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Highlights

- Fungal VOCs were evaluated for the control of an insect and a fungus.
- 1-octen-3-ol, the most active fumigant, followed by 3-octanol and 3-octanone.
- The fungal VOCs also showed repellent activity against *Sitophilus zeamais.*
- 1-octen-3-ol inhibited *Fusarium verticillioides* growth and FB_1 production.
- Fungal VOCs as potential biopesticides, could improve food safety of stored grains.

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Effect of fungal volatile organic compounds on a fungus and an insect that damage stored maize

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ABSTRACT

The silo is an environment where a large number of biological interactions take place such as: insect-microorganism-grain interactions, which can generate great economic losses due to the deterioration in quality of the grain and the presence of mycotoxins. In recent years, particular interest has been focused on the search for environmentally friendly insecticides that will provide pest control in stored grains. The volatile organic compounds (VOCs), of a fungal origin, were evaluated for the control of maize grain pests: the insect *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), the fungus *Fusarium verticillioides* and its mycotoxin, fumonisin B_1 (FB_1). The most active fumigant compound tested was 1-octen-3-ol (LD_{50} = 27.7 μL/L air), followed by 3-octanol and 3-octanone (LD_{50} = 43.2 and 219.7 μL/L air, respectively). The fungal VOCs also showed repellent activity against *S. zeamais*, with antifugal activity against *F. verticillioides* growth being inhibited at concentrations greater than 0.53 mM, while its mycotoxin production capacity was inhibited depending on the compound concentration. At the repellent concentration, the fungal VOCs showed low phytotoxicity activities. The results presented in this paper demonstrate the potential of fungal VOCs as biopesticides, because they may control granivorous insects, fungal growth and FB_1 production, which consequently is of economic importance and might improve food safety of stored grains.

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1. Introduction

The environment of a grain silo includes numerous interactions, such as grain-fungal, grain-insect and insect-fungal (Cox and Collins, 2001; Cox, 2004), which can generate great economic losses due to deterioration in quality of the grain and the presence of mycotoxins. Among the main pests found in stored maize grains in Argentina are the insect *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) and the filamentous fungus *Fusarium verticillioides* (Sacc.) Nirenberg (=*Fusarium moniliforme* Sheldon teleomorph *G. Fujikuroi* (Sawada) Ito in Ito & Kimura) (Bartosik, 2014; Chulze et al., 2000).

F. verticillioides is the main cause of maize ear rot in Argentina (Chulze et al., 2000) and is the major producer of the mycotoxin called fumonisin. This mycotoxin represents a major problem due to its toxicological implications in humans and farm animals (Theumer et al., 2010 and references therein). The fumonisins are subdivided into the groups FA_1, FA_2, FB_1, FB_2 and FB_3 (Abodo-Becognee et al., 1998; Zhang et al., 2013), with those of group B being the ones of most importance owing to their toxicity and the frequency that they appear in nature, especially fumonisin B_1 (FB_1) (Rheeder et al., 2002). Maize infection by *F. verticillioides* occurs mainly via grain stigmata or grain wounds, which at all stages of its development causes maize ear rot in the pre- and post-harvest stages (Munkvold and Desjardins, 1997; Martinez et al., 2010). However, the highest production of fumonisin takes place during grain storage, when the temperature, humidity and the presence of insect vectors such as *S. zeamais* favor fungal growth and secondary metabolite production (Chulze, 2010).

Sitophilus zeamais is an insect that causes extensive damage among stored grains, particularly maize (Trematerra et al., 2013). Although the coats of the grains form a defensive barrier against microbial action, the damaging action of *S. zeamais* on the maize grain coats can lead to fungal infection and subsequent mycotoxin production. The systematic and intensive use of synthetic

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insecticides, particularly when used recommended treatment concentrations, has caused the development of a resistent insect population (Boyer et al., 2012; references therein; Corrêa et al., 2011; Guerra Pimentel et al., 2008). Therefore, in recent years, increasing interest has been focused on the search for new insecticides and/or environmentally friendly strategies that can provide effective pest control in the stored grains (Abebe et al., 2009; Hardin et al., 2010). This has been the starting point of many studies that have used natural products such as essential oils as potential pesticides (Zunino et al., 2012).

