TITLE

Cell death induced by fumonisin B1 in two maize hybrids: correlation with oxidative status biomarkers and salicylic and jasmonic acids imbalances.

ABBREVIATED RUNNING HEADLINE

Fumonisin B1 phytotoxicity in maize

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ABSTRACT

Fungal and plant secondary metabolites modulate the host-pathogen interactions. However, the participation of fumonisin B1 (FB1) in the *Fusarium verticillioides*-maize pathosystem is unclear. In this work was studied the cell death, and the reactive oxygen species - phytohormone imbalance interplay and their correlation with the FB1 phytotoxicity in two maize genotypes with contrasting resistance to *Fusarium* ear rot. Resistant (RH) and susceptible hybrid (SH) maize seedlings, grown from uninoculated seeds irrigated with FB1 (1 and 20 ppm), were harvested at 7, 14 and 21 days after planting, and were examined for electrolyte leakage (aerial parts); and for oxidative stress biomarkers (aerial parts and roots). The salicylic/jasmonic acid levels associated to cytotoxicity were further explored in seedlings exposed 24 h to FB1 (1 ppm) in hydroponics, with and without pre-treatment with the antioxidant ascorbic acid (AA). Cell death increased in RH and SH watered with 1 and 20 ppm of mycotoxin, respectively. Both toxin concentrations were pro-oxidant, and the major perturbations were found in roots. The overall plant stress, estimated by an Integrated Biomarker Response index, was higher in plants treated with 20 ppm of FB1, while 1ppm was most stressful in RH at 21 days. Different phytohormone changes were found in both hybrids: salicylic acid increases (avoided by AA) in RH, and jasmonic acid reductions in both germplasms (although prevented by AA pre-treatment only in SH). Cell death induced by FB1 was associated to different phytohormonal regulatory mechanisms in both maize genotypes, some of them mediated by the redox status.

Keywords: mycotoxins; fumonisin B1; maize; phytotoxicity; oxidative stress; phytohormones

Abbreviations

CAT, catalase; dap, days after planting; EL, electrolyte leakage; FB1, fumonisin B1; GPOX, guaiacol peroxidase; MDA, malondialdehyde; $O_2^{\bullet-}$, superoxide radical anion; RH, resistant hybrid; ROS, reactive oxygen species; SH, susceptible hybrid; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; SA, salicylic acid; JA, jasmonic acid; AA, ascorbic acid.

Introduction

Fumonisins are a set of mycotoxins primarily produced by the secondary metabolism of toxicogenic strains of *Fusarium*, mainly *F. verticillioides* and *F. proliferatum*; even though, in recent years, it has also been observed that these fumonisins can be synthesised by some black aspergilli (Frisvad *et al.*, 2011; Susca *et al.*, 2014). The group includes fumonisin analogs belonging to four primary series (FA, FB, FC and FP), although fumonisin B1 (FB1) is undoubtedly the most relevant because of its incidence in maize and its toxicity to human beings and animals (Nelson *et al.*, 1983; Musser, 1996; Proctor *et al.*, 1999; Rheeder *et al.*, 2002; CAST, 2003).

Maize (*Zea mays L.*) has become one of the most important raw materials worldwide, because it is a key crop for food and feed production. It is also used in the industry for starch and oil extraction. Fungal diseases are one of the factors that limit the yield and quality of grains. *Fusarium verticillioides*, an hemibiotrophic fungus, infects maize all over the world causing severe pathologies such as ear, stem, root and grain rot (Lanubile *et al.*, 2014). This fungus attacks stalks, kernels, and seedlings in all stages of development, inducing pre- and post-harvest diseases. Sometimes the damage remains unnoticed, and the infection can spread to the root system and cause seedling underdevelopment. Under certain conditions, it causes root and stem rot, increasing the possibility of overturning. Likewise, *F. verticillioides* can attack and invade developing maize ears and kernels, causing ear rots (Lanubile *et al.*, 2017). Diseases are the result of complex interplays of environmental conditions, and the intrinsic characteristics of both the pathogen and the host (CAST, 2003; Lanubile *et al.*, 2014). About the latter, maize genotypes generally respond differently to the infection by *Fusarium* spp.; some of them are susceptible, while others exhibit greater resistance to the fungal phytopathology (Santiago *et al.*, 2015).

In a previous work we studied if *F. verticillioides* infection under greenhouse conditions, could reproduce the phenotypes of two maize hybrids classified as resistant (RH; LT 622 MG) and susceptible (SH; HX 31P77) to Fusarium ear rot in field (Arias et al., 2012). The fungal infection increased growth parameters in RH, while the opposite effects and higher fungal colonization rates were found in SH. The seedlings presented lesions similar to those observed in the disease developed by *F. verticillioides* in field. Besides, such disease symptoms were reproduced by the irrigation of uninoculated seedlings with FB1 solutions, strongly suggesting the involvement of this mycotoxin in the fungal pathogenesis.

A large number of low-molecular-weight secondary metabolites synthesized by both the fungi and the plants may be involved, at some extent, in the outcome of the plant-pathogen interactions (Pusztahelyi *et al.*, 2015; Selin *et al.*, 2016). While some fungal metabolites are essential for virulence over specific plants, others act as non-host selective toxins that may contribute to pathogenicity. The *F. verticillioides*-maize link at the molecular level is not known in depth; however, several plant and fungal metabolites, including FB1, must be involved in the biochemical communication in both senses. The toxicodynamics of this mycotoxin seems to be, at least, partially shared in animals and plants, and it

would be mainly related to the competitive inhibition of the toxin over the ceramide synthase activity, leading to imbalances in cellular lipids that have structural functions, and are involved in cell signalling (IPCS-WHO, 2000). In a previous work, we found that FB1 induced contrasting lipid imbalances depending on the hybrid resistance-susceptibility to the *F. verticillioides* invasion, mimicking those found in the fungal infection. The toxin significantly raised the sphinganine (Sa) and the phytosphingosine (Pso) levels in maize seedlings from resistant (RH; LT 622 MG) and susceptible (SH; HX 31P77) hybrids to Fusarium ear rot in field. However, in RH, the FB1 induced a greater increase of Sa, whereas in SH, higher levels of Pso were observed, and it was speculated that the Sa increase would favour the pathogen elimination in maize seedlings by activating localized cell death pathways (Arias *et al.*, 2016).

