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Possible contributions of volatile-producing bacteria to soil fungistasis

Chang-Song Zou[1], Ming-He Mo[1], Ying-Qi Gu, Jun-Pei Zhou, Ke-Qin Zhang*

Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, Kunming 650091, PR China

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Abstract

Soil fungistasis can adversely affect the germination and growth of most fungal species in the field. Among the inhibitors, volatiles of microbial origins are potentially very important. In this study, we investigated the frequency and identity of bacteria producing fungistatic volatiles. Among the 1018 bacterial isolates tested, 328 were found to produce antifungal volatiles that could inhibit spore germination and mycelial growth of two nematicidal fungi *Paecilomyces lilacinus* and *Pochonia chlamydosporia*. A phylogenetic analysis based on restriction fragment length polymorphism (RFLP) and 16S rDNA sequence placed the 328 bacteria in five groups: *Alcaligenaceae, Bacillales*, *Micrococcaceae*, *Rhizobiaceae* and *Xanthomonadaceae*. Volatile compounds of 39 bacterial isolates were identified by gas chromatography/mass spectrum (GC/MS). Tests with commercially available antifungal compounds suggested that seven volatile compounds of bacterial origins (acetamide, benzaldehyde, benzothiazole, 1-butanamine, methanamine, phenylacetaldehyde and 1-decene) likely play important roles in soil fungistasis.

Keywords: Soil fungistasis; Bacteria; Volatiles; GC/MS

1. Introduction

Plant-parasitic nematodes are important agricultural pests (Sasser and Freekman, 1987). Several strategies including crop rotations, resistant crop breeding, and chemical or biological nematicide applications are currently used to combat these pests. Among these, the use of biocontrol agents is preferred due to its environmental friendliness. Two nematophagous fungi *Pochonia chlamydosporia* (Butt et al., 2001) and *Paecilomyces lilacinus* (Liu et al., 2004) have been developed into commercial products and used to control nematodes in the field for years. Unfortunately, due to soil fungistasis, these fungal agents displayed variable effects against nematodes in the field (Zhou and Mo, 2002). As a result, fungal biocontrol agents have not reached a reliable and consistent level for farmers to want to use them routinely. The reluctance by farmers may have restricted their application and commercial exploitation in the scientific community.

Soil fungistasis results in the suppression of either germination or growth of fungi. This phenomenon occurs in most natural soils. Soil fungistasis was first described by Dobbs and Hinson (1953). The intensity of fungistasis has been found to vary depending on the physical and chemical properties of soils as well as on soil microbial activity (Dobbs and Gash, 1965; Lockwood, 1977; Mondal and Hyakumachi, 1998; Alabouvette, 1999). Two popular hypotheses, nutrient-deprivation (Lockwood, 1964, 1977) and antibiosis (Romine and Baker, 1973), have been proposed to explain the mechanism of soil fungistasis. The former states that exogenous energy sources may be unavailable for germinating spores due to strong competition by other soil microorganisms. The latter emphasizes that soil fungistasis can be caused by antifungal compounds produced by other soil microorganisms. Fungistasis has been found to influence both the total microbial population (Chinn and Ledingham, 1967) and the fungal population (Mishra and Kanaujia, 1973). Recent developments suggested that specific groups of

*Corresponding author. Tel.: +86 871 5034878; fax: +86 871 5034838.
E-mail address: kqzhang111@yahoo.com.cn (K.-Q. Zhang).
[1]Authors Chang Song Zou and Ming He Mo contributed equally to this work.

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microorganisms might play an important role in the process of soil fungistasis (Alabouvette, 1999; Ellis et al., 2000; De Boer et al., 2003).

