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Hanseniaspora uvarum prolongs shelf life of strawberry via volatile production

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ABSTRACT

Gray mold caused by *Botrytis cinerea* led to severe postharvest losses for strawberry industry. In recent years, some studies have shown that postharvest diseases of strawberry can be controlled by using bacterial, fungal and yeast strains. The yeast strain *Hanseniaspora uvarum* was shown as an effective antagonist against *B. cinerea* growth. Here, we further investigated the volatile organic compounds (VOCs) production of *H. uvarum* and how this could impact on postharvest gray mold control of strawberry. A total of 28 VOCs were detected by GC-MS in the headspace of *H. uvarum* and strawberry with/without *B. cinerea* (SI and RSI $\geq$800). Among these VOCs, 15 VOCs were detected in both conditions, 4 VOCs were *H. uvarum* and strawberry without *B. cinerea* and the other 9 VOCs were only detected when *B. cinerea* was inoculated. Two VOCs, ethyl acetate and 1,3,5,7-cyclooctatetraene, enhanced by inoculation of *B. cinerea*. In *in vitro* assay, *H. uvarum* significantly inhibited mycelial growth and spore germination of *B. cinerea* via VOCs production. Moreover, *in vivo* assay showed that *H. uvarum* reduced *B. cinerea* infection of strawberry and maintained fruit appearance, firmness and total soluble solids via VOCs production. Collectively, our results showed that *H. uvarum* VOCs significantly controlled postharvest gray mold of strawberry and prolonged the storage time and shelf life.

1. Introduction

Strawberries (*Fragaria ananassa Duch*) are highly perishable fruits due to their extreme tenderness, vulnerability to mechanical damage, high level of respiration and their susceptibility to fungal spoilage (Dennis, 1978). Among them, gray mold caused by *Botrytis cinerea* is responsible for severe preharvest and postharvest losses for strawberry industry (Romanazzi et al., 2001; Droby and Lichter, 2004). *B. cinerea* is a necrotrophic plant pathogen. It can secrete cell-wall-degrading enzymes such as cellulases, hemicellulases, polygalacturonases, pectin methylesterases, and phytotoxic metabolites such as botrydial, which facilitate infection of strawberry fruit and developments of soft rot symptoms. Thus far, there are no strawberry cultivars with high resistance to *B. cinerea* (Huang et al., 2011). More efforts have been made to minimize losses through developing a better understanding of mechanisms of postharvest diseases, as well as by developing adequate postharvest handing technologies and control strategies (Prusky and Gullino, 2010).

In recent years, biological control is becoming an increasingly effective measure to control postharvest diseases of fruit (El-Neshawy and Shetaia, 2003; Zhang et al., 2007; Fan et al., 2009; Costa et al., 2013; Maryam et al., 2014; Zhang et al., 2014; Parafati et al., 2015; Platania et al., 2012). The mechanisms of biocontrol agents interact with pathogens and fruit tissues including competition for limiting nutrients and space, induced resistance, produced of lytic enzymes, mycoparasitism and the role of oxidative stress are demonstrated (Spadaro and Droby, 2016). Biocontrol applications for postharvest disease control is now directed more towards the use of volatile organic compounds (VOCs) produced by microorganisms that are biodegradable, that do not leave toxic residues on the fruit surface (Mercier and Smilanick, 2005).

Biological fumigation, or biofumigation, with volatile

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compounds produced by microorganisms has shown promise for killing a wide range of storage pathogens and controlling fungal decay. Mercier and Smilanick (2005) indicated that VOCs produced by *Muscador albus* had possibility to control fungal decays of apple and peach by biofumigation. Li et al. (2012, 2010) found that volatile substances from *Streptomyces globisporus* JK-1 inhibited the spore germination and mycelial growth of *Botrytis cinerea* and *Penicillium italicum* on tomato and *Citrus microcarpa*, respectively. Zheng et al. (2013) and Chen et al. (2008) indicated the VOCs of *Bacillus* spp. were antagonistic to the *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Penicillium digitatum*, *Penicillium italicum* and *Penicillium crusto sum*. Garbeva et al., 2014 analyzed the composition of volatiles produced by *Collimonas* on agar under different nutrient conditions and studied the effect on fungal growth. The volatiles had a negative effect on the growth of a broad spectrum of fungal species. Antifungal volatiles produced by *Collimonas* played an important role in realizing its mycophagous lifestyle. Di Francesco et al. (2015) demonstrated that the conidia germination of *Penicillium* spp. was completely inhibited by VOCs produced by two *Aurebasidium pullulans* L1 and L8 strains (100% inhibition). Moreover, the production of VOCs could play an essential role in the antagonistic activity of two *A. pullulans* strains against five fruit postharvest pathogens *in vitro* and *in vivo* tests.

