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Volatile organic compounds from the interaction between *Fusarium verticillioides* and maize kernels as a natural repellents of *Sitophilus zeamais*

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ABSTRACT

Maize kernels are exposed to *Sitophilus zeamais* attack and *Fusarium verticillioides* infestation during storage, which can result in product deterioration and economic losses. The objective of this study was to evaluate the involvement of the Volatile Organic Compounds (VOCs) emitted by the fungi-corn system in grain-insect interactions. Volatiles emitted by healthy maize kernels were different from those emitted by fungal infected kernels, with the latter being enriched by alcohols, ketones and sesquiterpenes, which were considered early indicators of fungal contamination. The results demonstrated that the kernels exposed to the fungal VOCs and their pure compounds (1-octen-3-ol and 3-octanol) were less attractive and less damaged by *S. zeamais* than controls. In addition to compound adsorption, other processes may have caused the protective effect of exposed kernels against insect damage. This is the first contribution of the role of the fungal volatiles on the behavior of *S. zeamais*, and could provide an important contribution to the conservation of stored grains and pest management and an early indicator of fungal contamination.

1. Introduction

Grains are stored in bulk before their commercialization or consumption. In this environment biological interactions occur, such as grain-microorganism, grain-insect, insect-microorganism and/or grain-grain (Cox, 2004), which often produce economic losses. Among the main pests that affect stored maize grains are fungi and insects.

The fungal pathogens *Fusarium verticillioides* (Sacc.) Nirenberg (=*F. moniliforme* Sheldon teleomorph *G. Fujikuroi* (Sawada) Ito in Ito & Kimura) are the main cause of ear rot of corn in Argentina (Chulze, 2010) and one of the major producers of fumonisins (FBs) which can cause health problems to humans and farm animals (Theumer et al., 2010). The principal FBs production can takes place during grain storage, when temperature and humidity conditions allow the synthesis of these fungal secondary metabolites. *Sitophilus zeamais* is a primary pest that affects stored grain and, the damage produced by this represents a gateway to fungal infection acting as a vector of the fungal spores (Ferreira-Castro et al., 2012).

During maize storage, the competition for substrate between fungi and insects may occur and both pests must develop diverse strategies to compete and persist in sulk stored grain. The volatile organic compounds (VOCs) emitted by living organisms are known to play a critical role in tritrophic interactions, acting as a signal to unstressed plants to adjust their defensive systems (Wenda-Piesik et al., 2010). For instance, oxylipins, synthetized by lipoxygenases (LOXs), and the sesquiterpenes have function being related to the defense against pests and pathogens (Engelberth, 2011; Ghirardo et al., 2012). Although the involvement of VOCs in insect-plant-microorganism interactions has been well-described (Wenke

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et al., 2010), the role of fungal VOCs in grain-insect-pathogen interactions is poorly understood. Our previous studies have demonstrated the antifungal and antimycotoxicogenic activities of some fungal VOCs (1-octen-3-ol, 3-octanol and 3-octanone) (Herrera et al., 2015) on *F. verticillioides,* and also their insecticide effects on *S. zeamais.* These results have suggested that *Fusarium* can release these fungal VOCs to prevail over competitors present in the storage environment. Thus, the objective of this study was to evaluate the involvement of VOCs emitted by a fungi-maize grain system on the behavior of *Sitophilus zeamais,* and their role in grain-insect interactions under storage conditions.

2. Materials and methods

2.1. Kernels, insects, fungal strain and inoculum preparation

Maize kernels were obtained from Experimental Station Manfredi (INTA, Córdoba, Argentina) and kept in closed containers at -4 °C and 70± 5% relative humidity (r.h.). *Sitophilus zeamais* adults, without differentiation of age or sex, were used on maize kernels in the bioassays, which were maintained under laboratory conditions (28 ± 2 °C and 70± 5% r.h.). The *Fusarium verticillioides* strain M3125, provided by Dr. Robert Proctor, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL, USA (Leslie and Summerel, 2006) was employed in all assays. Inoculum was obtained by growing it on Czapek-dox agar Petri plates for 7 days at 28 °C in the dark to allow profuse sporulation.

