Infection and Fumonisin Production by *Fusarium verticillioides* in Developing Maize Kernels

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ABSTRACT

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Fusarium ear rot and fumonisin contamination are serious problems for maize growers, particularly in the southeastern United States. The lack of maize genotypes highly resistant to infection by *Fusarium verticillioides* or to fumonisin contamination emphasizes the need for management strategies to prevent contamination by this mycotoxin. Information on the initial appearance of infection and fumonisin contamination of kernels and their increase over time is needed to determine if early

harvest may be an appropriate control strategy. Maize ears from replicated studies at two locations in eastern North Carolina were harvested weekly, starting 2 weeks after pollination and continuing for 14 weeks. The percentage of kernels infected with *F. verticillioides* and the fumonisin contamination in the harvested samples were determined. Kernel infection by *F. verticillioides* and fumonisin contamination appeared as kernels neared physiological maturity and increased up to the average harvest date for maize in North Carolina. Beyond this date, the concentrations of fumonisin fluctuated. Under years conducive for fumonisin contamination, early harvest (greater than 25% grain moisture) may help reduce the level of contamination.

Fusarium verticillioides (Sacc.) Nirenberg. (synonym: *F. moniliforme* J. Sheld.; teleomorph: *Gibberella fujikuroi*, mating population A) commonly is associated with maize kernels and, under favorable conditions, causes a kernel decay known as *Fusarium* ear rot (23). Infection by *F. verticillioides* also can result in the contamination of kernels with fumonisins, a family of mycotoxins toxic to animals and suspected to be human carcinogens (3,5,7,14,15,19,24). Ingestion of maize kernels infected with *F. verticillioides* is associated with equine leukoencephalomalacia in horses and porcine pulmonary edema in swine (14). Recent studies have shown a strong correlation between consumption of fumonisin-contaminated tortillas and neural tube defects in humans (18,21). Because of the health concerns associated with the consumption of fumonisins, the Food and Drug Administration has established recommended guidelines for their content in human and animal food. For example, these guidelines recommend that degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, and corn flour) contain fumonisin at no more than 2 mg/kg and that fumonisin content of maize fed to horses not exceed 5 mg/kg.

Over the last 10 years, fumonisin contamination of maize in the United States has exceeded 5 mg/kg in some areas (14). For example, the 1998 growing season in North Carolina was highly favorable for fumonisin contamination and several truckloads of grain were rejected at buying stations. Many of these stations assayed maize for fumonisin contamination by enzyme-linked immunosorbent assay (ELISA) test kits and rejected grain with fumonisin concentrations exceeding 15 mg/kg. Other stations rejected grain based on an arbitrarily established concentration of fumonisin or on excessive visible kernel rotting.

Concern over the quality of maize for human and animal consumption has led to breeding efforts aimed at developing maize genotypes with resistance to *F. verticillioides* and subsequent fumonisin accumulation. Although heritable resistance has been identified in maize (9,20), no highly resistant genotypes are known. Furthermore, it has been difficult to incorporate genes for resistance into commercially desirable hybrids.

Because of the concern about fumonisin contamination, particularly in the southern United States, other management strategies, along with the most resistant hybrids available, must be explored to manage this disease. In North Carolina, fumonisin contamination has been associated with delayed harvest due to late-season rains. If fumonisin concentrations continue to increase in the field after kernel maturity, one management strategy may be early harvest, particularly in those years that are conducive for fumonisin contamination.

Several investigators have studied the infection of maize with *F. verticillioides* (6,10,11,13,16), but we are unaware of any studies that have followed both kernel infection and the resulting accumulation of fumonisin in the field. In a study designed to follow the number of kernels infected with *F. verticillioides* during the growing season, King (8) harvested maize ears at weekly intervals up to 10 weeks after mid silk and assayed surface-sterilized kernels for the presence of *F. verticillioides*. He detected *F. verticillioides* in surface-sterilized kernels 2 weeks after mid silk and found that the number of infected kernels increased until the end of the study. The potential threat of fumonisin contamination in U.S. maize was unknown at the time of King's study.

