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Potential use of *Streptomyces mycarofaciens* SS-2-243 as a biofumigant to protect maize seeds against two aflatoxin producing fungi

Sawai Boukaew · Wanida Petlamul · Purawich Phitthayaphinant · Poonsuk Prasertsan

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Abstract *Streptomyces mycarofaciens* SS-2-243 has been found to produce non-volatile compounds possessing antifungal activity against the growth of plant pathogens, but the effect of its volatile compounds remain unknown. Therefore, the efficacy of volatile compounds from *S. mycarofaciens* SS-2-243 (volatiles SS-2-243) grown on wheat seeds for 12 days was evaluated against four pathogenic fungi. In vitro studies using an antifungal bioassay tests on PDA dishes indicated that the volatiles SS-2-243 could totally suppress the growth of all four strains with most pronounced activity (100% inhibition) against *Aspergillus parasiticus* TISTR 3276 and *A. flavus* TISTR 3041. Identification of the volatiles SS-2-243 using gas chromatography–mass spectrometry (GC–MS) revealed 33 compounds, with the most abundant being 2-methylisoborneol. Effect of the inoculum size and spore concentration of *S. mycarofaciens* SS-2-243 prepared as a wheat seed inoculum on the suppression of the two aflatoxin producing fungi was studied. Complete growth inhibition (100%) was achieved at the optimum wheat seed inoculum size of at least 30 g L^{-1} and 10^7 spore mL^{-1}. Use of 30 g L^{-1} wheat seed culture of *S. mycarofaciens* SS-2-243 could completely kill the conidia of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 with 1 h and 3 h exposures, respectively. Based on the promising antifungal activity of the volatiles SS-2-243, fumigation with 30 g L^{-1} wheat seed culture of *S. mycarofaciens* SS-2-243 for 24 h completely controlled the growth of the two aflatoxin producing fungi infecting maize seeds, without adverse effects on maize seed germination. The main effect of the volatiles SS-2-243 was damage and complete inhibition of conidia germination as evident by scanning electron microscope (SEM) images. Therefore, this biofumigant has good potential to control the two aflatoxin producing fungi.

Keywords Aflatoxin producing fungi · Biofumigation · Maize seeds · *Streptomyces mycarofaciens* · Volatile compounds

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S. Boukaew (✉) · W. Petlamul
College of Innovation and Management, Songkhla Rajabhat University, Songkhla 90000, Thailand
e-mail: sawai.bo@skru.ac.th

P. Phitthayaphinant
Faculty of Technology and Community Development, Thaksin University, Phatthalung 93210, Thailand

P. Prasertsan (✉)
Research and Development Office, Prince of Songkla University, Songkhla 90112, Thailand
e-mail: poonsuk918@yahoo.com

Introduction

Maize (*Zea mays* L.) is among the most important crops around the world as human food and animal feed (Valdez-Ortiz et al. 2007; Shaista et al. 2011), and is now also used for bioethanol production

(Eckert et al. 2018). Fungal contamination of stored seeds, including those of maize, is a common problem for the warehouses in Thailand with humid and warm weather. *Aspergillus parasiticus* and *A. flavus* are the most common spoilage fungi in food and feed, and are known to produce aflatoxins (Ng'ang'a et al. 2016; Picot et al. 2017). Aflatoxins are well-known for being hazardous to animals and humans (Zain 2011). Infections by toxigenic fungi reduce the economic value and quality of stored seeds (Razzaghi-Abyaneh et al. 2013; Ng'ang'a et al. 2016).

Chemical fungicides are usually used to control the spoilage fungi. However, these agents have negative effects via toxic residues in food, environmental and human health effects, as well as selection effects favoring fungicide resistant microorganisms (Chen et al. 2016; Tamreihao et al. 2016). The development of efficient antifungal microbial agents could be an alternative method to control food spoilage pathogens. Among the various antagonistic microorganisms, *Streptomyces* spp. is most promising for controlling pathogenic fungi, because of producing both volatile (Wan et al. 2008; Li et al. 2010, 2012; Boukaew et al. 2013) and non-volatile antimicrobial compounds (Boukaew et al. 2011, Boukaew & Prasertsan, 2014; Elango et al. 2015; Li et al. 2015; Chen et al. 2016; Shakeel et al. 2016; Singh and Gaur 2017) with diverse biological activities.

Streptomyces species have been widely evaluated as biocontrol agents against plant pathogens, because of their ability to produce a large number of antibiotics and other secondary metabolites (Demain 2000; Chen et al. 2016). The volatile antifungal compounds from *Streptomyces* spp. could help control plant pathogenic fungi such as *Colletotrichum* spp. (Shimizu et al. 2009; Boukaew et al. 2018), *R. solani* (Boukaew et al. 2013), *Sclerotinia sclerotiorum* (Wan et al. 2008), *Penicillium italicum* (Li et al. 2010) and *B. cinerea* (Li et al. 2012; Boukaew et al. 2017a). As well as *Streptomyces*, the endophytic fungus *Muscodor albus* (Strobel 2011; Suwannarach et al. 2013) and yeasts such as *Candida intermedia* (Huang et al. 2011), and bacteria such as *Bacillus pumilus* and *B. thuringiensis* (Zheng et al. 2013) also produce volatile antifungal compounds that are applied to control food spoilage microorganisms worldwide (Arrebola et al. 2010; Huang et al. 2011; Li et al. 2012; Braun et al. 2012; Suwannarach et al. 2013, 2015).

Streptomyces mycarofaciens SS-2-243 was previously found to produce non-volatile antimicrobial compounds that inhibited the growth of *S. rolfsii* and *Ralstonia solanacearum* (Boukaew et al. 2011) but it has never been studied for its volatile antimicrobial compounds against aflatoxin-producing fungi. Therefore, it was the aim of this work to investigate the efficacy of the volatiles from *S. mycarofaciens* SS-2-243 (volatiles SS-2-243) as a biofumigant to control the growth of aflatoxin producing fungi. The specific objectives were: (i) to test the antifungal activity of the volatiles SS-2-243 against four pathogenic fungi and to determine the effects of the volatiles SS-2-243 at different wheat seed culture ages against the selected fungi; (ii) to identify the chemical nature of the volatiles SS-2-243; (iii) to assess the effects of wheat seed inoculum size and spore concentration of *S. mycarofaciens* SS-2-243 on the selected fungi grown on PDA dishes and maize seeds; (iv) to study fungicidal period of the volatiles SS-2-243 against the selected fungi; (v) to evaluate the efficacy of the volatiles SS-2-243 on protection of maize seed; (vi) to study the effect of the fumigation period of the volatiles SS-2-243 for controlling the selected fungi and its effect on seed germination; and (vii) to investigate the mode of action of the volatiles SS-2-243 by studying the ultrastructure of the selected fungi.

