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Fumonisin- and AAL-Toxin-Induced Disruption of Sphingolipid Metabolism with Accumulation of Free Sphingoid Bases[1]

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Fumonisins (FB) and AAL-toxin are sphingoid-like compounds produced by several species of fungi associated with plant diseases. In animal cells, both fumonisins produced by *Fusarium moniliforme* and AAL-toxin produced by *Alternaria alternata* f. sp. *lycopersici* inhibit ceramide synthesis, an early biochemical event in the animal diseases associated with consumption of *F. moniliforme*-contaminated corn. In duckweed (*Lemna pausicostata* Heglem. 6746), tomato plants (*Lycopersicon esculentum* Mill), and tobacco callus (*Nicotiana tabacum* cv Wisconsin), pure FB_1 or AAL-toxin caused a marked elevation of phytosphingosine and sphinganine, sphingoid bases normally present in low concentrations. The relative increases were quite different in the three plant systems. Nonetheless, disruption of sphingolipid metabolism was clearly a common feature in plants exposed to FB_1 or AAL-toxin. Resistant varieties of tomato (*Asc/Asc*) were much less sensitive to toxin-induced increases in free sphinganine. Because free sphingoid bases are precursors to plant "ceramides," their accumulation suggests that the primary biochemical lesion is inhibition of de novo ceramide synthesis and reacylation of free sphingoid bases. Thus, in plants the disease symptoms associated with *A. alternata* and *F. moniliforme* infection may be due to disruption of sphingolipid metabolism.

Fumonisins (Bezuidenhout et al., 1988) and AAL-toxin (Bottini et al., 1981) are sphingoid-like compounds produced by several species of fungi commonly associated with plant diseases (for recent reviews, see Gilchrist et al., 1992; Abbas et al., 1993; Nelson et al., 1993). The fumonisins have yet to be proven to be pathogenicity or virulence factors in *Fusarium moniliforme* (Nelson et al., 1993); however, there is some evidence that fumonisins may be a virulence factor for *F. moniliforme* (Sheldon) in jimsonweed (Abbas et al., 1992). AAL-toxin has been established to be a pathogenicity factor for *Alternaria alternata* f. sp. *lycopersici*-induced stem canker disease in tomato (*Lycopersicon esculentum* Mill) (Gilchrist and Grogan, 1976). Conversely, although AAL-toxin is clearly involved in stem canker disease, it is not associated with any natural outbreaks of animal disease, whereas the fumonisins are known to be the cause of several naturally occurring and experimentally induced diseases of animals (for a recent review, see Riley et al., 1993b) associated with the consumption of corn and feeds contaminated with *F. moniliforme*.

Because the fumonisins bear a structural similarity to free sphingoid bases, it was originally hypothesized that the mechanism of animal toxicity might be a result of disruption of sphingolipid biosynthesis (Wang et al., 1991). There are now considerable data to support this hypothesis (Wang et al., 1992; Yoo et al., 1992; Riley et al., 1993a). In animal cells, the primary target of fumonisins and the structurally related AAL-toxin is the enzyme sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase) (Merrill et al., 1993). In vivo, inhibition of ceramide synthase causes the marked accumulation of free sphingoid bases (Wang et al., 1992; Riley et al., 1993a). Thus, elevated levels of free sphinganine and the ratio of free sphinganine:free sphingosine have been proposed as a reliable diagnostic indicator for the consumption of fumonisins and AAL-toxin. In stationary cultures of yeast, fumonisin B_1 at noncytotoxic concentrations induced the accumulation of sphingoid bases. The underlying biochemical mechanism for the accumulation of sphingoid bases in yeast is unknown (Kaneshiro et al., 1992).

Recent studies indicate that addition of the free sphingoid bases phytosphingosine, sphinganine, and sphingosine to the growth medium caused phytotoxicity symptoms in duckweed (*Lemna pausicostata*) that were similar to those caused by fumonisins and AAL-toxin (Tanaka et al., 1993). Thus, it was hypothesized that the mechanism by which AAL-toxin and

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Abbreviations: OPA, orthophthalaldehyde; TMS, trimethylsilyl.

fumonisins act to cause toxicity in plants involves the disruption of sphingolipid metabolism via inhibition of ceramide synthesis. The purpose of this study was to determine if fumonisin B_1 and AAL-toxin cause an intracellular accumulation of free sphingoid bases in plants. The results indicate that treatment of plants with fumonisin B_1 or AAL-toxin results in the marked accumulation of free sphinganine and free phytosphingosine. The findings are consistent with the hypothesis that disruption of sphingolipid metabolism is an important factor in the development of the plant diseases associated with infection by *F. moniliforme* and *A. alternata* f. sp. *lycopersici*.

MATERIALS AND METHODS

Production and Purification of Phytotoxins

Fusarium moniliforme JW#1 and *Alternaria alternata* SWSL#1 were grown on rice as previously described (Abbas et al., 1991; Abbas and Vesonder, 1993). Fumonisin B_1 and AAL-toxin (T_A isomer) used in the studies with duckweed (*Lemna pausicostata*) and tomato (*Lycopersicon esculentum* Mill) plants were isolated from the rice culture material and purified (Vesonder et al., 1990; Abbas et al., 1991; Abbas and Vesonder, 1993). The purity of the fumonisin B_1 and AAL-toxin isolated from rice culture materials was determined to be ≥93% and ≥95%, respectively, by HPLC (Ross et al., 1991) and TLC in two different development systems (methanol:water, 3:1 [v/v] and ethylacetate:acetic acid:water, 60:30:10 [v/v/v]) (Vesonder et al., 1990; Abbas et al., 1991; Abbas and Vesonder, 1993). Mol wt was confirmed for both toxins by high-resolution fast atom bombardment MS by Mr. Tom Krick (Department of Biochemistry, University of Minnesota, St. Paul). Fumonisin B_1 used in the study with tobacco callus was purchased from the Division of Food Sciences and Technology, Council for Scientific and Industrial Research, Pretoria, South Africa. The reported purity of this fumonisin B_1 was ≥95% and it was used as supplied.

