Journal of Microbiology (2019) Vol. 57, No. 5, pp. 396–404
DOI 10.1007/s12275-019-8517-9
eISSN 1976-3794
pISSN 1225-8873

Biocontrol activity of volatile organic compounds from *Streptomyces alboflavus* TD-1 against *Aspergillus flavus* growth and aflatoxin production

Mingguan Yang, Laifeng Lu, Jing Pang, Yiling Hu, Qingbin Guo, Zhenjing Li, Shufen Wu, Huanhuan Liu, and Changlu Wang*

State Key Laboratory of Food Nutrition and Safety, Key Laboratory of Food Nutrition and Safety, Ministry of Education, College of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, P. R. China

(Received Sep 18, 2018 / Revised Nov 16, 2018 / Accepted Nov 30, 2018)

Aspergillus flavus **is a saprophytic fungus that contaminates crops with carcinogenic aflatoxin. In the present work, the antifungal effects of volatile organic compounds (VOCs) from *Streptomyces alboflavus* TD-1 against *A. flavus* were investigated. VOCs from 8-day-old wheat bran culture of *S. alboflavus* TD-1 displayed strong inhibitory effects against mycelial growth, sporulation, and conidial germination of *A. flavus*. Severely misshapen conidia and hyphae of *A. flavus* were observed by scanning electron microscopy after exposure to VOCs for 6 and 12 h, respectively. Rhodamine 123 staining of mitochondria indicated that mitochondria may be a legitimate antifungal target of the VOCs from *S. alboflavus* TD-1. Furthermore, the VOCs effectively inhibited aflatoxin B1 production by downregulating genes involved in aflatoxin biosynthesis. Dimethyl trisulfide and benzenamine may play important roles in the suppression of *A. flavus* growth and production of aflatoxin. The results indicate that VOCs from *S. alboflavus* TD-1 have tremendous potential to be developed as a useful bio-pesticide for controlling *A. flavus*.**

Keywords: volatile organic compounds, biocontrol, *Streptomyces alboflavus* TD-1, *Aspergillus flavus*, aflatoxin B1

Introduction

Aspergillus flavus, a common pathogen in agriculture, is ubiquitously distributed in agricultural crops, especially in peanuts (Wu *et al*., 2016) and corn (Hong *et al*., 2010; Garcia *et al*., 2012), causing severe crop damage and compromising food security. *A. flavus* infection is commonly considered a serious health hazard for humans and animals due to the threat of aflatoxins, which are highly toxic, carcinogenic, and teratogenic secondary metabolites (Razzaghi-Abyaneh, 2013). Aflatoxin B1 has been classified as class 1 carcinogen by the International Agency for Research of Cancer (WHO and IARC, 1993). Aflatoxins that occur in food or livestock feed via fungal contamination can bio-accumulate in humans and animals (Kumar *et al*., 2017). It is estimated that aflatoxins may contribute to 4.6–28.2% of all global hepatocellular carcinoma cases (Liu and Wu, 2010). Therefore, the control of aflatoxin contamination is an urgent task around the world.

Prevention of aflatoxin-producing fungi is an effective strategy for controlling the presence of aflatoxin in foods (Mellon *et al*., 2012). Traditionally, the prevention of *A. flavus* is mainly reliant on fungicides. However, the use of agrochemicals has been the subject of public concern in consideration of environmental and health concerns (Pimentel, 2005; Da *et al*., 2015), as well as the development of resistant strains due to the indiscriminate use of the chemical substances (Da *et al*., 2013). Moreover, the use of agrochemicals is becoming increasingly restricted because new synthetic pesticides are becoming expensive and increasingly difficult to synthesize (Glare *et al*., 2012). For reducing the negative effects generated by the abuse of chemical pesticides, biotechnological tools are currently considered as a viable alternative to combat fungal pathogens to agrochemicals (Heydari and Pessarakli 2010; Shams-Ghahfarokhi *et al*., 2013; Kifle *et al*., 2017).

Volatile organic compounds (VOCs) from microorganisms present enormous and fascinating potential to control fungal pathogens (Schalchli *et al*., 2016). VOCs typically constitute a complex mixture of low-molecular weight compounds that easily evaporate under environmental conditions (Hung *et al*., 2015). Consequently, they can be applied to protect crops without direct contact. These metabolites are more eco-friendly and innocuous than chemical agents, in addition to being rapidly biodegradable in the environment (Schalchli *et al*., 2016).

Numerous literature reports have shown that VOCs have significant antifungal activity against fungal pathogens (Minerdi *et al*., 2009; Ando *et al*., 2010; Glare *et al*., 2012; Cordovez *et al*., 2015; Gotorvila *et al*., 2017). Up to now, the most investigated VOC-producing species belong to the fungal genera *Muscodor* and *Trichoderma* (Schalchli *et al*., 2016). The VOCs emitted by the endophytic fungus *Muscodor albus* have been effectively applied to control several pathogens, including *Botrytis cinerea*, *Penicillium expansum*, *Sclerotinia sclerotiorum*, *Fusarium sambusinum*, and *Helminthosporium solani* (Ramin *et al*., 2005; Corcuff *et al*., 2011). In addition, some other microorganisms have shown great potential bio-

*For correspondence. E-mail: clw123@tust.edu.cn; Tel.: +86-22-60601154; Fax: +86-22-60601154

activity against plant pathogens via the emission of VOCs, such as the bacteria *Streptomyces* spp. (Li *et al*., 2010; Wu *et al*., 2015) and *Bacillus* spp. (Kong *et al*., 2014; Gao *et al*., 2017; Gotorvila *et al*., 2017).

For the control of *A. flavus* growth and aflatoxin production, efforts have been made with VOCs produced by *Shewanella algae* YM8 (Gong *et al*., 2015) and *Pichia anomala* WRL-076 (Hua *et al*., 2014). *Streptomyces* spp. are the most noteworthy producers of versatile and diverse antibiotics, including VOCs (Berdy, 2005). However, there is little literature concerning the control of *A. flavus* using VOCs from *Streptomyces* spp. Previously, we isolated a new strain, *Streptomyces alboflavus* TD-1, from the soil surrounding a granary in Tianjin, China. The VOCs emitted by *S. alboflavus* TD-1 showed great capacity to control various pathogens (Wang *et al*., 2013). Nevertheless, limited information is available on the activity of VOCs from *S. alboflavus* TD-1 against *A. flavus*. Therefore, the aim of this study was to evaluate the antifungal effects of VOCs from *S. alboflavus* TD-1 against *A. flavus* growth and aflatoxin production.