It has been shown that the fungistatic substances in soil include both volatile and non-volatile inhibitors. Volatile compounds may occur in the soil atmospheres at a range of concentrations (Wheatley et al., 1996) and their participation in soil fungistasis has been demonstrated (Hora and Baker, 1970; Smith, 1973; Lynch and Harper, 1974a, b; Balis, 1976; Lockwood, 1977; Xu et al., 2004). These volatile inhibitors can be both inorganic (Ko and Hora, 1974) and organic (Xu et al., 2004) in nature. Volatile inhibitors that have been identified include ethylene (Hora and Baker, 1970; Smith, 1973), ammonia (Ko and Hora, 1974; Ko et al., 1974), allyl alcohol, acrylic acid (Balis, 1976), trimethylamine, benzaldehyde, and *N*, *N*-dimethyloctylamine (Xu et al., 2004). The spectrum of volatile compounds produced appears to depend not only on environmental conditions, such as nutrients (Fiddaman and Rossall, 1994; Wheatley et al., 1996, 1997), temperature (Tronsmo and Dennis, 1978), pH and moisture, but also on the organism itself (Zechman and Labows, 1985; Giudici et al., 1990). However, to our knowledge, almost nothing is known about the relationship between the identity of bacteria and their volatile fungal inhibitors. The objectives of this study were to assay the bacterial volatile inhibitors involved in soil fungistasis and to identify the bacteria producing these volatile inhibitors. The 16S rRNA gene sequencing combined with RFLP analysis was used in characterizing the volatile producers.

2. Materials and methods

2.1. Soil sampling

Tobacco fields located in three counties: Mile county (24°39′N, 103°42′E), Yanshan county (23°77′N, 103°66′E), and Binchuan county (25°35′N, 100°36′E) were chosen for sampling. All three counties are in the province of Yunnan, in southwestern China, At each location, 50 soil samples, each about 200 g, were taken randomly over an area of about 100 m^2, from the top layer (2–15 cm) from several tobacco fields. All soil samples from one location were mixed to produce one representative sample for the location. Soils were sieved through 2 mm sieve to eliminate large soil particles and stored at 4 °C until use.

2.2. Fungi and growth conditions

Two nematocidal fungi, *Pae. lilacinus* (CGMCC no. 0241) and *Po. chlamydosporia* (CGMCC no. 0418), were used as models for the soil fungistasis test. Fungi were cultured on potato dextrose agar (PDA) plates for 7 d at 25 °C. Conidia were scraped from the colony surface and suspended in sterile distilled water. To remove mycelial fragments and germinated conidia, the suspension was filtered through three layers of lens wiping paper and washed twice by centrifugation at 1420*g* at room temperature for 10 min. The suspension was adjusted to 2×10^4 conidia ml^{-1} by adding sterile distilled water.

2.3. Isolation of soil bacteria

A modified BPSA medium (0.37 g beef extract, 1.2 g peptone, 0.6 g NaCl, 1 L soil extract, and 15 g agar) was used to isolate soil bacteria (Ulrich and Wirth, 1999). To prepare soil extract, 1 kg of soil from each corresponding site was suspended into 1 L of tap water and shaken for 30 min at 200 rev min^{-1}. After filtration, the soil suspension was adjusted to 1 L and autoclaved. For each sample, 10 g soil was dispersed in 90 ml of sterile distilled water and then diluted 10-fold. Aliquots (0.2 ml) of the diluted samples were spread onto BPSA plates. Ten plates were prepared for each sample. After incubation for 5 d at 25 °C, more than 300 bacterial colonies with at least 30 per plate were randomly picked for determination of their volatile fungistatic substances.

2.4. Assay for soil fungistasis and bacterial volatile inhibitors

The soil fungistasis test was performed following Xu et al. (2004). Volatile fungistatic activity of bacteria was identified following Fernando et al. (2005) with some modifications. Briefly, in a three-compartment Petri dish, an individual isolate from a fresh colony was streaked onto one compartment containing BPSA, while the two other compartments were poured with a layer of 1.5% water agar (WA). The plate was wrapped in Parafilm, so that volatile compounds could diffuse into the WA. After incubation for 7 d at 25 °C, the WA surface of one compartment was spread with 20 μl of a fungal spore suspension (approximately 400 spores) and the other compartment was inoculated with a 5 mm-diameter mycelial plug cut from the margin of an actively growing culture. The plate was re-sealed with Parafilm and incubated at 25 °C. Spore germination was calculated after 48 h and radial growth of the mycelial plug was measured every 2 d. After 12 d, mycelial plugs and spores from all treated plates were removed to a fresh WA plate for viability test. For those candidates producing volatile inhibitors, a 10 ml bacterial culture incubated for 16 h in BPSB (BPSA without agar) was washed twice by centrifugation and adjusted to a concentration of 10^8 ml^{-1} by adding sterile distilled water. Then, 1.2 ml suspension or an equal volume of BPSB as control was mixed thoroughly with 20 g of autoclaved soil. Three duplicates were used for each treatment. After incubation for 7 d at 25 °C, fungal spores were added and the fungistastic activity was measured following the method described above.

