, in darkness
Styrene	B	> 99.5 (GC)	4°C, in darkness
1-Tetradecyne	C	≥ 97.0 (GC)	4°C, in darkness

^a Sources: A, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China; B, Aladdin Reagent Database Inc., Shanghai, China; C, Sigma–Aldrich, St. Louis.

^b Grade: GC, gas chromatography; AR, analytical reagent.

containing 20 ml of PDA. Meanwhile, a piece of filter paper (1.6 by 1.5 cm, length by width) was placed in the center of another petri dish (9 cm in diameter). The synthetic VOCs tested were individually diluted to 0.1, 0.5, 1.0, 2.5, 5.0, 10, and 50% (vol/vol) with anhydrous ethanol. An aliquot of 100 μ l of a diluted solution of a given VOC was pipetted onto the filter paper piece (FPP) in the dish. After cover removal, the PDA dish inoculated with a mycelial agar plug of *B. cinerea* was covered above the FPP dish containing an investigated VOC and the set of the double dishes (DD) was sealed using double layers of parafilm (Parafilm M, Chicago) to make a closed DD chamber of ≈ 180 cm³

in volume (Fig. 1A). For the control treatment, an aliquot of 100 μ l of anhydrous ethanol was pipetted on the FPP in a petri dish, which was covered above a PDA dish inoculated with a mycelial agar plug of *B. cinerea*. The DD set was also sealed using the parafilm. There were three DD sets (replicates) for the control treatment and for the given VOC treatment at each dilution point. The DD sets were incubated at 20°C for 3 days. Diameter of the colony of *B. cinerea* in each DD set was measured. The percentage of inhibition of mycelial growth of *B. cinerea* by a given VOC at each dilution point was calculated on the basis of the difference of the colony diameter between the

Fig. 1. Effects of the volatile organic compounds (VOCs) produced by *Candida intermedia* C410, the VOCs of *C. intermedia* plus activated carbon (AC) and 2-nonanone on mycelial growth of *Botrytis cinerea* at different temperatures (bioassay 2). **A**, A schematic diagram showing the method for testing antifungal activity of the VOCs of *C. intermedia*, the VOCs of *C. intermedia* + AC and 2-nonanone; **B** and **C**, Effects of the VOCs of *C. intermedia*, the VOCs of *C. intermedia* + AC and 2-nonanone on mycelial growth of *B. cinerea* in the simultaneous and nonsimultaneous co-incubation trials, respectively. **D** and **E**, The yeast biomass of *C. intermedia* on yeast extract peptone dextrose agar in the simultaneous and nonsimultaneous co-incubation trials, respectively. Results in all the histograms are expressed as means $\pm$ standard errors ($n = 9$). Mean values for different treatments at each temperature labeled with different letters indicate significant difference ($P < 0.01$) according to Fisher's protected least significant difference test.

each control and tested VOC treatment. The concentration value for 50% inhibition of mycelial growth (IC_{50}) expressed as micro-liters per liter was inferred from the data about the inhibition percentages and the VOC doses applied to the DD sets (44).

For inhibition of conidial germination, aliquots of a conidial suspension (1×10^7 conidia/ml) of *B. cinerea* were pipetted onto WA in petri dishes (9 cm in diameter), 100 μ l/dish. The conidial suspension in each dish was evenly spread on the surface of WA. After cover removal, a WA dish inoculated with conidia of *B. cinerea* was covered above a FPP dish containing anhydrous ethanol alone (control) or each ethanol-diluted VOC. The DD set was sealed using the parafilm. There were three DD sets for the each control and tested VOC treatment. The DD sets were incubated at 20°C for 12 h. The frequency of conidial germination of *B. cinerea* was determined by randomly counting at least 150 conidia in each WA dish under a compound light microscope. A conidium was considered to have germinated when the length of the germ tube was equal to (or greater than) the diameter of that conidium. The percentage of inhibition of conidial germination of *B. cinerea* by a given VOC was calculated on the basis of the difference in the percentage of germinated conidia between the control and tested VOC treatments. The IC_{50} value for each investigated VOC was inferred from the data about the inhibition percentages of conidial germination and the VOC doses applied to the DD sets (44).

