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Identification of volatile organic compounds for the biocontrol of postharvest litchi fruit pathogen *Peronophythora litchii*

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ABSTRACT

Litchi is an important economic fruit in subtropical countries. The litchi downy blight (LDB) caused by the oomycete *Peronophythora litchii* severely affects the production and quality of litchi fruit, and is widespread in almost all litchi production regions of China. Therefore, there is an urgent need for effective and sustainable control strategies against LDB. Our previous study showed that *Bacillus amyloliquefaciens* LI24 and PP19, *B. licheniformis* HS10, *B. pumilus* PI26, and *Exiguobacterium acetylicum* SI17 are promising biocontrol agents (BCAs) in controlling LDB, and their volatile organic compounds (VOCs) could inhibit the growth of *P. litchii in vitro*. In this study, we found that pre-exposure of litchi fruit to VOCs produced by PP19, SI17 and PI26 can significantly reduce the severity of LDB during 36 h to 72 h post inoculation. We further analyzed VOCs produced from the three BCAs (*i.e.*, PP19, SI17, PI26) by solid phase microextraction gas chromatography-mass spectrometry (SPME-GC–MS), and found that their chemical compositions varied substantially over incubation time and between BCAs. In total, 70, 98, 101 chemicals were detected in PP19, SI17, PI26 from 24 to 72 h of incubation, respectively; 17 of them were commonly produced at more than one time points by PP19, and 11 were selected for further study. Two of the compounds 1-(2-Aminophenyl)ethanone (EA) and Benzothiazole (BTH) showed inhibitory activity against both *P. litchii* on plates and LDB on litchi fruit when the compounds were directly applied, while another compound α-Farnesene (AF) was able to suppress LDB *in vivo*, but did not exhibit antagonistic activity against the pathogen *in vitro*, suggesting that it may act through induction of host defense mechanisms. Our results showed that the bacterial VOCs and compounds of BTH or AF could be promising for the control of LDB on harvested litchi fruit.

1. Introduction

Litchi (*Litchi chinensis* Sonn.) is one of the most popular and delicious subtropical fruit. It is an important economic plant in the southern provinces of China, including Fujian, Guangdong, Guangxi, and Hainan, and is also grown as commercial crops in subtropical Asia, South Africa, Australia, Hawaii and Israel (Jiang et al., 2001). However, litchi fruit are highly perishable and suffer from postharvest diseases caused by various pathogens, including *Peronophythora litchii*, *Geotrichum candidum*, *Colletotrichum gloeosporiodes*. Particularly, litchi downy blight (LDB) caused by *P. litchii*, is the main postharvest disease of litchi, leading to seriously damaged fruit and dramatically shortened shelf-life (Wang et al., 2013). It may alone destroy 20–30% of the litchi fruit per year.

Current methods to control LDB disease mostly rely on physical or chemical strategies. Artificially controlled storage conditions such as temperature management, heat treatments, specialized packaging, and modified atmosphere are widely adopted, but they can only slow down the progression of LDB and usually result in reduction of fruit quality (Jiang et al., 2006). On the other hand, chemical fungicides have high

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efficiencies but would inevitably leave residues on fruit, thus suffering from major health and environmental concerns (Jiang et al., 2006). Therefore, it is necessary and urgent to find alternative methods such as biological control for reducing the decay of harvested litchi fruit, given its safe, environmentally friendly, and sustainable effects, especially meeting the requirements of the global strategic designs of green and/or organic planting agriculture, which represents an in-depth understanding of how today's mainstream green consumers differ markedly from yesterday's fringe activists in attitudes, behaviors, lifestyle, and corporate expectations (Ottman, 2011).

Biological control agents (BCAs) have been shown to be successful in the control of multiple postharvest diseases of fruit and vegetables (*e.g.* botrytis rot caused by *Botrytis cinerea* of strawberry or Rhizopus rot caused by *Rhizopus stolonifer* in postharvest peach fruit). Importantly, the VOCs produced by certain BCAs can also exhibit inhibitory activities against diseases. For instance, Ryu et al. (2004) and Yuan et al. (2012) found that VOCs from *Bacillus* spp. and *P. fluorescens* can effectively reduce crop diseases. Baldwin et al. (2006) reported that volatile organic compounds (VOCs) produced from BCAs could play different regulatory roles in different species, the defensive strategies were common among species. Park et al. (2015) suggested that VOCs from *P. fluorescens* SS101 could promote plant growth and induce systemic resistance. Ryu et al. (2004) reported that VOCs from GB03 (*B. subtilis*) and IN937a (*B. amyloliquefaciens*) could induce systemic resistance by the major component 2,3-butanediol. Zamioudis et al. (2015) indicated that VOCs from WCS417 played a role in promoting seed germination and seedling growth, enhancing resource competition, and inducing defense responses in *Arabidopsis* plants. Meanwhile the chemical components, such as 3-Aminobutanoic acid (BABA), 2,6-dichloroisonicotinic acid (INA) and Benzothiazole (BTH), would induce disease resistance in many different species (Cohen, 2002), which conferred broad-spectrum disease resistance (Zimmerli et al., 2000), often mediate priming defense responses upon challenge (Baldwin et al., 2006); Choi et al (2014) demonstrated that VOCs produced by *B. amyloliquefaciens* IN937a (and 3-pentanol can enhance the resistance to bacterial speck disease of pepper by activating salicylic acid (SA) and jasmonic acid (JA) signaling pathways through priming under field conditions. The research predicted that VOCs or its VOCs compounds played an important role in the ecological environment to plant disease defense.

Previous studies have reported that several bacteria suppressed *P. litchii* and the decay of postharvest litchi fruit, such as *Bacillus subtilis* (Dharini et al., 2008; Jiang et al., 2001; Sivakumar et al., 2007), endophytic bacterial strain *Bacillus amyloliquefaciens* TB2 and LY-1 (Cai et al., 2010; Wu et al., 2017), and *Lactobacillus plantarum* LAB (Martínez-Castellanos et al., 2011). It has been further demonstrated that VOCs produced by certain BCAs could act against *P. litchii*, including *Streptomyces fimicarius* BWL-H1 (Xing et al., 2018) and *Paecilomyces* sp. SC0924 (Xu et al., 2013); the active compounds have also been characterized for the latter two BCAs. However, there is limited information about the actual biocontrol efficacy of VOCs or the active compounds against LDB *in vivo*.

In this study, we evaluated VOCs produced by five BCAs for their efficacies in controlling LDB, examined the chemical compositions of the VOCs, and assessed the biocontrol activities of individual VOC components both *in vivo* (against LDB on litchi fruit) and *in vitro* (against *P. litchii* on plates). The results showed that volatile organic compounds produced by BCAs are promising for the control of the postharvest disease LDB, providing important resources for the future development of LDB biocontrol strategies.