Plant Culture and Bioassays

Cultures of *L. pausicostata* Helgelm. 6746 were initiated and grown and bioassays were performed with test agents as previously described (Tanaka et al., 1993). Briefly, 50 colonies of three fronds each were transferred to 6-cm polystyrene Petri dishes containing 0, 0.1, 0.33, 1.0, or 3.3 μM AAL-toxin or 1.0 μM fumonisin B_1. Plants were exposed for up to 48 h and then rinsed and frozen.

Tomato plants with the genotype (*asc/asc*) susceptible to *A. alternata* f. sp. *lycopersici*, the causal agent of stem canker disease, and plants resistant (*Asc/Asc*) to the pathogen were used in this study. Seeds of the tomato variety line #85–6 with genotype *asc/asc* (susceptible) and #85–14 with genotype *Asc/Asc* (resistant) were obtained from Dr. D.G. Gilchrist (Plant Pathology Department, University of California, Davis). The other tomato variety lines used in these studies were cv Earlypak #LA12 with genotype *asc/asc* (susceptible) and Ace #LA2992 with the genotype *Asc/Asc* (resistant) obtained from Dr. C.M. Rick (Tomato Genetics Resource Center, Department of Vegetable Crops, University of California, Davis).

Plants were grown for 1 month (six-leaf stage) in plastic pots (20 cm i.d.) containing Jiffy Mix (Jiffy Products, Batavia, IL):soil in a 3:2 ratio in the greenhouse. Conditions included a photoperiod of 14 h at 1600 to 1800 μE m^{-2} s^{-1} PPFD at midday, and temperatures were maintained between 28 and 32°C with 40 to 60% RH. When plants were 1 month of age they were treated with rice culture material (Abbas et al., 1991) or pure toxins. Briefly, suspensions of rice culture material (filtered through cheesecloth) containing both the fungus and the toxin produced by the fungus (0.1 g/mL) or solutions of pure toxins were applied by spraying with an atomizer until runoff occurred. AAL-toxin and fumonisin B_1 were applied at 5 and 70 μM, respectively, in both rice culture filtrates and as pure toxins dissolved in distilled water. The 5- and 70-μM concentrations were chosen because in preliminary studies these concentrations of AAL-toxin and fumonisin B_1, respectively, caused similar symptoms of phytotoxic injury after 48 to 72 h and caused plant mortality within 5 d. Treated plants were observed daily for symptom development (wilt and necrotic lesions on leaves and stems). Plants were removed after 24, 48, and 72 h, frozen, and freeze-dried.

Tomato leaf discs (#LA12) were used to correlate time course and dose response profiles for free sphingoid bases with electrolyte leakage. Briefly, 4-mm diameter tomato leaf discs were cut with a cork borer from large plants at the six- to eight-leaf stage and washed in 1% Suc, 1 mL of Mes (pH 6.5) and then placed in 6-cm diameter polystyrene Petri dishes with 5 mL of wash medium with or without AAL-toxin. Discs were then incubated at 25°C under continuous light for up to 48 h at 500 μE m^{-2} s^{-1} in a growth chamber. The discs were observed for visual signs of phytotoxic effects and the conductivity of the bathing medium was monitored at each sample time. The time-course experiment was conducted with 1 μM AAL-toxin (0–48 h) and samples from the dose-response study were taken at 24 h (0, 0.1, 0.33, and 1.0 μM AAL-toxin). At the appropriate sample times leaf discs were removed, frozen, freeze-dried, and stored at −80°C until they were analyzed for free sphingoid bases. Electrolyte leakage measurements (50 discs/replicate) and samples of leaf discs (15–25 discs/replicate) were determined in triplicate.

Tobacco callus cultures (*Nicotiana tabacum* cv Wisconsin) were purchased from Carolina Biological (Burlington, NC) and maintained on agar containing callus initiation medium (Carolina Biological). The callus was subcultured every 2 weeks for 2 months. When a relatively uniform cell suspension was achieved, callus was subcultured evenly onto plates (60 × 15 mm) containing 10 mL of solid medium. Fumonisin B_1 (14 μM) dissolved in liquid medium (filter sterilized) was added to the surface of the agar containing the experimental callus samples. An equivalent volume of medium only was added to the agar surface of control cultures. Control and experimental samples were extracted at the indicated intervals over 9 d.

Extraction and Purification of Free Sphingoid Bases

Duckweed and tomato plants were freeze-dried and reduced to a fine powder with a mortar and pestle after cooling

in liquid N_2. Approximately 10 to 20 mg of powdered material was transferred to a glass tube, and lipids were extracted and base hydrolyzed, and free phytosphingosine and free sphinganine were quantified by HPLC as described by Merrill et al. (1988) with slight modification (Riley et al., 1994). Briefly, the powdered tissue was rehydrated with cold phosphate buffer (50 mM phosphate, pH 7.0), C:20 internal standard (Nimkar et al., 1988) was added, and then lipids were extracted in a mixture of $CHCl_3$:CH_3OH (1:2, v/v) made alkaline by the addition of 2 N NH_4OH. The $CHCl_3$:CH_3OH:NH_4OH extracts were washed three times with alkaline water and then the remaining water was removed from the organic solvent ($CHCl_3$) by passing over Na_2SO_4 packed in columns. The $CHCl_3$ was removed using a SpeedVac (Savant Instruments, Farmingdale, NY). The lipid residue was dissolved in dilute KOH (0.1 N) in CH_3OH and $CHCl_3$, and then the lipids were base hydrolyzed for 1 h at 37°C. Again, the mixture was washed and dried, and $CHCl_3$ was removed as described above.

