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Toxin distribution and sphingoid base imbalances in *Fusarium verticillioides*-infected and fumonisin B1-watered maize seedlings

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ABSTRACT

Fusarium verticillioides is a major maize pathogen and there are susceptible and resistant cultivars to this fungal infection. Recent studies suggest that its main mycotoxin fumonisin B1 (FB1) may be involved in phytopathogenicity, but the underlying mechanisms are mostly still unknown. This work was aimed at assessing whether FB1 disseminates inside the plants, as well as identifying possible correlations between the maize resistant/susceptible phenotype and the unbalances of the FB1-structurally-related sphingoid base sphinganine (Sa) and phytosphingosine (Pso) due to toxin accumulation. Resistant (RH) and susceptible hybrid (SH) maize seedlings grown from seeds inoculated with a FB1-producer *F. verticillioides* and from uninoculated ones irrigated with FB1 (20 ppm), were harvested at 7, 14 and 21 days after planting (dap), and the FB1, Sa and Pso levels were quantified in roots and aerial parts. The toxin was detected in roots and aerial parts for inoculated and FB1-irrigated plants of both hybrids. However, FB1 levels were overall higher in SH seedlings regardless of the treatment (infection or watering). Sa levels increased substantially in RH lines, peaking at 54-fold in infected roots at 14 dap. In contrast, the main change observed in SH seedlings was an increase of Pso in infected roots at 7 dap. Here, it was found that FB1 disseminates inside seedlings in the absence of FB1-producer fungal infections, perhaps indicating this might condition the fungus–plant interaction before the first contact. Furthermore, the results strongly suggest the existence of at least two ceramide synthase isoforms in maize with different substrate specificities, whose differential expression after FB1 exposure could be closely related to the susceptibility/resistance to *F. verticillioides*.

1. Introduction

Fusarium verticillioides is a major maize pathogen limiting crop productivity worldwide, causing significant economic losses year after year. Although this hemi-biotrophic fungus can spend most of its life cycle in maize, it can also exist as a facultative saprophyte and infect seeds and/or lateral roots from plant residues or soils (Oren et al., 2003). The fungus can either remain as a plant endophyte or move toward a pathogenic phase (Brown et al., 2014), thereby causing alterations in growth parameters and symptoms such as seed, root and stem rot. In addition, *F. verticillioides* can synthesize significant amounts of secondary metabolites including the fumonisin mycotoxins, among which fumonisin B1 (**1**) (FB1 (**1**), Fig. 1) is considered to be the most significant because of its toxicity. With respect to *Fusarium* ear rots, previous research has suggested a correlation between symptom severities and FB1 (**1**) concentrations in a wide range of host genotypes (Butrón et al., 2015; Clements et al., 2004; Presello et al., 2008). One approach to prevent contamination with fungal toxins is by developing and using cultivars more resistant to diseases. However, to date, neither commercial maize hybrids nor inbred lines, have achieved the desired resistance levels (Lanubile et al., 2011).

Previously, it was found that FB1 (**1**) reproduces some of the maize seedling disease symptoms observed in its *F. verticillioides* infection (Arias et al., 2012). Moreover, other studies have shown

Abbreviations: CS, ceramide synthase; dap, days after planting; FB1, fumonisin B1; LCB, long-chain base; LC–MS, liquid chromatography–mass spectrometry; PCD, programmed cell death; Pso, phytosphingosine (t18:0); RH, resistant hybrid; Sa, sphinganine (d18:0); SH, susceptible hybrid; So, sphingosine (d18:1).

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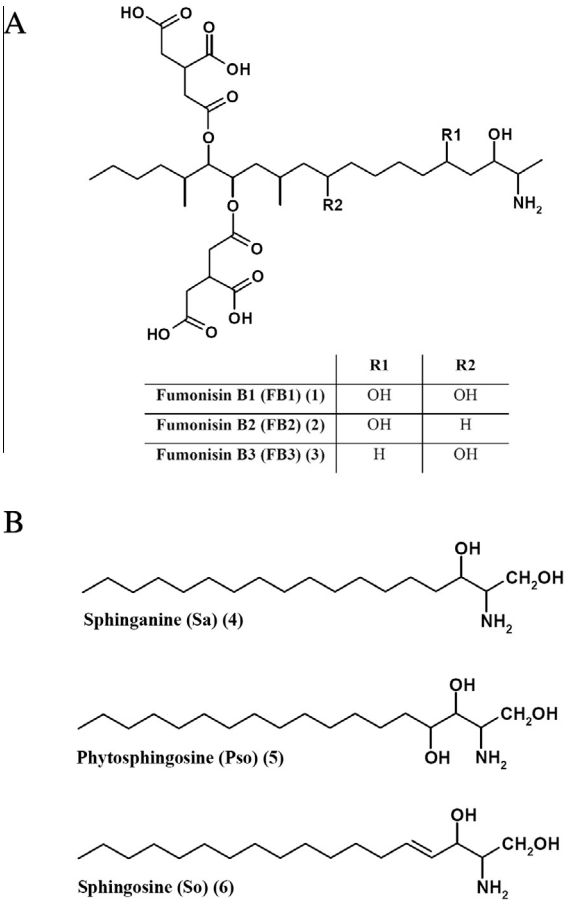

	R1	R2
Fumonisin B1 (FB1) (1)	OH	OH
Fumonisin B2 (FB2) (2)	OH	H
Fumonisin B3 (FB3) (3)	H	OH

Fig. 1. Chemical structures of (A) fumonisins B1 (FB1) (**1**), B2 (FB2) (**2**) and B3 (FB3) (**3**); and (B) the structurally related sphingoid bases sphinganine (Sa) (**4**), phytosphingosine (Pso) (**5**) and sphingosine (So) (**6**).

that fumonisin B1 (**1**) production contributes to the fungal pathogenicity in maize seedlings (Glenn et al., 2008; Myung et al., 2012), but still there are conflicting results concerning this issue (Desjardins et al., 1998, 2002). Related to this, fumonisins may not be necessary for maize ear rot development in the plant maturity stage. However, in another study, Glenn et al. (2008) restored, by molecular genetic complementation, fumonisins synthesis and pathogenicity toward maize seedlings in a fumonisin non-producing isolate originally obtained from banana. Such banana-associated isolates are not pathogenic toward maize and are now recognized as *Fusarium musae* (Van Hove et al., 2011), a cryptic species of *F. verticillioides*. Conferring mycotoxin production and pathogenicity supported the conclusion that FB1 (**1**) was necessary for development of foliar disease symptoms on susceptible maize seedlings.

Similarly to *F. verticillioides*, FB1 (**1**) has been isolated from corn stover and soil (Abbas et al., 2008; Nelson, 1992; Williams et al., 2006), so it is possible that this hydrosoluble and light- and heat-stable toxin can contaminate water reservoirs near cultivation areas.

There is some evidence that FB1 (**1**) can accumulate in various parts of maize plants, such as roots, stems, leaves, ears and grains (Almeida et al., 2002; Baldwin et al., 2014; Presello et al., 2011; Williams et al., 2007) from inoculated seeds. However, there is limited information about toxin mobility within uninoculated plants, when it is introduced via watering. Zitomer et al. (2010) suggested that although FB1 (**1**) may be absorbed from the soil and accumulated in roots, the toxin might not be translocated to aerial parts.

FB1 (**1**) can alter the host physiology, possibly weakening defense mechanisms and therefore favoring fungal invasion. Regarding the latter, results of a previous study (Arias et al., 2012) strongly suggest the existence of plant detoxification mechanisms, efficiently operating but only up to a certain threshold FB1 (**1**) contamination level, beyond which, the plant is unable to control FB1 (**1**) toxicity effectively.