Suppression of mycelial growth of *B. cinerea* by the VOCs of *C. intermedia* (bioassay 2). Two trials, the simultaneous co-incubation (SI) trial and the nonsimultaneous co-incubation (NSI) trial, were conducted in this bioassay. There were four treatments in each trial: (i) negative control, (ii) *C. intermedia*, (iii) *C. intermedia* + activated carbon (AC), and (iv) positive control (2-nonanone). AC was used as the absorbent of the VOCs of *C. intermedia* in the *C. intermedia* + AC treatment (15,50). In the SI trial, mycelial agar plugs (6 mm in diameter) of *B. cinerea* were inoculated in the center of petri dishes, each containing 20 ml of PDA, one mycelial agar plug per dish. For the negative-control treatment, a PDA dish with *B. cinerea* was covered above a YEPDA dish containing uncolonized YEPDA to make a DD set. For the *C. intermedia* treatment, aliquots of the yeast cell suspension of *C. intermedia* (1×10^9 yeast cells/ml) were pipetted to petri dishes each containing 20 ml of YEPDA and 100 μ l of yeast cell suspension per dish. The suspension drop in each dish was evenly spread on the surface of YEPDA using a sterilized glass spatula. Then, a PDA dish with *B. cinerea* was covered above a YEPDA dish with *C. intermedia* to make a DD set (Fig. 1A). For the *C. intermedia* + AC treatment, a piece of cotton gauze (warp $\times$ weft = $21^S \times 21^S$; Nanchang Xiangyi Medical Apparatus Co., Ltd., Nanchang, Jiangxi Province, China) with an average opening size of ≈ 1 mm² was covered above the YEPDA dish with *C. intermedia*. TT AC particles (6 g, ≈ 1 cm in diameter) (Guangzhou Huana Automobile Products Co., Ltd., Guang Dong, China) were placed on the gauze. Then, a PDA dish with *B. cinerea* was covered above the gauze and YEPDA dish to make a DD set. For the positive-control treatment, a PDA dish with *B. cinerea* was covered above a dish with an FPP soaked with 2.5 μ l of undiluted 2-nonanone to make a DD set. All the DD sets were individually sealed with parafilm (Fig. 1A). For each treatment, there were nine DD sets which were divided into three groups, three DD sets per group. Finally, the three groups of DD sets were incubated at 4°C for 6 days, 10°C for 4 days, and 20°C for 3 days, respectively, for determination of the colony diameter of *B. cinerea*. Meanwhile, yeast cells of *C. intermedia* in each YEPDA dish for the *C. intermedia* and *C. intermedia* + AC treatments were washed off with sterile distilled water (10 ml of water per dish) and the concentration of the resulting yeast cell suspensions was determined using a hemacytometer and a compound light microscope.

In the NSI trial, aliquots of the yeast cell suspension of *C. intermedia* C410 were pipetted and plated on YEPDA in petri

dishes, 100 μ l of yeast cell suspension per dish, and the yeast cultures were incubated at 20°C for 24 h. Mycelial agar plugs of *B. cinerea* were inoculated on PDA in petri dishes, one mycelial agar plug per dish. They were then individually covered above dishes containing uncolonized YEPDA, YEPDA cultures of *C. intermedia* (24 h old), YEPDA cultures of *C. intermedia* (24 h old) + AC, and 2-nonanone (2.5 μ l/dish) for the treatments negative control, *C. intermedia*, *C. intermedia* + AC, and positive control, respectively. The DD sets were individually sealed with parafilm (Fig. 1A). For each treatment, there were nine DD sets, which were divided into three groups, three DD sets per group. Finally, the three groups of DD sets were incubated at 4°C for 6 days, 10°C for 4 days, and 20°C for 3 days, respectively, for determination of the colony diameter of *B. cinerea*. The bioassay was repeated three times.

Suppression of conidial germination of *B. cinerea* by the VOCs of *C. intermedia* (bioassay 3). Four treatments—negative control, *C. intermedia*, *C. intermedia* + AC, and positive control (2-nonanone)—were included in both the SI trial and the NSI trial of this bioassay. The experiment procedures for this bioassay were similar to the procedures described for bioassay 2 of this study. In both trials, aliquots of the conidial suspension of *B. cinerea* (1×10^7 conidia/dish) were pipetted and plated on WA in petri dishes, 100 μ l per dish. They were individually covered above dishes containing uncolonized YEPDA, YEPDA cultures (0 or 24 h old) of *C. intermedia*, YEPDA cultures (0 or 24 h old) of *C. intermedia* + AC, and 2-nonanone (2.5 μ l/dish) for the negative control, *C. intermedia*, *C. intermedia* + AC, and positive control treatments, respectively. The DD sets were individually sealed with parafilm (Fig. 1A) and incubated at 4°C for 48 h, 10°C for 48 h, or 20°C for 24 h for determination of germinated conidia and the length of germ tubes of *B. cinerea* on WA in each dish. Meanwhile, the yeast cells of *C. intermedia* in each YEPDA dish were washed with sterile distilled water for determination of the yeast biomass. The bioassay was repeated three times with three replicates for each treatment at each time.

