Antibacterial effects of volatiles produced by *Bacillus* strain D13 against *Xanthomonas oryzae* pv. *oryzae*

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SUMMARY

Recent investigations have demonstrated that bacteria employ the volatile compounds they produce during interactions with other organisms, such as plants, fungi, nematodes and bacteria. However, studies focused on the antibacterial activity of plant growth-promoting rhizobacteria (PGPR) volatiles against bacterial phytopathogens are still rare. In this study, *Bacillus* strain D13, which is antagonistic to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), was isolated and screened. Volatile compounds emitted from strain D13 reduced the colony diameter and cell motility of *Xoo* cultured in divided Petri plates. Transmission electron micrograph analysis showed concentration in cytoplasm and altered surface morphology in the majority of *Xanthomonas* cells after co-cultivation with strain D13. Transcriptional expression of virulence-associated genes in *Xoo* was repressed. Based on gas chromatography/mass spectrometry (GC/MS) analysis, 12 volatile compounds specifically produced by strain D13 were identified. Among them, decyl alcohol and 3,5,5-trimethylhexanol inhibited the growth of *Xoo* at minimum inhibitory amounts of 0.48 and 2.4 mg, respectively. Furthermore, transcriptional expression of virulence-associated genes was also repressed by decyl alcohol and 3,5,5-trimethylhexanol. These results are useful for a better understanding of the biocontrol mechanisms of *Bacillus*.

Keywords: antibacterial activity, *Bacillus*, volatile compounds, *Xanthomonas oryzae* pv. *oryzae*.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight, a vascular pathogen that grows in the xylem vessels, causing yellow/white lesions along the leaf veins. Bacterial leaf blight disease is one of the major diseases of rice in Asian countries, where high-yielding rice cultivars are often susceptible to the disease, leading to 50%–60% failure in severely affected crops. Several virulence-related factors have been identified, such as the hypersensitive response and pathogenicity (*hrp*) gene (Zhu

et al., 2000), products depending on type II and III secretion systems (Ray *et al.*, 2000), extracellular polysaccharides (EPSs) (Rajeshwari *et al.*, 1997), motility (Yu *et al.*, 2014), diffusible signal factors (DSFs) (He *et al.*, 2010) and extracellular enzymes (Rajeshwari *et al.*, 2005; Sun *et al.*, 2005).

It has long been recognized that bacteria emit characteristic scents, and humans have exploited microbial volatiles as aroma components of cheese, yogurt and wine. Recent investigations have demonstrated that bacteria employ volatiles during interactions with other organisms, such as plants (Ryu *et al.*, 2003; Velázquez-Becerra *et al.*, 2011; Zou *et al.*, 2010), fungi (McCain, 1966; Zheng *et al.*, 2013), nematodes (Dainty *et al.*, 1985; Zechman and Labows, 1985) and bacteria (Kim *et al.*, 2013). Ryu *et al.* (2003) demonstrated for the first time that volatiles released by bacteria can affect plant growth. Over the past decade, active volatiles have been identified as acetoin, 2,3-butanediol (Ryu *et al.*, 2003), dimethylhexadecylamine (Velázquez-Becerra *et al.*, 2011) and 2-pentylfuran (Zou *et al.*, 2010). In addition, volatiles released by plant growth-promoting rhizobacteria (PGPR) have also been confirmed to induce plant resistance to abiotic and biotic stresses, including drought stress, salt stress and nutrient deficiency (Ryu *et al.*, 2004; Yang *et al.*, 2009). For example, bacterial volatile organic compounds (VOCs) help plants to increase iron uptake (Zhang *et al.*, 2009) and modulate sodium homeostasis (Zhang *et al.*, 2008). In addition, Ryu *et al.* (2004) first reported that VOCs released from PGPR induce systemic resistance in *Arabidopsis*. 2,3-Butanediol from *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a significantly increase plant resistance against *Pectobacterium carotovorum* ssp. *carotovorum* (Ryu *et al.*, 2004). Moreover, the tridecane produced by *Paenibacillus polymyxa* E681 also induces systemic resistance in plants (Lee *et al.*, 2012).

Bacterial volatiles affecting fungal growth and development were reported as early as 1966 (McCain, 1966). This study demonstrated that volatiles from *Streptomyces griseus* reduced *Gleosporium aridum* sporulation and induced *Rhizoctonia solani* sclerotial formation. Numerous reports have demonstrated that fungal growth is inhibited by bacterial volatiles, including dimethyl disulfide, 1-undecene, benzaldehyde, benzothiazole, dimethyl trisulfide, cyclohexanol, decanal, 2-ethyl-1-hexanol, methyl pyrazine and some mid- and long-chain alkanes, alkenes and alcohols (Kai *et al.*, 2009).

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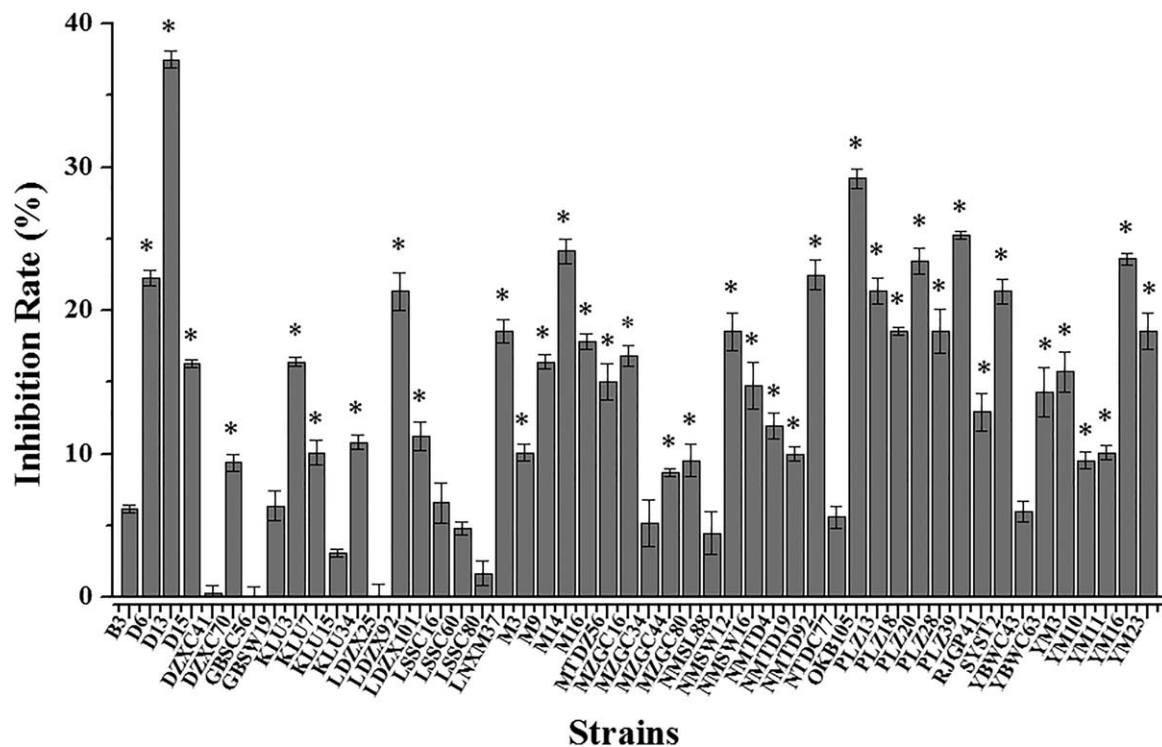†These authors contributed equally to this work and share first authorship.

