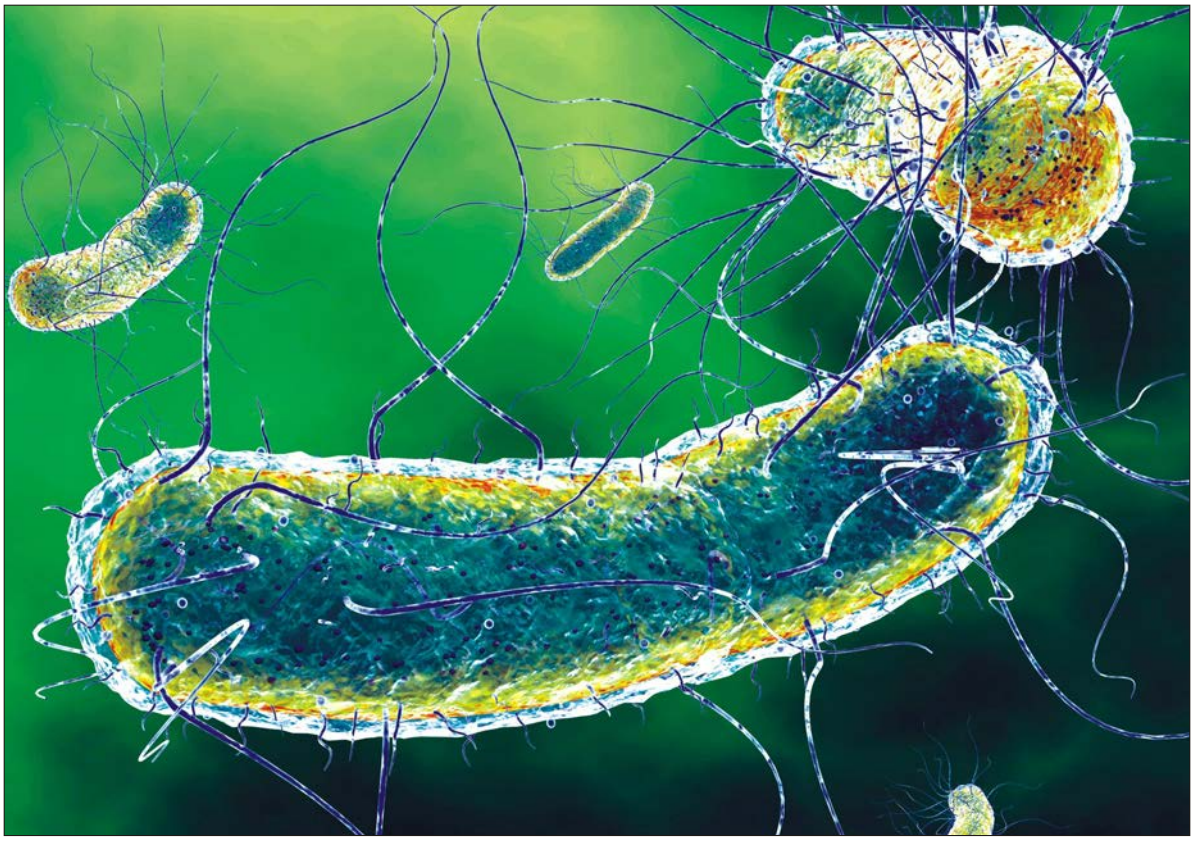

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Antifungal Activities and Components of VOCs Produced by *Bacillus subtilis* G_8

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Abstract: *Bacillus subtilis* G_8, isolated from soil in China, produced antifungal volatile organic compounds (VOCs). Bioassay in sealed dishes revealed that these volatiles significantly inhibited the mycelial growth and completely prevented the pigment production of all tested soil-borne plant pathogens (43-93% inhibition, respectively) and effectively controlled the overwintered sclerotoid germination of *Sclerotinia sclerotiorum*. Such effective antifungal VOCs were extracted using Solid Phase Microextraction (SPME) and given further identification by Gas Chromatography-Mass Spectrometry (GC-MS) technique. The detected volatile compounds included alkyls, alcohols, esters, ketones, acid, amine, oxime, phenols and heterocyclic compounds. Present results demonstrate that soil bacteria are rich resources of bioactive volatiles and may play an important role in reducing disease levels.

