JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Fumonisins: Probable Role as Effectors in the Complex Interaction of Susceptible and Resistant Maize Hybrids and *Fusarium verticillioides*

Silvina L. Arias, Martin G. Theumer, Veronica S. Mary, and Hector R. Rubinstein*

Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina

ABSTRACT: *Fusarium verticillioides* is best known for its worldwide occurrence on maize resulting in highly variable disease symptoms, ranging from asymptomatic to severe rotting and wilting and fumonisin production. The aim of this study was to investigate the influence of hybrid genotypes in the early stages of *F. verticillioides* infection, and the role of fumonisins as effectors in the outcome of this complex interaction. Disease symptoms, growth parameters, root morphology, and fungal colonization were evaluated at 7, 14, and 21 days after planting in seedlings from maize seeds of resistant (RH) and susceptible (SH) hybrids inoculated with *F. verticillioides* or watered with solutions of fumonisins. *F. verticillioides* induced growth enhancement or retardation depending on the plant genetic background and the fungal colonization rate, while fumonisins caused severe reduction in biomass and fitness. Seedlings watered with high fumonisin concentrations displayed lesions similar to those seen in *F. verticillioides* maize seedling disease, and also elicited inhibitory effects on root growth and morphology and on functional properties. In summary, these data strongly suggest a dual role for fumonisins in the *F. verticillioides*–maize interaction, acting as pathogenic factors at high concentrations, or triggering the plant detoxification mechanisms at low levels.

KEYWORDS: *Fusarium verticillioides, fumonisins, Zea mays L., resistant maize hybrid, susceptible maize hybrid, effectors*

INTRODUCTION

Fusarium verticillioides is one of the most prevalent seedborne fungi associated with corn (*Zea mays* L.), whose effects can result in highly variable disease symptoms, ranging from asymptomatic plants to severe rotting and wilting. In addition, *F. verticillioides* is a prolific fumonisin producer, with animal and human health problems related to these mycotoxins being almost exclusively associated with the consumption of contaminated maize or of products made from maize worldwide. The fumonisins B1 (FB1), FB2, and FB3 are the most common forms, with FB1 being the most important of the group due to its prevalence and toxicological potency.[1] As *F. verticillioides* and fumonisins are found in maize debris and soil, it is reasonable to expect that maize seedlings could become infected with the fungus and also be potentially exposed to fumonisins in the field. Moreover, it was reported that *F. verticillioides* is also able to produce biologically available fumonisins in natural soils.[2]

On the plant host side, genetic resistance appears to be the best preventive action against fumonisin contamination, although at present no commercial maize hybrids have been found to be completely resistant.[3,4] The resistance to *Fusarium* ear and kernel rot of corn and the mechanisms associated with the infection have been examined.[5−7] Fumonisin concentration in grain was demonstrated to be moderately correlated with the severity of *Fusarium* ear and kernel rot,[5] with fumonisins also having been detected at levels of concern in asymptomatic corn kernels.[6] With respect to this, Presello et al.[7] tested a set of Argentinean hybrids in Canada and Argentina for resistance against *F. verticillioides* and *F. graminearum,* and concluded that genotype effect was more important than genotype-by-fungal species or genotype-by-fungal species-by-environment interaction effects. Nevertheless, subsequent results indicated that the role of fumonisins depends on the complex environmental and genetic contexts present in this plant–pathogen interaction.[8]

On the microorganism side, many pathogens employ an array of strategies to distress, weaken, or kill the plant host in order to gain access to nutrients, and several "effectors" could be involved in the process of infection and establishment of a parasitic fungal–plant interaction (virulence factor or toxin). Furthermore, these molecules may trigger defense responses (avirulence factors or elicitors). Many proteic factors have been described in pathogenic processes, such as cell wall degrading enzymes (cutinases, hydrolytic enzymes, etc.) and pathogenicity effector proteins that can be transferred into host cells by haustoria (specialized feeding structure) during infection.[9] However, effectors are not restricted to polypeptides, but also include secondary metabolites such as phytotoxins, which often participate in the mechanisms of virulence and pathogenicity. Fungal compounds, for example the trichothecenes, have been shown to act as virulence factors in infection of plants by *Fusarium* spp. Moreover, a differential phytotoxic action of trichothecenes among their wide molecular structure spectrum was found. Researchers have suggested that type B trichothecenes (i.e., nivalenol, deoxynivalenol) can suppress the defense response, whereas type A trichothecenes (T-2 toxin, diacetoxyscirpenol, for instance) trigger cell death by activation of an elicitor-like signaling pathway in *Fusarium*-susceptible *Arabidopsis*.[10] Likewise, *Alternaria alternata lycopersici* toxin (AAL) has been described as a pathogenicity factor for

Received: December 29, 2011
Revised: May 10, 2012
Accepted: May 11, 2012
Published: May 11, 2012

A. alternata, due to its inducing stem canker disease on susceptible tomato plants.[11] Interestingly, AAL bears toxicological properties and structural characteristics similar to those of FB1, strongly indicating a direct participation of FB1 in the *Fusarium*–maize interaction and phytotoxicity. Nevertheless, toxicological research suggests that many phytotoxins can have entirely different effects on target organisms at both low and high concentrations; in particular, low concentrations of toxins can often result in improving growth and survival rather than causing growth inhibition or mortality.[12,13]

In the complex interaction between *F. verticillioides* and maize, investigation of the potential role of fumonisin production on the disease and development has produced controversial results.[14,15] Glenn et al.[16] and Williams et al.[2,17] demonstrated that only strains of *F. verticillioides* that produced fumonisins were able to cause foliar disease symptoms on seedlings of the sweet maize hybrid 'Silver Queen', with this hybrid henceforth being used as the standard susceptible cultivar in these assays. However, no measurements were made of root functionality.

Fungal diseases affect plant growth via several different processes. A pathogen that infects parts of the leaves can reduce the foliage area, thereby affecting the amount of assimilate available for growth. Shoot and stem pathogens may reduce growth by interfering with the movement of water, nutrients, and assimilates in the stem and branches. A pathogen that infects and kills part or all of the roots of a plant reduces the ability of the plant to absorb water and nutrients, thus affecting growth indirectly, and can result in its wilting and death.[18]

The present research focused on an evaluation, in a comparative study, of the influence of hybrid genotypes at the early stages of *F. verticillioides* infection and also investigated the role of fumonisins as effectors in the outcome of the interaction. The specific objectives were to (i) test whether the time course for expression of disease symptoms differed between resistant and susceptible hybrids infected by fumonisin-producing strain of *F. verticillioides* (infection model); (ii) discover whether disease symptoms were consistent with a direct fumonisin exposure (phytoxicity model); and (iii) determine the contribution made to the uptake by localized physiological and morphological responses of the root systems. Consequently, it is possible using these data to reconstruct the events that had contributed to the exploitation of the nutrient and water by the plants.

MATERIALS AND METHODS

Chemicals and Reagents. Fumonisin standards were purchased from PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, Republic of South Africa). Methanol and acetonitrile were of HPLC grade (Sintorgan, Buenos Aires, Argentina). The reagents 2-mercaptoethanol and *o*-phthaldialdehyde were purchased from Merck and J.T. Baker (Buenos Aires, Argentina), respectively. 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). All other chemicals were provided by Sigma-Aldrich (Buenos Aires, Argentina).