Materials and methods

Microorganisms

Streptomyces mycarofaciens SS-2-243 was isolated by Boukaew et al. (2011) and grown on GYM agar dishes at 28 ± 2 °C for 10 days before use. *A. parasiticus* TISTR 3276, *A. flavus* TISTR 3041, *A. niger* ATCC 6275, and *Penicillium* sp. were maintained on potato dextrose agar (PDA 39 g L^{-1}) medium at 4 °C. The four pathogenic fungi were provided by Microbiology Laboratory of the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkhla Province, Thailand. *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 reportedly produce aflatoxins (Rammanee and Hongpattarakere 2011; Sangmanee and Hongpattarakere 2014).

Preparation of wheat seed culture of *S. mycarofaciens* SS-2-243 and spore inoculum of four pathogenic fungi

Wheat seed culture of of *Streptomyces mycarofaciens* SS-2-243 was prepared by inoculating a spore suspension (10^7 spores mL^{-1}) on sterile wheat seeds (autoclaved at 121 °C / 15 min) in conical flasks (250 mL) at 1 mL per 100 g of wheat seeds and incubated at 28 ± 2 °C on a rotary shaker (150 rpm) for 12 days.

Spore inoculum of the four pathogenic fungi were prepared by inoculating 5 mm diameter mycelial plug cutting from the periphery of a growing cultures (at 28 ± 2 °C for 10 days) from each strain and mixed with 5 mL sterile distilled water. The spore counts was done using a hematocytometer. Inoculums were prepared by dilution with sterile distilled water to the required spore concentration of each experiment.

Antifungal activity of the volatiles SS-2-243 against four pathogenic fungi and the effect of wheat seed culture age

The influence of the volatiles from 12 days old wheat seed culture of *Streptomyces mycarofaciens* SS-2-243 on the mycelial growth of the four pathogenic fungi was investigated using an antifungal bioassay (Li et al. 2012). The bioassay was performed following the procedure as previously described (Boukaew et al. 2013). The inhibition of mycelial growth was calculated using the equation:

$$\text{Percentage of inhibition} = [\{(\text{Control}-\text{treatment})/\text{Control}\} \times 100].$$

Effects of the volatiles SS-2-243 from different wheat seed culture ages (0, 3, 6, 9, 12 and 15 days) against the selected pathogenic fungi (based on the above results) was studied. In this experiment, the wheat seed culture of *S. mycarofaciens* SS-2-243 was prepared as described above except that the amount of spore suspension added was the autoclaved wheat seeds 1 mL per 30 g of wheat seeds and incubated (at 28 ± 2 °C) on a rotary shaker (at 150 rpm) for 15 days. Wheat seed culture samples were taken every 3 days for use in antifungal assays (Li et al. 2012) as described above. The colony size in each treatment was recorded and inhibition of mycelial growth was calculated as above.

Identification of the volatiles SS-2-243 by gas chromatography–mass spectrometry

Identification of the volatilesSS-2-243 was carried out following the procedure as described by Wan et al. (2008) with minor modifications. Briefly, volatile compounds from the head space of a 100 ml sealed Duran bottle containing 30 g autoclaved wheat seeds or wheat seed cultures of *Streptomyces mycarofaciens* SS-2-243 were collected by means of solid-phase micro-extraction (SPME) and analyzed with a gas chromatograph mass spectrometer (GC-MS) (Trace GC Ultra/ISQMS, Thermo Scientific Inc., USA) equipped with a TR-5MS column (30 m × 0.25 mm, film thickness 0.25 μm, Agilent, Santa Clara, CA, USA). Mass spectra were obtained using the scan modus (total ion count, 35–500 *m/z*). Confirmation of the compound identities was achieved by comparison of the mass spectra and retention times with those of available standards from the Library of the National Institute of Standards and Technology (NIST). In addition, any baseline volatile compounds from autoclaved wheat seeds were measured and subtracted. The experiments were conducted three times.

Effects of wheat seed inoculum size and spore concentration of *S. mycarofaciens* SS-2-243 on mycelial growth, conidia germination and sporulation of the selected pathogenic fungi growing on PDA dishes or on maize seeds

Effects of wheat seed inoculum size tested on agar dishes

The effect of inoculum size (1, 5, 10, 15, 30, 45 and 60 g) of wheat seed culture of *Streptomyces mycarofaciens* SS-2-243 per L of airspace in the flask) on the mycelial growth, conidia germination and sporulation of the selected pathogenic fungi were studied on PDA dishes, using an antifungal bioassay (as described above). Autoclaved wheat seeds (not inoculated with *S. mycarofaciens* SS-2-243) in similar amounts were used as a control treatments. The diameters of the colonies in the smaller dishes were recorded after incubating (at 28 ± 2 °C) for two days. The inhibition of mycelial growth was calculated as above.

For the conidia germination, 50 μL of spore inoculum (10^4 spore mL^{-1}) of the selected pathogenic fungi were spread on three of the smaller dishes containing 5 mL PDA and the fourth dish had each concentration of the seven inoculum sizes tested. After 24 h incubation at 28 ± 2 °C, the conidia germination in the smaller dishes was recorded. The inhibition of conidia germination was calculated using the equation:

$$\text{Percentage of conidia germination inhibition} = [\{(\text{Control} - \text{treatment})/\text{Control}\} \times 100].$$

For the sporulation of the selected pathogenic fungion PDA dishes, the experiment was as described above (for conidia germination). After incubation at 28 ± 2 °C for 5 days, the total number of conidia per plate was assessed by diluting with sterile water before counting the number density of conidia under a compound microscope (Omano OM88, USA).

Effects of wheat seed inoculum size tested on maize seeds

Maize seeds were prepared by soaking in distilled water (100 mL) for 5 h, and then autoclaving at 121 °C for 15 min (Yang and Chang 2010). Five maize seeds were transferred on each of the three smaller dishes, a 50 μL spore inoculum (10^4 spore mL^{-1}) of the selected pathogenic fungi was dropped on each maize seed. Wheat seed inoculum sizes of *S. mycarofaciens* SS-2-243 at various concentrations (1, 5, 10, 15, 30, 45 and 60 g L^{-1}) were added to the fourth Petri dishes. The same concentration of wheat seeds (not inoculated with *S. mycarofaciens* SS-2-243) was used in the control cases. After incubation at 28 ± 2 °C for five days, the total number of conidia per maize seed was determined by flushing the infected maize seeds with sterile water, then counting the number of conidia in the flush water under a compound microscope.