It has been proposed that the biological interactions generated during the storage of grains, and among grains, insects and fungi, are mediated by volatile organic compounds (VOCs) (Germinara et al., 2008; Trematerra et al., 2013 and references therein). These studies have revealed the importance for an organism to be able to recognize the chemical signals from the environment that surrounds it, because an incorrect identification could result in poor nutrition, in its intoxication, or it being the target of a predator. Certain VOCs (oxylipins) are originated mainly from the oxidation of unsaturated fatty acids by the lipoxygenase (LOX) enzyme. Related to this, the plant LOX was reported to oxidize linoleic acid to yield 13-hydroperoxide or 9-hydroperoxide and their related hexanal and (3Z)-nonenal, compounds, respectively (Combet et al., 2006). Mushrooms and fungi can also generate VOCs by fungal LOX activity (10-LOX), which catalyzes the stereospecific oxidation of linoleic fatty acid to (8E, 12Z, 10S)-hydroperoxy-8,12-octadecadienoic acid and is finally decomposed to (8E)-10-oxo-8-decenoic acid and 1-octen-3-ol, and their derivatives, 3-octanol, 1-octen-3-one and 3-octanone (Buśko et al., 2010; Combet et al., 2006; Husson et al., 2001).

The extensive biological activity against different organisms demonstrated by the fungal VOCs has been used to try to explain the biological interactions that occur inside the silo. It has been reported that 1-octen-3-ol compound inhibits the growth of *Botrytis cinerea* Pers.:Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel], *Fusarium oxysporum* Schlechtend.:Fr. (Zhao et al., 2011) and *Aspergillus flavus Link* (Cleveland et al., 2009), as well as the conidia germination of *A. flavus* and *Penicillium* species (Chitarra et al., 2005; Cleveland et al., 2009). Although, the compounds 1-octen-3-ol and octanol have no apparent effect on mycotoxin synthesis (Cleveland et al., 2009), 1-octen-3-ol has shown insecticidal activity against *Tribolium castaneum* (Zhao et al., 2011), a pest in the food industry, and has also demonstrated attractant activity on several flies and beetles (Faldt et al., 1999). This broad spectrum of activities demonstrates the potential of these compounds for use in the preservation of foods, such as in stored grains in silos. In the present work, we have selected for our experiments the compounds 1-octen-3-ol, 3-octanol and 3-octanone, as they represent over 70% of the fungal VOCs produced by *Aspergillus, Penicillium, Fusarium* and *Alternaria* species (Combet et al., 2006; Jelén and Wasowicz, 1998).

One potential function of fungal VOCs in the interactions between fungus, insect and grain that occur in stored grains is discussed in this manuscript. Another objective of this investigation was to determine the insecticidal, repellent and acetylcholinesterase (AChE) activities of these VOCs against *S. zeamais,* as well as the inhibition of growth and fumonisin production by *F. verticillioides*, in order to establish a basis from new biopesticides.

2. Materials and methods

2.1. Organisms

Sitophilus zeamais were reared on sterilized whole maize grain in sealed containers. Insect rearing was carried out under controlled temperature and humidity (28 °C and 60–70%) and a light/dark regime of 12:12 (FAO, 1974). Adults of a strain of *S. zeamais* were obtained from Metán, Salta province, Argentina. The colony was maintained in our laboratory for one year without exposure to insecticides before testing. The unsexed adult weevils used in all the experiments were approximately 2 weeks old. All experiments were conducted under complete darkness in a controlled environment chamber (28 °C and 60–70% r.h.).

The fungal strain *F. verticillioides* M3125, provided by Dr. Robert Proctor, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL, United States, was used for all experiments. It was isolated from maize in California and is a fumonisin-producing strain (Leslie et al., 1992).

As inoculum in the antifungal test, a conidia suspension (1×10^6 conidia/ml) was prepared adding sterile distilled water to a culture of *F. verticillioides* M 3125 grown in Czapek-dox agar Petri plates for 7 days at 28 °C in the dark.

2.2. Chemicals

The VOCs selected for use in the current work were: 1-octen-3-ol (98%) (Cat. N° 05284, Aldrich); 3-Octanone (98%) (Cat. N° 136913, Aldrich) and 3-octanol (98%) (Cat. N° W358118, Aldrich). The dichlorvos (DDVP, technical grade, >98% purity) was purchased from Chemotécnica S.A. (Buenos Aires, Argentina).

