Volatile organic compounds produced by a soil-isolate, *Bacillus subtilis* FA26 induce adverse ultra-structural changes to the cells of *Clavibacter michiganensis* ssp. *sepedonicus*, the causal agent of bacterial ring rot of potato

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**Abstract**

Rhizobacterial volatile organic compounds (VOCs) play an important role in the suppression of soil-borne phytopathogens. In this study, the VOCs produced by a soil-isolate, *Bacillus subtilis* FA26, were evaluated in vitro for their antibacterial activity against *Clavibacter michiganensis* ssp. *sepedonicus* (Cms), the causal agent of bacterial ring rot of potato. The VOCs emitted by FA26 inhibited the growth of Cms significantly compared with the control. Scanning and transmission electron microscopy analyses revealed distorted colony morphology and a wide range of abnormalities in Cms cells exposed to the VOCs of FA26. Varying the inoculation strategy and inoculum size showed that the production and activity of the antibacterial VOCs of FA26 were dependent on the culture conditions. Headspace solid-phase microextraction/gas chromatography–mass spectrometry analyses revealed that FA26 produced 11 VOCs. Four VOCs (benzaldehyde, nonanal, benzothiazole and acetophenone) were associated with the antibacterial activity against Cms. The results suggested that the VOCs produced by FA26 could control the causal agent of bacterial ring rot of potato. This information will increase our understanding of the microbial interactions mediated by VOCs in nature and aid the development of safer strategies for controlling plant disease.

**INTRODUCTION**

Bacterial ring rot of potato, caused by the Gram-positive phytopathogen *Clavibacter michiganensis* ssp. *sepedonicus* (Cms), is a serious threat to the potato industry and is a regulated quarantine disease [1]. The disease has become widespread among all major potato-growing areas worldwide [2], except Australia [3], where no such outbreak has been reported. However, in China alone, up to 60 % yield losses have been recorded [4]. Cms is highly infectious and may lead to extensive losses of the infected crops [5]. Cms is mainly tuber-borne; however, soil serves as the second most important source for the establishment and survival of the pathogen [6, 7].

Chemical bactericides have been widely used to curb this disease [8]. However, these bactericides are of environmental concern. The biocontrol of bacterial ring rot of potato using microbial antagonists has been achieved and is considered an environmentally friendly alternative to chemical bactericides [9]. The antibacterial mechanisms of microbial antagonists include the induction of resistance in plants and the production of antibiotics, hydrolytic enzymes and volatile organic compounds (VOCs). Among them, the production of VOCs is considered an important biocontrol mechanism for short- and long-distance pathogen control. VOCs have several environmentally friendly properties, such as a low molecular weight, meaning that they can be evaporated easily at normal temperature and pressure, they diffuse through the atmosphere and soil over short and long distances, and they are biodegradable and do not leave toxic residues on plant surfaces [10, 11].

*Bacillus* spp. are among the most widely distributed rhizospheric bacteria in soil and are important biocontrol agents against phytopathogens. Biocontrol mechanisms by this
genus include the induction of systemic resistance in the host plants and production of antimicrobial compounds, such as lipopeptides, hydrolytic enzymes and VOCs [12]. Rhizobacterial VOCs produced by Bacillus spp. or by other rhizobacteria have been studied for nearly a decade. Previous studies reported that these VOCs have a good potential to control phytopathogenic fungi [10, 11, 13, 14], bacteria [15–17] and nematodes [18]. In addition, some researchers found that rhizobacterial VOCs showed biocontrol activity against certain post-harvest diseases caused by phytopathogenic fungi [19–21]. However, the identification and activity of antibacterial VOCs produced by rhizobacteria against the pathogen of bacterial ring rot of potato have not been characterized. Therefore, it is necessary to explore the possible biocontrol mechanisms of rhizobacterial VOCs against bacterial phytopathogens. The aims of this study were: (1) to evaluate a soil-isolate, Bacillus subtilis FA26, for its in vitro antibacterial volatile activity against the pathogen of bacterial ring rot of potato; (2) to examine the effects of rhizobacterial VOCs on the pathogen at the ultra-structural level using electron microscopy; (3) to identify the VOCs using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC-MS); and (4) to screen individual volatile compounds for their antibacterial activity against Cms.