Efficacy of the VOCs of *C. intermedia* in control of BFR of strawberry (bioassay 4). The bioassay was done in closed glass desiccators (18 by 23 cm, diameter by height, ≈ 5.8 liters in volume). Aliquots of the yeast cell suspension of *C. intermedia* (1×10^9 yeast cells/ml) were pipetted and plated on YEPDA in petri dishes (9 cm in diameter) at 100 μ l of yeast cell suspension per dish. The dishes were incubated at 20°C for 24 h and the yeast biomass of *C. intermedia* reached $\approx 1 \times 10^{10}$ yeast cells per dish. The covers of the petri dishes were removed and the dishes with the cultures of *C. intermedia* were placed at the bottom of the desiccators at two, four, six, and eight dishes per desiccator. For the control treatment, two uncovered dishes with uncolonized YEPDA were placed in a desiccator. Mature and healthy strawberry fruit (*Fragaria $\times$ ananassa* ‘Feng Xiang No. 5’) were harvested from plants grown in a greenhouse in Wuhan, China. Fourteen unwounded strawberry fruit of similar size (3.0 to 3.5 by 2.0 to 2.5 cm, length by width, 16 to 18 g/fruit) were surface sterilized in 70% ethanol for 3 s, washed in sterile distilled water, blotted on paper towels, and individually inoculated with the conidial suspension ($\approx 1 \times 10^7$ conidia/ml) of *B. cinerea* on the surface of strawberry fruit, 100 μ l of conidial suspension per fruit. The fruit were then placed on the perforated ceramic clapboard above the uncovered dishes containing *C. intermedia* cultures or uncolonized YEPDA in a desiccator. There were four desiccators (replicates) for the control treatment or the *C. intermedia* treatments (two, four, six, and eight dishes of *C. intermedia* culture). The desiccators were individually covered and placed in an incubator at 20°C under a regime of 12 h of light and 12 h of darkness for 8 days. Strawberry fruit showing symptoms of soft rot and gray mold in each desiccator were individually rated for disease severity using a scale of 0 to 8, where 0 represents healthy and 1, 2, 3, 4, 5, 6, 7, and 8 represent <12.5, 12.6 to 25.0, 25.1 to

37.5, 37.6 to 50.0, 50.1 to 62.5, 62.6 to 75.0, 75.1 to 87.5, and 87.6 to 100% of the area rotted, respectively.

An additional experiment was conducted to verify the suppressive effect of the VOCs of *C. intermedia* on development of BFR of strawberry. There were three treatments in this experiment: (i) *B. cinerea*, (ii) *B. cinerea* + *C. intermedia*, and (iii) *B. cinerea* + *C. intermedia* + AC. There were three desiccators (replicates) for each treatment. For the *B. cinerea* treatment, eight uncovered petri dishes with uninoculated YEPDA were placed at the bottom of each desiccator. For the *B. cinerea* + *C. intermedia* treatment, eight uncovered dishes with 24-h-old YEPDA cultures of *C. intermedia* (20°C) were placed at the bottom of each desiccator. For the *B. cinerea* + *C. intermedia* + AC treatment, eight uncovered dishes with the YEPDA cultures of *C. intermedia* and 100 g of Hua Cao Si Ya AC powder (Hubei Huanggang Lv Yuan Jiajia Daily Necessities Manufacturer, Huang Gang, Hubei Province, China) were placed at the bottom of each desiccator. Healthy strawberry fruit were surface sterilized, individually

inoculated with conidia of *B. cinerea* ($\approx 1 \times 10^5$ conidia/fruit), and placed on the clapboard of each desiccator, 14 fruit per desiccator. The desiccators were individually covered and incubated at 20°C for 8 days. Strawberry fruit in each desiccator were individually rated for incidence and severity of BFR using the rating scale described previously.

Control of BFR of strawberry by the VOCs from *C. intermedia*-infested fruit (bioassay 5). Three treatments—blank control, strawberry fruit (SF) with water (SF-water), and strawberry fruit with *C. intermedia* (SF-*C. intermedia*)—were included in this bioassay. There were three desiccators for each treatment representing three replicates. For the blank control treatment, the bottom space underneath the clapboard of each desiccator was left empty. For the SF-water treatment, 20 healthy strawberry fruit were dipped in sterile distilled water for 5 s, blotted on paper towels, and loaded underneath the clapboard of a desiccator. For the SF-*C. intermedia* treatment, 20 healthy strawberry fruit were dipped in a yeast cell suspension of *C. intermedia* ($\approx 1 \times 10^8$ yeast

