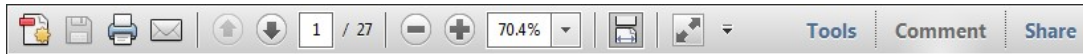
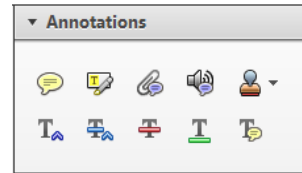


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1. Replace (Ins) Tool – for replacing text.

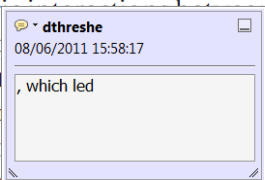


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How to use it

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standard framework for the analysis of microeconomic activity. Nevertheless, it also led to the development of a number of strategic substitutes. The number of competitors in an industry is that the strategic substitutes are the main components of the industry. At the level, are exogenous variables and important words on entry by firms (M henceforth) we open the 'black b



2. Strikethrough (Del) Tool – for deleting text.



Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
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there is no room for extra profits as mark ups are zero and the number of firms (net) values are not determined by Blanchard ~~and Kiyotaki~~ (1987), perfect competition in general equilibrium of aggregate demand and supply in the classical framework assuming monopoly. An exogenous number of firms

3. Add note to text Tool – for highlighting a section to be changed to bold or italic.



Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the [Add note to text](#) icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

dynamic responses of mark ups consistent with the **VAR** evidence

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4. Add sticky note Tool – for making notes at specific points in the text.



Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the [Add sticky note](#) icon in the Annotations section.
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and supply shocks. Most of a number of competitors and the impact is that the structure of the sector



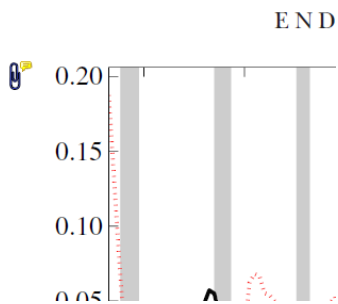
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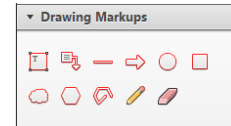
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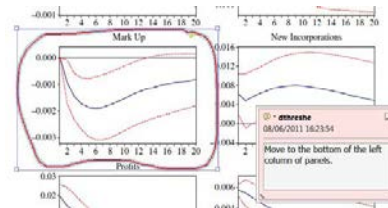
6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



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- Click on one of the shapes in the Drawing Markups section.
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- Double click on the shape and type any text in the red box that appears.



2 1 Volatile compounds released by maize grains and silks in 3 response to infection by *Fusarium verticillioides* and its 4 association with pathogen resistance

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16 Many plants respond to fungal infections by producing and/or emitting a specific blend of oxylipins, some of them
17 through the lipoxygenase (LOX) pathway. *In vitro* bioassays revealed that volatiles from silks and grains of a set of six
18 maize genotypes with variable resistance to *Fusarium verticillioides* affected fungal growth in different ways. Analyses
19 by solid phase microextraction and GC-MS showed different volatile profiles in silks and grains of each genotype. Sus-
20 ceptible genotypes with higher concentrations of precursor polyunsaturated fatty acids produced more volatiles, mainly
21 C9 compounds, in comparison to moderately resistant ones. Real-time PCR from several lipoxygenase transcripts
22 revealed that levels of gene expression depended on the genotype and the inoculation level and suggested that *F. verti-*
23 *cillioides* could use compounds from the 9-LOX pathway to promote infection in grains. On the other hand, volatiles
24 produced by the 13-LOX pathway were more important in moderately resistant genotypes and could be associated to
25 field resistance. Results from this set of genotypes indicate that LOX-regulated volatile compounds might be important
26 in *F. verticillioides* infection and should be targeted in further research to understand disease resistance.

27
28
29 **Keywords:** *Fusarium verticillioides*, lipoxygenase, maize, oxylipin, volatile compounds

30 Introduction

31
32 *Fusarium verticillioides* (synonym: *F. moniliforme*; teleo-
33 morph: *Gibberella moniliformis*) causes fusarium ear rot
34 in maize (*Zea mays*), which is an important disease in
35 Argentina and the world (Chulze *et al.*, 2000; Presello
36 *et al.*, 2007). This pathogen enters into the grain mainly
37 through the silks or grain wounds, affecting yield and
38 grain quality. In addition, the fungus produces mycotox-
39 ins, mainly fumonisins, with a large variety of toxicologi-
40 cal effects for human and animal health (Marasas, 1995;
41 Hussein & Brasel, 2001). Developing plant genotypes
42 less susceptible to ear rots is one of the most effective
43 techniques for disease management.

44
45 Disease resistance depends on several factors, including
46 biochemical compounds such as complex mixtures of
47 volatile organic compounds (VOCs) released by the plant
48 (e.g. monoterpenes, sesquiterpenes, shikimic acid path-
49 way derivatives such as methyl salicylate, as well as the
50 LOX derivatives, methyl jasmonate and C6-volatiles;
51 Prost *et al.*, 2005; Becker *et al.*, 2014). Some of those
52 VOCs are plant oxylipins, a group of oxygenated lipids
53 derived from polyunsaturated fatty acids (Prost *et al.*,
54 2005; Christensen & Kolomiets, 2011). Most oxylipins
55 are produced through the lipoxygenase (LOX) pathway
56 (Feussner & Wasternack, 2002). Lipoxygenase enzymes

57 catalyse the incorporation of molecular oxygen into free
58 polyunsaturated fatty acids, primarily linoleic (C18:2)
and linolenic (C18:3) acids, at either position 9 or 13,
giving 9- or 13-hydroperoxides with antimicrobial activi-
ties (Howe & Schilmiller, 2002; Prost *et al.*, 2005;
Engelberth *et al.*, 2007; Gao *et al.*, 2008). LOX-derived
fatty acid hydroperoxides can be further metabolized
into volatile aldehydes and jasmonates in plants by at
least seven multienzyme pathways (Mosblech *et al.*,
2009). In maize grains, infections of *F. verticillioides*
induce the synthesis of LOX derivatives promoting or
inhibiting fungal development or mycotoxin production
(Maschietto *et al.*, 2015).

Oxylipins regulated by 9-LOX and 13-LOX enzymes
have antimicrobial activities (Prost *et al.*, 2005; Engel-
berth *et al.*, 2007; Gao *et al.*, 2008). The pathway catal-
ysed by 13-LOX enzymes leads to the synthesis of
alcohols, C6 aldehydes and jasmonates, including jas-
monic acid, among others (Feussner & Wasternack,
2002). The branch involving 9-LOX enzymes is still
under study and several works suggest that, contrary to
13-LOX products, these enzymes favour plant infections
by pathogens (Gao *et al.*, 2007).