For duckweed and tomato, a silica gel clean-up step was used prior to GC-MS analysis to remove Chl. Briefly, a column (about 1 × 5 cm) of silica gel (60–100 mesh, powder, Baker 3405) was equilibrated by sequential addition of 5 mL of $CHCl_3$, 5 mL of $CHCl_3$:CH_3OH (5:1), and 5 mL of $CHCl_3$:CH_3OH:CH_3COOH (100:20:1). The dried base-hydrolyzed lipid residues were dissolved in 0.25 mL of $CHCl_3$:CH_3OH:CH_3COOH (100:20:1) and then sonicated for 1 min. The resulting solution was loaded on the equilibrated silica gel column and the Chl was eluted with 10 mL of $CHCl_3$ followed by 10 mL of CH_3OH:2 N NH_4OH (10:1) to elute the free sphingoid bases. The $CHCl_3$ eluate was discarded and the CH_3OH:2 N NH_4OH eluate was collected in a 30-mL glass tube and $CHCl_3$ and alkaline water were added to achieve phase separation. The $CHCl_3$:CH_3OH mix was washed twice with alkaline water, the remaining water was removed by passing over Na_2SO_4, and the $CHCl_3$ was removed using the SpeedVac. Dried samples were stored under N_2 at −80°C.

Chromatography and MS of Free Sphingoid Bases

The HPLC procedure was essentially as previously described (Merrill et al., 1988) and as modified (Riley et al., 1994). Authentic standards of D,L-*erythro*-dihydrosphingosine (sphinganine), D-sphingosine, and phytosphingosine purchased from Sigma and C:20 sphinganine (Nimkar et al., 1988) were chromatographed daily. The mobile phase was 11 to 12% 5 mM potassium phosphate buffer, pH 7.0, 88 to 89% CH_3OH.

Confirmation of the presence of phytosphingosine and sphinganine in extracts from fumonisin B_1 and AAL-toxin-treated duckweed was accomplished by a combination of HPLC, TLC, GC, and MS. Briefly, samples for TLC were dissolved in ethanol and spotted on a 10 × 10 cm, prewashed, silica gel 13179 plate (Kodak) divided into 1.5-cm lanes. Authentic standards of sphingosine, sphinganine, and phytosphingosine were run on the end lanes alongside the plant extracts. Chromatograms were developed using $CHCl_3$:CH_3OH:NH_4OH (70:30:2). The end lanes containing the standards were sprayed with ninhydrin (0.2% in 95% ethanol), taking care to cover the center lanes, which contained the plant extracts. The silica gel in the lanes spotted with plant extracts, corresponding to the authentic standards, was removed and the materials migrating in those areas were eluted with the HPLC mobile phase (CH_3OH:5 mM phosphate buffer). Aliquots of the eluate were reacted with OPA and analyzed for free sphingoid bases by HPLC. The HPLC retention times for OPA-derivatized standards of phytosphingosine and sphinganine were compared to the retention times of the OPA-derivatized materials isolated from the TLC plates.

Extracts from duckweed treated with 1 μM fumonisin B_1 or AAL-toxin for 24 h were analyzed for free sphingoid bases by GC and MS. GC-MS was conducted on a Hewlett-Packard (HP) 5890 Series II gas chromatograph with a HP-5970 mass selective detector, HP-59970 MS ChemStation, and a HP-7673A automatic injector. A 25 m × 0.2 mm × 0.33 μm (length × i.d. × film thickness; HP Ultra-1) methyl silicone, fused silica capillary column was programmed as follows: initial temperature was 100°C for 2 min, then programmed at 25°C/min to 200°C and held for 2 min, then programmed at 15°C/min to 280°C and held for 7 min. Injection port and transfer line temperatures were 250 and 280°C, respectively.

The crude, dried extracts from fumonisin B_1 or AAL-toxin-treated duckweed (corresponding to approximately 200 ng of sphinganine or phytosphingosine as determined by HPLC analysis) were reacted with 20 μL of *N*-methyl-*N*-TMStrifluroacetamide (Pierce, Rockford, IL) in a conical vial (Pierce), capped (under N_2) at 70°C for 15 min, and 1 μL was injected into the GC-MS system.

RESULTS AND DISCUSSION

Representative HPLC profiles of the OPA-positive substances in base-hydrolyzed lipids extracted from control, fumonisin B_1, and AAL-toxin-treated duckweed are shown in Figure 1. Similar profiles were obtained for extracts from intact tomato plants, tomato leaf discs, and tobacco callus treated with fumonisin B_1, AAL-toxin, or rice cultures from *F. moniliforme* or *A. alternata* (data not shown). In extracts of duckweed, tomato, and tobacco callus treated with fumonisin B_1 or AAL-toxin, two fluorescent peaks were consistently found to be markedly elevated relative to their concentration in the control extracts. The retention times of these two peaks were identical to those of the authentic standards for phytosphingosine and sphinganine. Although other OPA-positive substances with retention times similar to other known sphingoid bases were also present, these peaks were either not increased relative to controls or were only slightly increased relative to the magnitude of the increases in putative phytosphingosine and sphinganine peaks. The relative mobilities of the putative phytosphingosine and sphinganine determined by TLC (data not shown) and GC (Fig. 2A) were identical to those of authentic standards. Also, the amount of phytosphingosine and sphinganine recovered after elution from the TLC plates and analysis by HPLC was calculated to be the same as the amount applied to the plates based on the concentration determined from the HPLC analysis of the crude extracts.