2.3. Fumigation toxicity assay

The insecticidal activity against *S. zeamais* was evaluated using fumigant toxicity assay described by Huang et al. (2000), with some modifications. Briefly, different amounts of pure VOCs at doses corresponding to 20–600 μL/L air were placed onto Whatman filter paper disks of 2 cm diameter. Only the lowest concentrations were diluted in n-hexane, and in these cases each filter paper disk was air dried for 2 min and placed on the underside of the screw cap of a glass vial (30 mL). To avoid direct contact of the weevils with VOCs, a nylon gauze piece was fitted 1 cm under the screw cap of each glass vial. Ten adult *S. zeamais* were placed into each vial, with the experiment being repeated five times/dose. Control treatments were performed under the same conditions with dichlorvos compound 0.06–20 μL/L air (positive control), or without pure compounds (negative control). Dichlorvos was used as a positive control due to their high vapor pressure and their known insecticide activity. Insect mortality was checked after 24 h, and the mortality percentages and LD_{50} values were calculated.

2.4. Anti-acetylcholinesterase test

Untreated *S. zeamais* adults (0.5 g) were separately homogenized in 5 ml of 0.1 M ice-cold phosphate buffer (pH 7.4) using a Teflon glass tissue homogenizer. The homogenates were centrifuged (5000 rpm for 20 min at 0 °C), and the supernatants used as the enzyme source for determination of AChE activity. Inhibition of AChE was determined by the colorimetric method of Ellman et al. (1961) using acetylthiocholine iodide (ATChI) at 0.25 mM (Sigma Aldrich Co., St. Louis, MO USA) as the substrate. Enzyme aliquots (100 μL) and 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB) (100 μL of 0.01 M) were added to 0.1 M phosphate buffer (pH 7,4; 600 μL), and volatile compound test solutions (100 μL) prepared in absolute ethanol were added to this mixture. Control treatments were prepared by the addition of absolute ethanol (100 μL) instead of a volatile compound. These mixtures were incubated at 35 °C for 15 min, and the reactions were started by adding ATChI (100 μL). Absorbance was measured at 412 nm using a UV/VIS Spectrometer

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(Lambda 25, Perkin Elmer). Tested compounds were examined at the two concentrations of 1 mM and 5 mM, with each test and control being corrected by blanks for nonenzymic hydrolysis. All the experiments were performed three times for each dose. The inhibition percentage of AChE activity was calculated as follows:

AChE inhibition % = (ODC − ODT/ODC) × 100, where ODC is the optical density of control and ODT is the optical density of the treatment.

2.5. Two-choice olfactometer bioassay

The behavioral response of *S. zeamais* adults to individual VOCs was measured by using a two-choice olfactometer bioassay similar to that described by Dal Bello and Padín (2006) with some modifications. Briefly, two flasks (250 mL) were connected with a glass tube of 30 × 1 cm of diameter. In the middle (15 cm from the two flasks), a small hole was made of 1 × 1 cm. The connections between the two flasks and the tube were sealed with rubber plugs, which were covered with parafilm to prevent gas leakage (shown in supplementary material, S1). A filter paper of 2 cm diameter was placed within each flask where the compounds were added. Twenty insects, deprived of food for at least 4 h, were placed in the hole of the glass tube. These were then released and tested for 2 h in a climatic chamber, the experiments being carried out between 10:00 A M. and 4:00 P M. The position of the flasks was changed at every replication, and insects that did not show any response in the experiment were not taken into account. Preliminary experiments were conducted, which confirmed that the movement of the beetles towards either flask was random, by introducing the beetles into the hole of the glass tube without any test compounds. It was observed that nearly an equal number of insects moved to each flask when 20 insects were introduced into the hole of the glass tube (six times).

Insects were given a choice between a specific dose of the test compound and the solvent (n-hexane) used as a control. The experiments were performed five times for each assay, with insects only being used once.

In each trial, a response index (RI) was calculated by using the equation RI = [(T − C)/Tot] × 100, where T is the number responding to the treatment, C is the number responding to the control, and Tot is the total number of insects released (Phillips et al., 1993) Positive values of RI indicate attraction to the treatment, while negative ones indicate repellence.

