Distribution of Deoxynivalenol in *Fusarium graminearum*-Infected Maize Ears

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ABSTRACT

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The distribution of the mycotoxin deoxynivalenol in maize ears infected with *Fusarium graminearum* was determined. In 1990 and 1991, individual ears of five maize inbreds (CO272, F7, F2, A641, and CO266) and one hybrid (CO272 × CO266) were inoculated at approximately 6 days after silk emergence by the injection of a macroconidial suspension into the silk channel. Ears were harvested in mid-October and disease severity was visually evaluated using a rating scale of 1 to 7, in which 1 = no infection and 7 = > 75% of the kernels exhibiting symptoms of infection. Deoxynivalenol concentrations were determined in three ear tissue fractions: symptomless kernels, symptomatic kernels, and the cob. Disease severity ratings and deoxynivalenol concentrations were higher in 1990 than in 1991. In both years and for almost all genotypes, the highest concentration of deoxynivalenol was in the cob, followed by the symptomatic kernels and the symptomless kernels. Disease severity ratings of the ears at harvest were highly correlated to deoxynivalenol concentrations of kernels and whole ears.

Additional keywords: corn, vomitoxin, *Zea mays*.

Fusarium graminearum Schwabe (sexual state: *Gibberella zeae* (Schwein.) Petch) causes ear rot of maize or corn (*Zea mays* L.) in Canada and many other maize-producing areas of the world with cool, wet growing seasons (24). *F. graminearum* inoculum is dispersed via wind, rain, insects, and birds. Spore entry into maize ears can occur through wounds or by growth of mycelium down silks to the kernels and cob from spores germinating on the silks (9,10,24). Mycelial growth on the kernels has a characteristic pinkish color and the cob (rachis) becomes soft and spongy with rot.

Although *F. graminearum* ear rot occurs sporadically, it can represent a serious problem because of mycotoxins which are produced by this pathogen (13). This is of considerable concern for livestock producers (15). In 1990, an epidemic in the northeastern United States resulted in symptoms of toxicity in poultry and cattle, including decreased performance, decreased production, and death in the cattle (4). Swine are the most sensitive to *F. graminearum* mycotoxins. Two major mycotoxins are produced by this pathogen: zearalenone and deoxynivalenol.

Zearalenone is an estrogen-like metabolite which tends to accumulate in corn ears in storage rather than in the field (12,23,25). This mycotoxin causes swine estrogenic syndrome, as well as male infertility, reduced litter size, feed refusal, and haemorrhagia (13). The trichothecene toxin, deoxynivalenol (DON, vomitoxin), is more likely to be produced in the field than in storage (6,26). DON induces emesis in swine which is characterized by vomiting, feed refusal, and decreased weight gain (26).

The distribution of mycotoxins in the ear has important implications during harvesting since the cob, and probably many of the lighter infected kernels, are passed through the combine and not into the feed. Studies on the mycotoxin content of infected ears have usually measured toxin levels of the kernels alone or of the whole ear, sometimes including the husk. Few studies have examined the distribution of toxin in the ear. Visconti et al. (27) looked at maize ears naturally infected with *F. graminearum* and found higher levels of DON and zearalenone in the cobs than in the kernels. In contrast, it has been reported that for another ear-rotting pathogen, *Aspergillus flavus* Link:Fr., higher levels and a greater incidence of aflatoxin occurs in the kernels than the cob (22).

The objective of the present study was to determine, after inoculation of the silk channel of maize ears, the distribution of DON in three ear tissue fractions (symptomless kernels, symptomatic kernels, and the cob).

MATERIALS AND METHODS

In 1990 and 1991, five maize inbreds (CO272, F7, F2, A641, and CO266 ranging from least to most susceptible, respectively [19]) and one hybrid (CO272 × CO266) were planted in a randomized complete block design with four blocks at the Central Experimental Farm, Agriculture and Agri-Food Canada, Ottawa, Ontario. Within each block, each genotype was grown in a three-row plot, the middle row of which was inoculated and the two outer rows were left as borders. An additional plot of the most resistant inbred (CO272) and the most susceptible inbred (CO266) were planted in each block and inoculated with sterile water to serve as natural infection controls. Each row was 3.8-m long (76 cm between rows) with 14 plants, the center 10 of which were inoculated.