A better knowledge of the importance of VOCs in
host-pathogen interactions might accelerate the develop-
ment of hybrids less susceptible to fusarium ear rot. This
work aimed to analyse relations between disease resis-
tance and (i) production of VOCs in silks and grain

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tissues; (ii) effects of VOCs on fungal growth; and (iii) expression of genes related to LOX-9 and LOX-13 VOCs in a group of maize genotypes with variable reaction to *F. verticillioides*.

Materials and methods

Plant materials and field assays

Six maize genotypes, representing a range of variability for disease resistance to fusarium ear rot (Presello *et al.*, 2007), were evaluated after silk inoculation with *F. verticillioides* (Table 1). A complete randomized block design experiment with three replicates was conducted in Pergamino, Buenos Aires Province, Argentina. The six genotypes and two inoculation levels (inoculated with *F. verticillioides* and non-inoculated) were randomized in a 6 × 2 factorial arrangement. Each plot consisted of eight 5 m rows sown at a rate of five plants per metre. P364, a fumonisin-producing isolate of *F. verticillioides* (Iglesias *et al.*, 2010) was used to produce the inoculum. For inoculation, 2 mL of a conidial suspension (10⁶ conidia mL⁻¹) were injected into the silk channel with a cattle vaccinator when silks exhibited brown tips, approximately 4–6 days after silking. Disease severity was visually assessed in each plant as the ear area covered by mould (%). Data were arcsine-transformed into $\sqrt{[\text{percentage of disease severity}]}$ to normalize errors. Levels of grain fumonisin and ergosterol, a fungal-specific marker molecule to measure fungal biomass, were assessed following Iglesias *et al.* (2010).

For *in vitro* experiments, silk and grain samples were collected from 10 plants of each plot. Three days after inoculation, silks were taken from inoculated and non-inoculated plots. A fraction of each sample was used immediately and the remnant fraction was stored at -80 °C. At maturity (18–20% grain moisture), ears were manually harvested from the two central rows of each plot, naturally dried to reach equilibrium moisture (nearly 12%), shelled and bulked. Grain samples were stored at -20 °C for less than 2 months.

All statistical tests were conducted using INFostat v. 1.1 (Infostat, 2002) at $\alpha = 0.05$.

Table 1 Pedigree, origin and *Fusarium verticillioides* reaction of six Argentinian maize genotypes

Genotype	Pedigree	Origin	<i>Fusarium</i> reaction
L4674	Inbred	Commercial hybrid Ax924	Susceptible
L4637	Inbred	LP561 × LP611	Moderately resistant
L943 × L944	Single cross between related inbreds	LP915 × LP2541	Moderately resistant
L897 × L900	Single cross between related inbreds	LP915 × LP2541	Susceptible
ARZM05040	Landrace	Argentinian germplasm collection	Moderately resistant
SPF051	Open pollinated variety	Selection from an Argentinian germplasm collection	Moderately resistant

Grain fatty acid content analysis

Fatty acid contents in grains were assessed following Borras *et al.* (2006) with some modifications. The germs of 10 random grains from each genotype were homogenized. The oil from 200 mg flour was extracted in hexane. The method for the preparation of fatty acid methyl esters was performed as described in Official Method Ce 2-66 (AOCS, 1997). A gas chromatograph (Aerograph model 2740-10; Varian Instrument Division) equipped with a flame-ionization detector and a stainless steel Supelco GP 10% SP-2330 column (cyanosilicone phase) in chromosorb WAW 100/120 mesh 1/8 i.d. (Supelco) was used. The column temperature was set at 200 °C and the injector and detector ports were set at 220 °C. The flow rate for the N₂ carrier gas was 30 mL min⁻¹ and the detector gases were set at 30 mL min⁻¹ for H₂ and 300 mL min⁻¹ for air. Peak areas were measured by using a Hewlett Packard model 3365A integrator. The relative amounts of 16:0, 18:0, 18:1 and 18:2 fatty acids were determined as a percentage of the total peak area. The data were mean values of triplicate determination.

In vitro effects of grain and silk volatiles on fungal growth

Culture media were prepared by adding 15 mL potato dextrose agar (PDA) to Petri dishes (Zeringue *et al.*, 1996). A 10 mm agar plug extracted from PDA that had been inoculated with P364 was placed in the well centre. At the same time, milled grain or silk samples (1 g) were placed in the inverted lid. Inverted Petri dishes were sealed with Parafilm. Control plates were prepared in the same way, but no plant tissue was added. Five replicates of each treatment were assayed. Radial fungal growth was assessed after incubation for 7 days at 25 °C.

Profile of volatiles from silks and grains

Volatile profiles were assessed in grains from the six genotypes and silks of L4674 and L4637, two inbreds with significant differences in resistance to *F. verticillioides*. Two grams of milled grains or intact silks were placed into 10 mL vials. Chlorobenzene (Sigma) was used as the internal standard (50 ng kg⁻¹ final concentration). Vials were sealed with a steel crimp cap fitted with a Teflon/silicone septum conditioned at 100 °C. Vials containing samples were placed in a heating block at 50 °C with the solid phase microextraction fibre inserted into the headspace above the sample. Adsorption was timed for 50 min. Carboxen/PDMS (Supelco) fibre was used. Fibre was desorbed at 270 °C for 5 min on a Varian Saturn 2000 GC-MS system. The initial oven temperature was 40 °C, held for 3 min, ramped at 8 °C min⁻¹ to 240 °C and ramped at 30 °C min⁻¹ to 290 °C and held for 5 min. The GC was equipped with a capillary Cpsil 8 ms column (0.25 mm diameter × 30 m length × 0.25 µm film thickness; Varian) coupled with a capillary VF 17 ms (0.25 mm diameter × 30 m length × 0.25 µm film thickness; Varian). The injection port was operated in splitless mode with a constant He carrier gas flow of 1.0 mL min⁻¹. Positive identification of component was performed by mass spectral library (NIST, 1998). Integrated areas were normalized on chlorobenzene.

A principal component analysis (PCA) from standardized data was performed using percentages of disease severity, *in vitro* fungal growth and concentrations of VOCs of each genotype as input traits.