TABLE 2. Volatile organic compounds (VOCs) released from *Candida intermedia* strain C410

RT (min) ^a	Possible compound ^b	RA (%) ^c	MW (Da) ^c
0.80	1,6-Octadiene, 5,7-dimethyl-, (R)-	0.100	138
1.41	4,5-Nonadiene	0.028	124
1.50	1-Tetradecyne	0.100	194
2.13	5-Formyl-6-methyl-4,5-dihydropyran	0.021	142
3.54	1-Tridecyne	0.014	180
3.97	3-Octyne	0.010	110
4.86	1,19-Eicosadiene	0.190	278
6.31	1,2-15,16-Diepoxyhexadecane	0.155	254
6.70	19,19-Dimethyl-eicosa-8,11-dienoic acid	0.207	336
6.91	7-Oxabicyclo[4.1.0]heptane, 3-oxir	0.045	140
6.94	1,3,4-Hexatriene, 3-methoxy-	0.003	110
7.09	Ethyl acetate	1.086	88
7.31	Acetic acid, pentyl ester	0.083	130
8.12	Z,Z-6,13-octadecadien-1-ol acetate	0.045	308
9.55	Propanoic acid, ethyl ester	0.431	102
10.58	1-Butanol, 3-methyl-	38.957	88
13.11	Butanoic acid, ethyl ester	0.841	116
14.21	2-Ethylacridine	0.038	207
16.80	1-Butanol, 3-methyl-, acetate	1.738	130
17.69	1,3,5,7-Cyclooctatetraene	29.410	104
18.39	Styrene	0.203	104
20.66	Benzeneacetonitrile, α , -hydroxy-	0.034	133
20.92	Benzeneethanamine, α , 2,6-trimethyl-	0.038	163
21.41	Pentanoic acid, 4-methyl-, ethyl ester	1.962	144
22.08	2,6-Dimethylbenzaldehyde	0.038	134
23.17	Hexanoic acid, ethyl ester	0.438	144
23.45	2-Carbamoylphenoxyacetic acid	0.024	195
23.83	4-Ethylbenzoic acid, oct-3-en-2-yl ester	0.024	246
24.17	Benzene, 1,3-dichloro-	0.059	147
24.24	Benzene, 1,4-dichloro-	0.028	147
24.31	Benzene, 1,2-dichloro-	0.017	147
24.69	Spiro[3.5]nona-5,7-dien-1-one,5,9,9-trimethyl-	0.010	176
26.13	Heptane, 4-methyl-	0.121	114
26.39	10,12-Octadecadienoic acid	0.076	276
27.37	Benzeneethanamine, β -methyl-	0.007	135
27.64	Pyrazine, 2,6-diethyl-	0.345	136
27.77	4-Pyridinamine, N,N,2-trimethyl-	0.028	136
28.10	2-Nonanone	0.210	142
29.34	Cis,cis-7,10,-hexadecadienal	0.007	236
30.23	Phenylethyl alcohol	2.248	122
32.32	Preg-4-en-3-one, 17. α -hydroxy-	0.017	316
32.62	Cis-8-methyl-exo-tricyclo[5.2.1.0(2,6)] decane	0.041	150
32.85	Cis-11-Hexadecenal	0.021	238
33.62	11,14-Eicosadienoic acid, methyl ester	0.024	322
33.98	E,E-1,9,17-docasatriene	0.010	304
34.47	8-Dodecen-1-ol, acetate	0.010	226
34.60	Cyclodecene	0.010	166
34.71	Bicyclo[2.2.1]heptane, 2-methyl-	0.028	110
35.12	9-Octadecynoic acid, methyl ester	0.028	294

^a Retention time in gas chromatography–mass spectrometry.

^b VOCs of *C. intermedia* with the relative peak areas of <0.001% are not included in this table.

^c RA, relative peak area; MW, molecular weight.

cells/ml) for 5 s, blotted on paper towels, and loaded underneath the clapboard of each desiccator. Then, 14 healthy strawberry fruit were surface sterilized in 70% ethanol, rinsed in sterile distilled water, individually inoculated with aliquots of the conidial suspension of *B. cinerea* (1×10^7 conidia/ml), 100 μ l per fruit, and placed on the clapboard of each desiccator for these three treatments. The desiccators were individually covered and incubated at 20°C for 8 days. Strawberry fruit on and beneath the clapboard in each desiccator were individually rated for incidence and severity of fruit rot using the rating scale described in bioassay 4.

