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Fumonisin Disruption of Ceramide Biosynthesis in Maize Roots and the Effects on Plant Development and *Fusarium verticillioides*-Induced Seedling Disease

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The fungus *Fusarium verticillioides* infects maize and produces fumonisins, inhibitors of ceramide synthase. Seeds of the cultivar Silver Queen were inoculated with fumonisin-producing or non-fumonisin-producing strains of *F. verticillioide*s. Leaf lesion incidence and severity of effects on root and stalk growth were significantly correlated with fumonisin in roots and disruption of sphingolipid metabolism in roots. Uninoculated seeds grown in soil watered with solutions of fumonisin B_1 exhibited above-ground symptoms indicative of *F. verticillioides*-induced seedling disease and dose-dependent reduction in root mass that was inversely correlated with fumonisin B_1, sphingoid bases, and sphingoid base 1-phosphates in roots. There was also evidence of an adaptive response to disrupted sphingolipid metabolism in both the virulence and watering assays, suggesting induction of pathways responsible for metabolism of sphingoid base 1-phosphates after prolonged exposure. The results suggest that fumonisin, and its effects on sphingolipids, could contribute to all aspects of *F. verticillioides* maize seedling disease.

KEYWORDS: Fumonisin; *Fusarium verticillioides*; maize; *Zea mays*; seedling disease

INTRODUCTION

Fumonisins are water-soluble mycotoxins produced by the fungus *Fusarium verticillioides*, a nonobligate plant pathogen commonly associated with maize (*Zea mays*) (*1*, *2*). Diseases of maize associated with *F. verticillioides* include seed rot, root rot, stalk rot, kernel or ear rot, and seedling blight (*3*, *4*). In addition to the fumonisins, *F. verticillioides* produces a number of other fungal metabolites (*1*); however, only the fumonisins are known to cause diseases in farm animals and are possible human carcinogens (*5*, *6*). At present, at least 28 different fumonisins have been reported (*7*). Fumonisin B_1 is the most abundant and is believed to be the most toxic of the fumonisins (*5*).

The role that fumonisins play in pathogenicity in maize is not understood. Although they are found to contribute to *F. verticillioides* virulence in seedlings grown from inoculated maize seeds, based on the ability of a non-fumonisin-producing strain to reduce seedling emergence and shoot growth, it was concluded that fumonisins were neither necessary nor sufficient for virulence (*8*). The structurally similar compound, *Alternaria alternata lycopersici* (AAL) toxin, has been shown to be a pathogenicity factor for *Alternaria alternata* f. sp. *lycopersici*-induced stem canker disease in tomato (*Lycopersicon esculentum*) (*9*). In tomato protoplasts and leaflets, the mechanism of cell death induced by AAL toxin and fumonisin B_1 is apoptotic-like (*10*). Studies in protoplasts and infiltrated leaflets from *Arabidopsis thaliana* indicate that fumonisin-induced programmed cell death is light-dependent and requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways (*11*). Interestingly, *Arabidopsis* seedlings grown on agar medium containing fumonisin developed similar lesions on leaves, but root growth was largely unaffected (*12*). In susceptible tomato plants, fumonisin B_1 or mycelia and spores of *F. verticillioides* sprayed on aerial parts have been shown to mimic both the symptoms of AAL toxin and *A. alternata* f. sp. *lycopersici*-induced toxicity (*13*). Similar experiments that involved spraying maize plants with concentrations of fumonisin B_1 as high as 1387 nmol/mL did not produce any symptoms of disease (*14*). However, fumonisin B_1 did cause significant reductions in growth of maize seedlings and callus tissue (*15*, *16*). Thus, the pathological consequences of exposure to fumonisins in plants, as in animals, are species-specific and the route of exposure plays a large role in disease expression. The common denominator in animals is disruption of sphingolipid metabolism.

The structure of fumonisin B_1 is very similar to that of the free sphingoid base sphinganine, which led to the hypothesis that its mechanism of action might be related to disruption of

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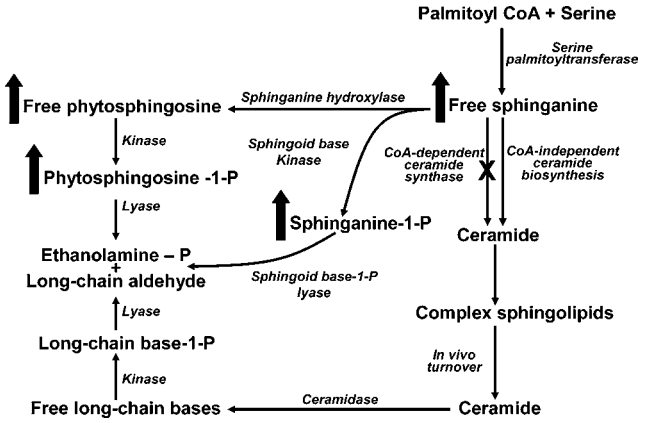

Figure 1. Proposed de novo sphingolipid biosynthesis and turnover pathway in plants, showing point of disruption by fumonisin B_1 (X) [coenzyme A- (CoA-) dependent ceramide synthase]. Bold arrows indicate known effects of fumonisin B_1 inhibition of ceramide synthase on accumulation of free sphinganine (d18:0; dihydrosphingosine), free phytosphingosine (t18:0; 4-hydroxysphinganine), sphinganine 1-phosphate, and phytosphingosine 1-phosphate in roots from maize seedlings (*23*). Key enzymes or enzymatic steps in the pathway are indicated in italic type. The key steps in the pathway are derived from the review of Lynch and Dunn (*31*) and from Williams et al. (*23*).

either sphingolipid metabolism or function (*17*). Today, there is considerable evidence that fumonisin-induced disruption of sphingolipid metabolism is important in the cascade of events leading to altered cell growth, differentiation, and cell injury observed both in vitro and in vivo (*18*, *19*). The most important piece of evidence is that fumonisin B_1 and AAL toxin are inhibitors of ceramide synthase, a key enzyme in the de novo sphingolipid biosynthetic pathway (**Figure 1**) (*17*, *20*). Fumonisin inhibition of ceramide synthase, as evidenced by elevation of free sphingoid bases (*21*), has been shown in several plant models (*13*, *22*) including roots of maize seedlings (*23*) and excised maize shoots (*24*). The toxicity of AAL toxin in tomato is closely associated with disruption of sphingolipid metabolism (*13*), and tomato varieties resistant to *A. alternata* f. sp. *lycopersici* are also resistant to AAL toxin and fumonisin-induced accumulation of free sphingoid bases (*13*). In contrast, even though maize tissues (cob, stalks, and kernels) can contain detectable levels of fumonisins, fumonisin-induced disruption of sphingolipid metabolism has not been shown to be a mechanism of any disease in maize.