Maize Seedlings. The maize seedlings (*Zea mays* L.) were obtained by sowing seeds of resistant hybrid (RH, LT 622 MG) and susceptible hybrid (SH, HX 31P77), which have shown resistance to and also susceptibility to *Fusarium* ear rot in the field, respectively.[19] The plants were grown under controlled conditions in a greenhouse with a 14/10 h light/dark cycle at 22 °C.

Fungal Strain. A wild-type toxigenic isolate of *Fusarium 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 Rio Cuarto, Cordoba, Argentina (RC), culture collection center. Cultures were maintained in 15% glycerol at 80 °C. The ability to produce fumonisins was quantitatively analyzed by HPLC as described below.

Inoculum Preparation. Conidia suspensions, prepared with *F. verticillioides* RC2024 culture grown at 25 °C for 7 days in potato dextrose agar (PDA, Oxoid) (toxicogenicity in maize and seed infections) or in V-8 juice agar (fumonisin production in bioreactor) and Tween 20 at 2.5% (v/v) in sterile water, were used as inocula.[20] The conidial concentration was standardized using a spectrophotometer set at an optical density 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).

Assessment of *F. verticillioides* RC2024 Toxicogenicity in Maize. The toxigenicity of *F. verticillioides* RC2024 was obtained by measuring the fumonisin levels in the aqueous extracts prepared from maize kernels inoculated with the fungus and fermented for 28 days according to a procedure described by Theumer et al.[21]

Fumonisin Production in Bioreactor. The fermentor vessel (10 L glass stirred jar) (New Brunswick Scientific Co., Inc., Edison, NJ) containing sterilized MYRO medium (1 g of $(NH_4)_2HPO_4$, 3 g of KH_2PO_4, 2 g of $MgSO_4 \cdot 7H_2O$, 5 g of NaCl, 40 g of sucrose, and 10 g of glycerin in 1 L of distilled water)[22] was inoculated with the conidia suspension and maintained at 28 °C with 120 rpm agitation. Dissolved oxygen was maintained using a stir rate and an air flow rate of 2 standard liters per minute (SLPM). A pH of 3.5 was sustained by automatic adjustment with 0.1 M H_3PO_4 or 0.1 M NaOH, and incubation was carried out for 28 days. The fermented liquid medium was autoclaved and then filter clarified through a 0.45 μm filter. A sample of the filtrate was used for fumonisin quantitation, and the remaining volume was kept at −20 °C until use.

Quantitation of Fumonisins. The fumonisins were quantitated in aqueous extracts from fermented maize as well as in the fermented MYRO medium. The samples were diluted with acetonitrile HPLC grade at a 1:1 ratio, and the quantitation of the diluted extracts was determined following a methodology proposed by Shephard et al.[23] and Sydenham et al.[24] The latter method was successfully validated through collaborative studies and has been adopted as the official method by the Association of Official Analytical Chemists International (AOAC). Briefly, an aliquot (50 μL) of the diluted samples was derivatized with 200 μL of an *o*-phthaldialdehyde solution obtained by adding 5 mL of 0.1 M sodium tetraborate and 50 μL of 2-mercaptoethanol to 1 mL of methanol containing 40 mg of *o*-phthaldialdehyde. The derivatized samples were analyzed by means of a Hewlett-Packard series 1100 HPLC system, having a loop of 50 μL, with an isocratic pump (G1310A) coupled with a fluorescence detector (Agilent Technologies series 1200), using wavelengths of 335 and 440 nm for excitation and emission, respectively. The column used was a 150 mm × 4.6 mm i.d., 5 μm, Luna 100 RP-18, with a 20 mm × 4.6 mm i.d. guard column of the same material (Phenomenex, Torrance, CA, USA). The mobile phase was methanol:0.1 M NaH_2PO_4 at a 75:25 ratio (v/v), the pH was 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 HP Chemstation Rev. A.07.01 software.

The HPLC method was verified using both pure standards and spiked samples. Limit of detection (LOD) was established at a signal-to-noise ratio of 3 (S/N = 3), while a S/N = 10 was considered for limit of quantification (LOQ). Therefore, considering sample dilution, LOD was 0.01 μg/g while LOQ was 0.03 μg/g. Quantitation was performed using linear regression from a calibration plot constructed with analytical standards (r^2 = 0.995). Spiked samples were used to evaluate the recovery percentage of the method, which was above 90% considering two different concentrations (0.1 μg/g and 1.0 μg/g).

The FB1:FB2:FB3 ratio produced by *F. verticillioides* RC2024 in maize was 88:5:7.

Table 1. Seedling Growth Changes of Susceptible and Resistant Maize Hybrids at 7 Day Intervals after Inoculation with *F. verticillioides*, as Assessed by the Length of the Aerial and Root Plant Parts[a]

	height (cm)			primary root length (cm)		
variety (treatment)[b]	7[c]	14[c]	21[c]	7[c]	14[c]	21[c]
RH (ni)	1.9 ± 0.1	10.9 ± 0.4	20.8 ± 1.3	4.8 ± 0.4	7.4 ± 0.4	10.6 ± 0.6
RH (i)	3.3 ± 0.3 d[d]	16.5 ± 0.7 d	25.6 ± 1.7 c	5.8 ± 0.4	12.8 ± 0.4 d	14.2 ± 0.6 d
SH (ni)	3.5 ± 0.2	13.3 ± 0.2	27.8 ± 0.6	6.7 ± 0.2	12.6 ± 0.3	17.1 ± 0.5
SH (i)	3.2 ± 0.2	13.8 ± 0.6	25.2 ± 0.9 c	7.1 ± 0.2	11.4 ± 0.4 c	15.4 ± 0.6 c

[a]Data are means ± standard error ($n \geq 10$). [b]RH (ni): Resistant hybrid noninoculated. RH (i): Resistant hybrid inoculated. SH (ni): Susceptible hybrid noninoculated. SH (i): Susceptible hybrid inoculated. [c]Days after inoculation. [d]Letters c and d indicate that value is significantly (c, $p < 0.05$; d, $p < 0.0001$) different from the corresponding noninoculated (ni) group.

EXPERIMENTAL DESIGN

In order to evaluate the *F. verticillioides*–maize interaction and the participation of fumonisins as effectors, HR/HS maize seedlings from seeds infected with *F. verticillioides* (infection model) and noninfected seeds watered with fumonisin solutions (phytotoxicity model) were harvested on days 7, 14, and 21. Previously, seeds had been surface-disinfected for 2 min in 100% bleach (0.4% hypochlorite), rinsed three times with sterile water, and blotted dry on paper toweling. To avoid potential problems with fungicide residues, maize seed without fungicide treatments was used. In the infection model, inoculations were performed by placing sterilized seeds in a Petri dish (100 mm) and flooding them with 15 mL of sterile phosphate buffered saline (PBS, 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 ClO_2 and the percentage of infection were evaluated in grains randomly selected and seeded directly on Petri dishes containing DRBC. The infected and noninfected seeds (three replicates of 10 seeds each) were sown in 24 cm diameter pots containing washed autoclaved sand.

