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REGULAR ARTICLE

Production of anti-fungal volatiles by non-pathogenic *Fusarium oxysporum* and its efficacy in suppression of Verticillium wilt of cotton

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Abstract
Aims The study aimed at identifying volatile organic compounds (VOCs) produced by the non-pathogenic *Fusarium oxysporum* (*Fo*) strain CanR-46, and to determine the anti-fungal spectrum and the control efficacy of the *Fo*-VOCs.
Methods The *Fo*-VOCs were identified by GC-MS. The antifungal activity of the *Fo*-VOCs was tested using the double-dish method. Soil contaminated with *Verticillium dahliae* was fumigated with the *Fo*-VOCs to test the control efficacy. The GFP-tagged derivative strain CanR-46GFP was tested to colonize cotton roots for prevention of infection by *V. dahliae*.
Results Nineteen VOCs were identified with eremophila-1 (10),11-diene being the most abundant. The *Fo*-VOCs inhibited mycelial growth of 14 fungal species including *V. dahliae*, delayed *V. dahliae* conidial germination, suppressed *V. dahliae* germ-tube elongation, and caused *V. dahliae* hyphal shriveling and collapse. Four synthetic chemicals, 5-hexenoic acid, limonene, octanoic acid and 3,4-2H-dihydropyran, in the *Fo*-VOCs profile showed antifungal activity against *V. dahliae*. The *Fo*-VOCs significantly ($P<0.05$) reduced severity of Verticillium wilt of cotton compared to the control. CanR-46GFP extensively colonized the cotton root tissues, thus effectively prevented *V. dahliae* infection.

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Conclusions The VOC-producing fungus *F. oxysporum* CanR-46 is a promising biocontrol agent against *V. dahliae*.

Keywords *Fusarium oxysporum* CanR-46 · Volatile organic compounds · *Verticillium dahliae* · Biological control · Fumigation

Introduction

Volatile organic compounds (VOCs) are carbon-based small chemicals with molecular weight usually lower than 300 Da and low polarity, but with high vapor pressure (approximately 0.01 kPa) at room temperatures (Vespermann et al. 2007; Morath et al. 2012). They are ideal infochemicals functioning in above-ground and underground communications between living organisms (Rasmann et al. 2005; McCormick et al. 2012). According to a recent review by Morath et al. (2012), approximately 250 fungal VOCs belonging to hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters, benzene derivatives and cyclohexanes have been identified. Previous studies showed that a mixture of VOCs of the endophytic fungus *Muscodor albus* can be used as a mycofumigant for control of many post-harvest fruit diseases (Mercier and Jiménez 2004; Mercier and Manker 2005; Mercier and Smilanick 2005; Schnabel and Mercier 2006) and soilborne plant diseases (Stinson et al. 2003). The success of *M. albus* inspired numerous researchers to explore use of VOCs produced by other fungi and bacteria to control plant diseases. Minerdi et al. (2009) reported that the VOCs produced by the non-pathogenic strain MSA 35 of *Fusarium oxysporum* together with its ectosymbiotic bacterial consortium (e.g., *Serratia*, *Achromobacter*, *Bacillus* and *Stenotrophomonas*) could inhibit growth of several pathogenic *formae speciales* of *F. oxysporum* on agar media.

Two mechanisms, namely direct inhibition of mycelial growth and/or spore germination (Strobel et al. 2001; Huang et al. 2011, 2012), and/or induction of plant resistance (Ryu et al. 2004), have been proposed to be involved in suppression of plant pathogens by the microbial VOCs. Furthermore, previous studies reported that the VOCs of certain bacterial species, including *Bacillus subtilis* GB03, *B. amyloliquefaciens* IN937a and *E. cloacae* JM22 (Ryu et al. (2003); *Arthrobacter agilis* UMCV2 (Velázquez-Becerra et al. 2011), *Proteus vulgaris* JBLS202 (Yu and Lee 2013) can promote plant growth. Similarly, Minerdi et al. (2011) found that the VOCs of *F. oxysporum* MSA 35 can promote growth of lettuce (*Lactuca sativa* L.).

Verticillium dahliae Kleb. is a destructive soilborne fungus causing Verticillium wilt on many economically important crops including cotton (*Gossypium* spp.) in temperate and subtropical regions (Pegg and Brady 2002). Stinson et al. (2003) reported that mycofumigation with the VOCs of *M. albus* and *M. roseus* effectively reduced inoculum density of *V. dahliae* in soil, thereby leading to suppression of the severity of Verticillium wilt of eggplant (*Solanum melongena* L.).

Strain CanR-46 of *F. oxysporum* is an endophytic fungus isolated from a healthy root of oilseed rape (*Brassica napus* L.) in our previous study (Zhang et al. 2014). It was stored in China Center for Type Culture Collection (CCTCC) with the accession number of CCTCC M2014440. It was detected to be non-pathogenic on oilseed rape and cotton, and to be able to produce antifungal VOCs inhibitory to growth of the plant pathogenic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum* on agar media (Zhang et al. 2014). However, the chemical components of the VOCs of *F. oxysporum* CanR-46 and the efficacy of *F. oxysporum* in suppression of *V. dahliae* on cotton remain unknown.

The objectives of this study are: (i) to identify the chemical nature of the VOCs produced by *F. oxysporum* CanR-46; (ii) to determine the antifungal spectrum of the VOCs of *F. oxysporum* CanR-46; and (iii) to evaluate efficacy of the VOCs of *F. oxysporum* CanR-46 in suppression of Verticillium wilt of cotton.

Materials and methods

Fungal isolates and cultural media

The endophytic strain CanR-46 of *F. oxysporum* was originally isolated from a healthy root of oilseed rape grown in Wuhan of central China (Zhang et al. 2014). It was sub-cultured on potato dextrose agar (PDA). Strain CanR-46GFP harboring the green fluorescent protein gene was derived from strain CanR-46 (Supplementary material Fig. S1). Both strains are similar in growth rate on PDA (25 °C), in conidial germination rate on water agar (WA, 25 °C), and in production

of the antifungal VOCs on autoclaved wheat grains (AWG). Sixteen plant pathogenic fungal strains were used in this study and their origins were listed in Supplementary material Table S1. Stock cultures of these fungal strains were maintained on PDA and stored at −80 °C in 40 % glycerol (*v/v*). Working cultures were prepared by transferring mycelia from storage onto PDA. The cultures were incubated in the dark at 20 or 25 °C for 3 to 15 days depending on the mycelial growth rates of the investigated fungal strains.

Three cultural media, including AWG, PDA and water agar (WA), were used in this study. AWG was made of wheat grains (50 % water content, *w/w*). PDA was made of peeled potato (200 g peeled potato tuber, 20 g glucose, 15 g agar, 1000 ml water). WA contained agar alone in distilled water (1.5 %, *w/v*).