Sphinganine and phytosphingosine were identified in the

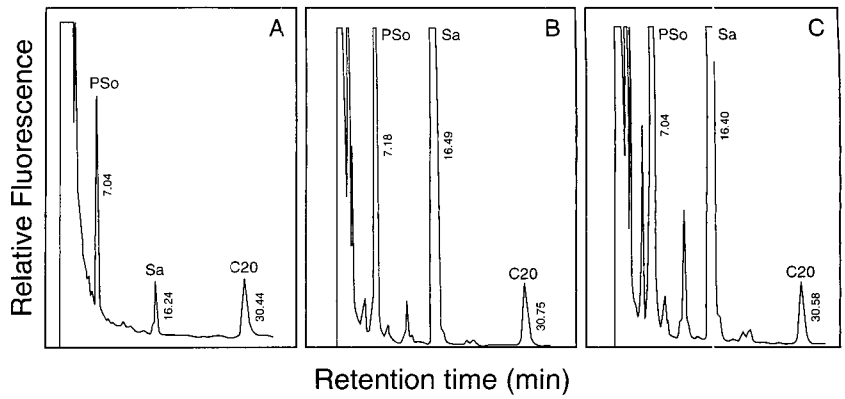

Figure 1. HPLC profile of free phytosphingosine (PSo) and free sphinganine (Sa) from extracts of duckweed control cultures (A) and cultures treated with 1 μM fumonisin B_1 (B) or AAL-toxin (C) for 24 h. C:20 = internal standard.

crude extracts of treated duckweed as their TMS-ethers. Confirmation of the TMS-ethers was based on GC retention times (Fig. 2A) and mass fragmentation patterns (Fig. 2, B and C) compared to the TMS-ethers of the authentic standards. Additional confirmation was obtained by enrichment procedures that provided co-chromatography retention times and spectra consistent with the authentic standards (data not shown).

Diagnostic fragments for the TMS_3-ether of sphinganine (retention time = 15.25 min) and TMS_4-ether of phytosphingosine (retention time = 16.84 min) (Fig. 2, B and C) occur at m/z 204 (100%; $TMSN^+H{=}CHCH_2OTMS$) with other characteristic fragments occurring at sphinganine-TMS_3 (m/z 502, 1%, $M^+ - 15$; m/z 414, 8%, $C_{15}H_{31}CH[OTMS]CH = N^+HTMS$; m/z 313, 1%, $C_{15}H_{31}CH = O^+TMS$); phytosphingosine-TMS_4 (m/z 590, 0.1%, $M^+ - 15$; m/z 502, 2%, $M^+ - TMSO^+{=}CH_2$; m/z 412, 9%, $M^+ - TMSOH - 103$; m/z 299, 5%, $C_{14}H_{29}CH$ = OTMS).

Additionally, the TMS_3-ethers of authentic sphingosine and C:20 sphinganine gave the characteristic fragmentation patterns expected (i.e. m/z 204, 100%; sphingosine-TMS_3 m/z 500, 412, 311; C:20 sphinganine-TMS_3 m/z 530, 442, 306) with retention times at 14.87 and 17.51 min, respectively.

Although the amount of phytosphingosine and sphinganine was not quantified by GC, the ratio of the TMS-ethers for sphinganine and phytosphingosine observed with GC-MS analysis of the crude extracts was consistent with that observed by HPLC analysis of the OPA derivatives (data not shown). The MS analysis of control duckweed extracts was only able to confirm the presence of phytosphingosine (data not shown). Based on both the HPLC and GC analysis, it was clear that there could be other free sphingoid bases present in the extracts. However, the mass spectral fragmentation patterns were not consistent with any of the known or expected patterns for sphingoid bases or the fragmentation patterns of available standards. No attempt was made to identify these compounds.

The relative increase in free phytosphingosine and free sphinganine was different in the four test systems (Figs. 3, 4, 5, and 6; Tables I, II, and III). However, phytosphingosine and sphinganine were easily identified by HPLC of extracts from control tissues and both sphingoid bases were markedly elevated in fumonisin- or AAL-toxin-treated tissues from all test systems. For example, there was an 18- and 45-fold