F. graminearum inoculum was prepared as previously described (17) using a single isolate, DAOM194276, isolated from naturally infected corn and deposited in the Canadian Collection

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of Fungus Cultures, Agriculture and Agri-Food Canada, Ottawa, Ontario. Individual plants were inoculated by injecting 2 ml of a 5×10^5 macroconidia suspension into the silk channel of the primary ear using a graduated, 10-ml, self-refilling, automatic vaccinator attached to a 2.5-liter backpack container (Nasco Co., Fort Atkinson, WI). Inoculations were made when silks were elongated, pollinated, and had some tip browning (approximately 6 days after silk emergence). To maintain humid conditions after inoculation, plots were irrigated with 2 to 5 mm of water twice daily for 4 weeks.

At normal grain harvest in mid-October (average moisture content of 24%), ears were hand picked, husked, and the severity of ear rot symptoms was evaluated using a seven-class rating scale in which 1 = no infection, 2 = 1 to 3%, 3 = 4 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 = > 75% of the kernels exhibiting visible symptoms of infection such as rot and mycelial growth. Ears were grouped on a per-plot basis (10 ears per plot), bagged in mesh sacks, and air-dried for 2 weeks.

For DON analysis, ears (bulk of 10) were hand shelled, taking care to separate the symptomless kernels (no visible signs of infection) from the symptomatic kernels (visible signs of infection). The resulting three tissue fractions (symptomless kernels, symptomatic kernels, and cobs) were weighed. Each kernel sample was mixed thoroughly to obtain a random distribution of the kernels and a 50-g sample was ground to a fine powder in a Retsch ultra centrifugal mill type ZM1 (Brinkman Instruments, Inc., Rexdale, Ontario) with a 0.75-mm mesh. Cob fractions were ground in a Wiley mill and passed through a 0.75-mm wire mesh. From all samples, a 25-g subsample was sent to Paracel Laboratories, Ottawa, Ontario, for DON analysis by high performance liquid chromatography.

From each kernel sample, 20 kernels were randomly selected and plated for species identification. Kernels were surface sterilized in 10% sodium hypochlorite for 1 min and plated on pentachloronitrobenzene media amended with streptomycin sulfate (1.0 g/liter) (14,16). Plates were incubated at room temperature under natural daylight for 1 week, after which any *Fusarium* colonies present were transferred to a synthetic nutrient agar medium (16) in 6-cm-diameter petri dishes on which a 1-cm^2 triangular piece of sterile filter paper had been placed to enhance sporulation. These plates were incubated for 2 weeks at room temperature under one black tube (Sylvania Black Lite Bleu [F40 BLB]; GTE Corp., Stamford, CT) surrounded by four fluorescent tubes (Sylvania Cool White [F40 CW]; GTE Corp.), with a 12 h of light/12 h of dark photoperiod. Cultures were examined microscopically and identifications were made according to Nelson et al. (16) and with the aid of a computer-based identification key developed by K. Siefert (Centre for Land and Biological Resources Research, Agriculture and Agri-Food Canada, Ottawa, Ontario).

Statistical analyses. For disease severity data, residual error terms were generated and tested for normal distribution using the Kolmogorov *D* statistic (21). Disease severity and DON concentrations were analyzed separately by year. Because disease severity and DON concentrations were very low in water-inoculated treatments, these were omitted from the analysis of variance so that subplot effects and interactions would reflect only the differences among genotypes and tissues, and not include differences between inoculated and control plots. Since the lowest detectable limit of DON was 0.1 μg/g, for the purposes of data analysis, samples with this limit were given a value of 0.1.

Univariate analysis of variance was conducted to assess the significance of block and genotype effects on mean disease ratings and DON concentrations within tissue fractions. Partial correlation coefficients, reflecting correlation among tissues after the removal of block and genotype effects, were calculated from a multivariate analysis for overall genotype and block effects. Repeated measures analysis was used to analyze data on DON concentrations of the three tissue fractions, treating the toxin concentrations of the three tissue fractions as repetitions of the same measure within experimental units. Wilks' Lambda *F* statistic was used to test the significance of differences between tissues, tissue × block, and tissue × genotype interactions. Analysis of variance of contrast variables was used to examine differences between tissues and genotype effects on these differences.

