Relationship of Sphinganine Analog Mycotoxin Contamination in Maize Silage to Seasonal Weather Conditions and to Agronomic and Ensiling Practices

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ABSTRACT

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Sphinganine analog mycotoxins (SAMs) are reported in maize and maize based feeds. Our objectives were to detect and quantify fumonisins B₁ and B₂ and *Alternaria* toxins (AAL toxins) AAL-TA and AAL-TB and determine how agronomic practices, weather conditions, and ensiling affected the occurrence and levels in maize silage. Silage was collected at harvest and after ensiling in 2001 and 2002 from 30 to 40 dairies, representing four regions in Pennsylvania. SAMs were quantified using high pressure liquid chromatography (HPLC) with fluorescence detection and high pressure liquid chromatography-mass spectrometry HPLC-MS. The average concentrations and ranges were as follows: fumonisin B₁ 2.02 µg/g (0.20 to 10.10), fumonisin B₂ 0.98 µg/g (0.20 to 20.30), AAL-

TA 0.17 µg/g (0.20 to 2.01), and AAL-TB 0.05 µg/g (0.03 to 0.90). Fumonisin B₁ was the most frequently detected toxin (92%) in all samples, followed by fumonisin B₂ (55%), AAL-TA (23%), and -TB (13%). Temperature during maize development was positively correlated with fumonisin occurrence and levels and negatively with AAL-TA, while moisture events were negatively correlated with fumonisins and positively with AAL-TA. Fumonisin levels were higher in silage harvested at later developmental stages (dough through physiological maturity). Ensiling did not affect toxin concentration nor did agronomic practices (tillage system, inoculant use, or silo type) or silage characteristics (dry matter, pH, or organic acid concentration). This is the first report of AAL-TB in silage and on factors that affect SAM frequency and levels in maize silage.

Additional keywords: *Fusarium* ear rot, *Fusarium verticillioides*, *F. proliferatum*, *Gibberella moniliformis*, *G. fujikuroi*.

Sphinganine analog mycotoxins (SAMs) are structurally similar compounds (Fig. 1) produced by members of the fungal genera *Fusarium* and *Alternaria* (44,50,55). Two classes of SAMs, the fumonisins and *Alternaria* toxins (AAL-toxins) AAL-TA, have been reported in maize-based feeds, including silage (28,34,54,70). Although both classes of SAMs have similar cytotoxic activity, (59), the fumonisins are considerably more toxic (65), associated with several human and animal diseases (15,34,35,49,58), and are federally regulated in foods and feeds (43). To date, the cellular affects of AAL-toxin exposure have been studied and found to be similar to those caused by fumonisins (1,2); however, the toxicological impact on humans and animals is unclear. Feeding studies with fumonisin B₁ have revealed that although cattle are not acutely sensitive to this toxin (51), ingestion resulted in abnormal hepatic and immune system pathology (38). To our knowledge, no studies have investigated chronic effects on dairy cattle exposed to subacute levels of fumonisins or AAL-toxins. An additional concern is that SAMs do co-occur with other mycotoxins (70), increasing the risk of additive or synergistic toxicological affects (56).

SAM-producing fungi are diverse in terms of their behaviors toward maize and the niches they inhabit. The most commonly encountered fumonisin-producing *Fusarium* spp. on maize are *F. verticillioides* (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis*) and *F. proliferatum* (Matushima, T.) Nirenberg (teleomorph *Gibberella fujikuroi* mating population D). Although

both species cause an ear rot referred to as *Fusarium* ear rot (53), *F. verticillioides* is also an endophyte of maize (7,23,30,49). This endophytic association is of concern, as fumonisins may still be present in infected plant parts even in the absence of symptoms (17). In contrast to *Fusarium* spp., *Alternaria* spp., such as *A. alternata* and *A. tenuissima* (45) may be found as epiphytic saprophytes (10), although they may act as opportunistic pathogens if the plant is wounded (14,53), and as the plant

Fig. 1. Chemical structure of A, fumonisins and B, *Alternaria* (AAL) toxins.

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naturally senesces during the growing season. Both species have been reported on maize and in silage (3,29,45).