In our previous research, *Hanseniaspora uvarum* was an effective antagonist, which reduced the natural decay development of grape and strawberry and maintained the quality parameters (Qin et al., 2015; Cai et al., 2015). Meanwhile, *H. uvarum* has been reported as a potential biological control agent for control of chilli fruit rot (Basha and Ramanujam., 2015) and postharvest green mold of oranges (Li et al., 2016). *H. uvarum* has been reported to inhibit the growth of *B. cinerea* with multiple modes of action such as competition for nutrients and space, induction of host defense, morphology change and secondary metabolites (Liu et al., 2010; Qin et al., 2015; Cai et al., 2015; Romanazzi et al., 2012). Moreira et al. (2011) have identified different VOCs produced by *Hanseniaspora* yeasts during red wine vinifications included such as 3-methyl-1-butanol, ethyl acetate, phenylethyl alcohol and butanoic acid, ethyl ester. However, the effect of VOCs produced by *H. uvarum* as a mode of antagonism action for the control of postharvest disease is not described. Here, we identified VOCs from *H. uvarum* on strawberry with/without *B. cinerea*, and studied antifungal activity of VOCs against *B. cinerea in vitro* and *in vivo*. This work attempted to find a better strategy to control gray mold of strawberry, and prolong the storage time and shelf time.

2. Materials and methods

2.1. Fungal strains

The antagonist yeast was isolated from the surface of strawberries and identified as *Hanseniaspora uvarum* based on the similarity analysis of its morphologies, physiological-biochemical characteristics and 26S rDNA D1/D2 domain sequence (Genbank accession number: JX125041). The yeast was maintained on potato dextrose agar (PDA, 200 mL extract of boiled potatoes, 20 g dextrose and 20 g agar in 1000 mL distilled water) at 4 °C before use. Liquid cultures of the yeast were grown in 250 mL Erlenmeyer flasks with 100 mL potato dextrose (PDB, 200 mL extract of boiled potatoes, 20 g dextrose in 1000 mL distilled water) on a gyratory shaker at 180 r min^{-1}, 28 °C for 24 h. The yeast cells were acquired by centrifuging at 6000 g for 15 min at 4 °C, then washed with sterile distilled water twice and re-suspended in sterile distilled water with 0.05% Tween-20. Cells concentration was adjusted to a final concentration of approximately 1×10^9 CFU mL^{-1} using a hemacytometer (XB-K-25; Shanghai, China).

B.cinerea, from infected strawberry fruit, obtained from Kang Tu, Department of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China (Wei et al., 2014) and stored on potato dextrose agar (PDA) at 4 °C. Before use, *B. cinerea* was freshly cultured on PDA plates at 23 °C. Spore suspensions were prepared by removing the spores from a 7-day old culture with a sterile inoculator and then suspending in sterile distilled water to the required concentration of 1×10^5 spores·mL^{-1}, which was estimated using a hemacytometer.

2.2. Fruit

Fragaria ananassa 'Hong Yan', were grown in a greenhouse located in Yuhua district, Nanjing city, Jiangsu province, China. Diurnal temperature in the greenhouses ranges from 8 °C to 25 °C. Commercially mature fruit were harvested early in the morning, and then transported immediately to the laboratory in 2 h. All fruits were selected depending on the maturity, size color, and the absence of physical injuries or infections.

2.3. Gas chromatography-mass spectrometry analysis of H. uvarum VOCs

The equator of fruits were hit a wide 3 mm, deep 4 mm hole with a sterile hole puncher and placed 30 min. The experiments were divided into three groups: (Control) Inoculated with 100 μL sterile water into the wound of strawberry fruit; (H) Inoculated with 100 μL 1×10^9 CFU mL^{-1} *H. uvarum* into the wound of strawberry fruit; (HB) Inoculated with 100 μL 1×10^9 CFU mL^{-1} *H. uvarum* into the wound of strawberry fruit and then 100 μL 1×10^5 spore·mL^{-1} conidial suspension of *B. cinerea*. All groups were incubated for 3 days at 25 °C and 5 g wound samples of every group were transferred to a 20 mL extraction glass vial. The experiment was repeated twice per group (15 unwounded fruits/group).

Yeast VOCs composition was evaluated by SPME coupled with gas chromatography-mass spectrometry (GC-MS). For all subsequent experiments, an SPME fiber with 75 μm CAR/PDMS coating was used. Trapped compounds were desorbed into the GC injection port at 250 °C for 3 min, and separated in a gas chromatograph equipped with a TR-5MS fused silica capillary column (30 m by 0.25 mm inside diameter; 0.25 μm film thickness) connected to a quadrupole mass detector. The oven temperature was set at 40 °C for 2.5 min and then programmed to rise from 40 to 200 °C at 5 °C·min^{-1}, from 200 to 240 °C at 10 °C min^{-1} for 5 min. The transfer line was heated at 230 °C and the ion source at 250 °C. Helium carrier gas had a flow of 1 mL min^{-1}. The mass spectrometer was operated in electron impact mode at 70 eV, scanning the range of 40–400 amu. 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) and the Wiley Registry of Mass Spectral Database (Wiley 7.0) in the MS (SI and RSI $\geq$ 800).