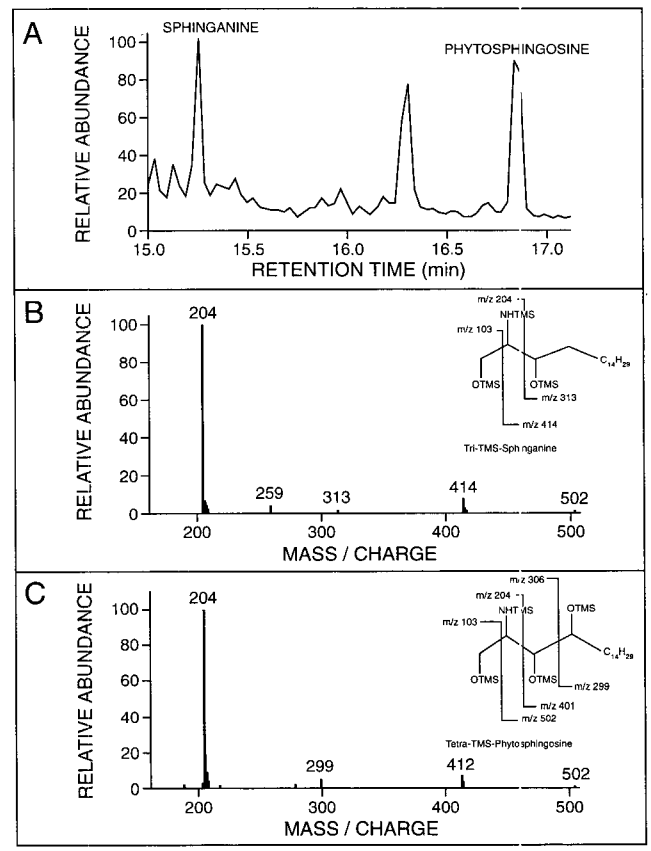

Figure 2. A, Representative gas chromatograms showing TMS phytosphingosine and sphinganine (dihydrosphingosine) in extracts from duckweed treated with 1 μM AAL-toxin for 24 h. B and C, Examples of the mass-spectral fragmentation patterns for the sphinganine and phytosphingosine peaks, respectively, in the AAL-toxin-treated duckweed. The fragmentation patterns were identical to the authentic standards (data not shown). Other compounds were present in the extracts and were resolved by GC; however, the mass spectral fragmentation patterns were not consistent with any of the known or expected patterns for sphingoid bases or the fragmentation patterns of available standards.

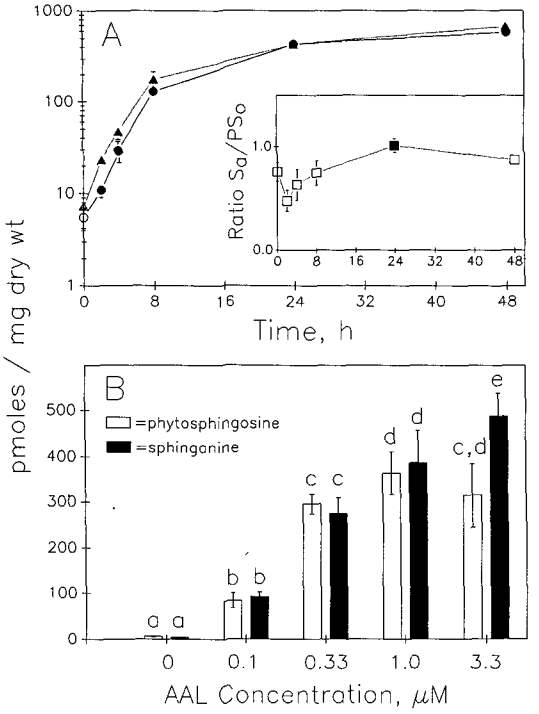

Figure 3. A, Time-course experiment for the change in free sphingoid base concentration (●, sphinganine; ▲, phytosphingosine) in duckweed treated with 1 μM AAL-toxin. Inset shows the change in the ratio of free sphinganine to free phytosphingosine. Values are the means ± SD (n = 3–6 experiments) at each time. Filled symbols indicate means significantly different ($P \leq 0.05$) from the time-0 control based on an analysis of variance using the general linear modeling procedure (SAS Institute, 1985). B, The dose-response in duckweed exposed to AAL-toxin for 24 h. Values are the means ± SD (n = 3 experiments) at each concentration. Different superscripts above vertical bars indicate means that are significantly different based on an analysis of variance using the general linear modeling procedure (SAS Institute, 1985). Comparison of conductivity measurements taken prior to exposure to AAL-toxin and at the time the duckweed was harvested indicated that no electrolyte leakage ($P > 0.05$, n = 3 experiments at each sample time or dose level) had occurred during the period of exposure in either the time-course (A) or the dose-response experiment (B).

increase in phytosphingosine, and a 76- and 129-fold increase in sphinganine, in duckweed treated for 24 h with 1 μM fumonisin B_1 or AAL-toxin, respectively (Table I). Similarly large increases in free phytosphingosine and free sphinganine were found in tomato leaf discs under similar conditions (Table I). However, the relative increase in free sphinganine appeared to be much greater in tomato than in duckweed (Table I; Figs. 3A, 4A, and 5A). This difference in relative response was clearly apparent when the time course data were plotted as the ratio of free sphinganine to free phytosphingosine (insets in Figs. 3A, 4A, and 5A).

In duckweed significantly elevated concentrations of both free phytosphingosine and free sphinganine were observed at 2 h (the first time sample) after exposure to 1 μM AAL-toxin (Fig. 3A). Free phytosphingosine and free sphinganine were significantly elevated ($P \leq 0.05$) relative to controls at all concentrations tested (Fig. 3B).

In tomato leaf discs significantly elevated concentrations of both free phytosphingosine and free sphinganine were observed at 0.5 h (Fig. 4A) or 0.8 h (Fig. 5A) after exposure to 1 μM AAL-toxin. As with duckweed (Fig. 3B), free phytosphingosine and free sphinganine were significantly elevated ($P \leq 0.05$) relative to controls at all concentrations tested (data not shown). The no-effect level in tomato leaf discs (as in duckweed) for significant elevation of free phytosphingosine and free sphinganine was less than 0.1 μM ($P < 0.05$, n = 3), which was the lowest concentration of AAL-toxin tested.