Effects on fungal growth of silk and grain volatiles

In vitro experiments were conducted to assess the effects on fungal growth of the most relevant VOCs observed in silk and grain tissues. The set of VOCs included aldehydes (hexanal, trans-2-heptenal, octanal, trans-2-octenal, nonanal), methyl jasmonate, benzothiophene and fatty acid methyl esters (hexanoic acid, heptanoic acid, octanoic acid, decanoic acid, lauric acid, palmitic acid, oleic acid and linoleic acid methyl esters). *Fusarium verticillioides* was cultured on PDA solid media. Seven millimetre plugs from actively growing regions were taken and placed into the centre of Petri dishes filled with 15 g PDA. Sterile Eppendorf caps containing 5, 20 or 50 µL of the volatiles were placed at the edge of the plate. The PDA dishes were then sealed with Parafilm. After incubation for 7 days at 25 °C, fungal radial growth was assessed. Control plates were prepared as described above without the addition of the volatile compounds. Experiments were conducted in triplicate.

qRT-PCR expression analysis

Gene expression profiles from LOX genes were analysed by quantitative reverse transcriptase (qRT)-PCR assays in maize silk samples from L4674 and L4637. Gene-specific primers were designed along the last exon and the 3' UTR region, using PRIMER3 software (Table S1).

Total RNA was extracted from 100 mg of silk pooled samples using the Trizol protocol (Invitrogen) and purified with the RNA Clean-up protocol (QIAGEN) according to the manufacturer's instructions. The amount and the quality of the total RNA were estimated in a NanoDrop spectrophotometer as well as by agarose gel electrophoresis. cDNA was synthesized using 4 µg of total RNA and oligo(dT) with Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions, and used for performing qRT-PCR.

Twenty nanograms of single strand cDNA were used for qRT-PCR. SYBR Green PCR Master Mix (Invitrogen) was used for the PCRs according to the manufacturer's protocol, with 2.5 mM MgCl₂, 0.25 µM of each primer and 0.025 U *Taq* Platinum polymerase (Invitrogen). Relative quantitative analysis was performed using a Stratagene device (Stratagene). Cycling parameters were as follows: initial denaturation at 95 °C for 2 min; then 45 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 20 s. Melting curves for each PCR were determined by measuring the decrease of fluorescence with increasing temperature (from 65 to 95 °C). The specificity of the PCR was confirmed by melting curve analysis using MxPro Mx3000p software as well as by agarose gel electrophoresis of the products. Relative quantification was normalized using *actin1* sequence (MAc1) [EMBL-EBI: J01238] as internal control and the expression ratio was calculated using the 2^{-ΔΔC_t} method (Livak & Schmittgen, 2001). Each assay was run in triplicate and repeated at least three times in different samples.

Results

Disease severity and grain content of ergosterol and fumonisins

Significant differences in disease severity and grain contents of ergosterol and fumonisins were observed among genotypes (Table 2). Disease severity ranged from 2.2 to 81.7% of the ear visibly affected by mould. Grain

Table 2 Ear rot severity, and fumonisin and ergosterol concentrations in grain of six maize genotypes subjected to silk channel inoculation with conidial suspensions of *Fusarium verticillioides*

Genotype	Disease severity (%) ^a	Fumonisin concentration (ppm)	Ergosterol concentration (ppm)	Disease reaction ^b
L943 × L944	4.8 a	17.7 c	1.2 a	MR
ARZM05040	3.2 a	19.4 c	0.9 a	MR
SPF051	2.5 a	4.3 a	0.6 a	MR
L4637	2.2 a	14.4 b	4.4 ab	MR
L4674	81.7 c	406.6 e	48.4 c	S
L897 × L900	16.3 b	62.9 d	7.6 b	S

Means followed by different letters are significantly different at $P < 0.05$.

^aPercentage of the ear visibly covered by mould.

^bMR: moderately resistant, S: susceptible.

fumonisin contents ranged from 4.3 to 406.6 ppm. Ergosterol concentration, which was used as an indicator of fungal biomass, correlated positively with disease severity ($R^2 = 0.98$) and grain fumonisin content ($R^2 = 0.99$). These results indicate that, for this isolate of *F. verticillioides*, visual evaluation of disease severity is an accurate parameter to assess fungal invasion and predict mycotoxin accumulation. Genotypes SPF051, ARZM05040, L943 × L944 and L4637 exhibited lower values of disease severity, ergosterol and grain fumonisin contents compared to those of L4674 and L897 × L900 (Table 2), which is consistent with previous information (Presello *et al.*, 2006, 2007, 2008).

Contents of fatty acids in grain

Grains of susceptible genotypes L897 × L900 and L4674 showed significantly higher amounts of linoleic acid and lower amounts of oleic acid, compared with those of moderately resistant genotypes (Table 3). A significant positive correlation was observed between the grain concentrations of linoleic acid and total volatiles ($R^2 = 0.92$), which is consistent with the fact that volatile compounds are synthesized from polyunsaturated fatty acids, such as linoleic or linolenic acid. No associations with disease resistance were observed for the other fatty acids assayed.

In vitro effects of grain and silk volatiles on fungal growth

Non-inoculated milled grains and silks were used to find differences in fungal growth caused only by constitutive volatiles. Although volatile compounds from infected samples might be important, resistance response from some of these genotypes depends on constitutive mechanisms (Campos-Bermudez *et al.*, 2013), and so the focus was on these. Volatiles generated from milled grains and

Table 3 Fatty acid composition of grains from six maize genotypes

Genotype ^a	% Fatty acid ^b					
	Palmitic acid (16:0)	Stearic acid (18:0)	Oleic acid (18:1)	Linoleic acid (18:2)	Linolenic acid (18:3)	Linoleic:oleic acid ratio
SPF051 (R)	14.7 c	1.7 a	33.8 cd	48.7 a	1.1 b	1.44 ab
L943 × L944 (R)	11.7 a	2.0 ab	35.1 d	50.3 ab	1.0 ab	1.43 a
ARZM 05040 (R)	13.6 b	2.1 b	32.3 bc	51.1 b	0.9 ab	1.58 b
L4637 (R)	13.2 b	2.0 ab	32.7 bc	51.4 b	0.8 a	1.57 b
L897 × L900 (S)	11.3 a	1.7 a	31.0 b	55.3 c	1.0 ab	1.78 c
L4674 (S)	11.2 a	1.8 a	27.3 a	58.6 d	1.2 b	2.15 d

^aR, moderately resistant; S, susceptible to field *Fusarium verticillioides* infection.

^bMeans followed by different letters are significantly different at $P < 0.05$ (*t*-test).

silks of each genotype exhibited differences in biological activities after exposure to solid cultures of *F. verticillioides* over 7 days. Fungal growth in Petri dishes was reduced by volatiles released from grains or silks of SPF051 and L4637 moderately resistant genotypes, but promoted by those released from silk or grains of L4674 and L897 × 900 susceptible genotypes (Table 4; Fig. 1). *In vitro* inhibition and enhancing effects for these genotypes were observed regardless of the tissue used for the assays. No effects were found for volatiles released from silks and grains of ARZM05040 and L943 × L944.

