Synthetic and Systems Biotechnology 8 (2023) 427–436

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

Volatiles of *Shiraia* fruiting body-associated *Pseudomonas putida* No.24 stimulate fungal hypocrellin production

Rui Xu [a], Qun Yan Huang [a], Wen Hao Shen [a], Xin Ping Li [a], Li Ping Zheng [b,**], Jian Wen Wang [a,*]

[a] College of Pharmaceutical Sciences, Soochow University, Suzhou, 215123, China
[b] Department of Horticultural Sciences, Soochow University, Suzhou, 215123, China

ARTICLE INFO

Keywords:
Shiraia
Pseudomonas putida
Volatiles
Reactive oxygen species
Hypocrellins
Elicitation

ABSTRACT

Hypocrellins are major bioactive perylenequinones from *Shiraia* fruiting bodies and have been developed as efficient photosensitizers for photodynamic therapy. *Pseudomonas* is the second dominant genus inside *Shiraia* fruiting bodies, but with less known actions on the host fungus. In this work, the effects of bacterial volatiles from the *Shiraia*-associated *Pseudomonas* on fungal hypocrellin production were investigated. *Pseudomonas putida* No.24 was the most active to promote significantly accumulation of *Shiraia* perylenequinones including hypocrellin A (HA), HC, elsinochrome A (EA) and EC. Headspace analysis of the emitted volatiles revealed dimethyl disulfide as one of active compounds to promote fungal hypocrellin production. The bacterial volatiles induced an apoptosis in *Shiraia* hyphal cell, which was associated with the generation of reactive oxygen species (ROS). ROS generation was proved to mediate the volatile-induced membrane permeability and up-regulation of gene expressions for hypocrellin biosynthesis. In the submerged volatile co-culture, the bacterial volatiles stimulated not only HA content in mycelia, but also HA secretion into the medium, leading to the enhanced HA production to 249.85 mg/L, about 2.07-fold over the control. This is the first report on the regulation of *Pseudomonas* volatiles on fungal perylenequinone production. These findings could be helpful to understand the roles of bacterial volatiles in fruiting bodies and also provide new elicitation method using bacterial volatiles to stimulate fungal secondary metabolite production.

1. Introduction

Shiraia bambusicola is a parasitic fungus of bamboos in southeast Asia [1,2]. Its fruiting body has long been used as a traditional Chinese medicine to treat rheumatic arthritis, sciatica, chronic bronchitis and tracheitis [3]. Their main bioactive components were perylenequinone pigments, including hypocrellin A-D (HA-HD) and elsinochrome A (EA) [4]. Recently, the application of hypocrellins as effective photodynamic therapy (PDT) agents has received intensive attention for the treatment of virus infections and cancers due to their efficient photooxidation properties [5]. However, due to the difficulty and complexity of the chemical synthesis of hypocrellins [6], wild *Shiraia* fruiting bodies are still the main resource for hypocrellin supply for medical application.

In the wild, diverse bacteria were observed in fungal fruiting bodies. The predominate bacteria *Stenotrophomonas* and *Pseudomonas* were found in fruiting body of wild Himalayan *Cantharellus* spp. [7]. Two *Pseudomonas* sp., belonging to the family Pseudomonadaceae, have been isolated and characterized as the dominant bacterial species in the fruiting body of *Tricholoma matsutake* [8]. A number of bacteria (*Bradyrhizobium*, *Variovorax*, *Pedobacter* and *Sphingobacterium*) colonized fruiting bodies of *Tuber melanosporum* [9]. In our previous study [10], 31 bacteria were isolated from *Shiraia* fruiting body, where *Bacillus* and *Pseudomonas* were the dominant genera. Although the bacteria associated with the fruiting bodies in nature are both abundant and diverse, their biological functions remain less known. Varese et al. (1996) found that *Pseudomonas* isolated from the fruiting body of *Suillus grevillea* was able to promote markedly fungal hyphae growth, but *Streptomyces* inhibited fungal growth [11]. Xiang et al. (2017) found that *Pseudomonas* DJ35 from fruiting body of *Agaricus bisporus* had potential to produce indole acetic acid and cellulase, which stimulate mushroom growth [12]. Citterio et al. (2001) reported that *P. fluorescens* isolated from *Tuber borchii* had the ability to degrade cellulose and chitin for

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author.
** Corresponding author.
E-mail addresses: lpzheng@suda.edu.cn (L.P. Zheng), jwwang@suda.edu.cn (J.W. Wang).

https://doi.org/10.1016/j.synbio.2023.06.004
Received 22 April 2023; Received in revised form 8 June 2023; Accepted 8 June 2023
Available online 20 June 2023
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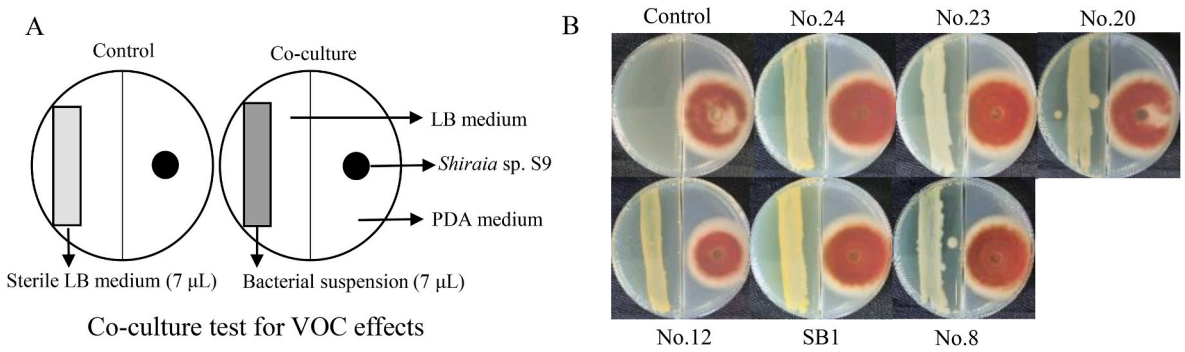

Fig. 1. Effects of *Pseudomonas* volatiles on the fungal red pigments accumulation in solid cultures of *Shiraia* sp. S9. The mode diagram (A) and plates cultures (B) of bacterial volatiles and fungus S9. The fungus was kept on PDA treated with bacterial volatiles for 6 days at 28 °C.

Table 1
The effects of *Pseudomonas* volatiles on HA accumulation of *Shiraia* sp. S9 in solid-state culture[a].

Co-culture		HA contents (mg/plate)
Host fungus	Associated bacterium	
S9	/	0.25 ± 0.08
S9	*P. putida* No.8	1.18 ± 0.03**
S9	*P. fulva* SB1	1.50 ± 0.24**
S9	*P. fulva* No.12	0.91 ± 0.12**
S9	*P. parafulva* No.20	0.98 ± 0.11**
S9	*P. putida* No.23	1.19 ± 0.29**
S9	*P. putida* No.24	1.67 ± 0.26**

[a] The co-culture of live bacteria and fungi was maintained on PDA plate at 28 °C for 6 days. The bacterial inoculum was 7 μL (10^5 cells/mL). The condition of co-culture was the same as specified in Fig. 1. Values are mean ± SD from three independent experiments (**$p < 0.01$ versus fungal monoculture as the control group).

possible regulation in the development of the fruiting body [13]. Our previous reports showed that all *Pseudomonas* isolated from *Shiraia* fruiting body could enhance fungal HA production [14]. *Bacillus cereus* No.1, another bacterium dominating in *Shiraia* fruiting body was found to exhibit obvious suppression on fungal growth and HA production, but its volatiles could stimulate fungal perylenequinone production [15].