A soluble fertilizer was applied before planting and also twice a week thereafter. Pots were watered every 3 days with sterile water, except for the groups exposed to the fumonisins in the phytotoxity model, in which the plants were watered with 100 mL of fumonisin solutions (FB1: 1 and 20 μg/mL in sterile water) on days 2, 4, and 6 after planting, and then watered every 3 days with sterile water. Assays were performed under controlled greenhouse conditions. After harvest: (a) visual inspections of disease symptoms were assessed and images were taken using a Sony Cyber-shot DCS-S700 digital camera with a resolution of 7.2 megapixels; (b) the lengths of the primary root and shoot (where shoot length was defined from the seed attachment site to the tip of the longest leaf) were measured, and the roots were carefully separated from the aerial parts and the seeds removed; (c) digital pictures of the roots were obtained individually by HP Photosmart Scan 3110; (d) plant parts were oven-dried at 60 °C for 72 h for biomass determination; and (e) the leaf dry matter content (LDMC) indices were calculated as the ratio of leaf dry mass to fresh mass (only for groups exposed to 20 ppm fumonisin concentration).

In the infection model, parts of the plants were used for fungal isolation and others were stored in ethanol 50% (v:v) at 4 °C for microscopy studies.

Time-Course Assessment of the Infection: Fungal Isolation and Microscopy. *1. Fungal Isolation.* The fungal distribution was determined weekly, by placing duplicates of surface-disinfected segments from the root, mesocotyl, node, stem, and oldest leaf on the PDA. Segments from which the fungus grew on the PDA were scored as positive, and the percentage of positive responses was calculated for each plant part.[12]

2. Microscopic Examination of Roots and Leaves. Roots were thoroughly washed under running tap water and cut into 1 cm pieces. These segments were stained with 0.05% trypan blue dye following the procedure of Sahay and Varma.[25] Leaves were cleared with ethanol and stained following the same procedure. The samples were observed with a Nikon Eclipse TE2000-U (Tokyo, Japan) microscope. The pictures were taken by a color video camera Nikon DS-5 M with a supported resolution up to 2560–1920 pix (Capture).

Root Morphology. Root systems from each treatment were rinsed with water to remove any adhering sand, and they were then put into a tray submerged in water and spread out with the help of tweezers to minimize overlap. The roots were immediately scanned on a flatbed scanner at a resolution of 200 dpi, and a good contrast between "white" roots and a black background was obtained. Software Root Image Analyzer (USA)[26] was used to determine the total root length. The test was conducted using metal wires of known lengths as standards. The SRL was calculated as the ratio of total root length to root dry mass.

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 pull presented homoscedasticity. In some cases, due to lack of homoscedasticity, a nonparametric comparison was also performed using the Kruskal–Wallis test ($p < 0.05$). The seedling growth of the infection model data were analyzed using the two-tailed Student's t test. Differences were considered 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 AND DISCUSSION

Analysis of Growth Responses in Infection Model. The growth effects related to *in vivo* resistance or susceptibility to a fumonisin-producing strain of *F. verticillioides* infection, for height and primary root length, at 7, 14, and 21 days after planting (dap), are summarized in Table 1. Growth-promoting activities were observed in RH-infected plants, with seedling height being significantly higher for infected than for noninoculated kernels ($p < 0.0001$ at 7 and 14 dap and $p < 0.05$ at 21 dap). Moreover, the roots from the treated plants at 14 and 21 days also exceeded that of the controls ($p < 0.0001$). Conversely, a reduced growth of aerial part plants (21 dap) and primary roots (14–21 dap) was recorded in SH-inoculated seedlings compared to noninfected ones. In Figure 1, representative images of the differential responses of root development can be observed for inoculated (Figure 1A) and noninoculated (Figure 1B) SH at 21 dap.

Similar results were also found from the measurements of the dry weight of the plant fractions (aerial parts and roots) and total biomass (Figure 2), with the latter data being useful for evaluating resource allocation patterns of the plants under environmental stress in order to maximize acquisition of the particular resource that limits growth. Related to this, the mass of the plant organ tends to reflect its cost of construction and thereby the effort expended on it. In the present research, *F. verticillioides* significantly enhanced the dry matter accumulation in RH, in which the RH total biomass of infected seedlings exceeded that of nontreated plants at 7 and 14 dap, with this increase being due to the above-ground biomass contribution. In contrast, *F. verticillioides* negatively affected the SH-seedling growth at 14 days, and this alteration persisted until day 21

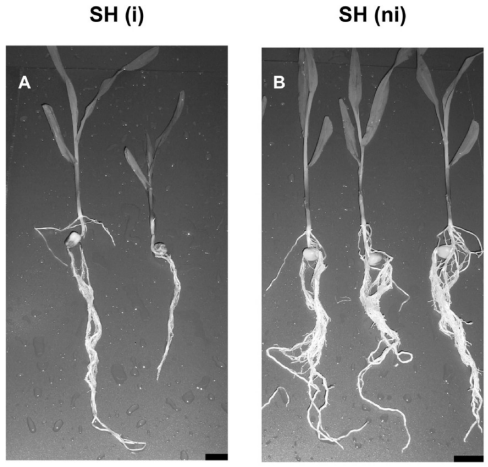

Figure 1. Morphological responses induced in seedling hybrids infected with *Fusarium verticillioides*. (A) Reduced root development in a susceptible hybrid 21 days after planting of seeds inoculated with 10^{12} cfu/mL of the fumonisin-producing *F. verticillioides* strain RC2024. (B) A control seedling growth from noninfected seeds. Bars: (A) 7.5 mm; (B) 10 mm.

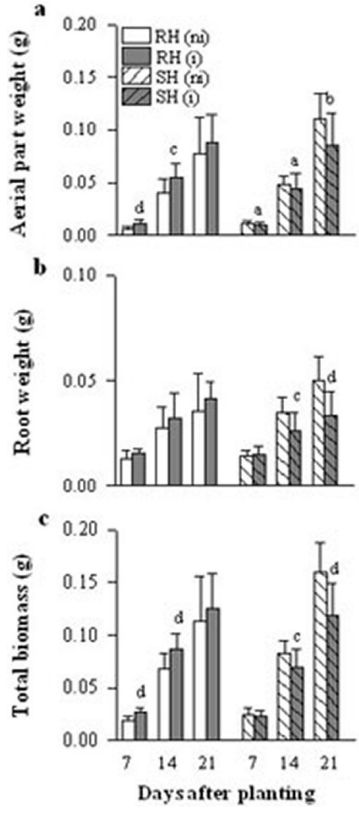

Figure 2. Effects of *Fusarium verticillioides* infection on the seedling growth as assessed by the mass of the aerial and root plant parts. Increased growth in height (A) and root mass (B) in the resistant hybrids grown from seeds inoculated with the fungus (RH (i)) and harvested after 7, 14, and 21 days. Reduced growth of (A) aerial plant parts (leaves and stems) and (B) roots was found in susceptible hybrids grown from seeds inoculated with *F. verticillioides* (SH(i)). (C) Changes in total biomass for both RH and SH in plants grown from uninoculated or inoculated seeds. Values are means ± SD ($n \geq 10$). $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, $^{d}p < 0.0001$, when the infected plants were compared with the noninoculated groups for the indicated harvest date.

after planting ($p < 0.001$ and $p < 0.0001$, respectively). The severe decrease in total biomass reflected a tendency to distribute fewer resources to both the roots and aerial parts in infected plants.