Volatile compounds from silks and grains

In order to identify VOCs that may be responsible for the bioactivity observed *in vitro*, GC-MS chromatographic profiles were analysed in grain samples from inoculated and non-inoculated treatments of the six maize genotypes and silk samples of L4674 and L4637.

Volatile compounds in grains

Seventy-two VOCs (Table S2) were identified, which consisted of alcohols, aldehydes, ketones, alkenes, carboxylic acids, sulphur-containing compounds and terpenoids,

Table 4 Effects on radial growth of 7-day-old cultures of *Fusarium verticillioides* exposed to volatiles released by silk or grain tissues from six maize genotypes

Genotype	Fungal radial growth (cm) ^a	
	Milled kernel	Silks
SPF051	1.97 a	2.06 a
L4637	2.02 a	2.11 a
L943 × L944	2.51 bc	2.19 ab
ARZM05040	2.61 bcd	2.41 cd
L4674	2.72 cd	2.55 de
L897 × L900	2.81 d	2.67 e
Control	2.36 b	2.27 bc

^aMeans followed by different letters are significantly different at $\alpha = 0.05$.

among others. Chromatographic patterns showed quantitative and qualitative differences among genotypes and inoculation levels. A PCA analysis based on disease severity, fungal growth and volatile concentrations revealed three major areas of the graph, and its distribution did not appear to be independent of genotype and inoculation level (Fig. 2). The relative contents of the most significant volatiles released by grains of the six genotypes in inoculated and non-inoculated treatments are displayed in Figure 3. From the PCA analysis it was observed that Group 1 included a large number of compounds released by L4674, regardless of the inoculation level, and by L897 × L900 after inoculation. The large size of the vectors in PC1, and the position of L4674 at the extreme of the axes, indicate that this genotype produced large amounts of this set of compounds. Vectors for disease severity and fungal growth were close to this group, indicating that these compounds might be associated with fungal growth promotion. The compounds grouped in this sector included several C9-compounds such as nonanal (V16), (E,E)-2,6-nonadienal (V24), 1-nonene (V32), (E)-2-nonen-1-ol (V34), 6-nonenal (V39) and 8-oxo-nonenal (V66). Group 2 was mainly defined by scores in PC2 and included volatiles released by moderately resistant genotypes SPF051 and L4637 without inoculation. This sector mostly included aromatic compounds such as benzothiophene (V47), naphthalene (V51), 2-methyl naphthalene (V53), 2,2-dimethyl-propanoic acid (V18) and benzoic acid (V42). Upon inoculation, these genotypes tended to generate a new class of volatile compounds (Group 3) including methyl jasmonate (V72). They were grouped at the other extreme of the PC1 axis and corresponded with several compounds released by the resistant genotypes and non-inoculated L897 × L900. According to their position in Figure 2, these compounds seem to be present in low concentrations in grains of L4674, the most susceptible line.

Volatile compounds in silks

Moderately resistant L4637 and susceptible L4674 genotypes that had exhibited differences in disease resistance and volatile effects on *in vitro* fungal growth were

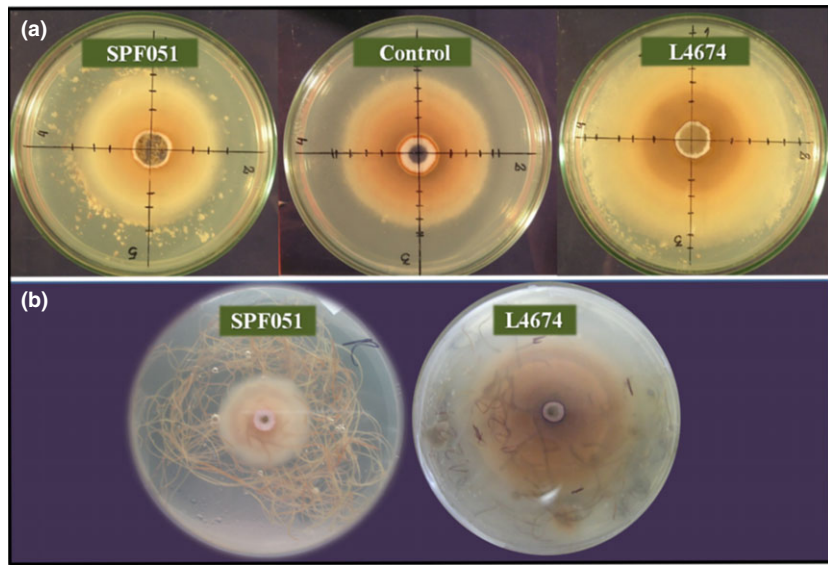


Figure 1 *Fusarium verticillioides* growth in Petri plate exposed to volatiles released from 1 g of milled grains (a) and silks (b) from field resistant (SPF051) and field susceptible (L4674) maize genotypes. The photograph was taken 7 days after inoculation with a conidial suspension of *F. verticillioides* into the centre well of the Petri plate. [Colour figure can be viewed at wileyonlinelibrary.com].

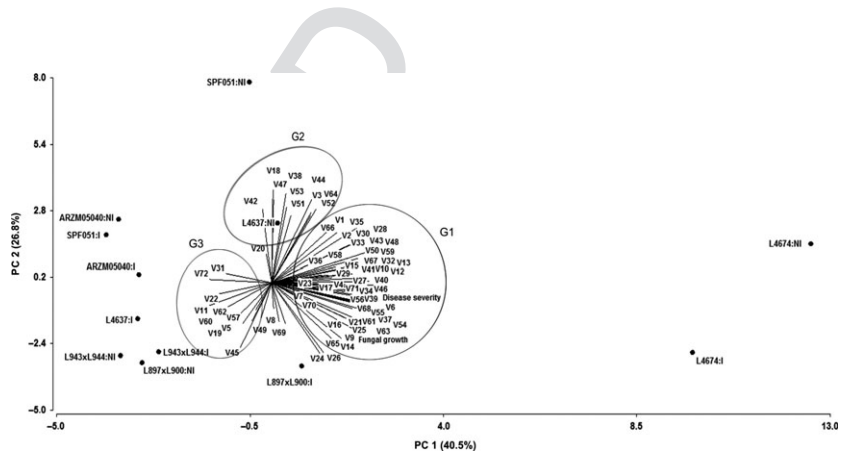


Figure 2 Principal component analysis from the volatile compounds released by grains of six maize genotypes inoculated (I) with *Fusarium verticillioides* and non-inoculated (NI). G1: Group 1, G2: Group 2, G3: Group 3.

evaluated. The chromatographic pattern analysis of VOCs obtained by GC-MS showed quantitative and qualitative differences between genotypes. Forty-three VOCs were identified, including alcohols, aldehydes, carboxylic acid, benzoic acids, terpenoids and fatty acid derivatives, among others (Table S3).