2.6. Effect of volatile compounds on fungal growth and fumonisin production

The antifungal activity of the VOCs was tested using a radial growth of the fungal colony following a methodology proposed by Neri et al. (2007). Briefly, a paper filter was placed on the inside cover of the maize meal extract agar (3%) Petri dish. The VOCs were added separately to 90-mm paper filter as pure liquid compounds, and the concentrations (0.03; 0.06; 0.13; 0.27; 0.53; 1.06; 2.12 and 4.24 mM) were expressed as 10^{-3} mol on filter paper per dish volume. A paper filter without VOCs was used as control. Then, 10 μL of a conidial suspension (1 × 10^6/mL) of *F. verticillioides* M3125 was added aseptically to the centre of each Petri dish. The Maize Meal Extract Agar (3%) petri dishes were then covered, wrapped in parafilm and incubated in the dark at 27 °C. The colony diameter of *F. verticillioides* was measured after 7 days of incubation, and the colony area calculated using the formula for the area of a circle (π *r^2). Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the VOCs compounds at which no fungal growth was observed. To study the effects of the VOCs on FB_1 production, the inoculated plates were incubated in the dark at 27 °C for 28 days. After this incubation, the parafilm and filter papers were removed and agar in the experimental plates were dried for 96 h at 60 °C in a forced-air oven before being ground to a fine dry powder. Finally, 5 mL of water was added to the dried agar from each disk, and FB_1 was extracted by shaking the dried dishes with water for 120 min on an orbital shaker, with the mixture then being centrifuged at 5000 rpm for 15 min. The experiments were performed twice in triplicate.

2.7. Fumonisin B_1 quantification

Samples (1000 μL) from the FB_1 extracts were diluted with acetonitrile (1:1), and quantification of the samples was performed following a methodology proposed by Shephard et al. (2000). Briefly, an aliquot (50 μL) was derivatized during 3.5 min with 200 μL of a solution, prepared by adding 5 ml of 0.1 M sodium tetraborate and 50 μL of 2-mercaptoethanol to 1 mL of methanol containing 40 mg of o-phthaldialdehyde. The derivatized samples were then analyzed by means of a Perkin Elmer HPLC equipped with a fluorescence detector, with the wavelengths used for excitation and emission being 335 nm and 440 nm, respectively. An analytical reverse phase column C_{18} (150 mm × 4.6 mm internal diameter and 5 μm particle size) connected to a precolumn C18 (20 mm × 4.6 mm and 5 μm particle size) was also used. The mobile phase was methanol and NaH_2PO_4 0.1 M (75:25), with the pH being set at 3.35 ± 0.2 with orthophosphoric acid and a flow rate of 1.5 mL/min used. The quantification of FB_1 was carried out by comparing the peak areas obtained from samples with those corresponding to FB_1 analytical standards (PROMEC, Program on Mycotoxins and Experimental Carcinogenesis, Tygerberg, Republic of South Africa).

2.8. Seed germination bioassay

The maize seeds were sterilized with 2% sodium hypochlorite for 5 min, before being rinsed with abundant distilled water. Two filter papers were placed on the bottom of each Petri dish (8 cm diameter) and 10 seeds of maize were placed on the filter papers. Then, 5 mL of distilled water were added to each Petri dish and aluminum foil of 2 cm diameter was placed in the centre of the Petri dish, with the corresponding compound being spotted onto the foil. The four concentrations used (volatile source) were: MIC of *Fusarium verticilloides*, LD_{50}, the repellence dose of *S. zeamais* (4 μL/L air) and none (control). Concentrations in the airspace within the Petri dish were calculated assuming that the spotted compounds volatilized completely, without any loss due to adsorption or leakage. The Petri dishes were closed and sealed with adhesive tape to prevent the VOCs from escaping, and then placed in a growth chamber maintained at 27 ± 2 °C temperature and a relative humidity of 60 ± 2% for 6 days. The assays were performed in a completely randomized design, with five replications including controls being used. Germination counts were made daily during the first three days, with germination considered to have occurred when the radical protruded 2 mm. After 6 days, the number of germinated seeds was also counted, and the rate of germination (seed vigour) was calculated by using the equation: $\sum$ (n d^{-1}), where n = number of seeds germinated on each day, and d = number of days from the beginning of the test (Agrawal, 1980).

2.9. Data analysis

Statistical analyses were conducted using InfoStat/Professional 2010p. (Di Rienzo et al., 2010) at P = 0.05.

In the antifungal and antifumonisin assays, the data were analyzed by a one-way analysis of variance (ANOVA), and the

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normality of the data was tested using the Shapiro–Wilk test. Comparisons between the control and treatment data sets were carried out using by the DGC test (Di Rienzo et al., 2002), with results giving P values < 0.05 being considered significant.