Fig. 1 Antibacterial activity of *Bacillus* spp. volatiles against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Each isolated *Bacillus* spp. strain was cultured in one compartment of the divided plates containing Luria broth (LB) medium. *Xoo* was cultured in a separate compartment containing nutrient agar (NA) medium. Control plates contained *Xoo* on one half and no *Bacillus*-inoculated LB medium on the other half. After incubation for 6 days, the rates of *Xoo* growth inhibition by volatiles emitted from *Bacillus* were measured. Inhibition rate (%) = (average colony diameter of control − average colony diameter of treatment)/average colony diameter of control × 100%. The data were statistically evaluated using analysis of variance, followed by Fisher's least-significant difference test ($P < 0.05$), employing SPSS software (SPSS, Chicago, IL, USA). All error bars represent standard deviations. Asterisks above the bars indicate a significant difference ($P < 0.05$).

Recently, a study has shown that VOCs emitted from *B. subtilis* reduce the motility and increase the antibiotic resistance of *Escherichia coli* (Kim *et al.*, 2013). However, few studies have focused on the antibacterial activity of PGPR volatiles. The aim of this study was to select effective antibacterial volatile-producing strains, and identify and screen the key volatile compounds produced by *Bacillus* spp. against *Xoo*.

RESULTS

Screening and identification of antagonistic bacterial strains

Fifty *Bacillus* spp. strains isolated from rhizosphere soils in Malaysia and Tibet were tested for antagonistic activity mediated by volatile compounds, using divided Petri plates. The sources of the isolated strains are listed in Table S1 (see Supporting Information). Preliminary screening showed that strain D13 had the strongest antagonistic activity towards *Xoo*, and the inhibition rate was about 37.5% after incubation for 6 days (Fig. 1).

Both 16S rRNA and the *gyrB* gene of strain D13 were amplified and sequenced. BLAST analysis consistently showed 99% identity

with *Bacillus cereus* when compared with the National Center for Biotechnology Information (NCBI) database. In addition, phylogenetic analysis performed using MEGA software indicated that strain D13 grouped with the *B. cereus* branch (Fig. 2). Based on these analyses, the strain D13 grouped with the *B. cereus* branch.

Motility deficiency of *Xoo* after co-cultivation with *Bacillus* strain D13

As shown in Fig. 3A, the swarming and swimming motilities of *Xoo* were significantly ($P < 0.05$) inhibited after exposure to *Bacillus* strain D13 volatiles for 3 days; the colony diameter and cell viability were unchanged in this time period (Fig. S1A,B, see Supporting Information). In addition, the transcriptional levels of *motA* and *motC* were correspondingly down-regulated (Fig. 3B). The genes *motA*, encoding flagellar motor component MotA, and *motC*, encoding flagellar motor protein, are required for motility. Nevertheless, the number of viable cells declined sharply after exposure for 6 days, suggesting that the activity of *Bacillus* antibacterial volatiles requires a certain time to achieve lethal concentrations. These observations suggest that VOCs released by strain
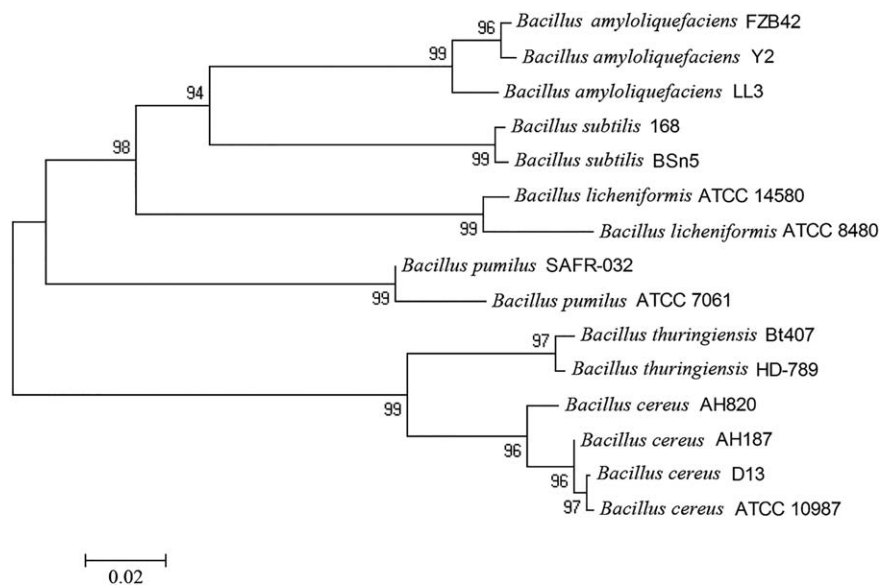
Fig. 2 Phylogenetic analysis of *Bacillus* strain D13 based on *gyrB* gene sequences. The *gyrB* gene of *Bacillus* strain D13 was amplified and sequenced, and phylogenetic analysis was performed with the neighbour-joining method using MEGA 4.0 software. Bars indicate the scales of genetic distances (0.02). Genetic distance measures are indicators of relatedness among species and are useful for reconstructing the historic and phylogenetic relationships among such groups.