2.4. In vitro VOCs antagonistic assay

The efficacy of the VOCs produced by the yeast on the mycelium growth and conidia germination of *B. cinerea* were tested. The method used was adopted by a double petri dish assay (Rouissi et al., 2013) with some modifications. The PDA plates were inoculated spreading 100 μL of antagonist cell suspension (1×10^9 CFU mL^{-1}). Equivalent amounts of sterile distilled water were used as the control. The lid of plate was replaced, by a base plate of PDA inoculated with an agar plug (5 mm) from the

periphery of an actively growing culture of *B. cinerea*. The two base plates were sealed immediately with double layer of parafilm and incubated for 5 days at 25 °C. The fungal hyphal diameter was assessed and the percentage of inhibition was calculated using the formula: (Control value − Treatment value)/Control value × 100%.

Spore suspensions (1×10^5 spore·mL^{-1}) were prepared from exposed to volatiles treatment and control on the fifth day and then inoculated in PDB. Four hours later, conidial germination and germ tube length were examined via optical light microscope. For each experiment, there were three replicates, and the experiments were repeated twice.

2.5. In vivo VOCs antagonistic assay

2.5.1. Effects of H. uvarum VOCs on control of gray mold in strawberry fruit at 25 °C

The bioassay was done in closed glass desiccators (6.5 cm by 15 cm, down diameter by up diameter). Aliquots of the yeast cell suspension of *H. uvarum* (1×10^9 CFU mL^{-1}) were pipetted and plated on PDA in petri dishes at 200 μL of yeast cell suspension per dish. Thirty unwounded strawberry fruits of similar size individually were inoculated with the conidial suspension ($\approx 1 \times 10^5$ spore·mL^{-1}) of *B. cinerea* on the surface of strawberry fruit for each treatment. The dishes with the culture of *H. uvarum* were placed at the bottom of the desiccators at six dishes per desiccator. The fruit were then placed on the perforated ceramic clapboard above the uncovered dishes containing *H. uvarum* cultures or uncolonized PDA in a desiccator. There were four different fumigation time treatments in each trial: (i) negative control (no fumigation), (ii) one day, (iii) two days, (iv) three days. After different fumigation time, strawberries were placed in an incubator at 25 °C (RH90%–95%). The decay index and weight loss were evaluated on the 6th day. The experiment was repeated twice with three replicates per treatment (30 fruits/treatment).

An additional experiment was conducted to verify the suppressive effect of the VOCs of *H. uvarum* on development of gray mold. Thirty unwounded fruits were inoculated with the conidial suspension ($\approx 1 \times 10^5$ spore·mL^{-1}) of *B. cinerea* on the surface of strawberry fruit for each treatment. There were three treatments in this experiment: (i) uninoculated with the yeast suspension on PDA, (ii) inoculated with the yeast suspension on PDA, (iii) inoculated with the yeast suspension on PDA and active carbon. There were three desiccators (replicates) for each treatment. For the (i) treatment, six uncovered dishes with uninoculated PDA were placed at the bottom of each desiccator. For the (ii) treatment, six uncovered dishes with culture of *H. uvarum* were placed at the bottom of each desiccator. For the (iii) treatment, six uncovered dishes with the PDA cultures of *H. uvarum* and 50 g of active carbon were placed at the bottom of each desiccator. The desiccators were individually covered and incubated at 25 °C (RH90%–95%) for 6 days. The decay index and weight loss were determined after incubation. The experiment was repeated twice with three replicates per treatment (30 fruits/treatment).

2.5.2. Effects of VOCs on postharvest quality of strawberry fruit during cold storage

The bioassay was done in closed glass desiccators (9 cm by 27 cm, down diameter by up diameter). Aliquots of the yeast cell suspension of *H. uvarum* (1×10^9 CFU mL^{-1}) were pipetted and plated on PDA in petri dishes at 500 μL of yeast cell suspension per dish. Control: no inoculated yeast suspension on PDA; Treatment: inoculated yeast suspensions on PDA. Seventy five of unwounded strawberry fruits were then placed on the perforated ceramic clapboard above the eight uncovered dishes containing *H. uvarum* cultures or uncolonized PDA in a desiccator for each treatment. The desiccators were individually covered and incubated at 25 °C (RH90%–95%) for 3 days, Then all groups were taken out from the desiccators and placed at 2 ± 1 °C, RH90%–95%. Postharvest qualities of the fruit were evaluated at intervals of 5 days. Weight loss was determined before treatment and after storage, and the weight loss was calculated as (A-B)/A × 100%, A: weight before treatment, B: weight after storage. Commodity rate was calculated as (A + B)/C × 100%, where A was the number of no decay, B was the number of very slight decay that covering <10% of the fruit surface and C was the total number of fruit. The testing methods of decay index, firmness, pH and total soluble solids (TSS) were same with Cai et al., 2015. The experiment was repeated twice with three replicates per treatment (75 fruits/treatment).

2.6. Statistical analysis

All statistical analyses were performed in the SAS Software (Version8.2; SAS Institute, Cary, NC, USA). The data were analyzed by one-way analysis of variance (ANOVA). Comparison of means was performed by Duncan's multiple range tests. Statistical significance was assessed at the level of $P \leq 0.05$.