Statistical analysis. All the statistical analyses in this study were conducted using SAS/STAT software (version 8.0; SAS Institute, Cary, NC). Data on colony diameter, percentage of germinated conidia (arcsine transformed), length of germ tubes, disease incidence (arcsine transformed), and disease severity in different repetitions were pooled for analysis. Means of each parameter for different treatments in each bioassay were compared using Fisher's protected least significant difference test ($P < 0.01$).

RESULTS

Identification of the VOCs from *C. intermedia*. Results of GC-MS analysis showed that 49 VOCs released from 1-day-old YEPDA cultures of *C. intermedia* C410 were identified on the basis of mass spectral properties (Table 2). These compounds fell into classes of esters, alcohols, alkenes, alkanes, alkynes, organic acids, ketones, aldehydes, benzenes, and amines. The most abundant compounds in the VOC profile of *C. intermedia* C410 were 1,3,5,7-cyclooctatetraene and 3-methyl-1-butanol. The relative peak area (RA) for these two VOCs accounted for 29.4 and 38.8%, respectively. Eighteen VOCs were moderately abundant, with RA values of 0.1 to 2.2%. They included acetic acid, pentyl ester; 1-butanol, 3-methyl-, acetate; butanoic acid, ethyl ester; 1,2-15,16-diepoxyhexadecane; 5,7-dimethyl-, (R)-; 19,19-dimethyl-eicosa-8, 11-dienoic acid; 1,19-eicosadiene; ethyl acetate; hexanoic acid, ethyl ester; heptane, 4-methyl-; 2-nonanone; 1,6-octadiene; pentanoic acid, 4-methyl-, ethyl ester; phenylethyl alcohol; propanoic acid, ethyl ester; pyrazine, 2,6-diethyl-; styrene; and 1-tetradecyne. The other 29 VOCs were the least abundant, with RA values $<0.1\%$.

Antifungal activity of the selected VOCs from *C. intermedia* (bioassay 1). Thirteen highly to moderately abundant VOCs

produced by *C. intermedia* C410 were selected for testing their antifungal effects on *B. cinerea*. Artificially synthetic products of these VOCs were individually evaluated as fumigants in DD sets for inhibition of mycelial growth and conidial germination of *B. cinerea*. Results showed that 10 compounds (acetic acid, pentyl ester; butanoic acid, ethyl ester; 3-methyl-1-butanol; 1-butanol, 3-methyl-, acetate; 1,3,5,7-cyclooctatetraene; hexanoic acid, ethyl ester; 2-nonanone; pentanoic acid, 4-methyl-, ethyl ester; phenylethyl alcohol; and propanoic acid, ethyl ester) effectively inhibited mycelial growth of *B. cinerea*, with IC_{50} values of 6.5 to 70.2 μ l/liter (Table 3). Of these 10 compounds, 7 (acetic acid, pentyl ester; 3-methyl-1-butanol; 1-butanol, 3-methyl-, acetate; 1,3,5,7-cyclo-octatetraene; hexanoic acid, ethyl ester; 2-nonanone; and pentanoic acid, 4-methyl-, ethyl ester) effectively inhibited conidial germination of *B. cinerea*, with IC_{50} values of 2.5 to 90.8 μ l/liter. Styrene could not inhibit mycelial growth of *B. cinerea* ($IC_{50} > 260$ μ l/liter). However, it effectively inhibited conidial germination of this pathogen ($IC_{50} = 42.1$ μ l/liter). Ethyl acetate and 1-tetradecyne could not inhibit mycelial growth and conidial germination of *B. cinerea* ($IC_{50} > 280$ μ l/liter).

Suppression of mycelial growth of *B. cinerea* by the VOCs of *C. intermedia* (bioassay 2). After incubation at 4°C for 6 days, 10°C for 4 days, and 20°C for 3 days in both the SI and NSI trials, the average colony diameter of *B. cinerea* reached 11 to 18 mm in the positive control treatment (2-nonanone) and 83 to 89 mm in the negative control treatment (uncolonized YEPDA) (Fig. 1B and C). In the *C. intermedia* treatment (*C. intermedia* on YEPDA), due to the presence of the VOCs from the cultures of *C. intermedia* (Fig. 1D and E), the average colony diameter of *B. cinerea* was 63, 21, and 11 mm at 4, 10, and 20°C, respectively, in the SI trial (Fig. 1B), and 12, 12, and 9 mm at the same temperatures, respectively, in the NSI trial (Fig. 1C). A significant difference ($P < 0.01$) in the average colony diameter of *B. cinerea* was detected between the negative control and *C. intermedia* treatments at each temperature in each trial. However, the suppressive effect of the VOCs of *C. intermedia* on mycelial growth of *B. cinerea* was partially or completely nullified by AC in the *C. intermedia* + AC treatment. The average colony diameter of *B. cinerea* in this treatment reached 83 to 85 mm in the SI trial and 66 to 84 mm in the NSI trial. These colony-diameter values were significantly ($P < 0.01$) higher than those for the *C. intermedia* treatment but were not significantly ($P > 0.01$) different, or slightly different (although significantly different in terms of statistics), from those for the negative control treatment.