2.5. RFLP analysis of the 16S rRNA sequences of bacteria producing volatile inhibitors

The genomic DNA of bacteria was extracted using a bacterial genomic DNA extraction kit (BioTeke Corporation,

China, Cat#:DP2001). Bacterial 16S rRNA genes were amplified by PCR using a combination of universal primer 1492r (5′GGT TAC CTT GTT ACG ACT T 3′) and bacterial primer 27f (5′ AGA GTT TGA TCC TGG CTC AG 3′) (Lane, 1991). The amplified products were purified with agarose gel DNA purification kit (Takara, code DV805A). To select isolates for sequencing, the purification products were analyzed first by RFLP using separate enzymatic digestions with *Hha*I (TaKara), *Msp*I (TaKara) and *Hae*III (TaKara) endonucleases. The digested DNA fragments were electrophoresed in 4.0% agarose gels. After staining with ethidium bromide, the gels were photographed using an image-capture system UVI^{TEC} DBT-08, and scanning image analyses were performed manually.

2.6. DNA sequencing and phylogenetic analysis

One to six clones from each unique RFLP type were submitted to the Beijing Genomics Institute for sequencing. The resulting sequences (about 1430 bp from each) were compared with those available in GenBank by use of the BLAST network service to determine their phylogenetic affiliation. Phylogenetic analysis was performed using the MEGA software packages (Version 3.1) (Kumar et al., 2004) after multiple alignments of data by CLUSTAL_X (Thompson et al., 1997), with gaps treated as missing data. The relationships among sequences were analyzed using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by analyzing 1000 randomized data sets (Felsenstein, 1985).

The 16S rRNA gene sequences have been deposited in the NCBI nucleotide sequence database under Accession nos. DQ993294–DQ993332.

2.7. Identification of fungistatic volatile inhibitors of bacteria and soils

Volatiles were extracted from soil samples or fresh bacterial cultures in BPSB by solid-phase micro-extraction (SPME) method (Augusto and Valente, 2002; Wady et al., 2003; Xu et al. 2004) using a 75 μm fiber (Supelco, Bellefonte, PA, USA). After extraction, the SPME fiber was directly inserted into the front inlet of a gas chromatograph (GC, HP 6890A) connected to a mass spectrometer (MS, HP 5973, GC/MS: Agilent Technologies, USA) and desorbed at 250 °C for 2 min. GC conditions were similar to those described previously by Xu et al. (2004). Standard Wiley27 and NBS75 K MS-libraries were used to identify volatiles. Each sample was tested twice. The fungistatic activities of candidate volatile compounds were confirmed using pure commercial compounds following the method of Xu et al. (2004).

2.8. Data analysis

Inhibition of spore germination and mycelial growth from triplicate assays were calculated using the following formula: germination or growth inhibition = (germination or growth in the negative control–germination or growth in the treatment)/(germination or growth in the negative control) × 100%. The inhibitory effect of more than 75% was considered as strong inhibition, 50–75% as moderate, 25–50% as low, and 10–25% as slight. Data were analyzed using the analysis of variance (ANOVA), and means were compared by the test of least significant difference (LSD) at $P = 0.05$ using SPSS 11.0 for Windows (SPSS Inc., Chicago, USA).

3. Results

3.1. Volatile fungistasis of soils and soil bacteria

The three soil samples differed considerably in volatile fungistasis action against the two nematophagous fungi *Pae. lilacinus* and *Po. chlamydosporia*. Volatiles of the Mile and Yanshan samples exhibited equally strong fungistatic effects in terms of both conidial germination (92.1% and 94.1%) and radial growth inhibition (75.9% and 82.5%) for the two fungi. However, soil from the Binchuan displayed little volatile fungistasis both in terms of conidial germination (germination inhibition < 16%) and mycelial growth (growth inhibition < 11.2%), and its activity was significantly less than those of the other two soil samples ($P < 0.01$).