3. Results

3.1. Identification of VOCs from H.uvarum on strawberry with/without B.cinerea

A total of 28 volatile organic compounds were detected from *H. uvarum* on strawberry with/without *B. cinerea*, excluding the VOCs of strawberry (SI and RSI ≥ 800) (Table 1). Among these VOCs, 15 VOCs were detected in both conditions, 4 VOCs were *H. uvarum* and strawberry without *B. cinerea* and the other 9 VOCs were only detected when *B. cinerea* was inoculated. These volatiles were classified into alcohols, esters, organic acids, alkenes, ketones and aldehydes (Table 1). In the presence of *B. cinerea*, the relative peak areas of ethyl acetate and 1,3,5,7-cyclooctatetraene was higher than that of *H. uvarum* alone, approximately 1.6- and 17.4-fold (Table 1 and Fig. 1A&B).

Through analyzing the composition of *H. uvarum* VOCs, we detected several VOCs which have been demonstrated inhibiting fungal growth (specific annotation in Table 1). Therefore, we tested the antifungal activity of *H. uvarum* via VOCs production *in vitro* and *in vivo*.

3.2. Effect of H.uvarum VOCs against B.cinerea

Growth of *B. cinerea* was monitored and mycelial length was quantified daily for 5 days. The *in vitro* assay showed that *H. uvarum* was able to inhibit growth of *B. cinerea* via production of VOCs (Fig. 2A). A significant difference ($P < 0.05$) in the fungal hyphal diameter of *B. cinerea* was observed between the *H. uvarum* treatment and the control (Fig. 2B). *H. uvarum* affected *B. cinerea* development via VOCs production as well. In control *B. cinerea* started sporulating 3 days after inoculation, however, sporulation was not observed in the *H. uvarum* treatment (Fig. 2A). Futhermore, microscopy analysis revealed differences in hypha morphology of *B. cinerea* between *H. uvarum* treatment and control. The hypha of *B. cinerea* in control showed a thick morphology with a disperse surface. However, the stunted tips and morphological abnormalities on hypha of *B. cinerea* were observed in the *H. uvarum* treatment (Fig. 2C). These results suggested that the VOCs had a significant inhibitory effect on *B. cinerea* growth.

3.3. Effects of H. uvarum VOCs on control of gray mold in strawberry fruit at 25 °C

After incubation at 25 °C, RH90%–95% for 6 days, all strawberry fruits in the control treatment (uncolonized PDA) showed soft rot and gray mold symptoms, with the highest decay index and weight loss (Fig. 3). However, in the presence of the fumigation of *H. uvarum* VOCs increasing from 1st day to 3rd day, the decay index of strawberry decreased from 0.71 to 0.29 and the weight loss decreased from 2.45 to 0.73, which were significantly lower than those of the control fruit ($P < 0.05$) (Fig. 3).

The *H. uvarum* VOCs suppressed significantly decay index and weight loss of strawberry (Fig. 4). In contrast, the suppressive effect of the *H. uvarum* VOCs and active carbon treatment was greatly nullified by active carbon. The decay index reached 0.59 (same with the control) and weight loss reached 4.99% (significantly higher than the control) in this treatment. These values were significantly higher than those in the *H. uvarum* treatment ($P < 0.05$) (Fig. 4).

3.4. Effects of H. uvarum VOCs on postharvest quality of strawberry fruit during cold storage

The effects of *H. uvarum* via VOCs on strawberry fruit decay index, commodity index, weight loss, firmness, total soluble solid and pH were determined (Fig. 5). Results showed that the decay index of strawberry treated by the *H. uvarum* treatment was 0.65, which was significantly ($P < 0.05$) lower than that of the control (Fig. 5A) on the 25th day. Meanwhile, the commodity rate of strawberries treated by *H. uvarum* treatment was 33.3%, which was significantly ($P < 0.05$) higher than that of the control (0%) on the 25th day (Fig. 5B). The weight loss of fruit treated by the *H. uvarum*

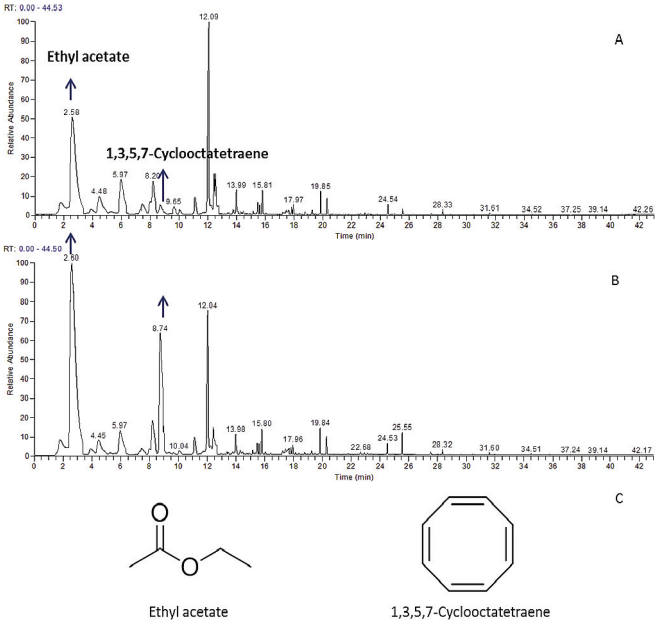

Fig. 1. **(A)** Total ion chromatogram of VOCs produced by *H. uvarum* on strawberry. **(B)** Total ion chromatogram of VOCs produced by *H. uvarum* on strawberry inoculated with *B. cinerea*. **(C)** The structures of ethyl acetate and 1,3,5,7-cyclooctatetraene.