Volatile organic compounds (VOCs) produced by various bacteria have significant potential to change plant growth and exert antifungal activity against pathogens [16–18]. However, there are less reports on the effects of bacterial volatiles on fungal secondary metabolism. Zhao et al. (2011) reported that citronellol produced by *Paenibacillus polymyxa* BMP-11 could suppress completely fungal pigment accumulation of *Fusarium oxysporum* [19]. After exposure to volatiles of a pathogenic bacterium *Ralstonia solanacearum*, *Aspergillus flavus* reduced conidia production and increased aflatoxin production [20]. A mixture of VOCs of endophytic *Bacillus* spp. could have biocontrol activity against *Sclerotinia sclerotiorum* by reducing fungal sclerotia biosynthesis [21]. In order to understand the interaction between fruiting body-associated bacteria and host fungi [10], and continue our work on the effects of bacterial VOCs on *Shiraia* perylenequinones [15], the present work was carried out on the effects of volatiles from the second dominate *Pseudomonas* species on *Shiraia* host. Both the chemical composition of VOCs produced by the active stain *P. putida* No.24 and their physiological elicitation on *Shiraia* HA biosynthesis were investigated in detail. The submerged volatile co-cultures were also established for biotechnological production of hypocrellins.

2. Materials and methods

2.1. Strains and culture conditions

The HA-producing strain *Shiraia* sp. S9 and its associated bacterium *P. putida* No.24 were isolated from *Shiraia* fruiting bodies on bamboo (*Brachystachyum densiflorum*) in our previous work [10], and registered in China General Microbiological Culture Collection Center (CGMCC) as CGMCC16369 and CGMCC1.61835, respectively. To initiate the liquid cultures, *Shiraia* sp. S9 was cultured on potato dextrose agar (PDA) medium at 28 °C for 8 days. The details of medium component and conditions for the fungal cultures were the same as described in our previous report [22].

2.2. The bacterial and fungal co-culture

The co-culture assay was carried out according to the method reported by Kai et al. (2007) with a slight modification [23]. Two-compartment plastic plates (90 mm) were loaded with 12-mL PDA media on one side and 10-mL Luria-Bertani (LB) media on the other side (Fig. 1A). After 24 h of cultivation, the prepared bacterial suspension (7 μL, 10^5 cells/mL) was streaked in parallelly on LB media. Simultaneously, a mycelial plug (5 mm) was taken from the margin of 8-day-old *Shiraia* colony and placed onto the center of PDA media. The equivalent sterile LB broth was used as the control group. The two-compartment plates were incubated at 28 °C in an incubator (GNP-9080BS-III, CIMO, Shanghai, China) for 6 days under darkness.

The submerged volatile co-culture was constructed to investigate the effects of bacterial volatiles in liquid culture according to our pervious report method [15]. Briefly, this co-culture system was composed of two 250 mL culture flasks for incubating fungus S9 and bacterium No.24, separately. Two flasks were connected through a glass tube (Supplementary Fig. S1). The S9 seed culture (10 mL) was added into a flask with 100-mL liquid medium. Bacterium at 500 cells/mL was added to the flask for bacterial volatile producer on day 2, 4, 6 of the fungal culture and then connected to the flask for mycelium culture. The co-culture was maintained on a ZD-8802 rotary shaker (Hualida, Suzhou, China) at 150 rpm at 28 °C for 8 days. Sterile LB broth instead of the bacterial suspension was added to the flask as a control group.

2.3. Microscopic morphology observation

The fungal colony in PDA plates was eluted with 8-mL distilled water. Then, the fungal spores were observed and counted under a light microscope (CKX41, Olympus, Tokyo, Japan). Fungal hyphae branches were observed using fluorescence inverted microscope (TS2R-FL, Nikon, Tokyo, Japan). In submerged volatile co-culture (day 2, 4, 6 and 8), the fungal pellets were harvested and observed using a stereoscopic microscope (SMZ1000, Nikon, Tokyo, Japan). Pellet diameters were