Materials and Methods

Microorganisms

Streptomyces alboflavus TD-1 used in this study was isolated from soil surrounding a granary in Tianjin (China) and was identified by morphological, physiological, and 16S rDNA sequencing (GenBank accession No.: JX915780). The strain was maintained on Gause's synthetic agar slants (Wang *et al*., 2013), which were supplemented with 20% glycerol and placed at -80°C for long-term storage. The strain was subcultured on fresh Gause's synthetic agar slants at 28°C for up to 5 days prior to use. For the preparation of volatile substances, autoclaved wheat bran in conical flasks (250 ml) were inoculated with a spore suspension of *S. alboflavus* TD-1 at a rate of 2 ml (1×10^6 conidia/ml) per 10 g of wheat bran, and the flasks were incubated at 28°C for 8 days.

Aspergillus flavus was purchased from the China Center of Industrial Culture Collection (CICC NO. 2219). The strain was cultured on potato dextrose agar (PDA) in Petri Dishes at 28°C for 3 days. The conidia were harvested and suspended in sterile distilled water containing 0.05% (v/v) Tween 80. Then, the suspension was filtered through eight layers of autoclaved medical gauze. The conidia concentration was adjusted by a hemocytometer to a final concentration of approximately 1×10^6 conidia/ml.

Antagonistic activity of VOCs from *S. alboflavus* TD-1 against *A. flavus*

The efficacy of the VOCs generated by *S. alboflavus* TD-1 against the mycelial growth and conidial germination of *A. flavus* was tested in two inverse face-to-face Petri Dishes according to Wu *et al*. (2015). The bioassay system was set up using the bottoms of two lidless Petri Dishes (60 mm in diameter). The upper plate contained 5 ml of PDA inoculated with a 6-mm-diameter fungal plug. The lower plate contained different amounts of wheat bran culture of *S. alboflavus* TD-1. The two base dishes were sealed immediately with double layer of parafilm to obtain a chamber with 75 ml of airspace and incubated at 28°C for 3 days. The colony diameter of each plate was measured in millimeters and the number of conidia per plate was assessed. Each treatment consisted of three replicates, and the experiments were performed in triplicate. The fungal growth inhibition was calculated using the following equation:

Fungal growth inhibition (%) = (Rc - Rt) / Rc × 100%

Where Rc is the colony diameter of the control and Rt is the colony diameter of the treatment.

To study the inhibition of VOCs on conidial germination of *A. flavus*, the inverse face-to-face Petri Dishes setup was used as described above. The difference was that the top dish containing PDA was laid over sterilized cellophane membranes onto which 100 µl of *A. flavus* conidial suspension at 1×10^6 conidia/ml were spread. After 6, 9, and 12 h post inoculation at 28°C, the germination per 100 conidia was observed for each plate using an optical microscope at 400× (Olympus CX41, Olympus Co.). Plates with equivalent amounts of autoclaved wheat bran were used as control. The conidia were considered germinated when the germ tube was at least twice the length of the conidia (Li and Xiao, 2008). The percentage inhibition of conidial germination was calculated according to the following equation:

Inhibition of conidial germination (%)
= (Gc - Gt) / Gc × 100%

Where Gc is the germination rate of the control and Gt is the germination rate of the treatment.

Morphological changes of the *A. flavus* treated by VOCs

Samples were prepared using the inverse face-to-face Petri Dishes setup as described above. For the observation of conidial morphology, 100 µl of conidial suspension at 1×10^6 conidia/ml was spread evenly on the top plate and wheat bran culture at a concentration of 40 g/L was placed in the lower plate. The samples were collected 6 h later. To obtain hyphae, 100 µl of conidial suspension was first incubated on PDA medium at 28°C for 16 h to allow the spores to completely grow into hyphae; then, the fresh hyphae were treated by the wheat bran culture. Samples were collected after 12 h.

The conidial and hyphal morphology of *A. flavus* was observed using a scanning electron microscope (SEM) (Li *et al*., 2015). 6-mm-diameter fungal plugs were cut from the PDA medium described above and then fixed with 2.5% glutaraldehyde at 4°C overnight. The materials were rinsed with phosphate buffer solution (0.1 M PBS, pH 7.2) three times and dehydrated in an ethanol gradient (at concentrations of 30, 50, 70, 80, 90, and 100%). Then, the fungal plugs were dried by utilizing vacuum freeze-drying method. Finally, the samples were sputter-coated with gold using an ion sputter (E-1010, Hitachi) and examined with the SEM (Su1510, Hitachi).

Determination of the mitochondrial membrane potential

The mitochondrial membrane potential was measured by staining with rhodamine-123 (Perry *et al*., 2011). In this assay, 100 µl of conidial suspension of *A. flavus* was pre-cultured for16 h on PDA medium and then treated by wheat bran culture of *S. alboflavus* TD-1 for 12 h. Treated mycelia

were taken off from the agar plate and placed on a clean slide. The samples were then mixed with 50 µl of rhodamine-123 at a final concentration of 10 µM. The mixtures were stained for 30 min in the dark at 37°C. Finally, the mycelia were washed with PBS and examined using an inverted fluorescence microscope (EVOS; AMG).

Analysis of AFB1

The amount of AFB1 was determined by high performance liquid chromatography (HPLC). In this assay, conidial suspension of *A. flavus* was pre-cultured on PDA medium for 3 days at 28°C. The plates were then exposed to 40 g/L of wheat bran culture of *S. alboflavus* TD-1 for 3 days. AFB1 extraction from PDA medium was performed according to Bavaro *et al.* (2016). Five agar plugs (0.6 cm diameter) were cut out from the edge of the colony and then extracted with 25 ml of 80% methanol by shaking for 60 min at room temperature. The samples were centrifuged at 4,500 rpm for 10 min. The supernatant was diluted with an equal volume of water and then filtered through a 0.22 µm syringe filter.

The samples were analyzed with an Agilent 1260 HPLC (Agilent) equipped with a C18 column (4.6 × 250 mm, 5 µm). Samples (20 µl) were injected and eluted with a mobile phase consisting of water: acetonitrile (75:25, v/v) at a flow rate of 0.5 ml/min. AFB1 was quantified by a fluorescent detector with excitation at 365 nm and emission at 435 nm.