In this study, *F. verticillioides* did not suppress maize resistant seedling growth and may even have enhanced plant development. This result was unexpected during the first few days after germination from infected seeds, since the fungus and seedling may compete for the same food reserves stored in the kernel endosperm, according to the findings of Yates et al.[12] However, these authors described this growth repression as temporary, and in fact noted increases in the shoot diameter, root dry weight, and plant weight of infected maize seedlings that exceeded noninfected seedlings at 4 weeks after planting. These effects of growth promotion are similar to results obtained in 2005 by this same group, in a trial realized under yield conditions.[27] On the other hand, the findings in treated SH are in agreement, at least in part, with the negative effects of infection in maize reported by Williams et al.[2,17] and Glenn et al.,[16] who observed that a fumonisin-producing strain of *F. verticillioides* (MRC 826) caused leaf lesions, developmental abnormalities, stunting, and sometimes death of susceptible sweet maize seedlings (Silver Queen).

Timing and Spatial Dynamics of Host Colonization: Fungal Isolation and Microscopy. A microscopic examination of plant tissues revealed a moderate to heavy infection with *F. verticillioides*. The fungal growth was mainly intercellular, with the structures being detected inside roots (Figure 3A). An

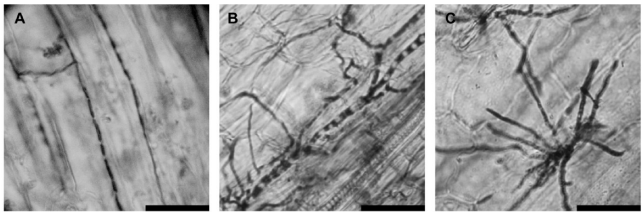

Figure 3. Systemic colonization by *Fusarium verticillioides* strain RC2024 in maize at 21 days after planting of susceptible seeds inoculated with 10^{12}cfu/mL. Hyphae were observed running in parallel within intercellular root spaces (A), among leaf tissue (B), and emerging through stomas (C). Stained in 0.05% trypan blue solution. (Magnification: 400×.) Bars: (A–C) 50 μm.

equally efficient invasive growth occurred on the leaf tissues, showing branching septate hyphae between cell walls (Figure 3B). In addition, hyphae were found throughout the stomas (Figure 3C).

The host colonization process of *F. verticillioides* at 7, 14, and 21 dap is shown in Figure 4. The endophytic colonization strategy of disseminating migration observed by microscopy was further investigated by fungal isolation of different plant sections (root, mesocotyl, node, stem, and oldest leaf), superficially disinfected and placed on top of PDA medium (Figure 4A). The fungal development was microscopically analyzed in order to confirm the presence of the fructification structures that characterize *F. verticillioides* (Figure 4B).

F. verticillioides was recovered from both below-ground and above-ground tissues of the infected RH and SH at each sampling date (Figure 4C), with the highest infection frequency being found in SH. For the specific segments, greater rates of isolation were evident in roots, mesocotyls (the tissue

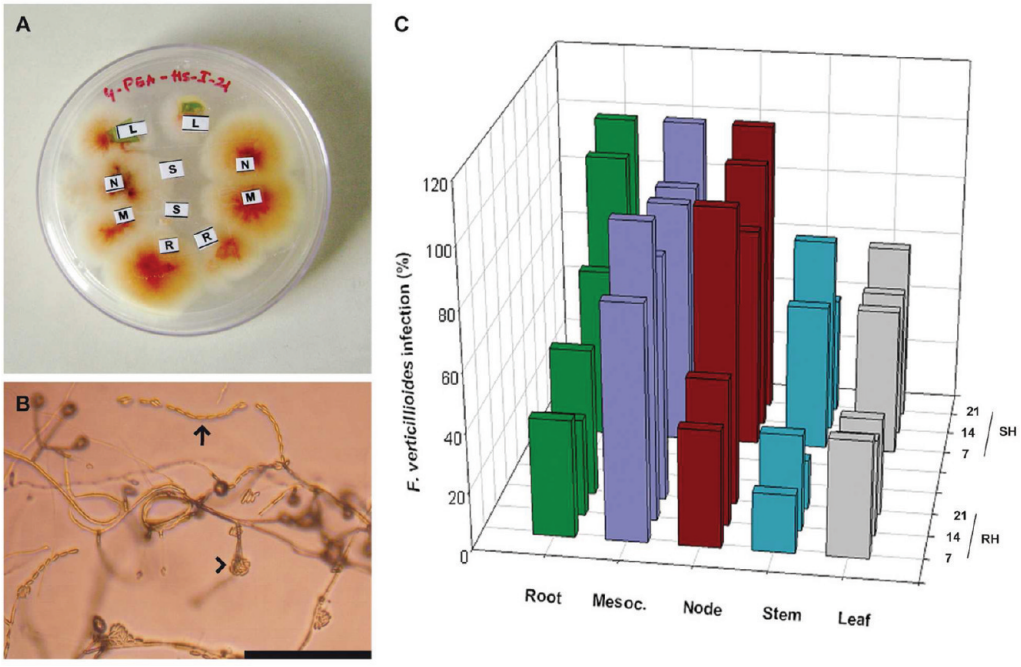

Figure 4. Time-course isolation of *F. verticillioides* in specific plant parts. (A) Frequency of fungal isolation from tissue segments of resistant and susceptible corn seedlings grown from inoculated kernels. (B) Recovery of *F. verticillioides* from the root (R), mesocotyl (M), node (N), stem (S), and oldest leaf (L) of susceptible plants grown from inoculated kernels and harvested after 21 days on potato dextrose agar (PDA). (C) Microscopic examination of mycelium developed on agar 1% medium, showing microconidia in long chains (arrow) and in false head (arrowhead) characteristic of this *Fusarium* species. (Magnification: 400×.) Bar: (B) 100 μm.