Among the 1018 bacterial isolates randomly picked from the three soil samples, 328 showed inhibitory effect against conidial germination and mycelial growth to varying degrees (Table 1). Of the 328 isolates, 149 (out of 347), 131 (out of 351), and 48 (out of 320), were from Mile, Yanshan and Binchuan soils, respectively. This result suggested that the soil with a stronger fungistasis had a higher proportion of fungistatic bacteria. All 328 isolates produced volatile inhibitors also inhibit mycelial growth, whereas only 219 isolates inhibited spore germination of both *Pae. lilacinus* (mean inhibition = 99.2%) and *Po. chlamydosporia* (mean inhibition = 95.4%). The fungistatic activity of the remaining 109 isolates was restricted only to inhibition of mycelial growth. There was a significant variability in inhibitory effects against mycelium growth among the 328 bacterial isolates. The proportion of bacterial isolates showing strong, moderate, low and slight fungistatic activities were 76.2%, 14%, 9.8% and 0 to *Pae. lilacinus*, and 71%, 19.2%, 9.2% and 0.6% to *Po. chlamydosporia*, respectively (Table 1). When mycelial plugs or conidia exposed to fungistatic volatiles of soils or bacterial cultures for 12 d were transferred to fresh agar plates, all fungal propagules grew or germinated normally, indicating the non-fungicidal nature of the volatiles.

Of the 328 isolates, 39 were randomly selected to determine their volatile fungistasis by growing them in the autoclaved soil. All bacteria showed similar antifungal intensities to those detected using bacterial cultures on BPSA (data not shown). This suggested that the artificial

Table 1
The intensity of antifungal activity by volatiles produced by 328 soil bacterial isolates

Model fungi	Proportion of soil bacteria with volatile antifungal activities (%)					Mean inhibition ± SD (%)	
	Spore germination	Mycelium growth					
		Strong[a]	Moderate	Low	Slight	Spore germination	Mycelium growth
Pae. lilacinus	66.8	76.2	14.0	9.8	0.0	99.2 ± 0.5	80.9 ± 2.5
Po. chlamydosporia	66.8	71.0	19.2	9.2	0.6	95.4 ± 0.3	83.3 ± 2.3

[a]Degree of fungistasis. Strong, 100–75% inhibition; moderate, 75–50% inhibition; low, 50–25% inhibition; slight, 25–10% inhibition.

medium used to evaluate the bacteria that produced volatile fungistatic inhibitors was suitable and effective.

3.2. RFLP analysis

The 16S rRNA gene of the 328 bacteria that produced volatile fungistatic substances was successfully amplified using the primer pair of 27F and 1492R. Each strain produced a characteristic single band of about 1500 bp. All PCR products were subjected to RFLP analysis using separate enzymatic digestions with three endonucleases *Hha*I, *Msp*I and *Hae*III. Totally, 20 different RFLP types were obtained, of which 12 were common albeit with varying abundances for the different soil origins (Table 2). The 149 and 131 isolates from Mile and Yanshan were found to contain 18 different RFLP types, respectively, whereas the 48 isolates from Binchuan were represented by 15 RFLP patterns. The two largest groups, type F and G, together accounted for 15.2% (8.2 and 7.0% for each) of the isolates analyzed. The prevalences of groups C, I, L, X and Y, which included 19–22 isolates, were also relatively large. Of the remaining 13 RFLP patterns, each was represented by 9–17 isolates (Table 2).

Table 2
16S rDNA RFLP types of bacteria identified from distinct sampling positions

RFLP type	Isolate number and origin			Total
	Mile	Yanshan	Binchuan	
A	4	3	3	10
B	6	8	2	16
C	10	5	6	21
E	0	11	5	16
F	17	8	2	27
G	11	9	3	23
H	9	4	0	13
I	5	11	3	19
J	9	0	0	9
K	7	2	0	9
L	11	6	3	20
M	0	11	1	12
N	8	9	0	17
P	11	4	0	15
U	6	7	2	15
V	5	8	3	16
W	5	5	4	14
X	9	11	2	22
Y	9	9	4	22
Z	7	0	5	12

3.3. Phylogenetic analysis

Based on RELP pattern, 39 isolates, at least one from each unique RFLP type, were submitted for 16S rDNA sequencing (Table 3). The resulting sequences, about 1430 nucleotides for each of the 39 strains, were compared with those available in the GenBank by the use of the BLAST network service to determine their approximate phylogenetic affiliation (Table 3). Our analysis showed that all the sequences have identical matches to those in the GenBank as of November 2006, and all were closely related to the cultivated *Bacteria* members (sequence identity of 97–100%). The 39 sequenced isolates were placed in five groups of the domain Bacteria: (i) *Alcaligenaceae*, (ii) *Bacillales*, (iii) *Micrococcaceae*, (iv) *Rhizobiaceae*, (v) *Xanthomonadaceae* (Table 3).