In the fumigation toxicity assay, the mortality percentages and LD_{50} values were calculated according to Finney (1971). The LD_{50} values were subjected to probit regression analysis using POLO-PLUS. (LeOra Software 2002–2014).

The significance of the mean RI in each treatment of the two-choice olfactometer bioassay was evaluated by the Student's t-test for paired comparisons (Phillips et al., 1993). The most significant positive or negative mean values of RI were first analyzed by an analysis of variance, and subsequently ranked by using the Duncan multiple range test ($P \leq 0.05$).

3. Results

The three VOCs (1-octen-3-ol, 3-octanol and 3-octanone) were evaluated as insecticides, repellents, antifungals, anti-mycotoxigenics and phytotoxics. The fumigant toxicity of the VOCs was evaluated against *S. zeamais* (Table 1), and the most active fumigant compound was 1-octen-3-ol with a LD_{50} value of 27.7 μL/L air, followed by 3-octanol and 3-octanone with LD_{50} values of 43.2 μL/L and 219.7 μL/L air, respectively.

The AChE inhibition of the most active compounds (1-octen-3-ol and 3-octanol) was evaluated for two different concentrations, 1 mM and 5 mM, with the results revealing that only the 3-octanol compound showed a higher AChE inhibition with the highest concentration tested. At the 1 mM concentration, 1-octen-3-ol and 3-octanol caused AChE inhibitions of 50% and 3%, respectively. In contrast, at 5 mM the most active inhibitor was 3-octanol with 40% inhibition, while 1-octen-3-ol only caused an inhibition of 15%. AChE inhibitory activity using 3-octanone was not performed due to its low insecticidal activity LD_{50} of 219.7 μL/L air. At the tested concentrations (0.2, 0.4 and 4 μL/L), the three VOCs caused repellent effects on *S. zeamais* (Table 2), which were dose dependent.

The effects of the VOCs on the radial growth of the *F. verticillioides* are shown in Fig. 1 and Table 1, where it can be observed that the extent of inhibition was strongly dependent upon the nature and concentration of the compounds. At high concentrations (greater than 0.53 mM), the radial growth of the *F. verticillioides* colony was reduced by all the evaluated fungal VOCs, with 1-octen-3-ol being the most active inhibitor with a MIC value of 0.53 mM. In contrast, at very low concentrations (smaller than 0.13 mM), 1-octen-3-ol and 1-octanone stimulated the fungal growth, whereas 3-octanol compound did not produce any fungal growth stimulation at any of the concentrations used.

The three evaluated VOCs caused inhibition of FB_1 production by *F. verticillioides* (Fig. 1), with 1-octen-3-ol being the most active inhibitor with FB_1 inhibition values higher than 74%, at all the evaluated concentrations (except at 0.03 mM), and 3-octanol causing FB_1 inhibition at 0.06, 0.27 and 0.53 mM. The total inhibition of mycotoxin production resulting from 1-octen-3-ol and 3-octanol (1.06 mM – 4.24 mM and 2.12 mM – 4.24 mM, respectively) was due to the total inhibition of fungal growth resulting from these compounds. Although 3-octanone caused an inhibition of FB_1 production at 0.13, 1.06 and 2.12 mM, this compound produced FB_1 stimulation evaluated concentrations of 0.03, 0.06 mM and 4.24 mM, respectively.

Finally, the effect of VOCs on the germination of maize kernels was evaluated, using the reference concentrations obtained for each bioassay (repellent dose = 4 μL/L air; insecticide = LD_{50} values and fungicide = MIC values). At the concentration corresponding to antifungal MICs, the three fungal VOCs inhibited grain germination, although these compounds exerted a slight stimulatory effect on maize growth when the repellent concentration was used. In addition, at the LD_{50} insecticide concentration, the 1-octen-3-ol and 3-octanone compounds inhibited 50% of the germination capacity of the grains (Table 3).

Table 2
Response of adult *Sitophilus zeamais* to increasing doses of volatile compounds in a two-choice olfactometer bioassay.