Thus far, studies on fumonisins have been limited to understanding factors that affect toxin contamination of the ear and grain rather than of the whole plant, which is significant for understanding toxin accumulation in silage. High levels of fumonisins in maize grain are often associated with warm weather (41,42,66); 30°C is optimal for toxin production *in vitro* (36,39). Fumonisin contamination is also dependent on other factors, including fungal strain (17), maize genotype (16,52), plant maturity (11,70), interactions with other fungi (37,64), and insect wounding (19,61). However, less is known about factors that affect growth of *Alternaria* spp., and there have been no published reports on conditions for toxin production on maize. Torres et al. (63) found that when *A. alternata* was grown on maize kernels at 20 and 30°C and water activities of 0.92, 0.95, and 0.98, the fungus was able to grow at all conditions studied, although optimal growth occurred at 30°C with 0.98 *a_w*. Under field conditions, high frequencies of *Alternaria* spp. have been found on cereals during sustained periods of warm, wet weather (24,45).

Current management of fumonisin contamination in maize relies on cultural practices (16,21,46,52) and resistant hybrids, although other strategies, such as the use of transgenic maize lines, microbial biocontrol, and in planta detoxification by genetically modified plants are under investigation (7–9,13,21,47,48). Natural resistance to *Fusarium* ear rot pathogens and fumonisin contamination appears to be contingent on a number of physical and genetic characteristics, which do not always provide consistent control (16,26,67). Transgenic hybrids, particularly Bt lines, have been recommended as their toxicity towards lepidopteran larvae results in less feeding damage and generally lower concentration of fumonisins than nontransgenic isolines (25,47). However, the cost-effectiveness of these hybrids over traditional lines may vary by region (18,20). The impact of cultural management practices has been the focus of few investigations, however, the information provided by Flett et al. (22) found that different tillage systems had no effect on *Fusarium* ear rot. Postharvest approaches, such as identifying microorganisms that degrade fumonisins (9,12,13), are also under investigation, although most studies are still in preliminary stages. Commercial binding agents have also received some attention and some show promise for reducing the biological availability of fumonisins (6), although this has yet to be tested with ruminants. As the overall risk of AAL-toxin contamination of maize is not well understood, to date, no effort has been made to address control measures.

The current inability to prevent or control SAM contamination of maize-based feeds, including silage, is limited by lack of understanding of factors conducive or inhibitory to toxin formation. Although there is a significant amount of information on fumonisin production in maize grain, it is unknown whether conditions affecting toxin occurrence in grain are the same for a whole plant feed such as silage. Additionally, the prevalence and levels of AAL-toxins in maize silage has not been studied rigorously, and little is known about stimuli that impact toxin occurrence. As such, our objectives were (i) to determine the levels and frequencies of fumonisins B₁, B₂, and AAL-toxins TA and TB; and (ii) to investigate how weather conditions, agronomic practices, and the process of ensiling affect the concentration and frequency of SAMs in maize silage.

MATERIALS AND METHODS

Silage collection. Maize silage was collected from four regions in Pennsylvania (Fig. 2) at dairies with a history of known or suspected mycotoxin contamination. Thirty-two and thirty-one farms participated in the study in 2001 and 2002, respectively. Samples were collected at harvest and 3 to 6 months after ensiling

to evaluate the effect ensiling had on the content of SAMs in silage. Collection of harvest and ensiled samples was performed as described by Mansfield et al. (33) where, at harvest, 1 to 2 kg were collected from a mechanical harvester as it made a pass through the field until samples, representative of several passes, had been collected. Ensiled samples were collected by either removing silage from several locations at the face of bunker or trench silos or from an upright silo as the material was being emptied during feed out. Sample collection from each type of silo was based on the amount of silage fed per day, where sites feeding 1 to 2 tons per day were sampled every other day for 14 days, while those feeding 3 to 4 tons (or more) per day were sampled for 7 days consecutively. After collection, 1 to 3 kg samples were stored at –80°C until analysis. Due to drought conditions during the 2001 and 2002 seasons, maize from some sites did not reach physiological maturity (R6), which is the recommended maturity rating for harvest (57). Therefore, some samples were harvested at the earlier maturity ratings of milk (R3), dough (R4), and dent (R5).