As with fumonisin- and AAL-toxin-induced disruption of sphingolipid metabolism in animal cells, the ratio of free sphinganine to free phytosphingosine in susceptible tomato plants (Figs. 4B, 5B, and 6C) may provide a simple, convenient dimensionless marker for disruption of sphingolipid metabolism in this species. However, in other species such as duckweed (Fig. 3A), it may have less utility. In animal cells and tissues the fumonisin-induced increase in free sphingosine concentration is much less than the increase in free sphinganine (Yoo et al., 1992; Riley et al., 1993a). The elevation in free sphinganine is clearly due to the inhibition of the microsomal enzyme sphinganine N-acyltransferase (Merrill et al., 1993). The elevation of free sphingosine, however,

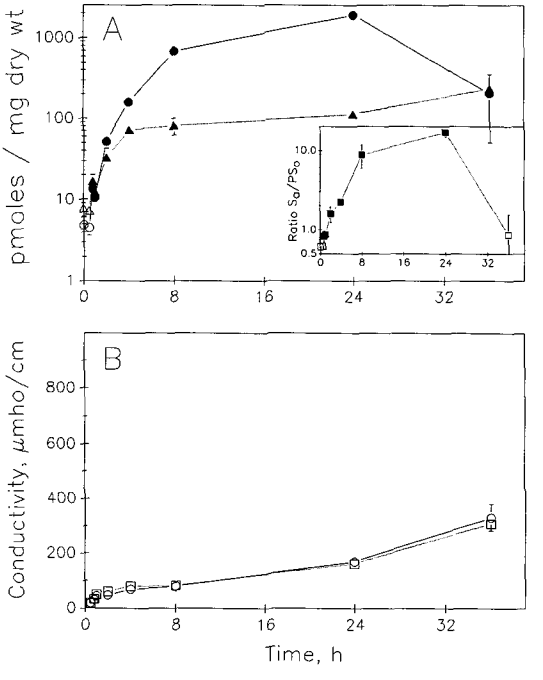

Figure 4. A, Time-course experiment 1 for the change in free sphingoid base concentration (●, sphinganine; ▲, phytosphingosine) in tomato leaf discs from a susceptible (*Asc/Asc*) tomato variety (LA12) treated with 1 μM AAL-toxin. Inset shows the change in the ratio of free sphinganine to free phytosphingosine. B, The change in conductivity of the culture medium for concurrent control (□) and 1 μM AAL-toxin-treated (○) leaf discs. Values are the means ± SD (n = 3) at each time. Filled symbols indicate means significantly different ($P \leq 0.05$) from the time-0 control based on an analysis of variance using the general linear modeling procedure (SAS Institute, 1985).

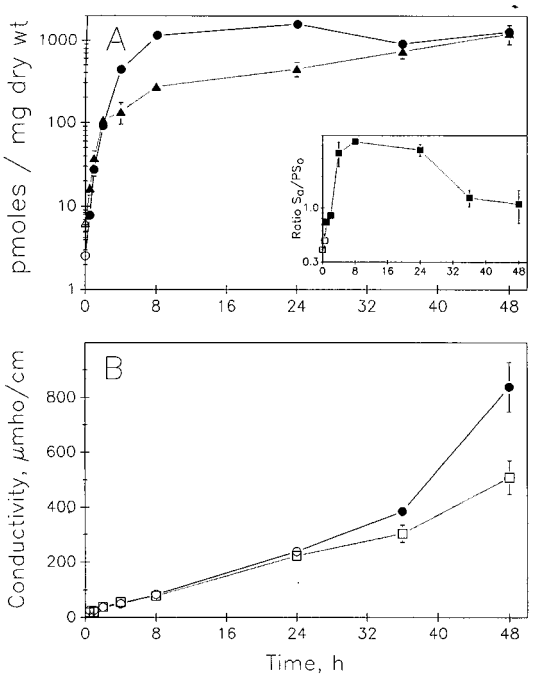

Figure 5. A and B, Time-course experiment 2 with tomato leaf discs. All details are the same as for Figure 4.

is believed to be due to the inability of cells to reacylate sphingosine generated as a consequence of the turnover of complex sphingolipids or the inhibited acylation of sphingosine from dietary sources. It is possible that the elevation of free phytosphingosine in fumonisin- and AAL-toxin-treated tomato tissues is (like free sphingosine in animal cells) due to the inability of cells to reacylate phytosphinganine generated as a consequence of complex sphingolipid turnover. The abrupt decline in the ratio in tomato leaf discs (Figs. 4A and 5A) after prolonged exposure to the toxins may be a consequence of disruption of the ER (the site of de novo sphingolipid biosynthesis in animal cells) resulting from phytotoxicity.

For the dose-response measurements, the duration of exposure to the toxins (24 h) was chosen so that the samples would be taken before electrolyte leakage increased significantly relative to concurrent controls in tomato leaf discs (Figs. 4B and 5B) and duckweed (Fig. 3 legend and Tanaka et al., 1993). Electrolyte leakage is a very early and sensitive measure of phytotoxicity of many toxins, occurring before visual symptoms can be observed or even before most other physiological symptoms can be detected (Kenyon et al., 1985; Duke and Kenyon, 1993). Thus, in duckweed and tomato leaf discs the elevation in free sphingoid bases occurs before the onset of toxin-induced cytotoxicity, as evidenced by increased electrolyte leakage relative to controls.

Both resistant (*Asc/Asc*) and susceptible (*asc/asc*) tomato plants at the six- to eight-leaf stage grown under greenhouse conditions and treated with AAL-toxin at 5 μM or fumonisin B_1 at 70 μM showed similar initial symptoms of only a few necrotic spots (<1 mm) at 24 h. In resistant plants, growth relative to controls was similar after 72 h and the necrotic lesions noted at 24 h did not increase in size or number. In the susceptible tomato plants necrotic lesions increased in both number and size. Wilting was observed at 48 h and plants began to die at 72 h. The concentrations of pure AAL-toxin (5 μM) and pure fumonisin B_1 (70 μM) and the exposure times (24–72 h) were chosen because at these dosages the visually observed response in the susceptible plants was similar with both toxins.

