Effect of Kernel Development and Wet Periods on Production of Deoxynivalenol in Wheat Infected With *Gibberella zeae*

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ABSTRACT

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Increasing the time wheat heads were enclosed in plastic bags after inoculation with *Gibberella zeae* increased the severity of infection and the level of deoxynivalenol in grain harvested immediately after removal of the plastic and in fully ripe grain. Deoxynivalenol was detected in ripe field-inoculated grain when heads were covered with plastic bags for 3 and 6 hr immediately after inoculation, even though there were no symptoms of infection. Inoculating heads after the kernels were filled resulted in less yield loss, but the production of deoxynivalenol was dependent on the hours of head wetness and not the stage of kernel development. When wheat was inoculated with *G. zeae* before kernel fill, grain weight was lower compared to wheat inoculated after kernel fill.

Deoxynivalenol is a cytotoxic trichothecene produced by *Fusarium graminearum* Schw., the conidial stage of *Gibberella zeae* (Schw.)Petch. Deoxynivalenol has been reported in mixed feeds (11), corn (8,11,17), barley (12,15,18), wheat (7,12,15), and milled fractions of wheat (7). Although less toxic than trichothecenes such as T-2 and diacetoxyscirpenol, it is known to cause feed refusal and vomiting in swine (5,17). Deoxynivalenol in wheat and other grains is not regulated in the U.S., although the Food and Drug Administration (FDA) has issued a "level of concern" of 2 μg of deoxynivalenol per gram of whole grain, and 1 μg of deoxynivalenol per gram of finished product. Deoxynivalenol is regulated in Canada at these levels.

Head scab of wheat, caused by *G. zeae*, was a major disease problem in 1980 (15) and 1982 (6), and deoxynivalenol was often found at high levels in scabbed wheat (6,15). Head scab occurs in years when there is an abundance of moisture (1,2,3,9,10,13,14). Early studies indicated that infections occurred during or soon after flowering (1,14). Later infections caused lower yield loss (14). Kernel development, temperature, and moisture have been studied in relation to infection and disease development (1,14), but little is known about their effect on the production of deoxynivalenol.

This paper reports on the effect of time of inoculation in relation to kernel development and extended periods of wetness on the production of deoxynivalenol in wheat kernels inoculated with G. zeae.

MATERIALS AND METHODS

Inoculum preparation. *G. zeae* isolate W-8 (Pennsylvania State University No. U-5373) was stored in sterilized soil and recovered on potato-dextrose broth (PDB) as needed. Inoculum was prepared by growing W-8 in 200 ml of PDB in 1-L Roux bottles for 1–2 wk. Cultures were filtered through two layers of cheesecloth, washed with 600 ml of H_2O, and the mycelia were comminuted with 200 ml of H_2O in a Waring blender for 1 min and transferred to plastic spray bottles. Wheat heads were sprayed with inoculum prepared in this way. The inoculum contained approximately 10^6 colony forming units per milliliter.

Wheat plants. Greenhouse-grown wheat (*Triticum aestivum* L. 'Genesee') was planted in 15-cm-diameter fiber containers in a steamed sand:peat:topsoil greenhouse mix (1:1:2, v/v). After emergence, plants were vernalized at 2 C under low intensity lights for 8 wk, then grown on greenhouse benches under a 14-hr-day photoperiod (6.6×10^4 lx at 15 cm below the light source). All of the heads in a container (eight to 12 heads per container) were sprayed with 5–10 ml of inoculum suspension and each container served as one replicate. Heads were examined prior to inoculation to determine the stage of kernel development.

In the field, seven commercial red and white soft winter wheat cultivars (Abe, Arthur, Augusta, Frankenmuth, Ionia, Tecumseh, and Yorkstar) were planted in the fall of 1981, and again in the fall of 1982, in seven-row plots (18 cm between rows). Each plot was 30 m long and was replicated three times. Plots were fertilized and limed according to soil test recommendations.

Greenhouse experiments. Deoxynivalenol production in the grain was compared in plants inoculated at various stages of kernel development, and in grain from heads covered with plastic bags for various times after inoculation. The stages of kernel development were: <1/4 filled, 1/4–1/2 filled, 1/2–3/4 filled, and fully filled (grain contents watery). After inoculation, heads were covered with plastic bags for 6 days. All grain was harvested when the kernels were ripe (45 days after inoculation).

In a second experiment, heads with full-size kernels in the watery, milk, and early dough stages of development were inoculated and covered immediately with plastic bags for 24, 48, 72, and 96 hr. Grain was harvested 45, 37, and 23 days after inoculation. In both experiments one and two, controls were sprayed with H_2O and covered for 6 days. Greenhouse temperatures were recorded with a recording hygrothermograph and ranged from 15–21 C between night and day.

The effect of infection on the grain was determined by weighing 100 kernels from each treatment after oven drying at 105 C for 48 hr. Ten to fifteen kernels from each replication in the second experiment were plated onto carnation leaf water agar (4) and rated for percent germination and the percentage of kernels from which *G. zeae* grew. Each treatment in experiments one and two was replicated three times, but analysis of deoxynivalenol within each treatment was made from a single subsample of the pooled replications (0.6–14 g per treatment).

Field experiments. *Experiment 1 (1982).* Fifteen to twenty heads in each replicate plot of the seven cultivars were inoculated as described previously, and the heads were covered with plastic bags for 72 hr. A different set of heads in each plot was inoculated every seventh day, beginning at day one, over a 3-wk period. The first inoculation was at the early milk stage of grain development, and the last of the mid-dough stage. Grain was harvested at 13% moisture content.