2. Materials and methods

2.1. Microorganisms

The pathogen *P. litchii* SC18 was isolated by the fungus laboratory of Department of Plant Pathology, South China Agricultural University. The fungus was cultured on carrot agar (CA) for 7 days, eluted with sterile water, filtered with four layers of sterile gauze, inoculation concentration was adjusted to 5×10^4 sporangia mL^{-1} observed under the electron microscope.

To identify potential biocontrol agents of LDB, 188 bacterial strains were isolated from different microenvironments around litchi trees, including the phyllosphere and the interior of healthy leaf, fruit pericarp, and fruit sarcocarp, as well as the rhizosphere and surrounding bulk soils associated with healthy plants. In total, five strains achieved efficacy greater than 30% in controlling LDB *in vivo* on litchi fruit in a small greenhouse at laboratory conditions, namely *B. amyloliquefaciens* LI24 (isolated from the interior of litchi leaves) and PP19 (isolated from the phyllosphere or carposphere of litchi fruit pericarp), *B. licheniformis* HS10 (isolated from mixed rhizosphere and surrounding bulk soils of healthy cucumber plants, provided by Jianhua Guo at Nanjing Agricultural University), *B. pumilus* PI26 (isolated from the interior of litchi fruit pericarp), and *E. acetylicum* SI17 (isolated from the interior of litchi fruit sarcocarp). Moreover, the performance of the VOCs produced by the five BCAs in inhibiting the growth of *P. litchii* mycelia on plates were 5%, 8.06%, 7.66% (Fig. 2 in Data in Brief), 14.17% and 6.05%, respectively (Zheng et al., unpublished result). So the five BCAs were selected in the experiments, which were plant promising bacteria in controlling LDB and browning (Zheng et al., unpublished result). The strains were grown in LB broth at 28 °C with 200 r min^{-1} of shaking speed for 24 h. The bacterial cell suspension was adjusted to 5×10^8 colony-forming units (CFU) mL^{-1} with sterile water, whose concentration was determined using a spectrophotometer at 600 nm.

2.2. In vivo test of biocontrol by bacterial VOCs

Freshly harvested, mature and healthy litchi fruit with approximate size and color were used in the experiment. The fruit were placed at equidistant points with a circular pattern in the incubation device, which was mounted upside-down on two 20 cm sterile glass petri dishes (18 cm sterilized filter papers wetted with a fixed amount of sterile water was placed on the bottom of each dish). Bacterial suspension (100 μL) was coated evenly on LB plates (9 cm sterile glass dishes containing 15 mL of LB solid medium), then were placed in the center of the glass petri dishes with no direct contact with the litchis, to ensure the bacterial VOCs to exchange gas in the container (Ton et al., 2005); Sterile water of the same volume replaced the bacteria cultures as the control. At 24 hpt (hours post treatment), removed away the sealing parafilm and the bacterial VOCs (the central 9 cm dishes); the pathogen *P. litchii* was inoculated at 5×10^4 sporangia mL^{-1} after the 24 h long exposure of litchi fruit to bacterial VOCs by spraying 50 mL totally per treatment. It should be noted that the pathogens were inoculated after the exposure of fruit to bacterial VOCs, therefore this test was designed to evaluate the VOCs for their elicitor capacities instead of direct antagonist activities. The 20 cm glass petri dishes were covered with grass-lid and incubated under illumination at 25 °C (in the small greenhouse, with the relative humidity of 85–90 % in the containers while 60–75 % in the air of the lab trial; parameters were monitored by the TH6 automatic humidity and temperature data logge, Hangzhou Meacon Automation Technology Co., Ltd.) and 24 h light cycle. The disease severity was observed from 36 hpi (hours post inoculation) to 96 hpi. Four replications of each treatment were performed with 15 fruit per replicate and the experiment was repeated two times in "Feizixiao" (about 80% ripening degree, a private farm, Huadu district, Guangzhou City, Guangdong Province) or "Huaizhi" (about 85% ripening degree, a private farm, Conghua district, Guangzhou City, Guangdong Province), respectively.

Disease severity was defined as follows: 0, 1, 3, 5, 7, and 9 represent 0, < 5, 6–10, 11–25, 26–50, and > 50% leaf area with symptoms, respectively. Disease index and biocontrol efficacy was calculated as follows: Disease index (%) = [Σ(Disease level × number of fruit in each

level) / (the highest level × total number of fruit)] × 100; Biocontrol efficacy (%) = [(Disease index of control − Disease index of treatment) /Disease index of control] × 100.

2.3. GC–MS analysis of bacterial VOCs

The three bacteria (*i.e.*, *B. amyloliquefaciens* PP19, *B. pumilus* PI26, and *E. acetylicum* SI17) whose VOCs showed inhibitory activities in our *in vivo* evaluation were further analyzed for the chemical compositions of their VOCs. Bacterial suspension (100 µL, 5×10^8 CFU mL^{-1}) was coated evenly on LB in sample vials (15 mL of LB solid medium with was added to sterile tissue culture flasks, with a height of 9.5 cm and a diameter of 5.5 cm); then the flasks were sealed with sterile foil and sealing film to prevent contamination. The volatiles produced by the three bacteria were collected after 24, 36, 48, 60, and 72 h of incubation at 25 °C. The LB medium without bacteria was set up as a control. The bacterial VOCs were collected using advanced headspace solid phase microextraction (SPME) technique (Farag et al., 2013) and analyzed by gas chromatography coupled with mass spectrometry. The bacteria were incubated in water bath at 45 °C for 80 min, and VOCs were extracted by headspace solid phase microextraction (SPME) (Supelco Co., Bellefonte, PA, USA; 50/30 µm DVB/CAR/PDMS, gray) during the last 40 min. After extraction, the SPME device was removed from the sample bottle and inserted into the injection port of the GC–MS system (SHIMADAZU GCMS-QP2010 Ultra). GC–MS was performed according to procedures outlined by Banerjee (2010) under chromatographic conditions described by Raza et al. (2016). The mass spectra of the resulting gas components were compared with those in a GC–MS library (NIST11S) to identify the components of VOCs. The composition of VOCs of the treatment was determined by data analysis in a GC–MS workstation (Software Version SHIMADZU GCMS solution) with LB as control.

2.4. Examination of identified volatile components for in vitro antagonism

Different amounts of 11 individual VOCs (Technical grade, Supplemental Fig. 2B) were determined to test the effect by mycelial growth assays. The volatile components were identified from bacterial VOCs, while BABA, SA and methyl jasmonate (MeJA), were selected as the positive controls from literature (Conrath et al., 2006). The protocol was as follows: a certain amount of each component was added to a certain volume of PDA medium to prepare plates with seven concentration gradients, punched the purified pathogen colonies (Φ_0 = 0.5 cm) from the periphery. Then the two lidless plates were sealed with parafilm. Equivalent amounts of sterile distilled water (mock) or 95% ethanol diluent (control) (the stock solution of each component was dissolved in 95% ethanol) were used as the controls. After incubation at 25 °C for seven days, the colony diameter was measured, and the inhibition rate was calculated. Five replicates were used per treatment and the experiment was repeated two times.