RESULTS

In the macroconidial suspension-inoculated ears, *F. graminearum* was isolated from 5 to 32% of the symptomless kernels, with less in the more resistant genotypes, and 85 to 97% of the symptomatic kernels. *F. moniliforme* J. Sheld. was the fungus most frequently isolated from symptomless kernels which had no apparent *F. graminearum* infection upon plating.

Some visible symptoms of *Fusarium* infection were found in the control plants which had been inoculated only with sterile water, especially in 1990 in the most susceptible inbred CO266 (Table 1). The presence of DON in these samples (Table 1), along with the identification of *F. graminearum*, confirmed natural infection by *F. graminearum*. *F. moniliforme* was also isolated from some samples.

Block effects were not significant for any of the variables. For mean disease severity ratings, differences among genotypes were significant ($P < 0.01$) in both years (Table 1). For all of the genotypes evaluated, ear rot symptoms were more severe in 1990 than in 1991 (Table 1). In both years, symptoms were least severe in the inbred CO272 and most severe in the inbred CO266.

As expected, there was a large difference in tissue weights between genotypes, with the hybrid (CO272 × CO266) consistently having the highest total ear, total kernel, and cob weights (Fig. 1). Genotypes with higher disease severity ratings had larger symptomatic kernel tissue fractions. Visual disease severity ratings were positively correlated with the total weight of symptomatic kernels in both years ($r = 0.872$, $P < 0.05$, 1990; $r = 0.991$, $P < 0.01$, 1991), but no correlations were found to any of the other weights.

The disease rating scale used employed estimates of the percentage of the ear that exhibited disease symptoms. For example, a rating of 3 should correspond to between 4 and 10% of the ker-

TABLE 1. Visual disease severity ratings and deoxynivalenol (DON) concentrations for six maize genotypes inoculated with *Fusarium graminearum*

Year	Genotype	Disease severity[a,b]	DON concentration in tissue fractions (μg/g)[a]		
			Symptomless kernels	Symptomatic kernels	Cob
1990	CO272	1.9 d	41.5 b	769.5 a	823.1 a
	F7	3.5 c	24.9 b	1226.7 a	1,621.7 a
	F2	3.3 c	17.6 b	776.4 a	1,333.7 a
	A641	4.3 b	40.6 b	984.8 a	1,191.3 a
	CO266	5.3 a	134.0 a	1563.6 a	2,271.3 a
	CO272 × CO266	3.4 c	6.5 b	1264.5 a	1,330.1 a
	CO272, water	1.2	0.3	52.5	9.8
	CO266, water	2.1	3.4	489.6	995.0
1991	CO272	1.2 e	0.1 c	0.2 a	0.1 d
	F7	1.4 de	0.6 c	23.7 a	92.4 cd
	F2	2.3 c	1.8 bc	55.3 a	169.1 bc
	A641	3.6 b	5.6 ab	112.0 a	342.5 a
	CO266	4.3 a	8.1 a	113.7 a	266.3 ab
	CO272 × CO266	1.5 d	0.1 c	5.1 a	3.2 cd
	CO272, water	1.0	0.1	...	0.1
	CO266, water	1.7	0.4	0.7	13.0

[a] Means followed by the same letter for a given year and variable are not significantly different at the 0.05 probability level based on the Fisher (protected) LSD test (water controls were not included in analysis).

[b] Disease severity ratings were based on a scale of 1 to 7 in which 1 = no infection and 7 = > 75% of the kernels visibly infected. ... = no tissue.

nels. In this study, ears were rated individually, and mean ratings were obtained for each plot. We have used this approach in numerous experiments (17,18,19,20) and have found it to be effective for differentiating maize genotypes with different levels of resistance. Here, since the kernels from each plot were separated into symptomless and symptomatic fractions, and the dry weight of these fractions was recorded, we had the opportunity to validate our disease rating scale. The proportion by weight of symptomatic kernels from each plot was graphed against the mean disease rating based on visual assessment (Fig. 2). The midpoints of the rating scale categories were also graphed for purposes of comparison. There was a correlation ($r = 0.83$ and 0.79, $P < 0.01$, 1990 and 1991, respectively) between the means of the visual estimates and the actual percentages by weight. Furthermore, the relationship between the two values was consistent across years. At low to moderate disease severity, there was a tendency for the visual assessments to underestimate the percentage by weight of diseased kernels. This was surprising; we expected that any bias would be in the opposite direction, because diseased kernels may weigh less than healthy ones. Overall, the results indicated that the disease rating scale used here was effective for estimating the proportion of diseased kernels.