In other studies, a phase III mechanism of xenobiotic detoxification in plants has been described, involving the sequestration of conjugated compounds into the vacuole, or their irreversible chemical binding to the cell wall. By this means, the detoxification products are permanently stored in the plant tissue, with root exudation being the only effective plant excretion process (Berthiller et al., 2013).

The sphingolipid metabolism disruption is currently widely accepted as being a major mechanism implicated in FB1 (**1**) toxicity in animal and plant cells. The toxin competitively inhibits the key enzyme ceramide synthase (CS), due to its structural similarity with the natural substrates sphinganine (Sa) (**4**) and phytosphingosine (Pso) (**5**), thereby causing not only the accumulation of these free sphingoid bases but also complex sphingolipid depletion.

In plants, sphingolipid metabolism disruption affects their development and response to biotic and abiotic stress (Chen et al., 2006; Nagano et al., 2012, 2014; Rivas-San Vicente et al., 2013; Spassieva et al., 2002; Ternes et al., 2011; Williams et al., 2007). Furthermore, from studies carried out with different *Arabidopsis* mutants, it was shown that disturbances in sphingolipid metabolism induced by AAL (*Alternaria alternata* f. sp. *lycopersici* toxin) and FB1 (**1**) may lead to cell death. However, the FB1 (**1**) phytotoxic effects in the natural environment where disease develops, and the specific lipids involved in triggering programmed cell death (PCD) have not been well characterized to date. Findings reported in response to exogenous long chain bases are not in agreement about the main sphingolipids responsible for PCD induction. Shi et al. (2007) showed d18:0 (Sa) (**4**), t18:0 (Pso) (**5**) and d18:1 (sphingosine, So (**6**)) to be cell death inducers in *Arabidopsis* spp. seedlings in agar, with Sa (**4**) having a weaker lethal activity than Pso (**5**). These results were consistent with those of Tanaka et al. (1993), who found that Pso (**5**) behaved as a more potent phytotoxin than Sa (**4**) in duckweed. Notwithstanding, Saucedo García et al. (2011) recently reported that Sa (**4**), but not Pso (**5**), induced PCD in *Arabidopsis* spp. seedlings. Hence, the precise role of the sphingoid bases in PCD induction still remains to be clarified, especially in maize where data concerning Sa (**4**) and Pso (**5**) participation in cell death program activation is scarce.

Within this context, the aim of this work was to evaluate both the kinetics and the extent of FB1 (**1**) accumulation in maize seedlings from hybrids resistant and susceptible to *F. verticillioides* ear rot, developed from seeds inoculated with a toxigenic *F. verticillioides* and from uninoculated seeds irrigated with this mycotoxin, with a special focus on identifying in the latter the potential toxin uptake and dissemination to distal tissues from the site of entry. Furthermore, the possible correlations between the Sa (**4**) and Pso (**5**) changes induced by FB1 (**1**) and the differential behavior of both genotypes in the maize-*F. verticillioides* pathosystem were evaluated.

2. Results

2.1. FB1 (*1*) accumulation in maize seedling tissues

In this work, quantifiable levels of FB1 (**1**) in root and aerial tissues, were detected for almost all experimental conditions, except for the controls and the aerial parts of the RH-infected plants at

7 dap (Fig. 2). FB2 (**2**) and FB3 (**3**) were found in a small number of samples of both plant tissues and sand, but at levels always below the quantification limits (defined as signal to noise ratio of 10). The toxin was located mainly in the roots, and was detected even at the earliest end point tested in both treatments and at levels up to 10 times higher than those found in the aerial parts.

Regarding the infection model, the kinetics of FB1 (**1**) accumulation in the roots were mostly similar in both hybrids, reaching peak levels at 14 dap and increasing up to seventeen times the toxin levels found at 7 dap in both RH and SH seedlings. However, the mean FB1 (**1**) concentration in SH seedlings was substantially higher than in those RH (137.21 and 25.74 nmol FB1/g dw, respectively). Furthermore, the FB1 (**1**) levels in the roots were always higher in SH seedlings. This could have been due to a greater fungal growth (biomass), to a stimulating effect of the "SH environment" on the FB1 (**1**) biosynthesis, or to a combination of both these issues. Hence, in order to characterize better which of these effects were occurring in this case, the fungal biomass was comparatively estimated by quantifying the ergosterol (**7**) levels in the infected roots of both hybrids cultured for 21 days (Fig. 3), which gave values of 12.88 ± 1.75 and 3.89 ± 0.76 mg/g dw, for SH and RH seedlings, respectively. Then, the ergosterol (**7**) values were used to correlate FB1 (**1**) production to fungal mass in infected maize seedling. Although the result indicated a greater fungal development in the former ($p < 0.01$), no significant differences were found in the FB1/ergosterol (**1/7**) ratio between SH and RH seedlings (6.49 ± 1.81 and 5.41 ± 1.91, respectively). It was also determined that FB1 (**1**) levels found in the aerial parts of the infected seedlings were always correlated with those detected in the corresponding roots, but at concentrations about 10 times lower. Despite the findings in roots and aerial parts, the FB1 (**1**) levels detected in sand (infection model) were slightly higher (however, without being statistically significant) in RH than in SH, with values of 11.71 ± 3.67 and 8.51 ± 1.76 pmol FB1 (**1**) per gram of sand at 21 dap, respectively.

In the phytotoxicity model, in which the plants were watered with 20 ppm-FB1 (**1**) solution on 2, 4 and 6 dap, some differences regarding toxin accumulation in the roots of both hybrids were observed. While the FB1 (**1**) levels found at 7 dap remained largely unchanged until 21 dap in the RH, in the SH they were relatively constant until 14 dap, after which their concentration increased significantly and reached the highest level at 21 dap (6 times higher than at 7 dap). In contrast with the results observed in the infection, the FB1 (**1**) levels in the aerial parts were only correlated