F. verticillioides is found in maize debris (*25*). Therefore it is reasonable to expect that fungus-infected debris and soil could serve as a source of fungal inoculum, and possibly fumonisins, in the soil and rhizosphere. In sandy loam soils, fumonisins are bound tightly but can be released under acidic conditions (*27*). Recently, it was reported that *F. verticillioides* can produce fumonisins in natural soils and that the fumonisin that is produced is biologically available, based on increased free sphingoid bases and sphingoid base 1-phosphates in the roots of maize seedlings (*23*). In addition, it was found that the amount of fumonisin B_1 in the natural soils was significantly correlated with the number of leaf lesions and the decrease in root mass (*23*).

The biochemical consequences of ceramide synthase inhibition have been closely correlated with fumonisin-induced diseases in animals (*19*). This is not surprising since sphingolipids are important signaling molecules and are involved in regulating numerous cellular processes critical to cell survival (*18*). There is increasing evidence for the importance of sphingolipids as signaling molecules in plants (*28*−*32*), and therefore it is likely that fumonisin disruption of sphingolipid metabolism in maize roots (*23*) is an important contributor to maize seedling disease in *F. verticillioides*-infected plants.

The purpose of these studies was to determine the role of fumonisin in *F. verticillioides*-induced maize seedling disease and to explore the possible role of ceramide synthase inhibition as the mechanistic basis for the effects of fumonisins. Effects on ceramide synthase were assessed by changes in free sphingoid bases and sphingoid base 1-phosphates as markers for fumonisin-induced disruption of sphingolipid metabolism. The specific objectives were to determine (i) the time course for expression of disease symptoms in *F. verticillioides* MRC826-infected maize seedlings and their relationship to fumonisin production and disruption of sphingolipid metabolism in roots; (ii) the ability of selected fumonisin-producing and non-fumonisin-producing strains of *F. verticillioides* to induce disease symptoms and disrupt sphingolipid metabolism in roots; and (iii) the ability of fumonisin alone to induce disease symptoms and disrupt sphingolipid metabolism in roots.

MATERIALS AND METHODS

Virulence Assay. Five strains of *F. verticillioides*, designated NRRL25059 [NRRL = Northern Regional Research Laboratory (=NCAUR), USDA-ARS, Peoria, IL], JFL-04516 (JFL = John F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan, KS), AEG1-1-57 (AEG = Anthony E. Glenn, Russell Research Center, USDA-ARS, Athens, GA), AEG3-A3-6, and MRC826 (Medical Research Council, Tygerberg, South Africa), were used in this study.

The phylogenetic placement of strain NRRL25059 is uncertain (*33*). Broad-scale phylogenetic assessment and mating studies (*34*−*37*) identify it as a *F. verticillioides* species; however, similarities with other banana isolates suggest it is a cryptic species having a close phylogenetic relationship with *F. verticillioides* (*37*−*39*). AEG1-1-57 was derived from a sexual cross between MRC826 and NRRL25059. AEG3-A3-6 was obtained from a backcross between AEG1-1-57 and MRC826. Strain JFL-A04516 has a mutation in *FUM1*, a polyketide synthase necessary for fumonisin production (*40*).

The conidia or hyphae from all strains were frozen at −80 °C in 15% glycerol until inoculated on potato dextrose agar and incubated at 27 °C in the dark to initiate experimental cultures. The inoculum (conidia and mycelia) was obtained by flooding the agar surface with 10 mL of sterile water and diluting the concentrated inoculum to 10^4 colony-forming units/mL. In order to determine the ability of the five strains to produce fumonisins on maize kernels, twice-autoclaved cracked maize (5 g hydrated with 45% water in a 20 mL glass vial) was inoculated with the conidial suspension ($\sim 2.5 \times 10^7$ conidia). Two vials of maize were inoculated for each strain. After 14 days of incubation at 27 °C in the dark, 10 mL of acetonitrile:water (1:1) was added to each vial, and the vials were shaken and allowed to stand and then analyzed for fumonisin as described below.

Maize seed (Silver Queen) (Gurney's Seed & Nursery Co., Yankton, SD) were surface-disinfected for 10 min in 100% bleach (5.25% hypochlorite), rinsed with sterile water, and allowed to imbibe for 4 h in sterile water. The seed were then heat-shocked by placing them in a 60 °C water bath for 5 min for internal sterilization (*41*). Inoculations were performed by placing sterilized seeds in a Petri dish (100 mm) and flooding them with 10 mL of the conidial suspension. Sterile water was used for the control group. The seed were incubated overnight at 27 °C. Samples of the seeds before and after inoculation were analyzed for fumonisins. Three or five replicates of 10 seeds each were planted in sterile 4-in. plastic azalea pots (Hummert International, Earth City, MO) containing twice-autoclaved commercial potting mix (45% sphagnum peat, Conrad Fafard Inc., Agawam, MA). Pots were watered from below for the first 6 days and then watered as needed from above throughout the duration of the assay. Assays were performed under aseptic conditions in a plant growth chamber at 30 °C under a 14 h

light (cool-white, high-output fluorescent tubes at an average of 254 μmol m^{-2} s^{-1}) and a 10 h dark regime at 22 °C.

Disease symptoms were visually assessed for indications of seedling blight (*35*) for up to 21 days after planting. The exposure time was chosen on the basis of other studies with the sweet maize cultivars Polar Vee (*35*) and Silver Queen (*42*). The earlier study (*35*) showed that seedlings grown from seeds inoculated with MRC826 or NRRL25059 had endophytic infection with the inoculated strains in all tested aerial tissues (leaf, whorl, and node). Control tissues from surface-sterilized kernels showed no infection with fungi. In the present study, two separate virulence assays were conducted: one was a time course study, with MRC826 only, that lasted 21 days, and seedlings were harvested on days 7, 14, and 21. The second virulence assay utilized all five strains of *F. verticillioides* and all seedlings were harvested at 21 days.