2.2. Maize inoculation

In order to determine the optimal experimental parameters for maize inoculations, preliminary evaluation were carried out using different days of exposure, grain weights and water contents, and varying the number and size of the mycelial discs. From these results, maize grains (25 g) were placed in a 250 ml Erlenmeyer flask and sterilized for 2 consecutive days in an autoclave for 15 min at 121 °C. Sterile distilled water (8 ml) was added to the autoclaved maize to obtain 35% humidity. Then, three 10-mm diameter mycelial discs of *F. verticillioides*, taken from the edge of the plate of a 7-day-old culture on Czapek-dox agar, were transferred aseptically to the conditioning corn. Flasks with inoculated maize were incubated for 7 days at 28 °C with manual periodic shaking to achieve a good homogenization, with flasks containing sterile corn without fungal inoculation being used as control. Four replications of each treatment were performed. These treatments were used as inductor of healthy grains.

2.3. Conditioning of kernels with VOCs from fungal infected kernels system

For the purpose of determining the capacity of the kernels to respond to VOCs emitted by *F. verticillioides* during infection, the conditioning of the kernels was carried out using a method described by Trematerra et al. (2013), with some modifications. Plastic containers (20 cm × 30 cm x 10 cm; 3 L) were divided by an interior wall into two compartments (A and B), connected by a free space between the dividing wall and the lid of the container. Inside each compartment, a glass plate (10 cm diameter x 3 cm high) was positioned. Then, on the plate in compartment A, 25 g of fungal infected kernels or a filter paper with 0.004 μl/L of 1-octen-3-ol (Sigma-Aldrich, ≥99%) and 3-octanol (Sigma-Aldrich, ≥98%), (minimum concentration of *S. zeamais* repellence previously determined) were placed, while on the plate from compartment B, 25 g of healthy whole maize kernels were placed to be exposed to the VOCs or pure compounds released from compartment A. For incubation, the plastic container was kept in a controlled room at 28± 2 °C with 70± 5% r.h. and continuous darkness for 6 days. As a control treatment, healthy whole maize kernels were deposited in both compartments (A and B) of another plastic container and maintained under the same conditions. The selection of the two fungal VOCs compounds was performed based on previous studies against *S. zeamais* in our laboratory (Herrera et al., 2015). In *in vitro* test, the 1-octen-3-ol and 3-octanol had greater insecticide activity and shown greater effect on seed germination considering that these compounds would affect the surrounding grains, even at very low concentration (Herrera et al., 2015). The 1-octen-3-ol was selected for the evaluation in this study because this is the first compound formed by fungal LOX activity (10-LOX) (Brash, 1999).

After the incubation period, the exposed maize kernels (compartment B) were used to study their effects on the dietary behavioral of *S. zeamais,* by testing the susceptibility of these kernels to insect attack and in the attraction-repellency tests (see sections 2.4 and 2.5). The kernels exposed to pure compounds were also used for volatile determination of these compounds (see section 2.6).

2.4. Repellent/attraction activity bioassay

To determine the effect of conditioned kernels on the behavior of *S.zeamais,* a Repellent/Attraction Activity bioassay was performed using a two-choice olfactometer, according to Herrera et al. (2015). This test allows the effect of conditioning related to the choice of *S. zeamais* between conditioned kernels and controls to be observed. Briefly, two flasks (250 ml) were connected by a glass tube of 30 cm × 1 cm diameter with a small hole (1 cm × 1 cm) in the middle (15 cm from the two flasks), with entry points between the flasks and the tube being sealed with rubber plugs, which were covered with parafilm to prevent gas leakage. Before connecting the flasks and the tube, maize samples were added to the flasks. Then, twenty insects, deprived of food for at least 24 h were placed in the hole of the glass tube, which were then released and tested for 2 h in a climatic chamber, with the experiments being carried out between 10:00 and 16:00 h and the response index (RI) calculated. The position of the flasks was changed at every replication. Insects were given a choice between the conditioned maize and control (treatments are listed in Table 2), and the experiments were performed five times for each assay, with each group of insect only being used once. For each trial, the RI was calculated by using the equation (1) RI = [(T-C)/Tot] x 100, where T is 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. (Phillips et al., 1993). Insects that did not show any response in the experiment were not taken into account. Positive values of RI indicate attraction to the treatment, while negative ones indicate repellence.