Table 1
Volatile organic compounds detected from *H.uvarum* on strawberry with/without *B.cinerea*.

Possible compounds	RA (%)[a]		SI/RSI[b]	MW(Da)[c]	Antifungal activity	References
	H. uvarum	*H. uvarum* and *B. cinerea*				
Ethanol	2.01	1.83	892/892	46	✓	Huang et al., 2011
Ethyl acetate	21.78	34.87	900/901	88	✓	Huang et al., 2011
Propanoic acid, ethyl ester	1.06	0.98	832/891	102	✓	Huang et al., 2011
3-Methyl-1-butanol	3.19	1.86	895/892	88	✓	Huang et al., 2011; Singh et al., 2011
1,3,5,7-Cyclooctatetraene	1.27	22.1	898/908	104	✓	Huang et al., 2011
Phenylethyl alcohol	2.14	1.55	944/946	122	✓	Huang et al., 2011
Acetic acid, 2-phenylethyl ester	1.41	1.14	923/923	164	✓	Huang et al., 2011
Butanoic acid, 2-methyl-, ethyl ester	0.19	0.12	867/908	130	✓	Huang et al., 2011; Kudalkar et al., 2012
1-Hexanol	0.81	0.32	894/906	102	※	
Butanoic acid 3-hydroxy-, ethyl ester	0.81	0.54	926/927	132	※	
Benzyl alcohol	0.12	0.16	915/917	108	※	
Hexanoic acid, 3-hydroxy-, ethyl ester	0.09	0.12	909/914	160	※	
Octanoic acid, ethyl ester	0.76	0.57	924/925	172	※	
Benzenepropanol	0.27	0.17	807/901	136	※	
n-Decanoic acid	0.06	0.17	909/913	172	※	
1-Octanol	0.25	–	906/911	130	※	
Acetophenone	0.08	–	888/888	120	※	
Acetic acid, octyl ester	0.07	–	901/904	172	※	
Hexanoic acid, 2-hexenyl ester, (E)-	0.06	–	897/973	198	※	
2-Nonanone	–	0.11	807/879	142	✓	Huang et al., 2011
3-Methyl-1-butanol, acetate	–	0.03	882/907	130	✓	Huang et al., 2011; Zheng et al., 2013
Phenol, 4-ethyl-	–	0.17	840/860	122	✓	Ren et al., 2010
Acetic acid 2-methylpropyl ester	–	0.21	820/820	116	※	
Hexanoic acid	–	0.41	800/800	116	※	
3-Phenyl-2-propenal	–	0.03	839/878	132	※	
Decanoic acid, ethyl ester	–	0.05	914/916	200	※	
3-Phenyl-2-propenoic acid, ethyl ester	–	0.93	907/912	176	※	
Dodecanoic acid	–	0.14	801/801	200	※	

–, Not detected or not in the scope (SI and RSI ≥ 800).
✓, These volatiles have been demonstrated the antifungal activity in other reference according to the above table.
※, These volatiles were detected in this study but not sure the antifungal activity.
[a] RA, relative peak area: the value for a volatile compound represented the percentage of the area of the peak for that volatile compound in the total area of peaks for all the detected volatile compounds.
[b] SI, Similarity index; RSI, Reverse similarity index; the chemical compounds (SI and RSI≥800) were analyzed.
[c] MW, molecular weight.