Fumonisin Preparation and Fumonisin Watering Assays. Fumonisin B_1 was prepared by the method of Meredith et al. (*43*), and the purity (>96%) was determined by the procedure of Plattner and Branham (*44*). Impurities were primarily other fumonisins and fumonisin dimers. Fumonisin B_1 was dissolved in 1 L of sterile distilled water to make concentrations of 1.4, 6.9, 13.9, and 27.7 nmol/mL to be used for the watering assay described below.

Maize seeds (Silver Queen) were surface-disinfected. Three replicates of 10 sterilized seeds each were planted in sterile 4-in. plastic azalea pots containing twice-autoclaved commercial potting mix, which was confirmed to lack any detectable fumonisins. Plants were watered with 100 mL solutions of each concentration of fumonisin B_1 on days 2, 4, and 6 after planting. The plants were then watered with sterile water as needed until the plants were harvested 8 and 21 days after planting. Control groups were similarly treated but with sterile distilled water. Assays were performed in a plant growth chamber, cycling as previously described; however, the daytime temperature was 27 °C. Upon harvest, disease symptoms were assessed. The number of surviving plants, height of surviving plants, dry weight of roots, number of plants with leaf lesions, and number of plants with leaf developmental abnormalities was recorded.

In order to more easily observe effects on the young root system, sterilized Silver Queen seed ($n = 3$/treatment) were grown on Murashige and Skoog (*45*) medium with 8 g/L Caisson plant culture gelling agent in the presence of 0 or 13.9 nmol/mL fumonisin B_1 at room temperature on the benchtop under fluorescent lights. The developing root system was observed daily, and after 14 days seedlings were removed from the tubes and the effects on root development was visually assessed.

Extraction of Fumonisins from the Soils and Roots. After harvest, the soils from each replicate in both the virulence and the fumonisin B_1 watering assays were carefully separated from the root mass, collected, allowed to air-dry in a fume hood, and then stored at −20 °C. The intact roots from each pot and treatment were immersed in an ice−water bath to remove any remaining soil. The washed soil-free roots were then allowed to drain and were blotted dry and placed in a −80 °C freezer overnight. Because fumonisins are water-soluble compounds (>27.7 mmol/L), most if not all fumonisins external to the roots should have been removed by the water washing. The plants were then freeze-dried, and the roots were separated from the leaves and stalks and placed into labeled zip-lock bags. Soils were also carefully inspected to remove all visible root materials. Fumonisins in the soil from the 21-day virulence assay (five strains of *F. verticillioides*) and the watering assay were extracted and analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/MS) as described by Williams et al. (*23*).

Prior to fumonisin extraction, the water-washed freeze-dried root tissues were carefully inspected to remove any remaining soil. Clean root tissues were weighed to determine the effects of fumonisin on root growth. The root tissues were then placed in round-bottom tubes, pulverized with a glass rod to a fine powder under liquid nitrogen, and stored in a vacuum desiccator with anhydrous calcium sulfate. To measure the amount of fumonisins in the roots, samples consisting of 10 mg of ground, homogenized root tissue from each replicate were placed into 2 mL polypropylene tubes and extracted by a procedure similar to that used for extracting soils. Briefly, distilled water (1 mL) was added to each tube and the tubes were shaken for 2 h. The tubes were then centrifuged for 10 min at 16 000 rcf and the supernatants were collected to be analyzed for water-extractable fumonisin that was defined as "loosely" associated with the roots. More "tightly" bound fumonisin was extracted by adding distilled water, formic acid, and acetonitrile to the pelleted roots to make a final volume of 1 mL of acetonitrile/5% formic acid in water (1:1 v/v). This approach was developed for extracting fumonisins from sandy loam soils (*25*) that retained significant amounts of fumonisin that was released by extraction with the acidic solution. The tubes were shaken for 2 h and centrifuged for 10 min at 16 000 rcf, and the supernatants were collected and placed into new polypropylene tubes. Distilled H_2O was added to the supernatant to make a final proportion of 30:70 acetonitrile/3% formic acid in water. Samples were analyzed for fumonisin by LC/MS (*23*). The detection limit for fumonisin B_1 is 0.006 nmol/g.

Extraction of Free Sphingoid Bases and Sphingoid Base 1-Phosphates. To determine the effect of treatments on sphingolipid biosynthesis, the ground root tissues were analyzed for sphinganine, phytosphingosine, sphinganine 1-phosphate, and phytosphingosine 1-phosphate. The extraction and LC/MS methods were the same as described by Williams et al. (*23*). Briefly, samples consisting of 20 mg of ground root tissue from each replicate were homogenized, and a solution of MeOH and $CHCl_3$ containing internal standards (C_{17} sphinganine 1-phosphate and C_{20} dihydrosphingosine) (Avanti Polar Lipids, Inc., Alabaster, AL) in ethanol was added to each sample. Samples were incubated overnight at 48 °C in a heating block, methanolic KOH was added, and the samples were incubated at 37 °C to hydrolyze glycerolipids. The samples were then centrifuged, and the supernatants were removed, neutralized, and then evaporated to dryness. Samples were reconstituted in acetonitrile/water/formic acid containing 5 mM ammonium formate and clarified by filter centrifugation and then analyzed by LC/MS (*23*). The detection limits for sphingoid bases and sphingoid base 1-phosphates were 0.06 and 0.4 nmol/g, respectively.

Some samples of the root extracts were also analyzed by LC/MS by data-dependent scanning of the two most intense ions (m/z 150−600). The purpose of the data-dependent scans was to determine if free long-chain bases other than phytosphingosine and sphinganine were elevated by fumonisin treatment; specifically, sphingosine (d18:1; 4-sphingenine), 8-sphingenine (d18:1), 4,8-sphingadienine (d18:2), and 4-hydroxy-8-sphingenine (t18:1). These unsaturated sphingoid bases would most likely be derived from sphinganine with both hydroxylation and reduction occurring subsequent to ceramide formation (*31*).