Maschietto and collaborators showed the induction of oxidative stress in ears of resistant and susceptible maize lines inoculated with *F. verticillioides* (Maschietto *et al.*, 2016), and FB1 is probably involved in such outcome. The oxidative stress was induced as a plausible mechanism for the FB1 toxicity in animal and plant cells (Xing *et al.*, 2013; Wang *et al.*, 2016). Studies performed in *Arabidopsis thaliana* pointed out the involvement of reactive oxygen species (ROS) as chemical mediators of lipid-induced cell death, whose levels are increased by exposure to FB1 (Saucedo-Garcia *et al.*, 2011). Moreover, Zhao and co-workers (2015) showed that ROS accumulation caused by FB1 was reduced by breakdown products of indole glucosinolate with antioxidant behaviour.

Despite the fact that several studies showed the phytotoxicity of FB1, the data available about the involvement of this mycotoxin in the phytopathogenesis of maize diseases by *F. verticillioides* are not conclusive. For instance, there is differing information regarding the distribution of the toxin in plants. While some studies suggested that the fungus-plant interaction is necessary for FB1 translocation in maize seedlings (Zitomer *et al.*, 2010), in a recent work conducted by our group, it was observed that the toxin disseminated to the aerial parts of the maize plants when administered via watering (Arias *et al.*, 2016).

Regardless of the toxin distribution throughout the plants, symptoms indicative of disease induced by *F. verticillioides* were found in maize seedlings grown from uninoculated seeds irrigated with FB1 solutions (Williams *et al.*, 2007; Arias *et al.*, 2012), showing that the toxin is probably involved in the pathogenicity of this fungal infection. Moreover, Glenn and collaborators (2008) reported that the ability to develop foliar disease symptoms on maize seedlings by FB1 non-producing strains of *F. verticillioides* was restored in fumonisin-producing transformants, therefore indicating that the toxins contribute to the fungal pathogenesis. Conversely, other studies suggest that *F. verticillioides* do not require the synthesis of fumonisins to cause maize root and ear infections, or to produce ear rot (Desjardins and Plattner, 2000; Dastjerdi and Karlovsky, 2015). Therefore, further research must be conducted in order to elucidate the participation of fumonisins in the *F. verticillioides* invasion and pathogenesis in maize as well as the mechanisms underlying their effects.

Previous studies show that FB1 is an inducer of cell death (Asai *et al.*, 2000; Igarashi *et al.*, 2013; Glenz *et al.*, 2019) by mechanisms not fully elucidated. In *Arabidopsis* spp., FB1 produces a cell death that resembles a hypersensitive response, constituting a plant defense mechanism effective against biotrophic pathogens, which obtain nutrients from the living host, but it is not for necrotrophic pathogens that benefit from dead tissue (del Pozo and Lam, 1998; Govrin and Levine, 2000; Balint-Kurti, 2019; Glazebrook, 2005). In this regard, salicylic acid (SA) is a phytohormone commonly associated with the positive regulation of hypersensitive response-type cell death. It has a central role in defence and induces the activation of pathogenesis-related genes (PR), which generates resistance to a wide range of pathogens (Loake and Grant, 2007; Klessig *et al.*, 2018). The cell death induced by FB1 in *Arabidopsis* is dependent on both, the accumulation of ROS and the synthesis of SA (Xing *et al.*, 2013). Jasmonic acid (JA) may contain the spread of lesions caused by ROS, having this phytohormone an antagonistic effect on SA (Overmyer *et al.*, 2003). However, Zhang *et al.* (2015) showed that the signaling pathway of JA is inhibited by FB1. Despite these studies show a central role of SA in the phytotoxicity of FB1 in *Arabidopsis*, the cell death induced by this mycotoxin, and how ROS and phytohormones, such as SA and JA, modulate this process, must still be explored in depth in plants of agronomic interest such as maize.

In this work we studied the cell death, and the reactive oxygen species (ROS) - phytohormone imbalance interplay and their correlation with the phytotoxicity of FB1 in seedlings of two maize hybrids with contrasting resistance to *Fusarium* ear rot in the field.

Materials and methods

Chemicals and reagents

Fumonisin B1 (FB1) analytical standard (purity > 95 %) was purchased from PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, Republic of South Africa). 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). Acetonitrile and methanol were of HPLC quality (Sintorgan, Argentina), and the other solvents used in this work were of analytical grade. 1,1,3,3-tetramethoxypropane (TEP, ≥ 97 %), 2-thiobarbituric acid (TBA, ≥ 98 %), superoxide dismutase (SOD), guaiacol peroxidase (GPOX) and trichloroacetic acid (TCA) were all purchased from Sigma-Aldrich, Buenos Aires, Argentina. The Bradford reagent was obtained from Bio-Rad Laboratories (Buenos Aires, Argentina). Ultrapure water (Millipore, Milli-Q system) was used to prepare standard solutions, dilutions and blanks.