Analysis of the VOCs of *F. oxysporum* CanR-46

The chemical components of the VOCs of *F. oxysporum* CanR-46 were analyzed using GC-MS. Strain CanR-46 was inoculated in a 250-mL Erlenmeyer flask containing 30 g AWG at 3×10^4 microconidia g^{-1} AWG. The culture was incubated at 20 °C in the dark for 5 days. Preliminary experiments showed that VOC emission reached the plateau in 5 days under such cultural conditions. The VOCs in the flask were collected using the headspace solid-phase microextraction technique (HS-SPME) (Wan et al. 2008). The VOC components were identified using a GC-MS machine (6890N-5975B, Agilent Technologies Inc., CA, USA) following the procedures recommended by the manufacturer. Meanwhile, the VOCs released from the non-inoculated AWG were also collected and identified in the same manner. The experiment was conducted two times. The VOCs appearing in the HS-SPME extract from the AWG cultures of *F. oxysporum* in both trials, but not appearing in the HS-SPME extract from the non-inoculated AWG, were considered to the components produced by *F. oxysporum*.

Antifungal activity of selected synthetic VOCs

Five synthetic chemicals (β-caryophyllene, 3,4-dihydro-2H-pyran, (±)-limonene, 5-hexenoic acid, and octanoic acid) were selected based on the VOC profile of *F. oxysporum* CanR-46, as only these compounds were available from the chemical companies. β-Caryophyllene and 3,4-dihydro-2H-pyran were purchased from TCI (Shanghai) Development Co., Ltd. (Shanghai, China). (±)-Limonene and 5-hexenoic acid were purchased from Tokoyo Chemical Industry Co., Ltd. (Tokyo, Japan). Octanoic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All these synthetic compounds were pure by 90 % or higher. They were individually tested for inhibition of mycelial growth and conidial germination of *V. dahliae* using the double-dish method described in our previous studies (Wan et al. 2008; Huang et al. 2011). The concentration value for 50 % inhibition of mycelial growth and conidial germination (IC_{50}) expressed as microliters per liter (μL L^{-1}) was inferred from the data about the inhibition percentages and the corresponding VOCs doses applied in the double-dishes assay (Huang et al. 2011).

Antifungal activity of the VOCs of *F. oxysporum* CanR-46

Inhibition of mycelial growth and conidial germination of *V. dahliae* by the VOCs of *F. oxysporum* CanR-46 was tested using the double-dish method as previously described (Wan et al. 2008; Minerdi et al. 2009; Huang et al. 2011). In the mycelial growth experiment, *F. oxysporum*-inoculated AWG (3×10^4 microconidia g^{-1} AWG) was transferred to Petri dishes (9 cm in diameter) at 0 (empty dish), 2, 4, 8, 16 or 32 g per dish with three replicated dishes per treatment. Fresh non-inoculated AWG (8 g per dish) served as the control. The dishes with the *F. oxysporum* culture and the control AWG were incubated at 25 °C in the dark for 3 days to allow fungal growth and production of the VOCs. A bottom dish with PDA was inoculated with a mycelial agar plug (6 mm in diameter) of *V. dahliae* removed from the colony margin area of a PDA culture. It was placed over facing a bottom dish with the fresh AWG or with the AWG culture of *F. oxysporum* to create a double-dish set, which was immediately wrapped with parafilms. The double-dish sets were placed at 25 °C for 21 days. Diameter of the colony of *V. dahliae* was measured on the daily basis and the mycelial growth rate (mm day^{-1}) was calculated. The difference in mycelial growth rate between the control treatment (non-inoculated AWG) and the *F. oxysporum* treatment (*F. oxysporum*-inoculated AWG) was used to calculate the percentage of mycelial growth inhibition.

To understand the mechanisms involved in suppression of mycelial growth of *V. dahliae* by the VOCs of

F. oxysporum, morphological characteristics of the hyphae of *V. dahliae* treated by the VOCs were observed under scanning electron microscope. A double-dish set for the treatment of *F. oxysporum*-*V. dahliae* contained 8 g AWG culture of *F. oxysporum* in one dish and *V. dahliae* on a cellophane film in the other dish with PDA. A control double-dish set contained 8 g fresh AWG in one dish and *V. dahliae* on a cellophane film in another PDA dish. The double-dish sets were individually wrapped with parafilms and placed in an incubator at 25 °C in the dark for 7 days. The cellophane film with the colony margin of *V. dahliae* for each treatment was cut into small pieces (about 1×1 mm, length×width) using a sterilized razor blade. The mycelial specimens were immediately fixed, dehydrated, critical-point dried, gold-coated and examined under a scanning electron microscope (JSM-6390/LV, NTC, Tokyo, Japan) using the standard procedures.

In the conidial germination experiment, a double-dish set for the treatment of *F. oxysporum*-*V. dahliae* contained 8 g AWG culture of *F. oxysporum* in one dish and the conidia of *V. dahliae* (1×10^4 conidia dish^{-1}) on water agar in another dish. A double-dish set for the treatment of *V. dahliae* alone contained 8 g fresh AWG (control) in one dish and the conidia of *V. dahliae* on water agar in the other dish. There were 24 double-dish sets for each treatment. The double-dish sets were individually wrapped with parafilms and placed in an incubator at 25 °C in the dark. After incubation for 3, 6, 9, 12, 15, 18, 21 and 24 h, three double-dish sets for each treatment were taken out from the incubator. Germination of the *V. dahliae* conidia in each dish was observed under a light compound microscope by randomly counting 100 conidia and length of 30 germ tubes were measured. A conidium was regarded germinated when the germ tube length was equal to or longer than the diameter of that conidium. Data about the conidial germination rates or germ-tube length over time were analyzed using Slogistic 1 function in Growth/Sigmoidal Category in the Origin 8.0 software (OriginLab, Northampton, MA, USA).

Antifungal spectrum of the VOCs of *F. oxysporum* CanR-46

Effects of the VOCs of *F. oxysporum* CanR-46 on mycelial growth of 15 other fungal strains belonging to nine genera (Supplementary material Table S1) were tested using the above-mentioned double-dish method. *V. dahliae* was included as reference in this bioassay. The Petri dishes each containing 8 g AWG cultures of *F. oxysporum* CanR-46 or 8 g fresh AWG (control) and the Petri dishes each containing a tested fungus on PDA were used to set up the double-dish sets. There were three double-dish sets for each tested fungus and *V. dahliae*. All the double-dish sets were placed in the dark at 20 or 25 °C for 3 to 21 days. Colony diameter of each tested fungus in a double-dish set was measured daily and the mycelial growth rate (mm day^{-1}) was calculated. At the end of the experiment, the viability of the tested fungi was determined by removing the dishes containing *F. oxysporum* CanR-46 from the double-dish sets. The other dishes with the tested fungi were covered again and placed in the incubator for another 7 days. Appearance of new mycelial growth indicated that the mycelia were still viable after exposure to the VOCs.