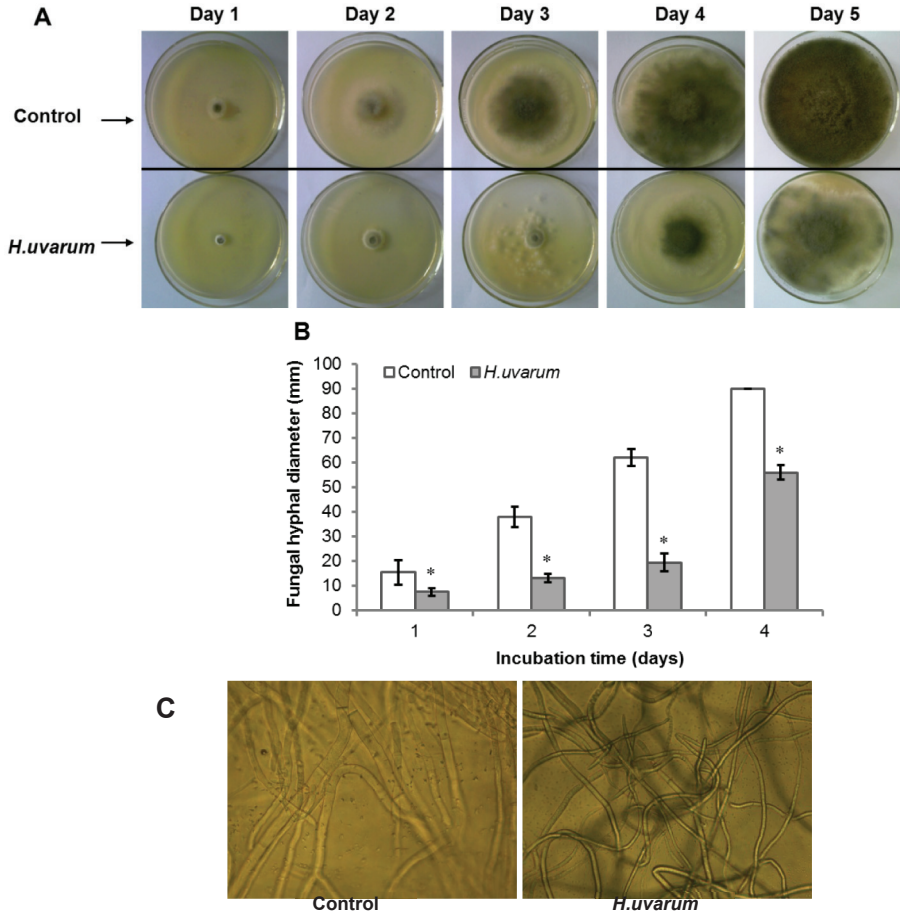

Fig. 2. **(A)** Fungal growth after exposure to *H. uvarum* VOCs. Photographs of all the plates were taken with a fixed distance between the plate and the camera. **(B)** *In vitro* antifungal activity with *H. uvarum* VOCs. Bars represent standard errors of the mean of 3 independent replicates. Asterisks indicate statistical differences compared to control according to Duncan's multiple range test at $P \leq 0.05$ level. **(C)** Microscopic view of *B. cinerea* hyphae exposed to *H. uvarum* VOCs on the 5th day (40 ×).

treatment was lower than that of control significantly ($P < 0.05$) (Fig. 5C). Besides, the treatment of *H. uvarum* maintained greatly the fruit firmness, total soluble solid and pH of strawberry (Fig. 5D–F), which were significantly ($P < 0.05$) higher than that of control on the 25th day.

4. Discussion

In previous research, *H. uvarum* played an important role in biocontrol of gray mold of strawberry by nutrient and space competition, suppression of conidial germination and hyphal growth of *B. cinerea*, hyperparasitism and induction of host defense (Qin et al., 2015; Cai et al., 2015). However, the antagonistic mechanisms of *H. uvarum* were not fully understood. In this study we focused on antifungal activity of the secondary metabolites, the volatile organic compounds (VOCs) produced by *H. uvarum*. Currently, most studies on microbial volatiles are performed *in vitro* under nutrient rich conditions (Kai et al., 2009; Weise et al., 2012; Garbeva et al., 2014) and may not represent the conditions that prevail in the microbial environment. So in this work we studied the *H. uvarum* VOCs on strawberry. Furthermore, to study the antifungal activity of *H. uvarum* VOCs, we inoculated the *B. cinerea* on the wound strawberry for further research.

All VOCs produced by microorganisms could generally be chemically grouped into alcohols, esters, alkanes, alkenes, alkynes, organic acids, ketones, terpenoids, aldehydes and sulfur compounds (Corcuff et al., 2011; Wan et al., 2008). In this study, 28 volatiles were classified into alcohols, esters, organic acids, alkenes, ketones and aldehydes, which were in accordance with the above categories. We just presented the volatiles in every group (SI and RSI ≥800), and deducted the components of control. The rest VOCs were shown in Table 1. However, some researchers demonstrated that VOCs such as ethyl acetate; 3-methyl-1-butanol, acetate and hexanoic acid were detected in strawberries (Pelayo et al., 2003; Kim et al., 2013). Similarly, in our study, these volatiles were also detected in control (strawberries), but which were not in the scope (SI and RSI ≥800). Thus, these volatiles were not included in control when we analyzed the results. VOCs from strawberry inoculated *B. cinerea* alone were also detected in this work (data not shown), we found some volatiles such as 2-undecenal; 2-undecanone; 2-decenal, (Z)-2-heptenal, (Z)-; 2H-pyran-2-one, tetrahydro-4-hydroxy-4-methyl-; 2,4-decadienal; benzene, 4-ethenyl-1,2-dimethyl- were not in other groups. Meanwhile, Ethyl acetate and 1,3,5,7-cyclooctatetraene were not detected in the strawberry inoculated with *B. cinerea* alone without *H. uvarum* (SI and RSI ≥800). The VOCs assay on strawberry was quite complex due to the interaction between strawberry and fungi. Some volatiles may be from yeast or strawberry induced by yeast and pathogen. In our previous work, volatiles from *H. uvarum* and *B. cinerea* on pure PDB cultures were detected. The results showed that *H. uvarum* VOCs including ethanol; ethyl acetate; propanoic acid, ethyl ester; 3-methyl-1-butanol, 1-pentanol; 3-methyl-1-butanol, acetate;

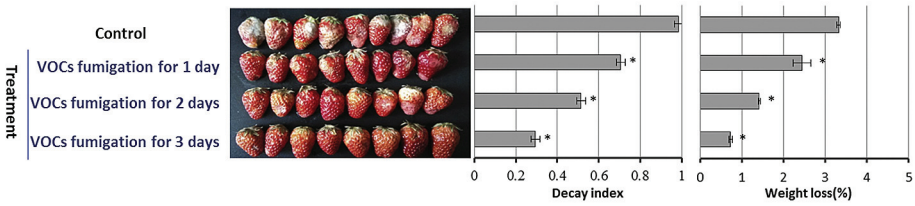

Fig. 3. Effect of the *H. uvarum* VOCs treatment on gray mold of strawberry. All groups were stored at 25 °C after fumigation. Decay index and weight loss were determined on the 6th day. Bars represent standard errors of the mean of 3 independent replicates. Asterisks indicate statistical differences compared to control according to Duncan's multiple range test at $P \leq 0.05$ level.