Fungal strain and inoculum preparation

A wild-type toxigenic isolate of *Fusarium verticillioides* (RC2024) obtained from carnation leaf-agar by monosporic isolation was used for fumonisins production. This strain was isolated from maize in Argentina, and stored in the Culture Collection Centre of the National University of Río Cuarto (RC),

in Córdoba, Argentina. All cultures were maintained in 15 % glycerol at -80 °C. The ability of this strain to produce fumonisins was assessed using maize as the substrate, as previously described (Theumer *et al.*, 2008). The RC2024 strain produced fumonisins at a ratio FB1:FB2:FB3 of 88:5:7.

Conidia suspensions were prepared with *F. verticillioides* RC2024 cultures grown at 25 °C for 7 days in V8 juice agar and Tween 20 at 2.5 % (v/v) in sterile water, and were used as inoculum.

Fumonisin production in bioreactor

FB1 used in the maize seedling assay were produced in culture media, due to the large amount of fumonisin necessary for watering. This strategy has been used in others studies (Zitomer *et al*., 2010) and previously in our group (Arias *et al.*, 2012; Arias *et al.*, 2016). The fermentor vessel (10-L glass stirred-jar) (New Brunswick Scientific Co., Inc. Edison, NJ, USA) containing sterilised Myro medium ($(NH_4)_2HPO_4$ (1 g), KH_2PO_4 (3 g), $MgSO_4.7H_2O$ (2 g), NaCl (5 g), sucrose (40 g) and glycerine (10 g) in 10 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 litres per minute. The pH was continually monitored during 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.

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 performed following a methodology proposed by Shephard *et al.* (1990). An aliquot (50 µL) of the diluted samples was derivatised 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 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 x 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-0.1M 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 was used. The quantitation of fumonisins was carried out by comparing the peak areas obtained from samples with those corresponding to analytical standards of FB1, FB2 and FB3 (purity > 95 %), using an HP Chemstation Rev. A.07.01 software.

Maize seedling assays

Phytotoxicity of FB1 in maize seedlings grown in pots

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).

The maize seeds were surface-disinfected for 2 min in 10 % bleach (0.4 % NaClO), rinsed three times with sterile H_2O, and blotted dry on paper towelling. Then, seeds (three replicates of 10 seeds each) were sown in 24-cm diameter pots containing washed autoclaved sand, thus mimicking the simplest soil system with very little organic material or mineral nutrients (Arias *et al.*, 2012; Arias *et al.*, 2016). A soluble fertilizer was applied before planting and also twice a week thereafter. Pots were watered with FB1 solutions (1 and 20 ppm 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 days after planting (dap). Maize seedlings from all endpoints were collected for measuring electrolyte leakage and oxidative status biomarkers: H_2O_2, antioxidants enzymes (superoxide dismutase, SOD; and guaiacol peroxidase, GPOX) and thiobarbituric acid reactive substances (TBARS). Upon harvesting, leaf discs were immediately obtained from some seedlings (n=6 per group) for electrolyte conductivity measuring. The remaining seedlings were gently washed, and the roots were separated from the aerial parts of the plants. Both roots and aerial parts were ground to a powder after freezing with liquid N_2 and kept at -80 °C until use.

Mechanisms involved in the phytotoxicity of FB1: Hydroponic model

The maize seeds were surface-disinfected as described above. Hydroponic cultures were assayed as described by Zörb *et al.* (2013), with minor modifications. Briefly, SH and RH maize seeds were submerged in a 1 mM $CuSO_4$ solution at 25 °C for 24 hours. Then, they were incubated for 3 additional days between filter paper layers moistened with the same solution. Subsequently, the germinated seeds were transferred to 15 mL Falcon tubes (one per tube), containing hydroponic solution (0.25X). The concentration of this solution was gradually increased to 0.5X and 1X after 2 and 4 days of hydroponic culture, respectively. A hydroponic solution was used (2.5 mM $Ca(NO_3)_2$, 1.0 mM K_2SO_4, 0.2 mM KH_2PO_4, 0.6 mM $MgSO_4$, 5.0 mM $CaCl_2$, 1.0 mM NaCl, 1.0 µM H_3BO_4, 2.0 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.005 µM $(NH_4)_6Mo_7O_{24}$, 200 µM Fe–EDTA), which was changed every two days to avoid total consumption of nutrients. After 14 days, the aerial part of the seedlings was sprayed with 0 and 1 mM ascorbic acid (AA), 2 hours before the mycotoxin treatment (Xing *et al.*, 2013). Then, the seedlings were exposed to 0 (Control) and 1 ppm of FB1 (dissolved in hydroponic solution). They were harvested at 24 hours post-treatment (hpt) with the mycotoxin, conditioned and stored as described above.

Electrolyte leakage

Cell death was assayed by measuring electrolyte leakage (EL) from leaf discs as described by Rizhsky *et al.* (2002), with minor modifications. Briefly, six leaf discs (6-mm diameter) were floated on 10 mL of ultrapure water and shaken at 60 rpm for 2 h at room temperature. Following incubation, the conductivity of the bathing solution was measured with a conductivity meter (CD 4301, Lutron). The solutions were then boiled at 95°C for 25 min to completely lyse the plant cell walls. The electrolyte conductivities of boiled solutions were recorded as the absolute conductivity. The percentage of EL was calculated as the initial conductivity / absolute conductivity x 100.