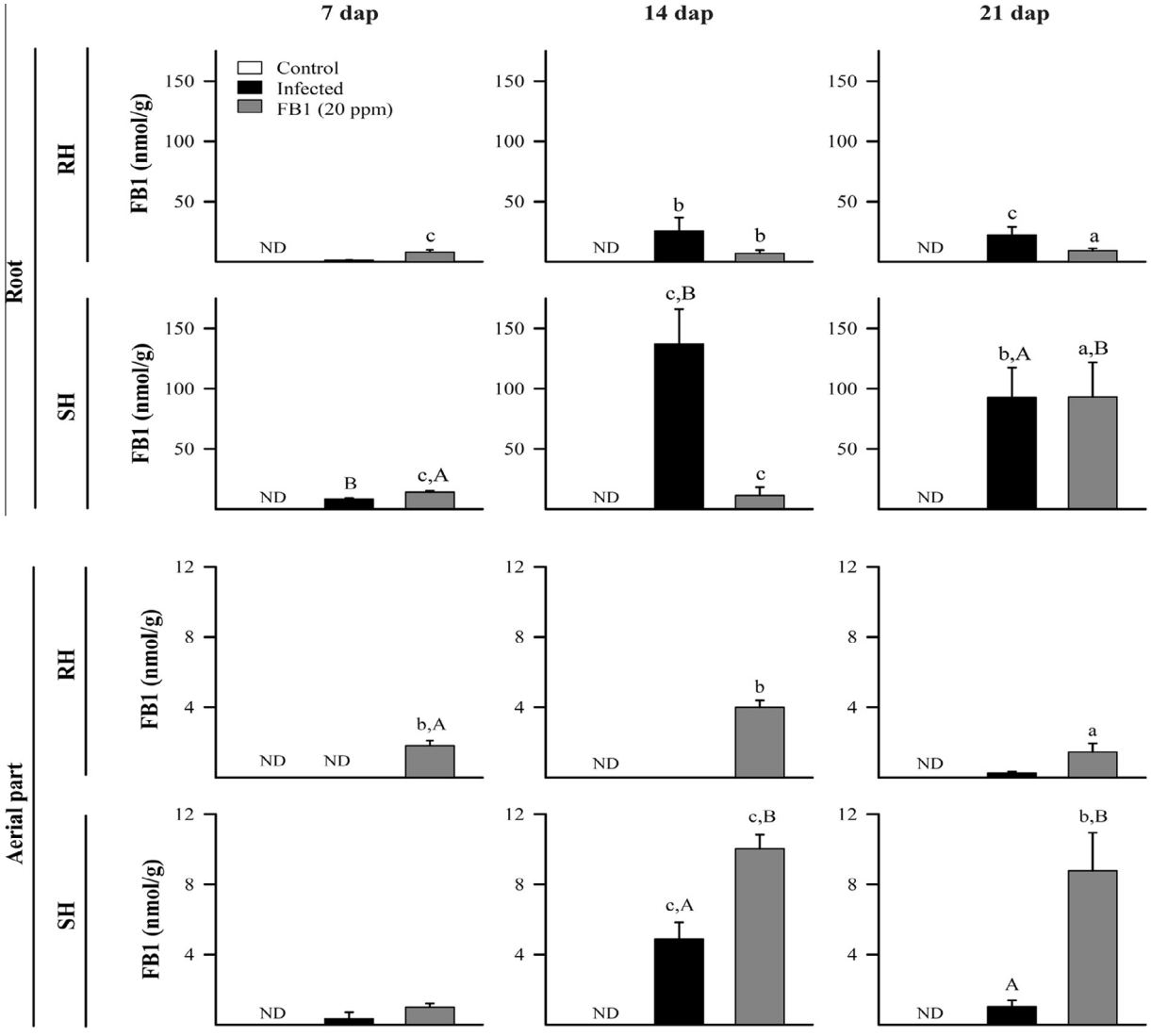

Fig. 2. Fumonisin B1 (**1**) accumulation in root and aerial parts of the RH and SH maize seedlings grown from kernels inoculated with *F. verticillioides* RC2024 and from uninoculated seeds watered with FB1 (1). Data are represented as means ± s.e. $^{a/A}p < 0.05$; $^{b/B}p < 0.01$; $^{c}p < 0.001$. Lower case letters denote differences between treatments and control. Capital letters indicate differences between hybrids for the same treatment. ND: Not detected.

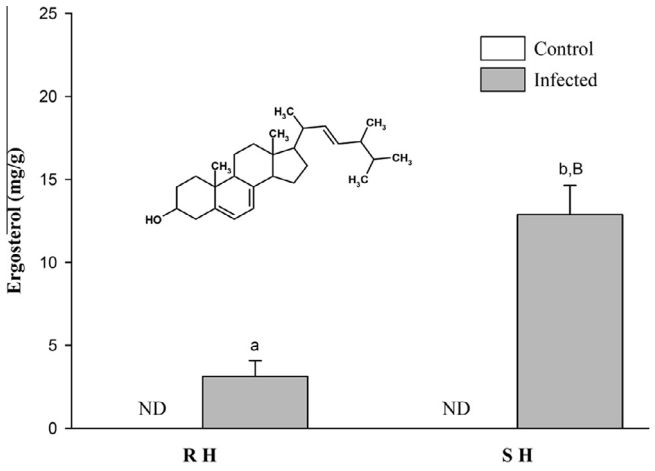

Fig. 3. Ergosterol (**7**) accumulation in roots from RH and SH maize seedlings grown from kernels inoculated with *F. verticillioides* RC2024 at 21 dap. Ergosterol (**7**) chemical structure and levels in roots. Data are represented as means ± s.e. $^{a}p < 0.05$; $^{b/B}p < 0.01$. Lower case letters denote differences between treatments and control. Capital letters indicate differences between hybrids for the same treatment. ND: Not detected.

with those in the roots at 14 and 21 dap. Moreover, while the FB1 (**1**) levels in infected roots of SH and RH seedlings were higher, or at least similar, to those found in the watering with FB1 (**1**) at 14 and 21 dap, the opposite was observed in aerial parts.

Summing up, in general the FB1 (**1**) levels were higher in SH than in RH seedlings, regardless of treatment (inoculation or watering with FB1 (**1**)).

2.2. Sa (*4*) unbalances in seedlings infected or watered with FB1 (*1*)

With the aim of analyzing FB1 (**1**)-induced imbalances in the sphingolipid metabolism, specific endogenous levels of Sa (**4**) and Pso (**5**) were quantified in maize seedlings of both hybrids inoculated with *F. verticillioides* or irrigated with this mycotoxin using LC–ESI-MS. The superposition of representative extracted ion chromatograms of Sa (**4**) at *m*/*z* 302.2 from a control root with that of FB1 (**1**)-irrigated and infected roots at 14 dap, indicated the increased peak heights in the last two samples compared with the control, with higher levels observed in RH than in SH seedlings (Fig. 4). The Sa (**4**) basal levels were similar in both hybrids at the three endpoints studied, both in the roots and aerial parts (Fig. 5). At 7 dap, only the irrigation with FB1 (**1**) increased the concentrations of this sphingoid base, while seedling infection did not alter this parameter.

Notably, the Sa (**4**) peaks were observed at 14 dap, increasing their concentrations 56-fold in RH infected roots, whereas this rise was much less for SH (seven fold compared to control). The imbalances were weaker in the aerial parts than in underground tissue, with the maximum Sa (**4**) value also being observed in RH at 14 dap, but in seedlings watered with the toxin. Infecting this hybrid, however, in general did not modify the Sa (**4**) contents in aerial parts, not just at 14 dap, but also throughout the period studied. In contrast, SH infection induced accumulation of Sa (**4**). Overall, at 21 dap, the Sa (**4**) levels remained elevated in roots and aerial parts in both experimental models (infection and phytotoxicity), although at lower concentrations than those observed at 14 dap.

2.3. Pso (*5*) changes in seedlings infected or watered with FB1 (*1*)

Fig. 6 depicts the superposition of representative extracted ion chromatogram of Pso (**5**) at *m*/*z* 318.2, from control roots with that of FB1 (**1**)-irrigated or infected roots, with the increases induced by both treatments being shown. The Pso (**5**) basal concentrations were higher in roots than aerial parts, and slightly greater in RH seedlings (Fig. 7). The Pso (**5**) bioaccumulation kinetics were similar to those observed for Sa (**4**), as they increased at 14 dap, before descending by the end of the two experimental models (infection or phytotoxicity).

At 7 dap, the infection raised the Pso (**5**) concentrations 4.8 times higher in SH roots, but had no effect on RH roots. Irrigation with FB1 (**1**) also modified the Pso (**5**) levels, but in this treatment the increases were recorded in both the roots of SH (11.8-fold) and