In the PCA analysis, the two first principal components explained 80.2% of the total variance (Fig. 4). Inoculation levels were discriminated by scores in PC1 and genotypes by scores in PC2. Volatile compounds with higher scores in PC1 represented most of the variation, indicating that *F. verticillioides* inoculation caused a stimulation of volatile liberation. The most important volatiles from this group were oxazole [2-(1-naphthalenyl)-5-phenyl] (V17), isojasnone (V23), 2-hexyl-1-decanol (V4), nonanoic acid, 9-oxo-, methyl ester (V22), heneicosene (V3), 1-icosanol (V2), nonadecane (V5), oleic acid, methyl ester (V10), hexadecanoic acid, 14-methyl-, methyl ester (V13), isocaryophyllene (V26), linoleic acid, methyl ester (V12) and nonadecanoic acid, 18-oxo-, methyl ester (V18). The relative content of the most significant volatiles released by silks of the two

genotypes in inoculated and non-inoculated treatments are shown in Figure 5. Some compounds, including 2,4,4-trimethyl-1-hexene (V37), 7-methyl-5-octen-4-one (V36), naphthalene (V39) and hexanal (V41), exhibited lower scores in PC1 (i.e. exhibited prevalence in the non-inoculated treatment). Hexanal was released by the non-inoculated resistant genotype (Fig. 4). Scores in PC2 indicated that inoculated susceptible and resistant genotypes produced different classes of volatiles. L4674 showed higher concentrations of C6 volatiles, including trans-2-octenal (V8) and trans-2-hexenal (V43), volatiles derived from the 13-LOX pathway and (E,E)-2,4-nonadienal (V35) and (Z)-6-nonenal (V7), which are derived from the 9-LOX pathway. These results indicate a stimulation of both 9- and 13-LOX pathways. This moderately resistant line also released fatty acid methyl and ethyl esters, such as linoleic acid, methyl ester (V6), linoleic acid, ethyl ester (V12) and oleic acid, methyl ester (V10); 4-methyl-2-propyl-furan (V34); 1,3,5-cycloheptatriene (V42) and 2,2,4-trimethyl-3-penten-1-ol (V28). On the other hand, inoculated silks of L4674 released alkyl derived fatty acids, such as methyl ester fatty acid like

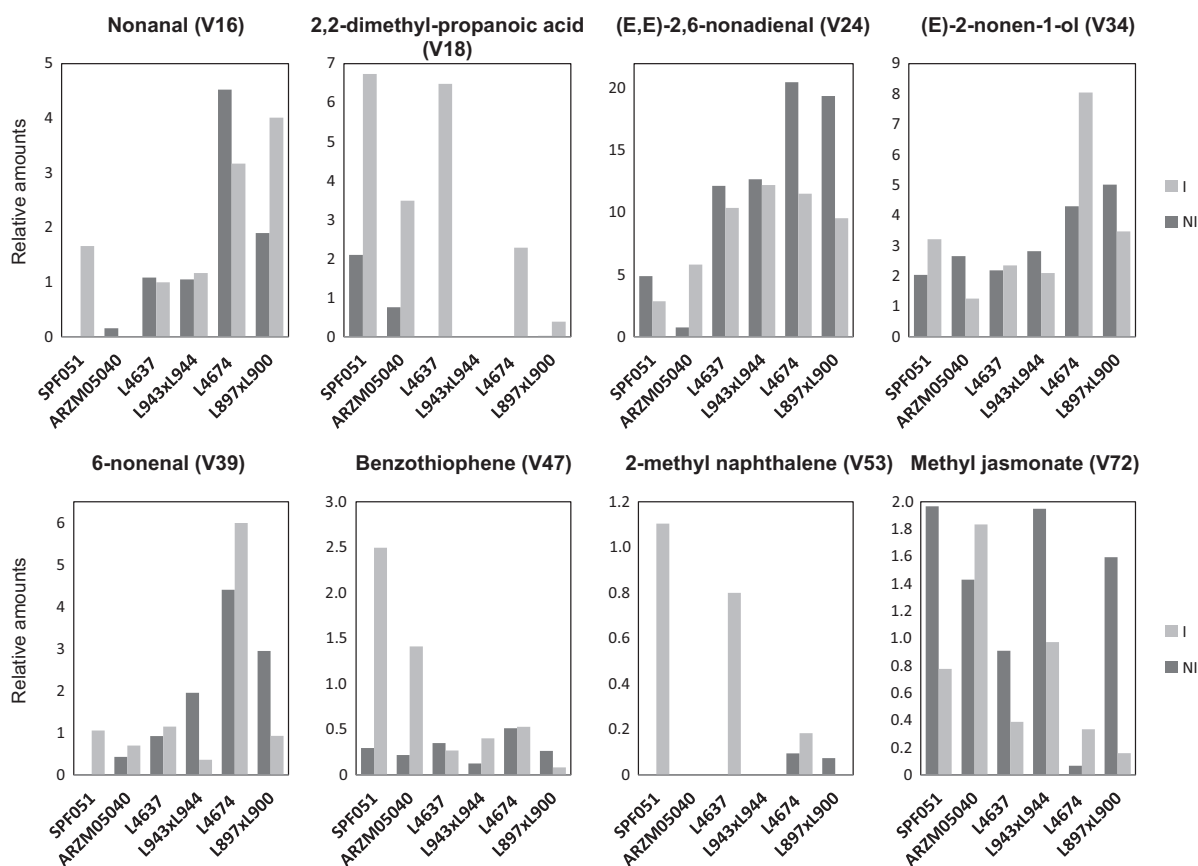


Figure 3 Relative content of the most significant volatiles released by grains of six maize genotypes inoculated (I) with *Fusarium verticillioides* and non-inoculated (NI).

6,11-eicosadienoic acid, methyl ester (V11), hexadecanoic acid, 14-methyl-, methyl ester (V13), 9-dodecanoic acid, methyl ester (V14), nonadecanoic acid-, 18-oxo-, methyl ester (V18), nonanoic acid, 9-oxo-, methyl ester (V22), 2-methyl decanoic acid (V31), germacrene B (V25) and aromatic hydrocarbons like naphthalene (V39) and azulene (V38).