Key words: Antifungal activity, *Bacillus subtilis*, soil-borne diseases, SPME-GC/MS, VOCs

INTRODUCTION

Soil-borne disease is one of the most serious diseases of vegetables and crops in the world. Several strategies such as crop rotations, breeding resistant varieties and applying chemical fungicides may be useful, however, these cultural practices alone are rarely adequate and resistant cultivars are effective only against certain specific fungi species. Chemical fungicides, though usually more effective than other strategies, have caused significant environmental problems and fungicide-resistance. These years, biocontrol, for example, the application of antagonistic fungi and bacteria (Paulitz and Bélanger, 2001; Minuto and Spadaro, 2006), seems to arouse our great interest because it is eco-friendly, safe and may provide long-term protection to the crop (Fernando *et al*., 2005).

Recently, volatile compounds produced by fungi and some bacteria have been demonstrated with the potential antifungal or nematicidal nature by several studies (Alstrom, 2001; Wheatley, 2002; Xu *et al*., 2004; Zou *et al*., 2007; Gu *et al*., 2007) and the application of antifungal volatiles from fungi had been carried out in greenhouse (Mercier and Manker, 2005; Koitabashi, 2005). However, little is known about the effective volatiles produced by *B. subtilis* in the broad-spectrum control of soilborne plant diseases. In this study, eight pathogenic fungi that can colonize a wide range of host plants were chosen for bioassay and the method of SPME-GC/MS was expatiated for the extraction and identification of bacterial VOCs.

MATERIALS AND METHODS

Bacterial Culture

Bacillus subtilis G_8 was isolated from the soil in greenhouse in China and maintained in Nutrient Broth (NB) supplemented with 20% (v/v) glycerol at -20°C before use. For bioassay and analysis of volatile compounds, bacterium was inoculated into 25 mL TSB-YE (Bacto-tryptone 15 g L^{-1}, soya

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peptone 5 g L^{-1}, yeast extract 6.5 g L^{-1}, NaCl 5 g L^{-1}, pH 7.2) in 50 mL sample vial. The vial was rapidly sealed with parafilm (Menasha) and cultured at 30°C under agitation (180 rpm) in the dark up to an OD_{600} of 1.0-1.5.

Pathogenic Fungi and Storage Conditions

Eight pathogenic fungi that can colonize a wide range of host plants and have consistent high virulence, including *S. sclerotiorum*, *Botrytis cinerea*, *Alternaria brassicae*, *Alternaria solani*, *A. citrullina*, *Fusarium oxysporum*, *Cercospora kikuchii* Chupp, *Rhizoctonia solani*, were used for the bioassay in sealed dishes (Fig. 1). For short-term maintenance, fungi were cultured on PDA plates at 22~25°C under darkness. For long-term storage, tiny pieces of an actively growing colony were brought into Potato Dextrose Agar (PDA) slants at 4°C.

Antifungal Activities of Bacterial Volatiles Against Mycelial Growth

Briefly, in sealed dishes (Fernando *et al*., 2005), 200 µL bacterial cultures (10^8 cfu mL^{-1}) described above were spread onto the bottom dish of a sterile Petri-dish containing TSA-YE (Bacto-tryptone 15 g L^{-1}, Soya peptone 5 g L^{-1}, yeast extract 6.5 g L^{-1}, NaCl 5 g L^{-1}, agar 15 g L^{-1}, pH 7.2). A 5 mm mycelial plug was taken from the margin of the colony and placed in the centre of a second bottom dish containing fresh PDA. The bacterial dish was immediately inverted over the fungi dish and the dishes were rapidly sealed with parafilm. The dishes were incubated at 25°C in the dark (Fig. 2). Volatiles from TSB-YE and indoor sterile air instead of bacterial volatiles serve as controls. The diameter (mm) of the fungal colony was measured in a crisscross fashion when the radial mycelium of the controls extended to 3/4 plate. There were four replicates for each treatment and the experiments were repeated twice.