RNA extraction and reverse transcription

Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. RNA was treated with RNase free DNAse I (Qiagen) to eliminate possible trace amounts of contaminating DNA. The purity and concentration of RNA were determined by measuring the absorbance at 260 and 280 nm. The synthesis of cDNA was performed using a TaKaRa RNA PCR Kit (TaKaRa Dalian).

Quantitative Real-time PCR analysis

To investigate the expression level of aflatoxin biosynthesis genes, quantitative real-time PCR (qRT-PCR) was performed. The *β-tubulin* gene was used as an internal control. Each reaction was carried out in a final volume of 20 µl containing 10 µl SYBR Premix Ex Taq, 8 µl Nuclease-Free water, and 0.5 µl 10 mM primers (forward and reverse) and an appropriate amount of cDNA (100 ng). The program included an initial denaturation step at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and a final annealing step at 60°C for 30 sec. The relative quantification of gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Collection and identification of VOCs produced by *S. alboflavus* TD-1

VOCs from wheat bran culture of *S. alboflavus* TD-1 were collected and analyzed by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography- mass spectrometry (GC-MS). Briefly, the volatile compounds were entrapped in a HS-SPME fiber (DVB/CAR/PDMS, Supelco) at 45°C for 30 min. Trapped compounds were thermally desorbed at 250°C for 2 min in the injection port of GC-MS (QP 2010 Ultra, Shimadzu), and separated by a capillary column (VF-5MS, 30 m × 0.25 mm i.d., 0.25 µm -thick film; Varian Co.). Helium was used as carrier gas at a flow rate of 1 ml/min. The oven temperature was initially adjusted to 40°C for 3 min, and then 1 min isothermal increased to 150°C at a rate of 4°C /min, and finally raised to 250°C at a rate of 8°C /min and held at 250°C for 6 min. The mass spectrometer was operated in the electron ionization mode at 70 eV with a source temperature of 230°C, and mass spectra were obtained with a scan range of 35 to 500 m/z. Volatile compounds were tentatively identified by comparing their mass spectra with those presented in the Library of the National Institute of Standards and Technology (NIST 11).

Antagonistic activity of screened VOCs against *A. flavus*

Five technical grade volatiles, namely, anisole, dimethyl trisulfide, β-pinene, benzenamine, and 1,5-cyclooctadiene were purchased from Sigma-Aldrich and individually tested for the ability to suppress the mycelial growth of *A. flavus* with inverse face-to-face Petri Dishes setup (Wu *et al.*, 2015). A 6-mm-diameter fungal plug was placed on the PDA plate, and a piece of autoclaved filter paper containing the volatile compound was placed in the other base plate. The concentration of volatiles was adjusted to 1, 10, 100, and 1,000 µl/L. Then, the two dishes were double sealed with parafilm and incubated at 28°C for 3 days. The percentage inhibition of mycelial growth was calculated according to the equation described above. Filter papers with equivalent volume of sterile distilled water were used as control. Each experiment consisted of three replicates and the experiments were performed in triplicate.

Statistical analyses

The data were submitted to analyses of variance (ANOVA) using SPSS software version 20.0 for Windows (SPSS Inc.). Statistical significance was evaluated by DMRT (Duncan's Multiple Range Test) and $P < 0.05$ was considered to be significant. PLS analysis was carried out using SIMCA- P 13.0 software (Umetrics AB).

Results and Discussion

Inhibitory effects of VOCs from *S. alboflavus* TD-1 against *A. flavus*

The ability of wheat bran cultures of *S. alboflavus* TD-1 to control *A. flavus* was evaluated using the two inverse face-to-face Petri Dishes method. The results indicated that the antifungal ability of VOCs gradually increased along with the fermentation time, and tended to be stable after 8 days fermentation. Eight-day-old wheat bran culture of *S. alboflavus* TD-1 exhibited 70.01% inhibition of mycelial growth against *A. flavus* (Fig. 1A). The 8-day-old wheat bran culture of *S. alboflavus* TD-1 was used in the following study.

In addition, increasing concentrations of wheat bran culture from 5 to 80 g/L resulted in a significant increase in the growth inhibition of *A. flavus* in range of 22.9 to 100%