Effects of spore concentration

The effect of spore concentration of *Streptomyces mycarofaciens* SS-2-243 on the mycelial growth, conidia germination and sporulation of the selected pathogenic fungi were investigated on PDA, using the antifungal bioassay (as stated above) using spore concentrations of 10^4, 10^5 10^6, 10^7 and 10^8 spores mL^{-1} at 1 mL per 30 g wheat seeds inoculated. For the conidia germination, 50 μL of spore inoculum (10^4 spores mL^{-1}) of the selected pathogenic fungi were spread on three of the smaller dishes containing 5 mL PDA, and the fourth dish had 30 g of wheat seed inoculum at five spore concentrations of *S. mycarofaciens* SS-2-243. The incubation and calculation of inhibition were as described above. The sporulation of the selected pathogenic fungi was tested on PDA dishes or on maize seeds. After incubation at 28 ± 2 °C for five days, the total number of the conidia per PDA plate was determined as described above. In the experiment on maize seeds, five maize seeds were placed on each of the three smaller dishes, then a 50 μL of spore inoculum (at 10^4 spore mL^{-1}) of the selected pathogenic fungi were dropped on each maize seed, and the fourth dish contained wheat seed inoculum of *S. mycarofaciens* SS-2-243 with five spore concentrations tested. The same concentrations of wheat seed (without inoculation of *S. mycarofaciens* SS-2-243) were used in the control. After incubation at 28 ± 2 °C for five days, the total number of conidia per maize seed was determined by flushing the infected maize seeds with sterile distilled water, then counting the number of conidia in the flush water with the use of a compound microscope.

Fungicidal period of the volatiles SS-2-243 against the selected pathogenic fungi

To assess the fungicide period of the volatiles SS-2-243, fifty μL of spore inoculum (10^4 spore mL^{-1}) of the selected pathogenic fungi were spread on PDA. The cultures were fumigated with 30 g L^{-1} wheat seed inoculum of *Streptomyces mycarofaciens* SS-2-243 for 0, 1, 3, 6, 9, 12, or 15 h. Equivalent amounts of autoclaved wheat seeds not inoculated with *S. mycarofaciens* SS-2-243 were used as the control. The spore fumigant was removed after each fumigation period and the cultures were incubated at 28 ± 2 °C for 48 h. For each treatment, there were three replicates. The

total number of conidia alive on PDA dishes were determined (Li et al. 2013).

The fungicidal period was defined as the minimum time required for fumigation to effect total inhibition of a pathogen.

Efficacy of the volatiles SS-2-243 at various inoculum sizes and spore concentrations of *S. mycarofaciens* SS-2-243 to protect maize seeds against the selected aflatoxin producing fungi

Evaluation of the volatiles SS-2-243 at different inoculum sizes and spore concentrations of *Streptomyces mycarofaciens* SS-2-243 to protect maize seed against contamination by the selected aflatoxin producing fungi was done using the antifungal bioassay. Five maize seeds were transferred on each of the three smaller dishes, then 50 μL of spore inoculum (10^4 spore mL^{-1}) of the selected aflatoxin producing fungi was dropped on each maize seed, and the fourth dish had some amount (1, 5, 10, 15, 30, 45 and 60 g L^{-1}) of wheat seed culture from varied spore concentrations (inoculated at 1 mL of 10^4, 10^5 10^6, 10^7 or 10^8 spores mL^{-1} on 30 g wheat seeds) of *S. mycarofaciens* SS-2-243. Equivalent amounts of autoclaved wheat seeds not inoculated with *S. mycarofaciens* SS-2-243 were used as the control. The dishes were incubated at 28 ± 2 °C for 5 days. Then, the maize seeds in the dishes were individually analyzed for infection under a stereo-binocular microscope (Sumalan et al. 2013) and the protection against infection of seeds was calculated: Percentage of protection = [{(Control-treatment)/ Control} × 100]. The experiment was repeated twice with three replicates in each.

Effects of the fumigation period with the volatiles SS-2-243 for controlling the selected aflatoxin producing fungi on maize seeds, and its effect on seed germination

The fumigation period with the volatiles SS-2-243 for controlling the aflatoxin producing fungi on maize seeds using the antifungal bioassay was investigated. The minimum dose (30 g L^{-1} wheat seed culture) of *Streptomyces mycarofaciens* SS-2-243 that completely controlled the infection of maize seeds was chosen for all treatments. The four treatments all had aflatoxin producing fungal infection, followed by 0, 6, 12 or 24 h of *S. mycarofaciens* SS-2-243 fumigation. The fungal fumigant was removed after each fumigation period and the Petri dishes were restored to incubation at 28 ± 2 °C. The contamination of maize seeds was observed every 2 days for 12 days. The growth of fungi on maize seeds was visually assessed using a stereo-binocular microscope. The seed contamination index (SCI) was obtained using the formula: % SCI = [(Number of contaminated seeds / Total number seeds) × 100] (Doolotkeldieva 2010).

Streptomyces mycarofaciens SS-2-243 with strong antifungal activity against the selected aflatoxin producing fungi was tested on the effects on maize seed germination. One hundred seeds were fumigated with 30 g L^{-1} of *S. mycarofaciens* SS-2-243 for 24 h and placed on moist paper on the bottom of a plastic box (120 mm width × 170 mm length × 68 mm height with 1 L inner volume). Equivalent amounts of autoclaved wheat seeds not inoculated with *S. mycarofaciens* SS-2-243 were used in the control. The effects of the volatiles SS-2-243 on maize seed germination were assessed after 10 days of incubation at 28 ± 2 °C.

Mode of action of the volatiles SS-2-243 by studying the ultrastructure of the selected aflatoxin producing fungi

Fungal biomass obtained from a 5-day-old culture grown on PDA with or without fumigation exposure to the volatiles SS-2-243 was subjected to scanning electron microscopy (SEM) to assess possible effects of the volatile compounds on the ultrastructure of the fungi. The mycelium affected by the volatiles SS-2-243 was transferred to a glass cover slip, then fixed with 1.5% glutaraldehyde and dehydrated with a graded series of ethanol washes followed by drying in a desiccator (Walter and Crawford 1995). Samples were affixed to SEM stubs using carbon tape followed by a thin coating with gold and examined using a SEM (FEI Quanta 400, SEM-Quanta).

Statistical analysis

All experiments were repeated twice with three replicates. The data were analyzed using Statistical Package for the Social Sciences (SPSS) software version 15 for

Windows. Statistical significance was evaluated using Duncan's Multiple Range and accepted at a $p = 0.05$.

Results

Antifungal activity of the volatiles SS-2-243 against four pathogenic fungi and the effect of wheat seed culture age

The volatiles generated by the 12 days old wheat seed culture of *Streptomyces mycarofaciens* SS-2-243 could inhibit the mycelial growth of the four pathogenic fungi. The growth inhibitions on *A. parasiticus* TISTR 3276, *A. flavus* TISTR 3041, *Penicillium* sp. and *A. niger* ATCC 6275 (on PDA) were 100%, 100%, 49%, and 22%, respectively. The least inhibited was *A. niger* ATCC 6275 while the most pronounced inhibition (100% inhibition) was against the two aflatoxin producing fungi *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041. Therefore, the two aflatoxin producing fungi were selected for further studies as they were completely inhibited by the volatiles SS-2-243.