Inhibition ratio (%) = $[(\Phi_{CK} - \Phi_0) - (\Phi_{TREATMENT} - \Phi_0)]/(\Phi_{CK} - \Phi_0) \times 100$. Φ is the inhibition zone diameter; Log concentration (X) and percentage inhibition of colony growth (Y) were calculated from the measured data. Toxicity regression equation (Y = a + bX), EC_{50}, and correlation coefficient (r^2) of each agent against the pathogen were calculated by the least square method.

2.5. Assays to assess the in vivo biocontrol activity of individual chemicals components

Six chemicals were evaluated for their *in vivo* biocontrol efficacies individually on "Huaizhi" fruit (about 85% ripening degree, a private farm, Conghua district, Guangzhou City, Guangdong Province) and leaves (a private farm, Huadu district, Guangzhou City, Guangdong Province). 1-(2-Aminophenyl)ethanone (EA), BTH, SA, and MeJA which showed antagonistic activity in our *in vitro* assay were evaluated at the concentrations of 500, 200, and 100 mg L^{-1}, α-Farnesene (AF) and BABA which did not exhibit *in vitro* antagonistic activity were tested at concentrations of 1000, 500, 100 mg L^{-1}. The corresponding solvent-only dilutions were used as control for each chemical and concentration tested. There were three replicates per treatment, and 30 fruit "Huaizhi" or 5 branches with at least 10–20 leaves per replicate. Fruit or leaves were placed in preservation containers (323 × 220 × 100 mm; Hualong Plastic Factory, Foshan, China). The bottom of the containers was covered with sterile filter papers (D = 18 cm), moistened with sterile water. 300 mL was used for each treatment by spray. After 24 hpt, the pathogen *P. litchii* was inoculated at 5×10^4 sporangia mL^{-1} by spraying. The small greenhouse culture condition and the methods of disease investigation and biocontrol analysis were the same as described in Section 2.2.

2.6. Data analysis

Data on plate antagonism assay, disease index, control efficacy were processed and analyzed in Microsoft Excel. Least significant difference test ($P < 0.05$) was performed using the statistical software data processing system (DPS version 7.05, Zhejiang University, Hangzhou, China). One-way ANOVA was used to compare the factors investigated.

3. Results and discussion

3.1. In vivo suppression of LDB by bacterial biocontrol agents and their VOCs

Here, we first carried out an *in vivo* test to further examine these five bacteria for the effectiveness of their VOCs in controlling LDB. Litchi (cultivar "Huaizhi") fruit were first exposed to VOCs produced by each bacterium for 24 h and then inoculated with *P. litchii*; the disease severity was recorded from 36 hpi to 72 hpi at 12 h interval (Fig. 1A). Our results showed that the development and expansion of fruit brown or white mildews were substantially suppressed upon pre-exposure of fruit to VOCs produced by PP19, SI17, PI26 (Fig. 1B and C). Notably, VOCs produced by SI17 resulted in significantly lower disease indices and the highest biocontrol efficacies across all four time points. VOCs produced by PP19 and PI26 also performed well in the test, resulting in significantly reduced disease severity at the first two and three time points, respectively. Overall, VOCs produced by the three bacteria achieved relatively high average efficacies (SI17: 42.53%; PP19: 34.19%; PI26: 32.04%), particularly at the first time point (36 hpi) where the efficacies of all three VOCs were greater than 50%. In contrast, pre-exposure to the VOCs produced by HS10 and LI24 only slightly reduced the disease severity, yet the differences were not significant at any time point (Fig. 1B and C). We performed the same *in vivo* test on the fruit of a different litchi cultivar ("Feizixiao") and observed the same patterns (Supplemental Fig. 1).

The use of VOCs produced by BCAs has long been recognized as an effective strategy in the biocontrol of postharvest diseases (Fiddaman and Rossall, 1993). Some of the known examples include VOCs produced by *B. amyloliquefaciens* CPA-8 against fruit pathogen *M. fructicola* of cherry (Gotor-Vila et al., 2017); or produced by two antagonistic strains of *B. pumilus* and *B. thuringiensis* on the anthracnose pathogen in postharvest mangos (Zheng et al., 2013); or from *B. amyloliquefaciens* PPCB004 on postharvest decay in citrus by *Penicillium crustosum* (Eva et al., 2010). VOCs can act directly on pathogens to inhibit their growth, or as signals to indirectly induce plant defense responses (Heil and Ton, 2008). Ryu et al. (2004) found that VOCs from GB03 (*B. subtilis*) and IN937a (*B. amyloliquefaciens*) induced systemic resistance in plants and were effective in controlling *Erwinia carotovora* subsp. carotovora. Furthermore, VOCs might promote the recruitment and establishment of microbial communities around host plants, which in turn facilitate the disease defense process. Yi et al. (2016) reported pretreatment of pepper roots with *B. subtilis* and its product, 2,3-