Less is known about the accumulation of fumonisin in the field. Warfield and Gilchrist (22) examined the effect of kernel development on fumonisin production in laboratory studies. They inoculated detached kernels at four stages of development: blister, milk, dough, and dent. All stages were able to support fumonisin production. Dent stage kernels were the most conducive for fumonisin production and blister stage kernels supported the least amount of fumonisin.

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The focus of this study was to determine both the initial onset of infection and the appearance of fumonisin, and the progression of infection and accumulation of fumonisin in maize kernels grown in the field. Both of these parameters are important to determine if early harvest is a possible control strategy for fumonisin contamination.

MATERIALS AND METHODS

Maize was grown at two locations in North Carolina: the Central Crops Research Station at Clayton (Clayton), and the Tidewater Research Station at Plymouth (Plymouth). Pioneer hybrid 3394 was planted in 1999 and 2000, and two additional hybrids, Pioneer hybrid 34K77 and an experimental hybrid, X1106D, were planted in 2000. Hybrids were planted during the last week of April or the first of May at a population of 59,000 plants/ha. These are typical planting dates for corn-growing regions of North Carolina. The average mid silk dates for the hybrids were 4 July 1999 and 3 July 2000.

A split-plot experimental design was employed with hybrids arranged in a randomized complete block, with four blocks as the main plots and harvest date as the subplots. Plot size was one row at Clayton and two rows at Plymouth. Maize was harvested from rows 3.8 m long and 1.0 m apart. A 0.9-m alley separated each of the four replicates. These plots were exposed to naturally occurring inoculum of *F. verticillioides*.

To determine if artificial inoculation would alter the infection and fumonisin accumulation profile, additional plots of Pioneer hybrid 3394 were inoculated with a fumonisin-producing isolate of *F. verticillioides* collected from North Carolina. A 5-ml suspension containing 2×10^5 conidia/ml was injected into the side of all ears in the plot 2 weeks after mid silk by the pinbar method of Campbell and White (1).

In each year of the study, 10 visibly sound ears were arbitrarily selected each week from the designated row in each replicate plot, starting 2 weeks after pollination and continuing for 14 weeks. Ears were dried in a forced-air drier at 35°C for 1 week and shelled, and the kernels from the 10 ears in a replicate were pooled and stored at room temperature. Kernel moisture content for the ears harvested from weeks two to six was determined by comparing the weight of three representative ears of each hybrid before and after drying. Kernel moisture content of kernels harvested from week seven through the end of the study was determined using a Dickey John (Model GAC 2100; Auburn, IL) moisture meter.

Incidence of kernel infection was determined using a modified protocol described by Zummo and Scott (25). Kernels randomly selected from the pooled sample of 10 ears harvested from each plot were surface disinfested using a 70% ethanol dip, followed by submersion in 1% NaOCl for 3 min. Kernels (13 per plate) were placed in 18-cm petri plates containing 15 ml of Czapeks Agar supplemented with 7% NaCl. In 1999, 390 kernels from each replicate plot were randomly sampled to detect fungal colonization. The number of kernels plated per replicate plot in 2000 was reduced to 195. Statistical analysis of the 1999 data showed that this sample size was sufficient to estimate mean percent kernel infection within $\pm 5\%$ (4). Kernels were incubated at 28°C for 7 days and the percentage of kernels with visible growth of *F. verticillioides* was recorded.

Because a high-salt selective medium was used for the plating assay, few fungal species were observed growing from plated kernels. Most of the species were from three genera: *Fusarium*, *Penicillium*, and *Aspergillus*. The fungus predominately observed exhibited consistent and predictable colony morphology and produced chains of microconidia typical of *F. verticillioides* (17). To confirm that these colonies were *F. verticillioides*, three randomly selected strains were sent to the Fusarium Research Center (FRC, The Pennsylvania State University, State College, PA). All three isolates were confirmed to be *F. verticillioides* and were deposited

at the FRC as strains M-8632, M-8633, and M-8634. Three isolates of another species of *Fusarium* observed growing from less than 1% of the kernels also was sent to FRC. All three isolates were identified as *F. semitectum* and deposited at FRC as strains R-9666, R-9667, and R-9668).