Hydrogen peroxide

Hydrogen peroxide was measured spectrophotometrically following a procedure published by Alexieva *et al.* (2001). Ground tissues (0.3 g) were homogenized with 0.1 % trichloroacetic acid (1.5 mL), and then centrifuged (12,000 x g for 15 minutes at 4 °C). The reaction mixture consisted of 160 µL of 0.1 % TCA tissue extract supernatant, 160 µL of 100 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.8) and 680 µL of 1 M KI solution in distilled water. Trichloroacetic acid (0.1 %) in absence of tissue extract was used as blank. The reaction was developed for 1 h in darkness, and absorbance measured at 390 nm using a microplate reader (Bio-Tek, Synergy HT). The amounts of H_2O_2 in samples were calculated using a standard curve (range: 0 – 1 mM), and the results were expressed as µmol (Fig 2) H_2O_2/g fresh weight (FW).

Enzyme extraction and measurement

Enzyme extracts were prepared from individual plants according to Monferrán *et al.* (2009), with minor modifications. Ground tissues were homogenized with rupture buffer containing 0.1 M Na_2HPO_4/NaH_2PO_4 pH 6.5, 20 % glycerol, 1 mM EDTA, and 1.4 mM dithioerythritol. After removal of cell debris (10 min at 13,000 g), the supernatant was used for protein (Bradford, 1976) and enzyme measurements, which were determined by spectrophotometry using a microplate reader (Bio-Tek, Synergy HT).

The SOD activity was determined in 96 well plates according to the procedure described by Aiassa *et al.* (2010). Under illumination, riboflavin loses an electron and induces superoxide anion radical ($O_2^{\bullet -}$), which reduces the nitroblue tetrazolium (NBT), but this last step was prevented by the SOD activity. The reaction mixture consisted of 10 µL of protein extract, SOD standard (calibration curve) or rupture buffer (blank); 30 µL of methionine 47.7 mM, 10 µL of NBT 0.825 mM in PBS, 30 µL of EDTA 0.367 µM and 30 µL of riboflavin 7.33 µM. The microplate was exposed to 20W fluorescent light for 30 minutes, and the colour developed was spectrophotometrically measured at 595 nm. The SOD activities in samples were expressed in units/mg protein, extrapolating the readings from samples in a calibration curve made with an analytical standard of SOD (0.25-1.00 µg/mL, equivalent to 1.14-2.56 SOD units/mL).

The GPOX activity was determined using H_2O_2 and guaiacol according to a procedure previously described (Bertrand *et al.*, 2016). Briefly, 180 µL of Na_2HPO_4/NaH_2PO_4 (0.1M, pH 5.0) were mixed with 8.5 µL of guaiacol (100 mM in DMSO) and 8.0 µL of H_2O_2 (200 mM in DMSO). Then 10 µL of protein extract or rupture buffer (blank) were added, and the reaction mixture was incubated at 37 °C. Absorbances (436 nm) were recorded up to 4 minutes of reaction. The GPOX activity was expressed as the $\Delta Abs_{436\ nm}$.mg protein^{-1}.min^{-1}.

Thiobarbituric acid reactive substances

TBARS were determined as indicators of lipid peroxidation according to a methodology proposed by Heath and Packer (1968), with minor modifications. Briefly, 0.5 g of ground tissue (aerial parts and roots) was homogenised with 2.5 mL of TCA 20 % (w/v) and centrifuged at 12,000 g for 4 minutes at 4 °C. Equal volumes of supernatant and reagent (thiobarbituric acid, TBA, 0.5 % dissolved in TCA 20 %) were then mixed. The samples were heated at 95 °C for 25 minutes, cooled in an ice bath, and then centrifuged at 9,000 g for 6 minutes at 4 °C. The absorbance at 532 nm was measured in the supernatant against a TBA blank, subtracting the absorbance of turbidity at 600 nm. The amounts of TBARS were calculated from a calibration curve based on the acid hydrolysis of TEP (0-100 µM) and the reaction with TBA, and the results were expressed as nmol TBARS/g of fresh weight tissue.

Quantification of phytohormones by LC-MS/MS.

The levels of jasmonic acid (JA) and salicylic acid (SA) in the aerial portion of plants were quantified. The extraction was carried out according to the method of Pan *et al.* (2008), with some modifications. Briefly, 0.5-1.0 g of tissue previously pulverized with liquid N_2 were weighed, homogenized with 500 µL of 1-propanol/H_2O/concentrated HCl (2:1:0.002; v/v/v), and stirred for 30 minutes at 4 °C. Then 1 ml of dichloromethane (CH_2Cl_2) was applied, stirred for 30 min at 4 °C, and centrifuged at 13000 g for 5 min. The lower organic phase (approx. 1 mL) was collected in vials, which was evaporated in a gaseous N_2 sequence. Finally, it was re-dissolved with 0.1 - 0.15 mL of 100 % methanol (HPLC grade), and stirred slightly with vortex.

The system of the 1200 series of Agilent technologies (Agilent Technologies, Santa Clara, CA, USA) is equipped with a gradient pump (Agilent G1312B SL Binary), solvent degasser (Agilent G1379 B), auto sampler (Agilent G1367 D SL+WP) and a reversed phase column (C18 kinetex 2,6 µm, 100 mm x 2,1 mm, Phenomenex, Torrance, CA, USA). It is used as a mobile solvent system composed of water with 0.1 % CH_2O_2 (A) and MeOH with 0.1% CH_2O_2 (B), with a correction flow of 0.25 mL/min. The initial gradient of B was maintained at 30% for 2 min, and then linearly increased to 100% at 28 min. For identification and quantification purposes, a mass spectrometer of the microTOF-Q11 Series QTOF (Bruker, Billerica, MA, USA) coupled to the above mentioned HPLC (LC-MS / MS) was used. The ionization source was used with electrospray (ESI) and the Compass (version 3.1) and Data Analysis (version 4.1) programs were used for data acquisition and processing, respectively. The mass spectra of

the data are recorded in negative mode. The mass/charge ratio (m/z) for each metabolite were: SA: 137.02 and JA: 209.12. The quantification of the activity was done by respecting the calibration curves with the linear adjustment, obtaining the results in a nanogram phytohormone/gram of fresh weight.