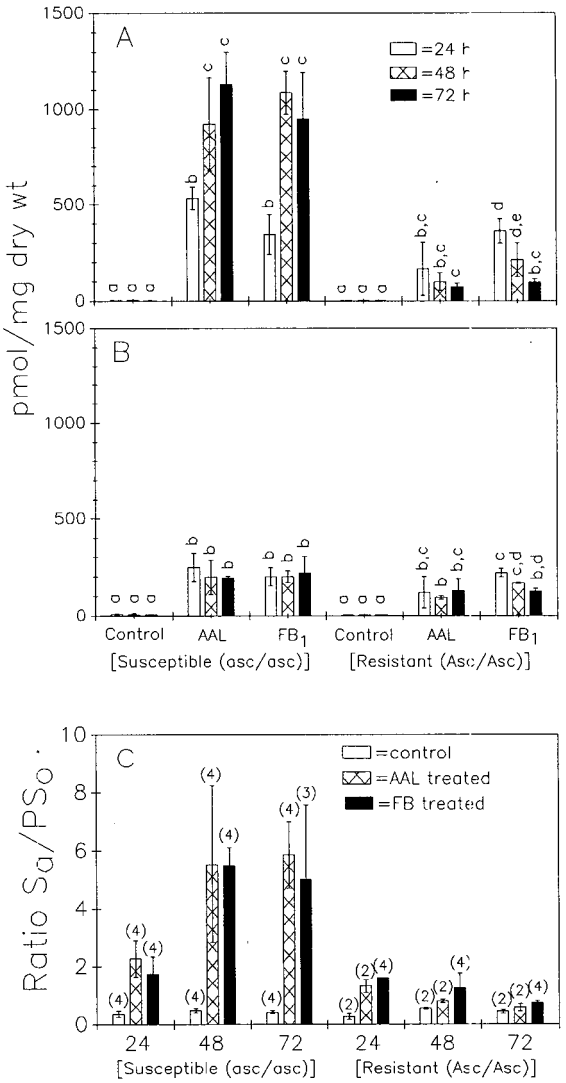

Figure 6. Time-course experiment for the change in free sphinganine (A), free phytosphingosine (B), and the ratio of free sphinganine to free phytosphingosine at 24, 48, and 72 h in susceptible (*asc/asc*) and resistant (*Asc/Asc*) tomato varieties sprayed until run off with distilled water (controls), 5 μM AAL-toxin in distilled water (AAL-toxin), or 70 μM fumonisin B_1 in water (C). The number of individual plants treated is shown in parentheses in C. Statistical differences between treatments and time within the susceptible and resistant varieties are shown only in A and B. Different superscripts indicate means that are significantly different within the susceptible or resistant varieties based on an analysis of variance using the general linear modeling procedure (SAS Institute, 1985).

Table I. *Concentration (pmol/mg dry weight) of free phytosphingosine and free sphinganine in duckweed treated with 1 μM fumonisin B_1 or AAL-toxin, and susceptible tomato leaf discs (LA12) treated with 1 μM AAL-toxin for 24 h*

Values are means ± SD from three to six independent experiments. Controls were treated the same as plants exposed to AAL-toxin or fumonisin B_1 but without addition of toxins. Asterisks (*) indicate means within a column that are significantly different ($P < 0.05$) from the corresponding control (within species) based on a one-way analysis of variance (SAS Institute, 1985).

Treatment	Phytosphinganine	Sphinganine
Duckweed		
Control	17 ± 10	6 ± 4
Fumonisin B_1	309 ± 108*	454 ± 186*
AAL-toxin	770 ± 242*	776 ± 501*
Tomato leaf discs		
Control	7 ± 2	4 ± 2
AAL-toxin	279 ± 188*	1749 ± 227*

In both susceptible and resistant tomato varieties, free sphinganine and free phytosphingosine were significantly elevated at all sample times relative to concurrent controls (Fig. 6, A and B). However, in the susceptible variety free sphinganine concentration increased with time, whereas in the resistant variety free sphinganine concentration decreased with time over the 24- to 72-h time period (Fig. 6A). Regression analysis of the change in free sphinganine content over the 24- to 72-h period indicated that in the susceptible variety there was a statistically significant correlation (positive slope) between time and free sphinganine content in both the AAL-toxin- and fumonisin B_1-treated plants ($P < 0.0001$ and 0.005, respectively). In the resistant variety the change in free sphinganine content was also correlated with time (negative slope) in the fumonisin B_1-treated plants ($P < 0.0001$) but was not significantly correlated in the AAL-toxin-treated plants ($P = 0.064$).

Table II. *Concentration (pmol/mg dry weight) of free phytosphingosine and free sphinganine in extracts from susceptible (asc/asc) tomato plants treated with either pure toxin or rice culture material for 40 to 60 h*

Control plants were sprayed with distilled water until runoff and treated plants were sprayed with 70 μM fumonisin B_1, 5 μM AAL-toxin (pure toxins), or equivalent concentrations of toxin in rice culture material (culture material) suspended in distilled water, until runoff.

Treatment	Phytosphingosine	Sphinganine
Control[a]	5 ± 2	6 ± 3
Pure toxins[b]		
Fumonisin B_1	154	1176
AAL-toxin	123	1274
Culture materials		
F. moniliforme	93	920
A. alternata	72	1148

[a] Values for controls are the mean ± SD from four experiments. [b] Values represent the mean of two replicate analyses of the powdered leaves from one experiment. No statistical analysis was done.