Of the five groups, members of the *Bacillales* were dominant in the 328 isolates that produced fungistatic volatiles. The *Bacillales* group included 251 isolates represented by 15 RFLP types (A, B, C, E, F, G, H, K, L, N, U, V, W, Y and Z) and were phylogenetically associated with four genera of *Bacillales* (98–100% similarities): *Bacillus* (208 isolates, 12 types), *Planomicrobium* (12, 1), *Sporosarcina* (17, 1) and *Staphylococcus* (14, 1) (Tables 2 and 3). The remaining four groups, *Alcaligenaceae*, *Micrococcaceae*, *Rhizobiaceae* and *Xanthomonadaceae*, included 12, 15, 19 and 31 isolates, respectively. Each of the four groups was represented by a unique type except the *Xanthomonadaceae* by two (Tables 2 and 3).

3.4. Identification and confirmation of fungistatic volatiles of soils and bacteria

Analysis by the SPME-GC/MS method revealed that volatiles of the two strongly (Mile and Yanshan) and one weakly (Binchuan) fungistatic soils consisted of 18, 16 and 10 compounds, respectively (Table 4). Dimethyl disulfide, methyl pyrazine, 2,5-dimethyl-pyrazine and dodecane were present in all samples. These compounds did not inhibit the spore germination or mycelial growth of *Po. lilacinus* and *Pae. chlamydosporia*, suggesting that they are not involved

Table 3
Similarity of 16S rDNA sequences of representative volatile-producing soil bacterial isolates with those of culturable strains in the NCBI Genebank database

Isolate	RFLP type	Accession no.	Origin	Closest NCBI library strain and accession no.	Similarity (%)
MHS002	A	DQ993324	BC	*Bacillus pumilus* KT1012 AB115957	99
MHS034	A	DQ993306	ML	*Bacillus pumilus* TUT1009 AB098578	100
MHS030	B	DQ993322	ML	*Bacillus flexus* IFO15715 AB021185	100
MHS031	C	DQ993321	YS	*Bacillus licheniformis* SSH4 AB219153	99
MHS006	E	DQ993317	YS	*Bacillus subtilis* KL-077 AY030330	99
MHS007	E	DQ993316	BC	*Bacillus subtilis* KL-077 AY030331	100
MHS008	E	DQ993315	BC	*Bacillus subtilis* KL-077 AY030331	99
MHS009	F	DQ993314	YS	*Bacillus litoralis* SW-211 AY608605	99
MHS012	G	DQ993311	ML	*Bacillus weihenstephanensis* MC67 DQ345791	99
MHS010	G	DQ993313	ML	*Bacillus cereus* G8639 AY138271	99
MHS003	G	DQ993323	YS	*Bacillus cereus* G8639 AY138271	99
MHS001	G	DQ993325	YS	*Bacillus cereus* G8639 AY138271	99
MHS032	G	DQ993320	ML	*Bacillus cereus* JUN 7 DQ152243	100
MHS011	G	DQ993312	YS	*Bacillus cereus* JUN 7 DQ152244	100
MHS004	H	DQ993319	ML	*Bacillus megaterium* AC46b1 AJ717381	99
MHS016	H	DQ993308	ML	*Bacillus megaterium* AC46b1 AJ717381	100
MHS017	H	DQ993307	ML	*Bacillus megaterium* KL-197 AY030338	100
MHS039	H	DQ993302	YS	*Bacillus megaterium* KL-197 AY030338	99
MHS029	H	DQ993295	YS	*Bacillus megaterium* WN591 DQ275181	99
MHS018	H	DQ993305	YS	*Bacillus megaterium* WN591 DQ275181	99
MHS020	I	DQ993332	BC	*Ensifer adhaerens* 5D19 AJ420773	99
MHS019	J	DQ993326	ML	*Stenotrophomonas maltophilia* LMG 10857 AJ131117	99
MHS021	J	DQ993329	ML	*Stenotrophomonas maltophilia* LMG 10857 AJ131117	99
MHS015	K	DQ993309	ML	*Bacillus koguryoae* SMC 4352-2 AY904033	99
MHS022	L	DQ993304	ML	*Bacillus psychrodurans* DSM 11713 AJ277984	98
MHS013	M	DQ993330	YS	*Alcaligenes faecalis* WM2072 AY548384	99
MHS033	M	DQ993328	BC	*Alcaligenes faecalis* WM2072 AY548384	99
MHS026	N	DQ993301	ML	*Sporosarcina ginsengisoli* Gsoil 1433 AB245381	99
MHS027	P	DQ993331	ML	*Arthrobacter nitroguajacolicus* CCM 4924T AJ512504	100
MHS023	U	DQ993303	ML	*Bacillus simplex* WN579 DQ275178	100
MHS038	U	DQ993299	BC	*Bacillus simplex* WN579 DQ275178	100
MHS035	U	DQ993300	ML	*Bacillus simplex* WN570 DQ275175	99
MHS014	U	DQ993310	YS	*Bacillus simplex* WN570 DQ275175	99
MHS005	U	DQ993318	YS	*Bacillus simplex* WN570 DQ275176	100
MHS024	V	DQ993298	BC	*Bacillus silvestris* HR3-23 AJ006086	98
MHS025	W	DQ993297	YS	*Staphylococcus cohnii* CV38 AJ717378	100
MHS036	X	DQ993327	YS	*Lysobacter gummosus* KCTC 12132 AB161361	97
MHS037	Y	DQ993294	YS	*Bacillus humi* LMG 22168 AJ627209	97
MHS028	Z	DQ993296	BC	*Planomicrobium okeanokoites* IFO 12536T D55729	98