The effect of the volatiles SS-2-243 at different wheat seed culture ages against the two aflatoxin producing fungi was investigated. The results indicated that the growth inhibition increased with the wheat seed culture age from 0 to 15 days (Fig. S1; Supplementary data). Growth of both pathogens was inhibited >60% after 6 days incubation and complete inhibition (100%) occurred at 9 days of cultivation for *A. flavus* TISTR 3041 and at 12 days for *A. parasiticus* TISTR 3276. Therefore, *A. parasiticus* TISTR 3276 was more resistant to the volatiles SS-2-243 than *A. flavus* TISTR 3041.

Identification of the volatiles SS-2-243

Identification of the chemical composition by GC–MS revealed 33 volatile compounds from the 12 days old wheat seed culture of *S. mycarofaciens* SS-2-243 (Table 1). *Streptomyces mycarofaciens* SS-2-243 excreted multiple antimicrobial compounds, and the two major constituents were 2-methylisoborneol (29.7%) followed by 3,7-dimethylocta-2,6-dien-1-ol (geosmin) (10.4%). The identified volatile compounds fell into several categories, including alcohols, alkenes, aromatic hydrocarbons, ketones, alkanes and carboxylic acids.

Effect of wheat seed inoculum size and spore concentration of *S. mycarofaciens* SS-2-243 on mycelial growth, conidia germination and sporulation of the selected pathogenic fungi growing on PDA dishes or on maize seeds

Effect of wheat seed inoculum size

The effects of inoculum size (1, 5, 10, 15, 30, 45 and 60 g L^{-1}) of the wheat seed culture of *Streptomyces mycarofaciens* SS-2-243 was tested against the two selected aflatoxin producing fungi. The reduction on mycelial growth, conidia germination and sporulation were dependent on the wheat seed inoculum size (Fig. 1). The optimum wheat seed inoculum size was 10 g L^{-1} for total growth inhibition (100%) of *A. parasiticus* TISTR 3276 and 30 g L^{-1} for *A. flavus* TISTR 3041 (Fig. 1a). In addition, the optimum inoculum size for conidia germination was 15 g L^{-1} for both aflatoxin producing fungal strains (Fig. 1b). The sporulation of the two strains was tested on PDA and on maize seeds using various inoculum sizes of *S. mycarofaciens* SS-2-243. The wheat seed inoculum with 15 g L^{-1} of *S. mycarofaciens* SS-2-243 completely inhibited sporulation (100%) of both *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 on PDA plate (Fig. 1c) and on maize seeds (Fig. 1d). The inoculum size 30 g L^{-1} was chosen for further study as it could completely inhibit growth, conidia germination and sporulation of both alfatoxigenic strains.

Effects of spore concentration

Effects of the volatiles SS-2-243 from different spore concentrations (10^4, 10^5, 10^6, 10^7 and 10^8 spore mL^{-1}) of *Streptomyces mycarofaciens* SS-2-243 in the selected wheat seed inoculum size (30 g L^{-1}) on mycelial growth, conidia germination and sporulation of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 were studied (Fig. 2). Increasing the concentration from 10^4 to 10^7 spore mL^{-1} increased the growth inhibition from 55.8% to 100% for *A. parasiticus* TISTR 3276 and from 34.5% to 100% for *A. flavus* TISTR 3041, respectively (Fig. 2a). Therefore, the optimum spore concentrations of *S. mycarofaciens* SS-2-243 were 10^7 and 10^6 spore mL^{-1} to completely inhibit the growth of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041, respectively. In addition, conidia germination was completely inhibited (100%) when the spores of these

Table 1 GC-MS analysis of the volatile compounds produced by *S. mycarofaciens* SS-2-243 after cultivation on wheat seeds at 28 ± 2 °C for 12 days

Retention time (min)	Total area (%)	Possible compound [a]
12.85	0.69	s-Methyl −3-methyl butanethioate
14.78	2.44	1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-
17.35	6.65	Cyclohexane, 1-*trans*-isopropenyl-4-
18.14	0.34	4-Isopropenyl-1-methyl-1-cyclohexen
19.48	5.32	Acetophenone
21.63	1.48	4,8-Dimethyl-nona-1,3,7-triene
21.81	3.17	Phenylethyl alcohol
23.66	0.40	Octane,6-ethyl-2-methyl
29.42	29.7	2-Methylisoborneol
31.85	4.46	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-
32.10	2.42	Cyclohexane, 1,1,4,4-tetramethyl-2,5-dimethyle
33.43	1.00	2(1H)-Azulenone, 4,5,6,7,8,8a-hexahydro-8a-m
34.56	1.77	Dodecane,2,7,10-trimethyl pentadecane
34.72	0.80	*trans*-anti-Tricyclo[7.3.0.0(2,6)]-8-dodecene
40.14	0.54	Cycloundecane, 1,1,2-trimethyl-
40.57	0.97	2-Ethyl-1-dodecanol
41.01	0.35	6-Dodecanone
42.24	0.24	á Elemene
44.07	10.4	3,7-Dimethylocta-2,6-dien-1-ol (geosmin)
46.31	1.81	Valencene
47.97	0.85	Hexadecane, 2,6,10,14-tetramethyl-
48.99	0.69	ç-Gurjunene
49.61	0.87	2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,
50.75	0.67	Tetradecane, 2-methyl-
52.86	0.56	2-Tetradecanone
57.33	0.94	á-Eudesmol
58.60	0.93	2-Pentadecanone
59.32	1.64	1H-Indene, 2-butyl-4-hexyloctahydro-
60.07	1.42	Hexadecane, 2,6,10,14-tetramethyl-
61.08	1.03	(E,1'RS,2'RS,3'SR)-1-(1′,2′-epoxy-3′-methoxy-2
61.11	0.64	Pentadecanoic acid
63.94	1.13	Verticellol
64.66	0.93	Hexadecanoic acid

[a] Minor compounds detected from autoclaved wheat seeds are not included in this table

T_R, retention time

pathogenic fungi were treated with 10^4 spore mL^{-1} of *S. mycarofaciens* SS-2-243 (Fig. 2b). The spore concentration of 10^7 spore mL^{-1} of *S. mycarofaciens* SS-2-243 completely inhibited sporulation of *A. parasiticus* TISTR 3276 (Fig. 2c) and *A. flavus* TISTR 3041 (Fig. 2d). Therefore, the spore concentration 10^7 spore mL^{-1} was chosen for further study, as it could completely inhibit growth, conidia germination and sporulation of both *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041.

Fungicidal period of the volatiles SS-2-243 against the two aflatoxin-producing fungi

The growth inhibition kinetics of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 by the volatiles SS-2-243 is presented in Fig. S2 (Supplementary data). Exposure of the two aflatoxin producing fungal spores to the volatiles SS-2-243 for a period of 0–15 h decreased conidia germination with exposure time. The volatiles SS-2-243 totally inhibited (100%) spore germination of *A. parasiticus* TISTR 3276 at 1 h of exposure, while it look 3 h for *A. flavus* TISTR 3041.