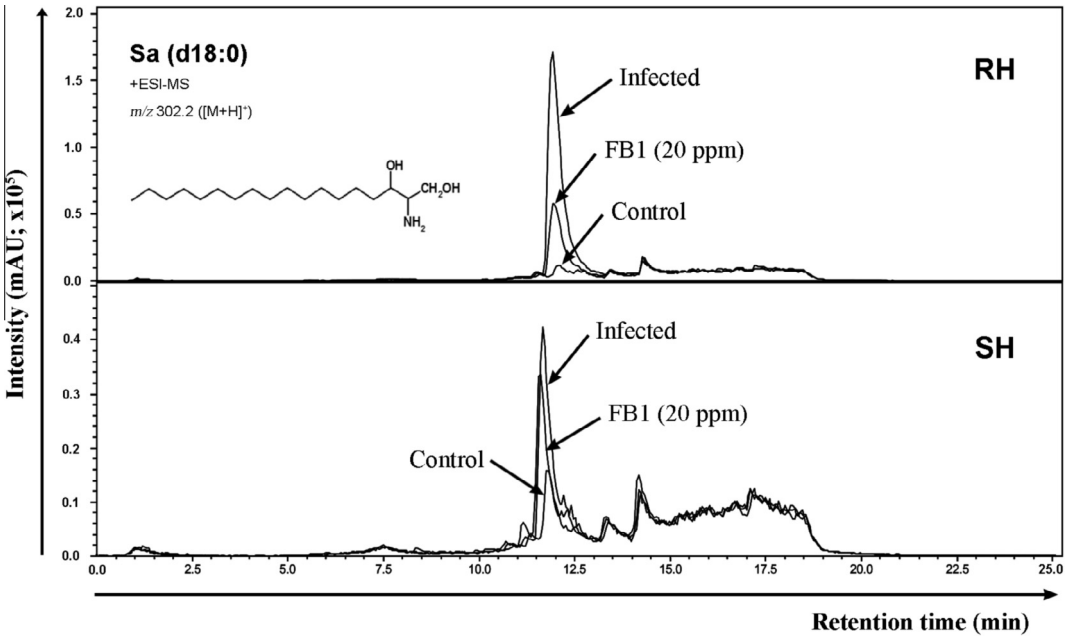

Fig. 4. Effects of *F. verticillioides* inoculation and FB1 (**1**) watering of RH and SH on Sa (**4**) levels in roots at 14 dap. Sa (**4**) chemical structure and typical LC-ESI-MS extracted ion chromatograms at *m*/*z* 302.2 (Sa) (**4**).

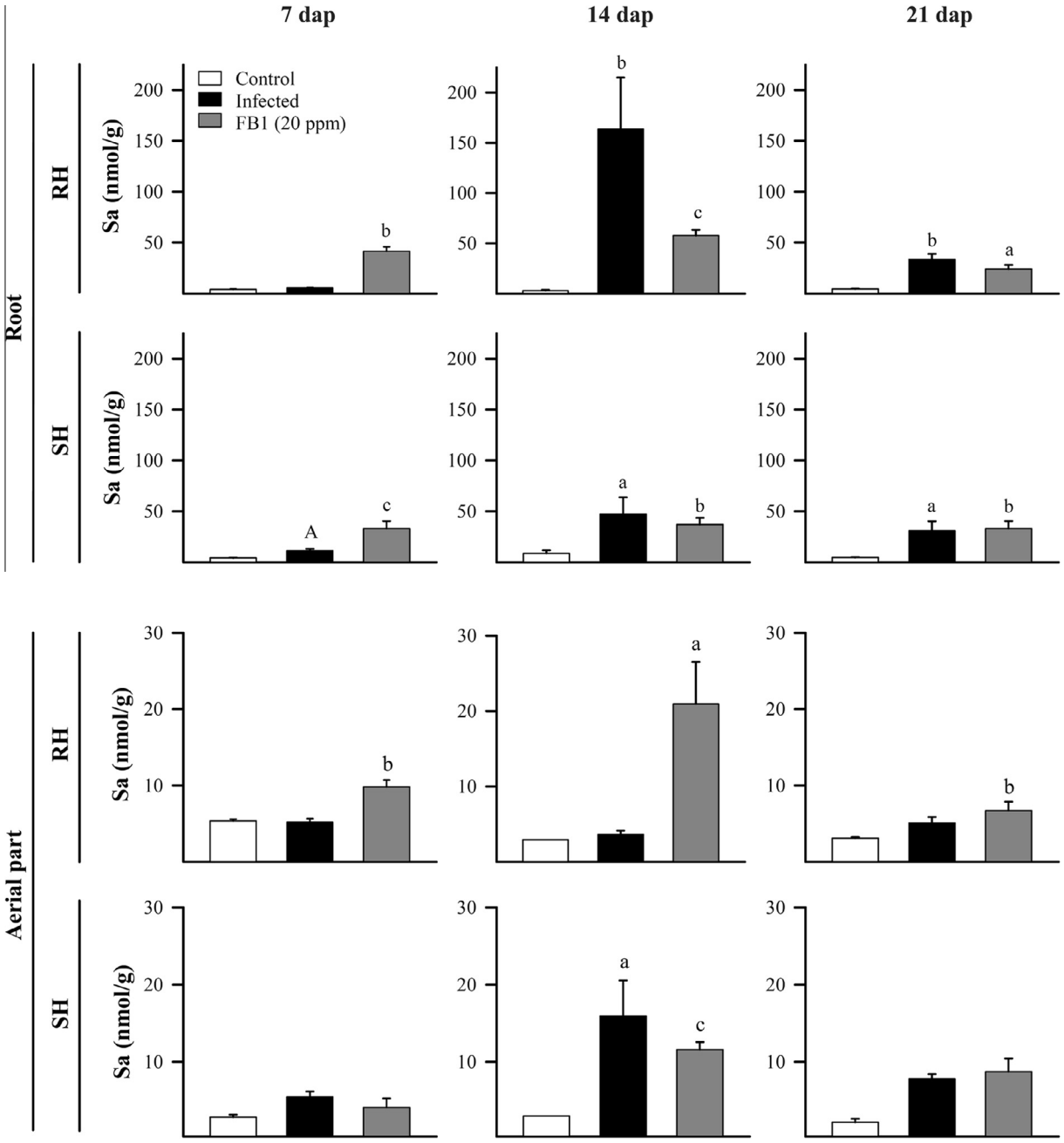

Fig. 5. Free Sa (**4**) accumulation in root and aerial parts of the RH and SH maize seedlings grown from kernels inoculated with *F. verticillioides* RC2024 and from uninoculated seeds watered with FB1 (**1**). Data are represented as means ± s.e. $^{a/A}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$. Lower case letters denote differences between treatments and control. Capital letters indicate differences between hybrids for the same treatment. ND: Not detected.

in the roots and aerial parts of RH (5.1 and 2.5-fold, respectively). At 14 dap, important results were found in the aerial parts. While inoculation with *F. verticillioides* and irrigation with FB1 (**1**) increased the Pso (**5**) basal levels in SH by 7.3 and 10.1 times, respectively, these increases in RH were significantly weaker (only 2 and 3 times, respectively). These differences were maintained at 21 dap.

3. Discussion

In the present study, FB1 (**1**) was detected at measurable levels in both the roots and aerial parts of maize seedlings resistant and susceptible to *F. verticillioides* ear rot, developed from *F. verticillioides* RC2024-inoculated seeds and from uninoculated seeds irrigated with this mycotoxin. However, using a similar experimental design, Zitomer et al. (2010) found no FB1 (**1**) or FB2 (**2**) in the aerial parts of susceptible maize seedlings irrigated with an aqueous solution of fumonisins, suggesting that these mycotoxins were not transported from the roots to other parts of plants. Recently, Baldwin et al. (2014) detected FB1 (**1**) in the first and second leaves of maize seedlings grown from seeds inoculated with an aconidial toxigenic mutant of *F. verticillioides*, which was unable to colonize aerial tissues, suggesting that root infection, but not leaf fungal colonization, is necessary for FB1 (**1**) accumulation in aerial parts. Nevertheless, the results of our study are not in line with this hypothesis, since FB1 (**1**) was found in leaf tissues of maize seedlings from both hybrids watered with the toxin. The possibility of seedling infection by FB1 (**1**)-producing strains was monitored