METHODS

Micro-organisms and their growth conditions

The antagonistic strain FA26, isolated from the rhizosphere of rice grown in Pakistan, was characterized previously as a broad-spectrum antagonist against several phytopathogens and was identified as Bacillus subtilis (NCBI accession no. KJ646017) through 16S rRNA gene sequencing [22]. The strain was grown routinely in Luria–Bertani (LB) medium [10 g of tryptone, 10 g of NaCl, 5 g of yeast extract, 15 g of agar per 1 l of Milli-Q water (Millipore, Bedford, MA, USA) and the pH was adjusted to between 7.0 and 7.2] at 37 °C overnight, unless stated otherwise. Cms, the causal agent of bacterial ring rot of potato, was obtained from the Laboratory of Plant Quarantine and Applied Immunology (College of Plant Protection, Nanjing Agricultural University, Nanjing) and was grown routinely on nutrient agar (NA) medium (5 g of polypeptone, 10 g of sucrose, 1 g of yeast extract, 2 g of beef extract, 15 g of agar per 1 l of Milli-Q water; and the pH was adjusted to between 7.0 and 7.2) at 28 °C for 3 days. Both FA26 and Cms were maintained on their respective medium supplemented with 30 % glycerol at −20 °C as working stocks.

Antibacterial activity assay of VOCs produced by Bacillus subtilis FA26

The volatile-mediated antibacterial activity of B. subtilis FA26 against Cms was evaluated under the I-plate system [23]. I-plates consist of centrally partitioned plastic Petri dishes (100 x 15 mm) that do not allow any physical contact between the two micro-organisms grown on either side. FA26 was grown in LB broth and incubated in an orbital shaker at 200 r.p.m. and 37 °C for 16 h. Five sterile filter papers (4 mm) were placed on one half of the I-plate containing LB agar and each filter paper was impregnated with 10 µl suspension of FA26. A fresh colony of Cms was inoculated in 20 ml of NA broth and shaken at 200 r.p.m. and 28 °C for 18–24 h [24]. One sterile filter paper was placed at the centre of the other half of the I-plate containing NA agar medium and impregnated with 10 µl suspension of Cms. I-plates were then double-sealed tightly with Paraflim M (Pechiney, Neenah, WI, USA) and incubated at 28 °C for 5 days. During incubation, Cms colonies were collected every 24 h and subjected to 10-fold serial dilution to count viable cells. Control plates had Cms grown on NA agar medium on one half of the I-plate and non-inoculated LB medium on the other. The experiment was repeated three times and each treatment had at least five replicates. Samples were also prepared for electron microscopy. For the viable cell counts, the inoculated colony of Cms was cut from the surrounding cultivation medium, picked up carefully using a sterile scalpel without damaging the colony and placed in a 50 ml conical flask containing 10 ml of sterile water. Flasks were swirled very carefully and Cms cells were allowed to mix homogeneously with the water. 100 µl of the suspension of Cms cells was then diluted up to 10-fold and plated onto NA agar. Plates were sealed and incubated at 28 °C for 3 days followed by counting the viable cells of Cms. The results were expressed as the log of c.f.u. per millilitre. To determine the bacteriostatic or bactericidal effects of antibacterial VOCs by FA26 on the growth of Cms, after incubation at 28 °C for 5 days, three I-plates were unused and incubated back at 28 °C for 3 days after removing FA26 along with agar medium from one half of the I-plates [15].

Scanning electron microscopy (SEM)

SEM was used to observe internal morphological changes in the Cms colony when exposed to rhizobacterial VOCs of B. subtilis FA26. Under sterile conditions, filter papers were removed from the inoculated colony of Cms using flame-sterilized micro-tweezers and Cms cells were then collected directly into an Eppendorf tube. Cells were then washed three times with 0.05 mol l⁻¹ PBS at pH 7.0 and fixed with 2 % glutaraldehyde (Solarbio, Beijing) for 30 min at 4 °C, following the previous protocol for SEM [25]. Electron micrographs were taken using a Hitachi Science System SEM (Hitachi S-3000N, Tokyo, Japan).