Efficacy of the volatiles SS-2-243 at various inoculum sizes and spore concentrations of *S. mycarofaciens* SS-2-243 to protect maize seeds against the selected aflatoxin producing fungi

Various concentrations of the wheat seed inoculum (1, 5, 10, 15, 30, 45 and 60 g L^{-1}) and spore concentrations (10^4, 10^5, 10^6, 10^7 and 10^8 spore mL^{-1}) of *Streptomyces mycarofaciens* SS-2-243 in the fumigation of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 on maize seeds were investigated for effects on fungal growth (Fig. 3). The percentages of protection on maize seeds significantly ($p < 0.05$) increased with the concentration of the wheat seed inoculum and with the spore concentration of *S. mycarofaciens* SS-2-243. The most effective wheat seed inoculum dose and spore concentration were 30 g L^{-1} (Fig. 3a) and 10^4 spore mL^{-1} (Fig. 3b), respectively, as the fumigation could completely protect (100%) maize seeds against infection by either alfatoxigenic fungus after 5 days of incubation at 28 ± 2 °C. Therefore, the optimum wheat seed inoculum size and spore concentration of *S. mycarofaciens* SS-2-243 were 30 g L^{-1} and 10^4 spore mL^{-1}, respectively, providing complete growth inhibition of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 on maize seeds.

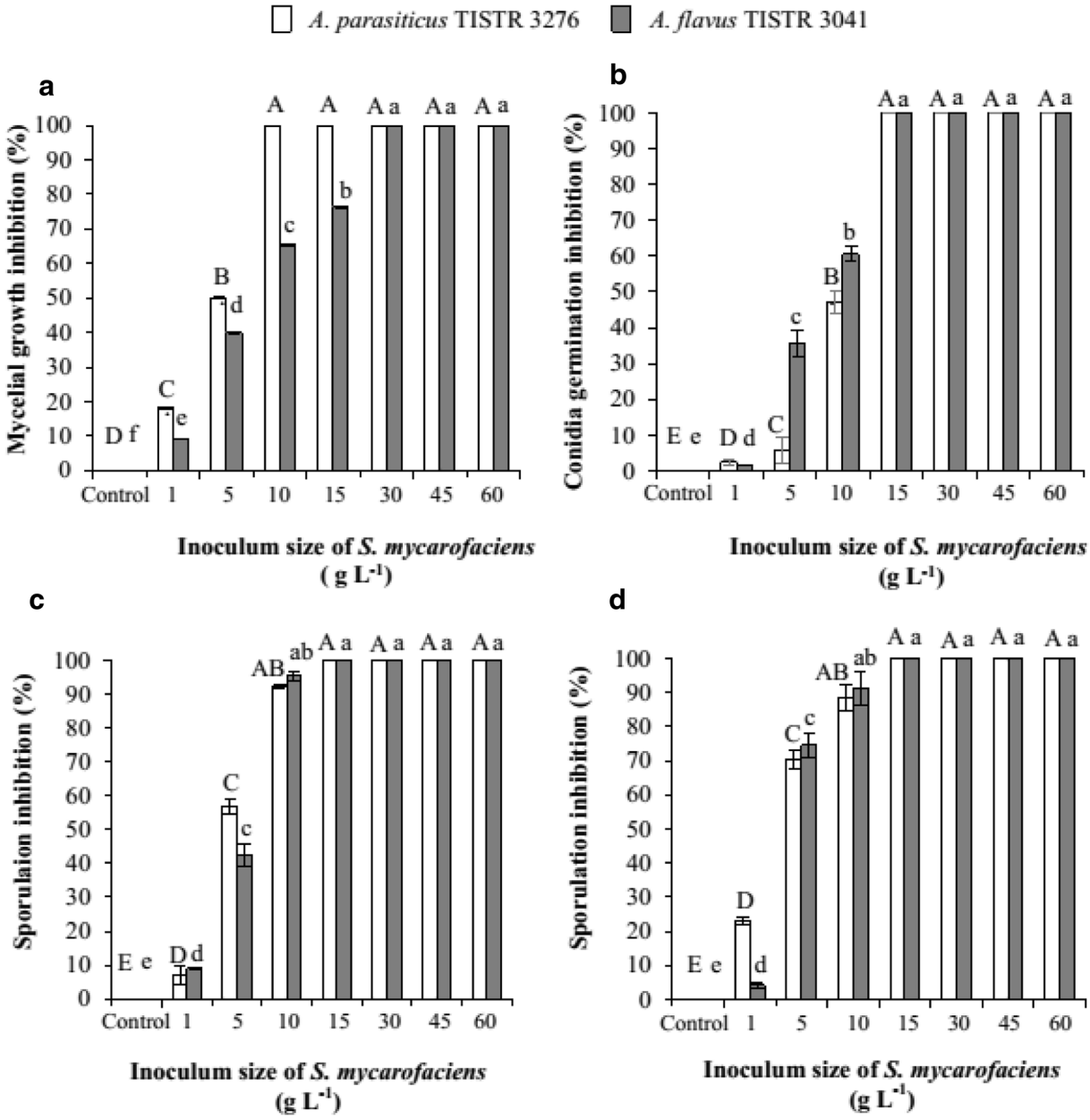

Fig. 1 Effects of wheat seed inoculum size on suppression of mycelial growth (**a**), conidial germination (**b**), sporulation on PDA plate (**c**), and sporulation on maize seeds (**d**) of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041, when fumigated with the volatiles SS-2-243. Different letters above bars indicate significant differences ($p < 0.05$) using ANOVA after the Duncan's Multiple Range Test in each group. Each bar represented mean value with standard error of three replicates

Effects of the fumigation period of the volatiles SS-2-243 for controlling the selected aflatoxin producing fungi on maize seeds, and its effect on maize seed germination

The fumigation period (0, 6, 12 or 24 h) had an influence on the level of infection by either pathogen (Table 2). Treated and non–fumigated maize seeds showed initial wheat seed contamination symptoms after 2 days, with contamination levels 46.6% for *A. parasiticus* TISTR 3276 and 33.3% for *A. flavus* TISTR 3041. With 6 h of fumigation, the initial wheat seed contamination (< 30%) of both pathogens occurred on the 8th day. Interestingly, the 24 h