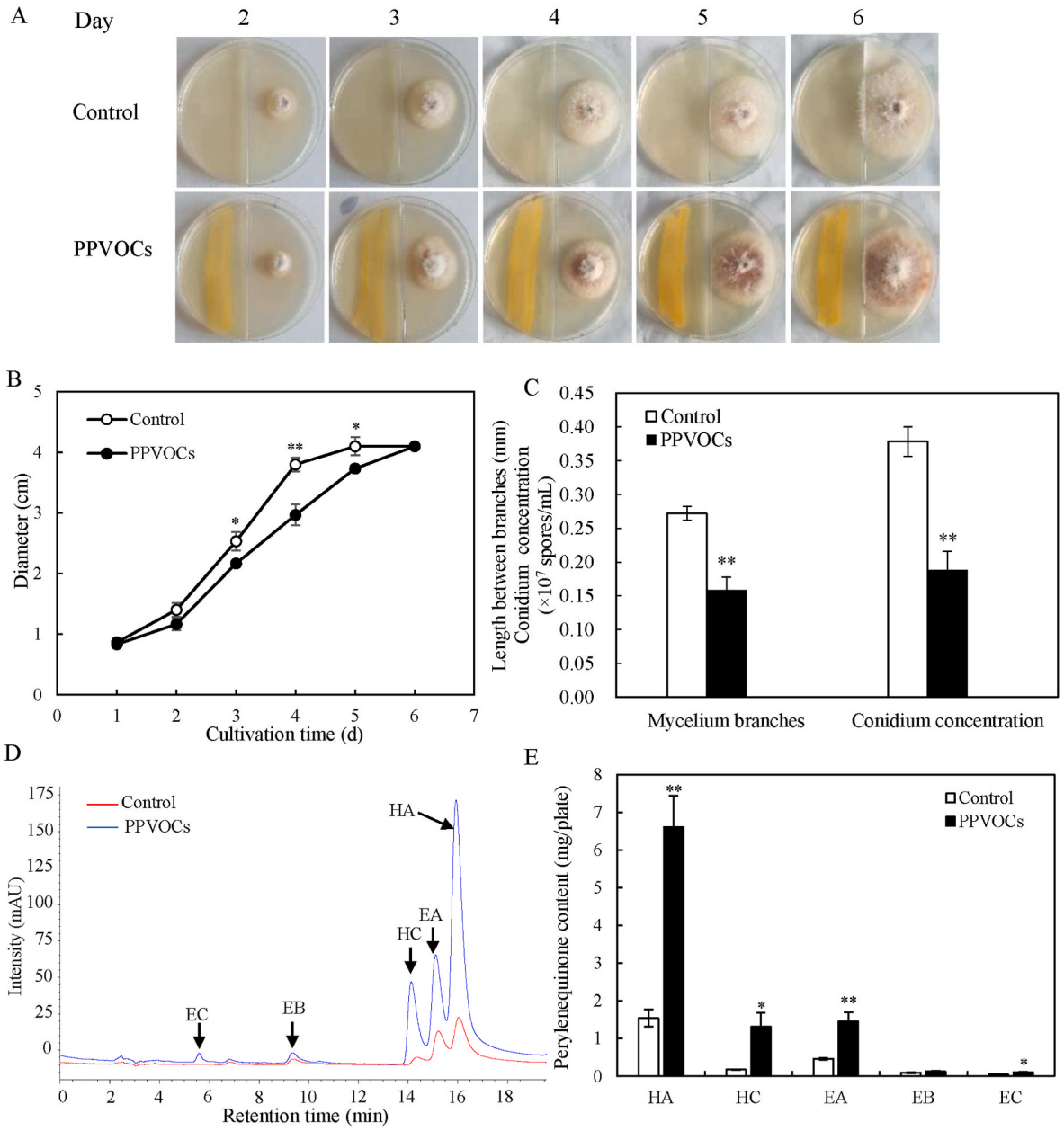

Fig. 2. The effects of *P. putida* No.24 volatiles (PPVOCs) on the fungal growth and perylenequinone accumulation of *Shiraia* sp. S9. The effects of bacterial volatiles on fungal colony diameter (A, B), mycelial branches and conidium concentration (C). The chromatogram of individual perylenequinone in mycelium (D) and its contents (E). The bacterial suspension (7 μL, 10^5 cells/mL) and fungal mycelial plugs were inoculated simultaneously. The fungus was kept on PDA at 28 °C treated with bacterial volatiles for 6 days. Values are mean ± SD from three independent experiments (*$p < 0.05$, **$p < 0.01$ versus the control group).

calculated in triplicates (50 objects for each determination).

2.4. Analysis of bacterial VOCs by GC-MS

After culture of 24 h in LB medium at 37 °C in a constant temperature incubator (GNP-9080BS-III, CIMO, Shanghai, China), VOCs from *P. putida* No.24 were collected by headspace solid-phase microextraction (HS-SPME) with a polydimethylsiloxane fiber (100 μm) and analyzed according to the method described by Li et al. (2010) [24]. VOCs were analyzed by gas chromatography-mass spectrometry (GC-MS, Agilent 6890N–5975B, Santa Clara, USA) equipped with an HP-5MS column. Mass spectra were acquired in the range 50–500 *m/z*. VOCs were identified initially by comparing mass spectra with data from the library of the National Institute of Standards and Technology (NIST) and also verified by the comparison of retention times and mass spectra with authentic standards.

2.5. Detection of ROS and activities of antioxidant enzymes

The ROS-specific fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime, Haimen, China) was added at 10 μM in the mycelia cultures in the darkness for 30 min, then the ROS accumulation in mycelia was detected under a CKX41 fluorescent microscope (Olympus, Tokyo, Japan) with an excitation/emission wavelength of 485/528 nm [25]. The content of hydrogen peroxide (H_2O_2) in mycelia was determined as previously described by Mirshekari et al. (2019) [26]. The activities of superoxide dismutase (SOD), NADPH oxidase (NOX) and catalase (CAT) were determined using the Enzyme Activity Assay Kit (Beyotime, Nanjing, China) according to the manufacturer's protocols and previous reports [27,28]. The activity of

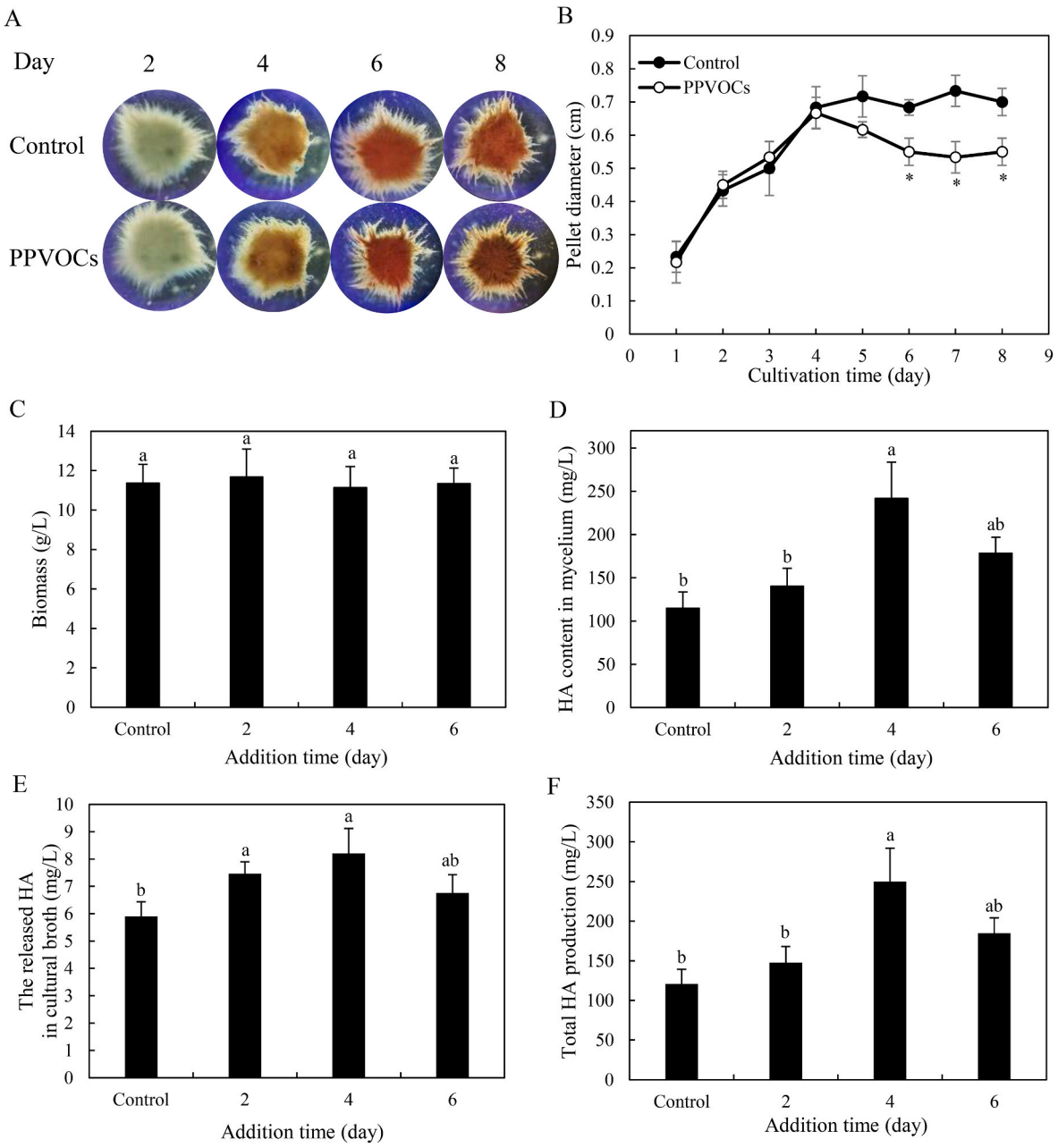

Fig. 3. Effects of *P. putida* No.24 volatiles (PPVOCs) on fungal growth and HA production in the submerged volatile co-cultures. (A) Morphology (80 ×) of the pellets after VOC treatment. (B) Time profiles of average pellet diameters during the culture. *P. putida* No.24 was added on day 4 of the culture. The fungal dry biomass (C), HA contents in mycelium (D), the released HA in cultural broth (E) and total HA production (F) was measured on day 8 in the culture. The arrow represents the addition time of *P. putida* No.24. An equal volume of sterile LB broth instead of bacterial suspension added to flask was used as control group. Values are mean ± standard deviations of three replicates (*$p < 0.05$ and **$p < 0.01$ versus control group). Different letters above the bars mean significant differences ($p < 0.05$).

peroxidase (POD) was determined as previously described method by Wu et al. (2002) [29].