In 1999, fumonisin B1 concentration was quantified by Optimum Quality Grains (Des Moines, IA) using an ELISA. The protocol used was a proprietary modification to the protocol described in Kulisek and Hazebroek (12). The values reported from Optimum Quality Grains were for fumonisin B1. Fumonisin B1 concentrations in 2000 were quantified by the NCSU Mycotoxin Lab using the fumonisin protocol FUM-LC1 developed by Romer Labs, Inc. (Union, MO). Briefly, a 454-g subsample of harvested kernels was selected randomly from each replicate and then individually ground to mesh size 20. A 25-g subsample was extracted for 1 h with 100 ml of CH₃CN/H₂O (50:50, vol/vol) and 2 ml of the extract was diluted with 8 ml of MeOH/H₂O (3:1, vol/vol). The diluted extract was added to a column conditioned with 5 ml of MeOH followed by 5 ml of 3:1 MeOH/H₂O. The column was washed with 8 ml of MeOH/H₂O (3:1, vol/vol) followed by 3 ml of MeOH. The wash solvent was discarded and the sample was eluted with 10 ml of MeOH/HOAc (99:1, vol/vol) and dried overnight on a Speedvac System SS3 (Savant, Holbrook, NY).

To derivatize fumonisin B1 for detection, the residue was dissolved in 1 ml of MeOH and the following reagents were added in order: 1 ml of 0.05 M sodium borate buffer (pH 9.5), 0.5 ml of sodium cyanide reagent (13 mg/liter of H₂O), and 0.5 ml of naphthalene-2,3-dicarboxaldehyde reagent. The sample was sealed and heated for 15 min at 60°C, cooled to room temperature, and diluted with 7 ml of solution (40:60, vol/vol) of 0.05 M phosphate buffer (pH 7)/CH₃CN. Next, 20 μ l of the sample was placed in the high-performance liquid chromatography (HPLC) system. The HPLC system consisted of a Model LC-600 HPLC pump and a SIL-9A auto injector (Shimadzu Corporation, Norcross, GA), a Brownlee column (0.4 by 10 cm; Perkin-Elmer Corp., Norwalk, CT), and a model RF-551 programmable and scanning fluorescence HPLC monitor (Shimadzu) set at 420 nm excitation and 500 nm emission.

All data were analyzed using the general linear models (GLM) procedure of SAS (version 8.1; SAS Institute, Cary, NC). Kernel infection data were transformed using a Logit transformation and fumonisin B1 concentration was transformed using a log transformation. Appropriate statistical comparisons among means were made using the Fisher's least significant difference ($P = 0.05$) statistic. Each experiment was analyzed separately, because there were significant interactions of treatments with year and location. In addition, the rates of increase in kernel infection for each of the three hybrids grown in 2000 were estimated by logistic regression of kernel infection data from 4 to 11 weeks postanthesis and compared. In this analysis, data were combined over both locations, because no significant hybrid-location interactions were detected for infection rates.

RESULTS

F. verticillioides was first detected 4 weeks after pollination (milk stage of kernel development) in maize kernels grown at Plymouth in 1999 (Fig. 1). The number of infected kernels increased rapidly to a peak 3 weeks later (17% kernel moisture). Following the peak at 7 weeks, there was an apparent decrease in the number of infected kernels. Fumonisin B1 also was detected in kernels 4 weeks after pollination and steadily increased until week six (Fig. 1). A second peak in the concentration of fumonisin was observed in kernels harvested 9 weeks after pollination.