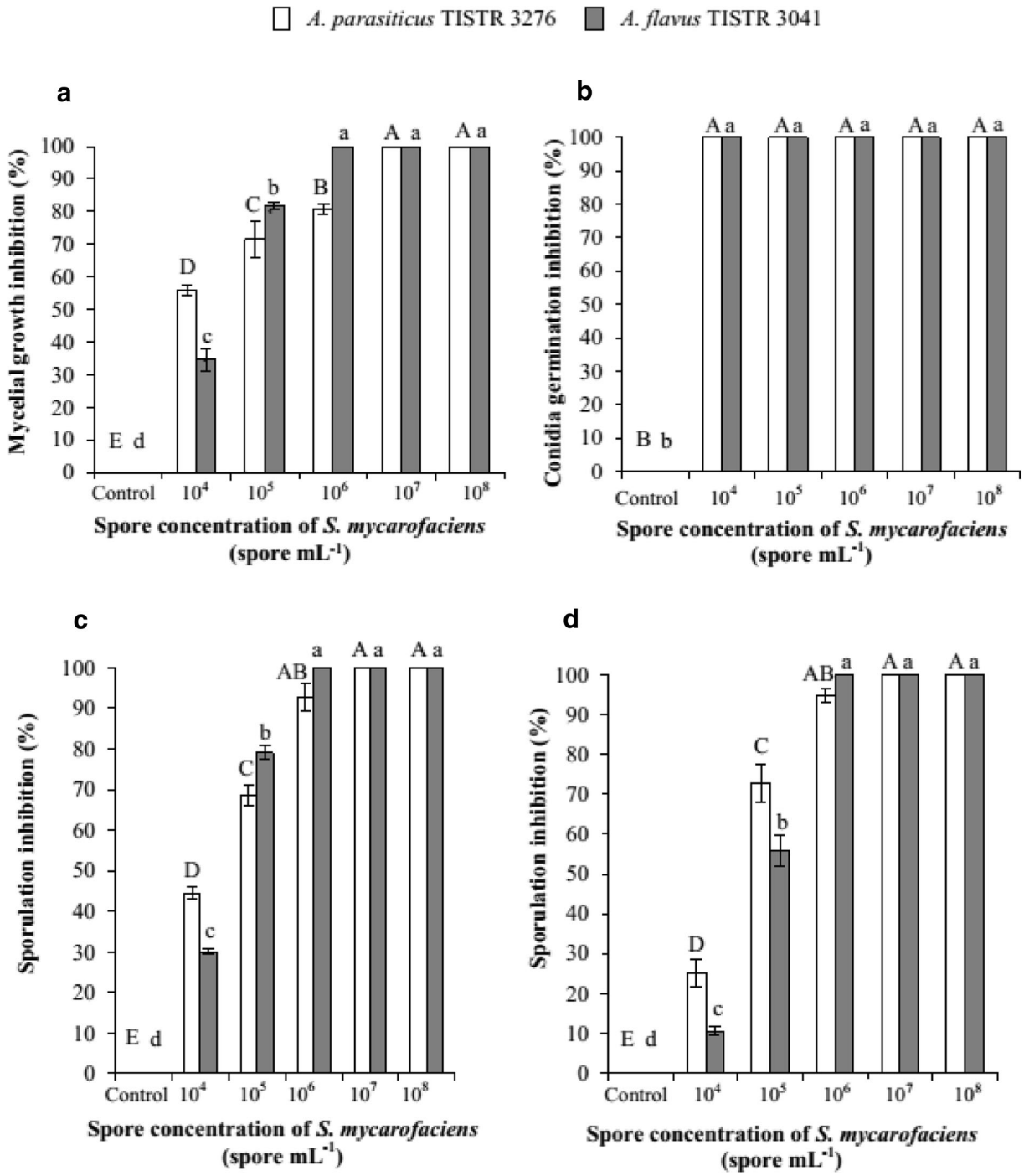

Fig. 2 Effects of spore concentration on suppression of mycelial growth (**a**), conidial germination (**b**), sporulation on PDA plate (**c**), and sporulation on maize seeds (**d**) of *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041, when fumigated with the volatiles SS-2-243. Different letters above bars indicate significant differences ($p < 0.05$) using ANOVA after the Duncan's Multiple Range Test in each group. Each bar represented mean value with standard error of three replicates

fumigation treatment completely controlled (100%) the two aflatoxin producing fungi on maize seeds. Therefore, 24 h fumigation was sufficient to control the two aflatoxin producing fungi on maize seeds.

The maize seeds were fumigated with 30 g L^{-1} wheat seed culture of *Streptomyces mycarofaciens* SS-2-243, incubated for 24 h, and placed in moist plastic boxes for 10 days. The volatiles SS-2-243 did not significantly ($p < 0.05$) affect maize seed germination relative to the control (data not shown). Therefore, the fumigation with *S. mycarofaciens* SS-2-243 can be applied to control aflatoxin producing fungi during maize seed storage.

Mode of action of the volatiles SS-2-243 by studying the ultrastructure of the selected aflatoxin producing fungi

Ultrastructure alterations of the two aflatoxin producing fungi after five days of treatment with the volatiles SS-2-243 were imaged by SEM (Fig. 4). In the control, there were no morphological changes in *A. flavus* TISTR 3041 (Fig. 4a) and *A. parasiticus* TISTR 3276 (Fig. 4d). The individual ordered mycelia could be clearly distinguished in the control samples, and a large quantity of well formed conidiophores grew at the fungal tips. On the other hand, images of the two aflatoxin producing fungi samples exposed to the volatiles SS-2-243 showed evident damage and completely inhibited conidia germination of *A. flavus* TISTR 3041 (Fig. 4b, c) and *A. parasiticus* TISTR 3276 (Fig. 4e, f). The two aflatoxin producing fungi were markedly shriveled, with crinkled cell-walls and flattened hyphae in the SEM images (Fig. 4c and f).

Discussion

Streptomyces mycarofaciens SS-2-243 was previously reported to produce non-volatile compounds with antimicrobial activity in a culture broth (Boukaew et al. 2011, 2017a), but production of volatile compounds by this strain SS-2-243 has never been assessed. In this study, *S. mycarofaciens* SS-2-243 was found to produce volatile compounds while growing on autoclaved wheat seeds, and the volatiles significantly ($p < 0.05$) affected the mycelial growth of four phytopathogenic fungi on PDA agar dishes. The volatile compounds were most active against two aflatoxin producing fungal strains (*A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041). *Streptomyces* spp. is known to produce volatile compounds that inhibit and kill plant pathogenic fungi (Wan et al. 2008; Li et al. 2010, 2012; Boukaew et al. 2013, 2018; Wang et al. 2013). Among the 33 volatile compounds from *S. mycarofaciens* SS-2-243 grown on autoclaved wheat seeds and identified by GC-MS analysis, 2-methylisoborneol was found as the largest component (29.7%) followed by 3,7-dimethylocta-2,6-dien-1-ol (geosmin) (10.4%). They could be chemically grouped into alcohols, alkenes, aromatic hydrocarbons, ketones, ester and alkanes, some of which were known antimicrobial agents (Wan et al. 2008). The 2-methylisoborneol was also the most abundant compound among 27 volatile compounds from *S. alboflavus* TD-1 (Wang et al. 2013). In contrast, trans-1,10-dimethyl-trans-9-decalol (geosmin) was the major component among 16 volatile substances released by cultures of *S. platensis* F-1 grown on autoclaved wheat seeds (Wan et al. 2008). Geosmin was also the most abundant compound among 41 volatile organic compounds from *S. globisporus* JK-1 growing on wheat seeds (Li et al. 2010). Unlike these cases, 3,7-dimethylocta-1,6-dien-3-ol (L-linalool) was the major compound among 36 volatile compounds from *S. philanthi* RM-1-138 growing on autoclaved wheat seed (Boukaew et al. 2013). Some of the volatiles SS-2-243, such as s-methyl −3-methyl butanethioate, 4-isopropenyl-1-methyl-1-cyclohexen, 4,8-dimethyl-nona-1,3,7-triene, octane,6-ethyl-2-methyl, pentadecanoic acid, verticellol and hexadecanoic acid have not been previously reported from *Streptomyces* spp. (Wan et al. 2008; Li et al. 2010; Boukaew et al. 2013; Wang et al. 2013). Thus, different *Streptomyces* species may produce similar or different major components of volatile compounds, with various effects. Three components of the volatiles SS-2-243 have been reported to possess antimicrobial activities: phenylethyl alcohol in *S. globisporus* JK-1 (Li et al. 2010) and *Muscodor albus* (Grimme et al. 2007; Strobel et al. 2007), acetophenone in *S. globisporus* JK-1 (Li et al. 2010) and hexadecanoic acid in *Holarrhena antidysenterica* (Preethi et al. 2010). In addition to their antimicrobial function, the volatile compounds from *Bacillus amyloliquefaciens* IN937 and *B. subtilis* GB03 were reported to promote the growth of *Arabidopsis thaliana* (Ryu et al. 2003; Vespermann et al. 2007) and induce systemic resistance in *A. thaliana* against *Erwinia carotovora* subsp. *carotovora* (Ryu et al. 2004). In addition, volatile compounds from bacteria and yeast inhibited pigment production by sapstain fungi (Bruce et al. 2003). Finally, volatile compounds can regulate aflatoxin biosynthesis in *Aspergillus parasiticus* (Roze et al. 2007).