D13 may also inhibit *Xoo* swarming and swimming motility below the lethal concentration.

Assay of the effects of *Bacillus* strain D13 volatiles on *Xoo* cell virulence

The effect of strain D13 volatiles on *Xoo* virulence was also evaluated. As shown in Fig. 4, the lesion lengths observed in rice plants (cultivar IR24) inoculated with *Xoo* cells after co-cultivation with strain D13 volatiles were significantly reduced ($P < 0.05$). In *Xoo*, the *rpf* (regulation of pathogenicity factor) gene cluster is required for biofilm formation and virulence, the core genes of which are *rpfF*, *rpfC* and *rpfG* (Chatterjee *et al.*, 2008; Guo *et al.*, 2012; He *et al.*, 2006). *rpfF* encodes a protein similar to enoyl-CoA hydratase which catalyses the synthesis of DSF. DSF is then sensed by a two-component signal transduction system consisting of RpfC and RpfG. Real-time polymerase chain reaction (PCR) showed that the transcriptional expression of *rpfC* was also repressed in the presence of volatile compounds (Fig. 3B).

Ultrastructural changes in *Xoo* cells after co-cultivation with *Bacillus* strain D13

To investigate the changes in shape and ultrastructure of *Xanthomonas* cells, cells were examined after co-cultivation with strain D13 by transmission electron microscopy. As shown in Fig. 5A, the membrane of the non-inoculated control cells was intact with uniformly distributed cytoplasm and electron density inside the cells. In contrast, more than 30% of *Xoo* cells showed concentrated cytoplasm and altered cell morphology after co-cultivation with strain D13 (Fig. 5B). However, the effects of volatile treatment on *Xoo* cells were variable. In some cells, the walls and

membranes were badly distorted, which may increase membrane permeability and cause leakage of intracellular substances. In other cases, the membrane permeability may decrease and result in concentrated cytoplasm.

Identification and antibacterial activity of volatile compounds produced by *Bacillus* strain D13

The volatiles released by strain D13 and Luria broth (LB) medium were analysed by solid-phase microextraction-gas chromatography/mass spectrometry (SPME-GC/MS). More than 30 compounds produced by strain D13 cultured in LB medium were detected; nevertheless, LB medium can also produce many volatile compounds. Identical volatile compounds produced by D13 and LB medium were deducted, and 12 volatile compounds specifically produced by strain D13 were identified, including four acids, three alcohols, two nitrogen-containing compounds, one benzene, one ketone and one sulfur-containing compound (Table S2, see Supporting Information). Eight volatiles, including lauric acid (LA), tridecanoic acid (TA), myristic acid (MA), palmitic acid (PA), 3-methyl-1,2-cyclopentanediol (MCP), 3,5,5-trimethylhexanol (TMH), decyl alcohol (DA) and 2-pentadecanone (PCO), were purchased and assayed for antibacterial activity in divided Petri dishes. The other four VOCs, including 1,4-dihydro-2-methyl-1,4-ethenoisoquinolin-3(2H)-one, *N,N*-dimethyl octanamide, 4-octadecyl morpholine and bis(1,1,3,3-tetramethylbutyl)disulfide, are unavailable as pure chemicals.

Among the eight volatile compounds mentioned above, only DA and TMH inhibited the growth of *Xoo* by 53.6% and 60.7%, respectively; the remaining compounds showed weak or no inhibitory activity (Fig. 6A). Different amounts of DA and TMH were tested for further investigation. The degree of inhibition
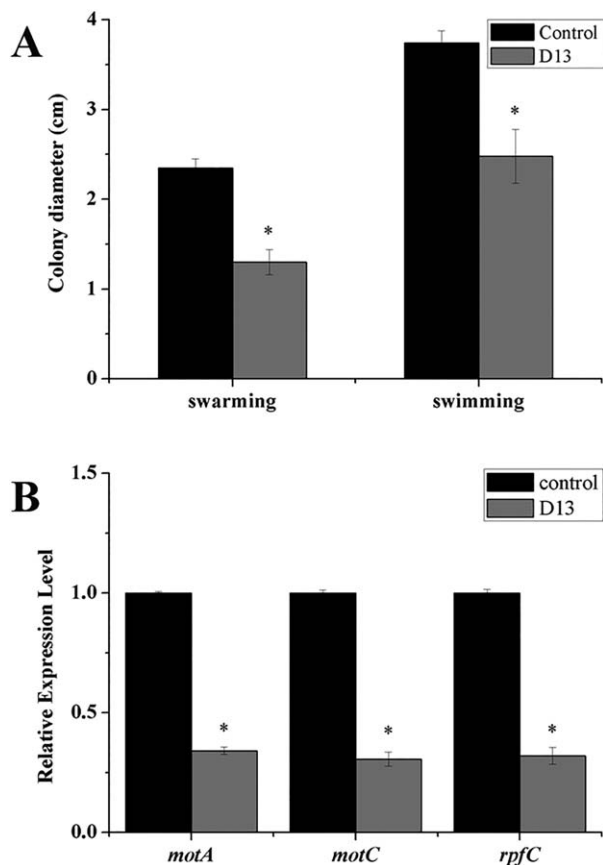
Fig. 3 Effects of volatiles produced by *Bacillus* strain D13 on cell motility in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). *Xoo* cultures were spotted onto the compartments of divided plates containing 0.7% (w/v) or 0.3% (w/v) nutrient agar (NA) medium; strain D13 was cultured in a separate compartment containing Luria broth (LB) medium. Control plates contained *Xoo* on one half and no *Bacillus*-inoculated LB medium on the other half. (A) Swarming and swimming haloes of *Xoo* exposed to strain D13 volatiles for 3 days. (B) Real-time polymerase chain reaction (PCR) analysis of *motA*, *motC* and *rpfC* expression in *Xoo* cells after co-cultivation with strain D13 for 36 h. Values were normalized to the levels of 16S rRNA, an internal reference gene. Three replicates were used for each treatment, and the experiment was repeated three times. Vertical bars represent standard errors. Asterisks above the bars indicate a significant difference ($P < 0.05$).

varied depending on the amount of DA and TMH, and the minimum inhibitory amounts were 0.48 and 2.4 mg, respectively (Fig. 6B,C).