Transmission electron microscopy (TEM)

TEM was conducted to determine the effects of antibacterial VOCs emitted from B. subtilis FA26 on the cells of Cms at the ultra-structural level. Cells were collected, washed and fixed with 2 % glutaraldehyde for 30 min at 4 °C, as mentioned above. The specimens were then prepared according to the reported protocol for TEM analysis [24]. Ultra-structural changes in the cells were observed using a Hitachi transmission electron microscope (H-600, Hitachi, Tokyo, Japan).
Effects of inoculation strategy and inoculum size on the production and activity of antibacterial VOCs of B. subtilis FA26 against Cms

This experiment was designed to determine the effects of inoculation strategy and inoculum size on the production and activity of antibacterial VOCs produced by B. subtilis FA26 against Cms. In this experiment, five or nine sterile filter papers (4 mm) were placed on one half of the I-plate containing LB agar and each filter paper was impregnated with 10 µl suspension of FA26. In addition, 20 µl of the same culture suspension was spread over one half of the I-plate. The other halves of the I-plates were inoculated with Cms, as described previously. Plates without FA26 served as controls. Plates were sealed and incubated as mentioned above. The plates were incubated at 28 °C, and Cms colonies were sampled every 24 h and subjected to 10-fold serial dilution to count viable cells.

Collection and analysis of rhizobacterial VOCs using HS-SPME/GC-MS

HS-SPME/GC-MS was used to characterize the volatile fraction of B. subtilis FA26. The SPME fibre used to collect volatile compounds was 2 cm 50/30 µm divinylbenzene/carboxen on polydimethylsiloxane (57 348 U, Supelco, Fluka). To extract the volatile compounds, a 20 µl suspension of FA26 (16 h old) was grown in 30 ml of LB agar in a 100 ml headspace vial at 28 °C for 3 days. The SPME fibre was inserted into the headspace vial just above the inoculated or non-inoculated (control) medium and allowed to equilibrate at 50 °C for 40 min in an incubator. GC-MS analysis was performed using a Bruker 450-GC gas chromatograph in combination with a Bruker 320 MS mass spectrometer. A capillary GC column, Supelcowax10 (30 m×0.25 mm inside diameter, 0.25 µm) (24 079, Supelco), was used to separate the volatile compounds. Helium was used as the carrier gas under a constant flow of 1 ml min⁻¹. The SPME fibre was inserted and desorbed at 220 °C for 1 min into the injection port of the injector. The injector temperature was maintained at 250 °C. The working temperature of the column was set as follows: 35 °C for 3 min at the beginning; increased to 180 °C at 10 °C min⁻¹ and held for 1 min; a further increase to 240 °C at 4 °C min⁻¹ and held for 4 min. The mass spectrometer was operated in an electron ionization mode at 70 eV with a source temperature of 220 °C, and a continuous scan ranging from 50 to 500 m/z. The obtained mass spectra of the volatile compounds were compared with those stored in the NIST 11 Mass Spectral Library (NIST11/2011/EPA/NIH) by using NIST MS Search software version 2.0g (2011–05).

Screening of individual VOCs against Cms

The VOCs identified by the GC-MS analysis were purchased as synthetic chemicals from Sigma-Aldrich or Aldrich. Each of the VOCs was first tested as a pure chemical for its antibacterial activity against Cms under the I-plate system. Solid-state VOCs were dissolved in DMSO at a final concentration of 1 mg ml⁻¹. For the antibacterial assay, freshly grown Cms (18–24 h) in nutrient broth was used. A 5 µl suspension of Cms was dropped in the centre of one half of the I-plate containing NA agar. On the other half, one piece of sterile filter paper (20 mm) was placed in the centre and impregnated with 100 µl of each of the tested VOCs or DMSO only. DMSO alone was used as a control since it does not inhibit micro-organisms [26] and has been used previously as a solvent control against Cms [24]. Plates were then immediately double-sealed with Parafilm M and incubated. Following 72 h of incubation at 28 °C, the colonies of Cms exposed to each individual VOC were subjected to 10-fold serial dilution to count viable Cms cells.

Based on the results of the initial screening of individual VOCs, four VOCs (benzaldehyde, nonanal, benzothiazole and acetonaphone) were chosen for further detailed study of their antibacterial activity against Cms at different concentrations. Selected VOCs were diluted in DMSO to give final concentrations of 1, 10 and 100 µl ml⁻¹. 100 µl of each pure VOC was also applied and compared. DMSO alone was applied as a control. The antibacterial volatile assay was carried out as described above. After 72 h of incubation at 28 °C, the colony morphology of Cms was observed and photographed digitally using an Olympus Research System Stereomicroscope SZX10 equipped with an Olympus DP72 digital camera. The experiment was repeated three times and each individual VOC had at least three replicates.