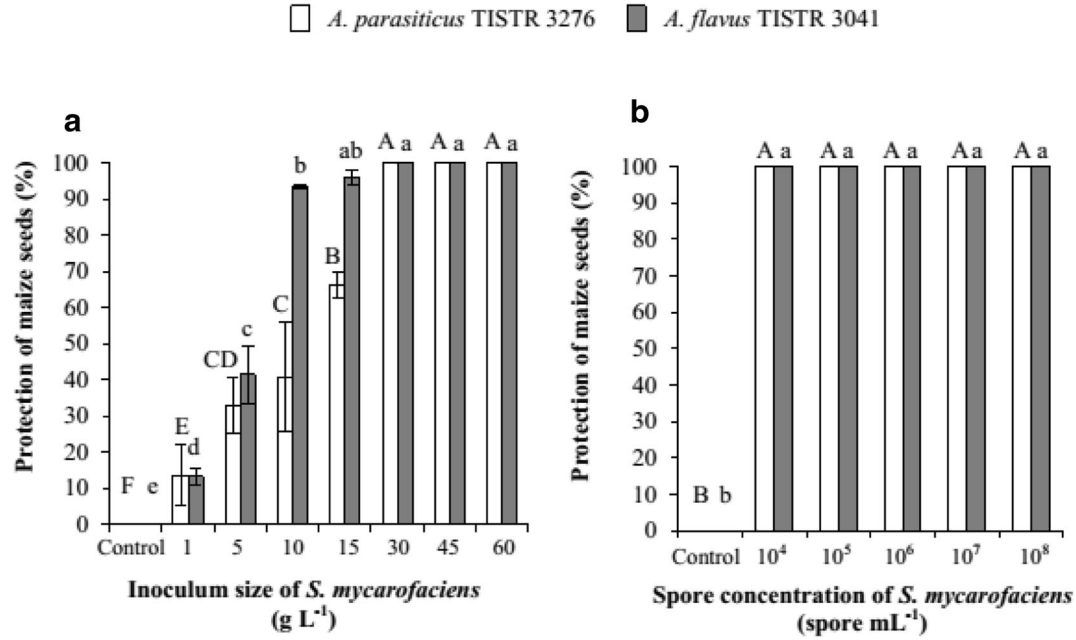

Fig. 3 Effects of inoculum size (**a**) and spore concentration (**b**) on the protection of maize seeds inoculated with *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 when fumigated with the volatiles SS-2-243 after cultivation at 28 ± 2 °C for five days. Different letters above bars indicate significant differences ($p < 0.05$) using ANOVA after the Duncan's Multiple Range Test in each group. Each bar represented mean value with standard error of three replicates

The wheat seed inoculum size and the spore concentration of *S. mycarofaciens* SS-2-243 had significant ($p < 0.05$) effects on growth, conidia germination, and sporulation of both aflatoxin producing fungal strains growing on PDA or on maize seeds. The efficacy of the volatile compounds of this strain increased with concentration and size of the inoculum, and the effective dose was found to be at least 30 g L^{-1} inoculum size with spore concentration of 10^7 spore mL^{-1}. Spores are important for the survival and

Table 2 Contamination index (%) after different fumigation periods with 30 g L^{-1} of *S. mycarofaciens* SS-2-243 for controlling *A. parasiticus* TISTR 3276 (A), and *A. flavus* TISTR 3041 (B) on maize seeds

Treatments	Contamination index (%)					
	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
(A)						
Inoculated + fumigated for 6 h	0^b	0^b	0^b	29.6 ± 4.3^b	53.3 ± 4.2^b	100^a/MS
Inoculated + fumigated for 12 h	0^b	0^b	0^b	0^c	26.6 ± 9.4^c	73.3 ± 9.4^b
Inoculated + fumigated for 24 h	0^b	0^b	0^b	0^c	0^d	0^c
Inoculated + non-fumigated	46.6 ± 9.3^a	80.0 ± 6.3^a	100^a/MS	100^a/MS	100^a/MS	100^a/MS
(B)						
Inoculated + fumigated for 6 h	0^b	0^b	0^b	26.6 ± 9.4^b	53.3 ± 4.3^b	80.0 ± 0^b
Inoculated + fumigated for 12 h	0^b	0^b	0^b	0^c	25.6 ± 2.1^c	50.3 ± 9.3^c
Inoculated + fumigated for 24 h	0^b	0^b	0^b	0^c	0^d	0^d
Inoculated + non-fumigated	33.3 ± 8.4^a	80.0 ± 16.3^a	93.3 ± 9.4^a	100^a/MS	100^a/MS	100^a/MS

Values shown are mean ($n = 3$) ± standard error. Seed contamination index (SCI) was calculated as %SCI = [(Number of contaminated seeds / Total number seeds) × 100]. MS, Maize seeds completely spoiled