Compounds	Response Index (RI)[a] (Mean ± SE)		
	0.2 (μL/L air)	0.4 (μL/L air)	4 (μL/L air)
1-octen-3-ol	−46.2 ± 13.1*b	−32.8 ± 11.9*b	−84.3 ± 5.5***a
3-octanol	−42.4 ± 12.4*b	−57.8 ± 14.9*b	−78.7 ± 3.5***a
3-octanone	−48.5 ± 8.9**b	−55.1 ± 12.3*b	−91.2 ± 2.2***a

*$P \leq 0.05$; **$P < 0.01$; ***$P < 0.001$ (significant response to experimental stimulus; paired-sample t-test).
[a] RI was calculated by using the equation RI = [(T − C)/Tot] × 100, where T is the number of insects responding to the treatment, C is the number of insects responding to the control, and Tot is the total number of insects released.

4. Discussion

The 1-octen-3-ol, 3-octanol and 3-octanone compounds are originated by the fungal LOX that catalyzes the stereoespecific oxidation of fatty acid (Buśko et al., 2010; Combet et al., 2006; Husson et al., 2001). In recent years, the VOC (R)-1-octen-3-ol has been described to act as an aggregation kairomone for different beetle species (Mozga et al., 2009), and may facilitate fungal spore dispersion of *Aspergillus* and *Penicillium* species (Combet et al., 2006; Ferreira-Castro et al., 2012; Nesci et al., 2011). This is in agreement with McFarlane et al. (2009), who suggested that infected maize grains with *F. verticilloides* may increase insect attack, because the fungal colonized tissues offer an environment in which complex carbohydrates and other cellular tissues are partially broken. Moreover, a previous hypothesis has suggested that beetles might use spores of fungi being the basis of their alimentation (Niewiada et al., 2005). Nevertheless, these reports

Table 1
Effect of volatile organic compounds against *Sitophilus zeamais* (LD values and AChE inhibition) and *Fusarium verticilioides* (MIC values).

Compounds	LD_{50} (μL/L air)	95% Confidence interval (μL/L air)	LD_{95} (μL/L air)	95% Confidence interval (μL/L air)	Slope ± S.E.	(X^2)[c]	MIC		AChE Inhibition[a]	
							(μL/L air)	(mM)	1.0 (mM)	5.0 (mM)
1-octen-3-ol	27.7	25.10–31.0	42.4	37.4–52.2	5.829 ± 0.884	0.345	81.5	0.53	50%	15%
3-octanol	43.2	39.7–46.6	60.2	55.5–67.8	8.605 ± 1.022	0.966	337.1	0.53	3%	40%
3-octanone[b]	219.7	171.61–291.8	378.2	301.6–649.7	1.970 ± 0.337	51.471	662.9	4.24	ND	ND
Dichlorvos	<0.06									

ND: Not determined.
[a] All the experiments were performed in triplicate. Inhibition percentage of AChE activity was calculated as follows: AChE inhibition % = (ODC–ODT/ODC) x 100, where ODC is the optical density of control and ODT is the optical density of treatment.
[b] The AChE inhibitory activity by 3-octanone was not performed due to of its low insecticidal activity.
[c] X^2: chi-square value, significant at $P < 0.05$ level.

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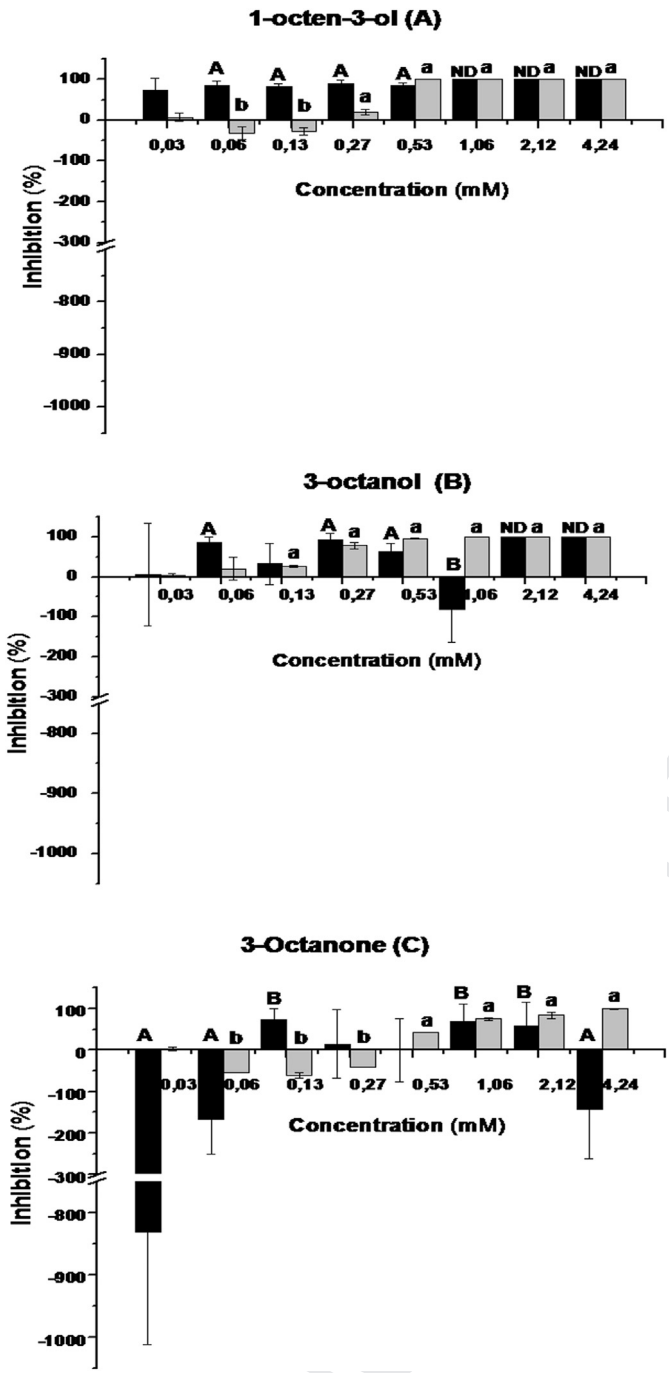