Extraction and purification of SAMs. Prior to toxin extraction, silage samples were dried at 55°C for 5 to 7 days, milled to a flourlike consistency with a Wiley mill, mixed, and subsampled in 500 g quantities for analysis. Using a method modified from Sydenham et al. (62), 25 g of ground silage was extracted with 100 ml of HPLC-grade methanol/water (3:1, vol/vol) and placed on a rotary shaker at 310 rpm for 1 h. The samples were then centrifuged at 6,000 × *g* for 10 min. Purification was performed using 8-ml polypropylene columns (Alltech, State College, PA) packed with 500 mg of strong anion exchange media (SAX) (Varian, Palo Alto, CA) and polypropylene frits with a 20 µm pore size (Alltech). Columns were primed by passing 5 ml of methanol through, followed by 5 ml of methanol/water (3:1, vol/vol). The pH of each sample was adjusted to a range of 5.8 to 6.5 with two to three drops of 1 M NaOH, and then 10 ml of the adjusted sample was passed through the SAX column. The column was then washed with 5 ml of methanol/water (3:1, vol/vol) and 3 ml of methanol. SAMs were eluted by adding 10 ml of methanol amended with 1% glacial acetic acid. Sample eluate was concentrated to dryness under a stream of nitrogen, resuspended in 200 µl of methanol, and stored at –20°C prior to analysis. Toxin extraction was done twice from two subsamples of each silage sample. The results of each extraction were then compared and if the difference was greater than 1.0 µg/g for the fumonisins, and 0.5 µg/g for AAL-TA and -TB, the extraction procedure was repeated with a third subsample.

SAM standards. A mixed standard of fumonisin B₁ (100 µg/ml) and B₂ (75 µg/ml) (Romer Labs Inc., Union, MO) was used to determine toxin frequency and quantity by HPLC analysis. AAL toxins TA and TB were generated from culture ATCC 28329 of *A. arborescens* to assess method recovery and

Fig. 2. Map of the relative locations of dairies where silage samples were collected. Symbols represent the four major dairy production regions: Northeast, ★; Northwest, ▲; Southeast, ■; and Southcentral, ●.

quantitative limitations. The fungus was grown on potato dextrose agar (Difco, Sparks, MD) for 7 days at room temperature, and then 2 ml of sterile distilled water was added to the plate to create a spore suspension. Rice media was prepared by adding 2 g of rice (Uncle Ben's White Rice, Mars, Inc., McLean, VA) to a 15-ml polypropylene tube, adding 2 ml of water, incubating at room temperature for 4 h, and autoclaving at 121°C for 30 min. After autoclaving, 500 µl of spore suspension was added to the media, which was then incubated in the light at 25 ± 2°C for 14 days. Cultures were extracted by adding 8 ml of HPLC-grade methanol/water (3:1, vol/vol) and shaking as described above. Cleanup and purification were performed as described above and the samples were resuspended in 200 µl and stored at -20°C until analysis.

SAM derivatization. For fluorescence HPLC detection, samples were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) (Sigma-Aldrich, St. Louis, MO) using a method modified from Manica et al. (32). For each silage extract, 12.5 µl was transferred to a new autosampler vial and desolvated under nitrogen. The sample was resuspended in 179 µl of 10 mM borate buffer (pH 9.2) to which 8 µl of 80 mM potassium cyanide solution (borate buffer) was added, followed by 16 µl of 55 mM NDA solution (acetonitrile). Samples were then incubated for 15 min at 25 ± 2°C and filtered with a 0.2 µm PTFE filter (Pall Life Sciences, East Hills, NY).

Detection of fumonisins B₁ and B₂. Fumonisins were detected and quantified using an Agilent 1100 HPLC with fluorescence detection (Agilent, Palo Alto, CA). Chromatographic separation was achieved using a reverse phase Thermo Betasil C-18 column (150 × 2.1 mm; 5 µm particle size, pore size 60 Å) (Thermo, Bellefonte, PA). Solvents consisted of A, 1% acetic acid in water; B, 1% acetic acid in acetonitrile; and C, methanol. The program followed a linear gradient beginning with A at 100 to 70% and B from 0 to 30% over 5 min, followed by A at 70 to 24% and B from 30 to 76% from minutes 5 to 38, holding at C from 38 to 42 min and re-equilibrating at A from 42 to 46 min before the next injection. Total run time was 46 min. Fluorescence detection was set at 420 nm excitation with a 480 nm emission for NDA-derivatized SAMs. Data were analyzed using ChemStation for LC Rev. A.08.01 analysis software (Agilent).