2.6. Measurement of apoptosis by TUNEL assay and caspase-3 activity

To determine the occurrence of cell apoptosis after VOC treatment, One Step TUNEL Apoptosis Assay Kit (Beyotime Biotechnology, Nanjing, China) was used [30] and the hyphae were observed under fluorescence microscopy (BX51, Olympus, Tokyo, Japan) with an excitation/emission wavelength of 480/515 nm. The activity of caspase-3 was measured using Caspase 3 Activity Assay Kit (Beyotime Biotechnology, Nanjing, China) according to a previous study [31].

2.7. Membrane permeabilization assay

The permeabilization of fungal membrane was observed using the fluorescence dye SYTOX Green (Molecular Probes, Eugene, USA) [32]. After 5 days of culture, the harvested hyphae were incubated with SYTOX Green at 0.50 μM for 30 min. Then, the fluorescence was observed under a CKX41 fluorescent microscope (Olympus, Tokyo, Japan) with excitation wavelength of 488 nm and emission wavelength of 538 nm. To analyze the effect of ROS on VOCs-induced fungal membrane permeabilization of *Shiraia* hyphae, the medium was added with exogenous H_2O_2 (200 μM) and vitamin C (Vc, 20 μM), respectively.

Table 2
The analysis of selected VOCs from strain *P. putida* No. 24 by headspace solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS).

Compound[a]	Identity[b] (%)	Peak area[c] (%)	RT[d] (min)	MW[e]	MF[f]
Thiocyanic acid, methyl ester	10	3.72	2.056	73.12	C_2H_3NS
Disulfide, dimethyl	78	63.89	2.508	94.2	$C_2H_6S_2$
Phenylethyl alcohol	76	0.42	8.264	108.14	C_7H_8O
2-Nonanone	93	4.93	9.729	142.24	$C_9H_{18}O$
2-Undecanone	94	2.78	14.558	170.29	$C_{11}H_{22}O$
2-Tridecanone	98	1.53	18.907	198.34	$C_{13}H_{26}O$
Butylated Hydroxytoluene	95	3.92	19.296	220.35	$C_{15}H_{24}O$

[a] The compounds for the activity test in our study are listed.
[b] Spectra similarity of analyte compounds with those available in the spectral library (NIST).
[c] Relative area of detected compounds as a percentage in reference to the total spectra peaks.
[d] Retention time (RT) of the compounds in the GC-MS analysis.
[e] Molecular weight (MW) of detected compounds.
[f] Molecular formula (MF) of identified compounds.

2.8. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from the fungal mycelia using the RNAprep pure Plant Kit (Tiangen, Beijing, China). Specific primers for each gene were listed in Supplementary Table S1. The qRT-PCR condition and procedure were set and performed using CFX96-C1000 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, USA) according to our previous report [33]. The relative transcript levels were determined according to the $2^{-\Delta\Delta Ct}$ method described by Livak et al. (2001) [34].

2.9. Statistical analysis

All treatments were performed in triplicate independent experiments. Student's *t*-test and one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests were performed for experimental results. All results are expressed as mean ± standard deviation (SD). The differences between the compared samplings were considered statistically significant ($p < 0.05$).

3. Results

3.1. Effect of Pseudomonas volatiles on fungal HA production in solid culture

The six previously isolated *Pseudomonas* strains from the fruiting body [10] were selected in the co-culture tests (Fig. 1). The experiment showed the volatiles from all tested *Pseudomonas* stains could promote HA accumulation (Table 1). When bacterial suspension (7 μL, 10^5 cells/mL) was added to the separated compartment in the co-culture plates, No.24 stain could promote HA production markedly to 1.67 mg/plate on day 6, about 6.56-fold higher than that of the control group (fungal mono-culture) (Fig. 1B, Supplementary Fig. S2). The identification based on the ITS ribosomal gene (Supplementary Fig. S3) and biochemical characteristics (Supplementary Table S2) indicated that No.24 was a strain of *Pseudomonas putida*. Volatiles from other *Pseudomonas* such as *P. putida* No.8, *P. fulva* SB1, *P. fulva* No.12, *P. parafulva* No.20, *P. putida* No.23 could also stimulate HA production in PDA plates, about 4.63-, 5.88-, 3.56-, 3.86-, 4.66-fold over the control group.

3.2. Effects of the bacterial volatiles on individual perylenequinone production in solid culture

In the co-culture between *P. putida* No.24 and the host fungus *Shiraia* sp. S9, the fungal growth diameter was suppressed by VOCs of *P. putida* No.24 (PPVOCs) from day 3–5 (Fig. 2A and B). The distance of hyphal branches was shortened and fungal conidiation were also suppressed by