Data analysis

Integrated biomarker response

In order to achieve a more complete understanding of the seedlings reactions to treatments, an integrated biomarker response (IBR) was calculated with the aim to identify the level of response or stress expressed by the exposed organisms (the higher IBR value, the greater stress and vice versa). In our study, stress biomarkers (EL, from aerial part; H_2O_2, TBARS, SOD and GPOX, from aerial part and root) with greater ability to segregate tested conditions were selected by a discriminant analysis (forward method) using the Statistica Software (version 8.0). The significance of the discriminant analysis was verified considering the Wilk's Lambda values. Because for the calculation of IBR, it is recommended to use a maximum of 9 biomarkers for each experimental condition, but the RStudio statistical software only allows the analysis of up to 7 biomarkers, in cases where the amount of biomarkers selected by the discriminant analysis exceeds this value, the selection was performed based on the partial lambda (λ) values found in the discriminant analysis, since a lower partial λ value represents a greater contribution of the biomarker at the time of segregating the groups.

This IBR was performed according to Beliaeff and Burgeot (2002), with modifications by Devin *et al.* (2014). Briefly, the mean value (Xi), the general mean (mi) and the standard deviation (SDi) were calculated for each biomarker of each hybrid, with each irrigation with FB1, both root and aerial part. The Xi value was then standardized to obtain Yi, where Yi = (Xi - mi) / SDi. Subsequently, Zi = −Yi or Zi = Yi was computed according to the biological effect of inhibition or activation, respectively. The minimum (mini) value of Zi of each biomarker was obtained for each condition. Finally, the S *score* was calculated as Si = Zi + | mini |, where | mini | is the absolute value. The IBR was calculated for each condition using the following formula:

$$IBR = S1 * \frac{S2}{2} + S2 * \frac{S3}{2} + \ldots + Sn - 1 * \frac{Sn}{2} + Sn * \frac{S1}{2}$$

Where the value obtained for each biomarker (Sn-1) is multiplied by the value obtained for the successive biomarker (Sn), ordered and dividing each calculation by 2 and adding all the values obtained.

The calculations were carried out using R Studio Software Version 0.99.902. With the same data set, several IBRs were obtained, through successive permutations that were exchanging the order of the biomarkers. A matrix was obtained with (k-1)! IBR values from which a median could be calculated. The number of permutations varied according to the amount of biomarkers used, obtaining

a matrix of 6 IBR values when using 4 biomarkers, one of 24 values with 5 biomarkers and one of 120 values with 6 of them.

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 < 0.05. The GraphPad InStat software version 3.01 (La Jolla, CA 92037 USA) was used for the analyses.

Results

Phytotoxicity in maize seedlings watered with FB1

Conductivity

Different profiles of cell death were observed between hybrids and levels of exposure to FB1 (1 and 20 ppm). The electrolyte leakage decreased at 14 dap in SH watered with 1 ppm of FB1, and increased in RH, at the same endpoint and mycotoxin concentration (Fig. 1). These alterations were transient, since conductivities remained unaltered in both hybrids at 21 dap. The highest toxin level tested (20 ppm) increased cell death in SH at 21 dap, but had no previous effects on this hybrid or on RH.

Hydrogen peroxide

H_2O_2 was quantified in maize seedlings exposed or not to FB1. In general, little effects were observed in SH watered with the lowest toxin concentration (Fig. 2). H_2O_2 decreased at 7 dap in roots of both hybrids, whereas in aerial parts, a similar outcome was observed only in RH. Moreover, this ROS increased at 14 and 21 dap in aerial parts of RH, while, in roots, similar and lower H_2O_2 levels were found, respectively.

Watering with FB1 20 ppm increased the H_2O_2 in roots of both hybrids in almost all endpoints assessed, except for RH at 21 dap. Nevertheless, in aerial parts, such effects were only found at 7 dap in SH, and at 14 dap in RH.

Antioxidant enzymes

The maize genotype susceptible to infection by *F. verticillioides* was characterized by higher basal SOD and GPOX activities compared with RH, which was evidenced in both roots and aerial parts, and in all endpoints assessed (Table 1). Furthermore, the effects of FB1 on these antioxidant activities were markedly different in both hybrids. In SH, the irrigation with 20 ppm of toxin increased at 7 dap the GPOX enzymatic activities in roots, and SOD in aerial parts; while at 14 dap, the lowest concentration of mycotoxin increased SOD activities in stems and leaves. However, the FB1 effects on

this hybrid were mainly inhibitory of both enzymes. Minor SOD and GPOX activities were recorded at 7 dap in roots of seedlings irrigated with 1 ppm of FB1. Similar changes were caused by both toxin concentrations in roots at 14 and 21 dap and, in aerial parts, in the last endpoint assessed.

Unlike the findings in SH, FB1 increased both antioxidant activities in RH, except for the GPOX decreases registered in roots at 14 dap, and at 21 dap in aerial parts of seedlings exposed to 20 and 1 ppm of toxin, respectively. Both toxin concentrations increased the GPOX throughout the seedling at 7 dap; while 20 ppm had the same effect in stems and leaves at 14 dap, and in both plant parts at 21 dap. In addition, the irrigation with 20 ppm of FB1 increased SOD throughout the seedlings at 7 dap, and in roots at 14 dap.