Table 2. Effect of Treatment with Fumonisins on the Development of Resistant and Susceptible Maize Seedlings, as Assessed by the Length of the Aerial and Root Plant Parts[a]

		height (cm)			primary root length (cm)		
variety[b]	FB1 (μg mL $^{-1}$)	7[c]	14[c]	21[c]	7[c]	14[c]	21[c]
RH	0	15.4 ± 0.7	38.7 ± 0.4	52.4 ± 2.5	16.0 ± 0.5	23.4 ± 2.2	32.3 ± 4.0
	1	7.2 ± 0.4 d[d]	29.4 ± 2.1	53.9 ± 1.3	12.6 ± 0.9	20.3 ± 0.9	28.9 ± 2.6
	20	10.7 ± 1.2 c	16.6 ± 1.5 d	24.0 ± 2.1 df[e]	7.1 ± 1.3 d	5.2 ± 0.8 df	7.2 ± 1.3 df
SH	0	16.6 ± 0.6	38.2 ± 1.1	49.5 ± 0.9	17.6 ± 0.7	20.0 ± 0.9	32.6 ± 0.9
	1	7.4 ± 0.5 d	32.3 ± 1.1 c	49.0 ± 1.1	9.2 ± 0.7 c	20.2 ± 1.3	27.4 ± 1.5
	20	10.3 ± 0.5 de	18.4 ± 2.1 bf	27.0 ± 2.6 df	4.9 ± 0.2 de	8.3 ± 0.9 df	7.9 ± 1.9 df

[a]Data are means ± standard error ($n \geq 10$). Plants were watered with 100 mL solutions of each concentration of fumonisins on days 2, 4, and 6 after planting. [b]RH: resistant hybrid. SH: susceptible hybrid. [c]Days after planting. [d]Letters c and d indicate that value is significantly (c, $p < 0.05$; d, $p < 0.001$) different from the corresponding control group. [e]Letters e and f indicate that value is significantly (e, $p < 0.05$; f, $p < 0.001$) different from the corresponding 1 μg mL $^{-1}$ FB1 treated group.

connecting the emerging root with the emerging shoot), and nodes, with lower values found in leaves and stems.

Analysis of Growth Responses in Phytoxicity Model. Phytotoxic effects were observed on seedling growth and development. The shoot growth and primary root length measured in RH and SH watered with 0, 1, and 20 μg/mL of FB1 are shown in Table 2. Seedlings exposed at 1 μg/mL of FB1 showed a massive-effect of growth inhibition in maize shoots in both RH and SH, but no effects of the toxin on day 21 were detected. A statistically significant ($p < 0.05$) decrease in root length was only observed at 7 days in the SH. The treatment with the highest FB1 concentration induced a severe inhibition of both parameters, with the strongest effects on plant growth in the fumonisin-treated SH group. Nonetheless, the height was significantly ($p < 0.01$) increased in RH for the 20 ppm treated group at 21 days compared with this group at 7 days, indicating that, although inhibited, shoot growth continued after 7 days at a reduced rate. No difference was registered in primary root lengths of RH when the 20 ppm treated group at 21 dap was compared to this group at 7 days, denoting growth inhibition. The comparison of both growth parameters between the 20 ppm treated group at 21 dap vs this group at 7 dap also was valid for SH.

The phytotoxic action of fumonisins in the growth and morphology of host plants was investigated. Visual symptoms of disease included stunting, wilting, mild bleaching, leaf-rolling, and necrotic leaf and root lesions, which were evident as early as 7 days and persisted for at least 21 dap in RH and SH watered with 20 μg/mL of FB1 (Figure 5). The root system exhibited a reduced growth of the primary, seminal, and lateral roots. In addition, a lack of root hair development was observed. The lesions were similar to those seen in *F. verticillioides* maize seedling disease, in the absence of the pathogen.

The effect of both concentrations of the fungal toxin on the dry weight accumulation of the whole plant or plant parts was

dx.doi.org/10.1021/jf3016333 | J. Agric. Food Chem. 2012, 60, 5667−5675

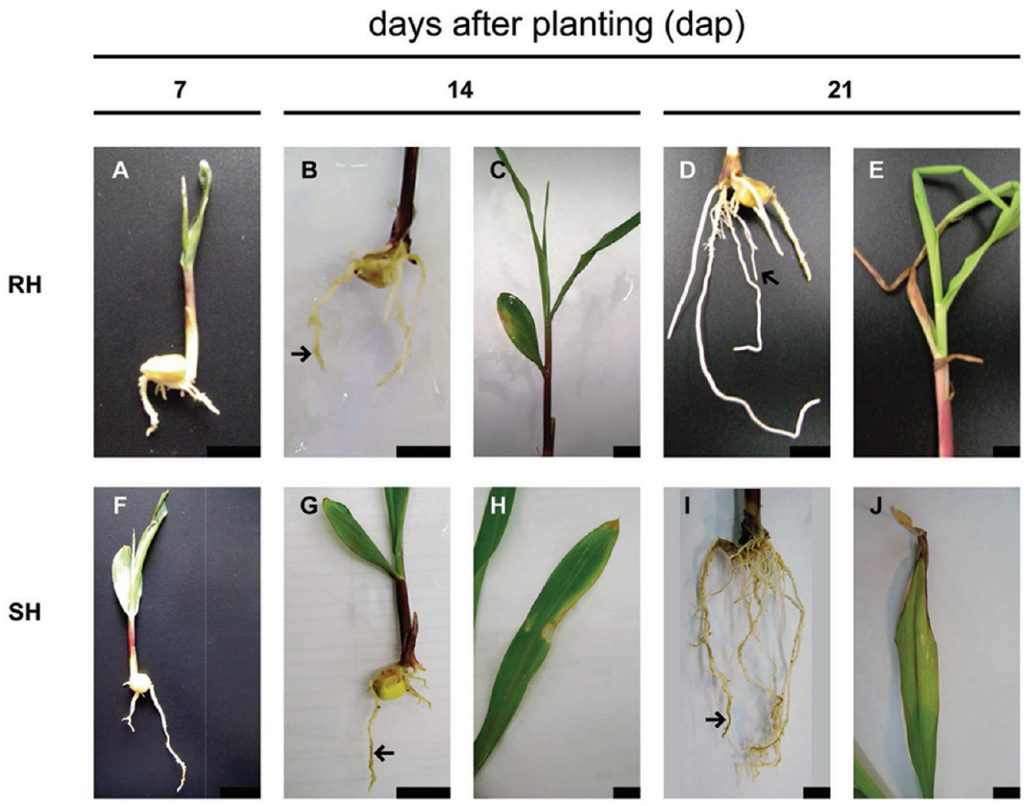

Figure 5. Morphological responses induced in resistant and susceptible hybrids (RH/SH) watered with fumonisin solutions (FB1: 20 μg/mL) at 7 day intervals after planting (dap). Reduced growth of the primary, seminal, and lateral roots and the lack of root hair development were observed (A, B, F, G, and I). Necrotic root lesions are denoted by the arrows at 14 and 21 dap (B, D, G, and I). For the aerial part, reduced shoot growth and wilting are shown (A, F). Also, leaf-rolling (A, E, F, and J), mild bleaching (C, H, and J) and necrotic leaf lesion (E, J) were observed. Bars: (C, E, H, I, J) 5 mm; (D, F) 7.5 mm; (A, B, G) 10 mm.

evaluated (Figure 6). Statistically significant decreases of dry weight of root, but not aerial part growth, were registrated from 7 dap in SH and 14 dap in RH (Figure 6A and 6B), whereas their contribution to the total biomass was much lower (Figure 6C). On the 21st dap, above-ground biomass (dry matter) declined significally in both hybrids.

Despite all of the fumonisin-treated seedlings presenting a severe reduction in biomass and of the fitness parameters, the plants watered with a lower concentration of fumonisins were able to reverse the negative effects to a toxin-untreated phenotype on 21 dap.