Statistical analysis

The results were analysed statistically using analysis of variance (ANOVA) with the Tukey–Kramer honestly significant difference (HSD) multiple range test (P=0.05) in JMP software version 10.0 (SAS Institute, Cary, NC) for Mac.

RESULTS AND DISCUSSION

Antibacterial volatile activity of B. subtilis FA26 against Cms growth

B. subtilis FA26 was evaluated for the production of antibacterial VOCs against Cms under the I-plate system. The results showed that, at 24 h, the cell numbers of Cms in control and VOC-treated colonies were not significantly different (Fig. 1). Afterwards, the cell numbers of Cms in control were increased continuously until 120 h; in the VOC-treated colonies, the cell numbers of Cms were not increased and remained statistically the same until 120 h compared to that after 24 h. These results suggested a bacteriostatic effect of the VOCs produced by FA26 against Cms. The I-plates containing Cms, which were incubated back at 28 °C for 3 days after removing paraffilm and FA26 along with agar medium from one half of the I-plates, resumed normal growth rate, which further confirmed the bacteriostatic nature of the VOCs of FA26 against Cms. Recently, similar effects were observed onRalstonia solanacearum by the VOCs of a biocontrol agent, Bacillus amyloliquefaciens strain SQR-9 [15].

The antimicrobial activity of rhizobacterial VOCs has been studied frequently. Many researchers have reported that the rhizobacterial VOCs could not kill phytopathogenic fungi such as Fusarium oxysporum [11, 27], Fusarium
Sclerotinia sclerotiorum VOCs might be able to kill the phytopathogenic fungus, but could inhibit the mycelial growth of the fungi significantly. Only a few reports stated that the rhizobacterial VOCs of *Bacillus subtilis* [15], and the nematode, *Bursaphelenchus xylophilus* [18], suggesting fungicidal and nematicidal activities, respectively. Furthermore, the antibacterial VOCs of *B. amyloliquefaciens* strain SQR-9 showed good potential against the growth and virulence traits of *Xanthomonas campestris* [11], and the nematode, *R. solanacearum* [15]. We have also documented recently that the antibacterial VOCs of *B. amyloliquefaciens* strain SQR-9 showed good potential against the growth and virulence traits of *R. solanacearum* [15]. We have also documented recently that the antibacterial VOCs of *B. amyloliquefaciens* strain SQR-9 showed good potential against the growth and virulence traits of *R. solanacearum* [15].

**Fig. 1.** Antibacterial activity of VOCs produced by *Bacillus subtilis* FA26 against Cms. Five sterile filter papers were placed on one of the two halves of the I-plate containing LB agar medium and each filter paper was impregnated with 10 µl of a suspension of FA26 (16 h old), while one sterile filter paper was placed at the centre of the other half and was impregnated with 10 µl suspension of Cms. The plates were incubated at 28 °C and every 24 h colonies of Cms were collected and viable Cms cells were counted using the serial dilution technique. The experiment was repeated three times with at least five replicates of each treatment. The results were expressed as log c.f.u. ml⁻¹. Error bars indicate means ± SEM. Similar letters on the error bars of means indicate that the means are not significantly different from each other according to the Tukey–Kramer HSD multiple range test at *P*=0.05.