Table 4
Volatile compounds from three tobacco field soils as determined by the SPME-GC/MS method

Soil origin	Volatile compounds		Sum
	In common	In difference	
Mile	Dimethyl disulfide, methyl pyrazine, 2,5-dimethyl-pyrazine, dodecane	Benzaldehyde, phenylacetaldehyde, benzothiazole, phenylenediamine, *N*,*N*-dimethyloctylamine, nonadecane, 2-octanone, heptedecanol, 1-phenyl-Ethanone, 1H-Indole,3-ethyl-1,5-octadiene, nonanol, 2-Butanone, 2,2,4,6,6-Pentamethyl-3-heptene	18
Yanshan	Dimethyl disulfide, methyl pyrazine, 2,5-dimethyl-pyrazine, dodecane	Benzaldehyde, phenylacetaldehyde, benzothiazole, nonadecane, nonanol, 1-pentanol, cyclohexanol, 3-methyl-propanol, 2-heptanone, 9-eicosene, 1H-Indole,1-phenyl-2-propanone	16
Binchuan	Dimethyl disulfide, methyl pyrazine, 2,5-dimethyl-pyrazine, dodecane	Caryophyllene, octanal, 2-decenal, decanal, pentadecane, tributyl ester	10

Table 5
Antifungal assay of commercial compounds involving in volatiles from soils or soil bacteria

Compounds	The lowest dosage of complete inhibition[a] ($mg\,L^{-1}$)			
	Pae. lilacinus		P*o. chlamydosporia*	
	A[b]	B	A	B
Acetamide	2±0.1	53±0.2	4±0.2	67±0.5
Methanamine	7±0.4	21±0.5	9±0.7	33±0.9
1-Butanamine	20±0.9	32±1.0	21±0.8	44±0.6
Benzaldehyde	4±0.3	16±0.6	6±0.2	40±0.4
Phenylacetaldehyde	6±0.2	17±0.4	8±0.4	34±0.7
1-Decene	6±0.3	22±0.7	6±0.5	37±0.8
Benzothiazole	NI	29±0.8	NI	158±0.7
Methyl pyrazine	NI	NI	NI	NI
Dimethyl disulfide	NI	NI	NI	NI
2,5-Dimethyl-pyrazine	NI	NI	NI	NI
Dodecane	NI	NI	NI	NI
Nonadecane	NI	NI	NI	NI

NI, not inhibition with concentration = $200\,mg\,L^{-1}$.
[a]Inhibition % ⩾99%.
[b]Treatment: A, test with spore; B, test with mycelium; Inhibition concentration ± SE.

in fungistasis (Table 5). Three other compounds, benzaldehyde, phenylacetaldehyde, and benzothiazole, present in both strongly fungistatic soils, were fungistatic (Table 5).