Fig. 1. Effects of 1-octen-3-ol (A), 3-octanol (B) and 3-octanone (C) on FB_1 production (■) and *Fusarium vertillioides* growth (□) in maize meal extract agar (3%) at 28 °C. Bars with different letters are statistically different from each other according to the DGC multiple range test at $P < 0.05$ ($n = 5$). Capital letter indicates significant difference in FB_1 production and lowercase significant difference in *F. verticillioides* growth. ND: Not determined. FB_1 inhibition was not determined due to there being no fungal growth. Variables of FB_1 total content (μg/plate) were transformed to square roots in order to comply with the assumptions of normality. For illustrative purposes, the results are represented in the graph as percentage of inhibition over control.

are not in agreement with the results presented in the present work, which showed repellent and insecticide effects of the VOCs on *S. zeamais*, even at concentrations as low as 0.2 μL/L air. In fact, the insecticidal effect of 1-octen-3-ol against *T. castaneun* has also been previously described by Zhao et al. (2011), in agreement with Mozga et al. (2009), who suggested that the presence of 3-octanol and 1-octen-3-ol in the faeces of *Sitophilus* species could indicate an insect overpopulation, and hence a decrease of nutrients. To our knowledge, this study is the first one dealing with the insecticidal activity and repellent effects of VOCs (1-octen-3-ol, 3-octanol and 3-octanone) against *S. zeamais.* The discrepancies found between our data and previous reports may be explained by the differences in the behavioral responses among the different *Sitophilus* species (Germinara et al., 2008; Niewiada et al., 2005).

Table 3
Seed vigour (%) of maize seed treated with volatile compounds at different concentrations in comparison with controls[a].

Treatment concentrations	Seed vigour (%)[b]
1-octen-3-ol	
LD_{50}: 27.7 μL/L air	52.3 (10.8)*
MIC: 81.5 μL/L air	15.1 (4.9)*
Repellence: 4 μL/L air	111.1 (2.9)
3-octanol	
LD_{50}: 43.2 μL/L air	82.2 (6.9)
MIC: 337.1 μL/L air	0 (0)
Repellence: 4 μL/L air	108.2 (4.1)
3-octanone	
LD_{50}: 219.7 μL/L air	53.3 (6.5)*
MIC: 662.9 μL/L air	14.0 (4.3)*
Repellence: 4 μL/L air	118.6 (12.6)

*Mean significant differences with Control at the 5% level by Duncan's multiple-range test. Statistical analyses were carried out on arcsin-transformed data, and results were extrapolated to original data. In order not to induce homoscedasticity errors, only data with variability were included in the statistical analysis.

[a] Values are means ± *SE* (in parentheses) of five experiments of 10 seeds each.

[b] Seed vigour (rate of germination) was calculated by using the equation: $\sum (n \cdot d - 1)$, where n = number of seeds germinated on each day and d = number of days from the beginning of the test.