Suppression of conidial germination of *B. cinerea* by the VOCs of *C. intermedia* (bioassay 3). After incubation at 4°C for 48 h, 10°C for 48 h, and 20°C for 24 h, the percentage of germinated conidia of *B. cinerea* reached 1, 86, and 96%, respectively, in the positive control treatment (2-nonanone) and 100% in the negative control treatment (uncolonized YEPDA) (Fig. 2). The average length of germ tubes of *B. cinerea* reached 19, 52, and 70 μ m at 4, 10, and 20°C, respectively, in the positive control treatment and 289, 429, and 332 μ m at the same three temperatures, respectively, in the negative control treatment. In the *C. intermedia* treatment, the yeast biomass of *C. intermedia* in the SI trial (0.3 to 9.6×10^9 yeast cells/dish) was lower than that in the NSI trial (1.9 to 3.0×10^{10} cells/dish). As a result, the percentage of germinated conidia of *B. cinerea* in this treatment was $>90\%$ (92 to 99%) in the SI trial but $<30\%$ (0.6 to 29.0%) in the NSI trial. The average length of germ tubes was 56 to 106 μ m in the SI trial and to 10 μ m in the NSI trial. However, in the *C. intermedia* + AC treatment, the suppressive effect of the VOCs of *C. intermedia* on conidial germination or extension of germ tubes of *B. cinerea* was partially or completely nullified by AC. The average percentage of germinated conidia of *B. cinerea* in this treatment reached 99 to 100% in the SI trial and 94 to 99% in the NSI trial. These percentage values were not significantly ($P < 0.01$) or slightly different from those in the negative control treatment. The average

TABLE 3. Values of the 50% inhibition concentration (IC_{50}) of the synthetic volatile organic compounds for suppression of mycelial growth and conidial germination of *Botrytis cinerea*

Compound	IC_{50} value (mean $\pm$ standard error) (μ l/liter)	
	Mycelial growth ^a	Conidial germination ^b
2-Nonanone	6.5 $\pm$ 0.5	2.5 $\pm$ 1.3
Pentanoic acid, 4-methyl-, ethyl ester	25.1 $\pm$ 7.2	11.9 $\pm$ 1.9
1,3,5,7-Cyclooctatetraene	16.5 $\pm$ 1.3	28.8 $\pm$ 4.1
1-Butanol, 3-methyl-, acetate	21.0 $\pm$ 2.4	28.2 $\pm$ 4.7
Acetic acid, pentyl ester	46.5 $\pm$ 3.6	17.4 $\pm$ 3.5
Hexanoic acid, ethyl ester	67.3 $\pm$ 12.2	18.6 $\pm$ 3.7
1-Butanol, 3-methyl-	70.2 $\pm$ 1.6	90.8 $\pm$ 0.4
Propanoic acid, ethyl ester	15.2 $\pm$ 1.1	>300.0
Butanoic acid, ethyl ester	18.6 $\pm$ 6.4	>280.0
Phenylethyl alcohol	29.9 $\pm$ 5.0	>500.0
Styrene	>260.0	42.1 $\pm$ 3.6
Ethyl acetate	>280.0	>580.0
1-Tetradecyne	$>1,000.0$	$>1,000.0$

^a Mycelial growth of *B. cinerea* was determined on potato dextrose agar in petri dishes after the cultures were incubated at 20°C for 72 h.

^b Conidial germination of *B. cinerea* was determined on water agar in petri dishes after the cultures were incubated at 20°C for 12 h.

length of germ tubes of *B. cinerea* reached 212 to 299 μm in the SI trial and 136 to 174 μm in the NSI trial. These values were significantly ($P < 0.01$) higher than those in the *C. intermedia* treatment, although they were still significantly ($P < 0.01$) lower than those in the negative control treatment.

Efficacy of the VOCs of *C. intermedia* in control of BFR of strawberry (bioassay 4). After incubation at 20°C for 8 days, all the strawberry fruit in the control treatment (uncolonized YEPDA) showed soft rot and gray mold symptoms, with the BFR severity reaching 7.8 (Fig. 3). However, in the presence of the cultures of *C. intermedia* C410 increasing from 2×10^{10} to 8×10^{10} yeast cells/desiccator (5.8 liters in volume), the average BFR incidence of strawberry decreased from 93 to 18% and the average BFR severity value decreased from 3.9 to 0.3.