Detection of AAL toxins TA and TB. Preliminary analysis indicated that although the fumonisins were well resolved from the silage matrix and from one another by the chromatography method described above, the AAL toxins were not easily separated from components of the silage matrix. Therefore, we chose to analyze these toxins by high pressure liquid chromatography-mass spectrometry HPLC-MS (HPLC-MS). Twenty-five microliters of silage extract was transferred to an amber autosampler vial, desolvated under nitrogen to dryness, and resuspended in 496 µl of acetonitrile. Four microliters of 1 µg/µl retinoic acid in acetonitrile (Sigma-Aldrich) was then added to each sample as an internal standard. The solvent delivery system consisted of a LC10ADvp ternary solvent delivery system (Shimadzu, Columbia, MD) to which a Quattro II mass spectrometer (Micromass, Beverly, MA) was attached for detection. Solvents for HPLC-MS analysis were the same as A and B described above. The method began with A at 100% following a linear gradient to 70% and B at 0%, going to 30% from time 0 to 2 min; followed by A from 70 to 24% and B from 30 to 76% from 2 to 12 min; a wash with 100% B from 12 to 14 min; followed by a re-equilibration step at 100% A from 14 to 15 min. Negative electrospray ionization (ESI-) was used to detect AAL-TA and -TB by MS analysis and the instrument was set up for selected ion monitoring for retinoic acid (*m/z* 299), AAL-TA (*m/z* 520), and TB (*m/z* 504). Data analysis was performed using Mass Lynx version 3.5 mass spectrometry software (Milford, MA).

When silage samples (*N* = 12) were spiked with each of the four toxins at a range of 0.5 to 5 µg of toxin per gram of silage,

the percent recoveries were as follows: FB₁ = 79 to 85%, FB₂ = 74 to 88%, AAL-TA = 71 to 84%, and AAL-TB = 77 to 82%. The limit of detection for the fumonisin- HPLC method was 30 ng of toxin per gram of silage and 20 ng/g of silage for the AAL-toxins with the HPLC-MS method.

Evaluation of agronomic practices, weather data, and silage characteristics. Information on agronomic practices used in crop management was collected from each of the participating sites and included crop rotation with a broadleaf crop, type of tillage system, type of inoculant of lactic acid bacteria or an organic acid added to silage prior to ensiling, and type of silo used for storage.

Estimated daily weather data such as minimum, maximum, and average temperature, total precipitation, average relative humidity, total wetness hours, growing degree day (base 10°C), accumulated growing degree day (day 1 planting date), precipitation minus evaporation, accumulated precipitation minus evaporation (day 1 planting date), and a hybrid growth prediction estimate (based on planting date) from tasselling (VT) through physiological maturity (R6) was provided by ZedX Inc. (Bellefonte, PA) for each of the study sites (4,31,57).

Ensiled samples were evaluated for percent dry matter, pH, ammonia content, and the concentrations of lactic, acetic, propionic, butyric, and isobutyric acids. These analyses were performed by Cumberland Valley Analytical Services Inc. (Hagerstown, MD) (5).

Statistical analyses. All statistical tests were performed at a 95% confidence level (71). The Mann-Whitney test was used to compare SAM frequency between the 2001 and 2002 growing seasons and between harvest and ensiled samples (Minitab ver. 13.32, Minitab Inc., State College, PA). The Kruskal-Wallis test was used to compare SAM frequency (2001 and 2002 harvest and ensiled samples) between silage production regions in Pennsylvania. SAM concentrations were compared between samples from 2001 and 2002 and the harvest and storage samples with the paired Student's *t* test. SAM levels (2001 and 2002 harvest and ensiled samples) between the four regions of Pennsylvania were compared using a general linear model.

Kendall's correlation, with the PROC CORR procedure in SAS statistical software (SAS Institute Inc., Cary, NC), was used to determine which of the surveyed agronomic practices appeared to impact SAM frequencies and levels in harvest and ensiled samples. Significant relationships were further evaluated with the Tukey-Kramer test to determine differences between categorical levels.

Daily weather variables for each site were averaged for each growth stage prior to statistical analysis. Pearson's correlation was used to study the relationship between weather variables during each growth stage and the SAM levels in 2001 and 2002 harvest samples. The relationship between SAM frequency in 2001 and 2002 harvest samples and weather variables was assessed with Kendall's correlation.

The Pearson correlation coefficient was used (SAS Institute Inc.) to examine the relationship between selected characteristics of ensiled samples from 2001 and 2002 (described above) and SAM concentrations. The Kendall correlation coefficient was used (SAS Institute Inc.) to evaluate the relationship between these characteristics of ensiled samples and the frequency of SAM contamination.