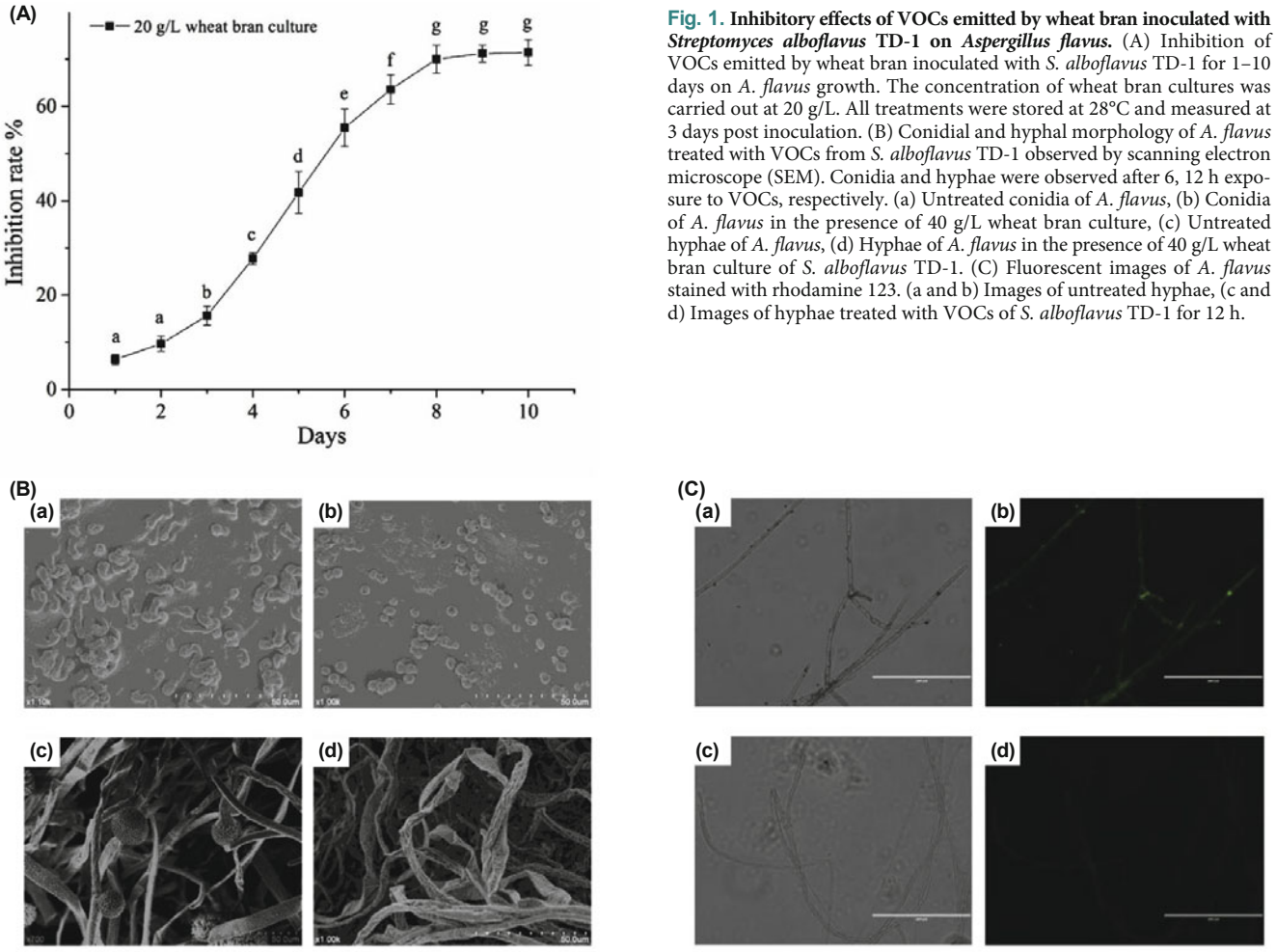

Fig. 1. Inhibitory effects of VOCs emitted by wheat bran inoculated with *Streptomyces alboflavus* TD-1 on *Aspergillus flavus*. (A) Inhibition of VOCs emitted by wheat bran inoculated with *S. alboflavus* TD-1 for 1–10 days on *A. flavus* growth. The concentration of wheat bran cultures was carried out at 20 g/L. All treatments were stored at 28°C and measured at 3 days post inoculation. (B) Conidial and hyphal morphology of *A. flavus* treated with VOCs from *S. alboflavus* TD-1 observed by scanning electron microscope (SEM). Conidia and hyphae were observed after 6, 12 h exposure to VOCs, respectively. (a) Untreated conidia of *A. flavus*, (b) Conidia of *A. flavus* in the presence of 40 g/L wheat bran culture, (c) Untreated hyphae of *A. flavus*, (d) Hyphae of *A. flavus* in the presence of 40 g/L wheat bran culture of *S. alboflavus* TD-1. (C) Fluorescent images of *A. flavus* stained with rhodamine 123. (a and b) Images of untreated hyphae, (c and d) Images of hyphae treated with VOCs of *S. alboflavus* TD-1 for 12 h.

(Table 1). Untreated *A. flavus* mycelia grew to a diameter of 46.7 mm and produced 1.15×10^8 conidia/ml at 3 days post inoculation (dpi). In presence of 5, 10, and 20 g/L wheat bran cultures of *S. alboflavus* TD-1, the average colony diameters were 36.0, 20.3, and 16.7 mm, respectively. Likewise, conidial production were much lower than that of the control, and no conidia were generated in 20 g/L wheat bran culture. No mycelial growth was observed at wheat bran cultures concentration of 40 and 80 g/L.

The inhibitory activity of the VOCs generated by wheat bran cultures of *S. alboflavus* TD-1 against the germination of *A. flavus* conidia was also investigated (Table 2). After exposure to 5 and 10 g/L wheat bran cultures for 9 h, the percentage of germinated conidia of *A. flavus* was 69.3 and 48.9%, respectively. In contrast, conidia in the untreated control were completely germinated at the same time. There was only 12.4% inhibition of conidial germination in 10 g/L of wheat bran culture after 12 h. Inhibition rate rose to 98.8% when the *S. alboflavus* TD-1 culture was increased to 20 g/L. The conidial germination of *A. flavus* was completely inhi-

Table 1. Effects of volatile organic compounds produced by wheat bran cultures of *Streptomyces alboflavus* TD-1 on the mycelial growth and sporulation of *Aspergillus flavus*

S. alboflavus TD-1[a] g/L	Mycelial growth[b]		Sporulation[b]
	Diameter (mm)	Inhibition (%)	Spores ($\times 10^4$ per plate)
0	46.7 ± 1.5a	0e	11461.1 ± 2781.8a
5	36.0 ± 1.3b	22.9 ± 0.8d	1211.7 ± 216.9b
10	20.3 ± 0.9c	56.6 ± 0.8c	9.4 ± 1.7c
20	16.7 ± 0.9d	64.2 ± 1.5b	0c
40	0e	100a	0c
80	0e	100a	0c

The concentrations of wheat bran cultures were carried out at 0, 5, 10, 20, 40, and 80 g/L, respectively. Data are the mean of nine replicates ± SD. Means followed by different letter within each column are significantly different ($P < 0.05$) according to ANOVA and DMRT.
[a] Grams of culture per liter of airspace.
[b] Mycelial growth and sporulation were measured at 3 days post inoculation.

Table 2. Effects of volatile organic compounds produced by wheat bran cultures of *Streptomyces alboflavus* TD-1 on the conidial germination of *Aspergillus flavus*

S. alboflavus TD-1[a] g/L	Conidial germination (%)[b]		
	6 h	9 h	12 h
0	24.1 ± 2.8a	100a	100a
5	15.3 ± 0.9b	69.3 ± 2.7b	100a
10	3.8 ± 0.4c	48.9 ± 2.5c	87.6 ± 2.7b
20	0d	0d	1.2 ± 0.2c
40	0d	0d	0c
80	0d	0d	0c

The concentrations of wheat bran cultures were carried out at 0, 5, 10, 20, 40, and 80 g/L, respectively. Data are the mean of nine replicates ± SD. Means followed by different letter within each column are significantly different ($P < 0.05$) according to ANOVA and DMRT.
[a] Grams of culture per L of airspace.
[b] Conidial germination was determined at 6, 9, and 12 h post inoculation, no less than 100 spores per plate were counted.

bited in the presence of VOCs produced by 40 and 80 g/L of wheat bran cultures.