Statistical Analysis. Statistical analysis was performed with SigmaStat software (Jandel Scientific, San Rafael, CA). Where many groups were compared, one-way analysis of variance (ANOVA) was used, followed by post hoc multiple comparisons. Where only two groups were compared, a Student's *t*-test or Mann Whitney rank sum test was used. A χ^2 test was performed to determine the significance of leaf lesion frequency data. The Pearson product moment correlation was used to measure the strength of the association between pairs of variables. All data were expressed as mean ± standard deviation, and differences among or between means were considered significant if the probability (p) was ≤0.05.

RESULTS

Clear evidence of seedling disease was apparent as early as 7 days and persisted for at least 21 days after planting of seeds inoculated with the pathogenic *F. verticillioides* strain MRC826. Visual symptoms of disease included stunting, necrotic leaf lesions, abnormal leaf development, and reduced root development compared to the control group (**Figure 2**). Reduced growth of aerial plant parts and roots was apparent as early as 7 days after planting of seeds inoculated with 10^4 total colony-forming units (**Figure 3A,B**). Leaf lesions were not apparent on day 7 but were easily observed in almost all plants by day 14 (**Figure 3A**, inset). Fumonisins were detected in roots on day 7 and were maximal by day 14 (**Figure 3C**). When planted, the MRC826 inoculated seeds contained no detectable fumonisins (<0.006 nmol/g). Significant increases in the levels of free phytosphingosine and free sphinganine were observed on day 7 and were

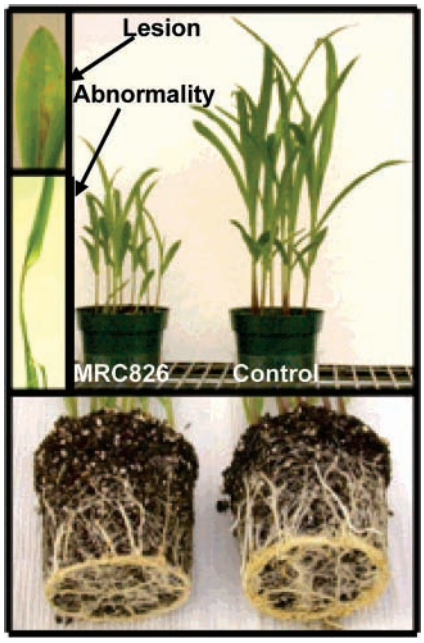

Figure 2. Example of stunting, necrotic leaf lesion, abnormal leaf development, and reduced root development in Silver Queen seedlings 21 days after planting of seeds inoculated with 10^4 spores/mL of the pathogenic fumonisin-producing *F. verticillioides* strain MRC826. A pot of control seedlings grown from uninoculated seeds is shown for comparison.

maximal on day 14 (**Figure 4A,B**). The maximal elevation in sphingoid base 1-phosphates was observed on day 7 (**Figure 4C,D**). Phytosphingosine 1-phosphate decreased slightly after day 14 but the decrease was not statistically significant (**Figure 4C**). The level of sphinganine 1-phosphate decreased significantly after day 7 (**Figure 4D**).

The five strains of *F. verticillioides* used in the 21-day virulence assay were tested for their ability to produce fumonisins on maize. Strains NRRL25059, JFL-A04516, and AEG1-1-57 did not produce fumonisins when grown on maize culture for 14 days. Analysis of 14-day cultures of strains MRC826 and AEG3-A3-6 produced on average 106 and 16 nmol of fumonisin B_1/g ($n = 5$), respectively, and the difference was statistically significant ($p < 0.001$).

In the 21-day virulence assay, there was no significant effect on seedling survival with any of the non-fumonisin-producing or fumonisin-producing strains of *F. verticillioides* compared to the controls (**Table 1**). Leaf lesions occurred only on the seedlings grown from seeds inoculated with the two fumonisin-producing strains, and the incidence was significantly greater in the MRC826 strain compared to the AEG3-A3-6 strain. All of the seedlings grown from seeds inoculated with fungi showed reductions in height and root mass at 21 days compared to controls. However, the changes were less pronounced in the non-fumonisin-producing strains. The reductions in both height and root mass of the MRC826-treated seedlings were significantly greater than for any of the other tested strains, including the other fumonisin-producing strain AEG3-A3-6 (**Table 1**). Fumonisin B_1 was detected only in extracts of the potting soil and roots of the maize seedlings grown from seeds inoculated with MRC826 and AEG3-A3-6, and significantly more fumonisin B_1 was present in both soil and roots of the MRC826 treatment group compared to the AEG3-A3-6 treated seedlings (**Table 1**). Fumonisins B_2 and B_3 were also detected (data not shown). The concentrations of free phytosphingosine and sphinganine were greatest in the roots of the seedlings treated with the two fumonisin-producing strains, and phytosphingosine

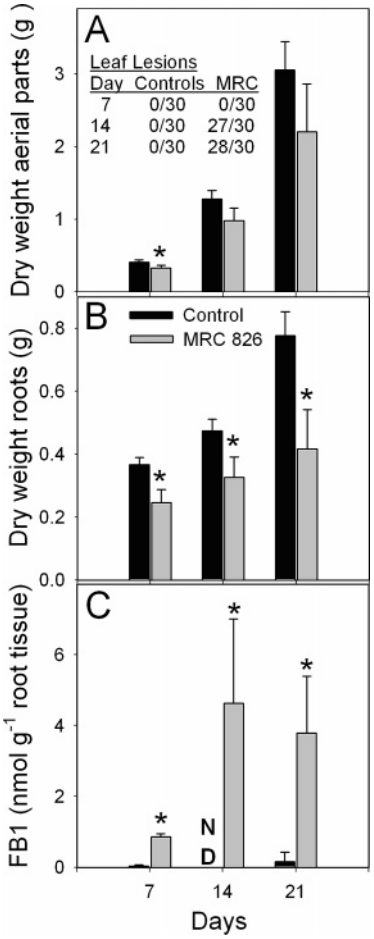

Figure 3. Reduced growth of (**A**) aerial plant parts (leaves and stems) and (**B**) roots and (**C**) increase in fumonisin B_1 in roots of plants grown from uninoculated seeds (control; black bars) or seeds inoculated with 10^4 spores/mL of the pathogenic fumonisin-producing *F. verticillioides* strain MRC826 (MRC; gray bars) and harvested after 7, 14, and 21 days. (A, inset) Incidence (three pots with 10 plants/pot) of leaf lesions (**Figure 2**) observed on days 7, 14, and 21. Leaf lesions were first observed on day 9. Values are means ± SD, $n = 3$ pots/treatment at each time point. An asterisk indicates a significant difference ($p \leq 0.05$) between the control and MRC826-treated pots at each time point; ND, not detected.