TBARS

TBARS were measured in order to estimate the lipidic oxidative damages induced by FB1. TBARS were higher at 7 dap, and decreased at 21 dap in roots from both hybrid seedlings watered with 1 ppm of FB1 (Fig. 3). Despite these findings, TBARS were higher in the aerial parts of the RH plantlets at 21 dap.

Similar phytotoxic effects were observed in roots from SH and RH seedlings exposed to 20 ppm of FB1, where the mycotoxin raised the TBARS at 7 and 14 dap. However, a major lipidic oxidation in aerial parts was estimated in both hybrids at 7 (but not 14) dap, and at the last endpoint assessed in SH, but in this case TBARS in roots were lower.

Discriminant analysis and integrated biomarker response

An Integrated Biomarker Response index (IBR) was calculated with the aim to obtain a more complete understanding of biological effects suffered by the tested hybrids. In our study, the biomarkers selected through a discriminant analysis and used to calculate IBR values are informed in the Supplementary Material (Tables S1 and S2).

The IBR calculated for every experimental condition is shown in Figure 4 and in Table 2. The grey areas shown in graphs, delimited by linking the IBR of control and FB1 (1 and 20 ppm) groups, allow a better visualization of the treatment that produced the greatest stress. Both FB1 concentrations used in this study caused significant IBR increases with respect to control. Irrigation with 20 ppm of FB1 induced the greatest IBR in both hybrids, and in almost all the endpoints assessed, with the exception of RH at 21 dap, where the greatest stress was caused by the lowest concentration of the mycotoxin.

Mechanisms involved in the phytotoxicity of FB1: Hydroponic model

Oxidative stress

We studied more deeply the mechanisms involved in the cell death caused by FB1 to maize seedlings. A hydroponic model was chosen for this purpose, due to the minimal interference of sample

manipulation in the results. First, we assessed if the oxidative stress was associated with the phytotoxicity caused by FB1 (1 ppm) in the hydroponic model. The H_2O_2 content was evaluated in both hybrids exposed to FB1, with and without pre-treatment with ascorbic acid (AA), a widely used ROS scavenger. As shown in Fig. 5a, the pre-treatment of seedlings with AA prevented the H_2O_2 increase induced by FB1 in both hybrids, therefore confirming its antioxidant activity.

We also studied if the cell death observed in seedlings grown in pots was also induced by FB1 in hydroponia, and its relation to the oxidative stress. The treatment with the mycotoxin increased the electrolyte leakage (EL) % at 24 hpt in both hybrids, but such outcomes were prevented by the pre-treatment of seedlings with AA (Fig. 5b). The consequences of the FB1 exposure in the SOD and GPOX antioxidant activities were similar to those found in seedlings grown in pots. While the mycotoxin decreased both activities in SH (Fig. 5c and d), the opposite was observed in RH. Nevertheless, such effects were prevented by the pre-treatment with the antioxidant.

FB1-induced cell death: Modulatory effects of ROS on phytohormones

In order to explore the modulatory effects of ROS on phytohormones in the FB1-induced cell death, the levels of SA and JA in both hybrids were quantified. SA remained unaltered in SH seedlings treated with FB1, but JA was decreased (Fig. 6a and b). Moreover, despite the mycotoxin had no effects on SA, it increased the JA levels in the seedlings pre-treated with the antioxidant (with respect to those untreated with AA). In RH, the toxin had opposed effects on SA and JA levels (increase and decrease, respectively), but although the pre-treatment with AA prevented such SA rise, it could not prevent the fall of JA caused by the mycotoxin (Fig. 6a and b).

Discussion

The evidence collected to date shows that different maize plant parts (embryos, seedlings, stalk, root, ears) offer diverse scenarios for FB1 toxicity (de la Torre-Hernandez *et al.*, 2010; Arias *et al.*, 2012; Arias *et al.*, 2016; Maschietto *et al.*, 2016). Fumonisin B1 is a phytotoxin apparently not essential for the pathogenicity of *F. verticillioides* in maize, although it may favour the fungal invasion of vegetal tissues. This mycotoxin is a potent inducer of programmed cell death in plants, and much of the progress in this field was done in *Arabidopsis thaliana* as experimental model (Abbas *et al.*, 1994; Stone *et al.*, 2000; Xing *et al.*, 2013; Glenz *et al.*, 2019).

Having in mind that FB1 can be found in ground with corn debris (Abbas *et al.*, 2008) and drainage water next to croplands (Waskiewicz *et al.*, 2015), the toxin would be present in soils and can debilitate maize seedlings growing on it, even in absence of fungal infection, since it can be absorbed from soil and disseminated throughout the plant to exert its toxicity (Arias *et al.*, 2016). Therefore, we carried out two experimental designs: i) a "chronic phytotoxicity" model to characterize cell death and oxidative status in seedlings grown in pots up to 15 days after exposure to 1 and 20 ppm of FB1 (21 dap); and ii) an "acute phytotoxicity" model in hydroponics to assess the modulating effects of ROS on

SA and JA levels associated to the cell death induced at 24 h of treatment with 1 ppm of FB1. The exposure levels used in this work were chosen on the basis of previous works, where 1 and 20 ppm of FB1 reproduced the phenotype of corn seedlings infected by *F. verticillioides*, although plants could apparently detoxify 1 ppm of FB1 (Arias *et al.*, 2012; Arias *et al.*, 2016). Due to the higher biological relevance of this concentration, it was used for studying the FB1 acute phytotoxicity in maize. Besides, it is important to emphasize that the use of only two hybrids, makes it difficult to extrapolate the results to contrast resistant vs. susceptible genotypes in general.