The results demonstrated that VOCs emitted by *S. alboflavus* TD-1 possesses a great capacity to suppress *A. flavus* growth. This result was similar to that observed by Gong *et al.* (2015), who reported that VOCs emitted by *Shewanella algae* strain YM8 completely inhibited the conidial germination of *A. flavus*, and no mycelial growth was observed at 5 dpi. We also noted that *A. flavus* renewed its growth after being transferred into fresh PDA plates. This phenomenon clearly indicates that *A. flavus* was suppressed but not completely killed post exposure to *S. alboflavus* TD-1 VOCs. This result in agreement with the results reported by others (Eva *et al.*, 2010; Li *et al.*, 2010, 2015), but differed with Boukaew who reported that VOCs of *Streptomyces philanthi* RM-1-138 could kill *Rhizoctonia solani* PTRRC-9 (Boukaew *et al.*, 2013). On one hand, this may be due to the different kinds and amount of volatile compounds generated by different microorganisms. On the other hand, given that diverse pathogens have different levels of resistance to VOCs (Gotorvila *et al.*, 2017), *R. solani* may be more susceptible to VOCs.

Morphological characteristics of treated *A. flavus* observed by SEM

SEM analysis were carried out to observe any morphological changes of treated *A. flavus* (Fig. 1B). In the absence of the wheat bran culture of *S. alboflavus* TD-1, germ tube has obviously protruded from the conidia of *A. flavus* after 6 h (Fig. 1Ba). However, no germ tubes were formed when the conidia were treated with VOCs. In addition, many sunken and irregular conidia were observed in the microphotograph (Fig. 1Bb). On the other hand, hyphae in the untreated control appeared a normal morphology with many typical conidiophores and tapered apices (Fig. 1Bc). In contrast, treated hyphae presented morphological alterations with wrinkled and irregular distortions. Moreover, apical growth was reduced and stunted (Fig. 1Bd) compared to the untreated control (Fig. 1Bc).

The cell membrane is vital to maintaining cell integrity and normal physiological function, and the destruction of cell membrane integrity of *A. flavus* indicated that the cell membrane may be a legitimate antifungal target of VOCs from *S. alboflavus* TD-1. Similar results were obtained for other pathogens treated by VOCs from microorganisms. The hyphae of *Rhizoctonia solani* PTRRC-9 underwent severe destruction with the loss of cytoplasmic material and displayed irregular shapes with curves after exposure to VOCs from *Streptomyces philanthi* RM-1-138 for 3 days (Boukaew and Prasertsan, 2014).

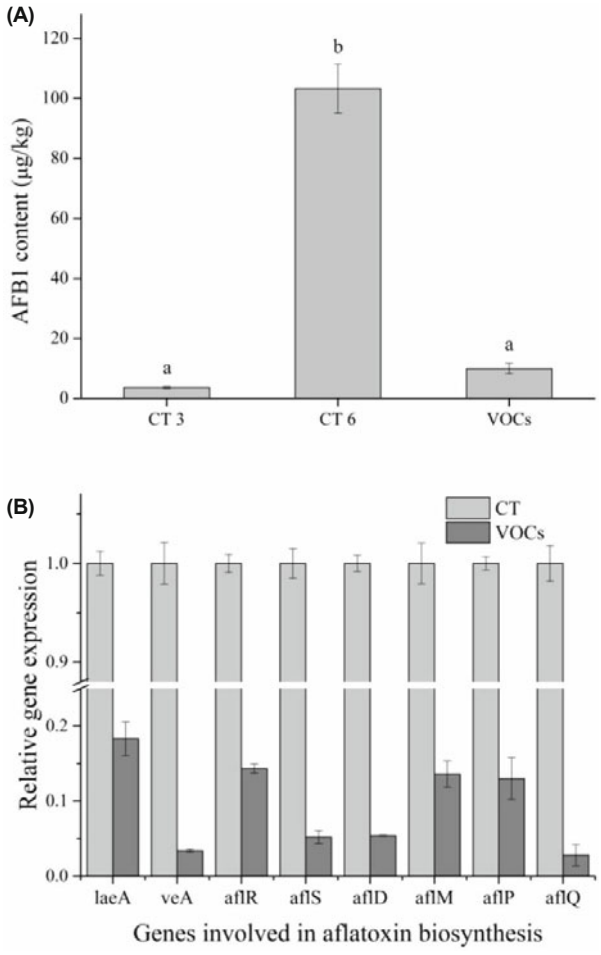

Fig. 2. Effects of VOCs from *Streptomyces alboflavus* TD-1 on aflatoxin B1 accumulation and expression of aflatoxin biosynthetic and regulatory genes. (A) Effects of VOCs on aflatoxin B1 accumulation. Aflatoxin B1 production by *A. flavus* after 3 days post inoculation (CT 3) and 6 days post inoculation (CT 6); Aflatoxin B1 production by *A.flavus* pre-cultured for 3 days, and then treated by VOCs for 3 days (VOCs). (B) Expression of aflatoxin biosynthetic and regulatory genes in *A. flavus* after the treatment of VOCs from *S. alboflavus* TD-1.