1-phosphate and sphinganine 1-phosphate were detected only in the roots of the seedlings from the groups treated with the two fumonisin-producing strains.

The results of the correlation analysis showed that there were highly significant positive correlations between the amount of fumonisin B_1 in the roots and the incidence of leaf lesions and the increase in free sphingoid bases and sphingoid base 1-phosphates in roots (**Table 2**). Conversely, there were highly significant negative correlations between fumonisin B_1 in the roots and the root weight and stalk height.

In the watering assay (plants watered with fumonisin solutions), germination and seedling survival was >93% for all treatment groups harvested on 21 days (**Table 3**). Stunting and leaf lesions were not seen at 8 days (data not shown) but were seen in the plants harvested on day 21 and first appeared on day 13. Leaf lesions were not observed in the control or 1.4 nmol/mL fumonisin B_1 treatment groups on day 21 but were seen in all other groups, and the total incidence of necrotic leaf lesions in the 6.9, 13.9, and 27.7 nmol/mL fumonisin B_1 treatment groups were 3/28, 4/28, and 10/30 (number of plants exhibiting lesions over total plants harvested), respectively. Abnormal leaf development was observed only in the 27.7 nmol/

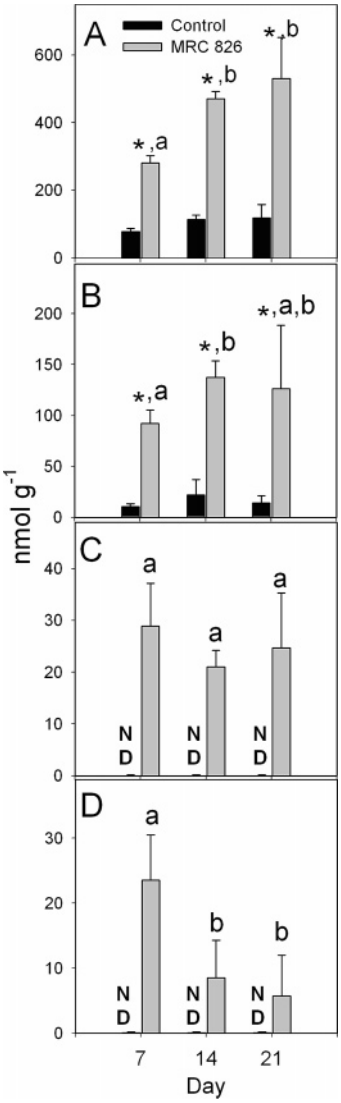

Figure 4. Change in (**A**) free phytosphingosine, (**B**) free sphinganine, (**C**) phytosphingosine 1-phosphate, and (**D**) sphinganine 1-phosphate in plants grown from uninoculated seeds (control) or seeds inoculated with 10^4 spores/mL of the pathogenic fumonisin-producing *F. verticillioides* strain MRC826 (MRC) and harvested after 7, 14, and 21 days. Values are means ± SD, $n = 3$ pots/treatment at each time point. An asterisk indicates a significant difference ($p \leq 0.05$) between the control and MRC826-treated pots at each time point. Differing letters (a, b) indicate significant differences ($p \leq 0.05$) among the MRC826 group at each sample time (there were no significant differences among the controls); ND, not detected.

mL fumonisin B_1 group harvested on day 21. The mean height of the seedlings on day 21 was decreased at 6.9, 13.9, and 27.7 nmol/mL fumonisin B_1 compared to the control group but was statistically significant only in the 27.7 nmol/mL fumonisin B_1 group ($p \leq 0.05$). A statistically significant ($p \leq 0.05$) decrease in root mass was observed at 8 days in the 27.7 nmol/mL fumonisin B_1 treatment group (data not shown). At 21 days there was a clear dose-dependent decrease in root mass (**Table 3**). The decrease was statistically significant in the 6.9, 13.9, and 27.7 nmol/mL fumonisin B_1 treatment groups compared to the control group. Nonetheless, total root mass was significantly ($p \leq 0.05$) increased in all groups at 21 days compared to 8 days (data not shown), indicating that, although inhibited, root growth continued after 8 days at a reduced rate.

In order to better visualize the effects of fumonisin B_1 on root development, sterilized Silver Queen seeds were germinated and grown on culture medium in the presence of 0 or 13.9 nmol/mL fumonisin B_1 for 14 days. Compared to the control, the root system of seedlings exposed to fumonisin B_1 exhibited reduced growth of the primary, seminal, and lateral roots. While the initiation of lateral roots was apparent, lateral root elongation was limited and there was a complete lack of root hair development in all roots that were in contact with the medium containing fumonisin. In addition to the effects on the development of the root system, there was reduced shoot growth, although it was much less marked compared to the reduced growth of the root system.

After harvest, the soils from each treatment were collected and analyzed for the presence of fumonisins. Fumonisin B_1 was detected in each treatment and at both time points at similar levels, and the increase was dose-dependent at 8 days (data not shown) and 21 days (**Table 3**). With the exception of the soils watered with the 1.4 nmol fumonisin B_1/mL solution, the levels of fumonisin B_1 detected in the soils at 8 and 21 days were not significantly different ($p > 0.05$). No fumonisin B_1 was detected in the control soils at either time point, and neither fumonisin B_2 nor fumonisin B_3 was detected in any of the soils watered with fumonisin B_1 solutions. The roots of the plants harvested at both time points were analyzed for fumonisin B_1; the toxin was detected in the roots at both time points, and there was a dose-dependent increase in the levels detected (**Table 3**). The levels of fumonisin B_1 in the roots at 21 days were significantly elevated, relative to the 8-day values, in the plants watered with 6.9, 13.9, and 27.7 nmol/mL solutions of fumonisin B_1 ($p \leq 0.05$).