In vitro effects of volatile compounds on fungal growth

In vitro experiments were conducted to test effects on fungal growth of the VOCs possibly involved in disease reaction. Fungal growth was affected not only by the type of volatile but also by the volatile concentration (Fig. 6). Aldehydes exhibited more inhibition effect than methyl esters. A complete inhibition of *F. verticillioides* growth was observed after addition of several amounts of trans-2-octenal. Trans-2-heptenal and nonanal showed a marked inhibitory effect on fungal growth with higher efficiency at low concentrations. The addition of 50 μ L of hexanal, and 20 and 50 μ L of octenal also reduced fungal growth. From the analysis of volatiles that, according to PCA, seemed to be associated with disease resistance, it was found that benzothiophene inhibited fungal growth, but no effect was observed for methyl

jasmonate regardless of the amount added. From the methyl esters assayed, only oleic acid showed no effect on fungal growth. The other methyl esters (hexanoic acid, heptanoic acid, octanoic acid, decanoic acid, lauric acid and palmitic acid methyl ester) displayed low inhibition effects.

Silk lipoxygenase expression in response to pathogen infection

Earlier reports have indicated that oxylipins produced by the LOX pathway are involved in plant-pathogen interactions (Burow *et al.*, 2000; Gao *et al.*, 2007; Christensen & Kolomiets, 2011; Maschietto *et al.*, 2015). Here, LOX gene expression from maize silks was analysed to compare gene expression with volatile profiles from inoculated and non-inoculated treatments of L4637 and L4674.

Several transcripts described as lipoxygenase were identified from the Maize Genetics and Genomics Database (<http://www.maizegdb.org>). Among them, six genes were chosen based on previous studies (*ZmLOX4*, 5, 6, 7, 10 and 11). *ZmLOX4* and 5 are predicted to be type 9-LOXs (Park *et al.*, 2010; Maschietto *et al.*, 2015) whereas *ZmLOX7*, 10 and 11 (Nemchenko *et al.*, 2006)

POOR QUALITY FIG

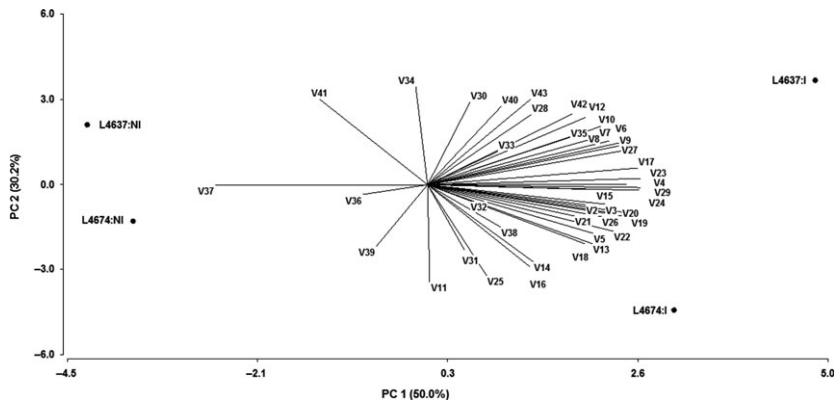


Figure 4 Principal component analysis from the volatile compounds released by silks of two maize inbreds L4637 and L4674 inoculated (I) with *Fusarium verticillioides* and non-inoculated (NI). Vectors stand for the expression of each volatile.

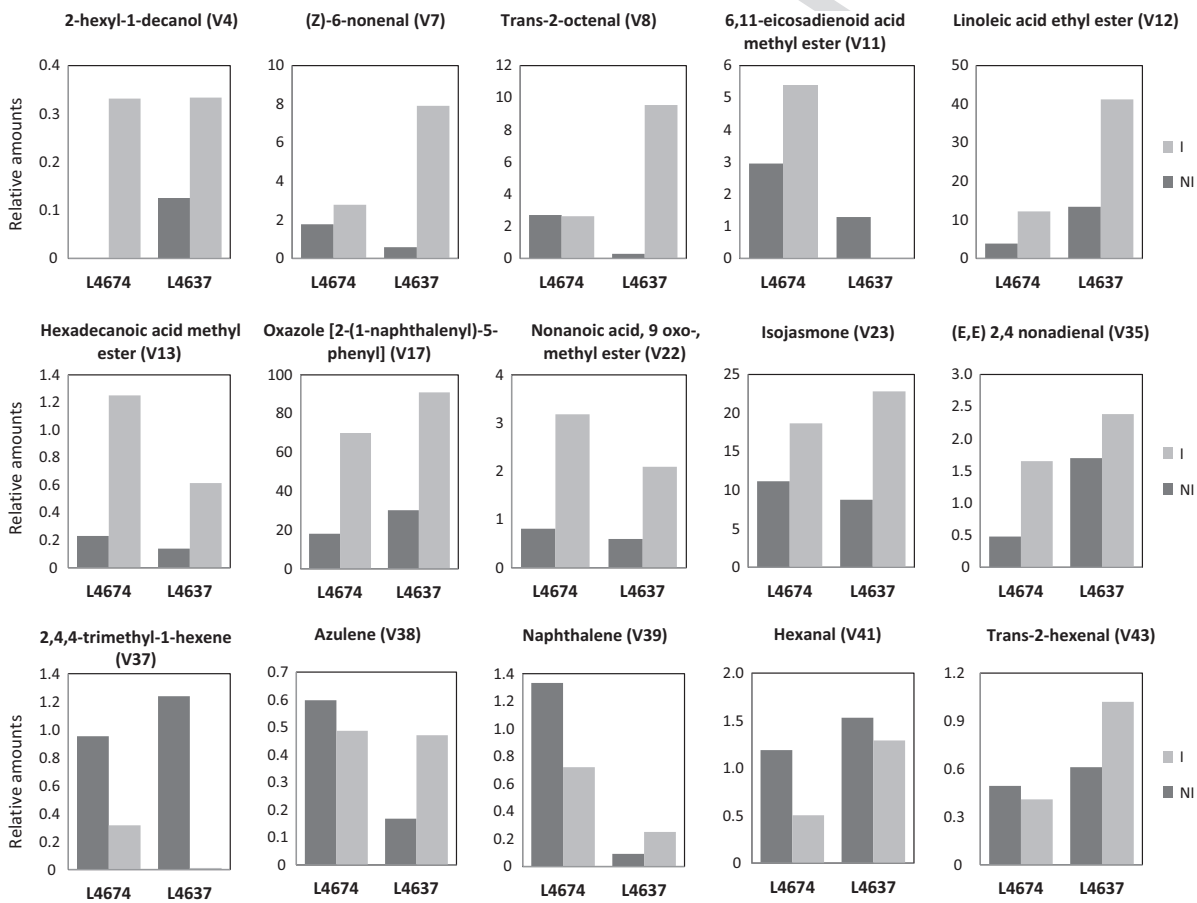


Figure 5 Relative content of the most significant volatiles released from silks of two maize genotypes inoculated (I) with *Fusarium verticillioides* and non-inoculated (NI).

are predicted to be 13-LOXs. Conversely, *ZmLOX6* has been reported to be a hydroperoxide lyase enzyme and seems to be involved in a branch of the 13-LOX pathway (Gao *et al.*, 2008).