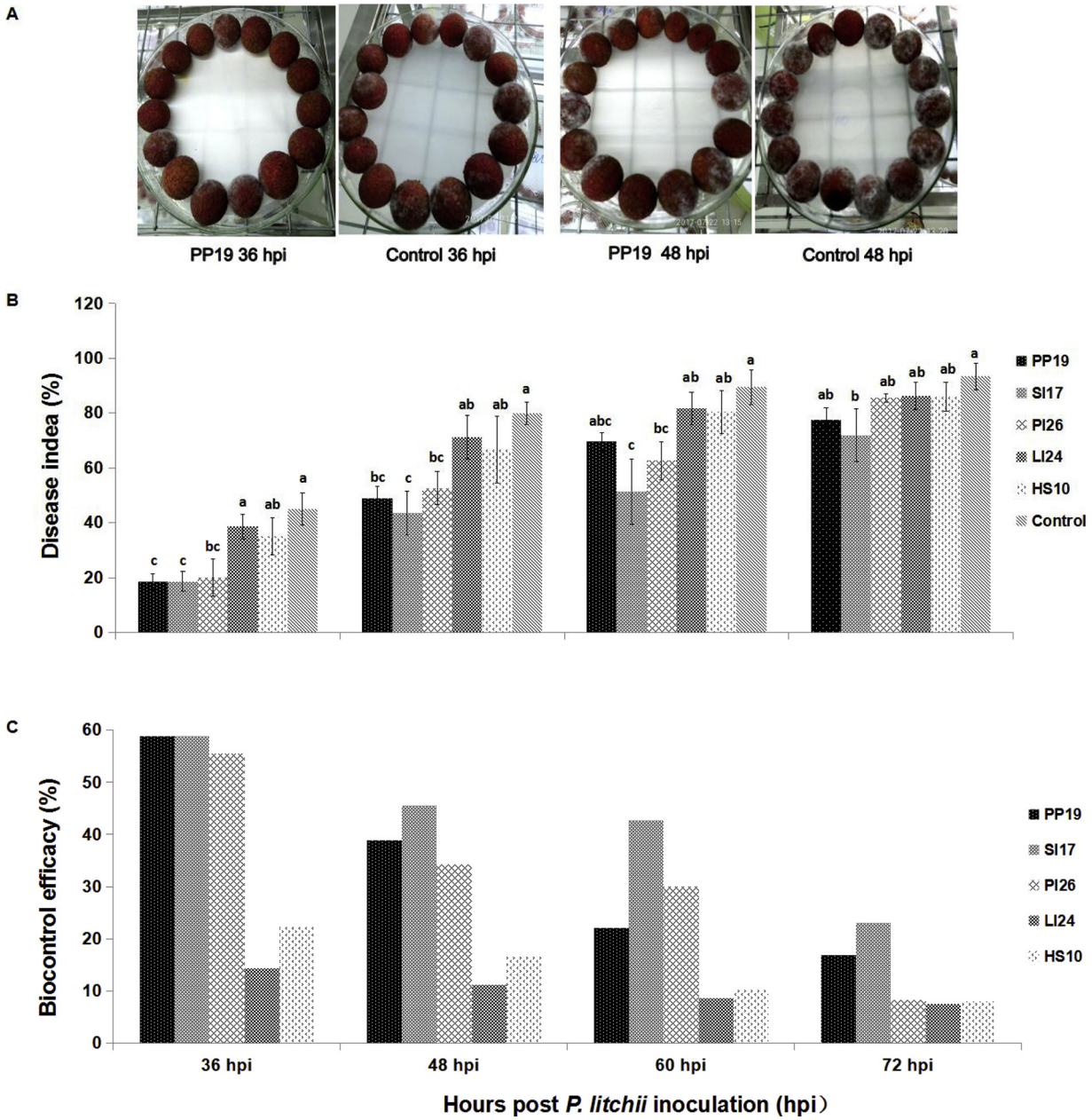

Fig. 1. Suppression of litchi downy blight (LDB) by five bacterial VOCs exposed 24 h to *in vivo* fruit "Huaizhi" in Guangzhou of 2017. Comparison between bacteria natural volatile organic compounds (VOCs) of PP19 and the control in disease index at 36–48 hpi (A); Disease index of litchi downy blight LDB treated with the bacterial VOCs produced by PP19, SI17, PI26, LI24, HS10 and the control LB media (**B**) and Biocontrol efficacy (**C**). Bacterial (5×10^8 CFU mL^{-1}, 100 μL) VOCs were elicited to fruit "Huaizhi" (about 85% ripening degree) in the small greenhouse of the airtight glass petri dish, 24 hpt (hours post treatment) the sealed parafilm was taken out with an open space, then the suspension of *P. litchii* at 5×10^4 sporangium mL^{-1} was sprayed onto the fruit each. Data are presented as means of four replicates ± standard errors; different letters indicate significant differences between treatments according to LSD test at $P < 0.05$.

butanediol, followed by inoculation with the pathogen *Ralstonia solanacearum*, induced expression of PR-related genes. It was speculated that VOCs from bacteria helped the bacteria colonize the rhizosphere, and acted as a mobile signal of inducing effective resistance. In our study, the biocontrol efficacies displayed by the VOCs produced by PP19, SI17 and PI26 may be attributed to one or more of the above-mentioned mechanisms, which requires further investigations to elucidate. On the contrary, the SI17 were effective against LDB (Zheng et al., in preparation), but did not show *in vitro* inhibitory activities against *P. litchii* on plates; the result suggest that the VOCs produced by SI17 might indeed act as signals for inducing plant disease defense or the colonization of microbial communities.

3.2. Identification of bacterial VOCs blends

The chemical compositions of VOC blends produced by *B. amyloliquefaciens* PP19, *B. pumilus* PI26, and *E. acetylicum* SI17 at 24, 36, 48, 60, and 72 h incubation times were characterized using GC–MS. In total, 101, 70, and 98 compounds were detected from the VOCs produced by PP19, PI26, and SI17 respectively (Fig. 1A in Data in Brief). The compositions of VOCs produced by each individual bacterium changed rapidly over time. For instance, for PP19, a total of 9, 33, 14, 28, and 17 compounds were detected at each of the 5 time points, respectively (Fig. 1A in Data in Brief), but only 2 compounds (*i.e.*, 2-Nonanone and 6-Methyl-2-heptanone) were common to all time points (Table 2 in Data in Brief). Similar patterns were observed for the other