Fumigation of soil with the VOCs of *F. oxysporum* CanR-46

F. oxysporum CanR-46 was inoculated in AWG in Petri dishes, 130 g AWG per dish. The dishes were placed in an incubator at 25 °C in the dark for 3 days. Then, the cover dishes were removed and a bottom dish was placed at the bottom of a desiccator. Meanwhile, autoclaved Organic Culture Mix (Zhengjiang Peilei Organic Manure Manufacturing Co. Ltd., Zheng Jiang, Jiangsu Province, China) was inoculated with *V. dahliae* to reach the final concentration of 2×10^8 conidia g^{-1}. The culture mix (approximately 40 % water content, *w/w*) was loaded on a piece of one-layered gauze on the clapboard of that desiccator. For the control treatment, a dish with 130 g fresh AWG was placed at the bottom of a desiccator and the *V. dahliae*-infested culture mix was loaded on a piece of one-layered gauze on the clapboard of that desiccator. The desiccators were individually covered and placed at 20 °C for 5 days. The culture mix of each treatment was then taken out and loaded in plastic pots (7×8 cm, diameter×height), and eight pots for each treatment. Two-true-leaf cotton seedlings (*Gossypium hirsutum* cv. E Kang Mian No. 12) were individually transplanted into the culture mix in the pots, one seedling per pot. The pots were maintained in a growth chamber at 25 °C under a lighting regime of 16-h light/8-h dark for 20 days. Then, the cotton seedlings were individually rated for disease severity using a 0 to 4 scale (Xu et al. 2012). Disease severity index for

each treatment was calculated with the formula described by Xu et al. (2012). The experiment was conducted two times.

Treatment of cotton seedlings with *F. oxysporum* CanR-46

F. oxysporum CanR-46GFP was used in this experiment to test its efficacy in suppression of Verticillium wilt of cotton and to determine colonization of *F. oxysporum* in the tissues of cotton roots. *V. dahliae* strain 4TM6-15 (Xu et al. 2012) and *F. oxysporum* CanR-46GFP (this study) were incubated on PDA in Petri dishes (25 °C) for 15 days. Conidia of each fungus were harvested washing the cultures with sterile distilled water, and the resulting mixtures were filtered through four-layered cheese cloth to remove the mycelial fragments in the conidial suspension. The conidial concentration was adjusted with sterilized water to 3×10^6 conidia mL^{-1} for *V. dahliae* and 3×10^7 microconidia mL^{-1} for *F. oxysporum*. Meanwhile, the two-true-leaf stage cotton seedlings were uprooted and the roots were washed under tap water to remove the remaining culture mix. Finally, the cotton seedlings were blotted dry on paper towels and used in the flowing treatments.

There were four treatments in this experiment: *F. oxysporum* CanR-46GFP + *V. dahliae* (*Fo*GFP + *V. dahliae*); *V. dahliae* alone; *F. oxysporum* CanR-46GFP alone (*Fo*GFP); and water control (CK). For the treatment of *Fo*GFP + *V. dahliae*, the conidial suspensions of *V. dahliae* and *F. oxysporum* were mixed at an equal volume (Final conidial concentration: *F. oxysporum* at 1.5×10^7 microconidia mL^{-1} and *V. dahliae* at 1.5×10^6 conidia mL^{-1}). For the treatments of *Fo*GFP alone and *V. dahliae* alone, the conidial suspensions were diluted by 1:1 with sterile water to give the same respective conidial concentrations as those used in the treatment of *Fo*GFP + *V. dahliae*. Roots of the cotton seedlings (10 per treatment) were dipped for 30 s in the respective conidial suspension, or in water for the control treatment. The seedlings were individually transplanted into a fine clay loam soil in plastic pots (12×10 cm, diameter×height), two seedlings per pot. The pots were maintained in a growth chamber at 25 °C under the regime of 16-h light/8-h dark for 10 days. Disease severity on each seedling was scored (Xu et al. 2012). Disease incidence and disease severity index for each treatment was then calculated. The experiment was conducted three times.

Laser scanning confocal microscopy

In order to detect colonization of cotton seedlings by *F. oxysporum* CanR-46GFP, three cotton seedlings for each treatment were randomly sampled. The tap root of each seedling was cut into slices either transversely or longitudinally using a razor blade. The root slices were examined under ZEISS LSM 510 Meta laser scanning confocal microscope (Carl Zeiss Jena GmbH, Module LSM 510, Germany) equipped with a Diode/Argon/HeNe1-HeNe2 laser. Green fluorescence in the cotton root tissue was excited at 488 nm. Images were analyzed and processed with the LSM Image Browser software (Version 4.2, Southwest Environmental Health Sciences Center, University of Arizona, Tucson, Arizona, USA) and Adobe Photoshop (Adobe Systems). Pure cultures of strains CanR-46 and CanR-46GFP were also examined under the UV light microscope for comparison purposes.

Data analysis

Statistical analyses were carried out using the SAS/STAT® software (SAS Institute, Cary, NC, version 8.0, 1999). Data on germ-tube length and disease severity index for different treatments were directly analyzed using analysis of variance (ANOVA). When significant treatment effects were detected, treatment means were separated using Duncan's Multiple Range test at $P=0.05$. Data on percentage of mycelial growth inhibition, conidial germination rate and disease incidence were individually arcsine-transformed to normalize the variance before ANOVA and Duncan's Multiple Range test. After analysis, means were individually back-transformed to numerical values.

Results

GC/MS identification of the VOCs of *F. oxysporum*

GC-MS analysis identified 19 volatile organic compounds (VOCs) in 5-day-old AWG cultures of *F. oxysporum* CanR-46 based on their mass spectrum properties (Table 1). These compounds fell into classes of alkenes, esters, alkanes, organic acids and alcohols. Most compounds (11/19) belong to alkenes.