Watering with fumonisin B_1 resulted in inhibition of ceramide synthase as indicated by the accumulation of sphingoid bases and sphingoid base 1-phosphates in the roots. The increase in the levels of both phytosphingosine and sphinganine in roots on days 8 (data not shown) and 21 (**Table 3**) were of a similar magnitude. The absolute amount of accumulated phytosphingosine was greater than sphinganine, and the levels of accumulation for both were clearly dose-dependent on day 21. With the exception of free phytosphingosine in the 27.7 nmol of fumonisin B_1/mL group, the differences in the levels of free phytosphingosine and free sphinganine on days 8 and 21 in the roots were not statistically significant ($p \leq 0.05$). However, there was a statistically significant reduction in the levels of both phytosphingosine 1-phosphate and sphinganine 1-phosphate on day 21 compared to the values at day 8 (**Figure 5**). Neither phytosphingosine 1-phosphate nor sphinganine 1-phosphate was detected in the roots of the control seedlings on days 8 or 21.

In order to determine if long-chain bases and base 1-phosphates other than phytosphingosine, sphinganine, phytosphingosine 1-phosphate, and sphinganine 1-phosphate were elevated by fumonisin B_1-induced ceramide synthase inhibition, root extracts were analyzed by data-dependent scanning of the two most intense ions (m/z 150−600). There was no evidence that any other long-chain bases or their 1-phosphates were present in the extracts of either fumonisin-treated or control-treated seedling roots, indicating that there was very little turnover of more complex sphingolipids. In addition, the precursor for sphinganine, 3-ketosphinganine, was not detected (data not shown).

The results of the correlation analysis showed that there were highly significant positive correlations between the amount of fumonisin B_1 in the roots and the incidence of leaf lesions and the increase in free sphingoid bases and sphingoid base 1-phosphates in roots (**Table 4**). Conversely, there were highly significant negative correlations between fumonisin B_1 in the roots and the root weight and stalk height.

Table 1. Summary of Phytotoxic Effects and Levels of Fumonisin B_1, Free Sphingoid Bases, and Sphingoid Base 1-Phosphates from the Virulence Assay[a]

treatment	survival[b]	leaf lesions[c]	height[d] (cm)	root weight (mg)	FB_1 roots[e]	FB_1 soil[e]	Pso[e]	Sa[e]	Pso-1-P[e]	Sa-1-P[e]
control	50/50	0/5 (0/50)	55[f] (100%)	736[f] (100%)	ND	ND	223[f]	5[f]	ND	ND
NRRL25059	49/50	0/5 (0/50)	54[f,g] (99%)	678[f,g,h] (92%)	ND	ND	232[f]	5[f]	ND	ND
JFL-A04516	50/50	0/5 (0/50)	49[g,h] (90%)	540[g,h,i] (73%)	ND	ND	259[f]	6[f]	ND	ND
AEG1-1-57	50/50	0/5 (0/50)	53[f,g] (97%)	516[h,i] (70%)	ND	ND	273[f,g]	7[f]	ND	ND
AEG3-A3-6	50/50	5/5 (23/50)	47[h] (86%)	442[i] (60%)	2.8[f]	16.6[f]	394[g,h]	14[g]	7[f]	2.2[f]
MRC826	48/50	5/5 (48/50)	38[i] (69%)	264[j] (36%)	16.6[g]	48.5[g]	490[h]	25[h]	9[f]	3.6[g]

[a] Phytotoxic effects, amounts of fumonisin B_1 (FB_1) in roots and soil, and amounts of phytosphingosine (Pso), sphinganine (Sa), phytosphingosine 1-phosphate (Pso-1-P), and sphinganine 1-phosphate (Sa-1-P) are tallied from the virulence assay with fumonisin-producing (MRC826 and AEG3-A3-6) and non-fumonisin-producing (NRRL25059, AEG 1-1-57, and JFL-A04516) strains of *F. verticillioides* at 21 days grown from seeds inoculated at 10^4 spores/mL. [b] Indicates the total number of seedlings harvested on day 21 over the number of seeds planted (10/pot). [c] Indicates the number of pots that had at least one seedling exhibiting leaf lesions over the total number of pots per treatment ($n = 5$). In parentheses is the number of plants exhibiting leaf lesions/total plants. On the basis of χ^2 analysis, there was a statistically significant difference ($p < 0.001$) in the incidence of leaf lesions between the fumonisin-producing strains (AEG3-A3-6 and MRC826) and the non-fumonisin-producing strains (NRRL25059, JFL-A04516, and AEG1-1-57), and there was also a statistically significant difference ($p < 0.001$) in the incidence of leaf lesions between the two fumonisin-producing strains. [d] Value is the mean ± standard deviation ($n = 5$) of the average stalk height of all plants harvested on day 21 from each treatment group. The change, expressed as a percent of the control, is given in parentheses for both stalk height and root weight. [e] Values for fumonisin B_1, sphingoid bases, and sphingoid base 1-phosphates are the mean nanomoles per gram ($n = 5$). ND, not detected. [f−j] Means within columns with differing letters are significantly different ($p \leq 0.05$).

Table 2. Pearson Product Moment Correlations in the Virulence Assay[a]

	leaf lesions	root weight	stalk height	Pso	Sa	Pso-1-P	Sa-1-P
correlation coefficient[b]	0.95	–0.73	–0.83	0.90	0.97	0.91	0.89
p value[c]	3.9×10^{-15}	1.1×10^{-5}	5.5×10^{-8}	4.7×10^{-11}	3.1×10^{-17}	3.4×10^{-11}	2.2×10^{-10}

[a] Correlations are given between the amount of fumonisin B_1 tightly associated with roots (FB, nanomoles per gram of roots) and the presence of leaf lesions, root weight, stalk height, and amounts of free phytosphingosine (Pso), free sphinganine (Sa), phytosphingosine 1-phosphate (Pso-1-P), and sphinganine 1-phosphate (Sa-1-P) on day 21 in the virulence assay with fumonisin-producing and non-fumonisin-producing strains of *F. verticillioides* ($n = 30$ pots). [b] Pairs of variables with positive correlation coefficients tend to increase together, and those with negative coefficients are inversely correlated. [c] The exact *p* values are presented in exponential format, and *p* values less than 0.05 (5×10^{-2}) are considered statistically significant.