Inhibition Effects of Volatiles on Sclerotoid Germination

The adult sclerotia of *S. sclerotiorum* were collected to a sterile Petri plate and stored at -4°C for two months before use. Briefly, the sclerotia with similar size and quality was selected and placed in the centre of a petri dish (90 mm diam) containing fresh PDA. Then 200 µL of bacterial cultures were spread onto another petri dish containing TSA-YE. The treatments with bacterial volatiles in sealed dishes were carried out as described above. The diameter of radial mycelial around germinated sclerotia was determined every 24 h until seven days. The control dishes had no bacterium. There were four replicates for each treatment and the experiments were repeated twice.

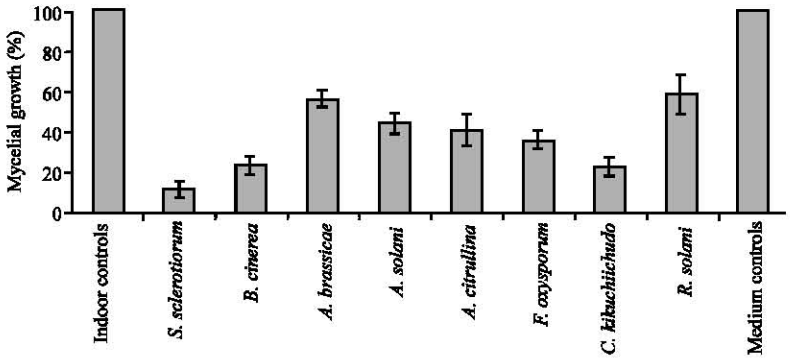

Fig. 1: Radial mycelial growth of different pathogenic fungi exposed to bacterial volatiles in sealed dishes. The growth of radial mycelium was determined and the percent inhibition compared to the controls (indoor controls and medium controls) was calculated. Error bars indicate±SD

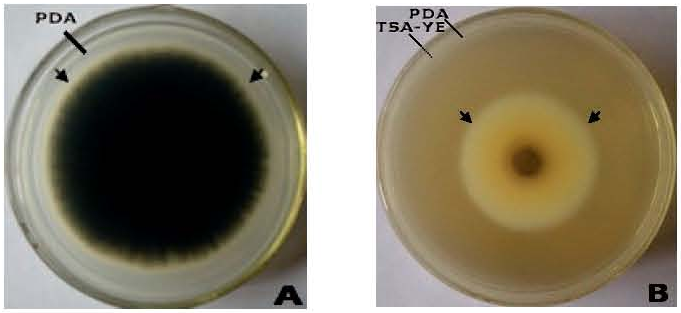

Fig. 2: Antifungal activities of bacterial volatiles against *Alternaria solani* in sealed dishes. Volatiles produced by G_8 on the TSA-YE dish presented visible inhibition effects on the mycelial growth and the pigment production of *A. solani* (B), whereas the mycelium grew normally and abundant of pigment was produced in the control dishes in absence of bacterial volatiles (A)

Volatile Organic Compounds Extraction

Bacterium was cultured as described above. Briefly, three SPME fibers (100 μm PDMS, 65 μm PDMS/DVB, 50/30 μm CAR/DVB/PDMS, purchased from SUPELCO) were chosen to extract volatiles and conditioned with helium at 250°C for 2 h before use, respectively. The sample vial was clamped inside a thermostatic water bath and placed on a hot stirrer. Samples were equilibrated at 40°C for 30 min. The SPME needle was allowed to pierce through the parafilm and the fiber was exposed to the headspace of the sample vial for 40 min. The VOCs from 25 mL TSB-YE medium was used as controls. After extraction, the SPME fiber was directly inserted into the front inlet of the gas chromatography.

GC/MS Parameters and Analysis

SPME fibers were desorbed at 210°C for 1 min in the injection port of GC/MS-QP2010 (70 ev; SHIMADZU) equipped with a Rtx-5 Capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness, Restek). GC/MS runs were 32 min and the fibers were conditioned at 210°C for 10 min before re-used. The injection port was operated in split mode at a ratio of 5:1. The carrier gas was He. The initial oven temperature was 40°C, held for 2 min, ramped at 6°C min^{-1} to 180°C and ramped at 10°C min^{-1} to 250°C and held for 3 min. The temperature of the transfer line and ion trap were 200 and 230°C, respectively. The ions were detected in the range 30-350 m/z. The mass spectra of the unknown compounds were compared with those in the NIST05 Library.