Results of the additional experiment showed that the VOCs of *C. intermedia* in the *C. intermedia* treatment were highly effective

in suppression of BFR of strawberry (Fig. 4). However, the suppressive effect of the VOCs of *C. intermedia* on BFR of strawberry in the *C. intermedia* + AC treatment was greatly nullified by AC (Fig. 4). The average BFR incidence reached 100% and the average BFR severity value reached 7.3 in this treatment. These values were significantly ($P < 0.01$) higher than those in the *C. intermedia* treatment but not significantly different ($P > 0.01$) from those in the control treatment (Fig. 4).

Control of BFR of strawberry by the VOCs from *C. intermedia*-infested fruit (bioassay 5). In the blank control and SF-water treatments, all the *B. cinerea*-inoculated strawberry fruit on the clapboards of the closed desiccators showed soft rot and gray mold symptoms (Fig. 5A). The BFR severity of strawberry reached 7.6 and 7.9 for these two treatments, respectively (Fig. 5B). Moreover, in the SF-water treatment, all the water-treated fruit of strawberry underneath the clapboard of the desiccators showed symptoms of soft rot and the average fruit rot severity

Fig. 2. Effects of the volatile organic compounds (VOCs) produced by *Candida intermedia* C410, the VOCs of *C. intermedia* plus activated carbon (AC) and 2-nonanone on conidial germination and germ-tube extension of *Botrytis cinerea* on water agar (WA) under different temperatures (bioassay 3). **A, B, and C,** The percentage of germinated conidia (**A**) and the length of germ tubes of *B. cinerea* on WA (**B**), and the yeast biomass of *C. intermedia* on yeast extract peptone dextrose agar (YEPDA) (**C**) in the simultaneous co-incubation (SI) trial. **D, E, and F,** The percentage of germinated conidia (**D**), the length of germ tubes of *B. cinerea* on WA (**E**), and the yeast biomass of *C. intermedia* on YEPDA (**F**) in the nonsimultaneous co-incubation (NSI) trial. Results in all the histograms are expressed as means $\pm$ standard errors ($n = 9$). Mean values for different treatments at each temperature labeled with different letters indicate significant difference ($P < 0.01$) according to Fisher's protected least significant difference test.

reached 7.6 (Fig. 5B). In contrast, in the SF-*C. intermedia* treatment, most of the *B. cinerea*-inoculated fruit of strawberry on the clapboards of the desiccators appeared healthy (Fig. 5A). The average BFR incidence was 14% and the average BFR severity value was 0.1 (Fig. 5B). Moreover, in this treatment, growth and proliferation of *C. intermedia* on the surface of *C. intermedia*-treated strawberry fruit underneath the clapboard of the desiccators were visible, showing slimy and milky symptoms (Fig. 5A). The average fruit rot incidence was 29% and the average fruit rot severity value was 0.6.

DISCUSSION

Numerous studies indicate that selected strains of many plant epiphytic yeast species, including *C. ciferrii* (49), *C. oleophila* (29,34), *C. saitoana* Nakase & M. Suzuki (14), and *C. sake* (Saito & M. Ota) Uden & H. R. Buckley ex S. A. Mey. & Ahearn (37), are effective agents for control of postharvest fruit diseases caused by fungal pathogens including *B. cinerea*. Liang and Chi (28) reported that strain C3 of *C. intermedia* isolated from decayed seed of corn was effective in controlling onion bulb rot caused by *Aspergillus niger* Tiegh. and fruit rot of citrus, orange, and apple caused by *Penicillium* spp. (28). Our study confirms the report of Huang et al. (18), who reported that strain C410 of *C. intermedia* isolated from a healthy strawberry leaf was effective in suppression of BFR of strawberry when yeast cells of this strain were inoculated on strawberry fruit (18). Numerous studies suggest that the mechanisms involved in biocontrol of *B. cinerea* and other plant fungal pathogens by yeasts include nutrient competition and site exclusion (10,28,29,37,41,57), restriction of conidial germination and hyphal growth of fungal pathogens (4,13), production of 1,3- β -exoglucanase (20,30), and induction of plant defense responses (12,14,46,57). Our study reveals that the VOCs produced by *C. intermedia* C410 grown on YEPDA and strawberry fruit can effectively suppress the development of BFR of strawberry through suppression of conidial germination and mycelial growth of *B. cinerea*. These results suggest that pro-

duction of antifungal VOCs by yeasts might be an important mechanism for suppression of infection of plant tissues by *B. cinerea* under airtight conditions.