Table 3. Summary of Phytotoxic Effects and Levels of Fumonisin B_1, Free Sphingoid Bases, and Sphingoid Base 1-Phosphates from the Watering Assay[a]

fumonisin B_1 (nmol/mL)	survival[b]	leaf lesions[c]	height (cm)[f]	root weight (mg)	FB_1 roots[e]	FB_1 soil[e]	Pso[e]	Sa[e]	Pso-1-P[e]	Sa-1-P[e]
0	29/30	0/3 (0/29)	67[f] (100%)	656[f] (100%)	0.14[f]	ND	77[f]	4[f]	ND	ND
1.4	30/30	0/3 (0/30)	70[f] (104%)	693[f] (106%)	0.42[f]	2.77[f]	150[f,g]	12[f,g]	17[f]	4[f]
6.9	28/30	2/3 (3/28)	64[f,h] (96%)	523[h] (80%)	1.66[g]	13.87[g]	178[g]	16[f,g]	33[f,g]	8[f,g]
13.9	28/30	2/3 (4/28)	60[h] (89%)	373[i] (57%)	2.77[h]	20.80[h]	194[g]	24[g,h]	45[g]	15[h]
27.7	30/30	3/3 (10/30)	52[i] (77%)	266[j] (41%)	6.80[i]	63.80[i]	225[g]	34[h]	50[g]	27[i]

[a] Phytotoxic effects, amounts of fumonisin B_1 (FB_1) in roots and soil, and amounts of phytosphingosine (Pso), sphinganine (Sa), phytosphingosine 1-phosphate (Pso-1-P), and sphinganine 1-phosphate (Sa-1-P) are tallied after the watering of maize seedlings with fumonisin B_1 at the indicated concentrations for 21 days. [b] Indicates the total number of seedlings harvested on day 21 over the number of seeds planted (10/pot). [c] Indicates the number of pots that had at least one seedling exhibiting leaf lesions over the total number of pots per treatment ($n = 3$). In parentheses is the number of plants exhibiting leaf lesions/total plants. On the basis of χ^2 analysis, there was a statistically significant increase in the incidence of leaf lesions with dose ($\chi^2 = 71.5$, degrees of freedom = 4, $p < 0.0001$). [d] Value is the mean ± standard deviation ($n = 3$) of the average stalk height of all plants harvested on day 21 from each treatment group. The change, expressed as a percent of the control, is given in parentheses for both stalk height and root weight. [e] Values for fumonisin B_1, sphingoid bases, and sphingoid base 1-phosphates are the mean nanomoles per gram ($n = 3$). ND, not detected. [f−i] Means within columns with differing letters are significantly different ($p \leq 0.05$).

DISCUSSION

The results of this study show that only the two fumonisin-producing strains were able to cause the full spectrum of symptoms indicative of *F. verticillioides*-induced maize seedling disease. In 1992, there was little evidence supporting a role of fumonisin in *F. verticillioides*-induced diseases in maize (*46*). In 1994, fumonisins were shown to cause dose-dependent reduction in root and shoot mass in maize seedlings grown on water agar (*15*). Desjardins et al. (*8*) compared the ability of fungal strains to produce fumonisin and virulence and concluded that fumonisin played a role in virulence but was not necessary or sufficient for virulence on maize seedlings. In the present

Table 4. Pearson Product Moment Correlations in the Watering Assay[a]

	leaf lesions	root weight	stalk height	Pso	Sa	Pso-1-P	Sa-1-P
correlation coefficient[b]	0.80	−0.86	−0.91	0.73	0.88	0.78	0.95
p value[c]	6.3×10^{-9}	4.8×10^{-5}	2.4×10^{-6}	1.9×10^{-3}	1.8×10^{-5}	6.6×10^{-4}	4.5×10^{-8}

[a] Correlations are given between the amount of fumonisin B_1 tightly associated with roots (FB, nanomoles per gram of roots) and the presence of leaf lesions, root weight, stalk height, and amounts of free phytosphingosine (Pso), free sphinganine (Sa), phytosphingosine 1-phosphate (Pso-1-P), and sphinganine 1-phosphate (Sa-1-P) on day 21 in the watering assay ($n = 15$ pots). [b] Pairs of variables with positive correlation coefficients tend to increase together, and those with negative coefficients are inversely correlated. [c] The exact *p* values are presented in exponential format, and *p* values less than 0.05 (5×10^{-2}) are considered statistically significant.

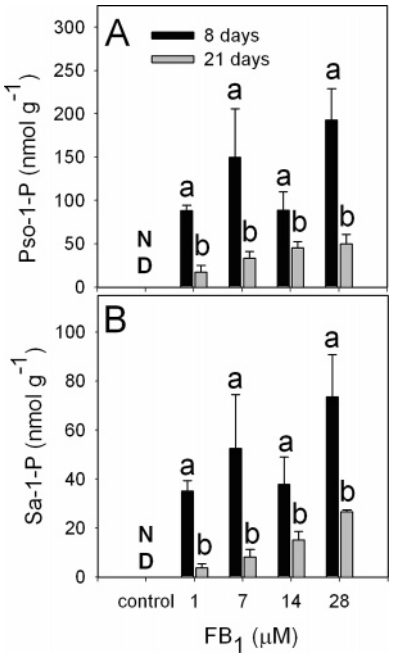

Figure 5. (**A**) Phytosphingosine 1-phosphate (Pso-1-P) and (**B**) sphinganine 1-phosphate (Sa-1-P) at 8 and 21 days in root tissues from maize seedlings after being watered with solutions of fumonisin B_1. Values are expressed as nanomoles per gram of root tissue (mean ± SD, $n = 3$ pots/treatment containing 8–10 seedlings). Differing letters (a, b) indicate a significant difference ($p \leq 0.05$) between treatments on days 8 and 21; ND, not detected.

study, maize seeds of the sweet maize cultivar Silver Queen were inoculated with two fumonisin-producing strains and three non-fumonisin-producing strains of *F. verticillioides*. Leaf lesions and abnormal leaf development were observed only in seedlings grown from seeds inoculated with the fumonisin-producing strains, and while non-fumonisin-producing strains caused reduced root and stalk growth, the extent of the reduction was greatest with the fumonisin-producing strains. The severity of the observed effects on maize seedlings was significantly correlated with the fumonisin levels in the roots and the degree of disruption of sphingolipid metabolism.