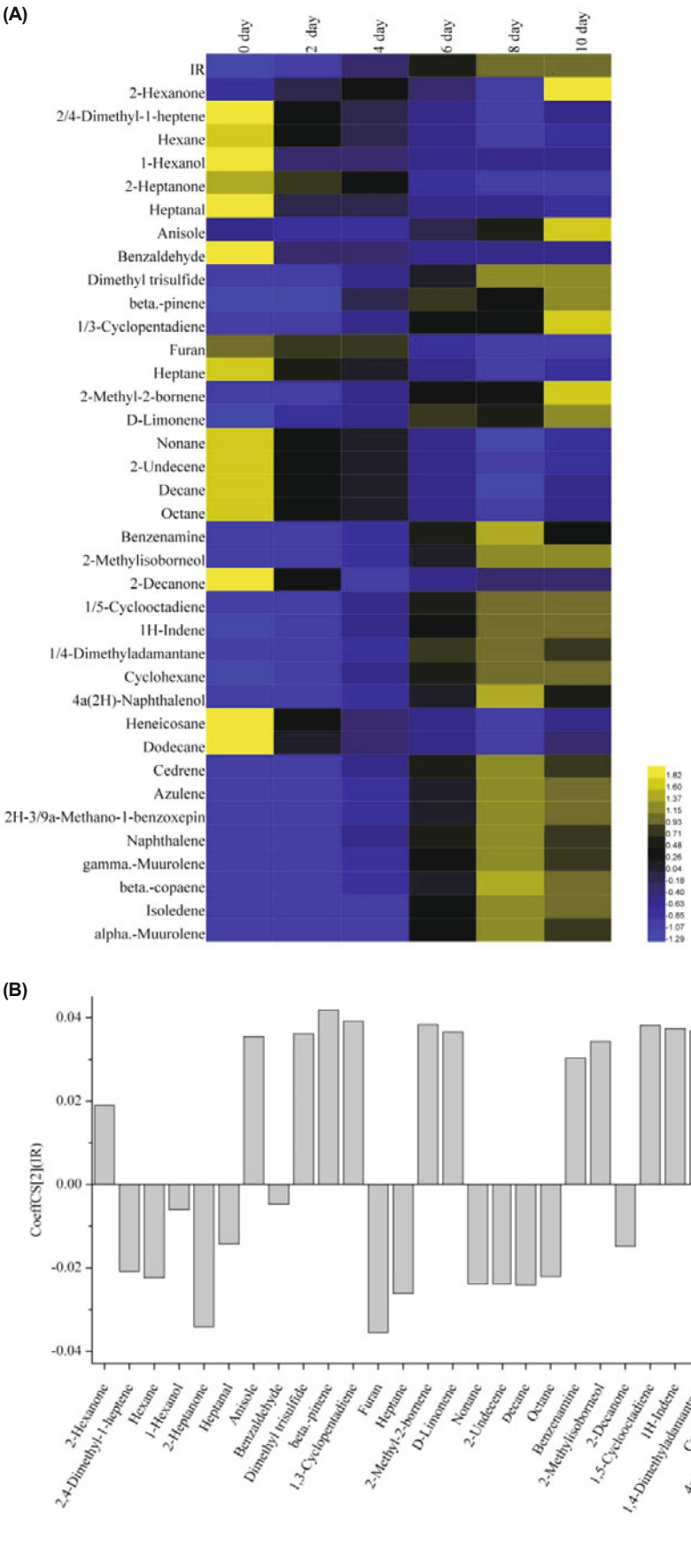

Fig. 3. Volatile organic compounds from wheat bran culture inoculated with *Streptomyces alboflavus* TD-1 at different days (0, 2, 4, 6, 8, and 10 days). (A) Heat map of dynamic changes of VOCs from *S. alboflavus* TD-1 during the fermentation process. Columns represent samples collected at different time, and rows represent the different identified VOCs, (IR) Inhibition rate. (B) Results of PLS analysis of the relationship between VOCs from *S. alboflavus* TD-1 and inhibition rate at different days.

Loss of mitochondria membrane potential

Rhodamine 123 is a typical probe of mitochondrial membrane potential, and active mitochondria in viable cells are stained bright green, while loss of gradients within nonviable cells results in quenching of fluorescence (Johnson *et al.*, 2013). The results of rhodamine-123 fluorescent staining are shown in Fig. 1C. Untreated *A. flavus* displayed a bright fluorescence intensity (Fig. 1Ca and b). While when the *A. flavus* was treated with VOCs of *S. alboflavus* TD-1, a significant decrease in the fluorescence intensity of the sample was observed (Fig. 1Cc and d). The results demonstrated that VOCs induced a loss of mitochondrial membrane potential and mitochondrial integrity of *A. flavus*, and proved that the mitochondria may be the important antimicrobial targets of the volatiles. The postulate is in agreement with conclusion previously reported by Tian *et al*. (2012).

Effect of VOCs on AFB1 accumulation and genes expression

The inhibition of VOCs from *S. alboflavus* TD-1 on AFB1 accumulation was shown in Fig. 2A. The results showed that AFB1 production increased significantly from 3-day to 6-day of cultivation. In contrast, VOCs of *S. alboflavus* TD-1 showed a significant ($P < 0.05$) reduction in AFB1 contamination, with an inhibition percentages of 93.68% in comparison to the controls.

To investigate whether the drastic reduction in AFB1 production by *A. flavus* was due to VOCs of *S. alboflavus* TD-1 down-regulated the expression of genes involved in aflatoxin biosynthesis, the expression of related genes were evaluated. In this study, we paid special attention to the global regulatory gene *laeA*, the developmental regulatory gene *veA*, the key two aflatoxin biosynthetic pathway specific regulatory genes *aflR* and *aflS* and the other four structural genes *aflD*, *aflM*, *aflP*, and *aflQ* (Amaike and Keller, 2009; Cleveland *et al*., 2009). As shown in Fig. 2B, the selected genes were significantly down-regulated by VOCs of *S. alboflavus* TD-1. *LaeA* and *vea* were down-expressed with 5.46- and 29.50-folds decrease compared to control. The two key transcriptional regulators, *aflR* and *aflS* were down-regulated with 6.98- and 19.27-folds decrease, separately. Furthermore, the other four structural genes, *aflD*, *aflM*, *aflP*, and *aflQ* were down-regulated in the range from 7.35- to 35.59-folds. The results confirmed that the VOCs directly reduced aflatoxin biosynthesis through reducing the abundance of gene mRNAs in *A. flavus*.

Dynamic changes of VOCs produced by wheat bran culture of *S. alboflavus* TD-1

Complex and dynamic VOCs were produced by *S. alboflavus* TD-1 during the fermentation (0, 2, 4, 6, 8, and 10 days after inoculation). As shown in Fig. 3A, these volatile compounds mainly fell into classes of alkanes, aldehydes, alcohols, ketones, terpenoids, and ethers. The contents of alkanes (e.g., hexane, heptane, octane, and dodecane), aldehydes (e.g., heptanal and benzaldehyde), alcohols (l-hexanol), and ketones (2-decanone) were declined along with the fermentation. Whereas the contents of terpenoids (e.g., 2-methylisoborneol, azulene, γ-muurolene, and β-copaene), ethers (e.g., anisole, dimethyl trisulfide, and benzenamine), and some ketones (2-hexanone) were increased.

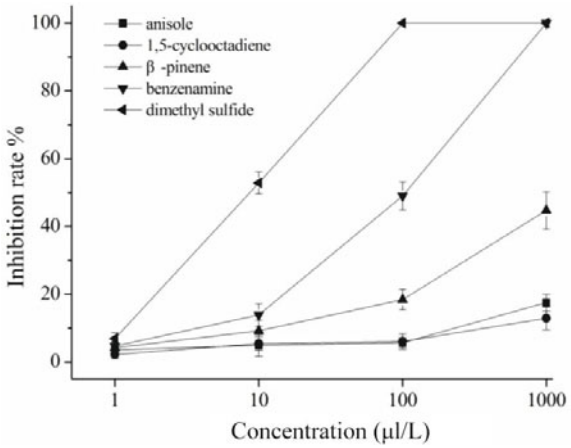

Fig. 4. Effects of technical grade volatiles on the mycelial growth of *Aspergillus flavus* at 3 days post inoculation. The concentrations of volatiles were carried out at 1, 10, 100, and 1000 μl/L, respectively.