All six genotypes had some kernels exhibiting disease symptoms (Fig. 1). In those kernels, DON concentrations did not differ significantly among genotypes (Table 1). DON was also detected in symptomless kernels and in cob tissue (Table 1). The DON concentrations in symptomless kernels differed significantly ($P < 0.01$) among genotypes in both years, while those in the cob tissue differed among genotypes only in 1991 (Table 1). The total ear DON concentrations ranged from 35.8 µg/g in CO272 to 1,169.4 µg/g in CO266 in 1990, compared with 0.1 µg/g in CO272 to 100.9 µg/g in A641 in 1991.

Repeated measures analysis of variance indicated significant variation among tissue fractions for DON concentrations in both years, and significant variation among genotype by tissue interactions for DON concentrations in 1991 (Table 2).

Analysis of variance of contrasts indicated that all contrasts among tissue fractions (symptomless versus symptomatic kernels, symptomless kernels versus cob, and symptomatic kernels versus cob) were significant in both years. For DON concentrations, genotype affected two contrasts (symptomless kernels versus cob and symptomatic kernels versus cob) in 1991. This may be explained by two of the genotypes (CO272 and CO272 × CO266)

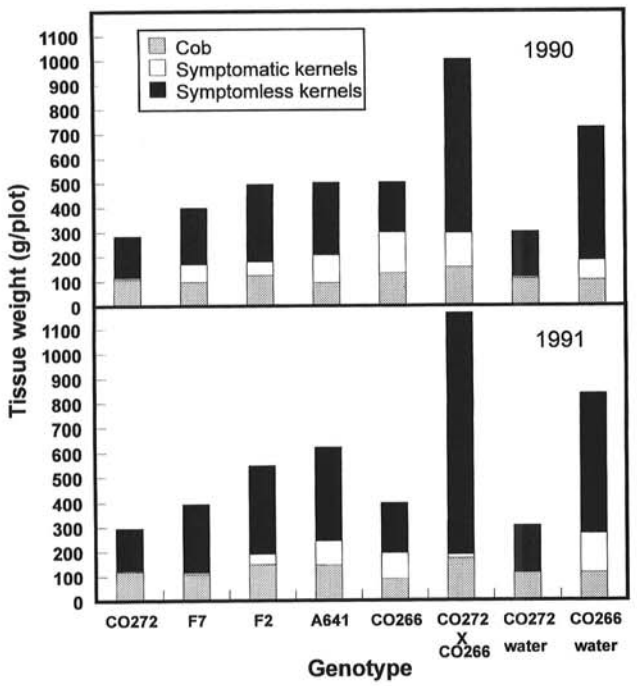

Fig. 1. Weight (total of 10 ears per plot) of three tissue fractions (symptomless kernels, symptomatic kernels, and cob) of six maize genotypes inoculated with *Fusarium graminearum* in 1990 and 1991.

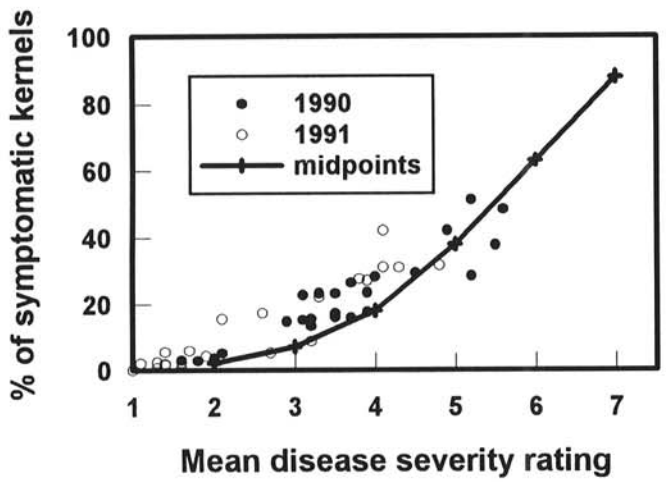

Fig. 2. Percentage (by weight) of symptomatic kernels versus mean disease severity rating compared with midpoints of disease rating categories for *Fusarium graminearum*-inoculated maize ears.