2.5. Susceptibility of kernels to insect attack. Determination of grain damage and weight

To determine the susceptibility of conditioned kernels to insect attack, the grain damage and loss weight was measured. This experiment was carried out as above using a two-choice olfactometer bioassay (see section 2.4), but for this test the experiment was run for 20 days, after which, kernels of both flasks were weighed on an analytical scale and the number of damaged kernels and dead insects and the RI were determined.

2.6. Volatile organic compound determination

To determine the composition of the VOCs emitted by the grain-fungi inductor system and its respective control (sterile maize grain), the methodology of Boué et al. (2005), with some modifications, was used. The VOCs were collected by SPME of the vials (100 ml) containing 25 g of grain (without breaking) were tightly capped, and placed in a water bath at 55 °C during 20 min. A SPME fiber (divinylbenzene/carboxen/PDMS; SUPELCO) was inserted into the headspace of the sample for 30 min, after which, it was desorbed at 250 °C in the injection module of the GC-MS. To identify the constituents in the VOC samples, electron impact mass spectra (EI-MS) were obtained using a gas chromatography-mass spectrometer, (GC-MS-FID Perkin Elmer 600) equipped with a capillary apolar column DB-5 (60 m × 0.25 mm i.d. and 0.25 μm coating thickness) and with a capillary polar column Elite-Wax (60 m × 0.25 mm i.d. and 0.50 μm coating thickness). The quantification of the compounds was performed by flame ionization detection (FID). The chromatography conditions employed were as follows: oven temperature profile of 40 °C for 2 min, ramped up to 200 °C at 10 °C min^{-1}, and then to 250 °C at 15 °C min^{-1}; injector temperature 250 °C; detector temperature, 250 °C. The injector was operated in splitless mode, with the carrier gas used being Helium at 45 cm s^{-1} and the ion source maintained at 70 eV. The volatile compounds were identified by comparing their retention index (determined on the basis of homologous n-alkane hydrocarbons (C8-C22)) and mass spectra with those of the standard compounds (n-hexane, 3-methyl-butanol, 3-octenone, 1-octen-3-ol, 3-octanol, limonene and 1.8-cineole) and the mass spectral databases of the Adams library, Wiley library and NIST 98 MS Library.

To determine the presence of the 1-octen-3-ol and 3-octanol fungal VOCs in the atmosphere of conditioned kernels, the residual concentrations was measured using the methodology described above. A calibration curve was then employed to define the VOC concentrations. This was made using vials of 100 ml with 25 g of kernels mixed with increasing concentrations of the both compounds.

2.7. Statistical analyses

The significance of the data in experiments of Repellent/Attraction Activity (see section 2.4) and Susceptibility of kernels to Insect Attack (see section 2.5) was evaluated using the *Student's t-test* for paired comparisons ($P \leq 0.05$). The statistical difference between the RI obtained was determined using an Analysis of variance (ANOVA) ($P \leq 0.05$). This analyzes were carried out with the Statistical software Infostat (2009). The assumptions of normality and homogeneity of variance were tested.

3. Results

3.1. Volatile organic compounds

The VOCs emitted by the inductor grain-fungi system were different from the control. The VOCs profiles of the inductor grain-fungi system and the control are shown in Table 1. The volatiles emitted by the control contained mainly the hydrocarbons n-hexane, 2-methylpentane and 3-methylpentane and the monoterpenes limonene and 1,8-cineole and an unknown hydrocarbon sesquiterpene. The compounds 1-octen-3-ol, 3-octanol and 3-octenone were also found in traces. However, the VOCs emitted by the inductor system were different, with alcohols and ketones being the main components, namely, 3-methyl-butanol, 3-octanol, 1-octen-3-one, 1-octen-3-ol/3-octenone.). In addition, this VOCs was enriched in hydrocarbon sesquiterpenes such as acora-3.7-(14)-diene, α-cedrene, β-funebrene and α-acoradiene, and the oxygenated sesquiterpene β-acorenol. In the profile of VOCs emmited by the inductor grain system two unknown compounds were also found. These unknown compounds showed a mass spectrum characteristic of sesquiterpenes (see Table 1) however, the retention index and the mass spectrum did not match with those mass spectrum of available libraries (Adams, Wiley library and NIST 98). The statistics analysis was not performed because the VOCs composition of the inductor system and the control had different compounds.