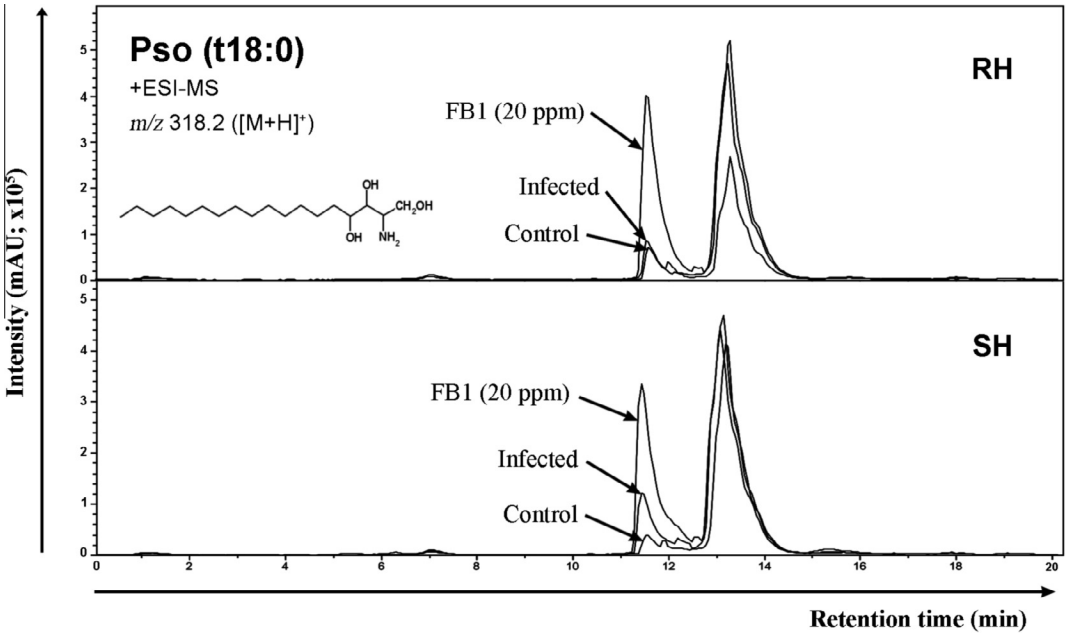

Fig. 6. Effects of *F. verticillioides* inoculation and FB1 (**1**) watering of RH and SH on Pso (**5**) levels in roots at 7 dap. Pso (**5**) chemical structure and representative LC-ESI-MS extracted ion chromatograms at *m/z* 318.2 (Pso) (**5**).

and excluded based on fungal isolation and microscopic examination from tissues, which indicated the plants were not infected (Arias et al., 2012). These findings show that the toxin can be distributed to tissues distal from the site of synthesis (infection), and entry (soil contamination) in maize seedlings. At the same time, our findings might not disagree with those recorded by Zitomer et al. (2010) as, in the latter study, several factors may have contributed to the fumonisins not being detected in the aerial parts of maize seedlings. These include the lower FB1 (**1**) doses administered (15.7 ppm FB1 (**1**); vs 20 ppm, this work), the possible interaction of fumonisin B1 (**1**) with organic soil components (commercial potting soil mix; vs sand, this work) that may therefore reduce its bioavailability, and the intrinsic phenotypic properties of the maize genotype (Silver Queen; vs HX 31P77 and LT 622 MG, this work) used by these researchers, among others.

There is limited information regarding the levels and half-life of FB1 (**1**) naturally occurring in soils. Abbas et al. (2008) reported 3.5–216 ppm of total fumonisins in soil-surface debris corn, and other toxin sources are not discarded. Since these toxins are heat and light stable, then it is possible that they accumulate to high levels in soils. Related to this, Waśkiewicz et al. (2015) found FB1 (**1**) in water courses adjacent to croplands. In this study, the FB1 (**1**) exposure level for watering experiments was chosen on the basis of previous work where it was found that 20 ppm of FB1 (**1**) reproduced some of the maize seedling disease symptoms caused by its *F. verticillioides* infection.

The results here further suggest that FB1 (**1**) entering through irrigation is more easily transported to the seedling aerial parts compared with that produced *in situ* by the fungus. Supporting this idea, while at 14 dap the FB1 (**1**) levels in RH roots were higher in the infection than in the toxicity model, the opposite was observed in the seedling aerial parts, where the toxin accumulation was significantly higher when administered through irrigation. This trend was repeated in the FB1 (**1**) levels in the SH at 14 dap, and a similar behavior was found in both hybrids at the final endpoint tested. However, this phenomenon was not identified at 7 dap, probably due to the low toxin levels synthesized in the infection for this short time of seedling growth. Nevertheless, at this endpoint, it is expected that the FB1 (**1**) administered via watering was quite higher than that produced by the fungus in the infection model.

In this work, the FB1 (**1**) levels in SH roots from infected seedlings at 14 dap were higher than those previously informed by Baldwin et al. (2014), Zitomer et al. (2008) and Williams et al. (2007) in similar experimental models. This could be explained, at least in part, by the probably higher fungal biomass infecting the SH roots from seeds inoculated with 10^{12} spores/ml (this work), with regard to those from seeds inoculated with 10^{4} - spores/ml (previous reports). Undoubtedly, several other factors including the fungal strains and the maize genotypes used in these studies may have contributed to such differences.

The FB1 (**1**) taken from the ground was distributed more efficiently than that produced in infected seedling tissues due to several possible factors. In the former, the toxin may enter as a contaminant of the water absorbed by the root through a permeation process, whose selectivity is determined primarily by the phenotype and by the root physiological state, easily accessing the plant vasculature. Moreover, during infection, the fungal metabolism might release the FB1 (**1**) slowly to other anatomical sites, with only the toxin fraction that exceeds the plant detoxification mechanisms (that include the toxin confinement and subcellular immobilization, the chemical transformations or masking, and the root exudation), being distributed to distal tissues. The FB1 (**1**) levels were lower at 21 dap than at 14 dap in both experimental models, but this might have been due to a further increase of plant biomass, rather than to higher FB1 (**1**) detoxification. Nevertheless, at 21 dap mycotoxin was detected in sand where infected maize seedlings were grown, possibly due to an extended fungal development from internal root tissues to their surfaces, although FB1 (**1**) detoxification by radical exudation is not discarded.

In general, the FB1 (**1**) analysis showed a higher toxin accumulation in SH than in RH, which was observed in both foliar and root tissues. Regarding infection, this can be determined by the hybrid chemical environment stimulus on fungal growth or FB1 (**1**) biosynthesis. Notwithstanding, the lack of significant differences in the FB1/ergosterol (**1/7**) ratio indicates that the higher FB1 (**1**) levels in SH may have been a consequence of an increased fungal