Effects of DA and TMH on different pathogenic bacteria

The inhibitory effects on the growth of different pathogenic bacteria were also investigated. *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) and *Ralstonia solanacearum* (*Rs*) were inhibited by TMH, whereas DA inhibited only *Xoc* (Fig. 7). These results suggest

that TMH may exert a general toxicity against pathogenic bacteria.

Effects of DA and TMH on the transcriptional expression of virulence-associated genes in *Xoo*

EPS production, motility and DSF have been confirmed to be associated with virulence in *Xanthomonas oryzae* (He *et al.*, 2010; Jeong *et al.*, 2008; Rai *et al.*, 2012). In this study, transcriptional expression of *gumD* (EPS synthesis), *motA* and *motC* (motility) and *rpfC* (DSF) was investigated by real-time PCR analysis. *motA*, *motC* and *rpfC* were all repressed following exposure to DA and TMH, whereas the expression of *gumD* was not affected (Fig. 8). The down-regulated expression of *motA*, *motC* and *rpfC* was consistent with the reduced virulence in *Xoo*.

DISCUSSION

In this study, we screened a collection of *Bacillus* strains isolated from soils for their antagonistic activity against *Xoo* via secretion of volatile compounds. We identified one strain, D13, which showed strong antagonistic activity against this pathogen. Based on the sequence analysis of 16S rRNA and *gyrB* gene, D13 was grouped with *B. cereus*, which is a Gram-positive bacterium belonging to *Bacillus* species. Several *Bacillus* spp., such as Quantum-400 (*Bacillus subtilis*, strain GB03), Serenade (*B. subtilis*, strain QST713) and Rhizovital (*Bacillus amyloliquefaciens*, strain FZB42), have been widely studied and applied as efficient, stable biocontrol agents because of their ability to form heat- and desiccation-resistant spores. *Bacillus* spp. have been shown to protect plants through antibiotic production, predation and parasitism, competition for ferric iron ions, competition for nutrients and niches, and induction of plant resistance (Pliego *et al.*, 2008; Van *et al.*, 1991). VOCs produced by *B. amyloliquefaciens* NJN-6 inhibit the growth and spore germination of *Fusarium oxysporum*. Volatile compound 2,3-butanediol from *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a significantly increases plant resistance against *Pectobacterium carotovorum* ssp. *carotovorum* (Ryu *et al.*, 2004).

Numerous reports of volatile compounds produced by bacteria have shown contrasting effects, including increased plant biomass (Ryu *et al.*, 2003; Zou *et al.*, 2010), improvement of plant resistance against abiotic and biotic stresses (Farag *et al.*, 2013), nematode death (Gu *et al.*, 2007) and inhibition of fungal mycelium growth and spore formation (Zou *et al.*, 2007). Moreover, bacterial volatiles can also function as info-chemicals for communication in and between organisms and cells (Kai *et al.*, 2009). Bacteria use a cell-to-cell communication system to monitor their population density (Ryan and Dow, 2008) in a system that involves the synthesis, release and subsequent detection of small diffusible signal molecules, called autoinducers (Miller and Bassler, 2001; Uzureau *et al.*, 2010). This system allows the
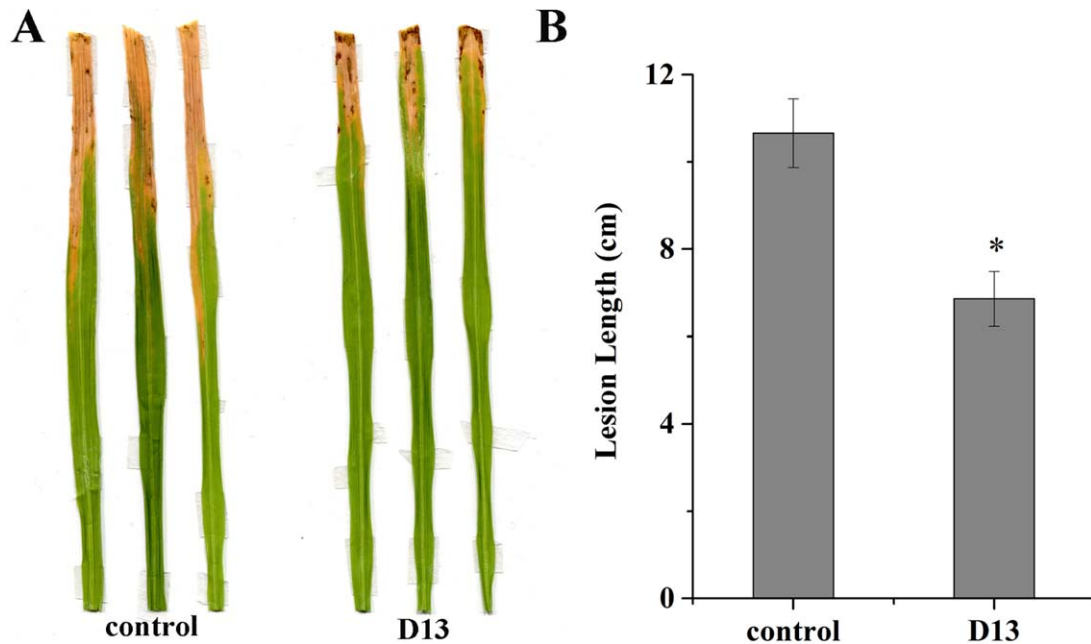
Fig. 4 Effects of *Bacillus* sp. D13 volatiles on the virulence of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). (A) Representative lesion lengths in rice seedling leaves (cultivar IR24, 2 months old) following inoculation with control *Xoo* cells and *Xoo* cells after co-cultivation with strain D13. (B) Analysis of lesion lengths. The *y*-axis values represent the mean lesion length $\pm$ the standard deviation ($n = 3$). Asterisk indicates significant difference compared with the control ($P < 0.05$).