The ability of the fungus to produce fumonisin is required for development of leaf lesions; however, in a related study we showed that endophytic colonization is neither required nor sufficient (unpublished data). Furthermore, gene deletion strains unable to produce fumonisins (Δ*FUM1* and Δ*FUM6*) did not produce the foliar disease symptoms, while genetic complementation of a non-fumonisin-producing *fum1* mutant with a wild-type *FUM1* allele conferred pathogenicity, and segregation analyses of progeny derived from sexual crosses between fumonisin-producing and non-fumonisin-producing parents had 100% linkage between production and pathogenicity. The fumonisin-producing strain AEG3-A3-6 used in this study does not colonize the aerial plant parts of maize seedlings when grown from inoculated seeds but does induce leaf lesions and disrupts sphingolipid metabolism in maize roots. Recently, we have found that although fumonisin accumulates in maize seedling roots, very little is detected in leaf tissue (unpublished data), and in a previous study elevation in free sphingoid bases was detected in roots but not stems of fumonisin-treated maize seedlings (*47*). Together the results suggest that while the ability to produce fumonisins is required, the development of leaf lesions is via a mechanism that does not require either endophytic fungal colonization or fumonisin translocation to the leaves. One possible explanation is that the development of leaf lesions is a consequence of changes induced by disruption of sphingolipid metabolism in the roots.

To further examine the hypothesis that fumonisin itself is an important contributor to *F. verticillioides* maize seedling disease, sterilized maize seeds were planted and watered with solutions containing purified fumonisin B_1, applied as an aqueous solution. The seedlings developed symptoms similar to those seen in seedlings grown from seeds inoculated with the pathogenic fumonisin-producing *F. verticillioides* strains MRC826 and AEG3-A3-6. In the watering assay, as in the virulence assay, there was clear evidence of disruption of sphingolipid metabolism and a close correlation between the fumonisin B_1 levels in roots, elevation in sphingoid bases and sphingoid base 1-phosphates, effects on seedling growth, and the incidence of leaf lesions; all results are consistent with the hypothesis that fumonisin by itself can induce the full spectrum of symptoms indicative of *F. verticillioides* maize seedling disease.

A recent study showed that the sensitivity of maize varieties to fumonisin B_1 is likely to be an ancestral trait in *Z. mays* and that insensitivity is a rare but inheritable trait in maize (*48*). The sensitivity of Silver Queen to *F. verticillioides* seedling disease could be dependent on lack of resistance to fumonisin inhibition of ceramide synthase as seen in tomato plants susceptible to fumonisin and AAL toxin. Genetic susceptibility to stem-canker disease in tomato is closely correlated with sensitivity to disruption of sphingolipid metabolism (*13*) and to expression of genes that confer resistance to fumonisin inhibition of the CoA-dependent ceramide synthase (*31*, *49*−*51*).

Prolonged fumonisin exposure appears to result in increased catabolism of sphingoid base 1-phosphates. On day 21 in the time-course virulence assay with MRC826, sphinganine 1-phosphate levels decreased significantly after day 7, and in the watering assay, both phytosphingosine 1-phosphate and sphinganine 1-phosphate were reduced by approximately 50−75% at every dose level compared to their levels on day 8, while free sphinganine and free phytosphingosine were not decreased. These results suggest that prolonged fumonisin exposure results in a change in the expression or relative activity of the enzymes responsible for metabolizing free sphingoid bases (**Figure 1**).

The data presented in this study are consistent with the hypothesis that inhibition of ceramide synthase is an important contributor to the adverse effects seen in the virulence and

watering assays with solutions of purified fumonisin B_1. The potential downstream cellular consequences of inhibition are too numerous to describe in detail, but there is increasing evidence that the cellular and physiological processes that are well documented in animal models and are the most likely to be affected by disruption of sphingolipid metabolism are also operative in plants; for example, (i) increased cell death and inhibited cell growth induced by elevation in free sphingoid bases (*52*, *53*), (ii) inhibition of plasma membrane ATPases (*54*), (iii) disrupted function of S1P receptor- (also known as G-protein coupled receptors and EDG receptors in animals) mediated responses induced by altered levels of ceramide and ceramide metabolites and increased levels of sphingoid base 1-phosphates (*29*, *30*, *32*, *55*), (iv) disruption of stress responses, cell−cell interactions, and defense responses associated with complex sphingolipids (*31*, *56*), and (v) altered function of glycosylphosphatidylinositol- (GPI-) anchored proteins via altered function of lipid rafts that are enriched in sphingolipids (*57*, *58*). It is quite likely that all of the downstream biochemical changes, collectively referred to as disruption of sphingolipid metabolism, occur concurrently at high dosages of fumonisin. In addition, the metabolism of sphinganine to phytosphingosine consumes NADPH/NADH and the phosphorylation to the 1-phosphates requires ATP. Thus, at the very least, persistent fumonisin inhibition of ceramide biosynthesis results in generation of toxic free sphingoid bases, elevation in sphingoid base 1-phosphates, depletion of critical membrane glycosphingolipids, altered function of GPI-anchored proteins and lipid rafts, and modulation of phospholipid signaling pathways and is an energy drain for affected cells.

In conclusion, the correlation between fungal pathogenicity, fumonisin B_1 production, ceramide synthase inhibition, the production of similar symptoms by both pathogenic strains of *F. verticillioides* and direct exposure to fumonisin B_1, and the known biological activity of sphingolipids as regulators of cell function in animals, yeast, and plants strongly support the hypothesis that fumonisin production is an important contributor to the amount of disease expression and is required for the induction of leaf lesions indicative of *F. verticillioides* maize seedling disease. In addition, the data offer additional evidence for the importance of sphingolipids in the physiological well-being of plants and indicate the need for developing a better understanding of the role of sphingolipids and their metabolism in physiological processes and disease resistance in plants.

ABBREVIATIONS

LC/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; HPLC, high-performance liquid chromatography; AAL toxin, *Alternaria alternata lycopersici* toxin; rcf, relative centrifugal force.

SAFETY

Fumonisin B_1 is a known liver and kidney carcinogen in rodents; therefore, it should be handled with proper precautionary measures.

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