TABLE 2. Significance probabilities (Wilks' Lambda F statistic) for sources of variation in repeated measures analysis for deoxynivalenol (DON) concentration in each of three tissue fractions of maize ears inoculated with *Fusarium graminearum* in 1990 and 1991

		Year[a]	
Source	df	1990	1991
Tissue	2	86.8**	22.2**
Block × tissue	6	1.8	0.9
Genotype × tissue	10	2.1	2.5*

[a] * = significant at the 0.05 probability level. ** = significant at the 0.01 probability level.

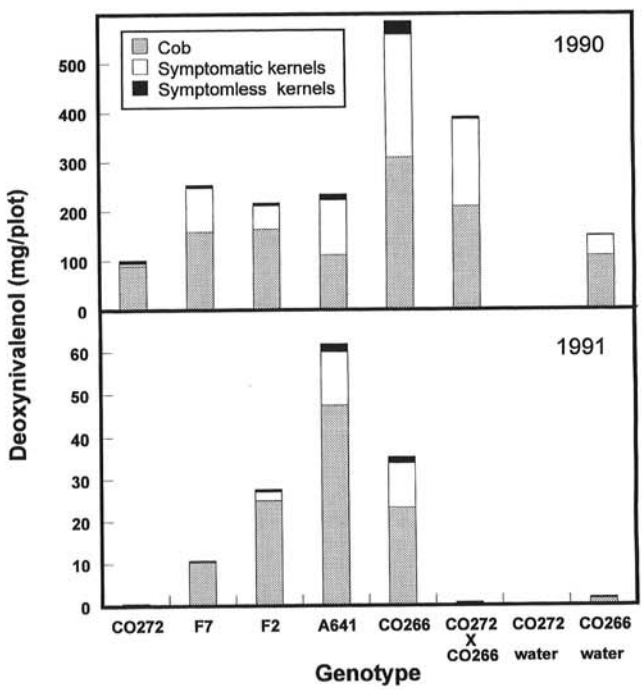

Fig. 3. Deoxynivalenol content (total of 10 ears per plot) in three tissue fractions (symptomless kernels, symptomatic kernels, and cob) of six maize genotypes inoculated with *Fusarium graminearum* in 1990 and 1991. N. B. 1990 y-axis scale is smaller than that of 1991.

which had little DON accumulation in the symptomless kernels and cob tissue.

Overall, DON concentrations were lowest in the symptomless kernels, intermediate in the symptomatic kernels, and highest in the cob tissue. To account for the large difference in tissue weights between cob and kernel tissue, DON contents (in milligrams) were calculated from the data on tissue weight and DON concentration (Fig. 3). In 1990, 47.7% (A641) to 88.3% (CO272) of the total ear DON was found in the cob; in 1991, 32.9% (CO272) to 96.0% (F7) of the total ear DON was found in the cob. In 1991, DON content of CO272 and CO272 × CO266 was very low, i.e., less than 0.8 mg total.

For DON concentrations, partial correlation coefficients among kernel fractions, corrected for genotype and block effects, were always positive. They were significant ($P < 0.01$) for symptomatic kernels versus cob tissue in 1990 ($r = 0.82$) and among all three tissue fractions in 1991 ($r = 0.77$ for symptomatic kernels versus cob tissue, $r = 0.68$ for symptomatic kernels versus symptomless kernels, and $r = 0.72$ for symptomless kernels versus cob tissue).

In both years, disease severity ratings were positively correlated with DON concentrations of the kernels (symptomless plus symptomatic) ($r = 0.89$ and 0.98, $P < 0.01$, for 1990 and 1991, respectively) and of the total ear (kernels plus cob) ($r = 0.81$ and 0.95, $P < 0.01$, for 1990 and 1991, respectively).

DISCUSSION

Significant differences were found in the distribution of DON in maize ears inoculated with a macroconidial suspension of *F. graminearum* injected into the silk channel. As expected, the lowest amount of DON was found in the symptomless kernels. Generally, DON concentrations were higher in the cob tissue than in either kernel fraction. In 1991, the resistant inbred CO272 and its hybrid with CO266 had very low DON concentrations in all three tissue fractions, and DON concentrations in the symptomatic kernels exceeded those in the cob. In these cases, disease severity was so low that neither the fungus nor the toxin could succeed in permeating much of the cob tissue. The general tendency seen here for DON to accumulate in the cob was in agreement with the findings of Visconti et al. (27). In contrast, Bennett et al. (3) conducted a similar study with one hybrid grown in a controlled environment chamber (Biotron) and toothpick inoculated, in which they reported less DON in the cob (48.4 to 149.6 μg/g) than in the severely rotted kernels (62.0 to 162.3 μg/g). With another major host of *F. graminearum*, wheat, Miller et al. (12) reported significantly higher levels of DON in the chaff than the kernels.