RESULTS

Using the HPLC with fluorescent detection and HPLC-MS methods described above, we were able to separate the fumonisins and AAL-toxins from one another as well as from the silage matrix, and to detect the toxins at levels well below those of concern for cattle. We found that the sensitivity and recovery ability of this method was comparable to the immunoaffinity-HPLC methods of Yu et al. (70) and Kim et al. (28). The retention times for FB₁ and FB₂ determined by the HPLC with fluorescence

detection method were 31.5 to 31.7 min and 35.0 to 35.2 min, respectively. Retention times determined by the HPLC-MS method for AAL-TA peak 1 were 4.2 to 4.4 min; for -TA peak 2, 4.5 to 4.7 min; for -TB peak 1, 5.5 min; for -TB peak 2, 5.9 to 6.1 min; and for retinoic acid, 14.1 to 14.3 min.

There was no statistically significant difference between silage at harvest and after ensiling in terms of the levels or frequency of SAMs detected. The average concentration and range (in parentheses) of SAMs in fresh and ensiled maize from 2001 and 2002 ($N=120$) are as follows: FB₁, 2.02 $\mu\text{g/g}$ (0.20 to 10.1); FB₂, 0.98 $\mu\text{g/g}$ (0.20 to 20.3); AAL-TA, 0.17 $\mu\text{g/g}$ (0.20 to 2.0); and TB, 0.05 $\mu\text{g/g}$ (0.03 to 0.90). FB₁ was the most frequently detected toxin (92%), followed by FB₂ (55%), AAL-TA (23%), and TB (13%).

The frequency of the fumonisins in 2002 was higher than in 2001 for most of the study regions. Conversely, the frequency of the AAL toxins was slightly higher in 2001 compared to 2002. In terms of concentration, although the southern regions had higher concentrations of the fumonisins and AAL-TA in 2002, this was not observed for the northern sites, nor consistently when all sites were grouped (Fig. 3).

The relationship we observed between FB₁ concentration and moisture suggests that warmer temperatures and decreased moisture availability lead to higher final concentrations of FB₁ in silage compared to the average. Although the relationship pattern was similar for toxin occurrence and concentration, the relationship of concentration to temperature and moisture was stronger than between these variables and occurrence.

The relationship between FB₂ occurrence and weather conditions appeared to be similar to that of FB₁, although environmental conditions during silking (VT) seemed to have a stronger impact

on occurrence of this toxin than they did on FB₁ occurrence (Table 1). During silking, there was a negative relationship between several moisture variables, including average precipitation, relative humidity, wetness hours, and precipitation minus evaporation, while temperature had a positive affect. Interestingly, while FB₁ occurrence was affected during most of the developmental stages of maize, no relationship was observed between FB₂ occurrence and weather conditions until the mid to later stages (R3 to R5) of kernel maturation, although this observation may be an artifact of detection. Again, temperature was found to be positively correlated, while moisture variables had a negative relationship to FB₂. The same pattern was observed between toxin concentration, moisture and temperature, although the relationship was stronger between environment and concentration than for occurrence (Table 2).

In contrast to the fumonisins, AAL-TA occurrence was affected by weather conditions during R2 and R6 where temperature during these stages was negatively correlated and moisture variables positively correlated. The same pattern was observed for AAL-TA toxin levels where temperature was negatively correlated during R2, R3, and R6, and positively correlated with precipitation. For AAL-TB, too few samples contained this toxin to achieve reliable correlation results.

As indicated by correlation analyses, conditions during the later stages of plant maturation appeared to have the strongest relationship with final FB₁ concentration. This is reflected by our observation that maize silage harvested at later (R5 and R6) maturation stages had slightly higher frequencies of contamination and significantly higher final FB₁ concentrations than silage harvested earlier (Fig. 4).

Fig. 3. Average concentration of sphinganine analog mycotoxins in maize silage: **A**, fumonisin B1; **B**, fumonisin B2; **C**, *Alternaria* toxin TA (AAL-TA); and **D**, *Alternaria* toxin (AAL-TB). The number of samples for each region were: Northeast (NE), 24; Northwest (NW), 16; Southeast (SE), 10; and Southcentral (SC), 10. Concentration is expressed in micrograms of SAM per gram of dried silage. Asterisks over bars indicate a season (2001 or 2002) with significantly ($P \leq 0.05$) higher toxin concentration.