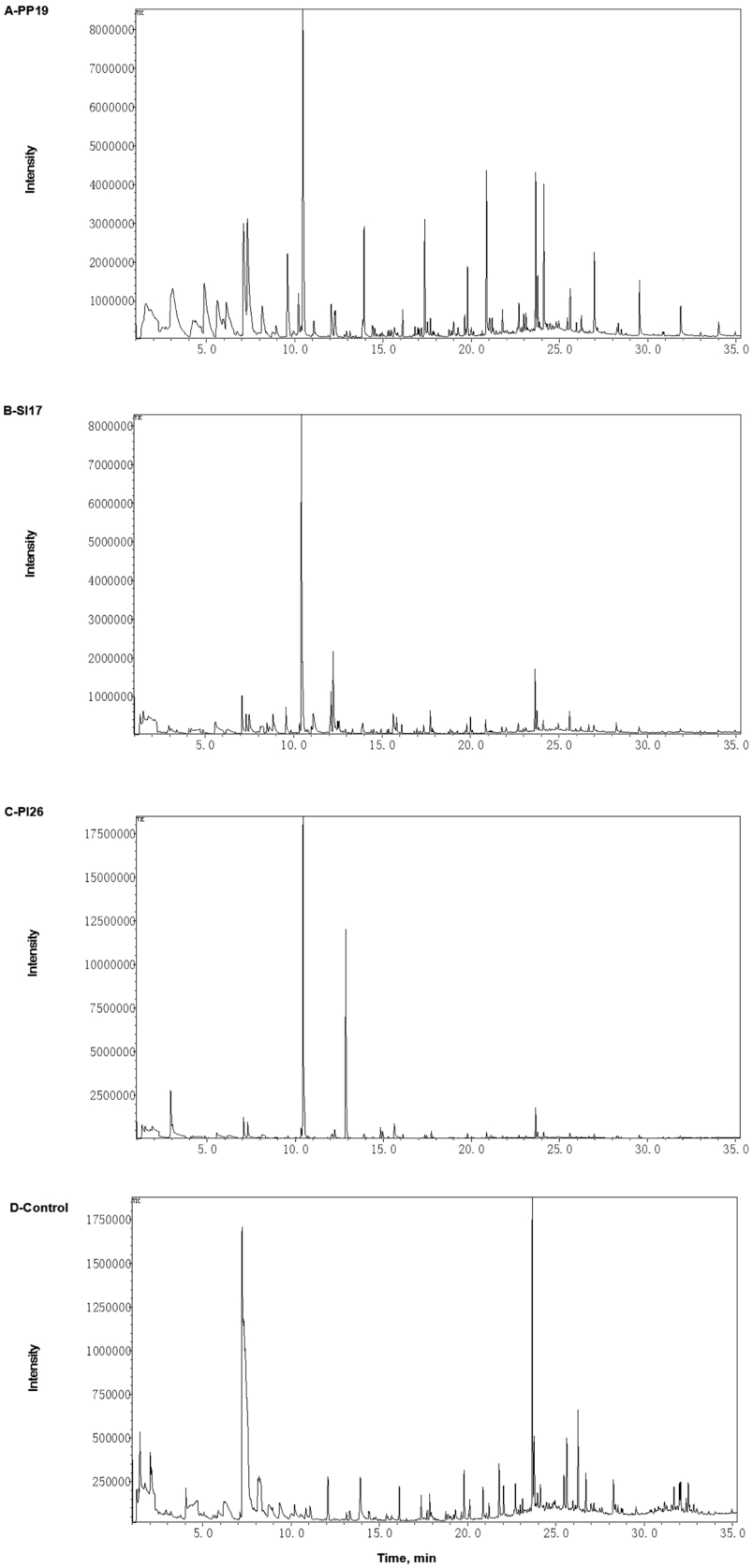

Fig. 2. GC–MS (Gas Chromatography-Mass Spectrometer) profiles of volatiles released on solid culture condition for 36 h at 28 °C. The VOCs from the isolate of PP19 (*B. amyloliquefaciens*) (**A**), SI17 (*E. acetylicum*) (**B**), PI26 (*B. pumilus*) (**C**) and uninoculated media (**D**). The BTH, EA and AF stand for Benzothiazole, 1-(2-Aminophenyl)ethanone, α-Farnesene, respecitively.

Table 1
In vitro inhibitory activity of individual VOC component against the pathogen *P. litchii*.

Tested VOCs composition[a]	Effective component/Dosage	CAS	Manufacturer	Concentration (mg L^{-1})	Toxicity regression equation	Correlation (r^2)	EC50 (mg L^{-1})
2,5-Dimethylpyrazine ($C_6H_8N_2$)	98% Liquid	123-32-0	Aladdin	2000; 1000; 667; 500; 400; 333; 286	y = 0.9159 + 1.4221x	0.8322	744.4996
Bicyclo [4.2.0] octa-1,3,5-triene (C_8H_8)[b]	Liquid	694-87-1	Aladdin	1000; 667; 500; 400; 333; 286; 250	y = 1.0446 + 1.0574x	0.6019	—
1-(2-Aminophenyl)ethanone (C_8H_9NO)	97% Liquid	551-93-9	Aladdin	1000; 500; 333; 250; 200; 167; 142	y=-7.3196 + 5.5215x	0.9489	170.2916
2-Undecanone ($C_{11}H_{22}O$)	99% Liquid	112-12-9	Aladdin	2000; 1000; 667; 500; 400; 333; 286	y = 0.0709 + 1.7095x	0.8785	764.3307
Benzothiazole (C_7H_5NS)	Liquid	95-16-9	Aladdin	500; 333; 250; 200; 167; 142; 125	y=-11.2053 + 7.4833x	0.9119	146.3947
Pentadecane ($C_{15}H_{32}$)	98%Liquid	629-62-9	Aladdin	2000; 1000; 667; 500; 400; 333; 286	y=-0.5862 + 0.4462x	0.0771	—
2-Ethylhexan-1-ol ($C_8H_{18}O$)	Liquid	106-67-2	Aladdin	2000; 1000; 667; 500; 400; 333; 286	y = 2.1580 + 1.2601x	0.8534	180.0630
2-Nonanone ($C_9H_{18}O$)	99% Liquid	821-55-6	Aladdin	2000; 1000; 667; 500; 400; 333; 286	y=-5.1826 + 2.3843x	0.3892	—
α-Farnesene($C_{15}H_{24}$)	Liquid	502-61-4	Sigma-aldrich	2000; 1000; 667; 500; 400; 333; 286	y=-2.5586 + 2.3004x	0.6973	—
1-Tridecene ($C_{13}H_{26}$)	Liquid	2437-56-1	Yuanye	2000; 1000; 667; 500; 400; 333; 286	y = 3.7550-0.1140x	−0.0870	—
6-Methyl-2-heptanone ($C_8H_{16}O$)	0.82 sg	928-68-7	TCI	2000; 100; 667; 500; 400; 333; 286	y=-8.3376 + 3.7480x	0.5733	—
3-Aminobutanoic acid ($C_4H_9NO_2$)	97% wp	541-48-0	Aladdin	2000; 1000; 667; 500; 400; 333; 286	y = 2.2635 + 0.5573x	0.2544	—
Salicylic acid ($C_7H_6O_3$)	99.5% wp	69-72-7	AR	500; 333; 250; 200; 167; 142; 125	y=-14.7277 + 8.7857x	0.9341	175.9676
Methyl jasmonate ($C_{13}H_{20}O_3$)	98% sg	39924-52-2	Sigma-aldrich	500; 333; 250; 200; 167; 142; 125	y = 0.1595 + 2.0901x	0.8190	206.9557

[a] Bacterial VOCs composition were tested in the lab of the glass petri dish. Toxicity regression equation, Correlation and EC_{50} were calculated by the DPS soft, significant differences between treatments according to LSD test at $P < 0.05$.
[b] VOCs composition printed in bold type were selected for the no inhibition in the petri dish.

two bacteria (Table 2 in Data in Brief). Notably, 17 compounds were detected in the VOCs produced by *B. amyloliquefaciens* PP19 (which was found to be a potent agent for many postharvest diseases, and also showed the best performance in our biocontrol assay) at more than one time points (Fig. 2; Table 2 in Data in Brief). Moreover, the most components of the VOCs produced by *B. pumilus* PI26 and *E. acetylicum* SI17 were also found in this set of 17 compounds (Fig. 2; Tables1 and 2 in Data in Brief).