Volatiles of the 39 isolates that had been subjected to the phylogenetic analysis were compared with that of the BSPB medium alone. Each sample produced 20–30 peaks when analyzed using the total ion current chromatography. A total of 38 volatile compounds were detected, covering a wide range of aldehydes, ketones, alcohols, aliphatic alkanes, amines, organic acids and phenolic compounds (Table 6). Among them, benzaldehyde was the most common, detected in 32 of the 38 isolates. These 32 isolates included all taxa isolated here except the genus *Sporosaricina*. The three volatiles, benzothiazole (produced by 29 isolates), 1-butanamine (produced by 14 isolates) and phenylacetaldehyde (produced by 12 isolates) were also dominant (Table 6).

To confirm the fungistatic nature of the bacterial volatiles, 29 commercial compounds with the same chemical structures as the volatiles of bacteria were tested. Among them, acetamide, benzaldehyde, benzothiazole, 1-butanamine, methanamine, phenylacetaldehyde and 1-decene exhibited antifungal activities (Table 5) suggesting their contribution to soil fungistasis.

4. Discussion

Microorganisms have been implicated as causal agents of soil fungistasis, with their mechanism of action being mediated either by limiting available carbon or producing antifungal compounds. Toyota et al. (1996) observed that the addition of a single microbial species to sterile soil aggregates never resulted in the same level of suppression against colonization by *Fusarium oxysporum* f. sp. *raphani* as observed for non-sterilized aggregates. They hypothesized that microbial diversity might be related to fungistasis. Based on a series of experiments, De Boer et al. (2003) concluded that microbial community composition was an important factor determining soil fungistasis and that the presence of antifungal activity of pseudomonads could be important in this aspect. Fungistasis can be attributed to the presence of antifungal compounds of microbiological origin (Romine and Baker, 1973; Fradkin and Patrick, 1985; Liebman and Espstein, 1992, 1994), and the production of fungus-inhibiting compounds has been described for a wide range of soil microorganisms (Fravel, 1988; Burgess et al., 1999; Behal, 2000). However, either for microbial community analysis or for discovering antifungal compounds, few studies have focused on the volatile inhibitors. Our previous study (Xu et al., 2004) had shown that volatiles of all 146 tested soils exhibited strong antifungal activity resulting in 50% reduction in spore germination and hyphal extension of three fungi. In this study, 32% of 1018 randomly selected bacteria produced fungistatic volatiles. These results suggest that volatile inhibitors of microbial origin may play an important role for determining soil fungistasis. These bacteria and the compounds that they produce should attract a greater attention in the future.

Soil fungistasis is a widespread phenomenon. However, fungistatic intensity varies with the physical and chemical properties of soil (Dobbs and Gash, 1965; Lockwood, 1977; Qian and Johnson, 1987; Mondal and Hyakumachi, 1998; Alabouvette, 1999). Other factors such as microbial community composition (De Boer et al., 2003) can also contribute to the suppression of germination and mycelial growth of soil fungi to a certain extent (Lockwood, 1977).

Members of *Bacillus* are ubiquitous in the environment (Nicholson, 2004), and have repeatedly found associated with antifungal activity by producing volatile compounds as well as non-volatile substances (Ryder et al., 1999; Bhaskar et al., 2005). Our results indicated that this group of bacteria was involved in soil fungistasis by producing volatile inhibitors. Actinomycetes are also known for their production of antifungal compounds (Andrade et al., 1994; Behal, 2000). However, in this investigation, only the genus *Arthrobacter* was found to produce fungistatic volatiles.

Hora and Baker (1974) suggested that abiotic factors could cause the production of volatile fungistatic compounds. However, we have shown that 39 bacterial isolates from soils produced antifungal compounds, with their activities demonstrated in both the three-compartment Petri dish tests and the soil tests. These results strongly suggest that fungistatic volatiles in soil are at least partially of microbial origin. These results support previous reports that many volatile organic compounds in the biosphere are probably microbial in origin (Stotzky and Schnenck, 1976; Stahl and Parkin, 1996; Fall, 1999; Xu et al., 2004).