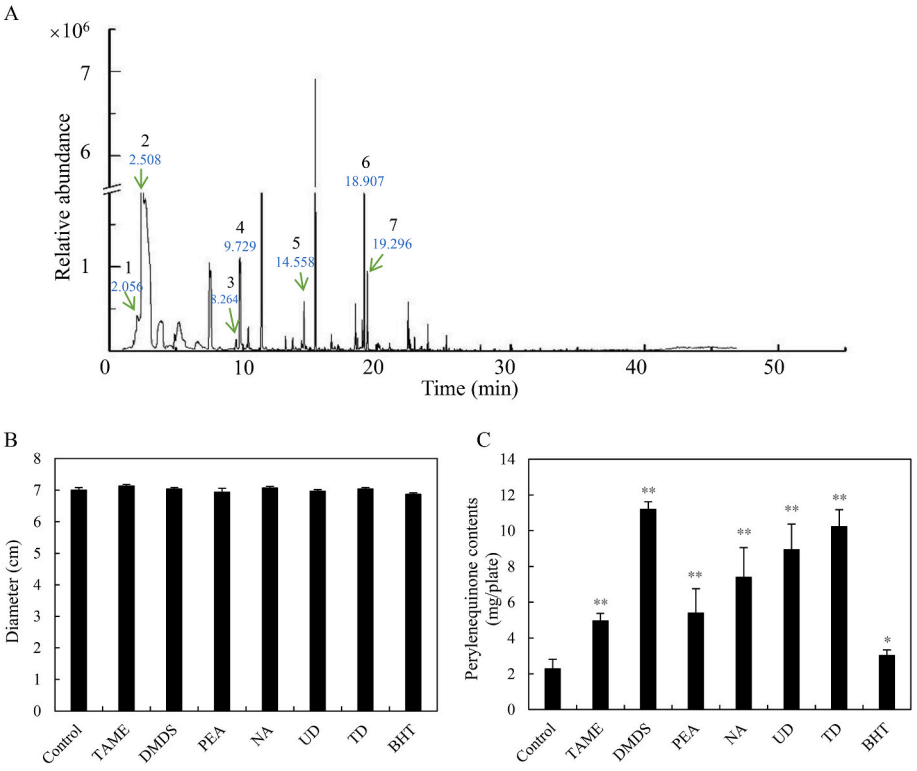

Fig. 4. The identification of *P. putida* No.24 volatiles (PPVOCs) and their effects on *Shiraia* sp. S9. (A) The total ion current diagram of PPVOCs after 24 h culture in LB medium at 37 °C. Main peaks: 1, thiocyanic acid, methyl ester (TAME, t_R 2.056 min); 2, dimethyl disulfide (DMDS, t_R 2.508 min); 3, phenylethyl alcohol (PEA, t_R 8.264 min); 4, 2-nonanone (NA, t_R 9.729 min); 5, 2-undecanone (UD, t_R 14.558 min); 6, 2-tridecanone (TD, t_R 18.907 min); 7, butylated hydroxytoluene (BHT, t_R 19.296 min). Effects of main VOCs on fungal growth diameter (B) and perylenequinone contents (C). Different VOCs was added at 10 mg/mL on day 5 of the plate cultures of fungus S9 at 28 °C and the measurement was taken after 2 days of the treatment. The conditions for the co-culture test were the same as specified in Fig. 1. Values are mean ± SD from three independent experiments (*$p < 0.05$, **$p < 0.01$ versus the control group).

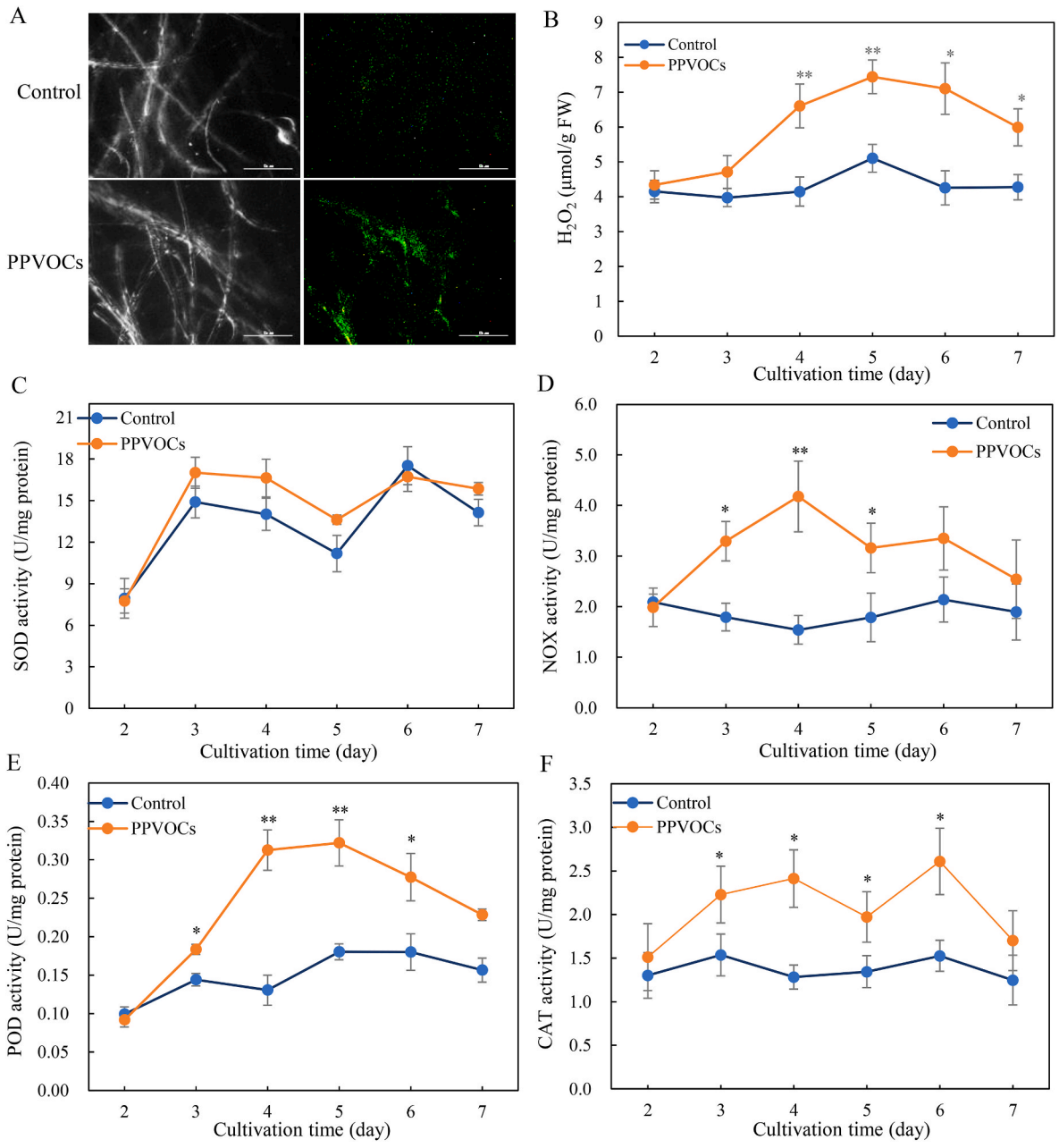

Fig. 5. Effects of *P. putida* No.24 volatiles (PPVOCs) on fungal ROS generation and the activities of antioxidant enzymes in *Shiraia* hyphae in solid culture. (A) Bright-field images (left) and fluorescence microscopy of DCFH-DA-stained mycelia (right) (400 ×) in the cultures. (B) Time course of H_2O_2 contents in S9 mycelia. Effects of PPVOCs on enzyme activities of superoxide dismutase (SOD) (C), NADPH oxidase (NOX) (D), peroxidase (POD) (E) and catalase (CAT) (F) in the mycelia of *Shiraia* sp. S9 in solid culture. The bacterial inoculum was 7 μL (10^5 cells/mL). The conditions for the co-culture test were the same as specified in Fig. 1. Values are mean ± SD from three independent experiments (*$p < 0.05$, **$p < 0.01$ versus the control group).

the bacterial volatiles (Fig. 2C). After 6 days, PPVOCs could enhance the secretion of red perylenequinone pigments in the plates. The content of individual perylenequinone (HA, HC, EA or EC) was enhanced significantly, about 4.28-, 7.53-, 3.15-, 2.02-fold higher than that of the control group, respectively (Fig. 2D and E). The total perylenequinone production reached 9.61 mg/plate, which was 4.13 times that of the control group.

3.3. Effects of PPVOCs on Shiraia HA production in liquid culture

A submerged volatile co-culture was established to analyze the influence of PPVOCs on fungus S9 in mycelium cultureSupplementary Fig. S3(Supplementray Fig. S1). Compared to the fluffier fungal pellets in control group, the smaller and more compact pellets emerged after the treatment of PPVOCs on day 4 (Fig. 3A and B). Although the fungal biomass was not altered during the co-culture (Fig. 3C), the bacterial volatiles promoted not only the accumulation of HA in mycelium (Fig. 3D), but also the released HA into the medium (Fig. 3E). The total HA production reached the maximum 249.85 mg/L on day 8, about 2.07-fold that of the control group (Fig. 3F).