Kernel infection by *F. verticillioides* appeared 5 weeks after pollination (milk stage of kernel development) in corn grown in Clayton in 1999 (Fig. 2). As was observed at Plymouth in 1999, the number of infected kernels increased rapidly and peaked

3 weeks later (20% kernel moisture). A hurricane on 16 September 1999 delayed harvest of the plots at Clayton for 2 weeks. Fumonisin B1 was detected in kernels 6 weeks after pollination at Clayton (Fig. 2) and reached a peak 2 weeks later. A second, higher peak in fumonisin contamination occurred in maize kernels assayed 13 weeks after pollination.

Weather conditions during the growing season in 2000 differed markedly from those in 1999, which was characterized by above-average temperatures and below-average rainfall until a hurricane in mid-September. The 2000 growing season was characterized by below-average temperatures and above-average rainfall throughout the season. In 2000, kernel infection was first detected 5 weeks after pollination, similar to that observed in 1999, but the rate of infection was slower and, at both locations, the maximum number

of infected kernels occurred later (Figs. 3A and 4A). Despite the slower rate in number of infected kernels, the maximum number of infected kernels was similar between the 2 years. These maximum observed levels in 2000 (38% at Plymouth and 58% at Clayton) were similar to those observed for 1999 (42% for Plymouth and 62% at Clayton).

Many factors may affect the number of kernels infected with *F. verticillioides*. To determine if the presence of additional inoculum would change either the time of initial infection or the progress of infection, maize ears were inoculated 2 weeks after pollination. The kernels were harvested at weekly intervals and assayed for the presence of *F. verticillioides* and fumonisin. We found that inoculated ears had $\approx 20\%$ more infected kernels (Fig. 5). However, the time of kernel infection (4 to 5 weeks after pollination) and the appearance of fumonisin were similar to that found for naturally infected ears. These data indicate that factors

Fig. 1. Infection by *Fusarium verticillioides* and fumonisin contaminated maize kernels grown at Plymouth in 1999 and harvested weekly after pollination. Bars show standard error.

Fig. 2. Infection by *Fusarium verticillioides* and fumonisin contaminated maize kernels grown at Clayton in 1999 and harvested weekly after pollination. Bars show standard error. Dotted lines indicate missing data points.

Fig. 3. A, Infection and B, fumonisin contamination by *Fusarium verticillioides* of three maize hybrids grown at Plymouth in 2000 harvested weekly after pollination. Bars show standard error.

other that inoculum concentrations influence the time at which kernels become infected with *F. verticillioides*.

To determine if host resistance may influence the time, rate, or maximum number of kernels infected and the accumulation of fumonisin, three maize hybrids differing in resistance to Fusarium ear rot were compared in 2000. In addition to hybrid 3394, which is designated as intermediately resistant to Fusarium ear rot, a more resistant hybrid (Pioneer hybrid 34K77) and a more susceptible experimental hybrid (X1106D) were compared. The time of initial infection was similar for the three hybrids at both locations. At Plymouth, Pioneer hybrid 34K77 had fewer infected kernels than the other two hybrids throughout the growing season. The time of initial infection and rate of infection for the three hybrids were similar at Clayton for the first 9 weeks after pollination but, after week nine, the susceptible hybrid X1106D had a higher percentage of infected kernels than the more resistant hybrids.

Fig. 4. A, Infection and B, fumonisin contamination by *Fusarium verticillioides* of three maize hybrids grown at Clayton in 2000 harvested weekly after pollination. Bars show standard error.

When infection rates were calculated over both locations for the period from 4 to 11 weeks postanthesis (the time period when kernel infection increased most rapidly), kernels of the susceptible hybrid X1106D were infected at a significantly higher rate than the other two hybrids (Table 1).