The Leaf Dry Matter Content (LDMC). Changes in a leaf dry matter content, in which high LDMC (the ratio of leaf dry mass (mg) to fresh mass (g)) might suggest slow-growing plants, were strongly influenced by the highest FB1 concentration treatments. The mean $\pm$ SD values of LDMC ($n \geq 10$) for fumonisin-exposed RH and fumonisin-nontreated RH, respectively, were 86.1 $\pm$ 7.3 and 68.0 $\pm$ 5.8 at 7 dap, 87.9 $\pm$ 7.5 and 79.2 $\pm$ 1.5 at 14 dap, and 99.0 $\pm$ 9.3 and 88.3 $\pm$ 3.5 at 21 dap. LDMC means were significantly affected by the toxin for the three dap but only slightly declined at 14 and 21 days ($p < 0.001$ (day 7), $p < 0.01$ (days 14, 21)). For fumonisin-treated SH and fumonisin-nontreated SH respectively, the mean $\pm$ SD values of LDMC ($n \geq 10$) were 89.9 $\pm$ 5.8 and 70.9 $\pm$ 3.2 at 7 dap, 102.8 $\pm$ 8.1 and 85.1 $\pm$ 5.9 at 14 dap, and 129.6 $\pm$ 16.2 and 89.3 $\pm$ 3.1 at 21 dap. These results showed a pronounced and significant increase of LDMC of the treated plant compared to the controls at 7, 14, and 21 dap ($p < 0.0001$). The toxin altered the leaf dry matter content in both hybrids, revealing a tendency for the plant to have similar strategies in retaining resources and having a slow turnover of plant parts (to a slightly greater extent in SH than in RH).

Measurement of Root Morphology. Due to nutrient uptake being more closely related to root length than to root biomass, a higher specific root length can imply a greater ability to obtain below-ground resources. The specific root length (SRL) showed an important differential functional response between hybrids (Figure 7), with this parameter being altered in RH only when the highest FB1 concentration was tested. However, the toxin, for SH treatment with 1 and 20 μg/mL of FB1, interfered with the nutrient and water uptake capacities of the roots, which was mainly due to a reduction in the SRL parameter.

In annual and fast-growing species such as maize, plants are expected to be able to maximize resource acquisition as this is imperative to fast growth and to complete their life cycle in a short period of time.[28] These species are characterized by a set of leaf traits that enable high carbon acquisition and roots with a high specific root length, which is usually associated with rapid rates of root elongation, high relative growth rate, large nutrient and water uptake capacities, and high metabolic activities.[29]

In this study, resistant and susceptible maize seedlings from seeds inoculated with the fumonisin-producing strain of *F. verticillioides* differed in terms of growth parameters, with the same strain inducing growth enhancement or retardation depending on the genetic background of the plant and the rate of colonization of the fungus. The resistant and susceptible

dx.doi.org/10.1021/jf3016333 | J. Agric. Food Chem. 2012, 60, 5667−5675

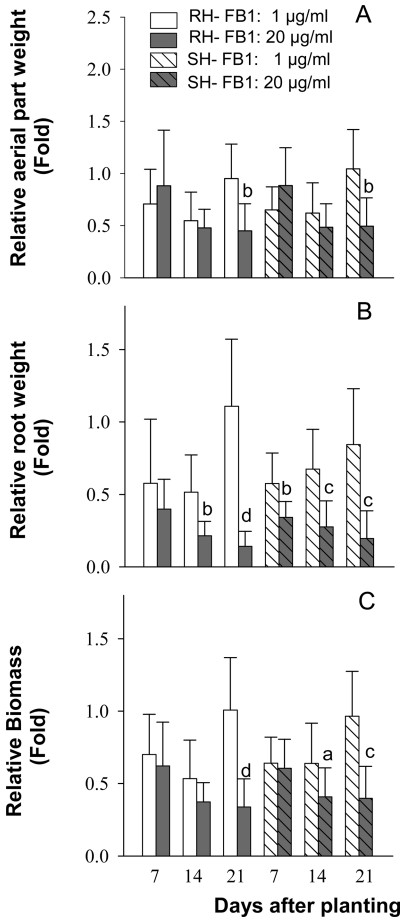

Figure 6. Comparative analysis of phytotoxic effects of FB1 (1 and 20 μg mL^{-1}) observed in resistant (RH) and susceptible hybrids (SH) harvested at 7, 14, and 21 days. Bars represent the means ± SE ($n \geq 10$) of the changes (fold) in the aerial part weight (A), the root weight (B), and the biomass (C) in seedlings watered with the FB1-containing solutions, with regard to their respective unexposed seedlings. $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, $^{d}p < 0.0001$, when plants watered with the smallest FB1 concentration were compared with their respective treatment with 20 μg mL^{-1} of FB1 for both RH and SH.

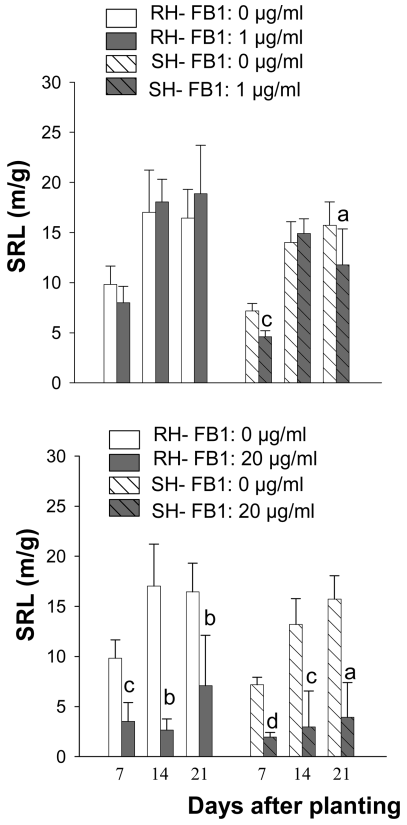

Figure 7. Specific root length (SRL) of maize resistant (RH) and susceptible hybrids (SH) harvested at 7, 14, and 21 days. Bars are means ± SE ($n \geq 10$). $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, $^{d}p < 0.0001$, when treated groups were compared with their controls.

assignments of the hybrids were consistent with previous experiments in the field[19] in the case of *Fusarium* ear rot, where conidial suspensions of *F. verticillioides* P364 were injected into the silk channel.

The development of the fungus in susceptible plants was correlated with a reduced seedling growth, which was more pronounced at root systems. Therefore, this may result in less efficiency in terms of the acquisition of mobile resources than in the case of the uninfected seeds and resistant hybrids.

These varying responses in the outcome of *F. verticillioides*–maize interaction may be mediated by a conflicting activity of effectors and has been widely studied in plant–microbial pathosystems,[30] in which signal molecules play an essential role during the early stages.[31] Coronatine, produced by *Pseudomonas syringae*, is an example of a compound with several identities, since, depending on the type of interaction, coronatine can be seen as a toxin, an elicitor, or a plant hormone in *Arabidopsis* plants.[30] There was contradictory evidence when attempting to elucidate a precise role for the fumonisins in maize pathogenicity. Experiments involving spraying maize leaves with concentrations of FB1 as high as 1,000 μg/mL did not cause any signs of pathology.[14] However, Lamprecht et al.[15] observed a significant reduction in the growth of maize seedlings grown on water agar containing 10 μM FB1. Desjardins et al.[32] concluded that fumonisin plays a role in virulence, but that was not necessary or sufficient for virulence on maize seedlings. In contrast, Williams et al.[17] and Glenn et al.[16] reported that fumonisin production is an important contributor to the *F. verticillioides* maize seedling disease expression and is required for the induction of leaf lesions in the sweet susceptible maize line.