Table 6
Antifungal activities and distribution among antifungal volatiles of 39 selected isolates

Isolate and taxonomic position[a]		Antifungal activities to *Pae. lilacinus*[b]		Antifungal volatiles							Potential fungistatic volatiles without confirmation		
		Germination	Growth (%)	A[c]	B	C	D	E	F	G	Amines	Aldehydes	Alkenes
Bacillus	MHS010	I	100.0±0.0	+			+	+				2	1
	MHS003	NI	82.2±6.4			+	+	+	+		2	3	1
	MHS001	NI	92.5±3.0				+		+		1	3	1
	MHS032	I	100.0±0.0	+			+					4	1
	MHS011	I	75.3±5.8			+	+	+			2		1
	MHS030	I	100.0±0.0	+			+	+				3	
	MHS037	I	100.0±0.0				+	+				1	
	MHS015	I	98.3±2.8	+			+	+				2	
	MHS031	I	100.0±0.0				+	+			1	2	
	MHS009	NI	61.8±5.5				+	+	+			2	
	MHS016	NI	79.6±4.1		+	+	+	+	+		3	1	
	MHS004	I	73.1±8.4	+	+		+	+	+	+	2	4	1
	MHS039	I	100.0±0.0				+	+				1	
	MHS017	I	92.5±6.3				+	+	+		1	3	
	MHS029	NI	82.7±2.3		+	+	+				1	1	1
	MHS018	NI	87.8±4.9		+	+	+		+		1	3	
	MHS022	I	81.5±1.9	+			+					1	
	MHS002	NI	65.2±2.8	+				+		+	1	4	1
	MHS034	I	100.0±0.0			+		+		+	1		
	MHS024	NI	46.3±4.4									2	
	MHS038	I	100.0±0.0			+		+			1	2	
	MHS023	NI	25.8±6.4				+	+	+		1	3	1
	MHS035	I	100.0±0.0			+	+	+	+		1	2	
	MHS014	I	82.6±11.6			+		+	+		1	1	1
	MHS005	I	71.0±4.3			+	+	+		+	2	2	1
	MHS006	I	67.7±2.6				+	+				1	
	MHS008	I	67.4±2.2				+	+				2	
	MHS007	NI	98.8±1.2				+	+			1	3	
	MHS012	I	98.3±2.7				+	+			1	1	1
Alcaligenes	MHS033	I	100.0±0.0		+	+					1	1	
	MHS013	I	80.9±2.4		+			+				3	
Stenotrophomonas	MHS019	I	86.8±3.0				+	+				3	
	MHS021	I	85.9±3.9					+			1		1
Arthrobacter	MHS027	I	77.8±2.4			+	+	+			2	1	
Ensifer	MHS020	NI	85.6±3.4				+	+				1	
Lysobacter	MHS036	I	100.0±0.0			+		+			1	2	
Planomicrobium	MHS028	NI	87.6±2.0					+				1	
Sporosarcina	MHS026	I	81.3±1.3	+			+					2	
Staphylococcus	MHS025	NI	38.4±5.6					+	+			1	

[a]The taxonomic position of isolates was determined according Table 3.
[b]Antifungal activities were tested by BPSB culture of isolate, and for spore germination. I means inhibition; ⩾95% while NI means no inhibition + volatile compound showing fungistatic activity.
[c]A, B, C, D, E, F, G, mean acetamide, methanamine, 1-butanamine, benzothiazole, benzaldehyde, phenylacetaldehyde, 1-decene, respectively.

Our study has demonstrated that soil bacteria can actively secrete antifungal volatiles during growth. Since most of commercial compounds of fungistatic volatile candidates are unavailable, the fungistatic effect of only seven compounds could be confirmed in vitro: acetamide, benzaldehyde, benzothiazole, 1-butanamine, methanamine, phenylacetaldehyde and 1-decene. Based on their chemical structures, acetamide, 1-butanamine and methanamine (this study), *N*,*N*-dimethyloctylamine and trimethylamine (Xu et al., 2004) and ammonia (Ko and Hora, 1974), are categorized into the amines group. Benzaldehyde (Xu et al., 2004) and phenylacetaldehyde (this study) are aldehydes group; and 1-decene (this study) and ethylene (Hora and Baker, 1970; Smith, 1973) are alkenes. In this study, additional volatiles were found belonging to the three dominant groups (Table 6). These were also detected in bacterial cultures, but the antifungal activities of these fungistatic candidates require further confirmation. Previous

studies reported that the amine group might change soil pH to more alkaline, which can lead to the production of ammonia and cause fungistasis (Lockwood, 1977; Kao and Ko, 1983; Liebman and Epstein, 1992; Ann, 1994). In contrast, aldehydes and alkenes do not significantly affect soil pH. They may be the causal agents for volatile fungistasis in acid soils. The three kinds of volatiles, amines, aldehydes and alkenes, may play important roles in the process of soil fungistasis.

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