The four silage production areas in Pennsylvania were significantly different from one another in terms of SAM occurrence and toxin levels (Fig. 5). For FB₁, there was a north-to-south variation in that sites SE and SC were more often contaminated and at higher average concentrations ($P \leq 0.05$). This pattern was not observed with FB₂ however, as northwestern (NW) and south-eastern (SE) sites both had lower average concentrations of this toxin compared to northeastern (NE) and southcentral (SC) sites. Again, this may be an artifact of detection rather than an actual observation. Although the AAL toxins were more often present in samples from NE and NW sites ($P \leq 0.05$) than from the southern part of the state, the levels were not significantly different between the regions. The differences in toxin concentration between the regions may be due in part to geographical differences in temperature. During the stages of maize development (VT to R6) the only consistent significant difference between the northern sites and those in the south was that the south had higher average temperatures from VT to R6. This suggests that temperature may be the overriding weather factor influencing levels of SAMs.

Correlation analysis between agronomic practices and SAM occurrence and levels revealed that none of the agronomic practices in question—additive use before ensiling, tillage system or silo type—yielded statistically significant differences in toxin incidence or concentration. Likewise, no relationship was observed between percent dry matter, pH, ammonia content, lactic acid, acetic, propionic, butyric, and isobutyric acid content and SAM incidence or levels in silage. As such, it may be that the pre- or postharvest strategies considered, such as tillage, crop rotation, pre-inoculant or other additives, do not have a significant effect on SAM reduction in silage.

TABLE 1. Relationships between weather variables and sphinganine analog mycotoxin occurrences in 2001 and 2002 maize silage

Toxin	Stage ^a	Variable ^b	Average ^c	Range (N = 60)	Correlation coefficient ^d
FB ₁	R1	RHUM	69.7	58.0 – 81.8	0.243
		WHR	8.4	0.0 – 17.5	0.241
	R2	TMAX	27.6	23.1 – 31.8	0.396
		ATEMP	22.1	17.4 – 26.0	0.321
	R3	TMAX	25.7	19.0 – 32.9	0.387
		TMIN	14.5	8.2 – 20.9	0.379
		ATEMP	20.0	13.7 – 27.0	0.381
		PVAP	-63.0	-82.0 – -46.2	-0.280
	R4	TMAX	23.7	15.3 – 34.7	0.396
		TMIN	12.6	2.8 – 21.0	0.395
		ATEMP	18.1	8.6 – 27.5	0.419
	R5	TMAX	23.6	15.6 – 29.0	0.324
FB ₂	R6	PREC	0.2	0.0 – 0.71	-0.453
		PVAP	-0.23	-0.84 – 0.43	-0.429
	VT	TMAX	28.8	24.8 – 32.8	0.396
		PREC	0.18	0.0 – 0.76	-0.266
		RHUM	73.1	60.2 – 83.2	-0.344
		WHR	10.5	0.0 – 19.3	-0.337
	R3	PVAP	-0.51	-0.91 – 0.76	-0.367
		RHUM	73.0	59.8 – 79.9	-0.259
	R4	TMAX	74.8	59.5 – 94.5	0.301
		RHUM	72.3	60.5 – 82.3	-0.392
	R5	PVAP	-0.48	-0.71 – 0.43	-0.287
		TMIN	12.8	8.8 – 18.8	0.313
		PREC	0.23	0.0 – 1.02	-0.389
AAL-TA	R2	TMAX	27.7	23.1 – 31.8	-0.287
	R6	PVAP	-0.23	-0.84 – 0.43	0.378

^a Growth stages are: VT = tassels; R1 = silking; R2 = blister; R3 = milk; R4 = dough; R5 = dent; and R6 = maturity (56).

^b Weather variables are: RHUM = relative humidity; WHR = wetness hours; TMAX = maximum temperature; TMIN = minimum temperature; ATEMP = average temperature; PVAP = precipitation minus evaporation; and PREC = precipitation.

^c Average values are expressed in the following units: RHUM, %; WHR, hours; ATEMP, °C; and PREC, centimeters.

^d Kendall's correlation coefficient.

DISCUSSION

As silage is a particularly difficult matrix to work with, several recent publications have focused on using antibody-based cleanup methods for SAMs in maize grain and silage (28,69,70). Although this approach was quite selective, it may not be the most cost-effective method for processing a large number of samples. Therefore, for our analysis we chose to exploit the structural similarity of the fumonisins and AAL-toxins (Fig. 1) to develop a more general, but less expensive, method to study the four toxins in question. Our extraction method was modified from Sydenham et al. (62), which was originally intended to extract fumonisins B₁, B₂, and B₃ from maize grain. This was successful as both classes of SAMs bind to strong anion exchange media within the pH range of 5.8 to 6.5, making this a simple and efficient method to extract and select for both groups of toxins simultaneously from silage.