The significant difference in the percentage of DON in the cob in 1990 and 1991 may reflect how the fungus enters the ear. In 1990, disease severity ratings and DON concentrations were higher than in 1991, and greater proportions of DON were found in the kernels. Temperatures during the months of July and August (the period of inoculation, infection, and grain filling) were similar in the 2 years, ranging from a maximum and minimum of 14.8 to 27.1°C in 1990 and 16.5 to 27.9°C in 1991. However, precipitation levels differed, with 252 mm in 1990 compared with 172.8 mm in 1991. It may be that under dry conditions fungal growth is not optimum, and the fungus may preferentially infect the cob rather than the kernels, possibly because moisture deficits delay or inhibit kernel development. In years more favorable to plant and fungal growth such as 1990, the pathogen may more frequently infect the kernels. This could be because of the availability of kernel tissue at the time of infection or the preferred movement of infection from the cob to the kernels.

The distribution of toxin in the cob and kernels could also be because of translocation. Young and Miller (30) reported that both the fungal metabolite ergosterol and DON were translocated from the ear to the husk, axial stem, and stalk after ears were inoculated with colonized toothpicks. Thus, increased levels of DON in the cob could, in part, be because of translocation from infected kernels. If the cob was invaded first, DON could be translocated into the kernels with grain filling. This may explain the presence of DON in symptomless kernels which were relatively free of infection upon plating. The amount of toxin translocation would depend upon the stage of grain filling at which infection took place and ultimately on the susceptibility of the genotype and the amount of infection. In more favorable years there would be active grain filling and increased translocation.

DON levels in the symptomatic kernels were positively correlated with that in the cob for both years. Shotwell et al. (22) also reported a positive correlation between aflatoxin in the kernels and in the cob ($r = 0.64$, $P < 0.05$).

In this study, visual evaluations of disease severity were highly correlated to DON concentrations in the ear. The correlations found in this research were consistent for those reported by other researchers using wound inoculation techniques (1,5,7,8).

In North American corn production, the average shelling percentage is approximately 18 kg of cobs for every 100 kg of grain (29). During grain harvest, the cob tissue passes through the combine and back into the field. According to the results presented here, much of the toxin in infected ears was found in cob tissue. During regular grain harvest, that fraction of the toxin is returned to the soil and does not present a risk to feed safety. However, a high level of DON in the cob may be of some concern when the cob itself is ground into a corncob mixture (CCM) or chopped and ensiled (mostly European corncob meal) to provide fiber and roughage for ruminant animal feed. Pith fractions are exceptional carriers of micronutrients in animal feed, and pelleted chaff and pith fractions are used as absorbents for molasses in beef and dairy rations (2). Corncobs also have some industrial uses (e.g., as fillers, carriers, and scrubbing agents), but for these, DON contamination would not likely be a serious problem.

Along with the cob tissue, small and light kernels are blown through the combine and returned to the soil. Since kernel-rotting pathogens consume nutrients from the kernels they infect, and since mycotoxins may have phytotoxic effects (28) that could lower grain filling, infected kernels may be smaller and lighter than average (11). Thus, the toxin concentration of mechanically harvested grain may be lower than would be estimated from analysis of all the kernels from hand-harvested ears.

Even though much of the DON may be returned to the soil during grain harvest, some will remain in the grain, where it can pose a threat to feed safety. In this study, we sometimes found high concentrations of DON even in symptomless kernels. Furthermore, we isolated *F. graminearum* from some symptomless kernels. Thus, even apparently clean grain may carry the pathogen with it into storage, so it is important that proper storage conditions be maintained to avoid further fungal growth, and the accumulation of additional toxin, particularly zearalenone, which is produced after harvest.

In conclusion, of the three tissue fractions examined (symptomless kernels, symptomatic kernels, and cobs) after silk channel inoculation of maize ears with *F. graminearum*, the highest levels of the mycotoxin deoxynivalenol were found in the cob fraction. In addition, levels of toxin were correlated to visual evaluations of disease severity.

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