Table 1
Volatile organic compounds emitted by the grain-fungi inductor system and it respective control.

Percentage similarity with spectrum of NIST library	Co-injection standard	RI $_{Kovats}$		Compounds	Relative percentage (mean ± E.E.)	
		Elite-Wax	DB-5		Grain-fungi inductor system	Control
96.3				2-methylpentane		7.14 ± 4.23
97.2				3-methylpentane		7.64 ± 2.43
99.0	Co			n-hexane		68.61 ± 2.64
99.1	Co	1206	736	3-methyl-butanol	16.91 ± 5.27	
89.3		1301	978	1-octen-3-one	2.47 ± 0.90	Tr
98.3	Co	1254	987	3-octenone	59.31 ± 8.39	Tr
91.1	Co	1444	987	1-octen-3-ol	1.58 ± 0.18	Tr
97.9	Co	1391	1000.0	3-octanol	11.65 ± 1.18	Tr
97.6	Co	1198	1033	Limonene		2.29 ± 2.02
98.0	Co	1211	1037	1.8-cineole		3.34 ± 1.95
89.8		1379	1107	1-octen-3-yl acetate	0.31 ± 0.07	
90.5		2034	1301	phenol-4-ethyl-2-methoxy	0.75 ± 0.14	
91.1		1719	1331	4-ethyl-1,2-dimethoxybenzene	1.54 ± 0.14	
88.7		1638	1433	acora-3,7-(14)-diene	0.19 ± 0.04	
99.4		1578	1443	α-cedrene	0.53 ± 0.17	
85.8		1601	1454	β-funebrene	0.50 ± 0.14	
96.7		1684	1487	α-acoradiene	2.51 ± 0.78	
			1505	unknown 1*	0.48 ± 0.30	
			1515	unknown 2*	0.71 ± 0.28	6.80 ± 2.16
87.9		2163	1675	β-acorenol	0.77 ± 0.49	

Components are listed in order of elution in the DB-5 column. Percentages were calculated from the peak area without correction. R.I. Kovats: retention index relative to homologous alkanes. Tr: traces (<0.10%). %). (*) Main ions, Unknown 1; MS, 70 eV, m/z (rel. int.): 240 (M+, 5), 136 (100), 121 (92), 93 (43), 107 (32). Unknown 2; MS, 70 eV, m/z (rel. int.): 235 (M+, 5), 123 (20), 97 (100), 83 (25).

3.2. Repellent/attraction activity bioassay

The repellent/attraction activity of conditioned kernels on *S. zeamais* is shown in Table 2. In this bioassay, we investigated the effect of maize kernels exposed to the VOCs produced by *F. verticillioides* (experiment b) or by two pure compounds (experiment d and e) on the behavioral responses of the insect. In a first ascertained was determined that the movement of the beetles towards either flask was random ($P > 0.05$) (experiment f) For the treatments, all revealed a repellent effect, with experiment b showing the highest value of repellence (71.4 ± 6.6%) because in this experiment the treated kernels were directly infected with the fungus, which was more repellent than the conditioned kernels. Experiment a revealed 56.1 ± 8.3% repellence, with experiment c showing 49.4 ± 6.2%. However, between experiments a and c there were no statistical differences, with the same being true for experiments with pure compounds (d and e). Finally, maize kernels induced by the two pure compounds presented lower values of repellence than those exposed to all the VOCs of *F. verticillioides* (d and e).