The present study reveals that 1,3,5,7-cyclooctatetraene, 3-methyl-1-butanol, 2-nonanone, and phenylethyl alcohol are the VOCs produced by *C. intermedia*. Strobel et al. (44) and Mitchell et al. (36) reported that the plant-endophytic fungi *M. albus* (44) and *M. crispans* (36) can produce a mixture of VOCs, including 3-methyl-1-butanol, 2-nonanone, and phenylethyl alcohol. The compound 2-nonanone was also found in the VOC mixture released from cultures of *Bacillus subtilis* (5) and even from mature fruit of strawberry (48). Stinson et al. (43) indicated that another plant-endophytic fungus (*Glilocladium* sp.) can produce 1,3,5,7-cyclooctatetraene (43). These findings suggest that some of the VOCs of *C. intermedia* are also produced by other organisms.

Previous studies indicate that antimicrobial VOCs produced by fungi or bacteria are effective biofumigants for control of plant diseases under airtight conditions. For example, the VOCs produced by strains of *M. albus* effectively controlled fruit decay caused by *Botrytis cinerea* in closed chambers (31,33,42), or soilborne plant pathogens such as *Rhizoctonia solani* Kühn, *Phytophthora capsici* Leonian (32), and nematodes (38) in soil. Koitabashi (23) reported that *Irpex lacteus* (Fr.) Fr., a wood-decaying basidiomycete, produces two antifungal volatiles, 5-pentyl-2-furaldehyde and 5-(4-pentenyl)-2-furaldehyde (23). Application of the cultures of *I. lacteus* in greenhouse effectively suppressed parsley powdery mildew caused by *Oidium* spp. (23). Our present study demonstrates that the VOCs released from cultures of *C. intermedia* or *C. intermedia*-infested fruit of straw-

Fig. 3. Effect of the volatile organic compounds from *Candida intermedia* C410 on suppression of botrytis fruit rot of strawberry (20°C, 8 days) (bioassay 4). Results in the two histograms are expressed as means $\pm$ standard errors. Mean values for different treatments with the same letters in each histogram are not significantly different ($P > 0.01$) according to Fisher's protected least significant difference test.

Fig. 4. Effect of the volatile organic compounds (VOCs) from *Candida intermedia* strain C410 and the VOCs of *C. intermedia* plus activated carbon (AC) on suppression of botrytis fruit rot of strawberry (20°C, 8 days) (bioassay 4). Results in the two histograms are expressed as means $\pm$ standard errors. Mean values for different treatments with the same letters in each histogram are not significantly different ($P > 0.01$) according to Fisher's protected least significant difference test.

Fig. 5. Effect of the volatile organic compounds from *Candida intermedia*-infested fruits of strawberry on suppression of botrytis fruit rot of strawberry (20°C, 8 days) (bioassay 5). **A**, Set-up of the experiment showing different treatments (T1/T2, T3/T4, and T5/T6) in three desiccators (the top row) and strawberry fruits with or without gray mold symptoms (the bottom two rows). The results are one of the three repetitions in this bioassay. T1, T3, and T5, strawberry fruits inoculated with conidia of *Botrytis cinerea* and placed on the clapboard of each desiccator; T2, blank control (empty) beneath the clapboard of the desiccator; T4, strawberry fruits treated with water and placed beneath the clapboard of the desiccator; and T6, strawberry fruits inoculated with *C. intermedia* and placed beneath the clapboard of the desiccator. Note the colonization of *C. intermedia* on the surface of strawberry fruits resulting in formation of slimy and milky appearance. **B**, Two histograms showing incidence and severity of botrytis fruit rot of strawberry. Results in the two histograms are expressed as means $\pm$ standard errors. Mean values for different treatments with the same letters in each histogram are not significantly different ($P > 0.01$) according to Fisher's protected least significant difference test.

berry are effective in reducing incidence and severity of BFR of strawberry. Therefore, strain C410 of *C. intermedia* can be potentially developed as a biofumigant for control of this important pre- and postharvest disease of strawberry fruit. Further tests for evaluation of the efficacy of application of *C. intermedia* C410 on a large scale for control of BFR of strawberry are warranted.

C. intermedia has been recorded as an opportunistic pathogen that causes, or may be related to, infection of human beings, although cases of human infection by this yeast species are rare (9,39,40). Strain C410 of *C. intermedia* investigated in this study was isolated from a healthy strawberry leaf (18). Whether or not this particular strain of *C. intermedia* is pathogenic or toxic to human beings remains unknown. Further studies to clarify the potential risk of strain C410 of *C. intermedia* as a biocontrol agent used for control of BFR of strawberry as well as Botrytis diseases on other economic crops are warranted.

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