3.4. Identification of PPVOCs and their effects on fungal perylenequinone contents

Twenty-five VOCs produced by *P. putida* No.24 were identified by HS-SPME/GC-MS, and the most abundant volatile metabolites were

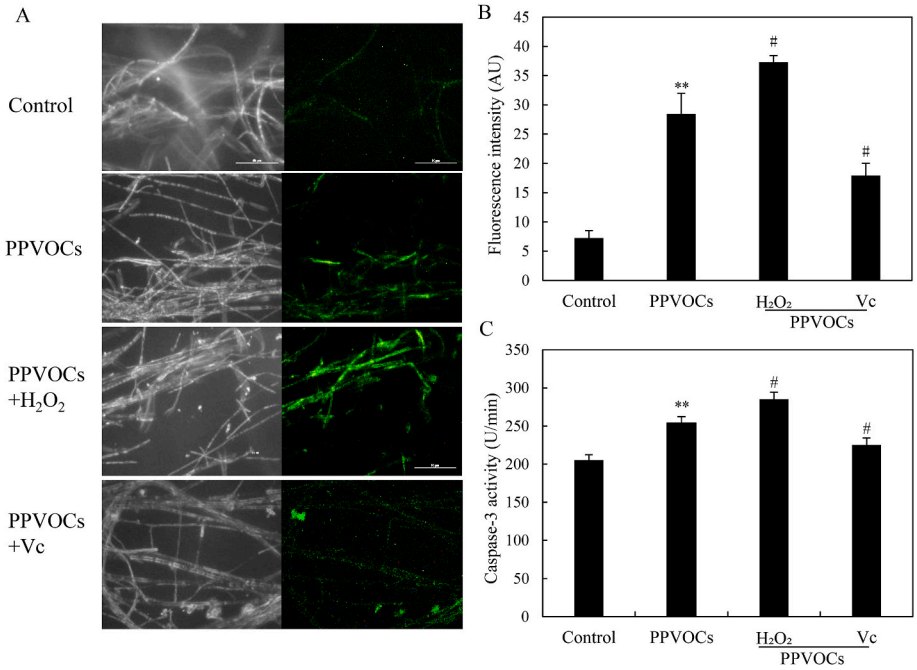

Fig. 6. Effects of H_2O_2 and vitamin C (Vc) on PPVOC-induced cells apoptosis of *Shiraia* hyphae in the co-culture. (A) Bright-field images (left) and fluorescence microscopy (right) by TUNEL stained mycelia (400 ×). The relative fluorescence intensity of cell apoptosis (B) and assays of caspase-3 activity (C) in mycelia. The bacterial inoculum was 7 μL (10^5 cells/mL). The conditions for the co-culture test were the same as specified in Fig. 1. H_2O_2 (200 μM) and Vc (20 μM) were added into PDA plates on day 1, and the measurement of apoptosis of fungus S9 was taken on day 5 in PDA plate at 28 °C. Values are mean ± SD from three independent experiments (**$p < 0.01$ vs. control, $^{\#}p < 0.05$ vs. PPVOC treatment).

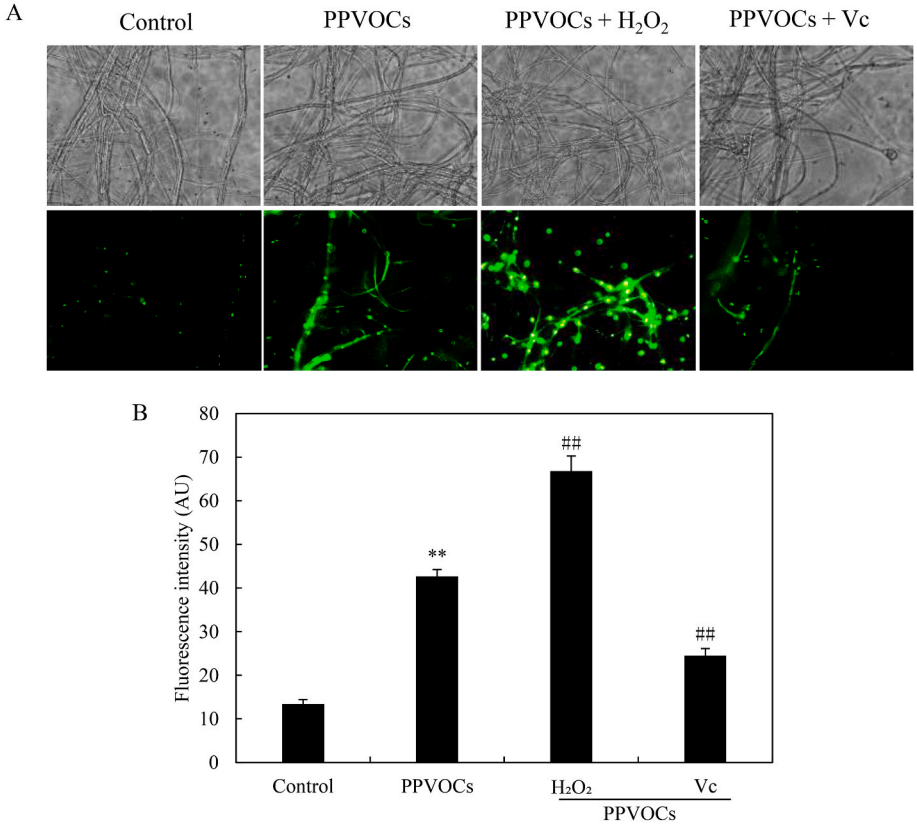

Fig. 7. Effects of H_2O_2 and vitamin C (Vc) on PPVOC-induced membrane permeability of *Shiraia* hyphae in the co-culture. (A) Bright-field images (top) and fluorescence microscopy (bottom) by SYTOX Green-stained hyphae (400 ×). (B) The relative fluorescence intensity. The bacterial inoculum was 7 μL (10^5 cells/mL). The conditions for the co-culture test were the same as specified in Fig. 1. H_2O_2 (200 μM) and Vc (20 μM) were added into PDA plates on day 1, and the measurement was taken on day 5 in PDA plate at 28 °C. Values are mean ± SD from three independent experiments (**$p < 0.01$ vs. control, $^{\#\#}p < 0.01$ vs. PPVOC treatment).

ketones, alkanes, phenols, alcohols and sulfides (Table 2, Supplementary Table S3). The methyl ester of thiocyanic acid(TAME), dimethyl disulfide (DMDS), phenylethyl alcohol (PEA), 2-nonanone (NA), 2-undecanone (UD), 2-tridecanone (TD), butylated hydroxytoluene (BHT) were selected for further investigation of their effects on fungal perylenequinone contents (Supplementary Fig. S4, Fig. 4). The tested VOCs at 1–20 mg/mL exerted no effect on the fungal growth, but most of them promoted fungal perylenequinone production markedly at 5–10 mg/mL, whereas both UD and TD exerted inhibitory effects at 20 mg/mL (Supplementary Fig. S4, Fig. 4). DMDS had the most significant effect on perylenequinone accumulation at 10 mg/mL, increasing perylenequinone content to 11.20 mg/plate on day 7, about 4.92 times that of the control group (Fig. 4C).