3.3. Susceptibility of kernels to insect attack

The effect of the conditioned kernels on the susceptibility to insect attack is shown in Table 3. In there, were no statistically significant differences between the RI of controls and treatments at the end of 20 days. However, kernel damage was lower in those exposed to the two pure compounds and *F. verticillioides* VOCs compared with their respective controls. Similar results were found with respect to weight loss in the maize kernel, with the exception of maize kernels exposed to 3-octanol.

3.4. Residual concentrations of the 1-octen-3-ol and 3-octanol fungal VOCs

To discard the possible repellent effect of the 1-octen-3-ol and 3-octanol for themselves, their residual concentrations in the atmosphere of the conditioned kernels were quantified. The maize kernels were exposed for 6 days to the fungal infected maize or kernels with either 1-octen-3-ol or 3-octanol at a concentration of 0.004 μL/L. The values observed in conditioned kernels of 1-octen-3-ol was 5.5×10^{-06} μL/L and that of 3-octanol was 6.8×10^{-07} μL/L in maize kernels, with the kernels without exposition to the compounds (control) also demonstrating residual concentrations of 1-octen-3-ol and 3-octanol (9.7×10^{-07} μL/L and 5.8×10^{-08} μL/L, respectively).

4. Discussion

Maize kernels are exposed during storage to insect attacks (such as *S.zeamais*) and fungal infection by filamentous fungi (for example *F.verticillioides*), resulting in diverse biological interactions such as grain-microorganism-insect. Here, the volatile profile emitted by the fungi-grain inductor system is enriched by alcohols and ketone compounds and also sesquiterpenes in contrast with the volatile profile of the control. This supports results from a previous investigation on the volatile emission of 1-octen-3-ol, 3-octanone, octen-3-ol and the hydrocarbon sesquiterpenes by *F. verticillioides* in relation with stored kernels (Becker et al., 2014; Dickschat et al., 2011). The volatile profiles reported in the present work could be used for early determination of fungal infection of stored maize kernels, as suggested by Girotti et al. (2012), who also reported on the use of fungal VOCs to predicted the infection of wheat by *F. graminearum.*

The VOCs form a large group of low molecular weight chemical compounds of different biochemical origins (Müller et al., 2013). Compounds such as 1-octen-3-ol, 3-octanone and 3-octanol are produced by the enzymatic action of fungal lipoxygenase activity (Balasubramanian et al., 2007; Brodhun and Feussner, 2011), whereas sesquiterpenes are produced by farnesyl pyrophosphate synthetase (Maffei et al., 2011). These fungal VOCs can vary depending on temperature, substrate and the features of the fungal environment (Heddergott et al., 2014; Maffei et al., 2011; Weikl et al., 2016). They have also been reported to have different ecological functions in fungal-insect-plant interactions, thereby allowing the fungi to prevail over their competitors (Nesci et al.,

Table 2
Two-choice olfactometer bioassay. Response Index (RI) of *S. zeamais* adults against maize kernels for different treatments.

Experimental sets	RI (±EE)	*P* value*
a) Kernels exposed to VOCs produced by *F. verticillioides* infected kernels (treatment) vs. healthy kernels (control)[a]	−56.1 ± 8.3	<0.0001
b) Kernels infected by *F. verticillioides* (treatment) vs. healthy kernels (control)[a]	−71.4 ± 6.6	<0.0001
c) Kernels exposed to VOCs produced by *F. verticillioides* infected kernels (treatment) vs. kernels exposed to kernels without *F. verticillioides* infection (control)[b]	−49.4 ± 6.2	0.0001
d) Kernels exposed to 1-octen 3-ol (treatment) vs. kernels exposed to kernels without these compounds (control)[b]	−26.0 ± 3.9	0.0001
e) Kernels exposed to 3-octanol (treatment) vs. kernels exposed to kernels without these compounds (control)[b]	−32.3 ± 7.3	0.0011
f) Healthy kernels (control 1) vs. healthy kernels (control 2)	−2.9 ± 6.7	0.6743

RI was calculated by using equation (1) (see Materials and methods). (n = 12).
*paired-sample *t-test* for raw data (inside each experimental set).
[a] healthy kernels maintained at ambient temperature.
[b] control kernels treated under the same conditions of temperature and humidity as the conditioned kernels (experimental model).