coordinated behaviour of a group of organisms in order to regulate processes contributing to virulence, biofilm formation and other developmental processes (Miller and Bassler, 2001; Whitehead *et al.*, 2001). Gram-negative bacteria use *N*-acylhomoserine lactones (N-AHLs), fatty acid derivatives and cyclic dipeptides for communication. Many autoinducers are compounds with small molecular masses that might act as volatiles. However, little is known about the antibacterial activity of volatiles produced by
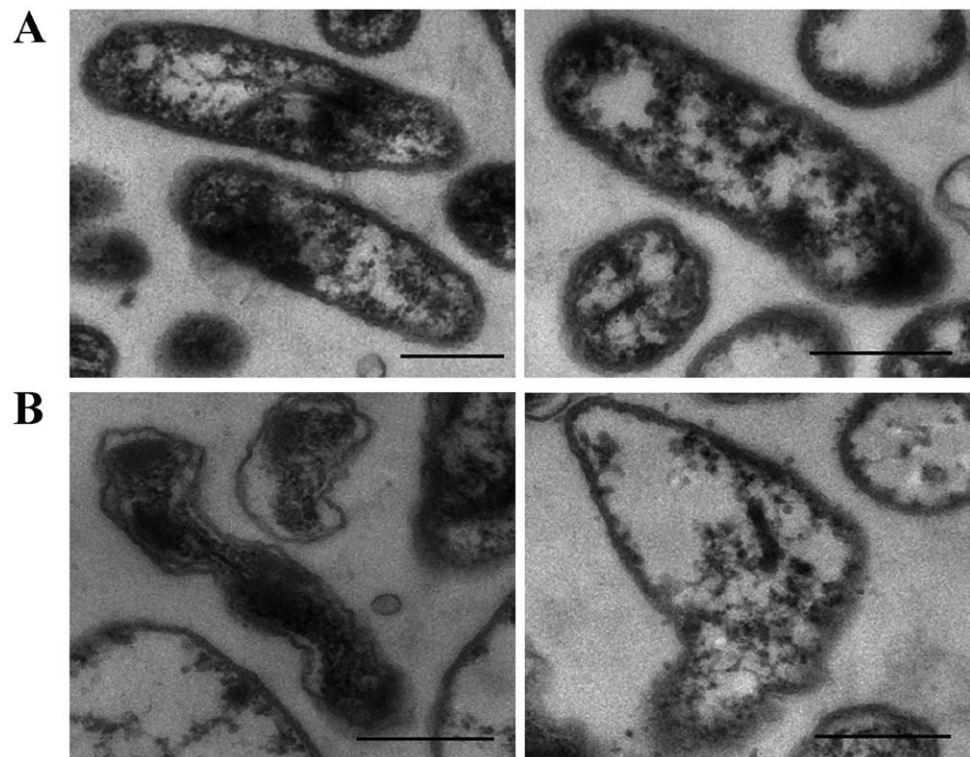

Fig. 5 Transmission electron micrographs of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) co-cultured with strain D13. The specimens were prepared from the samples taken directly from divided Petri plates after co-cultivation for 6 days. (A) Untreated control *Xoo* cells. (B) *Xoo* cells during co-cultivation. Scale bars indicate 500-nm lengths.
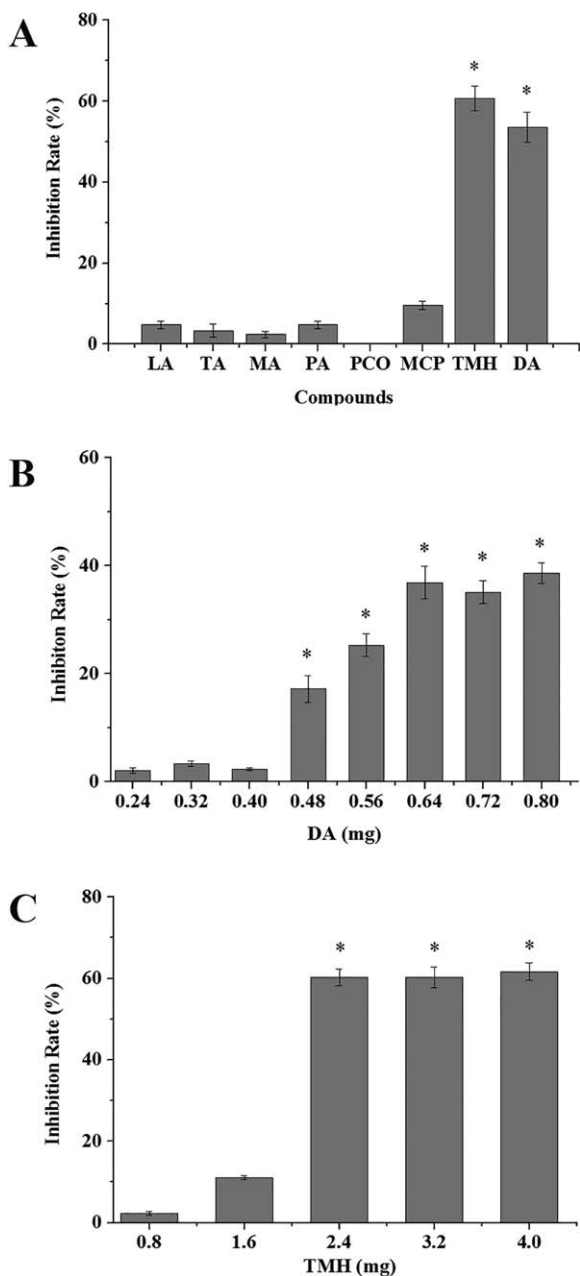
Fig. 6 Antibacterial effects of selected volatile organic compounds (VOCs) on *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) growth. (A) *Xoo* was incubated with eight volatile compounds, and the inhibition rates were measured after exposure for 6 days. (B) *Xoo* was incubated with DA at different amounts ranging from 0.24 to 0.8 mg, and the inhibition rates were measured after exposure for 6 days. (C) *Xoo* was incubated with TMH at different amounts ranging from 0.8 to 4 mg, and the inhibition rates were measured after exposure for 6 days. DA, decyl alcohol; LA, lauric acid; MA, myristic acid; MCP, 3-methyl-1,2-cyclopentanediol; PA, palmitic acid; PCO, 2-pentadecanone; TA, tridecanoic acid; TMH, 3,5,5-trimethylhexanol. The *y*-axis values represent the mean inhibition rate $\pm$ standard deviation ($n = 3$). *Significant difference compared with the control ($P < 0.05$).