PLS analysis was employed to evaluate the relationship between VOCs from *S. alboflavus* TD-1 and the inhibition rate of the fermentation process. The PLS coefficients plot as shown in Fig. 3B. The results demonstrated that terpenoids and ethers have higher regression coefficients compared to those of alkanes, alcohols, and aldehydes. Thus, three kinds of ethers (anisole, dimethyl trisulfide, and benzenamine) and two kinds of alkenes, including a terpenoid (β-pinene and 1,5-cyclooctadiene) were selected for testing the antimicrobial activity against *A. flavus*. As shown in Fig. 4, all five of the volatile compounds displayed inhibitory effects on *A. flavus*. Among those, dimethyl trisulfide was the most effective compound, showing complete suppression of *A. flavus* at the concentration of 100 μl/L. In addition, benzenamine also completely inhibited the mycelial growth of *A. flavus* at the concentration of 1,000 μl/L. β-Pinene showed a 44.7% mycelial growth inhibition at the highest tested concentration. In comparison, anisole and 1,5-cyclooctadiene exhibited similar, weaker antifungal activity against *A. flavus*, with percentages of inhibition of mycelial growth of 17.5 and 12.9%, respectively, when the concentration was 1000 μl/L.

The results demonstrated that dimethyl trisulfide and benzenamine may play an important role in bioactivity of *S. alboflavus* TD-1 against *A. flavus*. In addition, it is supposed that the bioactivity of VOCs is due to additive or synergistic action rather than the activity of a single component (Mercier and Jiménez, 2004; Gotorvila *et al*., 2017). Overall, the antimicrobial activity of other identified volatile compounds will be investigated in further research, and the synergistic effects of various volatile compounds in the mixture are needed to be evaluated.

Acknowledgements

This work was supported by the Key Technologies R & D Program of Tianjin [Grant number 16YFZCNC00700]; National Natural Science Foundation of China [Grant number

31701668]; Natural Science Foundation of Tianjin [Grant number 17JCQNJC14300] and Laboratory Innovation Fund of Tianjin University of Science and Technology [Grant number 1714A304].

Conflict of Interest

The authors declare no competing financial interest.

References

Amaike, S. and Keller, N.P. 2009. Distinct roles for VeA and LaeA in development and pathogenesis of *Aspergillus flavus*. *Eukaryot. Cell* **8**, 1051.

Ando, H., Hatanaka, K., Ohata, I., Yamashita-Kitaguchi, Y., Kurata, A., and Kishimoto, N. 2010. Antifungal activities of volatile substances generated by yeast isolated from Iranian commercial cheese. *Food Control* **21**, 472–478.

Bavaro, S.L., D'Antuono, I., Cozzi, G., Haidukowski, M., Cardinali, A., and Logrieco, A.F. 2016. Inhibition of aflatoxin B_1 production by verbascoside and other olive polyphenols. *World Mycotoxin J.* **9**, 1–10.

Berdy, J. 2005. Bioactive microbial metabolites: a personal view. *J. Antibiot.* **58**, 1–26.

Boukaew, S., Plubrukam, A., and Prasertsan, P. 2013. Effect of volatile substances from *Streptomyces philanthi* RM-1-138 on growth of *Rhizoctonia solani* on rice leaf. *Biocontrol* **58**, 471–482.

Boukaew, S. and Prasertsan, P. 2014. Suppression of rice sheath blight disease using a heat stable culture filtrate from *Streptomyces philanthi* RM-1-138. *Crop. Prot.* **61**, 1–10.

Cleveland, T.E., Yu, J.J., Fedorova, N., Bhatnagar, D., Payne, G.A., Nierman, W.C., and Bennett, J.W. 2009. Potential of *Aspergillus flavus* genomics for applications in biotechnology. *Trends Biotechnol.* **27**, 151–157.

Corcuff, R., Mercier, J., Tweddell, R., and Arul, J. 2011. Effect of water activity on the production of volatile organic compounds by *Muscodor albus* and their effect on three pathogens in stored potato. *Fungal Biol.* **115**, 220–227.

Cordovez, V., Carrion, V.J., Etalo, D.W., Mumm, R., Zhu, H., Wezel, G.P.V., and Raaijmakers, J.M. 2015. Diversity and functions of volatile organic compounds produced by *Streptomyces* from a disease-suppressive soil. *Front. Microbiol.* **6**, 1081.

Da, C.C.L., Fernández, P.V., and Patriarca, A. 2013. Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods. *Int. J. Food Microbiol.* **166**, 1–14.

Da, S.B.N., Nakassugi, L.P., Faggion, P.O.J., Kohiyama, C.Y., Mossini, S.A., Grespan, R., Nerilo, S.B., Mallmann, C.A., Alves, A.F.B., and Machinski, M.J. 2015. Antifungal activity and inhibition of fumonisin production by *Rosmarinus officinalis* L. essential oil in *Fusarium verticillioides* (sacc.) nirenberg. *Food Chem.* **166**, 330–336.

Eva, A., Dharini, S., and Lise, K. 2010. Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus. *Biol. Control* **53**, 122–128.

Gao, Z., Zhang, B., Liu, H., Han, J., and Zhang, Y. 2017. Identification of endophytic *Bacillus velezensis* ZSY-1 strain and antifungal activity of its volatile compounds against *Alternaria solani* and *Botrytis cinerea*. *Biol. Control* **105**, 27–39.

Garcia, D., Ramos, A.J., Sanchis, V., and Marín, S. 2012. Effect of *Equisetum arvense* and *Stevia rebaudiana* extracts on growth and mycotoxin production by *Aspergillus flavus* and *Fusarium verticillioides* in maize seeds as affected by water activity. *Int. J. Food Microbiol.* **153**, 21–27.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Köhl, J., Marrone, P., Morin, L., and Stewart, A. 2012. Have biopesticides come of age? *Trends Biotechnol.* **30**, 250–258.