From the qRT-PCR analysis, it was observed that 9-LOXs genes, *ZmLOX4* and *ZmLOX5*, were induced

after fungal inoculation in both genotypes, with higher expression in the L4674 (Fig. 7). On the other hand, *ZmLOX6* transcript was induced after inoculation in both lines, with higher expression in L4637. *ZmLOX7* expression was induced by inoculation in L4674 only, but higher basal expression levels were observed in

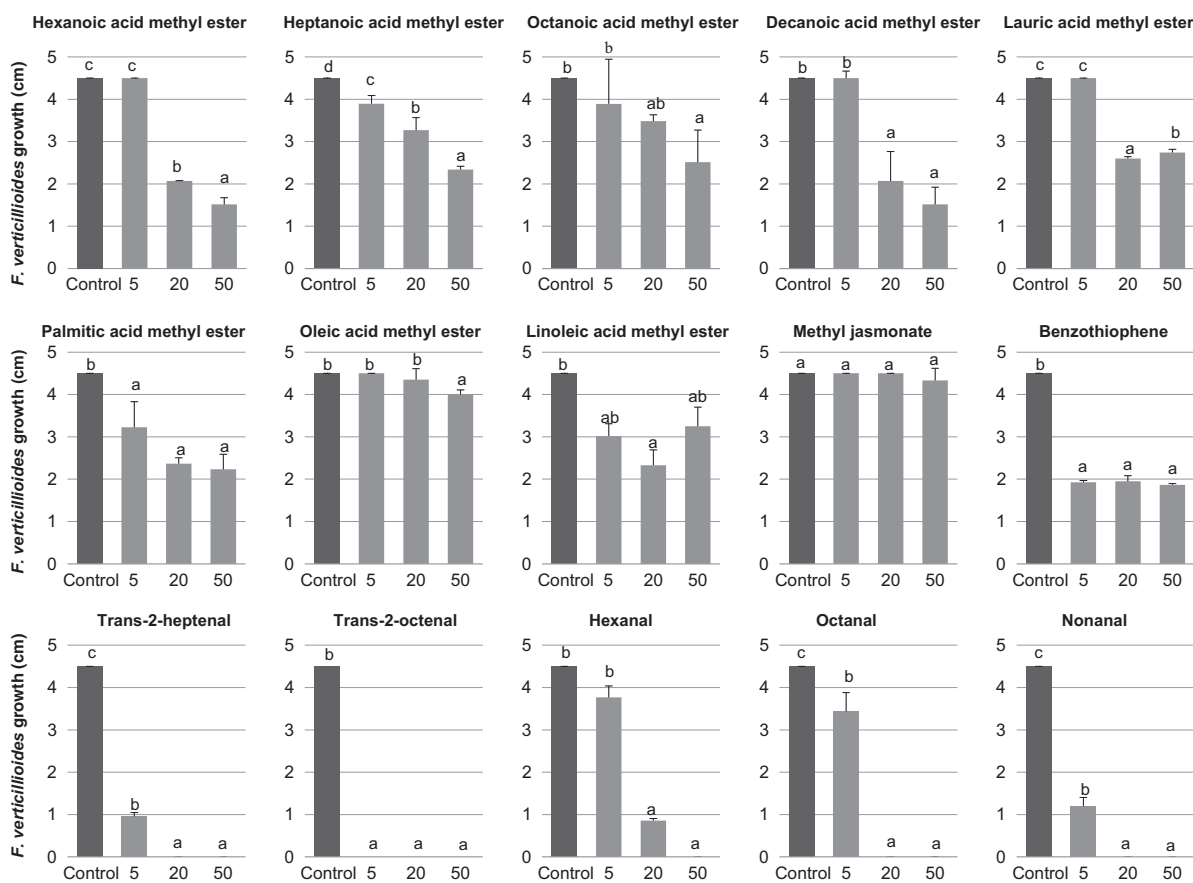


Figure 6 *In vitro* growth of *Fusarium verticillioides* after 7 days of continuous exposure to variable amounts (5, 20 and 50 μL) of volatile compounds

L4637. Expression of *ZmLOX10* and *ZmLOX11* genes was affected by inoculation. While both genes were induced by inoculation in L4637 only, expression of *ZmLOX10* seemed to be repressed after inoculation of L4674. These results indicate that 13-LOXs gene expression seems to be higher in the moderately resistant inbred while 9-LOXs gene expression, which was stimulated by inoculation, is higher in the susceptible inbred.

Discussion

Plants are capable of defending themselves by releasing complex mixtures of VOCs. Plant oxylipins, produced via the LOX pathway, are some VOCs released by plants as signals in defence and development (Prost *et al.*, 2005; Piesik *et al.*, 2015). *In vitro* experiments, where fungal growth was assessed after exposure to volatiles from silks or grains, showed that VOC blends released by each genotype affected fungal growth in different ways. Volatile blends from silks and grains of the most resistant genotypes inhibited, and those of the susceptible genotypes promoted, fungal growth. Those effects were observed regardless of the tissue, indicating that biological activity of volatiles is important in silks and grains.

Chromatographic analysis performed in grains of all genotypes and silks of L4637 and L4674 revealed

differences in amount and profiles of volatiles released by each genotype. Principal component analyses using grain volatile profiles, disease severity and *in vitro* fungal growth as input traits showed that volatile profile affected fungal growth. Considering that vectors for disease severity and fungal growth from PCA were closely related, and that the effects of volatile blends on fungal growth were similar in silks and grains, which can be colonized by *Fusarium*, volatile production might be a factor affecting field disease resistance.

The most susceptible genotypes, such as L4674, produced large amounts of VOCs with prevalence of C9 compounds, indicating that these volatiles might be associated with fungal growth promotion observed in Petri dishes. Thus, fungal growth could be positively regulated by 9-LOX products affecting susceptibility of the maize plant to *F. verticillioides*. These results are consistent with previous research on aspergillus ear rot reporting that 9-LOX-derived compounds induce aflatoxin biosynthesis in *Aspergillus* species (Burow *et al.*, 2000). For fusarium ear rot, evidence from a study of a 9-LOX gene mutant in maize, *ZmLOX3*, also supported this hypothesis, showing that the deficiency in 9-LOX derivatives significantly reduces production of pathogenic spores and mycotoxins of *F. verticillioides* in maize seeds (Gao *et al.*, 2007).