Cell death may be provoked by mycotoxins as part of the fungal strategies to invade plants. In this study, we observed changes in electrolyte leakage (EL) which depended on the FB1 concentration, the hybrid, and the age of the plants. The highest EL induced at 14 dap in RH watered with 1 ppm of FB1 probably shows that the toxin caused the loss of the plasma membrane integrity, leading to higher ion permeability as the ultimate step in cell death. A similar result could have been observed at 21 dap in SH irrigated with 20 ppm of FB1. However, while in the first case (RH watered with 1 ppm of FB1) the EL increase was transient, we could not clarify whether this parameter returned to values comparable to the control after the last point assessed in SH. The meaning of the lowest EL observed at 14 dap in SH is also unclear. Taken together, these data show that cell death is a chronic toxic effect induced by FB1 in maize seedlings regardless of the hybrid susceptibility or resistance to *Fusarium* ear rot in the field, although the severity and the kinetics of its chronic phytotoxicity may depend on the host genetic background. Moreover, the EL registered here is a probable consequence of the differential sphinganine and phytosphingosine imbalances reported in maize seedlings upon FB1 exposure (Arias *et al.*, 2016).

Maschietto and colleagues (2016) analyzed ears from two maize hybrids with contrasting resistance to *Fusarium* after 72 hours of infection, and proposed that the resistant phenotype is related to the higher constitutive expression of antioxidant enzymes and defence-related proteins. However, we found that the antioxidant enzyme profile in maize seedlings showed greater constitutive SOD and GPOX activities in SH, highlighting the need to study a greater number of hybrids in different developmental stages to reaffirm or discard such correlation. Also, it is important to note that the resistance to ear rot by *Fusarium* spp. is under polygenic control and strongly influenced by environmental factors (Presello *et al.*, 2006; Parsons and Munkvold, 2010; Cao *et al.*, 2013).

We observed that roots are the most affected plant parts when soils are contaminated by FB1. Further, considering the number and biological meaning of the alterations found in each condition, a major toxicity of the highest concentration of mycotoxin becomes evident. In general, the changes observed in SH were characterized by the inhibitory effects of the toxin (both concentrations) on the antioxidant enzymes, SOD and GPOX (except at 7 dap), as well as by the highest levels of H_2O_2 and TBARS induced by 20 ppm of FB1 (except al 21 dap). This toxin concentration (20 ppm) also induced the major changes in RH roots (mostly increases of H_2O_2 and TBARS, except at 21 dap), but unlike the findings from SH, SOD and GPOX activities generally increased in RH exposed to FB1 (except in

GPOX at 14 dap). The phytotoxic effects of FB1 on the aerial parts of SH and RH, were less evident than those found in the roots, although, with some exceptions (GPOX in RH at 21 dap and SOD in SH at 14 dap), in general they reflected the changes induced in roots. Maschietto *et al.* (2016) observed that the activities of antioxidant enzymes increased more rapidly in a resistant genotype after ear inoculation with *F. verticillioides*. We report a similar behaviour in maize seedlings watered with FB1, where the increases in the SOD and GPOX activities were evident throughout the RH plants, except in GPOX at 21 dap in aerial part (1 ppm) and at 14 dap in roots (20 ppm). Therefore, suggesting that these enzymes are generally produced in RH, but not in SH, in response to the mycotoxin. Moreover, the results of this work could show that the enzymatic antioxidant response of RH seedlings upon the FB1 contact, rather than the basal enzymatic activities, would be more closely related to *F. verticillioides* ear rot in the field. However, it is important to emphasize that, apart from FB1, other soluble or structural fungal components such as others phytotoxins, ergosterol, cell wall degrading enzymes, among others could modulate the plant-fungus interactions (Kamoun, 2006; Pusztahelyi *et al.*, 2015). Also, several plant secondary metabolites, quantitatively less important than enzymes in the antioxidant defences, contribute to maintaining the redox balance (Bartoli *et al.*, 2013; Noctor *et al.*, 2018).

Plant growth is strongly influenced by external conditions, and the cellular redox homeostasis was proposed as a key biochemical connection between plant metabolism and environment (Foyer and Noctor, 2009; Noctor *et al.*, 2018). In this sense, the integrated biomarker response (IBR) allowed us to get a comprehensive view of the stress produced by FB1 on the oxidative status of the plantlet cells. As it was expected, the stress evolved differently depending on the toxin concentration. The IBR pointed out that 1 ppm of FB1 was generally less stressful for both hybrids than the highest toxin concentration, with the exception of RH at 21 dap, where the opposite was observed. Using the same hybrids and experimental model, we reported that the biomass and fitness of maize seedlings irrigated with 1 ppm of FB1 were restored at 21 dap, so it was proposed that they would have efficient detoxification / excretion mechanisms for this level of exposure to FB1. However, the toxin accumulation and the incidence and severity of lesions, both in aerial parts and roots, were greater in SH, showing different biochemical responses to this mycotoxin, depending on the maize genotype (Arias *et al.*, 2012; 2016). High antioxidant enzymatic activities would let the plants reach the redox homeostasis sooner, by rapidly scavenging the excess of ROS, and diminishing their toxic effects (Caverzan *et al.*, 2016). In this sense, the results of this work suggest that the antioxidant enzymes of RH could contribute significantly to respond more efficiently against the oxidative stress caused by FB1. Nevertheless, the success of acclimation to the mycotoxin chronic stress would include a more complex universe of regulatory mechanisms of the cellular redox state, focused not only on the antioxidant system, but also on the signalling mediated by the ROS itself.