The three hybrids also were compared for the time of appearance and concentration of fumonisin in their kernels. While these three hybrids are known to differ in resistance to Fusarium ear rot, there are no available data on their resistance to fumonisin accumulation. Fumonisin was first observed after 5 weeks at Plymouth and 6 weeks at Clayton (Figs. 3B and 4B). At both locations, the accumulation of fumonisin in all hybrids was similar for the first 9 weeks after pollination. After 9 weeks, there was a rapid increase in fumonisin concentrations at both Plymouth and Clayton. After this time, hybrids differed in their concentrations of fumonisin. At Clayton, Pioneer hybrid 34K77 contained the greatest concentration of fumonisins; whereas, at Plymouth, hybrid X1106D had the highest concentration of fumonisin. Fumonisin concentrations in Pioneer hybrid 3394 did not differ greatly between the two locations.

A comparison of the fumonisin content of Pioneer hybrid 3394, which was planted both years of the study, showed that location did not appear to have a striking effect on fumonisin contamination. In contrast, the 1999 growing season appeared to be more favorable for fumonisin contamination than the 2000 season. Peak concentrations of fumonisin in Pioneer hybrid 3394 were 10 mg/kg in 1999 and 5 mg/kg in 2000.

DISCUSSION

This study addressed the time of initial appearance and rate of kernel infection by *F. verticillioides*, and the accumulation of

Fig. 5. Infection and fumonisin contamination of kernels from ears inoculated with *Fusarium verticillioides*. Bars show standard error.

TABLE 1. Rates of kernel infection by *Fusarium verticillioides* for three maize hybrids from weeks 4 to 11 postanthesis in 2000

Hybrid	Rate of infection
Pioneer brand 34K77	0.54126
Pioneer brand 3394	0.49507
Pioneer experimental X1106D	0.66339 ^a

^a Rate is significantly different from the other two hybrids; least significant difference = 0.0942.

fumonisin in maize kernels. A goal of the research was to determine if early harvest of maize may be an effective control strategy to reduce the contamination of grain with fumonisin. Our results show that both kernel infection and fumonisin contamination occur in North Carolina as kernels approach physiological maturity and increase during the season up to the average harvest date for maize. Initial infection of kernels with *F. verticillioides* occurred within 4 to 5 weeks after pollination and the percentage of infected kernels increased until ≈ 9 weeks after pollination. The average date at which 50% of the maize is harvested in this region of North Carolina is 12 September, which corresponds to ≈ 9 weeks after pollination.

Our data on the progress of kernel infection by *F. verticillioides* agree with those of King (8). In a study in Mississippi, he found *F. moniliforme* (= *F. verticillioides*) to be the fungus most commonly isolated from maize kernels. Infected kernels were detected 2 weeks after mid silk and the number of infected kernels increased until the end of the study at 10 weeks (35% kernel moisture). King found that the final percentage of infected kernels ranged from 35% in 1977 to 66% in 1978. The final infection rate of over the 2 years of our study ranged from 38 to 62%.

Weather conditions in 1999 differed greatly from those in 2000 and, consequently, the rate at which the kernels dried was different between the 2 years (Figs. 1, 2, 3A, and 4A). Kernel moisture content 6 weeks after pollination was $\approx 20\%$ in 1999 and 40% in 2000. Regardless, maximum kernel infection in both years of the study occurred when kernel moisture was $\approx 20\%$.

Fumonisin was detected in maize kernels within a week of the appearance of *F. verticillioides*, and reached an initial peak in concentration 2 to 3 weeks later. In both years and at both locations, fumonisin was present in maize at the average harvest date for maize in that region. After this date, fumonisin concentrations were prone to fluctuate, often reaching higher concentrations late in the season.

The maize kernels were in the dent stage of development (35 to 45% moisture) when fumonisin first appeared. Warfield and Gilchrist (22), in their study with inoculated detached kernels, found that dent-stage kernels supported the greatest concentration of fumonisin, whereas blister-stage kernels supported the least fumonisin. The differences observed between the two stages were not due only to moisture. Dent stage kernels supported the greatest fumonisin concentration when lyophilized kernels at four developmental stages were rehydrated to the same moisture content. From our data, it appears that kernels are conducive for fumonisin production as soon as they are infected with the *F. verticillioides*. The reasons for the late-season increase in fumonisin contamination are unclear. Although an increase in fumonisin was associated with late-season rains in Clayton in 1999, the moisture content of the kernels did not change greatly.