Eleven of the 17 compounds were commercially available and thus selected for further analysis, including 2,5-Dimethylpyrazine ($C_6H_8N_2$); Bicyclo[4.2.0]octa-1,3,5-triene (C_8H_8); 1-(2-Aminophenyl)ethanone (C_8H_9NO); 2-Undecanone ($C_{11}H_{22}O$); Benzothiazole (C_7H_5NS); Pentadecane ($C_{15}H_{32}$); 2-ethylhexan-1-ol ($C_8H_{18}O$); 2-Nonanone ($C_9H_{18}O$); α-Farnesene ($C_{15}H_{24}$); 1-Tridecene ($C_{13}H_{26}$); 6-Methyl-2-heptanone ($C_8H_{16}O$) (Table 1, Supplemental Fig. 2B); they belong to the categories of ketones, alcohols, hydrocarbons, benzene, or alkene. On the other hand, while the bacterial VOC blends differed considerably in their compositions, some compounds were commonly produced by all five bacteria, such as 1-(2-Aminophenyl)ethanone; 2-Nonanone; α-Farnesene; 1-Tridecene; 6-Methyl-2-heptanone; 5-Methyl-2-heptanone; 2-Dodecanone (Tables 2 and 3 in Data in Brief).

In previous research, the VOCs produced by microorganisms could generally be chemically grouped into esters, alcohols, alkenes, alkanes, alkynes, organic acids, ketones, terpenoids, aldehydes and disulfides (Fernando et al., 2005; Wan et al., 2008). Raza et al (2016) reported that 2-nonanone, 2-decanone, Tridecane, Hexadecanoic acid could be identified from VOCs produced by *B. amyloliquefaciens* SQR-9 on the modified MS medium, which was also be found in PP19 on LB medium with the same GC—MS condition. Asari et al (2016) found that it also can produce methylpyrazine, 2,5-dimethylpyrazine and hexanol at relatively high abundances, which were also detected in our GC–MS analysis. These two compounds may contribute to the negative effect on plants observed since pyrazine derivatives are known herbicides causing chlorosis to weeds (Doležal and Kráľová, 2011). Therefore, we discarded the two compounds in our subsequent analyses.

Gotor-Vila et al. (2017) examined VOCs produced by CPA-8 after 24 and 48 h of bacteria incubation, and did not find any substantial difference in the compositions and abundances of the VOCs at different incubation times, which is drastically different from the pattern observed in our study (Supplemental Table 2). The highly variable VOC compositions revealed by our GC–MS analyses (Fig. 2; Supplemental Tables 1, 2) were consistent with the results of Francesco et al. (2016) on yeasts where different VOCs were observed starting from 48 h of yeast incubation. Miyazawa et al. (2008) also found the production of VOCs depended on the growth progress of the culture time.

In the progress of *in vivo* co-culture of bacteria and fruit without being in physical contact, biofilms formed on the LB medium, which was the mainly form of most bacterial (Costerton et al., 1995). It remains to be seen whether it can affect the production of VOCs. In this study, the composition of VOCs varied over time for the same strain, with the highest numbers of components observed at the earliest three time points (*i.e.*, 24, 36, and 48 h). It is possible that the variations in VOC composition over time observed here may be related to the presence of biofilms. Furthermore, the difference in control efficacy between exposure time may also be related to the formation of biofilms, as biofilms can improve the colonizing capacity of rhizobacteria and thus increase the control efficacy (Chen et al., 2013).

3.3. Effect of identified volatile compounds on the mycelia growth in vitro and in vivo in the litchi fruit or leaves

We tested the aforementioned 11 components detected more than once by GC–MS for their capacities in inhibiting the growth of *P. litchii* mycelia. Each compound was tested at seven different concentrations. In addition, three previously reported compounds of BABA, SA, and MeJA were included as well (Table 1 and Fig. 1B in Data in Brief), as