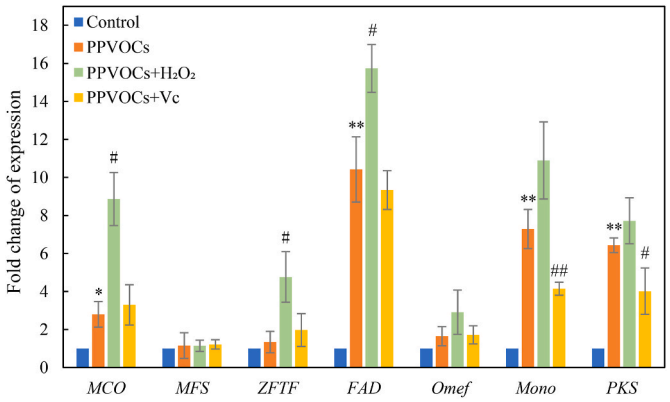

Fig. 8. Effects of H_2O_2 and vitamin C (Vc) on PPVOC-induced HA biosynthetic genes expression of *Shiraia* sp. S9 in the co-culture. The bacterial inoculum was 7 μL (10^5 cells/mL). The conditions for the co-culture test were the same as specified in Fig. 1. H_2O_2 (200 μM) and Vc (20 μM) were added into PDA plates on day 1, then the cultures were maintained on PDA plate at 28 °C for 6 days. Values are mean ± SD from three independent experiments ($^*p < 0.05$, $^{**}p < 0.01$ vs. control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. PPVOC treatment).

3.5. PPVOC-induced ROS generation and activities of antioxidant enzymes

After 5 days of PPVOC treatment, the green fluorescent signals of DCFH-DA were brighter and more intense (Fig. 5A). H_2O_2 content increased strikingly from 4.71 to 7.44 μmol/g FW (day 3–5) and then decreased (Fig. 5B). Although SOD activity was not significantly altered on day 5 (Fig. 5C), the activities of NOX (Fig. 5D) and other antioxidant enzymes (including CAT and POD) were increased significantly during the PPVOC treatment (Fig. 5E and F). The activity of NOX was enhanced quickly to 4.18 U/mg protein, about 2.71-fold over the control (Fig. 5D). The most significant increase of POD (Fig. 5E) and CAT activities (Fig. 5F) occurred on day 4 and day 6 respectively, about 139.39% and 70.92% higher over the control.

3.6. Detecting of PPVOC-induced apoptosis

In the co-culture, the bacterial volatiles induced even greener and more intense fluorescence of TUNEL stained hyphae on day 5 (Fig. 6A), suggesting the occurrence of apoptosis. When VOC-treated *Shiraia* sp. S9 was cultured in the presence of exogenous H_2O_2 at 200 μM, the relative intensities of fluorescence in hyphae exhibited much more notable increase of 31.04% (Fig. 6B). However, ROS scavenger vitamin C (Vc, 20 μM) reduced the relative intensities by 37.02% (Fig. 6B). To further confirm the occurrence of cell apoptosis after treatment of the bacterial volatiles, the activity of caspase-3 was determined on day 5. As shown in Fig. 6C, PPVOCs increased caspase-3 activity by 23.59%. ROS scavenger Vc at 20 μM significantly inhibited the induced caspase-3 activity (PPVOCs + Vc *vs.* PPVOCs in Fig. 6C). The exogenous H_2O_2 at 200 μM improved further the induced activity by 12.52%.

3.7. Effects of PPVOCs on fungal membrane permeability

We analyzed the alteration of hyphal cell membrane permeability of *Shiraia* sp. S9 under PPVOC treatment (Fig. 7). After 5 days of PPVOC treatment, the green fluorescence of SYTOX Green in hyphae became brighter and more intense (Fig. 7A), indicating a higher permeability of

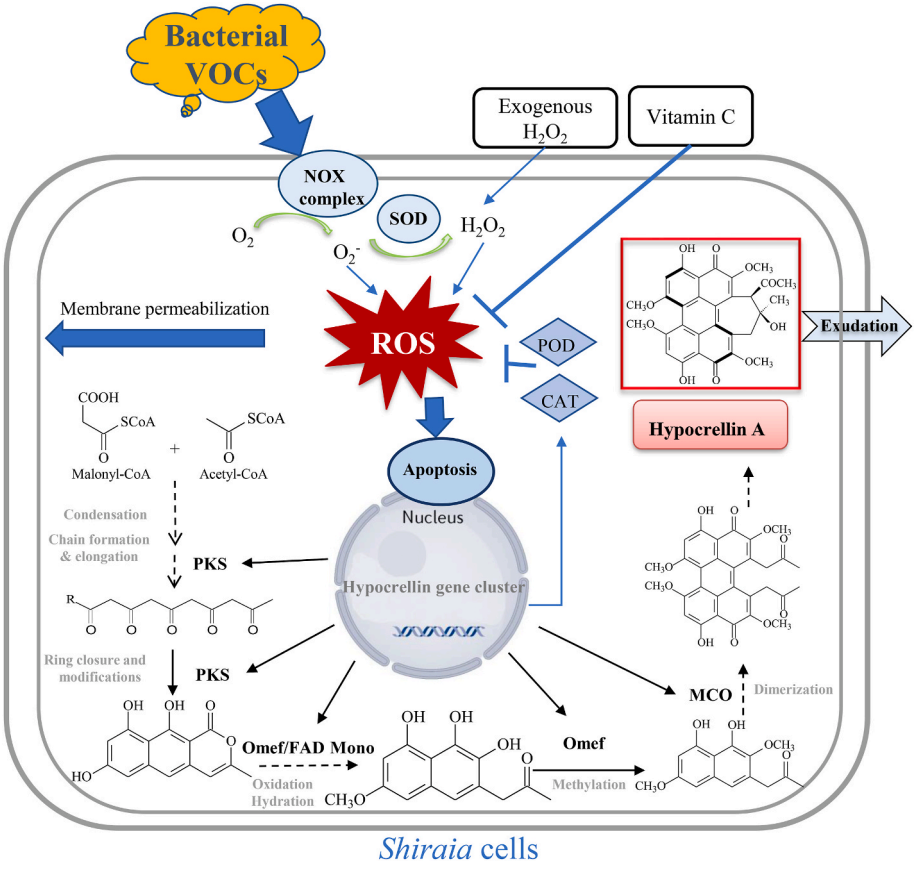

Fig. 9. Schematic representation of the hypothetical model of eliciting hypocrellin biosynthesis of *Shiraia* sp. S9 by PPVOCs. The solid arrows indicate data supported by our own experiments and experimentally supported in other fungal systems. The dotted arrows indicate more uncertain steps. PKS polyketide synthase, Omef *O*-methyltransferase, FAD FAD/FMN-dependent oxidoreductase, Mono monooxygenase, MCO multicopper oxidase.

fungal mycelia. To further investigate the effects of ROS on bacterial volatile-induced membrane penetration, exogenous H_2O_2 at 200 μM and ROS scavenger vitamin C (Vc, 20 μM) were added during the cultures. The green fluorescence induced by bacterial volatiles in hyphae was further stimulated by exogenous H_2O_2, but suppressed markedly by Vc (Fig. 7A and B).