In 2000, we compared three maize hybrids reported to differ in susceptibility to Fusarium ear rot. All three hybrids showed a similar pattern of infection at the two locations (Figs. 3A and 4A). However, the hybrid with the maximum number of infected kernels differed at the two locations. At Clayton, the resistant hybrid had a greater number of infected kernels than the intermediate hybrid from week 8 until the end of the study (Fig. 4). The trend is not as evident at Plymouth, but there were weeks in which the hybrid with greater resistance had a greater number of infected kernels than the more susceptible hybrid (Fig. 3, weeks 6, 8, and 10).

We also compared the rate of kernel infection among the three hybrids. For this comparison, we chose data points between weeks 4 and 11 because the rate of infection appeared to be the greatest for all hybrids during this time. The data from the Plymouth and Clayton locations were combined and a logit transformation performed. We found that the hybrid reported to be more susceptible to Fusarium ear rot had a significantly greater infection rate than either the resistant or intermediate hybrids.

King (8) compared two hybrids in his study and also found differences in the total number of infected kernels and the rate of kernel infection among three hybrids. He was unable to show any significant differences between the two hybrids at any one particular harvest date.

The three hybrids showed a similar pattern for fumonisin accumulation during the first 9 weeks of the study. After 9 weeks, differences in the concentration of fumonisin among the hybrids were observed, and location influenced the ranking of the hybrids. At Plymouth, the susceptible hybrid had more fumonisin contamination than the other hybrids (Figs. 3B and 4B), and at Clayton, the resistant hybrid had the highest fumonisin contamination levels. Log transformation of the fumonisin values did not change the ranking of the hybrids.

We did not observe extensive kernel rot in either year of our study. The percentage of moldy, rotten, or discolored kernels never exceeded 22% and, in most cases, the kernels were visibly sound (data not shown). Despite the relatively low occurrence of rot, the fumonisin concentrations in our study exceeded the recommended guidelines for fumonisin content in human and animal food.

Desjardins et al. (2) reported that maize inoculated with a high fumonisin-producing isolate had a high frequency of kernel infection without visible symptoms of disease. However, although they found that symptomless kernel infection often was very high, fumonisin levels in the symptomless kernels generally were low. We also found a high percentage of symptomless kernels naturally infected with *F. verticillioides*. Analysis of the symptomless and symptomatic kernels together revealed a relatively high concentration of fumonisin. This indicates that, under the conditions of our study, a few symptomatic kernels contained high concentrations of fumonisin or that symptomless kernels have higher levels of fumonisin than found in the study by Desjardins et al. (2).

In addition to *F. verticillioides*, other fungi were isolated from the surface-sterilized kernels. Three fungi, *Aspergillus flavus*, *F. semitectum*, and a species of *Penicillium* were the most common. Of these three, species of *Penicillium* were the most common and *A. flavus* was rarely found. Each of these fungi was found to co-infect kernels with *F. verticillioides*. There may have been other fungi present in the kernels, but the protocol used was optimized for *A. flavus* and *F. verticillioides* and the conditions present may have inhibited growth of other fungi and bacteria present.

Results from this study show that early harvest can be employed as a control strategy for fumonisin contamination. Fumonisin concentrations appear to increase from physiological maturity up to the average harvest date for maize in North Carolina. Beyond this date, the concentrations of fumonisin fluctuated. Under years conducive for fumonisin contamination, early harvest (greater than 25% grain moisture) may help reduce the level of contamination. Certainly, growers should avoid late harvesting of maize. These data also have implications for breeding programs. Because the levels of fumonisin can vary late in the season, a comparison of hybrids at a recommended harvest date may give a more accurate assessment of their susceptibility to fumonisin accumulation. Based on our studies, the ideal time to screen hybrids for kernel infection and fumonisin production appear to be between weeks 7 and 10, because the peak in infected kernels and fumonisin was within this range during both years of our study.

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