Bacillus spp. strains. In the present study, we evaluated the antibacterial activity of volatiles emitted from *Bacillus* strain D13 against *Xoo*. The cell motility and virulence of *Xoo* were significantly reduced ($P < 0.05$). Transmission electron microscopy showed the concentration in cytoplasm and altered surface morphology in the majority of *Xanthomonas* cells after co-cultivation with strain D13. In another study, transmission electron microscopy of fumigated and untreated *Botrytis cinerea* showed excessive vesiculation or thickened cell walls in exposed conidia, and increased vesiculation or marked retraction of the plasma membrane in exposed hyphae (Li *et al.*, 2012). These results indicate that the mode of action of volatiles can be explained, at least in part, by their ability to cause leakage or impede the interchange of materials through a change in the membrane permeability. In addition, the rhizosphere is a relative closed environment. The small space seems favourable for volatile-mediated communication, and volatiles are more likely to accumulate and reach their activity threshold than in the open environment. Moreover, the antibacterial effects of volatiles may inhibit *Xoo* infection via root wounds. These results may provide the basis of a potential method for the biocontrol of *Xoo*.

All of the active volatiles that have been reported so far can be grouped into alcohols, aldehydes, alkenes, alkanes, alkynes, esters, ketones, terpenoids and sulfur-containing compounds (Corcuff *et al.*, 2011; Fernando *et al.*, 2005; Wan *et al.*, 2008). For example, the growth of *Fusarium culmorum* mycelium is inhibited by dimethyl disulfide in a dose-dependent manner, and small effects have been obtained with 1-undecene (Kai *et al.*, 2009). Furthermore, 2-decanone, which is the second most dominant substance produced by *Bacillus pumilus* TB09 and *B. thuringiensis* T72, significantly inhibits the growth of *Colletotrichun gloeosporioides* mycelia (Zheng *et al.*, 2013). 2-Ethyl hexanol and n-decanal, produced by bacteria, have been confirmed to inhibit sclerotia and ascospore germination, and the growth of *Sclerotinia sclerotiorum* mycelium (Fernando *et al.*, 2005). Volatile compounds emitted from *B. amyloliquefaciens* NJN-6 inhibit the growth of *F. oxysporum* (Yuan *et al.*, 2012). Allyl alcohol inhibits carpogenic germination of sclerotia of *S. sclerotiorum* (Huang *et al.*, 1997) and also increases the populations of beneficial bacteria, such as *Pseudomonas fluorescens* and *P. putida* (Altman and Lawlor, 1966; Domsch, 1959).

Our results demonstrate that DA and TMH are the volatile components responsible for antibacterial activity against *Xoo* (Fig. 6). DA is a straight chain fatty alcohol with 10 carbon atoms. It has been confirmed to inhibit and disrupt biofilms of *Aspergillus fumigatus* in a concentration-dependent manner (Mowat *et al.*, 2010). DA also shows antifungal activity against *Zygosaccharomyces bailii* (Fujita *et al.*, 2008). TMH is a member of the fragrance structural group of branched chain saturated alcohols. TMH is a fragrance ingredient used in many fragrance compounds. It may
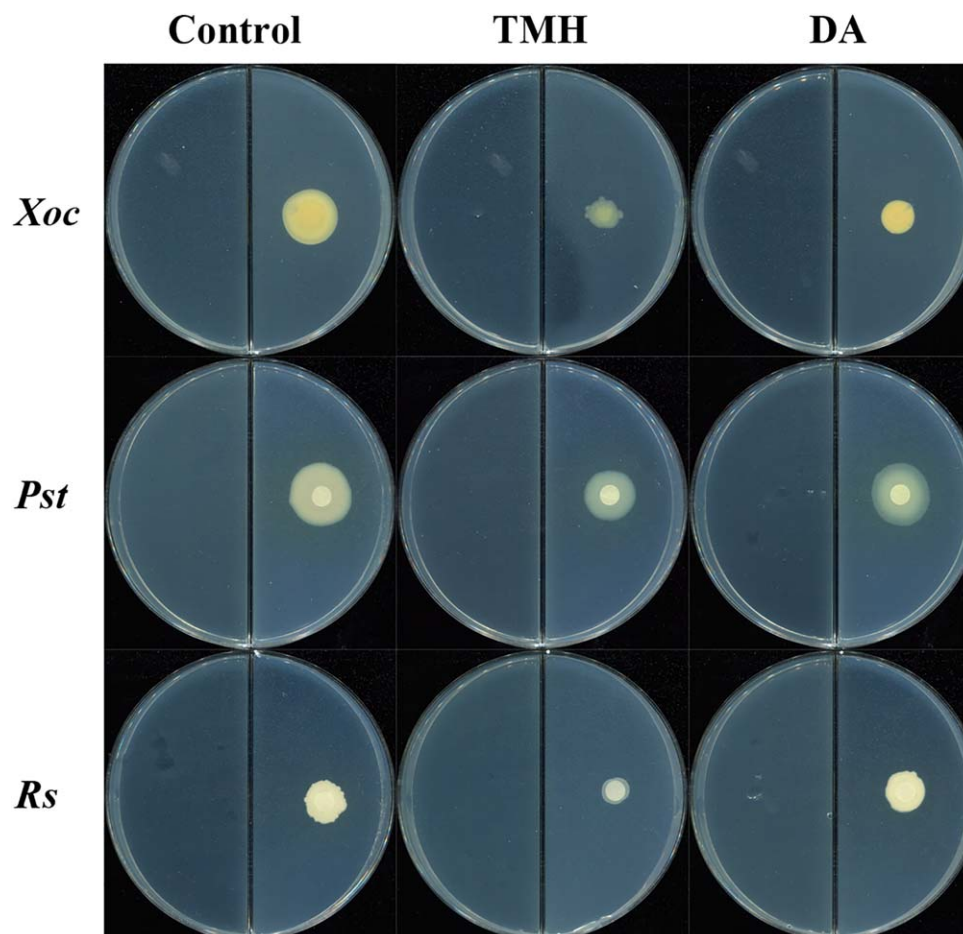
Fig. 7 Antibacterial activities of decyl alcohol (DA) and 3,5,5-trimethylhexanol (TMH) against *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) and *Ralstonia solanacearum* (*Rs*); 0.48 mg DA [dissolved in 100 μL dimethylsulfoxide (DMSO)], 2.4 mg TMH (dissolved in 100 μL DMSO) and DMSO were added to one compartment of the divided plates containing Luria broth (LB) medium; *Xoc*, *Pst* and *Rs* were cultured in a separate compartment containing nutrient agar (NA) or King's B (KB) medium. The diameters of *Xoc*, *Pst* and *Rs* colonies were measured after exposure for 14, 4 and 4 days, respectively. Three independent replicates of each experiment were performed.