Table 3
Means of Response Index (RI), weight loss and kernel damage of grains stored with 20 adult insects for 20 days.

	Weight loss of kernels (%)	Kernel damage (%)	RI
Maize kernels exposed to 1-octen-3-ol	19.4 ± 3.3***	30.2 ± 7.8*	−11.2 ± 14.7
Control	80.6 ± 3.3	69.8 ± 7.8	
Maize kernels exposed to 3-octanol	43.8 ± 9.0	33.2 ± 3.6**	−23.5 ± 14.2
Control	56.2 ± 9.0	66.8 ± 3.6	
Maize kernels exposed to *F. verticillioides* COVs	25.7 ± 5.3**	39.5 ± 4.3*	−10.1 ± 15.0
Control	74.3 ± 5.3	60.5 ± 4.3	

*P ≤ 0.05; **P < 0.01; ***P < 0.001 (significant response to paired-sample *t-test* compared with controls. n = 15).

2011). The results of the present work demonstrated that kernels exposed to the fungal VOCs were less attractive and consequently received less damage from *S. zeamais* compared to control kernels, with similar results being obtained when the kernels were exposed to the pure fungal VOC components 1-octen-3-ol and 3-octanol. Taken together, these findings suggest that in storage the fungal VOCs emitted during maize infection exert a protective effect on healthy maize grains against *S. zeamais*. The repellent effect of 1-octen-3-ol, 3-octanone and 3-octanol against *S. zeamais* has recently been reported by Herrera et al. (2015), who observed this type of activity at the concentration range of 0.2–0.4 μL/L air. However, in the present work, the concentrations of 1-octen-3-ol and 3-octanol in the atmosphere of exposed grains were 5.5×10^{-06} μL/L and 6.8×10^{-07} μL/L, respectively, suggesting that other processes could have caused the protective effect of the exposed kernels against insect damage.

Damaged plants possess the capability of inducing response pathways (Bate and Rothstein, 1998) such as emitting signal molecules including VOCs from the wound site, thereby alerting neighboring plants tot biotic stress and allowing them to adjust their defense phenotype (Bais et al., 2004; Bate and Rothstein, 1998). Among the VOCs emitted by plants, the main constituents are produced by the enzyme lipoxygenase, which acts to produce volatile aldehydes (León et al., 2002) or signal molecules such as jasmonic acid (Diezel et al., 2011). The role of these VOCs in plant-plant communication has been extensively explored (Bate and Rothstein, 1998; Piesik et al., 2011; Rhoades, 1983). However, although the LOX activity in grains has been previously reported (Brash, 1999; León et al., 2002; Žilić et al., 2012), to our knowledge there is no information available about VOC induction in maize kernels. The mature kernels are known to have metabolic activity in the aleurone layer, where metabolic activity is responsible for functions involved in maintaining the development of the seeds. In the present study, the kernels exposed to fungal VOCs or to the pure fungal components (1-octen-3-ol and 3-octanol) were more repellent and consequently suffered less damage by *S. zeamais*. Related to this, the small concentration of the pure fungal compounds present in the atmosphere of the exposed grains and the LOX activity reported in bibliography (Brash, 1999; León et al., 2002; Žilić et al., 2012) allows us to hypothesize that the observed protective effect could have been due to stimulation of VOC production by healthy maize grains. To investigate this, factors such as the VOC composition of conditioned kernels, LOX activity and gene expression should be evaluated in future studies.

The VOCs produced by fungal infected kernels could also have a role in the grain-grain interactions as they have been shown to have in plant-plant interactions. Summing up, to our knowledge, this is the first contribution about the role of the volatiles emitted in interaction grain-fungal on the behavior of the S. zeamais insect. These findings could provide an important contribution to the conservation of stored kernels and pest management and this appears as a promising tool for early detection of fungal infection and the mycotoxins presence in a silo using the VOCs as biomarker of fungal and/or mycotoxins presence.

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