The IBR also evidenced two clearly different responses of both germplasms to irrigation with the lowest concentration of toxin. In general, FB1 inhibited the antioxidant SOD and GPOX in SH, but

here IBRs were only slightly higher, which could be suggesting that the remaining enzymatic activities would be enough to control the pro-oxidant effect of the toxin at all endpoints assessed. This interpretation is also supported by the overall behaviour of H_2O_2 and TBARs, biomarkers that in SH were mostly unaltered by the toxin. A different scenario could have been occurring in RH, where the transient increase in cell death found at 14 dap might be showing that FB1 induced hypersensitive response (HR)-type cell death, an ordered process probably triggered by the oxidative stress (Stone *et al.*, 2000; Xing *et al.*, 2013). Such host response is not fundamental for the generation of resistance, but it is required for a rapid and strong activation of both local and systemic defence mechanisms (Heath, 2000). In this regard, the highest IBR found at 21 dap might be denoting a plant stress caused by its continuous response to alleviate the phytotoxicity of a contaminant that persists in soil.

Beside generating cell damage in the plant, ROS can act as second messengers by activating or inhibiting SA- and JA-mediated response mechanisms, respectively (Kwak *et al.*, 2006; Noctor, 2018). The hydroponic model allowed us to evaluate the participation of these phytohormones in the acute phytotoxicity of FB1, minimizing the effects of the collection and conditioning procedures of the samples. Alike the observations in pots, the mycotoxin also induced acute cell death in both hybrids. In RH, it was associated with an increase in SA and a reduction in JA levels, dependent or not on the accumulation of ROS, respectively. These results are in line with those reported by Xing *et al.* (2013), who observed that the pre-treatment of *Arabidopsis thaliana* leaves with ascorbic acid prevented the SA rise upon infiltration of the leaves with 10 µM (7.22 ppm) of FB1, suggesting that this phytohormone would be ROS-modulated. However, the cell death in SH was only related to a ROS-mediated decrease of JA. The toxin was phytotoxic to both hybrids, but the highest cell death observed in RH would be related to the SA increase mediated by ROS.

In summary, in this work we showed that FB1 caused cell death in maize seedlings by two different biochemical mechanisms in hybrids with contrasting susceptibility to *F. verticillioides* ear rot in the field. In addition, the results suggest that ROS has a dual role in the mycotoxin-induced cell death in maize plants, generating oxidative stress, and modulating phytohormone-mediated defence responses to reduce the phytotoxicity of FB1. From a fungal point of view, the cell death caused by FB1 would favor the beginning of the *F. verticillioides* necrotrophic phase and colonization. Moreover, if the pathogen succeeds, the activation and inhibition of SA and JA signaling pathways by FB1, respectively, would allow the infection progress (living from death tissue). But is important to recall that other mechanisms such as FB1 detoxification or elimination (Berthiller *et al.*, 2013; Arias *et al.*, 2016) may also be involved in the plant-pathogen biochemical war. Likewise, the balance between acclimation and cell death responses after the first contact with this mycotoxin would determine the fate of the plant. The RH would control more efficiently the FB1 phytotoxicity by rapidly inducing cell death, but the IBR index might be pointing out a major stress to mitigate the chronic exposure to the toxin. However,

future studies should be focused to confirm if the cell death, as well as the correlation with oxidative stress and SA and JA unbalances found in this work, are reproduced in other maize hybrids.

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Declarations

Conflicts of interest. The authors declare that they have no conflict of interest.

Data Availability Statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability. Not applicable.

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Supporting information

Figure and tables legends

Fig. 1 Effects of FB1 on electrolyte leakage in leafs of SH and RH maize seedlings. Data are represented as Mean ± SE. $^{a}p < 0.05$; $^{b}p < 0.01$ indicate differences between treatments and control. Capital letters indicate differences between both FB1 exposure levels.

Fig. 2 Effects of FB1 on hydrogen peroxide accumulation in aerial parts and roots of SH and RH maize seedlings. Data are represented as Means ± SE. $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$ indicate differences between treatments and control.

Fig. 3 Effects of FB1 on TBARS accumulation in aerial parts and roots of SH and RH maize seedlings. Data are represented as Means ± SE. $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$ indicate differences between treatments and control.

Fig. 4 Integrated Biomarker Response of SH and RH maize seedlings exposed at 0 (Control), 1 and 20 ppm of FB1 during 7, 14 and 21 days after planting (dap). Radar graph for the calculated IBR index. The spokes of the radar indicate the IBR index mean values for each studied treatment.

Fig. 5 Effects of 1 ppm of FB1 on the content of a) hydrogen peroxide, b) electrolyte leakage, c) Superoxide dismutase, and d) guaiacol peroxidase in SH and RH maize seedlings pre-treated or not with 1 mM AA. Data are represented as Means ± SE. $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$ indicate differences with the control. p-value indicated in the graph shows differences between FB1 vs AA + FB1 treatments.

Fig. 6 Effects of 1 ppm of FB1 on the content of SA and JA in SH and RH maize seedlings pre-treated or not with 1 mM AA. Means ± SE of the content of SA (a) and JA (b) are shown. $^{a}p < 0.05$; $^{c}p < 0.001$ indicate differences with the control. p-value indicated in the graph shows differences between FB1 vs AA + FB1 treatments.

Table 1 Effects of FB1 on SOD and GPOX activities in aerial parts and roots of SH and RH maize seedlings [1].

[1] SOD (units/mg protein) and GPOX ($\Delta Abs_{436\ nm}$.mg protein^{-1}.min^{-1}) activities are represented as Means ± SE. [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$. Lower case letters denote differences between treatments and control.

Table 2 Integrated biomarker response in irrigations of SH and RH of maize at different concentrations of FB1.

Median, mean, minimal and maximal values for each treatment. Different letters indicate Means ± SE. [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$. Lower case letters denote differences between treatments and control.