RESULTS AND DISCUSSION

Volatiles Produced by G_8 Displayed a Broad-Spectrum Antifungal Activity

It is obvious that all the mycelial growth in treatment dishes full of antifungal volatiles was significantly restricted, as compared to those in the two control dishes without bacterial volatiles (Fig. 1). However, it seemed that there was species-specificity among different fungi. Volatiles from G_8 inhibited mostly the growth of *S. sclerotiorum*, *B. cinerea* and *C. kikuchii* Chupp. (>75% inhibition, $p>0.05$), whereas the inhibition against *A. brassicae* and *R. solani* was lesser than 46% inhibition ($p>0.05$). This result implied the antifungal potential and the possibility of bacterial volatiles in soil plant diseases. The mycelium treated with volatiles from TSB-YE medium (medium controls) had the same growth rate with that in control dishes full of indoor sterile air (indoor controls, Fig. 1), which indicated that volatiles from TSB-YE had no antifungal activity.

Antifungal Effects of Volatiles on Fungal Pigments

Previously, the pigments of pathogenic fungi, such as melanin, were testified nearly interrelated with fungal pathogenicity and could endow fungi some special recovery function, such as anti-radiation, anti-oxidation, scavenging free radical, etc. (Cao and Yang, 2006; Souad *et al*., 2002). Some fungicides, such as tricyclazole and carpropamid, killed fungi mostly by inhibiting the production of melanin. In this study, we found that volatiles from G_8 strain exhibited visible inhibition to the pigments of all tested pathogenic fungi, including *B. cinerea*, *A. brassicae*, *A. solani*, *A. citrullina*, *F. oxysporum*, *C. kikuchii* Chupp., *R. solani*. A case in point was shown in Fig. 2. Therefore, it seems that these volatiles would be possible to play a significant role in reducing the pathogenic fungal infection ability. That could also be a positive support for bacterial volatiles in reducing disease level.

Inhibition Effects of Volatiles on Sclerotoid Germination

When transferred to fresh PDA culture medium, the overwintered sclerotia of *S. sclerotiorum* in control dishes gradually germinated within 24~48 h and their radial mycelium could overgrow the whole PDA dishes within four days after treatment (Fig. 3). However, the sclerotia in treatment plates full of antifungal volatiles could not germinate at all even if cultured on fresh PDA for seven days. The prevention of overwintering sclerotoid germination is the key in the control of *S. sclerotiorum*, which would reduce the apothecial formation, further decimate ascospore infection and would consequently reduce plant diseases (Abawi and Grogan, 1979). Therefore, if these volatiles could be applied in greenhouses, the diseases caused by *S. sclerotiorum* would be first suppressed effectively.

Determination and Analysis of Antifungal VOCs from G_8

Eventually, a 50/30 μm CAR/DVB/PDMS fiber was chosen to extract the VOCs from G_8, because of the most peak numbers and peak area of extracted compounds (Fig. 4). Totally, thirty organic compounds were determined by SPME-GC/MS, including alkyls, alcohols, esters, ketones, organic acid, amine, oxime, phenols as well as some heterocyclic compounds (Fig. 4, Table 1).

Mass spectra were obtained using the scan modus (total ion count, 30-350 m/z). The confirmation of compound identity was done by comparison of the retention time and mass spectra with those in the NIST05 library (similarity index >80, Fig. 5).

Here we selectively focused on these small organic molecules (molecular mass <300) that characteristically have a high vapour pressure and easily volatilize. Such VOCs are ideal infochemicals