In the present study, the dose–responses of fumonisin concentrations have been analyzed within a temporal context. In the case of a low dose (1 ppm), there was an adaptive response (21 dap) following an initial disruption in homeostasis, resulting in restored vegetative growth parameters in the affected systems. According to the results, the detoxification/excretion processes could be an adaptive strategy for the host to achieve the compensatory response. Such results are of special interest, and future research should be focused on further characterization of these effects. On the other hand, effects of the fumonisins (20 ppm), such as leaf yellowing, necrosis, and wilting, may be associated with the process called senescence. This is considered to be an indicator of life span in plants, which denotes the processes that lead to the programmed death of individual cells at the end of their life stage. In addition to age, senescence can be triggered by external factors such as pathogen attack, phytohormone application and continuous darkness.[33] By considering the toxins that may affect the developmental timing of the seedlings, it is possible to infer that fumonisins could induce early senescence in maize. Interestingly, this association is in agreement with previous study by

dx.doi.org/10.1021/jf3016333 | J. Agric. Food Chem. 2012, 60, 5667–5675

Brandwagt et al.,[34] who determined that the overexpression of genes that enhanced insensitivity to FB1 and *Alternaria* toxin (AAL) also increased resistance to the induction of senescence in tomato plants. The autoclaved culture materials containing known levels of fumonisins have been widely used to characterize the toxicity of these chemicals in laboratory and farm animals[35,36] as well as in plants.[32] However, in addition to fumonisins, *F. verticillioides* can produce other biologically active compounds, such as fusaric acid,[37,38] fusarin C, beauvericin (which is produced by a low percentage of isolates), and moniliformin (a weak mycotoxin and phytotoxin).[38] Although some phytotoxicity effects have been shown to be attributable to fusaric acid in the pathogenesis of corn seedling, on a liquid culture medium, only members of the mating population C (species of *Fusarium* isolated of rice), isolates of *F. oxysporum* and *F. solani* produced this toxin.[37]

Differences in fungal development in maize and the treatment with fumonisins may reflect variations in the growth rates, aggressiveness, and the time at which the appearance of disease symptoms occurred. In the present study, the data suggest that the production of fumonisins may favor pathogenic development and the switch to a more aggressive phase, thus accelerating the transition from a symptomless to a symptomatic phase. These observations are in agreement with and reinforce the results of Oren et al.[39] However, it would be important to determine the underlying events that convert the pathogen to the saprophytic state, where the produced fumonisins are not acting as phytotoxin. In this regard, and also taking into account that the phenomenon of competitive exclusion indicates that no two ecological homologues can occupy the same niche at the same time, novel strategies of control need to be developed to focus on modulating the toxicogenesis, but at the same time preserving the infection.

AUTHOR INFORMATION

Corresponding Author

*Phone: +54 351 4334164. Fax: +54 351 4333048. E-mail: hectorru@mail.fcq.unc.edu.ar.

Funding

This study was supported by grants from Secretaría de Ciencia y Tecnología-Universidad Nacional de Córdoba; Agencia Nacional de Promoción Científicay Tecnológica (PICT 2010-1232); and Ministerio de Ciencia y Tecnología de Córdoba (0279-005429/2006). S.L.A. and V.S.M. hold doctoral fellowships of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-Argentina). MGT is career investigator of the latter institution.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Paul Hobson for revision of the manuscript redaction.

REFERENCES

(1) IPCS-WHO. Fumonisin B1. Environmental Health Criteria 219. http://www.inchem.org/documents/ehc/ehc/ehc219.htm (accessed Aug 24, 2007).

(2) Williams, L. D.; Glenn, A. E.; Bacon, C. W.; Smith, M. A.; Riley, R. T. Fumonisin production and bioavailability to maize seedlings grown from seeds inoculated with Fusarium verticillioides and grown in natural soils. *J. Agric. Food Chem.* **2006**, *54*, 5694−5700.

(3) Lanubile, A.; Pasini, L.; Lo Pinto, M.; Battilani, P.; Prandini, A.; Marocco, A. Evaluation of broad spectrum sources of resistance to *Fusarium verticillioides* and advanced maize breeding lines. *World Mycotoxin J.* **2011**, *4*, 43−51.

(4) Munkvold, G. P. Cultural and genetic approaches to managing mycotoxins in maize. *Annu. Rev. Phytopathol.* **2003**, *41*, 99−116.

(5) Kleinschmid, C. E.; Clements, M. J.; Maragos, C. M.; Pataky, J. K.; White, D. G. Evaluation of food-grade dent corn hybrids for severity of *Fusarium* ear rot and fumonisin accumulation in grain. *Plant Dis.* **2005**, *89*, 291−297.

(6) Bullerman, L. B.; Tsai, W. Y. J. Incidence and levels of *Fusarium moniliforme*, *Fusarium proliferatum* and fumonisins in corn and corn-based foods and feeds. *J. Food Prot.* **1994**, *57*, 541−546.

(7) Presello, D. A.; Iglesias, J.; Botta, G.; Reid, L. M.; Lori, G. A.; Eyhérabide, G. H. Stability of maize resistance to the ear rots caused by *Fusarium graminearum* and *F. verticillioides* in Argentinian and Canadian environments. *Euphytica* **2006**, *147*, 403−407.

(8) Desjardins, A. E.; Busman, M.; Muhitch, M.; Proctor, R. H. Complementary host−pathogen genetic analyses of the role of fumonisins in the *Zea mays*−*Gibberella moniliformis* interaction. *Physiol. Mol. Plant Pathol.* **2007**, *70*, 149−160.

(9) Kamoun, S. A Catalogue of the effector secretome of plant pathogenic oomycetes. *Annu. Rev. Phytopathol.* **2006**, *44*, 41−60.

(10) Masuda, D.; Ishida, M.; Yamaguchi, K.; Yamaguchi, I.; Kimura, M.; Nishiuchi, T. Phytotoxic effects of trichothecenes on the growth and morphology of *Arabidopsis thaliana*. *J. Exp. Bot.* **2007**, *58*, 1617−1626.

(11) Abbas, H. K.; Tanaka, T.; Duke, S. O.; Porter, J. K.; Wray, E. M.; Hodges, L.; Sessions, A. E.; Wang, E.; Merrill, A. H., Jr.; Riley, R. T. Fumonisin- and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. *Plant Physiol.* **1994**, *106*, 1085−1093.

(12) Yates, I. E.; Bacon, C. W.; Hinton, D. M. Effects of endophytic infection by *Fusarium moniliforme* on corn growth and cellular morphology. *Plant Dis.* **1997**, *81*, 723−728.

(13) Calabrese, E. J.; Baldwin, L. A. Hormesis: the dose-response revolution. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 175−197.

(14) Abbas, H. K.; Boyette, C. D. Phytotoxicity of fumonisin B1 on weed and crop species. *Weed Technol.* **1992**, *6*, 548−552.