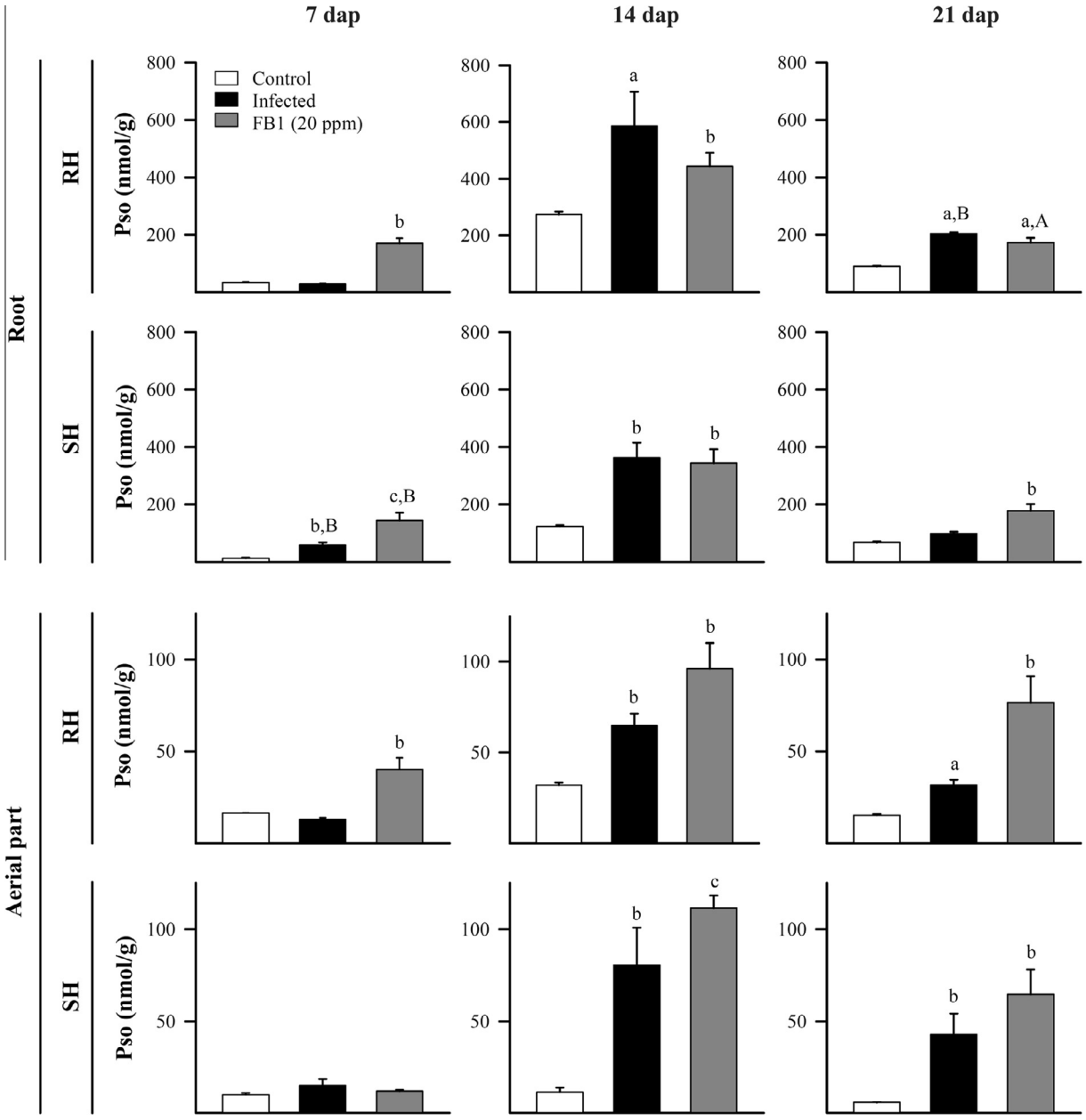

Fig. 7. Free Pso (**5**) accumulation in root and aerial parts of the RH and SH maize seedlings grown from kernels inoculated with *F. verticillioides* RC2024 and from uninoculated seeds watered with FB1 (**1**). Data are represented as means ± s.e. $^{a/A}p < 0.05$; $^{b/B}p < 0.01$; $^{c}p < 0.001$. Lower case letters denote differences between treatments and control. Capital letters indicate differences between hybrids for the same treatment. ND: Not detected.

development rather than to a presumptive modulation of the fumonisin synthesis. Moreover, the results in the toxicity model could be showing that the increased root non-selective permeability (Arias et al., 2012) and the lowest detoxifying capacity (including masking), had contributed to this toxin bioaccumulation in SH.

FB1 (**1**) accumulation was related to an altered sphingolipid metabolism in both experimental models (infection and phytotoxicity), which were mostly observed in roots. The sphingolipid imbalance occurred at FB1 (**1**) concentrations greater than 1.5 nmol/g, since at these contamination levels, roots of infected RH seedlings did not induce alterations in the sphingoid bases.

The levels in sphingoid bases (**2**, **3**) increased in a dose-dependent manner with regard to the mycotoxin, with some exceptions. In these cases, the absence of a correlation may have been due to several reasons, including a possible plant lipidic metabolism sensitization after the first contact with the fumonisins. This phenomenon has been described by Wang and colleagues (1999) in experimental animals, in which the authors showed that after the first FB1 (**1**) exposure, toxin levels 10 times lower were sufficient to cause the same effect on lipid imbalance in rats. Moreover, the sphingoid bases are presumably diffusible compounds able to be disseminated to different parts of the plant (Zitomer et al., 2010), thereby hampering the observation of a FB1 (**1**) localized toxic effect.

The alterations caused by FB1 (**1**) on lipid metabolism were different in both hybrids. In RH, the toxin induced a higher Sa (**4**) accumulation, which was particularly observed at 14 dap, while Pso (**5**) was the main sphingoid base that increased in SH, with this

effect, being detected at 7 dap. These results suggest that in maize, similar effects could be occurring as those described in *Arabidopsis thaliana*, where three CS isoforms (LOH1, LOH2 and LOH3) were identified, although LOH3 and LOH1 have high homologies and use the same substrate (Ternes et al., 2011). The CS-I (LOH2) preferentially acylates Sa (**4**) with C16 fatty acids (16:0-CoA) primarily for glucosylceramide synthesis, while CS-II (LOH1 and LOH3) acylates Pso (**5**) with very long chain fatty acid VLCFA-CoAs for glucosylceramide and glycosylinositolphosphoceramide synthesis.

Several mechanisms might contribute to the resistance or susceptibility of maize seedlings to *Fusarium* spp. infection. Among them, it could have been due to the differential expression of CS in the maize hybrids used in this work, although FB1 (**1**) may inhibit the same proportion of both isoforms. If this is the case, the results of this study may indicate an increased expression of CS-II in RH, leaving insufficient active CS-I enzyme to avoid an increase in Sa (**4**). Nevertheless, the remaining CS-II activity after the initial FB1 (**1**) toxic action might be only barely sufficient to maintain Pso (**5**) basal levels unchanged; subsequently, this sphingoid base would increase significantly by a strong CS-II inhibition secondary to a major accumulation of this mycotoxin. This hypothesis would then explain the results found in RH infected roots at 14 dap, where FB1 (**1**) induced a marked Sa (**4**) accumulation and a slight rise in Pso (**5**) amount relative to the control (54 and 2-fold, respectively). Moreover, the opposite may be happening in SH, where there would be a greater CS-I expression. If this were true, once FB1 (**1**) began to accumulate in the tissues, its toxic action would lead to scarce biologically active CS-II enzyme, resulting in accumulation of its substrate (Pso) (**5**). However, the results could also suggest that for SH, the CS-I expression may only be slightly higher than that of CS-II, since at 7 dap FB1 (**1**) increased 5 and 2.8-fold the Pso (**5**) and Sa (**4**) levels in infected roots, respectively.

Saucedo García et al. (2011), suggested that FB1 (**1**) may inhibit the different CS isoforms in *Arabidopsis*, at varying efficiencies with a higher toxin affinity for CS-I, and showing less inhibition of CS-II, with a consequent rise in Sa (**4**) levels. Although, this may also occur in maize, the sole difference of FB1 (**1**) affinity for CS-I and CS-II, does not explain the differential Sa (**4**) and Pso (**5**) imbalances registered in this work in RH and SH, respectively. Therefore, the results of our study support the idea that CSs may be differentially affected in RH and SH as a result of difference in expression levels after FB1 (**1**) exposure, but the connection with resistance and susceptibility is not clear. This shows the importance of exploring further the possible existence of more than one CS isoform in maize, and the probable relationships of their differential expressions with plant resistant and susceptible phenotypes in the pathosystem *Zea mays–F. verticillioides*.

Several studies have shown certain sphingolipid metabolites to be key molecules in the biochemical pathways that lead to death in animal (CAST, 2003) and plant (Berkey et al., 2012) cells. PCD appears to be promoted by the sphingoid bases increase and particularly by Sa (**4**), since the plant treatment with paraquat or FB1 (**1**) increased Sa (**4**) levels and produced a rise in cell death (Markham et al., 2013). Furthermore, the *Arabidopsis* spp. mutant *lcb2a-1* (gene encoding the serine palmitoyl transferase) prevented any increase in Sa (**4**) and PCD was observed in wild type plants irrigated with FB1 (1), suggesting the involvement of this sphingoid base in cell death induction (Saucedo García et al., 2011).