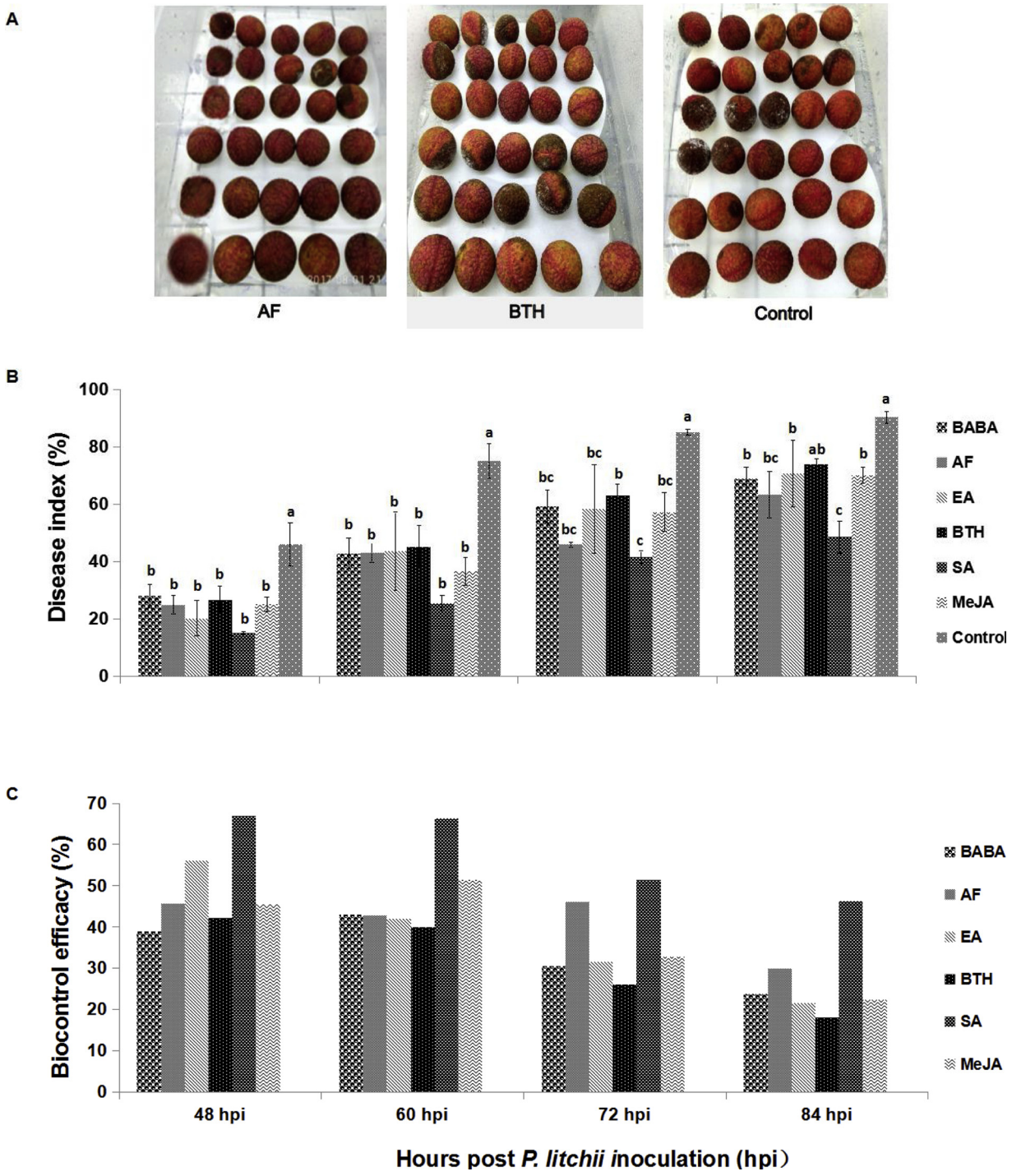

Fig. 3. Suppression of LDB by the VOCs blends of AF (α-Farnesene), BTH (Benzothiazole), EA (1-(2-Aminophenyl)ethanone), the positive control of SA (Salicylic acid), MeJA (Methyl jasmonate), BABA (3-Aminobutanoic acid) at 100 mg L^{-1}, and the corresponding solvent-only dilutions were used as control for each chemical and concentration tested in *in vivo* fruit "Huaizhi" in Guangzhou of 2017. Comparison between treatments and the control in disease index at 72 hpi (**A**); Disease index of litchi downy blight (LDB) treated with the volatile compounds and the controls (**B**) at 100 mg L^{-1} and Biocontrol efficacy (**C**). VOCs compounds were sprayed to fruit "Huaizhi" (about 85% ripening degree) in the small greenhouse of the preservation box, the suspension of *P. litchii* at 5×10^4 sporangium mL^{-1} was sprayed onto the fruit each at 24 hpt (hours post treatment). Data are presented as means of four replicates ± standard errors; different letters indicate significant differences between treatments according to LSD test at $P < 0.05$.

they are often associated with enhanced resistance to a broad spectrum of biotic or abiotic stresses or both (Jolanta et al., 2015; Ton et al., 2002; Park et al., 2007). In result, seven of the tested compounds showed significant antagonist activities, including two each of the GC–MS identified compounds (*i.e.*, EA and BTH; Table 1 and Fig. 2B in Data in Brief) and previously reported ones (*i.e.*, SA and MeJA, Table 1 and Fig. 2B in Data in Brief). The four active compounds were then further tested *in vivo* on litchi fruit and leaves, who showed the similar, relatively EC_{50} values (in the range between 146.39 mg L^{-1} and 206.96 mg L^{-1}; Table 1).

Interestingly, we found that α-Farnesene (AF) and the previously reported BABA showed no inhibitory effect on *P. litchii* mycelia growth

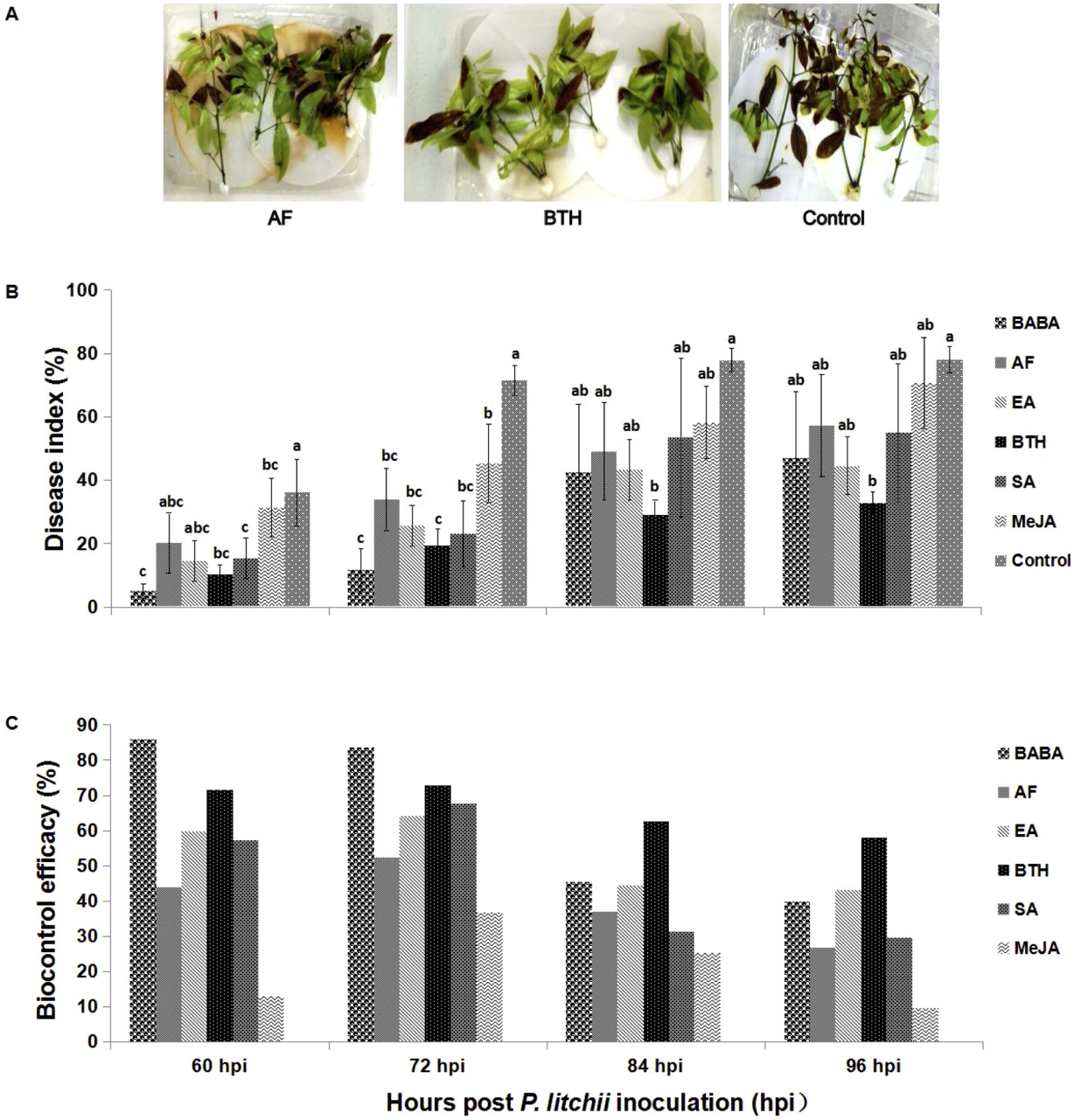

Fig. 4. Suppression of LDB by the VOCs blends of AF (α-Farnesene), BTH (Benzothiazole), EA (1-(2-Aminophenyl)ethanone) and the positive control of SA (Salicylic acid), MeJA (Methyl jasmonate), BABA (3-Aminobutanoic acid) at 100 mg L^{-1} in *in vivo* leaves "Huaizhi" in Guangzhou of 2017. Comparison between treatments and the control in disease index at 72 hpi (**A**); Disease index of litchi downy blight (LDB) treated with the volatile compounds and the controls (**B**) at 100 mg L^{-1} and Biocontrol efficacy (**C**). VOCs compounds were sprayed to leaves "Huaizhi" (about 85% ripening degree) in the small greenhouse of the preservation box, the suspension of *P. litchii* at 5×10^4 sporangium mL^{-1} was sprayed onto the leaves at 24 hpt (hours post treatment). Data are presented as means of four replicates ± standard errors; different letters indicate significant differences between treatments according to LSD test at $P < 0.05$.