The frequency at which we detected FB₁ and FB₂ in silage is comparable to what was reported by Kim et al. (28), although the average concentration and range for both toxins in our study was higher. This may be due to the fact that relatively severe droughts

TABLE 2. Relationships between weather variables and sphinganine analog mycotoxin concentrations in 2001 and 2002 maize silage

Toxin	Stage ^a	Variable ^b	Average ^c	Range (N = 60)	Correlation coefficient ^d
FB ₁	VT	TMIN	17.8	11.8 – 21.8	0.357
		ATEMP	23.1	18.7 – 26.4	0.345
	R2	TMAX	27.7	23.1 – 31.8	0.417
		TMIN	16.6	11.2 – 21.4	0.403
		RHUM	71.2	60.2 – 77.1	-0.491
		WHR	9.8	3.2 – 16.2	-0.439
	R3	PVAP	-0.46	-0.15 – -0.76	-0.426
		TMAX	25.7	19.0 – 32.9	0.602
		ATEMP	20.0	13.7 – 27.0	0.578
		RHUM	73.0	59.8 – 79.9	-0.433
	R4	PVAP	-0.36	-0.76 – 0.08	-0.429
		TMAX	23.8	15.3 – 34.7	0.415
		TMIN	12.6	2.9 – 21.0	0.434
		ATEMP	18.1	8.6 – 27.5	0.441
	R5	PVAP	-0.25	-0.76 – 0.74	-0.691
	R6	TMAX	23.2	14.2 – 33.8	0.506
		ATEMP	17.6	10.7 – 26.2	0.512
		PREC	0.20	0.0 – 0.71	-0.546
		PVAP	-0.23	-0.84 – 0.43	-0.728
FB ₂	VT	TMAX	28.8	24.8 – 32.8	0.296
	R3	TMAX	25.7	19.0 – 32.0	0.294
		TMIN	14.5	8.2 – 20.9	0.341
		ATEMP	20.0	13.7 – 27.0	0.335
	R4	PVAP	-0.36	-0.76 – 0.08	-0.258
		TMAX	23.8	15.3 – 34.7	0.333
		TMIN	12.6	2.9 – 21.0	0.495
		ATEMP	18.1	8.6 – 27.5	0.449
	R6	RHUM	72.3	60.5 – 82.3	-0.301
		WHR	8.5	2.2 – 17.8	-0.291
		PREC	0.28	0.05 – 0.74	-0.564
AAL-TA	R2	TMAX	81.8	73.6 – 89.2	-0.362
		RHUM	71.2	60.2 – 77.1	0.325
	R3	TMAX	25.7	19.0 – 32.9	-0.260
		PREC	0.20	0.05 – 0.43	0.264
		PVAP	-0.36	-0.76 – 0.08	0.274
	R6	TMAX	23.2	14.2 – 33.8	-0.392
		PREC	0.20	0.0 – 0.71	0.491
		PVAP	-0.23	-0.84 – 0.43	0.441

^a Growth stages are: VT = tassels; R1 = silking; R2 = blister; R3 = milk; R4 = dough; R5 = dent; and R6 = maturity (56).

^b Weather variables are: RHUM = relative humidity; WHR = wetness hours; TMAX = maximum temperature; TMIN = minimum temperature; ATEMP = average temperature; PVAP = precipitation minus evaporation; and PREC = precipitation.

^c Average values are expressed in the following units: RHUM, %; WHR, hours; ATEMP, °C; and PREC, centimeters.

^d Pearson's correlation coefficient.

occurred during both seasons of the study, a condition that is reported to favor fumonisin contamination (40). In comparison to the findings of Yu et al. (70), we detected FB₁ more frequently at higher levels, but AAL-TA less frequently at a slightly lower average concentration. To our knowledge, this is the first report of AAL-TB in silage.

The differences in frequency and concentration for the four toxins in the two seasons are likely a result of the weather conditions in each year. Although both the 2001 and 2002 growing seasons (April to October) had higher than average temperatures, 2001 ranked 53rd, and 2002 sixth, out of 108 recorded seasons as the warmest on record (National Climatic Data Center, Asheville, NC). The 2002 season was also drier than 2001, ranking 24th in terms of driest years compared to 2001, which was 28th. Previous studies have observed that fumonisin concentrations are higher during drought years (39,40) and have suggested that this may be due to greater insect activity, decreased plant resistance from water stress, and other conditions favorable for fungal proliferation and toxin production. Although little is known about conditions favorable for AAL toxin production, the warm, dry weather conditions prevalent during this study may have been deleterious to growth and toxin production of *Alternaria* spp., which may explain the low frequencies and concentrations we observed in both years. Interestingly, Torres et al. (63) found that *A. alternata* was able to grow at a wider range of temperatures and water activities than *F. verticillioides*. However, although *A. alternata* may be able to grow in vitro at a wide range of temperatures, based on our observations, favorable temperatures and moisture requirements for toxin production in the field may be more restrictive.