**Effect of inoculation strategy and inoculum size on the production and activity of antibacterial VOCs of FA26**

The effects of varying amounts of inoculum and different inoculation methods on the production and activity of antibacterial VOCs of FA26 on Cms were determined. The visual inspection showed changes in colour and size of the Cms colonies under different inoculation strategies and inoculum sizes of FA26 when compared with the control (Fig. 3b). The treated colonies appeared paler in colour than the non-treated colonies. The sizes of the treated colonies were also reduced but varied among the treatments when compared with the control. The smallest Cms colony was observed when the FA26 inoculum was 90 µl, applied as nine 10 µl drops. The colony size was smaller when 20 µl of the FA26 inoculum was spread over one half of the I-plate compared with dropping five 10 µl drops (50 µl). The Cms colonies were also serially diluted to count viable cells. The results showed a varying degree of growth inhibition of Cms by the VOCs of FA26 under different inoculation strategy and inoculum size of FA26 (Fig. 3a). The inhibition of Cms reached 43.92 and 39.94 % after 120 h when dropped (90 µl) or spread (20 µl), respectively. These results suggested that the production and activity of antibacterial VOCs correlated with an increase in the amount of the inoculum of FA26. In addition, spreading the inoculum, which needs a relatively small amount of inoculum, had similar effects to the 90 µl drops. This might be because the spread cells have greater access to nutrients as they cover a larger area, which could lead to a higher overall number of cells being present after growth. The present study showed that higher amounts of inoculum or spreading led to the maximum inhibition of Cms by antibacterial VOCs produced by FA26. These results were similar to those from a recent study on dose-dependent antibacterial activity of VOCs produced by *B. amyloliquefaciens* strain SQR-9 against *R. solanacearum* [15].

**Scanning and transmission electron microscopy**

The scanning electron micrographs of the samples, taken from the non-inoculated control after 120 h of incubation, showed normal growth of the cells (Fig. 2a). By contrast, the Cms colony was distorted internally and the cells were damaged in the presence of the VOCs of FA26 (Fig. 2b). The abnormal morphology of conidiophores and fungal hyphae of *Penicillium italicum* exposed to the VOCs of *Streptomyces globisporus* JK-1 was also observed under SEM [20].

The transmission electron micrographs of the cells, collected from the non-inoculated control, further showed apparently intact envelopes, electron dense cytoplasm and robust ultra-structure (Fig. 2c). Compared with the undamaged cells in the control, a wide range of abnormalities in Cms cells exposed to VOCs was observed (Fig. 2d, e), e.g. disintegration of cells, formation of inclusions, movement of cytoplasmic content towards the ruptured cell walls or cytoplasmic membranes, and lack of cytoplasmic content or fragmented cytoplasm. Some cells were misshapen and some electron dense spots were observed in the damaged cells. These results indicated the possible biocontrol mechanism of antibacterial VOCs from FA26 by inducing a wide range of Cms cell abnormalities at the ultra-structural level.

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References:

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**Fig. 2.** Abnormal ultra-structure of Cms. Scanning electron micrographs were collected and viable Cms cells were counted using the serial dilution technique. The experiment was repeated three times with at least five replicates of each treatment. The results were expressed as log c.f.u. ml⁻¹. Error bars indicate means ± SEM. Similar letters on the error bars of means indicate that the means are not significantly different from each other according to the Tukey–Kramer HSD multiple range test at *P*=0.05.
Fig. 2. SEM and TEM analysis of the ultrastructure of the colony and cellular morphology of Cms affected by the VOCs of B. subtilis FA26. The specimens were prepared from samples taken directly from the I-plates after 5 days of incubation. The Cms colony showed normal growth in the non-inoculated control (a). In contrast, the colony morphology of Cms was damaged in the presence of rhizobacterial VOCs of FA26 (b) and showed several deformed cells within the colony (white arrowheads). At the ultrastructural level, compared with the undamaged cells in the non-inoculated control (c), a wide range of abnormalities in the Cms cells affected by the VOCs of FA26 was observed (d and e), e.g. disintegration of cells (1), formation of inclusions (2), movement of cytoplasmic content towards the ruptured cell walls or cytoplasmic membranes (3), and a lack of cytoplasmic content or fragmented cytoplasm (4). Some cells were misshapen (5). Some electron dense spots were also observed in the damaged cells (6).