in vitro (Table 1 and Fig. 2B in Data in Brief). AF was commonly produced by the three BCAs at 24 h (see the results of GC–MS analysis in Table 1 in Data in Brief), while BABA is a potent priming agent of systemic acquired resistance (SAR) in plants, such as defense priming to *Phytophthora infestans* of potato (Jolanta et al., 2015) or developed an enhanced capacity of priming phenomenon in *Arabidopsis* (Ton et al., 2005). We speculated that these two compounds could also emit inducible SAR against the pathogen *P. litchii,* thus we also included them in our *in vivo* test.

We tested on "Huaizhi" the *in vivo* efficacy of EA, BTH, SA, MeJA (at 100, 200 and 500 mg L^{-1}) and AF, BABA (at 100, 500, and 1000 mg L^{-1}). Different concentrations of the six components showed different efficacy to litchi fruit or leaves. Interestingly, all the compounds at the lowest concentration tested (*i.e.*, 100 mg L^{-1}) showed over 30% efficacy from 48 hpi to 72 hpi on fruit (Fig. 3), and EA and BTH achieved about 40% efficacy from 60 hpi to 84 hpi on leaves (Fig. 4). AF and MeJA reduced disease severity only at the 72 hpi time point on leaves (Fig. 4B), whose efficacies were 52.43% and 36.62% (Fig. 4C).

We observed for multiple compounds (AF, BTH, EA, MeJA, and SA) that the best efficacies were achieved at the lowest concentration (*i.e.*, 100 mg L^{-1}, Figs. 3 and 4, Table 4 in Data in Brief), which was consistent with the findings of Ryu et al (2004) and Azami-Sardooei et al (2013) that the biocontrol efficacy of a chemical strongly depends on the concentration being used. Notably, the six compounds often had much reduced efficacies or even led to more severe disease symptoms at

higher concentrations (200, 500 mg L^{-1} for EA, BTH, SA, and MeJA, or 500, 1000 mg L^{-1} for AF and BABA) (Table 4 in Data in Brief). For instance, BABA showed efficacies of 4.0%–19.8% at the concentration of 1000 mg L^{-1}, much lower than the efficacies at lower concentrations (38.3%–58.2% at 500 mg L^{-1}, and 28.0%–68.9% at 100 mg L^{-1}). Additionally, the disease indices were increased by 90.8% to 198.7% after direct application of AF at the concentration of 1000 mg L^{-1}. Upon treatment with high concentrations of these chemicals, the fruit displayed yellow water-soaked spots and peel soften while the leaves became brown around lesion, indicating the occurrence of physiological injuries. It has been suggested that some of the compounds we identified from the bacterial VOCs (*e.g.*, BTH) may act as signaling molecules for environmental stresses and can sensitize plants for faster and/or stronger responses to successive pathogen invasions, a phenomenon commonly referred to as priming (Conrath, 2011). However, the same molecules may result in adverse effects on plant growth at high concentrations, causing cell necrosis and thus leading to more severe disease symptoms (Dietrich et al., 2005). Overall, our results suggest that appropriate concentrations are crucial for the function of biocontrol compounds.

Our results showed that AF and BABA can suppress LDB on fruit, despite of their lack of antagonistic activity against *P. litchii* on plates, suggesting that the two compounds might indeed function *via* triggering the induction of plant defense mechanisms. As reported, AF, is one of the simplest acyclic sesquiterpenes, was first discovered in the natural coating of “Crofton” and “Delicious” apples, “Packhm” pears and quinces; and was found to play a role in plant defense (Huelin and Murray, 1966; Paré and Tumlinson, 1999; Yang et al., 2011), or as an alarm pheromone as sexual or trail in *P. canalifrons* (Šobotník et al., 2008). We postulated that AF may also induce plant immunity through priming to protect postharvest litchi fruit from LDB.

For the other compounds that showed inhibitory effects both *in vivo* and *in vitro*, it is also possible that they may similarly activate plant disease defense. The mechanism of inducing plant defense and bacterial antagonists are not mutually exclusive (Francesco et al., 2016). For instance, BTH is a chemical analogue of SA that induces resistance in a variety of plants by activating the SAR pathway (Azami-Sardooei et al., 2013; Vallad and Goodman, 2004), it had been reported to induce priming of defense responses against *Pseudomonas syringae* pv *syringae* in Lima bean (Yi et al., 2009), induces resistance of postharvest peach (*Prunus persica* L. cv. Jiubao) fruit to infection by *Penicillium expansum* and enhances activity of fruit defense mechanisms (Liu et al., 2005). BTH referred to as priming at low concentrations (Conrath, 2011) while often resulted in direct activation of defenses and sometimes adverse effects on plant growth high concentrations (Heil et al., 2000; Walters and Heil, 2007). Plant defense activators, such as BTH, SA, MeJA, can induce priming of defense responses in susceptible hosts (Beckers and Conrath, 2007). In our study, these three compounds (*i.e.*, BTH, SA, and MeJA) also showed antagonistic activities against the pathogen *in vitro*; they inhibited the growth of the pathogen without disrupting the normal hyphae structures on petri dish. It is possible that the compounds may repress *P. litchii* infection by causing damage to appressoria and subsequently suppressing the penetration and formation of invasive hyphae, resulting in morphological and ultrastructure alteration on *P. litchii,* similar to the mechanisms reported by Xing et al (2018)

4. Conclusions

In this study, we showed that three biocontrol agents (*B. amyloliquefaciens* PP19, *B. pumilus* PI26, and *E. acetylicum* SI17), the VOCs they produce, and specific components of the VOCs (AF and BTH) were effective in controlling *P. litchii*, the pathogen that causes the postharvest disease LDB. Further biocontrol assays of individual compounds showed that, when applied directly, BTH might have antagonist effect against *P. litchii*, whereas AF might induce plant defense responses. The active compounds identified in our study are promising for the biocontrol of LDB, and our results are useful for the better understanding of the biocontrol mechanisms by BCAs and VOCs against *P. litchii*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2019.05.009.

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