Table 1 Volatile organic compounds produced by *Fusarium oxysporum* CanR-46 in 5-day-old AWG cultures detected by GC/MS analysis

RT	RA	Possible compound	MW (amu)
5.495	0.12	2H-Pyran, 3,4-dihydro-	84.12
26.55	0.08	Limonene	136.23
36.57	2.09	Octanoic acid	144.24
44.14	0.13	Sulfurous acid, dodecyl 2-propyl ester	292.20
44.16	0.20	Methoxyacetic acid, 2-tetradecyl ester	286.25
45.63	0.03	3,4-Dimethylbenzyl alcohol	136.09
45.68	0.04	Spiro[5.5]undeca-1,8-diene, 1,5,5,9-tetramethyl-, (R)-	204.20
45.72	0.03	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl) -4-(1- methylethylidene)-	204.19
45.81	0.03	Benzenemethanol, 3,5-dimethyl-	136.09
46.31	0.15	(+)-Epi-bicyclosesquiphellandrene	204.21
46.32	0.15	1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a -tetramethyl-, [1aR-(1a.alpha,7alpha, 7a.alpha, 7b.alpha)]-	204.19
46.34	0.11	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	204.19
46.38	0.15	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene −1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	204.20
46.78	1.70	Caryophyllene	204.36
46.84	0.46	Dihydromyrcene	188.26
46.88	0.65	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	204.18
47.01	0.65	1S,2S,5R-1,4,4-Trimethyltricyclo[6.3.1.0(2,5)]dodec-8(9)-ene	204.10
48.63	14.33	Eremophila-1(10),11-diene	204.35
49.87	0.66	5-Hexenoic acid	114.14

* *RT* Retention time, *RA* Relative peak area (%), the values in that column are the average of two repeats, *MW* Molecular weight; The VOCs of *F. oxysporum* CanR-46 with the relative peak areas less than 0.01 % are not included in this table

Antifungal activity of selected synthetic VOCs

Four (5-hexenoic acid; (±)-limonene; 3,4-2H-dihydropyran; octanoic acid) out of the five synthetic chemicals appearing in the VOC profile of *F. oxysporum* CanR-46 exhibited inhibitory activity against *V. dahliae* (Table 2). Among the tested synthetic chemicals, 5-hexenoic acid showed the highest antifungal activity with the lowest 50 % inhibition concentration values (IC_{50}) for growth inhibition (3.5 μL L^{-1}), and for conidial germination (9.7 μL L^{-1}). β-Caryophyllene did not show any detectable inhibitory activity against *V. dahliae*.

Table 2 Fifty percent inhibition concentrations (IC_{50}) of five synthetic compounds on the mycelial growth and conidial germination of *Botrytis cinerea* and *Verticillium dahliae*

Compound	IC_{50}, μL L^{-1}	
	Mycelial growth	Conidial germination
β-Caryophyllene	–*	–
5-Hexenoic acid	3.5	9.7
(±) -Limonene	6027.0	58.9
3,4-2H-dihydropyran	152.5	76.8
Octanoic acid	164.3	58.9

* The symbol "–" indicates no inhibitory effect detected

Inhibition of *V. dahliae* by the VOCs of *F. oxysporum*

While the VOCs from the fresh AWG did not inhibit growth of *V. dahliae*, the VOCs from the AWG culture of *F. oxysporum* effectively inhibited mycelial growth of *V. dahliae* (Fig. 1). With increase of the dosage of the AWG culture of *F. oxysporum* from 2 to 32 g per dish, the percentage of growth inhibition gradually increased from 60 to 100 % (25 °C, 21 days). SEM observation showed that while the hyphae of *V. dahliae* in the control treatment without the *F. oxysporum* VOCs appeared vigorous and turgid, the hyphae of *V. dahliae* in the treatment with the *F. oxysporum* VOCs became shriveled and collapsed (Fig. 2).

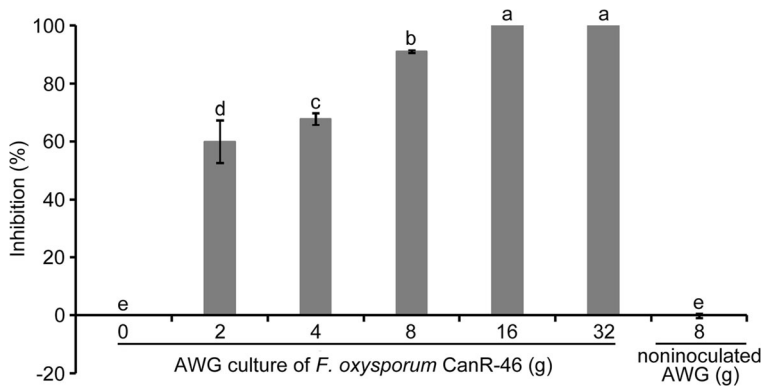

Fig. 1 Effects of the volatile organic compounds emitted from the AWG culture of *Fusarium oxysporum* CanR-46 on mycelial growth of *Verticillium dahliae*. AWG=Autoclaved Wheat Grains. Means±standard errors (n=3) labeled with different *letters* indicate significant difference (P<0.05) according to Duncan's Multiple Range Test

Results from the conidial germination experiment indicated that the VOCs of *F. oxysporum* CanR-46 had suppressive effect both on conidial germination rate and on germ-tube elongation of *V. dahliae* on WA (Fig. 3). In the absence of the VOCs, the average percentage of germinated conidia reached 26 % at 9 h post incubation (hpi) and 100 % at 21 hpi after incubation (Fig. 3a). The average length of germ tubes reached 37 μm at 9 hpi, and 145 μm at 21 hpi after incubation (Fig. 3b). In the presence of the VOCs, the percentage of germinated conidia was 1 % at 9 hpi and 96 % at 21 hpi (Fig. 3a). The average germ-tube length was lower than 5 μm at 9 hpi and 13 μm at 21 hpi (Fig. 3b).

Antifungal spectrum of the VOCs of *F. oxysporum*

The VOCs released from the 8 g AWG cultures of *F. oxysporum* CanR-46 inhibited growth of the 16 investigated fungal strains by 58 to 100 % (Table 3). The fungi belong to belonged to 14 fungal species in ten genera (*Aspergillus*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Magnaporthe*, *Monilinia*, *Penicillium*, *Rhizoctonia*, *Sclerotinia*, *Verticillium*). Interestingly, the VOCs suppressed *F. oxysporum* f.sp. *vasinfectum* and *F. oxysporum* f.sp. *niveum* by 58 and 78 %, respectively. The values were significantly lower (P<0.05) than those of 90 to 100 % for other fungal species (Table 3).

After removal of the dishes with *F. oxysporum* CanR-46 (the source of the VOCs) from the double-dish sets, 14 out of the 16 fungal strains recovered to grow on PDA at 20 or 25 °C (Table 3) and normal colonies were developed after incubation for 7 days (data not shown), suggesting that some suppressed mycelia were still viable after fumigation by the VOCs. However, *M. oryzae* WhMO-10 and *V. dahliae* 4TM6-15 did not grow after incubation for 7 days, suggesting that the hyphae of the two fungal strains were already dead under fumigation of the VOCs.