To date, few studies have reported the participation of Pso (**5**) in PCD activation. Shi and co-workers (2007) informed that Pso (**5**) efficiently induced cell death in a direct feeding experiment, with an *Arabidopsis* FB1-resistant mutant exhibiting attenuated sphingoid base formation and being incapable of initiating PCD when challenged by this mycotoxin. Other studies have indicated that Pso (**5**) is not an intermediary leading to the PCD (Chen et al., 2008; Saucedo García et al., 2011). Therefore, the precise Sa (**4**) and Pso (**5**) contributions to PCD activation in plants remain mostly unknown, although it is clear that subtle differences in sphingoid base structures determine cell fates, by conferring resistance or weakening the host against the pathogen.

4. Conclusions

In this investigation, it was shown that FB1 (**1**) can be absorbed by maize seedling roots from soils contaminated with the toxin, which is often disseminated to a greater or lesser extent to exert its phytotoxic action even in distal tissues, thereby conditioning the progress of the fungus–plant interaction before the first contact between *Fusarium* spp. and maize. The FB1 (**1**), taken from the soil or *in situ* synthesized in tissues infected by the pathogen, induced alterations in the sphingolipid metabolism suggesting the existence of at least two CS isoforms in maize with different substrate specificities, which might be differentially expressed, after FB1 (**1**) exposure, in hybrids susceptible and resistant to infection by *Fusarium* spp. In RH, FB1 (**1**) significantly raised the Sa (**4**) levels, which in turn could activate localized cell death pathways as a plant response, in an attempt to eliminate the pathogen, thus leading to a phenotypically resistant defense response. However, the Pso (**5**) level increase induced by the toxin in SH might stimulate biochemical pathways inefficient at eliminating the pathogen, thereby leading the susceptible phenotype to *Fusarium* spp. pathogenesis.

5. Experimental

5.1. Chemicals and reagents

A certified analytical standard soln. containing both fumonisin B1 (FB1) (**1**) and B2 (FB2) (**2**) was provided by Biopure (Tulln, Austria), and fumonisin B3 (FB3) (**3**) standard was purchased from PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, Republic of South Africa). Phytosphingosine (Pso) (**5**) and sphinganine or DL-erythrodihydrosphingosine (Sa) (**4**) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), with all other chemicals being provided by Sigma–Aldrich (Buenos Aires, Argentina). A soluble fertilizer, with a composition of 15% N [6.5% nitrate, 8.5% ammonia], 15% P as P_2O_5, 15% K as K_2O and 3.2% S was obtained from YARA (Buenos Aires, Argentina). CH_3CN and MeOH were of HPLC quality (Sintorgan, Argentina), and the other solvents used in this work were of analytical grade. Ultrapure H_2O was obtained from Arium 611 UV (Sartorius, Germany) purification system.

5.2. Fungal strain

A wild-type toxigenic isolate of *F. verticillioides* (RC2024), obtained from carnation leaf-agar by monosporic isolation, was used in all experiments. This strain was isolated from maize in Argentina and is deposited in the National University of Río Cuarto (RC), Córdoba, Argentina Culture Collection Centre. All cultures were maintained in 15% glycerol at −80 °C. The RC2024 strain produced fumonisins at a ratio of FB1:FB2:FB3 (1:2:3) of 88:5:7 (Arias et al., 2012).

5.3. Inoculum preparation

Conidia suspensions, prepared with *F. verticillioides* RC2024 cultures grown at 25 °C for 7 days in potato dextrose agar (PDA, Oxoid) (seed inoculation) or in V8 juice agar (fumonisin production in bioreactor) and Tween 20 at 2.5% (v/v) in sterile H_2O, were used as inocula. The conidial concentration was standardized using a spectrophotometer set at an absorbance of 0.1 at 600 nm, with this

density representing 10^{12} cfu/ml. Conidial viability was confirmed by the standard plate count method using dichloran rose bengal chloramphenicol agar (DRBC).

5.4. Fumonisin production in bioreactor

The fumonisins were produced in liquid Myro medium as previously described (Arias et al., 2012) for use in the seedling assay that is described below (Phytotoxicity model). The fermentor vessel (10-L glass stirred-jar) (New Brunswick Scientific Co., Inc. Edison, NJ, USA) containing sterilized Myro medium ($(NH_4)_2HPO_4$ (1 g), KH_2PO_4 (3 g), $MgSO_4{\cdot}7H_2O$ (2 g), NaCl (5 g), sucrose (40 g) and glycerin (10 g) in 1 L distilled-H_2O) (Dantzer et al., 1996) was inoculated with the conidia suspension and maintained at 28 °C with 120 rpm agitation. Aerobic conditions were maintained using a stir rate and an air flow rate of 2 standard liters per minute. The pH was continually monitored during the fermentation by a gel-filled pH probe, and maintained within the 3.5 ± 0.1 range by a controller which operates peristaltic pumps, assigned to perform 0.1 M H_3PO_4 or 0.1 M NaOH addition, and incubation was carried out for 28 days. The fermented liquid medium was autoclaved and then clarified through a 0.45 μm filter. A sample of the filtrate was used for fumonisin quantification.

5.5. Fumonisin quantification in fermented Myro medium

HPLC with fluorescence detection was used to quantify fumonisins produced in bioreactor. Samples of the fermented Myro medium were diluted with CH_3CN at a 1:1 ratio, and the quantification of the diluted extracts was determined following a methodology proposed by Shephard et al. (1990). An aliquot (50 μL) of the diluted samples was derivatized with *o*-phthaldialdehyde (200 μL) soln., obtained by adding 0.1 M sodium tetraborate (5 ml) and 2-mercaptoethanol (50 μL) to MeOH (1 ml) containing *o*-phthaldialdehyde (40 mg). The derivatized samples were analyzed by means of a Hewlett Packard series 1100 HPLC system, with a loop of 20 μL and an isocratic pump (G1310A) coupled with a fluorescence detector (Agilent Technologies series 1200), at wavelengths of 335 nm and 440 nm for excitation and emission, respectively. The column used was a 150 × 4.6 mm, 5 μm, Luna 100 RP-18, with a guard column of the same material (Phenomenex, Torrance, CA, USA). The mobile phase was MeOH-0.1 M NaH_2PO_4 (75:25), with the pH being set at 3.35 ± 0.20 with *o*-phosphoric acid, and a flow rate of 1.5 ml/min used. The quantitation of fumonisins was carried out by comparing the peak areas obtained from samples with those corresponding to analytical standards of FB1 (**1**), FB2 (**2**) and FB3 (**3**) (purity > 95%), using HP Chemstation Rev. A.07.01 software.

5.6. Maize seedling assay

The maize (*Z. mays* L.) seedlings were obtained by sowing seeds of a resistant hybrid (RH; LT 622 MG) and a susceptible hybrid (SH; HX 31P77), which have shown resistance and susceptibility to *Fusarium* ear rot in the field, respectively (Presello et al., 2009).

Maize kernels were treated as described previously (Arias et al., 2012). Briefly, the seeds were surface-disinfected for 2 min in 10% bleach (0.4% NaOCl), rinsed three times with sterile H_2O and blotted dry on paper toweling. To avoid any potential problems with fungicide residues, maize seed without fungicide treatments was used.

In order to evaluate the *F. verticillioides*-maize interaction and the participation of fumonisins as a factor of pathogenesis, RH/SH maize seedlings from seeds inoculated with *F. verticillioides* (Infection model) and non-inoculated seeds watered with fumonisin solutions (Phytotoxicity model) were grown up to 21 days after planting (dap). In the infection model, inoculations were performed by placing sterilized seeds in a Petri dish (100 mm) and flooding them with sterile phosphate buffered saline (PBS, (15 ml) pH: 7.4) (control) or with the fungal conidial suspension, prepared as previously described. The seeds were incubated overnight at 28 °C, and the decontamination efficiency of NaOCl and the percentage of infection were evaluated in grains randomly selected and seeded directly on Petri dishes containing DRBC.