Based on our correlation analyses, moisture and temperature variables appeared to have the strongest relationship on the occur-

rence and final concentration of SAMs. For the fumonisins, the relationship we observed is similar to previous reports on maize grain, where warm temperatures and low moisture availability positively affected final fumonisin concentrations (39-41,47,66). Because this study is unique in investigating how environment and plant maturity impact AAL toxin levels and occurrence, it is difficult to compare our results to previous studies.

Our observation that silage harvested at later maturities had higher average levels of FB₁ may indicate that the majority of FB₁ production may occur in the grain as higher fumonisin concentration occurred in crops harvested at later kernel maturation stages. A similar trend has been observed in grain, where maize harvested later in the season had higher average fumonisin concentration (11). Differential fumonisin production has also been observed in vitro on kernels harvested at different maturation stages (68) and it was found that kernels at R5 or dent stage supported the greatest production of fumonisin. Shim et al. (60) found that the degermed kernel tissue, containing the endosperm and pericarp, supported almost five times higher levels of fumonisin production than did the germ portion. As more mature kernels contain more endosperm, and thus more starch, than kernels at earlier stages of development (R1 to R3), this may explain why we observed higher fumonisin concentrations in silage harvested at later maturities. Similarly, Bush et al. (11) also observed that more mature kernels

Fig. 4. A, Frequency and **B,** average concentration of, sphinganine analog mycotoxin (SAM) in samples of maize silage harvested at different maturities. SAMs studied were: fumonisin B₁ (FB1); fumonisin B₂ (FB2); *Alternaria* toxin TA (AAL-TA); and *Alternaria* toxin TB (AAL-TB). Concentration is expressed in micrograms of SAM per gram of dried silage. Bars of the same toxin followed by a different letter are significantly different at $P \leq 0.05$.

Fig. 5. A, Frequency and **B,** average concentration of sphinganine analog mycotoxins (SAMs) in each of four maize silage-production regions. SAMs studied were: fumonisin B₁ (FB1); fumonisin B₂ (FB2); *Alternaria* toxin TA (AAL-TA); and *Alternaria* toxin TB (AAL-TB). The number of samples included in analysis for each region were: Northeast (NE), 24; Northwest (NW), 16; Southeast (SE), 10; and Southcentral (SC), 10. Concentration is expressed in micrograms of SAM per gram of dried silage. Bars of the same toxin followed by a different letter are significantly different at $P \leq 0.05$ between regions.

(R5 and R6) supported much greater fumonisin production compared to kernels at earlier stages of development.

Although all of the samples analyzed had levels lower than those recommended for grain for fumonisins (43), there are currently no guidelines set for a whole-plant feed such as silage. Also, because both fumonisins and AAL-toxins were present in the silage, this leads to questions of additive effects. In addition to the sphinganine analog mycotoxins, these samples also contained deoxynivalenol (33) and toxins produced by *Aspergillus* and *Penicillium* species (*unpublished data*). It is also unknown what the effects of chronic exposure may have on lactating animals.

Based on our observations, weather conditions during plant development and maturation appear to be the most influential factors affecting SAM occurrence and concentrations in silage within those considered in this study. Therefore, management of SAMs should focus on silage while the crop is in the field, although pre-harvest control of SAM contamination may be difficult to achieve due to the large and uncontrollable influence of weather. However, our observations may provide more detailed direction for efficiently collecting data to develop or improve models regarding SAM contamination. In turn, better descriptive models could assist in the development of improved control strategies such as the warning systems being developed for mycotoxins in other crops (27). As crop rotation and tillage did not appear to have an effect on toxin contamination, strategies such as using resistant hybrids or chemical controls should continue to be employed. In terms of postharvest management, we did not observe a reduction in SAMs from harvest to ensiling, indicating that microorganisms in silage may not be capable of degrading these toxins. However, as some microorganisms are known to degrade fumonisins, these organisms should continue to be studied so that their abilities can be exploited for postharvest detoxification.

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