(15) Lamprecht, S. C.; Marasas, W. F. O.; Alberts, J. F.; Cawood, M. E.; Gelderblom, W. C. A.; Shephard, G. S.; Thiel, P. G.; Calitz, F. J. Phytotoxicity of fumonisins and TA-toxin to corn and tomato. *Phytopathology* **1994**, *84*, 383−391.

(16) Glenn, A. E.; Zitomer, N. C.; Zimeri, A. M.; Williams, L. D.; Riley, R. T.; Proctor, R. H. Transformation-mediated complementation of a FUM gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. *Mol. Plant*−*Microbe Interact.* **2008**, *21*, 87−97.

(17) Williams, L. D.; Glenn, A. E.; Zimeri, A. M.; Bacon, C. W.; Smith, M. A.; Riley, R. T. Fumonisin disruption of ceramide biosynthesis in maize roots and the effects on plant development and *Fusarium verticillioides*-induced seedling disease. *J. Agric. Food Chem.* **2007**, *55*, 2937−2946.

(18) Agrios, G. N. Effects of pathogens on plant physiological functions. In *Plant Pathology*, 5th ed.; Elsevier Academic Press: London, U.K., 2005; pp 105−108.

(19) Presello, D.; Iglesias, J.; Fernández, M.; Fauguel, C.; Eyhérabide, G.; Lorea, R. Reacción de cultivares a hongos productores de micotoxinas en maíz. http://www.inta.gov.ar/PERGAMINO/info/documentos//t_maiz/09/artic73.htm (accessed April 07, 2010).

(20) Dambolena, J. S.; Zunino, M. P.; Lucini, E. I.; Zygadlo, J. A.; Banchio, E.; Biurrun, F.; Rotman, A.; Ahumada, O. Aromatic plants of northwest Argentina. Constituents of the essential oils of aerial parts of seven Verbenaceae: *Lantana* and *Aloysia*. *J. Essent. Oil Res.* **2010**, *22*, 289−293.

(21) Theumer, M. G.; Lopez, A. G.; Aoki, M. P.; Canepa, M. C.; Rubinstein, H. R. Subchronic mycotoxicoses in rats. Histopathological changes and modulation of the sphinganine to sphingosine (Sa/So) ratio imbalance induced by *Fusarium verticillioides* culture material, due

to the coexistence of aflatoxin B1 in the diet. *Food Chem. Toxicol.* **2008**, *46*, 967−977.
(22) Dantzer, W. R.; Pometto, A. L.; Murphy, P. A. Fumonisin B1 production by *Fusarium proliferatum* strain M5991 in a modified myro liquid medium. *Nat. Toxins* **1996**, *4*, 168−173.
(23) Shephard, G. S.; Sydenham, E. W.; Thiel, P. G.; Gelderblom, W. C. A. Quantitative determination of fumonisins B1 and B2 by high performance liquid chromatography with fluorescence detection. *J. Liq. Chromatogr.* **1990**, *13*, 2077−2087.
(24) Sydenham, E. W.; Shephard, G. S.; Thiel, P. G.; Stockenstrom, S.; Snijman, P. W.; Van Schalkwyk, D. J. Liquid chromatographic determination of fumonisins B1, B2, and B3 in corn: AOAC-IUPAC Collaborative Study. *J. AOAC Int.* **1996**, *79*, 688−696.
(25) Sahay, N. S.; Varma, A. *Piriformospora indica*: a new biological hardening tool for micropropagated plants. *FEMS Microbiol. Lett.* **1999**, *181*, 297−302.
(26) Smucker, A. J. M. Soil environmental modifications of root dynamics and measurement. *Annu. Rev. Phytopathol.* **1993**, *31*, 191−218.
(27) Yates, I. E.; Widstrom, N. W.; Bacon, C. W.; Glenn, A.; Hinton, D. M.; Sparks, D.; Jaworski, A. J. Field performance of maize grown from *Fusarium verticillioides*-inoculated seed. *Mycopathologia* **2005**, *159*, 65−73.
(28) Poorter, H.; Garnier, E. Ecological significance of inherent variation in relative growth rate and its components. In *Handbook of functional plant ecology*; Pugnaire, F. I., Valladares, F., Eds.; Marcel Dekker: New York, USA, 1999; pp 81−120.
(29) Roumet, C.; Urcelay, C.; Diaz, S. Suites of root traits differ between annual and perennial species growing in the field. *New Phytol.* **2006**, *170*, 357−368.
(30) Montesano, M.; Brader, G.; Palva, E. T. Pathogen derived elicitors: searching for receptors in plants. *Mol. Plant Pathol.* **2003**, *4*, 73−79.
(31) Pozo, M. J.; Van Loon, L. C.; Pieterse, C. M. J. Jasmonates—Signals in plant−microbe interactions. *J. Plant Growth Regul.* **2005**, *23*, 211−222.
(32) Desjardins, A. E.; Plattner, R. D.; Nelsen, T. C.; Leslie, J. F. Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays*) seedlings. *Appl. Environ. Microbiol.* **1995**, *61*, 79−86.
(33) Quirino, B. F.; Noh, Y. S.; Himelblau, E.; Amasino, R. M. Molecular aspects of leaf senescence. *Trends Plant Sci.* **2000**, *5*, 278−282.
(34) Brandwagt, B. F.; Kneppers, T. J. A.; Nijkamp, H. J. J.; Hille, J. Overexpression of the tomato Asc-1 gene mediates high insensitivity to AAL toxins and fumonisin B1 in tomato hairy roots and confers resistance to *Alternaria alternata* f. sp. *lycopersici* in *Nicotiana umbratica* plants. *Mol. Plant−Microbe Interact.* **2002**, *15*, 35−42.
(35) Asrani, R. K.; Katoch, R. C.; Gupta, V. K.; Deshmukh, S.; Jindal, N.; Ledoux, D. R.; Rottinghaus, G. E.; Singh, S. P. Effects of feeding *Fusarium verticillioides* (formerly *Fusarium moniliforme*) culture material containing known levels of fumonisin B1 in Japanese quail (*Coturnix coturnix japonica*). *Poult. Sci.* **2006**, *85*, 1129−1135.
(36) Theumer, M. G.; Canepa, M. C.; Lopez, A. G.; Mary, V. S.; Dambolena, J. S.; Rubinstein, H. R. Subchronic mycotoxicoses in Wistar rats: assessment of the *in vivo* and *in vitro* genotoxicity induced by fumonisins and aflatoxin B(1), and oxidative stress biomarkers status. *Toxicology* **2010**, *268*, 104−110.
(37) Bacon, C. W.; Porter, J. K.; Norred, W. P.; Leslie, J. F. Production of fusaric acid by *Fusarium* species. *Appl. Environ. Microbiol.* **1996**, *62*, 4039−4043.
(38) Bacon, C. W.; Glenn, A. E.; Yates, I. E. *Fusarium verticillioides*: managing the endophytic association with maize for reduced fumonisins accumulation. *Toxin Rev.* **2008**, *27*, 411−446.
(39) Oren, L.; Ezrati, S.; Cohen, D.; Sharon, A. Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Appl. Environ. Microbiol.* **2003**, *69*, 1695−1701.