The inoculated and non-inoculated seeds (three replicates of 10 seeds each) were sown in 24-cm diameter pots containing washed autoclaved sand, thus mimicking the simplest soil system containing very little organic material or mineral nutrients. A soluble fertilizer was applied before planting and also twice a week thereafter. Pots were watered every 3 days with sterile H_2O, except for the groups exposed to the fumonisins in the phytotoxicity model, in which the plants were watered with FB1 (**1**) soln. (20 μg/ml in sterile H_2O, 100 ml) on days 2, 4, and 6 after planting, and then watered every 3 days with sterile water. The plants were grown under controlled conditions in a greenhouse with a 14/10 h light/dark cycle at 22 °C, and harvested 7, 14 and 21 dap. Upon harvesting, the sand was carefully separated from the root mass, collected, allowed to dry in a fume hood, and then stored at −20 °C until FB1 (**1**) analysis (only for Infection model, 21 dap). Maize seedlings from all groups and endpoints were also collected for FB1 (**1**), free sphingoid bases (Sa (**4**) and Pso (**5**)), and ergosterol (**7**) quantification. The seedlings were gently washed and the roots were separated from the aerial parts of the plants. Both the roots and the aerial parts were ground to a powder after cooling with liq. N_2, before being lyophilized and kept at −80 °C until use. Because fumonisins are water-soluble compounds, then most if not all, the fumonisins external to the roots should have been removed by the water washing.

The fumonisin B1 (**1**) and ergosterol (**7**) levels were measured in soils and in maize root tissue samples, respectively, from groups inoculated (and uninoculated, control) with *F. verticillioides*, and harvested at the 21st dap. Furthermore, FB1 (**1**) and the free sphingoid bases Sa (**4**) and Pso (**5**) were measured in seedlings (roots and aerial parts) from all groups, at 7, 14 and 21 dap.

5.7. Ergosterol (**7**) quantification

This was performed following a methodology proposed by Young (1995). Samples (50 mg of lyophilized maize root tissue samples) were suspended in MeOH (2.0 ml), placed in a 17 ml tube, treated with 2 M aq. NaOH (0.5 ml) and tightly sealed with a Teflon-lined screw cap. These tubes were then placed within screw-capped 250 ml plastic bottles, which were then tightly sealed. This combination was then placed at the center of a household microwave oven operating at 2450 MHz and 750 W, maximum output, and heated at 50% power for 35 s, x3. After providing sufficient time for the samples to cool, CA. 15 min, the tubes were removed from the plastic outer bottles. Their contents were neutralized with 1 M HCl, before being extracted with pentane (3X CA. 2 ml). The combined pentane extracts were evaporated to dryness, and then made up to the appropriate volume (500 μl) in the mobile phase prior to analysis. Ergosterol (**7**) concentrations were assayed through a HPLC-Hewlett Packard 1100 equipped with a UV detector Konik Instrument UVIS 200 set at 282 nm. A C18-RP column (Phenomenex-C18, 150 × 4.6 mm, 5 μm) was used and the mobile phase was CH_3CN–MeOH (80:20) at a flow rate of 1.3 ml/min. The quantitation of ergosterol (**7**) was carried out by comparing the peak areas obtained from sam-

ples with those corresponding to the analytical standard (purity > 95%), using HP Chemstation Rev. A.07.01 software.

5.8. Extraction and quantification of FB1 (**1**), Sa (**4**) and Pso (**5**) from soils and maize seedlings

5.8.1. FB1 (**1**) extraction from soils

Fumonisins were extracted from the soil where *F. verticillioides*-inoculated seedling were grown, following a procedure published by Williams et al. (2006). Briefly, the dried sand (2 g) was placed into conical tubes (10 ml), and H_2O (5 ml) were added to each tube. These tubes were placed on a rotary shaker for 17 h, and then samples were centrifuged at 3000 rpm (20 min). A supernatant fraction (300 µl) was transferred to polypropylene tubes containing CH_3CN (300 µl). All samples were stored at 4 °C until analysis.

5.8.2. FB1 (**1**), Sa (**4**) and Pso (**5**) extraction from maize seedling roots and aerial parts

The fumonisins and the sphingoid bases (Sa (**4**) and Pso (**5**)) were extracted following a procedure used by Zitomer and colleagues (2008). Lyophilized root/aerial part tissue (ca 10 mg) was placed into a polypropylene tube (2 ml), after which, CH_3CN: H_2O + 5% HCO_2H (1 ml, 1:1, v/v) was added, and the tubes were briefly sonicated and then gently shaken for 3 h. The extracts were centrifuged at 12,000 rpm (10 min) and then supernatants were filtered using a PVDF membrane with 0.22 µm pores (Millipore-GVWPO 4700). Samples were stored at 4 °C until analysis.

5.8.3. FB1 (**1**), Sa (**4**) and Pso (**5**) quantification

Measurement of FB1 (**1**) and sphingoid bases in maize seedling roots and aerial parts was performed by LC–MS/MS, according to a methodology proposed by Zitomer et al. (2008) with minor modifications. LC–MS/MS analysis was carried out using a MicroTOFQ II apparatus (Bruker Daltonics, MA, USA), equipped with an ESI ion source operated in positive mode with N_2 as the nebulizing gas (3 psi) and drying gas (7.5 l/min, 200 °C), capillary 6000 V and end plate offset 280 V. Mass accuracy was verified by infusing Na-formate (10 mM, Sigma–Aldrich, Argentina) dissolved in iPrOH: H_2O (50:50). Pure compounds were first characterized by direct infusion to ESI using a syringe pump (Harvard Apparatus 11 Plus) and then recording both, the MS and MS/MS spectra. HPLC was used to separate the extract profiles, utilizing a C18-RP column (Agilent ZORBAX XDB-C18, 50 × 3.0 mm, 1.8 µm). Samples were introduced in the HPLC (20 µL) using an autosampler (Agilent HiP-ALSSL+) and a column oven (Agilent 1200 series, TCC-SL, G1316B) operated at 30 °C. Solvent delivery was performed at 0.3 ml/min by a binary pump (Agilent 1200 SL) using ultra-pure H_2O supplemented with 0.1% HCO_2H (A) and a HPLC-grade CH_3CN with 0.1% HCO_2H (B). The program was started with 10% B, changing to 90% B within 11 min, holding for 4.5 min, returning to 10% B in 0.5 min and keeping this condition for four additional minutes to achieve column stabilization before the next run (total run time 20 min). Eluted compounds were recorded using the auto MS–MS/MS mode with Ar in the collision cell. Resulting ions were analyzed by TOF, recording the total ionic current (TIC) spectra, with multiple reaction monitoring (MRM) modes being used to monitor the transitions from the precursor ions to the most abundant product ions (FB1 (**1**): 722.3 > 704.3, Sa (**4**): 302.2 > 284.3, Pso (**5**): 318.2 > 300.2).

The FB1 (**1**), Sa (**4**) and Pso (**5**) quantifications were carried out by extrapolating the peak areas registered in extracts into calibration curves constructed with commercial analytical standards.

5.9. Statistical evaluation

Data from the toxicity studies were analyzed by a two-tailed ANOVA followed by a post hoc test (Bonferroni Multiple Comparisons), when the data presented homoscedasticity. In some cases, due to a lack of homoscedasticity, a nonparametric comparison was also performed using the Kruskal–Wallis test ($p < 0.05$). Differences were considered to be statistically significant for p values $\leqslant 0.05$. The GraphPad InStat software version 3.01 (La Jolla, CA 92037 USA) was used for the analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2016.02.006. These data include MOL files and InChiKeys of the most important compounds described in this article.

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