Fig. 3. Effect of inoculation strategy and inoculum size on the production and activity of antibacterial VOCs of B. subtilis FA26 against Cms. In this experiment, five or nine sterile filter papers were placed on one of the two halves of the I-plate containing LB agar medium and each filter paper was impregnated with 10 µl of a suspension of FA26 (16 h old) or 20 µl of the same suspension was spread over the LB medium. Meanwhile, one sterile filter paper was placed at the centre of the other half and was impregnated with 10 µl suspension of Cms. Control plates had Cms only. The plates were incubated at 28°C and, every 24 h, colonies of Cms were collected and viable Cms cells were counted using the serial dilution technique. The experiment was repeated three times and each treatment had at least five replicates. The results were expressed as log c.f.u. ml⁻¹ (a). Error bars indicate mean±SD. Similar letters on the error bars of means indicate that the means are not significantly different from each other according to the Tukey-Kramer HSD multiple range test at P=0.05. The zoomed-in insets of the colonies (×10 magnification) indicate more distinct colony morphology of the treatments (b).
GC-MS analysis of VOCs produced by *B. subtilis* FA26

Collection and analysis of headspace VOCs of *B. subtilis* FA26 grown in LB medium was carried out by HS-SPME combined with GC-MS. The volatile fractions of FA26 were compared with VOCs retrieved from the control (non-inoculated LB medium), allowing us to select 11 VOCs of rhizobacterial origin for further analysis (Table S1, available in the online Supplementary Material). In this first screening, the results indicated that eicosane had no antibacterial effect on Cms, while 1,3-benzenediamine, catechol, 2-pentacosanone, hexadecane, pentadecane and oleic acid were not significantly effective in inhibiting the growth of Cms compared with the control (Fig. 4). However, only four VOCs (benzaldehyde, nonanal, benzothiazole and acetophenone) showed the growth inhibition of Cms significantly compared with the control. Thus, these four VOCs were selected for further detailed study. Interestingly, these four VOCs have already been reported to be produced by other rhizobacteria and showed good antimicrobial activities [11, 13, 15, 18, 20].

**Effect of benzaldehyde, nonanal, benzothiazole and acetophenone on Cms growth at different concentrations**

Benzaldehyde, nonanal, benzothiazole and acetophenone were further tested for their effects on Cms at different concentrations. The results showed varying degrees of Cms inhibition by the selected VOCs at different concentrations. Fig. 5(b) shows the reduced sizes of the Cms colonies with the increase in the concentration of all four VOCs. Rhizobacterial nonanal and benzothiazole have been characterized previously as antifungal VOCs that could completely inhibit the mycelial growth and sclerotia formation of *S. sclerotiorum* when applied at 150 µl of pure chemicals [13]. In this study, nonanal and benzothiazole showed strong antibacterial activity against Cms when applied at 100 µl ml$^{-1}$ (Fig. 5a). Rhizobacterial benzaldehyde has been shown previously to have 100 % nematicidal activity against the phytopathogenic nematode, *Bursaphelenchus xylophilus* [18]. The results also indicated that the effect of benzaldehyde on the growth of Cms reached its maximum level at 100 µl ml$^{-1}$ followed by the effects of nonanal, benzothiazole and acetophenone.

**Conclusions**

*B. subtilis* FA26 produced antibacterial VOCs that inhibited the bacterial phytopathogen, Cms, by inducing several cellular abnormalities. The production and activity of these VOCs depended on the inoculation strategy and inoculum size. The identification of rhizobacterial VOCs of FA26, such as benzaldehyde, nonanal, benzothiazole and acetophenone, revealed their antibacterial characteristics. Thus, they could be adopted as main ingredients to manufacture...
Fig. 5. The effects of four selected VOCs on Cms at different concentrations. During an initial screening, of all the identified VOCs against Cms, benzaldehyde, nonanal, benzothiazole and acetophenone were selected as potential antibacterial VOCs and were further tested at different concentrations (1, 10, and 100 µl ml\(^{-1}\) diluted in DMSO). 100 µl of undiluted pure VOCs were also applied. DMSO alone was applied as a control. The experiment was performed as described previously. The results were expressed as log c.f.u. ml\(^{-1}\) (a). Error bars indicate mean±SD. Similar letters on the error bars of means indicate that the means are not significantly different from each other according to the Tukey-Kramer HSD multiple range test at \(P=0.05\). The experiment was repeated three times with at least three replicates of each individual VOC. In addition, the colony morphology of Cms was observed and photographed digitally using a stereomicroscope (SZX10, Olympus) (b). All observations were made at the same magnification (×0.8) to ensure consistent and comparable results. Figures of the colonies were scaled at equal dimensions. Scale bar, 2 mm.
biopesticides against bacterial phytopathogens in the future. The results of this study suggested a long-distance biocontrol mechanism exerted by antibacterial VOCs produced by FA26 against Cms.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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