be found in fragrances used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries, as well as in non-cosmetic products, such as household cleaners and detergents
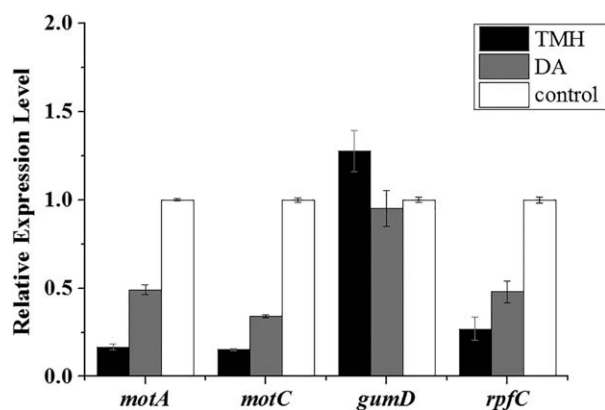

Fig. 8 Real-time polymerase chain reaction (PCR) analysis of *motA*, *motC*, *gumD* and *rpfC* expression in *Xanthomonas oryzae* pv. *oryzae* cells in response to 0.48 mg synthetic decyl alcohol (DA) or 2.4 mg 3,5,5-trimethylhexanol (TMH) for 24 h. Values were normalized to the levels of 16S rRNA, an internal reference gene. The *y*-axis values represent the mean expression ± standard deviation ($n = 3$) relative to the control.

(McGinty *et al.*, 2010). Moreover, the proportion calculated from the relative peak areas (TMH : DA) was 1 : 3. The growth of *Xoo* was assessed after exposure to different amounts of artificial mixtures of volatiles: the minimum inhibitory amount was 0.4 mg (Fig. S2, see Supporting Information), which was smaller than that of the individual compounds. TMH also inhibited the growth of *Xoc*, *Pst* and *Rs*, suggesting that TMH has a broad-spectrum resistance against pathogenic bacteria and has the potential for practical application.

In previous research, the antimicrobial mechanisms of *B. cereus* strains have been demonstrated. For example, *B. cereus* UW85 inhibits the growth of *Pseudopeziza medicaginis* by producing zwittermicin A and kanosamine (Emmert *et al.*, 2004; Milner *et al.*, 1996). Two antifungal chitinases (ChiCW and ChiCH) excreted by *B. cereus* 28-9 show inhibitory activity on the conidial germination of *Botrytis elliptica* (Huang *et al.*, 2005). The results of this study provide a better understanding of the mode of action of *B. cereus* as a biocontrol agent. Furthermore, unlike traditional chemical fungicides or bactericides, DA emitted by *Bacillus* strain D13 is generally non-phytotoxic (Kim *et al.*, 2004), thus strengthening its potential application for plant disease management.

EXPERIMENTAL PROCEDURES

Microorganisms and culture conditions

Fifty *Bacillus* spp. strains isolated from soils in Malaysia and Tibet were tested for antagonistic activity using divided Petri plates. The sources of the isolated strains are listed in Table S1. These strains were grown on LB medium incubated at 37 °C overnight. *Bacillus* strain D13 was stored in the China General Microbiological Culture Center (CGMCC, Beijing, China) under the registration number CGMCC No. 10243. The pathogenic bacteria *Xanthomonas oryzae* pv. *oryzae* PXO99A (*Xoo*) (Hopkins *et al.*, 1992), *Xanthomonas oryzae* pv. *oryzicola* RS105 (*Xoc*) (Zou *et al.*, 2006), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Whalen *et al.*, 1991) and *Ralstonia solanacearum* (*Rs*) (provided by Dr Qiao from Jiangsu-Provincial Academy of Agricultural Sciences, China) were used as target bacteria. *Rs* and *Xoo* were grown on nutrient agar (NA) medium at 28 °C for 24 and 48 h, respectively. *Pst* was grown on King's B (KB) medium at 28 °C for 24 h.

Antagonistic assay of VOCs against bacteria

One compartment of the divided plates containing LB medium was incubated with each of the isolated *Bacillus* spp. stains [30 μL; 1×10^8 colony-forming units (cfu)/mL]; the other compartment containing NA medium was cultured with 10 μL (10^8 cfu/mL) *Xoo* to test growth inhibition. Control plates contained *Xoo* on one half and no *Bacillus*-inoculated LB medium on the other half. The divided Petri plates were sealed and incubated for 6 days at 28 °C before the diameter of *Xoo* colonies was measured.

Motility assays

Xoo motility after co-cultivation with strain D13 was tested using divided Petri plates. *Xoo* cultures were normalized to an optical density at 600 nm (OD_{600}) of 1.0, and then 2 μL of the culture was spotted onto the compartment of the divided plates containing 0.7% (w/v) or 0.3% (w/v) NA medium. *Bacillus* strain D13 (30 μL; 10^8 cfu/mL) was cultured in another compartment containing LB medium. The motility halo was examined after incubation at 28 °C for 72 h.