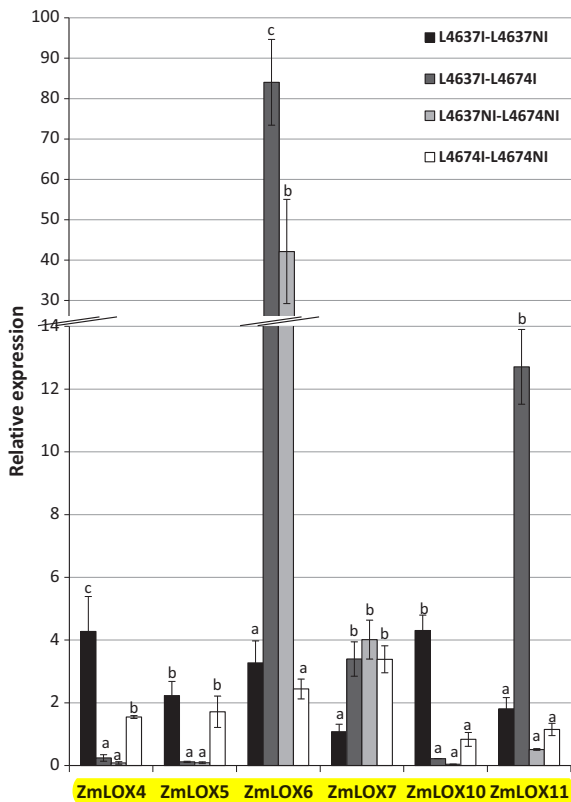


Figure 7 Relative expression of the transcripts of *ZmLOX* genes in silks of moderately resistant L4637 and susceptible L4674 maize genotypes. NI: not inoculated, I: inoculated with *Fusarium verticillioides*.

In studies of grain volatiles, moderately resistant L4637 and SPF051 were grouped by PCA in a cluster of genotypes producing grain aromatic VOCs, such as benzothiofene, naphthalene, 2-methyl naphthalene and benzoic acid. These compounds and their derivatives have antifungal effects in different species (Pinto *et al.*, 2008; Kim *et al.*, 2010; Salae *et al.*, 2010). Inoculation induced the synthesis of methyl jasmonate, a phytohormone inducing the expression of defence genes in plants in response to attack by several pathogens (Penninck *et al.*, 1998; Turner *et al.*, 2002) in these genotypes. In silk volatile experiments, most volatiles were produced after inoculation, indicating that the fungus caused a stimulation of volatile production. Inoculation increased the production of hexanal and trans-2-hexenal volatiles, particularly in silks from L4637, suggesting the contribution of 13-LOX pathway-derived volatile aldehydes in infection response. For aspergillus ear rot, downstream derivatives of 13-LOX, including methyl jasmonate and various C6–C12 alkanal and alkenal volatiles (Zeringue *et al.*, 1996; Mosblech *et al.*, 2009) also seem to be involved in inhibiting aflatoxin production.

The results here indicate that the 9-LOX pathway is activated in grains of susceptible genotypes, suggesting an important role of this pathway in susceptibility to *F. verticillioides*. Based on real-time PCR results, it seems

that 9-LOX genes (*ZmLOX4* and *ZmLOX5*) were triggered in response to pathogen attack in both genotypes, with higher expression levels in the susceptible line. Park (2011) reported that *ZmLOX5* contributes to susceptibility of maize to *Colletotrichum graminicola*, a leaf fungal pathogen. In contrast, it was found that both *lox4* and *lox5* mutants enhanced susceptibility to grain colonization and conidia production of *F. verticillioides*. These results are consistent with those from PCA indicating that the growth of *F. verticillioides* and fumonisin biosynthesis could be positively regulated by 9-LOXs and may be involved in susceptibility of the maize plant to this fungus. On the other hand, *ZmLOX10* transcript was induced after inoculation in L4637, indicating a possible role for LOX10 in disease defence. Furthermore, *ZmLOX6* transcript showed higher expression in L4637, indicating that this gene could also contribute to a resistance response. Conversely, Gao *et al.* (2008) reported that this gene was strongly induced by the fungal pathogen *Cochliobolus carbonum* during compatible interactions, suggesting that *ZmLOX6* may contribute to pathogen invasion. Therefore, further investigations are needed in order to determine the possible defence role of 13-LOX pathway derivatives in plants.

In vitro evaluations of the effect of specific volatiles on fungal growth revealed that aldehydes exhibited more inhibition effect than methyl esters. A complete inhibition of fungal growth was observed upon the addition of trans-2-octenal. Trans-2-heptenal and nonanal aldehydes showed high efficiency at low concentrations. It was found that benzothiofene inhibited fungal growth and no effect was observed for methyl jasmonate. This is consistent with previous results indicating antifungal effects of benzothiofene (Pauletti *et al.*, 2000). The absence of fungal growth inhibition for methyl jasmonate may be due to the fact that this compound is involved in signalling of defence pathways (Penninck *et al.*, 1998; Turner *et al.*, 2002) more than in specific inhibition mechanisms. These *in vitro* experiments show that several compounds have a direct inhibitory effect on fungal growth and could be targeted for further studies on their activities to prevent fungal infections in natural conditions.

This study indicates a correlation between VOC production and the grain profiles of fatty acids, which are volatile precursors. The most susceptible genotypes exhibited higher contents of linoleic acid and higher production of VOCs compared to those of moderately resistant genotypes. Recent studies have shown that the composition of total fatty acids can influence infections of *F. verticillioides* and fumonisin accumulation in maize under field conditions (Scala *et al.*, 2014). Similar results were reported by Dall'Asta *et al.* (2012) who found that hybrids with higher linoleic acid content also showed high fumonisin contamination. Thus, for the set of maize genotypes in the present study, concentration of polyunsaturated fatty acids seems to affect disease resistance.

The results from these experiments indicate that the amounts and profiles of volatiles produced in silks and grains depends on the genotype and infection level, and

seem to play a role in differences in disease resistance observed in this set of maize genotypes. The most resistant genotypes produce blends of VOCs that might be responsible for low disease severities observed in field conditions, and the susceptible genotypes produce VOCs promoting fungal growth, mostly via the 9-LOX pathway. This is new evidence for the role of specific 9-LOXs and their derivatives as possible factors affecting ear rot severity in maize. This result provides evidence that fungal infection changes plant LOX gene expression, leading to possible alterations in the fungal–host interaction.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Gene-specific primers used for qRT-PCR analysis.

Table S2. Relative amounts and identification of volatile compounds released from grains of six *Fusarium verticillioides* inoculated (I) and non-inoculated (NI) maize genotypes.

Table S3. Relative amounts and identification of volatile compounds released from silks of *Fusarium verticillioides* inoculated (I) and non-inoculated (NI) L4674 and L4637 maize genotypes.

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