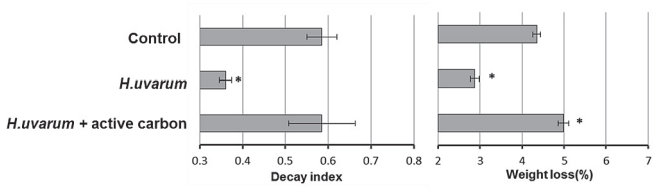

Fig. 4. Effect of the volatiles of *H. uvarum* plus activated carbon on decay index and weight loss of strawberry stored at 25 °C for 6 days. Bars represent standard errors of the mean of 3 independent replicates. Asterisks indicate statistical differences compared to control according to Duncan's multiple range test at $P \leq 0.05$ level.

1,3,5,7-cyclooctatetraene; phenylethyl alcohol; acetic acid; 2-methyl-1propanol; acetic acid, 2-methylpropyl ester; acetic acid, pentyl ester; octanoic acid; octanoic acid, ethyl ester; propanoic acid, 2-methyl; hexanoic acid were detected. *B. cinerea* VOCs including 2-methyl-1-butanol; butanoic acid, 2-methyl; 3-methyl-1-butanol, acetate; hexanoic acid, methyl ester; benzaldehyde; benzene, 1,4-dichloro-; benzeneacetaldehyde; 1-undecene; 2-nonanone; 2-nonanol were detected.

According to the references, some volatiles in Table 1 have been demonstrated antifungal activity, such as ethanol; ethyl acetate; 3-methyl-1-butanol; 1,3,5,7-cyclooctatetraene; phenylethyl alcohol; propanoic acid, ethyl ester; acetic acid, 2-phenylethyl ester; butanoic acid, ethyl ester; 2-nonanone; 3-methyl-1-butanol, acetate and phenol, 4-ethyl- (Table 1) (Huang et al., 2011; Singh et al., 2011; Kudalkar et al., 2012; Ren et al., 2010; Garbeva and De Boer, 2009; Garbeva et al., 2014; Chen et al., 2008; Druvefors et al., 2005; Stinson et al., 2003). Among these compounds, 1,3,5,7-cyclooctatetraene, an antifungal compound (Huang et al., 2011; Stinson et al., 2003), which was not detected in strawberry, was enhanced by the presence of *B. cinerea* as relative peak area increased to 22.1%. This indicated that volatile compound was