Gong, A.D., Li, H.P., Shen, L., Zhang, J.B., Wu, A.B., He, W.J., Yuan, Q.S., He, J.D., and Liao, Y.C. 2015. The *Shewanella algae* strain YM8 produces volatiles with strong inhibition activity against *Aspergillus* pathogens and aflatoxins. *Front. Microbiol.* **6**, 1091.

Gotorvila, A., Teixidó, N., Di, F.A., Usall, J., Ugolini, L., Torres, R., and Mari, M. 2017. Antifungal effect of volatile organic compounds produced by *Bacillus amyloliquefaciens* CPA-8 against fruit pathogen decays of cherry. *Food Microbiol.* **64**, 219–225.

Heydari, A. and Pessarakli, M. 2010. A review on biological control of fungal plant pathogens using microbial antagonists. *J. Biol. Sci.* **10**, 273–290.

Hong, L.S., Yusof, N.I.M., and Ling, H.M. 2010. Determination of aflatoxins B_1 and B_2 in peanuts and corn based products. *Sains Malays.* **39**, 731–735.

Hua, S.S., Beck, J.J., Sarreal, S.B., and Gee, W. 2014. The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. *Mycotoxin Res.* **30**, 71–78.

Hung, R., Lee, S., and Bennett, J.W. 2015. Fungal volatile organic compounds and their role in ecosystems. *Appl. Microbiol. Biotechnol.* **99**, 3395–3405.

Johnson, S., Nguyen, V., and Coder, D. 2013. Assessment of cell viability. Chapter 9, Unit 9.2.

Kifle, M.H., Yobo, K.S., and Laing, M.D. 2017. Biocontrol of *Aspergillus flavus* in groundnut using *Trichoderma harzianum* strain kd. *J. Plant Dis. Protect.* **124**, 51–56.

Kong, Q., Chi, C., Yu, J., Shan, S., Li, Q., Li, Q.T., Guan, B., Nierman, W.C., and Bennett, J.W. 2014. The inhibitory effect of *Bacillus megaterium* on aflatoxin and cyclopiazonic acid biosynthetic pathway gene expression in *Aspergillus flavus*. *Appl. Microbiol. Biotechnol.* **98**, 5161–5172.

Kumar, P., Mahato, D.K., Kamle, M., Mohanta, T.K., and Kang, S.G. 2017. Aflatoxins: a global concern for food safety, human health and their management. *Front. Microbiol.* **7**, 2170.

Li, Q., Ning, P., Zheng, L., Huang, J., Li, G., and Hsiang, T. 2010. Fumigant activity of volatiles of *Streptomyces globisporus* JK-1 against *Penicillium italicum* on citrus microcarpa. *Postharvest Biol. Technol.* **58**, 157–165.

Li, Q., Wu, L., Hao, J., Luo, L., Cao, Y., and Li, J. 2015. Biofumigation on post-harvest diseases of fruits using a new volatile-producing fungus of *Ceratocystis fimbriata*. *PLoS One* **10**, e0132009.

Li, H.X. and Xiao, C.L. 2008. Characterization of fludioxonil-resistant and pyrimethanil-resistant phenotypes of *Penicillium expansum* from apple. *Phytopathology* **98**, 427–435.

Liu, Y. and Wu, F. 2010. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environ. Health Persp.* **118**, 818–824.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Mellon, J.E., Zelaya, C.A., Dowd, M.K., Beltz, S.B., and Klich, M.A. 2012. Inhibitory effects of gossypol, gossypolone, and apogossypolone on a collection of economically important filamentous fungi. *J. Agric. Food Chem.* **60**, 2740–2745.

Mercier, J. and Jiménez, J.I. 2004. Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus*. *Postharvest Biol. Technol.* **31**, 1–8.

Minerdi, D., Bossi, S., Gullino, M.L., and Garibaldi, A. 2009. Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. *Environ. Microbiol.* **11**, 844–854.

Perry, S.W., Norman, J.P., Barbieri, J., Brown, E.B., and Gelbard, H.A. 2011. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques* **50**, 98.

Pimentel, D. 2005. Environmental and economic costs of the application of pesticides primarily in the United States. *Environ. Dev. Sustain*. **7**, 229–252.

Ramin, A.A., Braun, P.G., Prange, R.K., and Delong, J.M. 2005. *In vitro* effects of *Muscodor albus* and three volatile components on growth of selected postharvest microorganisms. *Hortscience* **40**, 2109–2114.

Razzaghi-Abyaneh, M. 2013. Aflatoxins: Recent advances and future prospects. *In* Tech open, Rijeka, Croatia.

Schalchli, H., Tortella, G.R., Rubilar, O., Parra, L., Hormazabal, E., and Quiroz, A. 2016. Fungal volatiles: an environmentally friendly tool to control pathogenic microorganisms in plants. *Crit. Rev. Biotechnol*. **36**, 144–152.

Shams-Ghahfarokhi, M., Kalantari, S., and Razzaghi-Abyaneh, M. 2013. Terrestrial bacteria from agricultural soils: versatile weapons against aflatoxigenic fungi. *In* Aflatoxins-recent advances and future prospects. InTech.

Tian, J., Huang, B., Luo, X., Zeng, H., Ban, X., He, J., and Wang, Y. 2012. The control of *Aspergillus flavus* with *Cinnamomum jensenianum* hand. -Mazz essential oil and its potential use as a food preservative. *Food Chem*. **130**, 520–527.

Wang, C., Wang, Z., Qiao, X., Li, Z., Li, F., Chen, M., Wang, Y., Huang, Y., and Cui, H. 2013. Antifungal activity of volatile organic compounds from *Streptomyces alboflavus* TD-1. *FEMS Microbiol. Lett*. **341**, 45–51.

WHO and IARC (International Agency for Research on Cancer). 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. *In* IARC monographs on the evaluation of carcinogenic risks to humans, vol. 56.

Wu, L.X., Ding, X.X., Li, P.W., Du, X.H., Zhou, H.Y., Bai, Y.Z., and Zhang, L.X. 2016. Aflatoxin contamination of peanuts at harvest in china from 2010 to 2013 and its relationship with climatic conditions. *Food Control* **60**, 117–123.

Wu, Y., Yuan, J., E, Y., Raza, W., Shen, Q., and Huang, Q. 2015. Effects of volatile organic compounds from *Streptomyces albulus* NJZJSA2 on growth of two fungal pathogens. *J. Basic Microbiol*. **55**, 1104–1117.