3.8. Effects of PPVOCs on genes expressions for fungal HA biosynthesis

The expression levels of seven key genes in HA biosynthetic cluster were determined by qRT-PCR (Fig. 8). After 6 days of PPVOC treatment, the bacterial volatiles up-regulated significantly the expression of multicopper oxidase (*MCO*), FAD/FMN-dependent oxidoreductase (*FAD*), monooxygenase (*Mono*) and polyketide synthase (*PKS*), about 2.80-, 11.38-, 6.63- and 5.20-fold of the control group, respectively. ROS scavenger Vc (20 μM) suppressed significantly the induced *PKS* and *Mono* expressions, while the transcriptional levels of *MCO*, zinc finger transcription factor (*ZFTF)* and *FAD* were further enhanced by exogenous H_2O_2 at 200 μM.

4. Discussion

Pseudomonas species are well known for their biocontrol abilities [35]. Chitinase from *Pseudomonas* was reported to have biocontrol activity against phytopathogenic *Macrophomina*, *Aspergillus* and *Phytophthora* spp. [36]. Phenazine-1-carboxylic acid produced by *P. aeruginosa* exerted its antifungal activity against *Botrytis cinerea* [37]. Tran et al. (2007) reported that cyclic lipopeptide massetolide A from *P. fluorescens* SS101 induced tomato resistance against late blight pathogen *Phytophthora infestans* [38]. Cyanogenic *Pseudomonas* strains from the rhizosphere and phyllosphere of potato could emit volatiles containing hydrogen cyanide to inhibit completely growth of *P. infestans* [39]. In our present study, volatiles from *P. putida* No. 24 inhibited fungal conidiation of *Shiraia* sp. S9 and shortened the distance of hyphal branches on solid plates (Fig. 2C). In mycelium culture, although *Shiraia* biomass was not altered by the volatiles, the fungal pellet diameter was suppressed markedly (Fig. 3B and C). This result suggested an antagonistic action of the VOCs from *P. putida* No.24 against the host fungus *Shiraia*. Furthermore, the VOCs of tested *Pseudomonas strains* from *Shiaria* fruiting bodies were found to enhance perylenequinone contents in the host fungus (Fig. 1B and Table 1). *Shiraia* species are pathogenic fungi in bamboo, which could utilize photoactive perylenequinones to induce ROS generation for the indiscriminate cellular damages during the infection [40,41]. We found the host *Shiraia* lived in association with a consortium of dominating bacteria belonging to *Bacillus* and *Pseudomonas* in the fruiting bodies [10]. The induced fungal perylenequinones by bacterial VOCs suggested a possible defensive response of *Shiraia* fungus to the presence of antagonize bacteria in the fruiting body. This is the first report of VOC production by *Pseudomonas* species in the fruiting bodies and their effects on fungal perylenequinone biosynthesis. Our results suggested a competitive or even more complex relationships between the bacteria and fungi in fruiting bodies.

In our previous studies, ROS generation and oxidative stress were found as early events for the abiotic elicitation on perylenequinone biosynthesis in *Shiraia* under the treatment of ultrasound [22], bamboo charcoal powder [42] and Triton X-100 [43]. Parmagnani et al. (2023) reported that VOCs produced by *Erwinia amylovora* enhanced shoot and root growth of *Arabidopsis thaliana* by triggering ROS burst from few minutes upon exposure [44]. The mixture of *Bacillus* spp. VOCs exhibited inhibitory effects on *S. sclerotiorum* via ROS accumulation in fungal hyphae cells [21]. In this study, PPVOC treatment also resulted in a significant ROS generation (Fig. 5A and B). The enhanced NOX and SOD activity induced by the bacterial volatiles (Fig. 5D) could be responsible for ROS generation [45]. The induced oxidative stress was also verified by substantial increasement in activities of antioxidant enzymes (POD and CAT) (Fig. 5E and F). Semighini et al. (2006) suggested that ROS participated in farnesol-induced apoptosis in *Aspergillus nidulans* [46]. The apoptosis-like cell death of *Fusarium oxysporum* f. sp. *cucumerinum* and *Penicillum digitatum* induced by the isooctanol was induced by ROS [47]. You et al. (2013) found further evidences to demonstrate that the apoptosis of *Ganoderma lucidum* induced by aspirin was essential to the improved biosynthesis of ganoderic acids [30]. In our present study, ROS was involved in the PPVOC-induced cell apoptosis (Fig. 6). The bacterial volatiles upregulated key gene expressions for perylenequinone biosynthesis [48,49], including *FAD*, *MCO*, *Mono* and *PKS* (Figs. 8 and 9). ROS production was demonstrated to mediate the enhanced transcriptional levels of *MCO*, *ZFTF* and *FAD* (Fig. 8). To the best of our knowledge, this is the first report showing that perylenequinone biosynthesis is linked to ROS generation and fungal apoptosis induced by bacterial VOCs (Fig. 9). In other hand, PPVOCs were found to increase membrane permeability of *Shiraia* sp. S9 (Fig. 7A and B). Zhang et al. (2021) reported that the VOCs produced by *P. chlororaphis* subsp. *aureofaciens* SPS-41 destroyed the integrity of cell membrane of *Ceratocystis fimbriata* [50]. Yue et al. (2022) found that *P. fluorescens* ZX-producing VOCs could increase the cell membrane permeability and malondialdehyde (MDA) content of *B. cinerea* [51]. In this study, ROS generation was proved to be involved in the induced membrane permeability (Fig. 7). Furthermore, the bacterial volatiles promoted not only the accumulation of HA in mycelium (Fig. 3D), but also the release of HA into the medium (Fig. 3E). These results still indicated that the efflux of perylenequinones was stimulated by enhancing fungal membrane permeability under the VOC treatment (Fig. 9).

5. Conclusions

In our study, the bacterial volatiles of *Pseudomonas putida* No.24 were found to have eliciting activity to induce fungal responses of *Shiraia* sp. S9, including ROS generation, cell apoptosis, increased membrane permeability and perylenequinone biosynthesis. *Pseudomonas putida* No.24 was one of dominant bacteria colonizing in the *Shiraia* fruiting body. The VOCs comprising dimethyl disulfide, 2-tridecanone, 2-undecanone, 2-nonanone, phenylethyl alcohol, thiocyanic acid methyl ester and butylated hydroxytoluene were verified to have eliciting activity on *Shiraia* perylenequinone production. The ROS generation induced by volatiles could serve as a signal to mediate the cell apoptosis, membrane permeability and the gene expression for fungal perylenequinone biosynthesis. In the submerged volatile co-culture, HA production reached 249.85 mg/L on day 8, about 2.07-fold that of the control group. From a biotechnological point of view, the increased production of HA, a PDT agent in mycelium culture is of great practical value. These findings can also facilitate further understanding in regulatory roles of bacterial VOCs in fruiting bodies.

CRediT authorship contribution statement

Rui Xu: Data curation, Methodology, Investigation, Writing – original draft, preparation. **Qun Yan Huang:** Investigation, Resources. **Wen Hao Shen:** Investigation. **Xin Ping Li:** Investigation. **Li Ping Zheng:** Methodology, Writing – review & editing, Supervision. **Jian Wen Wang:** Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

There are no conflicts of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 82073955 and 81773696) and the Priority Academic Program Development of the Jiangsu Higher Education Institutes (PAPD).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2023.06.004.

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