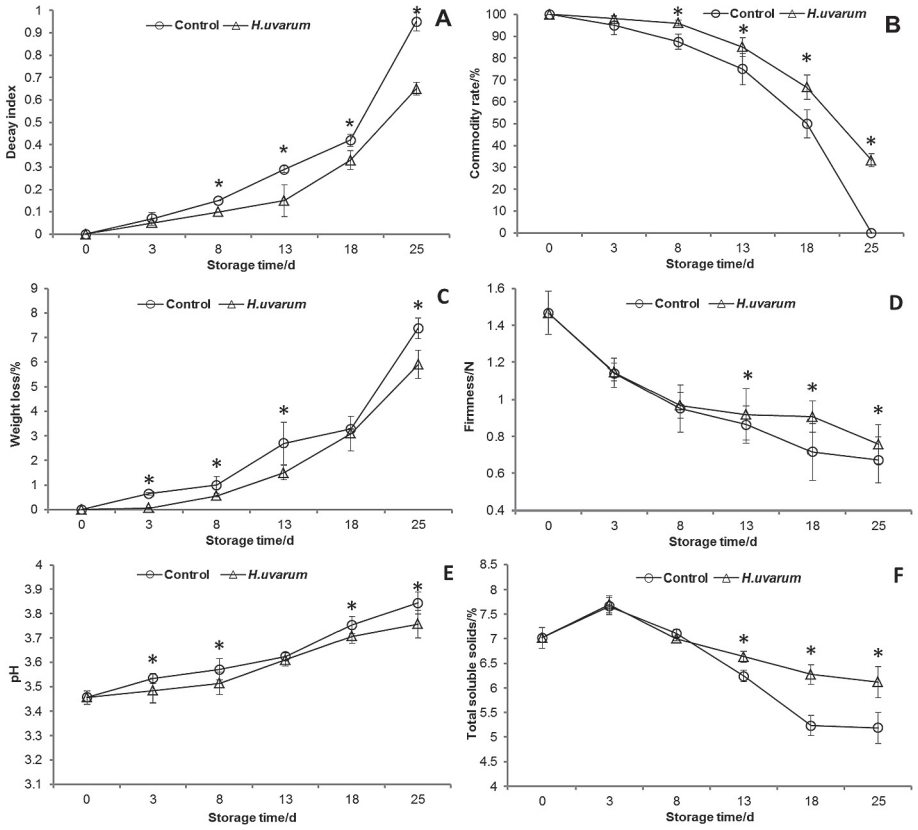

Fig. 5. Effect of fumigation with *H. uvarum* VOCs on Decay index (A), Commodity rate (B), Weight loss (C), Firmness (D), pH (E) and TSS (F) of strawberry during cold storage. Bars represent standard errors of the mean of 3 independent replicates. Asterisks indicate statistical differences compared to control according to Duncan's multiple range test at $P \leq 0.05$ level.

induced by *B. cinerea*. Some antifungal volatile compounds such as 2-nonanone, 3-methyl-1-butanol, acetate and phenol, 4-ethyl- were detected in the presence of *B. cinerea* on strawberry. However, other volatiles detected in this study were needed to determine further for their antifungal activity by pure standard substance. Overall, We could combine with the previous work and indicate that *H. uvarum* produced some antifungal volatiles such as ethanol; propanoic acid, ethyl ester; 1,3,5,7-cyclooctatetraene and phenylethyl alcohol on pure culture and strawberries. How do the *H. uvarum* VOCs inhibit the mycelial growth and spore germination of *B. cinerea*? It is likely that different volatiles occur synergistically among the antagonist, pathogen and fruit.

In vitro, *B. cinerea* grew the whole PDA plate almost on the 4th day and completely on the 5th day in the control. However, the mycelial growth and spore germination of *B. cinerea* were inhibited in the *H. uvarum* treatment. Furthermore, the antifungal effect of volatiles gradually increased with time (days) and the greatest inhibition (to 68.55%) of mycelial growth of *B. cinerea* was observed on the 3rd day (data not shown). Under optical light microscope, volatiles induced stunted tips and morphological abnormalities on the conidia of *B. cinerea*. This similar result was also found in other researches. For example, Using transmission electron microscopy, Li et al. (2012) revealed that fumigated and untreated *B. cinerea* showed excessive vesiculation or thickened cell walls in exposed conidia and increased vesiculation or strong retraction of plasma membrane in exposed hyphae. Bruce et al. (2003) showed that volatiles from bacteria and yeast inhibited pigment production by sapstain fungi. Thus, these results provided a better understanding of the volatiles' mode of action.

In order to verify the effect of *H. uvarum* VOCs on *B. cinerea* growth, spore suspensions (1×10^6 spore·mL^{-1}) were prepared from volatiles treatment and control on the 5th day and then inoculated in PDB. The results showed that there was no significant difference in spore production and germ tube length of *B. cinerea* between the control and the treatment in fresh PDB (data not shown). Thus, we speculated that *H. uvarum* only inhibited the mycelial growth and spore germination of *B. cinerea* via VOCs production, *B. cinerea* renewed growth when transferred to fresh PDB culture.

In vivo, our results obtained that the infection process of *B. cinerea* on strawberry fruit was suppressed in the presence of volatiles. The volatiles mainly affected the early stages of the infection process by inhibiting conidial germination and mycelial growth, which were verified the results *in vitro*. Results of different fumigation time showed that the longer the time of fumigation, the greater the inhibitory activity against the *B. cinerea*. The reason maybe the longer fumigation time produced more antifungal volatile substances. Active carbon was mainly used to absorb gas. In order to verify the function of volatiles, we designed active carbon combined with *H. uvarum* as another control group. The results showed decay index of strawberry in additional active carbon and control group were significantly ($P < 0.05$) higher than that of *H. uvarum* treatment. However, the weight loss of strawberry in additional active carbon were significantly ($P < 0.05$) higher than that of other groups, including control. This phenomenon suggested that *H. uvarum* could inhibit significantly *B. cinerea* infection of strawberry via VOCs production. Meanwhile, we speculated that the PDA cultures may produce some volatiles which inhibit *B. cinerea* growth slightly.

Some studies showed microbial VOCs display versatile functions: they inhibited bacterial and fungal growth, promote or inhibit plant growth, trigger plant resistance and attract other micro- and macro-organisms (Hagai et al., 2014; Schmidt et al., 2015). In the present study, the main quality parameters including firmness, total soluble solids, pH, weight loss, decay index and commodity of strawberry fruit were assayed. It was found that *H. uvarum* VOCs treatment maintained fruit weight loss, firmness, TSS and pH, and improved the commodity rate during cold storage. This also indicated that *H. uvarum* VOCs not only inhibited the *B. cinerea* growth, but decreased the fruit rot and prolonged the storage time.

In conclusion, the current work demonstrated that the volatiles from *H. uvarum* could inhibit *B. cinerea in vitro* and on strawberry fruit, and could potentially be an effective alternative for the control of postharvest diseases by fumigant action. Further studies are needed on improving the production of VOCs from *H. uvarum* and testing the inhibitory effect on other pathogens.

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Notes

The authors declare no competing financial interest.

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