Suppression of Verticillium wilt of cotton by soil fumigation with the VOCs

The treatment of soil fumigation with the *F. oxysporum* VOCs differed significantly from the control treatment (soil fumigated with the VOCs from the fresh AWG)

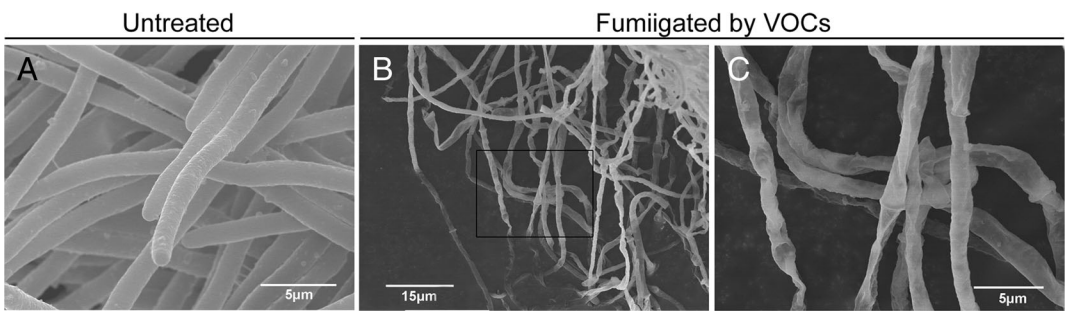

Fig. 2 Scanning electron micrographs showing malformation of the hyphae of *Verticllium dahliae* fumigated by the volatile organic compounds (VOCs) of *Fusarium oxysporum* CanR-46. **a** Normal hyphae of *V. dahliae* in the control treatment; **b**, **c** Shriveled hyphae of *V. dahliae* fumigated by the VOCs

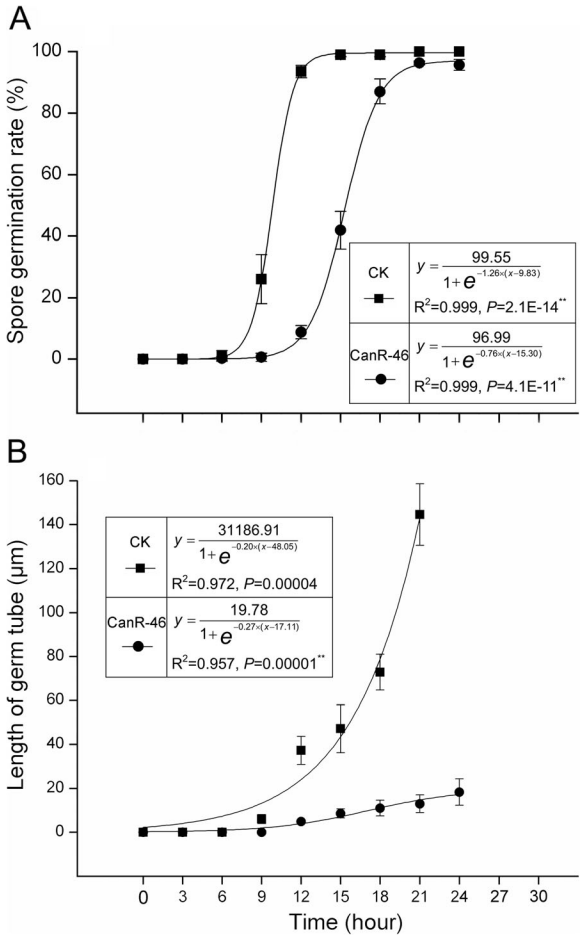

Fig. 3 Effects of the VOCs of *F. oxysporum* CanR-46 on conidial germination rate and germ-tube length of *Verticillium dahliae*. **a** spore germination rate (%); **b** Germ-tube length (μm); CK= Control

both in disease incidence and in disease severity index. After incubation at 25 °C for 20 days, the disease incidence was 27 % in the treatment of the *F. oxysporum* VOCs; significantly lower (P<0.05) than that of 80 % in the control treatment (Fig. 4). The disease severity index was 17 in the treatment of the *F. oxysporum* VOCs, also significantly lower (P<0.01) lower than that of 76 in the control treatment (Fig. 4). This result suggests that fumigation of the organic culture mix with the *F. oxysporum* VOCs of can effectively suppress *Verticillium* wilt of cotton.

Suppression of Verticillium wilt of cotton by *F. oxysporum*

After incubation at 25 °C for 10 days, while all the cotton seedlings in the treatments of water and *Fo*GFP were healthy, some cotton seedlings in the treatments of *V. dahliae* alone and *Fo*GFP + *V. dahliae* showed wilt symptoms. The treatment of *Fo*GFP + *Vdahliae* differed significantly from the treatment of *V. dahliae* both in disease incidence and in disease severity index. The disease incidence was 18 % in the treatment of *Fo*GFP + *V. dahliae*, significantly (P<0.05) lower than that of 94 % in the *V. dahliae* treatment (Fig. 5). The disease severity index was seven in the treatment of *Fo*GFP + *V. dahliae*, also significantly (P<0.05) lower than that of 93 in the *V. dahliae* treatment. Moreover, vascular discoloration was observed in the *V. dahliae* treatment, but was not observed in the other three treatments (Fig. 5). These results suggest that treatment of the cotton roots with the conidia of *F. oxysporum* CanR-46 can effectively control *Verticillium* wilt of cotton.

The hyphae and the conidia of strain CanR-46GFP of *F. oxysporum* (*Fo*GFP) exhibited green fluorescent color under UV light, whereas the hyphae and the conidia of the wild type strain CanR-46 (*Fo*) did not produce any green fluorescent color under the UV light (Supplementary material Fig. S1). Roots of the treated cotton seedlings were randomly sampled and observed under ZEISS LSM 510 Meta laser scanning confocal microscope for endophytic colonization by *Fo*GFP. Results showed that while no signs of green fluorescence in the treatments of water (Figs. 6a and b) and *V. dahliae* alone (data not shown), green fluorescence was consistently observed in the treatments of *Fo*GFP alone (Figs. 6c and d) and *Fo*GFP + *V. dahliae* (Fig. 6e and f), indicating that *Fo*GFP colonized the cotton roots. In both treatments, *Fo*GFP colonized both the root epidermis and the vascular bundles (xylem and phloem). *Fo*GFP grew in the intercellular space in most cases. Sometimes, growth of *Fo*GFP was observed inside the root cells.