Table III. *Concentration (pmol/mg dry weight) of free phytosphingosine and free sphinganine in extracts of tobacco callus treated with pure fumonisin B_1 (14 μM) for 3, 6, or 9 d*

Values are the means ± SD from three culture dishes for each treatment and day. Asterisks (*) indicate means that were significantly different ($P < 0.05$) from control means at the same time based on a one-way analysis of variance (SAS Institute, 1985).

Treatment	Phytosphingosine	Sphinganine
Control		
d 3	11.9 ± 3.8	4.7 ± 1.6
d 6	5.2 ± 0.8	3.1 ± 0.4
d 9	5.1 ± 0.6	2.4 ± 0.7
Fumonisin B_1		
d 3	50.9 ± 25.6	34.1 ± 17.7*
d 6	50.4 ± 14.4*	41.1 ± 14.6*
d 9	74.9 ± 40.3*	31.4 ± 19.1

Free phytosphingosine content in both the susceptible and the resistant tomato plants was much less variable with time compared to free sphinganine (Fig. 6B). Only the fumonisin-treated plants showed a statistically significant decrease in phytosphingosine with time ($P < 0.0002$).

Rice culture material (fungi plus toxins) diluted to concentrations of 5 μM AAL-toxin and 70 μM fumonisin B_1 was applied to susceptible tomato plants and had the same effect as pure compounds (5 μM AAL-toxin or 70 μM fumonisin B_1), both in regard to the pathogenicity (wilt and necrotic lesions on stem and leaves) and the increase in free phytosphingosine and free sphinganine (Table II). The elevation in free sphingoid bases was also qualitatively and quantitatively similar.

Tobacco callus (Table III) was the simplest plant system tested. Like the duckweed and tomato plants, fumonisin B_1 treatment resulted in elevation of both free phytosphingosine and free sphinganine. The increase in free phytosphingosine was greater than the increase in free sphinganine. No attempt was made to measure the physiological condition of the callus; however, visual observation suggested that there was no difference in growth, and the appearance of control and fumonisin-treated callus was similar.

Although the relative increases in the two sphingoid bases appears to be quite different in the three plant systems, the overall results show conclusively that disruption of sphingolipid metabolism is a common feature in plants exposed to fumonisins or AAL-toxin. Recently, it was reported that fumonisin B_1 inhibits ceramide synthesis in microsomal preparations from squash (Lynch et al., 1993). The report by Lynch et al. (1993) is consistent with the previous hypothesis, although phytosphingosine is not a substrate for sphinganine (sphingosine) *N*-acyltransferase (Lynch et al., 1993). Phytosphingosine has been shown to be formed by the hydroxylation of sphinganine in vivo in rats (Crossman and Hirschberg, 1984). Nonetheless, the marked elevation in free sphinganine in all three plant systems suggests that the diseases associated with *A. alternata* and *F. moniliforme* may have as an early biochemical lesion inhibition of ceramide synthesis via inhibition of *N*-acyltransferase(s).

In duckweed (Fig. 3) and tomato leaf discs (Figs. 4 and 5), the elevation of free sphingoid bases before the appearance

of any signs of electrolyte leakage supports the conclusion that disruption of sphingolipid metabolism is an early biochemical lesion. In tomato plants free sphingoid bases were significantly elevated in resistant and susceptible plants at times when only a few necrotic spots were evident. Future studies may relate the time and dose dependency of free sphingoid base alterations with physiological parameters such as Suc transport (Moussatos et al., 1993), which have been shown to be sensitive markers of exposure to AAL-toxin in tomato leaf discs.

There are several reasons to believe that the elevation in free sphinganine is a specific disease-induced response. First, free sphinganine occurs in very low levels in animal and plant tissues because it is an intermediate in the de novo sphingolipid biosynthesis pathway (Lynch et al., 1993). Intermediates in biosynthetic pathways do not normally accumulate unless a step in the pathway is blocked. Second, free sphinganine is unlikely to be elevated by the breakdown of complex sphingolipids because, as in animal cells, sphinganine is a minor component of plant complex sphingolipids (Cahoon and Lynch, 1991). It is possible that, like free sphingosine in animal cells (Merrill, 1991), free phytosphingosine in plant cells may be a product of regulated sphingolipid turnover. If the elevation in free phytosphingosine was a result of necrosis and the resulting metabolic chaos, then elevation of the much more abundant unsaturated di- and trihydroxy long-chain bases commonly found in glucocerebrosides (Cahoon and Lynch, 1991) would have been evident in the HPLC chromatograms. A third reason to believe that elevation in free sphinganine is a specific disease-induced response is that fumonisins and AAL-toxin are specific inhibitors of sphinganine (sphingosine) *N*-acyltransferase in cultured mammalian cells (for review, see Merrill et al., 1993). Therefore, it would seem unlikely that given the similarity between the known de novo sphingolipid biosynthesis pathway in animals (Merrill, 1991) and the proposed pathway in plants (Lynch et al., 1993), that the toxins would be specific in one system but not in the other.

The resultant fumonisin- and AAL-toxin-induced accumulation of free sphingoid bases and inhibition of complex sphingolipid biosynthesis could easily lead to the disruption of cellular regulation and cytotoxicity. Although there are substantial data demonstrating the role of sphingolipids in cellular regulation in animal cells (for review, see Merrill et al., 1994), there are only a few reports supporting equivalent roles in plants (Lynch and Steponkus, 1987; Dharmawardhane et al., 1989; Bille et al., 1992). Therefore, fumonisins and AAL-toxin will be useful research tools for studying the role of sphingolipids in plant cell regulation and the role of sphingolipids in plant disease.

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