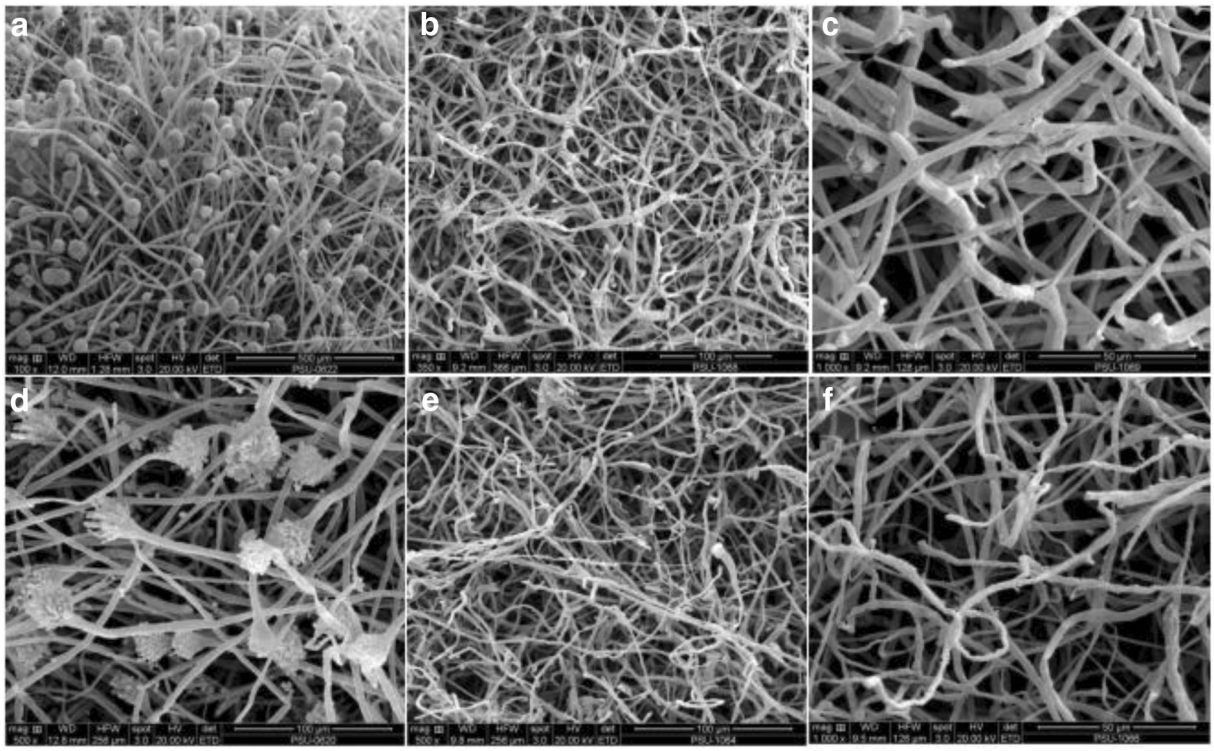

Fig. 4 Scanning electron micrographs (SEM) of normal hypha, conidiophores, and spores of and *A. parasiticus* TISTR 3276 (**a**) and *A. flavus* TISTR 3041 (**d**) without exposure to the volatiles SS-2-243 (the control) after incubation at 28 ± 2 °C for five days. Mycelia treated with the volatiles SS-2-243 of *A. parasiticus* TISTR 3276 (**b** and **c**) and of *A. flavus* TISTR 3041 (E and F) after incubation at 28 ± 2 °C for five days

spreading of aflatoxin producing fungi (Rabea et al. 2003). A significant ($p < 0.05$) decrease in their spore germination was observed for *A. parasiticus* TISTR 3276 and *A. flavus* TISTR 3041 with time of exposure to the volatiles. The kinetic study revealed that 1 h exposure to volatiles from 30 g L^{-1} wheat seed culture of *S. mycarofaciens* SS-2-243 could completely kill (100%) the conidia of *A. parasiticus* TISTR 3276, while the same effect occurred at 3 h for *A. flavus* TISTR 3041.

Although in vitro testing of the volatiles SS-2-243 was an important first step in checking their antifungal potential, in vivo testing is needed to confirm these results. In a maize seed assay, the volatile compounds exhibited 100% protection of maize seeds against contamination by the two aflatoxin producing fungi, with the effective wheat seed inoculum size (30 g L^{-1}) and spore concentration (10^7 spore mL^{-1}). This demonstrated the biofumigation effect. In this study, the wheat seed inoculum size differed from other biocontrol reports that have studied volatiles from *Streptomyces* spp. For example, fumigation with 15 g L^{-1} of wheat seed culture of *S. philanthi* RM-1-138 completely inhibited *Rhizoctonia solani* in in vitro and in vivo experiments (Boukaew et al. 2013). The effective inoculum size against *Botrytis cinerea* was a wheat seed culture with 120 g L^{-1} of *S. globisporus* JK-1 (Li et al. 2012), while biofumigation with 240 g L^{-1} of this culture completely inhibited *Penicillium italicum* (Li et al. 2010). *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea* could also be effectively inhibited by fumigation with 300 and 75 g L^{-1} of the wheat seed culture of *S. platensis* F-1 (Wan et al. 2008).

Furthermore, increasing the fumigation period, or exposure to the volatile compounds, from 6 to 24 h significantly ($p < 0.05$) reduced the fraction of infected maize seeds. Therefore, fumigation with 30 g L^{-1} wheat seed inoculum for at least 24 h provided complete control of maize seed infection by both *A. parasiticus* TISTR 3276 and *A. flavus* TISTR during the 12 days tested. Based on this

result, maize seeds must be fumigated with the volatiles SS-2-243 to protect against infection by the aflatoxin producing fungi before seed storage. In general, some compounds produced by micro-organisms (Qiming et al. 2006; Boukaew et al. 2011) or plant essential oils (Kordali et al. 2009; Young and Bush 2009; Boukaew et al. 2017b) have been reported to inhibit seed germination. In this study, *S. mycarofaciens* SS-2-243 volatile compounds had no significant ($p < 0.05$) effect on maize seed germination when compared to the control.

Results from both in vitro and in vivo observations showed that the volatiles SS-2-243 completely inhibited (by 100%) mycelial growth, conidia germination, and sporulation of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276 at 30 g L^{-1} of wheat seed inoculum. The SEM results confirmed that the suppression activity of the volatiles SS-2-243 on the two aflatoxin producing fungi incurred alterations in mycelial morphology, led to a gradual destruction of mycelia, and completely inhibited conidia germination. This confirms the potent inhibitory potential of these pathogenic fungi. Similar damage is induced by the volatile substances of *S. globisporus* JK-1 against *P. italicum* (Li et al. 2010) and *B. cinerea* (Li et al. 2012). Therefore, the main action of the volatiles SS-2-243 was on the conidia germination of *A. flavus* TISTR 3041 and *A. parasiticus* TISTR 3276.

Conclusion

Use of volatile compounds has been emerging as a possible alternative for the control of aflatoxin producing fungi. *Streptomyces mycarofaciens* SS-2-243 could be used as a biofumigant to protect maize seeds against the two aflatoxin producing fungi. The strain produced volatile compounds that have good potential as a biofumigant for controlling aflatoxin producing fungi, without any adverse effect on maize germination.

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Compliance with ethical standards

Conflict of interest The authors declare having no conflict of interest.

Human and animal studies This research did not involve human and/or animal participants.

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