Discussion

This study revealed that the volatile organic compounds (VOCs) produced by the non-pathogenic strain CanR-46 of *F. oxysporum* highly inhibited mycelial growth and germ-tube elongation of *V. dahliae* (Figs. 1,2,3; Table 3). Minerdi et al. (2009) reported that the VOCs produced by the wild type non-pathogenic strain WTMSA 35 of *F. oxysporum* inhibited growth of several pathogenic *formae speciales* of *F. oxysporum*. Freire et al. (2012) showed that VOCs produced by

Table 3 Antifungal activity of the volatile organic compounds produced by *Fusarium oxysporum* CanR-46

Tested fungus	Growth rate (mm/day)		Inhibition of growth rate, %	Viability after exposure to VOCs
	-VOC	+VOC		
Aspergillus flavus	6.6	0.1	98 abcd*	Alive (7 d at 20 °C)
Aspergillus parasiticus	4.9	0.1	98 abcd	Alive (7 d at 20 °C)
Botrytis cinerea	14.0	1.0	93 bcde	Alive (4 days at 20 °C)
Colletotrichum siamense	6.4	0.45	93 abcde	Alive (5 days at 20 °C)
Fusarium graminearum	9.8	1.0	90 e	Alive (3 days at 25 °C)
F. oxysporum f.sp. *vasinfectum*	6.0	2.5	58 g	Alive (4 days at 25 °C)
F. oxysporum f.sp. *niveum*	4.1	0.9	78 f	Alive (4 days at 25 °C)
Magnaporthe oryzae	3.2	0.0	100 a	Dead (18 days at 25 °C)
Monilinia fructicola	6.6	0.5	92 de	Alive (6 days at 20 °C)
Penicillium italicum	4.5	0.13	97abcd	Alive (7 days at 20 °C)
Rhizoctonia solani MaizeRs-1	17.9	0.2	99 ab	Alive (4 days at 20 °C)
Rhizoctonia solani WH-1	14.0	0.2	99 abc	Alive (4 days at 20 °C)
Sclerotinia nivalis	8.5	0.6	93 abcde	Alive (4 days at 20 °C)
Sclerotinia sclerotiorum	13.1	0.7	95 abcde	Alive (3 days at 20 °C)
Sclerotinia minor	16.4	1.3	92 cde	Alive (3 days at 20 °C)
Verticillum dahliae	2.4	0. 2	92 de	Dead (21 day at 25 °C)

* Means within the column followed by the same letters are not significantly different ($P>0.05$) according to Duncan's multiple range test

isolates 20a and 21 of *F. oxysporum* caused 88–96 % mortality of the second stage juveniles of the root-knot nematode *Meloidogyne incognita*. In this study, we found that the VOCs of *F. oxysporum* CanR-46 inhibited growth of all the 16 fungal strains belonging to the genera *Aspergillus*, *Botrytis*, *Collectrichum*, *Fusarium*, *Magnaporthe*, *Molinilia*, *Rhizoctonia*, *Sclerotinia* and *Verticillium* (Table 3). These results suggest that the VOCs produced by *F. oxysporum* may a wide antifungal spectrum.

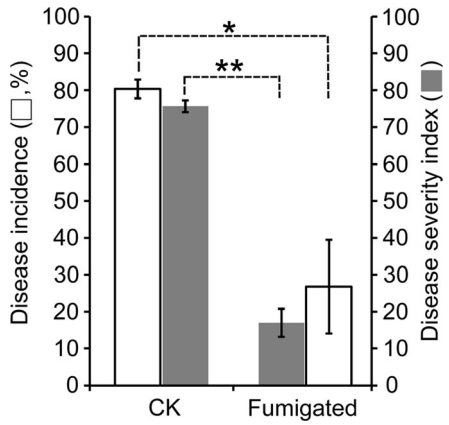

Fig. 4 Effects of fumigation of the culture mixture by the VOCs of *Fusarium oxysporum* CanR-46 on disease incidence and disease severity index of cotton seedlings caused by *Veriticillium dahliae*

The VOCs of strain MSA 35 was reported capable of changing surface hydrophobicity to hydrophilic. In this study, the SEM observation showed that hyphae of *V. dahliae* fumigated by the VOCs of *F. oxysporum* CanR-46 became shriveled and collapsed (Fig. 2). Ghannoum and Rice (1999) reported that the polyene antifungal substance amphotericin B can damage fungal cell membrane and increase membrane permeability, thus causing cytoplasm leakage. We found that 11 out of 19 VOCs produced by *F. oxysporum* CanR-46 are alkenes (Table 1). They might be responsible for malformation of the hyphae of *V. dahliae*.

Strain CanR-46 of *F. oxysporum* was detected capable of producing caryophyllene (Table **1**), a bicyclic sesquiterpene compound. Production of this compound by strains of *F. oxysporum* has been reported previously (Minerdi et al. 2009, 2011; Freire et al. 2012). β-Caryophyllene showed no antifungal activity against *V. dahliae* in this study (Table 2), and nor against *F. oxysporum* f. sp. *lactucae* (*FoL*) in a previous study (Minerdi et al. 2009). On the other hand, Minerdi et al. (2011) reported that the VOCs of WTMSA 35 could increase growth of lettuce (*Lactuca sativa*) and