Although there are no previous reports about the antifungal activity of 1-octen-3-ol, 3-octanol and 3-octanone against *F. verticillioides,* the activity of the evaluated VOCs against other filamentous fungi has been previously reported. For example, results published by Cleveland et al. (2009), reported an antifungal effect of 1-octen-3-ol and 3-octanol (around 3.82 mM) on the development and conidia germination of *A. flavus*, with Zhao et al. (2011) also reporting the effect of 1-octen-3-ol (around 0.11–0.66 mM) on the mycelium morphology of *F. oxysporum* and *B. cinerea,* in agreement with the results presented in this paper.

The antifungal activity of 1-octen-3-ol and 3-octanone probably involves an increase in membrane permeability (Chitarra et al., 2005). Consequently, the antifungal activity of alcohols respect to ketone compounds may be explained by the lower vapor pressure, which may generate a higher concentration of the compound around the mycelium, thus providing a hostile environment to fungal growth. Moreover, the aqueous environment of the culture medium might provide a better diffusion of alcohols respect to ketones. These hypotheses are in agreement with Yuan et al. (2012), who reported a negative relationship between antifungal activity and the length of the hydrocarbon chain of the ketone compounds.

The results presented in this study revealed that the effects of VOCs on FB_1 production were dependent upon the concentration, which was inhibitory at the highest tested concentration, whereas the 3-octanone compound stimulated FB_1 production at the lower concentrations. Importantly, the 1-octen-3-ol compound caused inhibition of FB_1 synthesis even at the lowest concentrations that were evaluated, which represents a strategically noteworthy result, because combined with its high repellent and insecticide effect on *S. zeamais*, this therefore reveals its potential to be used for the integrated control of pests in stored grains. Although, a slight effect on the inhibition of aflatoxin synthesis by VOCs has

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been previously reported, there are no precedents about the synthesis of FB_1. However, the stimulation of FB_1 production caused by 3-octanone VOCs at low concentrations is in agreement with Menniti et al. (2010) and Wright et al. (2000), who reported the inductive effect of other carboxylic compounds (trans-2-hexenal and octanal) on mycotoxin production. Although to our knowledge there are no references that explain the inductive activity of ketones on FB_1 production (which we did not find for the other compounds) this inductive effect might be explained as a survival response of the fungus to a stress situation (Reynoso et al., 2002 and references therein), and also as a protective effect of the fungus against the potential presence of insects. Related to this, some mycotoxins such as aflatoxin and OTA have insecticidal effects (Srivastava et al., 2009), although there is no background information on the insecticidal activity of FB_1 (Niu et al., 2009). The presence of seed-eating insects in grain storage system can be beneficial for the development of the fungus, because these might allow the dispersal of fungal conidias and the degradation of the grain coat, which represents the first line of antifungal defense (Ferreira-Castro et al., 2012; Nesci et al., 2011). On the other hand, the presence of seed-eating insects may be counterproductive since these may feed on fungal hyphae. These dual effects (inhibitory and stimulatory on fungal development and synthesis of FB_1) were dependent on the concentration of the VOCs, suggesting that the fungus can use these compounds and synthesize these mycotoxins effectively to control, according to their needs, their interactions with insects, grains and other microorganisms. While the exact concentration of these compounds reaching the targets was not measured, the results indicated that the bioactivity was dependent on the VOC initial concentration, hence these compounds may be effective in protecting maize from fungal and insect damage.

At the repellent concentration, none of the VOCs used in this paper demonstrated any effects on maize grain germination. However, an inhibitory effect on this was noted when the VOCs were evaluated at the antifungal concentration. Related to this, the inhibitory capacity of 1-octen-3-ol on seed germination of *Amaranthus retroflexus*, *Echinochloa crusgalli* and *Chenopodium album* has been previously reported (Zhao et al., 2011).

5. Conclusion

During grain storage, biological interactions such as grain-microorganism, grain–insect, insect–microorganism and grain–-grain should be taken into account. The high insecticidal, antifungal and antifumonisin biosynthesis activities of VOCs, reported in the present paper, suggests that the fungi could have yielded and released these VOCs and so prevailed over their competitors, in a tritrophic system including insects, grains and fungi. From an applied standpoint, the results presented and discussed in this paper demonstrate the great potential of VOCs to be used as biopesticides, because they can control granivorous insects, fungal growth and FB_1 production, which is of strategic importance to maintaining the quality of stored grains.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jspr.2015.04.006

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