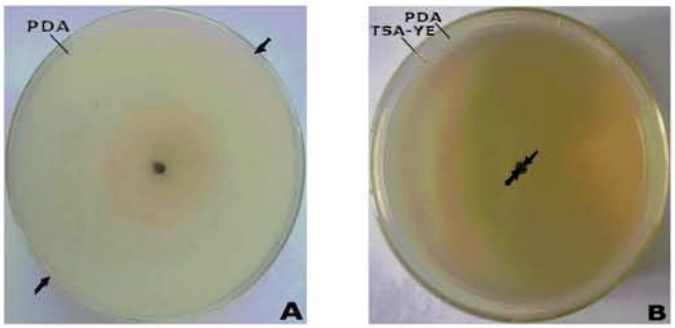

Fig. 3: Inhibition effects of bacterial volatiles against sclerotoid germination of *S. sclerotiorum* in sealed dishes. The sclerotia exposed to the antifungal volatiles from G_8 could not germinate at all (B) even if cultured on fresh PDA for seven days (The diam of radial mycelium was 0 mm). However, the sclerotia in control dishes germinated normally and the mean diam of the radial mycelium had reached 21 mm (A) after cultured for four days

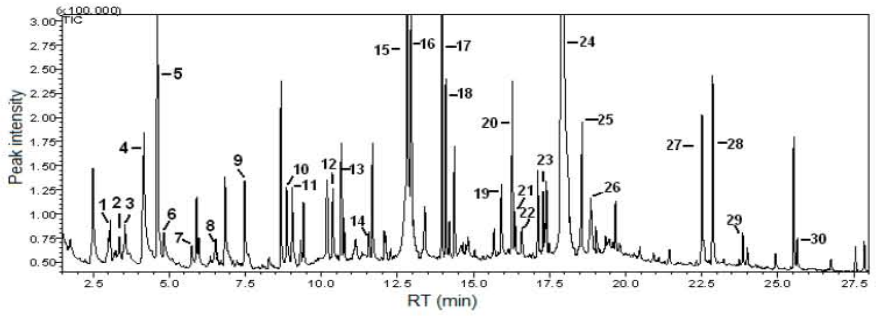

Fig. 4: GC profiles of bacterial volatile. These headspace volatiles were extracted by SPME and analyzed by a GC/MS-QP2010 from Shimadzu. The determined peaks of organic compounds were orderly numbered from 1 to 30

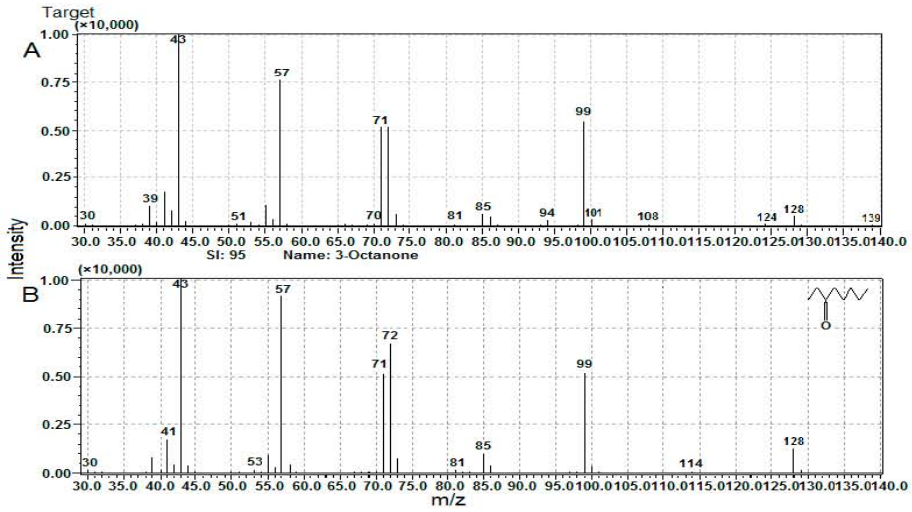

Fig. 5: GC-MS analysis of 3-Octanone. The mass spectrum of determined target and the compound 3-Octanone with 95% similar index searched by NIST05 Library was shown as A and B, respectively

because they can act over a wide range of distances and their spheres of activity will extend from proximal interactions to greater distances via diffusion in air, including in soil pores (Wheatley, 2002).