Pathogenicity assays

Xoo cells were collected after co-cultivation with *Bacillus* strain D13 VOCs for 3 days, resuspended in distilled water (final concentration, 10^8 cfu/mL) and inoculated into 2-month-old rice cultivar IR24 (Mazzola *et al.*, 1994) by the leaf clipping method (Kauffman *et al.*, 1973). After 14 days, the lesion lengths were measured. Twelve plants were inoculated with the *Xoo* strain in each treatment, and the experiment was repeated three times.

Transmission electron microscopy

Xoo cells were co-cultured with *Bacillus* strain D13 VOCs to investigate the changes in shape and ultrastructure. After 6 days, *Xoo* cells were collected and washed three times with sterilized water and then fixed with 2.5% glutaraldehyde. Fixed cells were rinsed three times for 10 min with 100 mM phosphate buffer, post-fixed for 3 h in 1% osmium tetroxide and dehydrated through an ethanol gradient. Samples were then embedded in Epon 812, sectioned with an ultramicrotome and observed using a Hitachi H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Collection of VOCs with SPME-GC/MS analysis

Volatiles were collected by a 2-cm, 50/30 μm divinylbenzene/carboxen on polydimethylsiloxane fibre (57348-U, Supelco, Bellefonte, PA, USA). For VOC extraction, the *Bacillus* strain (20 μL) was inoculated onto 30 ml LB medium in a 100-mL vial. After incubation at 28 °C for 6 days, the SPME fibre was inserted into the headspace of the vial and then placed at 50 °C for 30 min. GC/MS analysis was performed using a Bruker 450-GC gas chromatograph (Bruker diatonic, MA, USA) in combination with a Bruker 320-MS mass spectrometer, and helium gas was used as carrier (flow rate, 1 mL/min). The SPME fibres were desorbed at 220 °C for 5 min and the oven temperature was programmed as follows: 35 °C for 3 min at the beginning, increasing to 180 °C (10 °C/min), further increasing to 240 °C (4 °C/min) and held for 5 min (Yuan *et al.*, 2012). The mass spectrometer was operated in the electron ionization mode at 70 eV with a source temperature of 220 °C, and a continuous scan from 50 to 500 *m/z* was used. Mass spectral data of the volatile compounds were compared with data in the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health (NIST/EPA/NIH) Mass Spectrum Library.

Assessment of the effects of volatile compounds on *Xoo* growth

Eight high-purity individual VOCs (>98%) identified through GC/MS analysis were purchased and assayed for antibacterial activity in divided Petri dishes. These volatile compounds included LA (L110737, Aladdin, Shanghai, China), TA (T114032, Aladdin), MA (M108284, Aladdin), PA (P101053, Aladdin), MCP (S352810, Sigma-Aldrich, St. Louis, MO, USA), TMH (289485, Sigma-Aldrich), DA (74723, Sigma-Aldrich) and PCO (76535, Sigma-Aldrich). Liquid-state volatile compounds (100 μL) or solid-state volatile compounds [100 μL; final concentration, 1 g/mL dissolved in dimethylsulfoxide (DMSO)] were added to LB plates and 10 μL (10^8 cfu/mL) *Xoo* cultures were spotted onto another plate containing NA medium. Equivalent amounts of DMSO were used as control. After incubation at 28 °C for 6 days, the colony diameter was assessed.

According to the results of initial screening assays, TMH and DA were chosen for further investigation of their antibacterial activity against *Xoo*. The effects of different amounts of TMH and DA, ranging from 0.08 to 4 mg, against *Xoo* were evaluated. In addition, assays of the antibacterial activity of TMH and DA against *Xoc*, *Pst* and *Rs* were carried out. Amounts of 0.48 mg DA (dissolved in 100 μL DMSO), 2.4 mg TMH (dissolved in 100 μL DMSO) or DMSO were added to one compartment of the divided plates containing LB medium; 10 μL (10^8 cfu/mL) of *Xoc*, *Pst* or *Rs* were cultured in a separate compartment containing NA or KB medium. The diameters of *Xoc*, *Pst* and *Rs* colonies were measured after exposure for 14, 4 and 4 days, respectively. Three independent replicates of each experiment were performed.

Real-time PCR

Xoo cells were harvested after exposure to 2.4 mg TMH or 0.48 mg DA. Total RNA was extracted using the Bacterial RNA Kit (Omega Bio-Tek,

Norcross, GA, USA) according to the manufacturer's instructions. First-strand cDNA was obtained using reverse transcriptase (TaKaRa Bio, Dalian, China) with random hexamer primers. Real-time PCR was performed with SYBR Premix Ex Taq™ (TaKaRa Bio) using an ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). 16S rRNA was used as an internal reference, and the transcriptional levels of *motA*, *motC*, *gumD* and *rpfC* were detected. The specific primers used are listed in Table S3 (see Supporting Information).

Statistical analysis

Three independent experiments were performed for each assay. The data were statistically evaluated using analysis of variance, followed by a Fisher's least-significant difference test ($P < 0.05$), employing SPSS software (SPSS, Chicago, IL, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 *Bacillus* spp. isolated from different regions in Malaysia and Tibet.

Table S2 Gas chromatography/mass spectrometry (GC/MS) volatile profile of *Bacillus* strain D13.

Table S3 Oligonucleotide DNA primers used in this study.

Fig. S1 Colony diameters (A) and cell viability (B) of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) exposed to *Bacillus* strain D13. *Bacillus* strain D13 [30 μL; 1×10^8 colony-forming units (cfu)/mL] was cultured in one compartment of the divided Petri dishes containing Luria broth (LB) medium, except for control plates. Another compartment containing nutrient agar (NA) medium was used for *Xoo* (10 μL; 10^8 cfu/mL) to test the growth inhibition and cell viability. The colony diameters of *Xoo* were measured and viable cells were counted by the dilution method at 24-h intervals during exposure to volatiles. The experiment was repeated three times with three replicates each time.

Fig. S2 The colony diameters of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) exposed to different amounts of the artificial mixture of volatile compounds. The proportion calculated from the relative peak areas [3,5,5-trimethylhexanol : decyl alcohol (TMH : DA)] was 1 : 3. The diameters of *Xoo* colonies were measured after exposure to 0.08, 0.2, 0.4, 0.6, 0.8, 1, 3 and 6 mg of an artificial mixture of volatiles for 6 days. The experiment was repeated three times with three replicates each time.