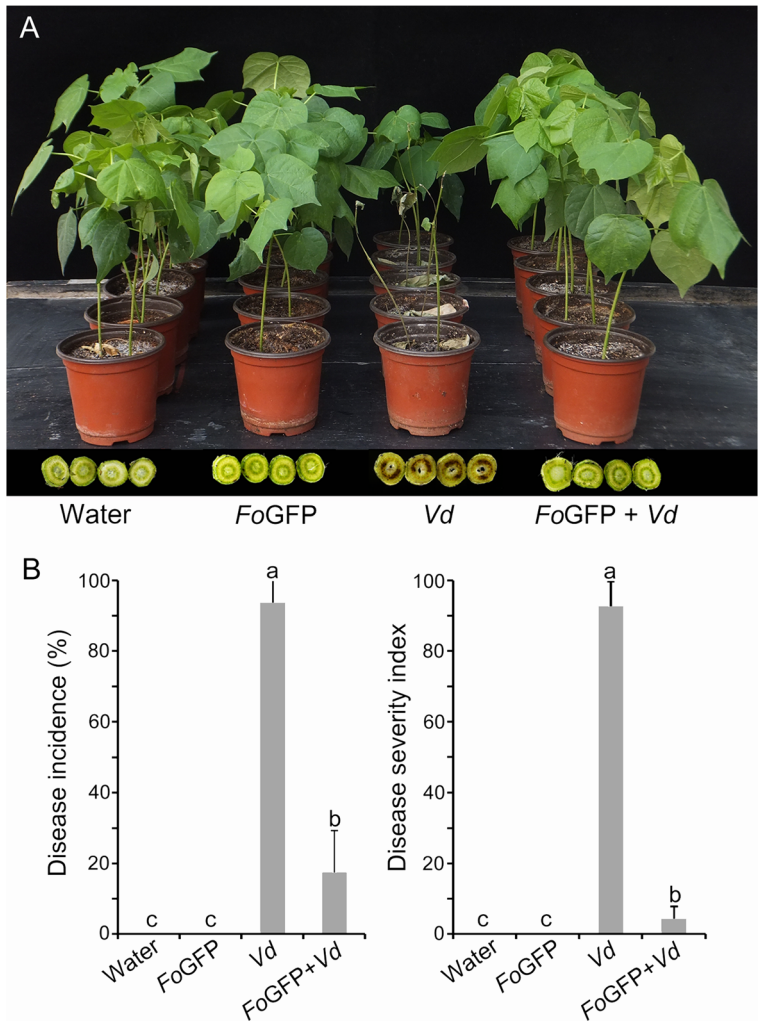

Fig. 5 Efficacy of *Fusarium oxysporum* CanR-46GFP in suppression of Verticillium wilt of cotton caused by *Verticillium dahliae* (25 °C, 10 days post inoculation). **a** Cotton seedlings treated with water, *F. oxysporum* CanR-46GFP alone (*Fo*GFP), *V. dahliae* alone (*Vd*), and *F. oxysporum* CanR-46GFP plus *V. dahliae* (*Fo*GFP + *Vd*). Stem cross sections of the treated cotton seedlings were shown. Note severe defoliation and vascular discoloration on cotton seedlings treated with *V. dahliae* alone, but no leaf yellowing, wilting and defoliation, and vascular discoloration on cotton seedlings treated with water, *Fo*GFP and *Fo*GFP + *Vd*; **b** Disease incidence and disease severity index of Verticillium wilt of cotton for the four treatments. Means±standard errors (n=10) for each treatment labeled with different *letters* indicate significant differences (P<0.05) according to Duncan's Multiple Range Test

expression of the expansin A5 gene in lettuce, and β-caryophyllene was confirmed to be responsible for the plant-growth promotion effect (Minerdi et al. 2011). Yamagiwa et al. (2011) reported that β-caryophyllene significantly enhanced seedlings growth of *Brassica campestris* and resistance to *C. higginsianum*, the causal agent for anthracnose disease of brassicaceous plants. Whether or not the VOCs of *F. oxysporum* CanR-46 can promote plant growth remains unknown and needs further clarification.

Except caryophyllene, other VOC composition produced by the strain CanR-46 of *F. oxysporum* appears different from the VOC composition of strains WTMSA 35 and 21 of *F. oxysporum* reported in previous studies (Minerdi et al. 2009; Freire et al. 2012). Minerdi et al. (2009) reported that α-humulene was abundant in the VOCs produced by strain WTMSA 35, but was not detected in the VOCs of strain 21 (Freire et al. 2012). α-Humulene was not detected in the VOC profile produced by strain CanR-46 in this study either (Table 1). Different genetic backgrounds of the strains of *F. oxysporum* and nutrients/cultural conditions might be responsible for the difference. Although strains CanR-46, WTMSA 35 and 21 were all identified to be *F. oxysporum* based on selected criteria, they have different origins, strain CanR-46 from a healthy root of oilseed rape in China (Zhang et al. 2014), strain WTMSA 35 from a *Fusarium* suppressive soil in Italy (Gilardi et al. 2005) and strain 21 from the coffee rhizosphere in Brazil (Freire et al. 2012). Different origins imply that these *F. oxysporum* strains might be different in niche adaptation, which might be regulated by related genes. Regarding the nutrients/cultural conditions for production of the VOCs, strain CanR-46 was

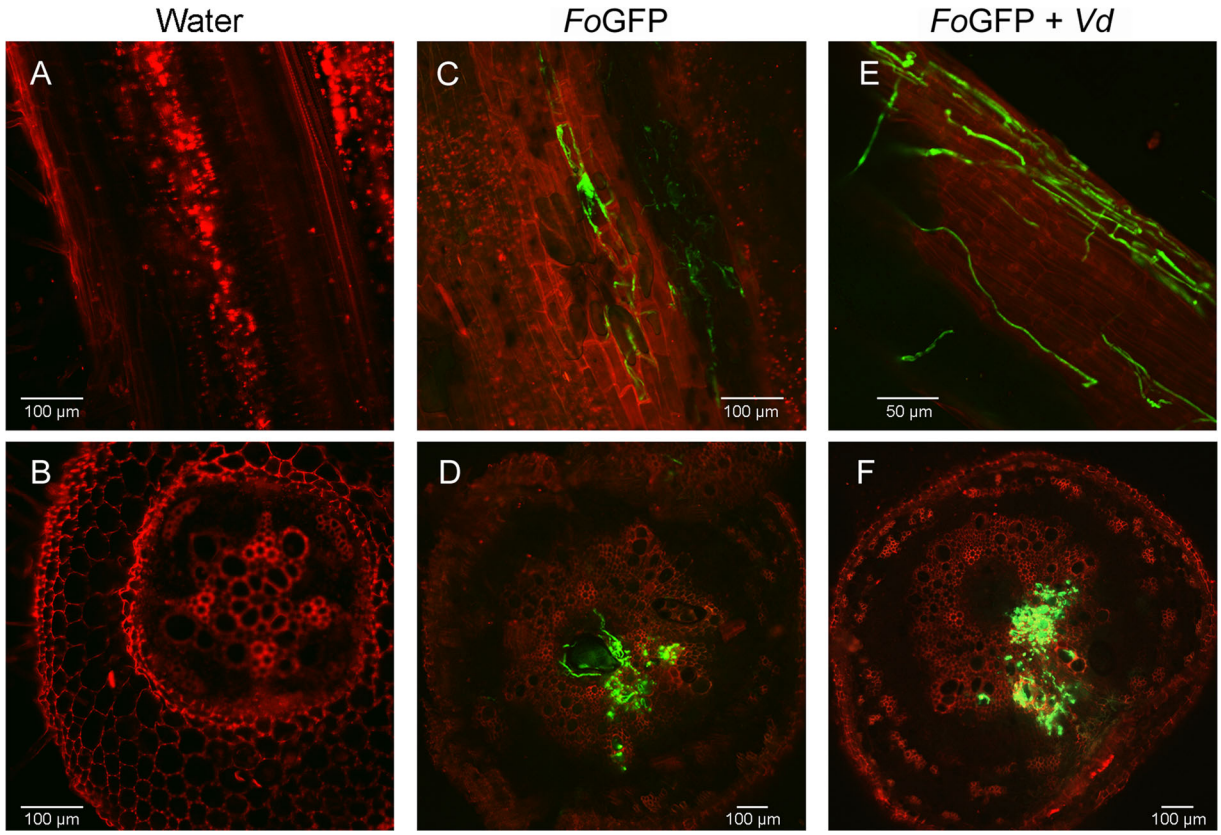

Fig. 6 Laser scanning confocal microscopic micrographs showing endophytic colonization of cotton roots by *Fusarium oxysporum* CanR-46GFP (25 °C, 10 days post inoculation). **a** and **b** Longitudinal and cross sections of the roots treated with water, respectively; **c** and **d** Longitudinal and cross sections of the roots inoculated with *F. oxysporum* CanR-46GFP alone (*Fo*GFP), respectively; **e** and **f** Longitudinal and cross sections of the roots inoculated with *Fo*GFP and *V. dahliae* (*Fo*GFP + *Vd*), respectively. Green fluorescence color and red fluorescence color indicate hyphae of *F. oxysporum* CanR-46GFP and cotton root cells, respectively

statically incubated in autoclaved wheat grains in this study. However, strains WTMSA 35 and 21 were shake-incubated in liquid media (Minerdi et al. 2009; Freire et al. 2012). Different nutrients in cultural media and in cultural conditions for strain CanR-46 might result in production of the VOCs differing in composition from those produced by strains WTMSA 35 and 21.