It seems that volatiles produced by G_8 contain more than one kind of bioactive compounds and the different compounds may determine the different antagonistic natures of bacterial volatiles. Gu *et al.* (2007) found that phenol, 2-octanol, 2-nonanone and 2-undecanone displayed 100% nematicidal activities to both free-living nematode *Panagrellus redivivus* and pinewood nematode *Bursaphelenchus xylophilus*. Among those, phenol is known for its toxic effects on cells and has been used as an antiseptic in clinical applications for a long time. Besides, benzothiazole, cyclohexanol, 2-ethyl-1-Hexanol and nonanol could completely inhibit the mycelial growth of *S. sclerotiorum* (Fernando *et al.*, 2005), but some ketones like 2-udecanone and 2-tridecanone had no inhibition. In our study, such effective compounds were also determined, whereas the antifungal natures of other tested novel compounds (such as oxime and morpholine) need to be further studied. The analysis for these

Table 1: VOCs from *B. subtilis* G_8 determined by SPME-GC/MS method

Peak	RT (min)	Name of compound	Molecular formula	Classify
1	3.063	Acetic acid, diethyl-	$C_4H_8O_2$	Acid
2	3.362	o-Xylene	C_8H_{10}	Alkyl
3	3.549	p-Xylene	C_8H_{10}	Alkyl
4	4.168	2-Heptanone	$C_7H_{14}O$	Ketone
5	4.617	Oxime-, methoxy-phenyl-	$C_7H_9O_2N$	Oxime
6	4.700	Ethanone, 1-(2-furanyl)-	$C_6H_7O_2$	Ketone
7	5.748	2-Heptanone, 6-methyl-	$C_8H_{16}O$	Ketone
8	6.490	3-Octanone	$C_8H_{16}O$	Ketone
9	7.482	1-Hexanol, 2-ethyl-	C_8H_{18}	Alcohol
10	8.863	2-Nonanone	$C_9H_{18}O$	Ketone
11	9.048	2-Nonanol	$C_9H_{20}O$	Alcohol
12	10.317	2-Decanone	$C_{10}H_{20}O$	Ketone
13	10.369	2-Octanol, 3-methyl-	$C_9H_{20}O$	Alcohol
14	11.562	Benzothiazole	C_7H_5NS	Heterocyclic
15	12.827	2-Undecanone	$C_{11}H_{22}O$	Ketone
16	12.953	2-Tridecanol	$C_{13}H_{28}O$	Alcohol
17	13.968	2-Dodecanone	$C_{12}H_{24}O$	Ketone
18	14.089	2-Dodecanol	$C_{12}H_{26}O$	Alcohol
19	15.917	1-Dodecanol	$C_{12}H_{26}O$	Alcohol
20	16.280	2-Tridecanone	$C_{13}H_{26}O$	Ketone
21	16.369	2-Heptadecanol	$C_{17}H_{36}O$	Alcohol
22	16.592	Butylated Hydroxytoluene	$C_{15}H_{24}O$	Phenol
23	17.288	2-Tetradecanone	$C_{14}H_{28}O$	Ketone
24	17.908	Diethyl Phthalate	$C_{12}H_{14}O_4$	Ester
25	18.503	Phenol,2,6-bis(1,1-dimethylethyl)-4-(1-methylpropyl)-	$C_{18}H_{30}O$	Phenol
26	18.800	4-Ethylcyclohexanol	$C_8H_{14}O$	Alcohol
27	22.512	Morpholine, 4-octadecyl-	$C_{22}H_{45}NO$	Heterocyclic
28	22.861	1-Hexadecanamine, N,N-dimethyl-	$C_{18}H_{39}N$	Amine
29	23.851	Dibutyl phthalate	$C_{16}H_{22}O_4$	Ester
30	25.645	9-Octadecenoic acid, methyl ester	$C_{19}H_{38}O_2$	Ester

novel structures could give us a revelation that some of the bacterial volatiles could be used as main skeleton for developing novel fungistatic or nematicidal agents by further chemical modifications.

The *B. subtilis* G_8 strain cultured in the TSB-YE medium with rich nutrition produced different bioactive compounds, therefore, such antagonistic volatiles-producing bacterium may have the potential to be effective biocontrol agents against soil-borne pathogens (such as fungi and nematodes) and less likely to select for resistance than synthetic fungicides composed of a single compound. However, the application of bacterial volatiles in the control of pathogens in greenhouse was under way.

In addition, the method used in this study may be useful for the extraction and identification of volatile metabolites produced by other microorganism (bacteria or fungi etc).

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