Additionally, previous studies conducted by Minerdi et al. (2009, 2011) showed that bacterial ectosymbiosis affected VOC composition produced by *F. oxysporum* WTMSA 35. Minerdi et al. (2008) reported that the non-pathogenic strain WTMSA 35 harbored a consortium of ectosymbiotic bacteria belonging to the genera *Serratia* (predominant), *Achromobacter*, *Bacillus* and *Stenotrophomonas*. The bacteria-cured strain CUMSA 35 from WTMSA 35 became pathogenic to lettuce (Minerdi et al. 2008). Minerdi et al. (2009, 2011) reported VOC composition produced by strain WTMSA 35 different from that produced by strain CUMSA 35. For example, sesquiterpenes (e.g., caryophyllene and α-humulene) were detected in the headspace extract of strain WTMSA 35, but was not detected in the headspace extracts of strain CUMSA 35, or in the ectosymbiotic bacteria *Achromobacte*r sp. strain MM1 and *Serratia* sp. strain DM1 (Minerdi et al. 2009, 2011). These results imply that the bacterial ectosymbiosis may stimulate or induce *F. oxysporum* WTMSA 35 to produce the sesquiterpene VOCs. In the present study, strain CanR-46 was sub-cultured several times on agar media and ectosymbiotic bacteria were not observed on the hyphae of *F. oxysporum* CanR-46 under scanning electron microscope (Supplementary material Fig. S2). Thus, production of the VOCs by *F. oxysporum* CanR-46 is not affected by bacterial ectosymbiosis.

Previous studies have demonstrated that the VOC-producing fungi can be used to treat soil as biofumigants

(or mycofumigants) for control of soilborne plant diseases. For example, Stinson et al. (2003) reported that incorporation of the two VOC-producing fungi *M. albus* and *M. roseus* into soil resulted in significant reduction of root rot diseases of sugar beet (*Beta vulgaris*) caused by *Aphanomyces cochlioides*, *P. ultimum* and *R. solani*. A further study conducted by Grimme et al. (2007) showed that a biorational synthetic mixture of organic VOC components mimicking the VOCs of *M. albus* was more effective than the live culture of this fungus in control of the three fungal pathogens on sugar beet and the root-knot nematode, *Meloidogyne incognita*, on tomato (*Lycopersicon esculentum*). In the present study, we found that the VOCs of *F. oxysporum* CanR-46 inhibited mycelial growth and germ-tube elongation of the soilborne pathogen *V. dahliae* (Fig. 3, Table 3), and suppressed Verticillium wilt of cotton by fumigation of the organic culture mix (Figs. 4, 5). These results suggest that the mixture of the VOCs produced by *F. oxysporum* CanR-46 can be used as a biofumigant to control *V. dahliae*. This study also showed that treatment of cotton roots with the conidia of *F. oxysporum* CanR-46 effectively prevented the roots from infection by *V. dahliae* (Fig. 5). These results suggest that *F. oxysporum* CanR-46 is a promising biocontrol agent of *V. dahliae*.

Numerous studies reported that non-pathogenic strains of *F. oxysporum* are effective agents for control of pathogenic *formae speciales* of *F. oxysporum* on various crops (Ogawa and Komada 1984; Paulitz et al. 1987; Postrna and Rattink 1992; Larkin et al. 1996; Silva and Belltiol 2005; Panina et al. 2007). Competition for nutrients/space, and induction of systemic resistance have been proposed as mechanisms involved in biocontrol of pathogenic *F. oxysporum* with non-pathogenic *F. oxysporum* (Fravel et al. 2003). Minerdi et al. (2009) reported that the VOCs (e.g., α-humulene) of the strain WTMSA 35 of *F. oxysporum* inhibited growth of several pathogenic *formae speciales* of *F. oxysporum* including *FoL* Fuslat10 and repressed expression of two putative virulence genes coding for a MAP kinase and Class V chitin synthase in *FoL* Fuslat10. The results about inhibition of *V. dahliae* on media, in soil and on cotton plants observed in this study provided an example of possible involvement of VOC production in biocontrol of fungal diseases by non-pathogenic *F. oxysporum*.

Pantelides et al. (2009) and Veloso and Díaz (2012) reported that the non-pathogenic *F. oxysporum* strains F2 and Fo47, when treated onto roots of eggplant and pepper (*Capsicum annuum*), respectively, provided effective control of *V. dahliae*. The two biocontrol agents showed different mechanisms for disease suppression (Gizi et al. 2011; Veloso and Díaz 2012). Strain F2, after being injected into eggplant stems, was capable of colonizing root surface and vascular tissues of eggplant, resulting in a significant reduction of vascular colonization by *V. dahliae,* suggesting a mechanism of competition for nutrients and space (Pantelides et al. 2009; Gizi et al. 2011). It was detected to be unable to induce resistance against *V. dahliae* on eggplant (Pantelides et al. 2009). Strain Fo47 was found to be able to induce resistance against *V. dahliae* on pepper plants (Veloso and Díaz 2012). In the present study, endophytic colonization of the cotton root tissues by *F. oxysporum* CanR-46 was observed either in the presence of *V. dahliae* or in the absence of *V. dahliae* (Fig. 6). This result suggests possible involvement of competition for nutrients and space as a mechanism, in addition to production of antifungal VOCs by *F. oxysporum* CanR-46. Whether or not *F. oxysporum* CanR-46 can trigger defense response of cotton against *V. dahliae* remains unknown and needs further study.

In conclusion, the present study showed that the non-pathogenic endophyte *F .oxysporum* CanR-46 is a VOC-producing antagonist with a wide spectrum of antifungal activity. The VOCs of *F. oxysporum* CanR-46 exhibited high efficacy in suppression of mycelial growth and germ-tube elongation of *V. dahliae*; and in suppression of Verticillium wilt of cotton caused by *V. dahliae*. Treatment of cotton roots with the conidia of *F. oxysporum* CanR-46 provided effective control of Verticillium wilt. Therefore, *F. oxysporum* CanR-46 is a promising biocontrol agent of *V. dahliae*.

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Compliance with ethical standards

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