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Experiment 2 (1983). Randomly selected heads from each cultivar were inoculated at the early dough stage, and covered immediately with plastic bags for 3, 6, 24, 48, 72, or 96 hr. Each treatment was replicated four times, and each replication consisted of 15–20 heads. Controls consisted of uninoculated heads enclosed in plastic bags for the same time as inoculated heads.

All of the replications from each treatment in both years' field experiments were analyzed separately for deoxynivalenol. The dry weight of 100 kernels was determined for grain harvested in 1983. Rainfall data were collected daily throughout the experiments in 1982 and 1983.

Deoxynivalenol analysis. Identification and quantitation were made by comparing samples with standards of deoxynivalenol of known concentration on TLC plates. The procedure for analysis of deoxynivalenol has been described previously (7) and was used to analyze the ripe grain from wheat cultivars inoculated over a 3-wk period in field experiments in 1982. This procedure was modified for all the other experiments as follows: the liquid and solid fractions were separated by centrifugation for 5 min at 1,200 *g* rather than by filtration through filter paper; TLC plates were dipped in 15% $AlCl_3$ (15 g $AlCl_3 \cdot 6H_2O$ in 100 ml of methanol:water, 85:15, v/v) before spotting and development (16); and after spotting and development, TLC plates were heated (120 C for 6 min) and viewed under 365-nm ultraviolet light (16). An intense blue fluorescence indicated the presence of deoxynivalenol.

RESULTS

Greenhouse experiments. In the first experiment, 100-kernel weights were less when inoculations were made before the kernels were fully filled (Table 1). In the second experiment, the dry weight of 100 kernels from heads inoculated at the watery stage of grain development was 1.9 g and was significantly less ($P = 0.05$) than the weight of 100 kernels of ripe grain from heads inoculated at the milk (3.2 g) and early dough stages (3.5 g). Percentage germination of wheat kernels, incidence of *G. zeae*, and levels of deoxynivalenol in the grain were not significantly different among the watery, milk, and early dough stages of kernel development in the second experiment. Therefore, subsequent analyses were performed on the pooled data (Table 2). The regression for the length of time heads were covered with plastic bags after inoculation and on weight was not significant (Table 2). However, the regressions for the time heads were covered with plastic bags on either the percentage of kernels germinated, or the incidence of *G. zeae* were significant (Table 2). Water had accumulated inside the plastic bags within 2 hr after inoculation.

TABLE 1. Kernel weight and production of deoxynivalenol (DON) in winter wheat inoculated with *Gibberella zeae* at different stages of kernel maturity

Treatment[a]	Dry wt (g) (100 kernels)	DON (μg/g)[b]	15-acetyl-DON (μg/g)
Control—H_2O	3.12	0	0
<1/4	0.61	218	18
1/4–1/2	0.66	348	23
1/2–3/4	0.83	271	11
Filled	1.58	160	0

[a] Stage of kernel development at the time of inoculation. Treatments indicate the size of the immature berry. All inoculated and control heads were covered with plastic bags for 6 days after inoculation. The filled kernels were at the early water stage of development.
[b] DON (μg) per gram dry weight of kernels.

Increasing the number of hours heads were covered with plastic bags after inoculation resulted in significantly increasing levels of deoxynivalenol in the grain (Table 2). The levels of deoxynivalenol in grain inoculated before (experiment 1) or after kernel fill (experiment 2) could not be compared because the heads were covered with plastic bags for different times, but the concentration of deoxynivalenol in grain inoculated before kernel fill (Table 1) was about twice the value predicted by the regression equation (Table 2). A metabolite of deoxynivalenol reported previously only in culture (17) was identified as 15-acetyl-deoxynivalenol by C. Mirocha (*personal communication*). Levels of 15-acetyl-deoxynivalenol were variable, but were detected consistently only in grain from heads covered with plastic bags for 72 hr or longer (Table 2). 15-acetyl deoxynivalenol was found in only a single replication among all replications from treatments of <72 hr. Concentrations of 15-acetyl-deoxynivalenol were determined by comparing the intensity of the fluorescent spots with deoxynivalenol standards, since a standard of 15-acetyl-deoxynivalenol was not available. Deoxynivalenol was also found in one control sample that had been covered with plastic for 6 days.

In a similar experiment to determine if deoxynivalenol was in the grain immediately after the incubation period, heads with kernels at the watery stage of development were inoculated as described, and then covered with plastic bags for 24, 48, 72, or 96 hr. The heads were harvested immediately after removal of the plastic bags, dried at 60 C, and the kernels were separated and weighed. Each treatment was replicated three times, and each replication was analyzed separately for deoxynivalenol. The experiment was repeated.

Deoxynivalenol content of the grain immediately after removal of the plastic bags, μg/g of grain, in the control, and the 24-, 48-, 72-, and 96-hr moist period treatments, and the percent of deoxynivalenol compared to grain collected after maturity was 0, 0, 1.2 (9.4%), 21.3 (51.1%), and 59.0 (76.3%), respectively, averaged over all six replications in both experiments. Although the results

TABLE 2. Kernel weight, production of deoxynivalenol (DON), germination percentage, and the incidence of *Gibberella zeae* in wheat heads inoculated in the full-kernel stages of development and kept wet for 24–96 hr

Treatment[a]	Dry wt (g) (100 kernels)	DON (μg/g)[b]	15-acetyl-DON (μg/g)	Germinated kernels (%)	Isolation of *G. zeae* from kernels (%)
Control	3.5	0.6	0	94.3	11.0
24	3.4	0.3	0	68.0	32.0
48	2.9	12.7	3.3	38.3	55.7
72	2.3	41.7	8.0	38.0	55.7
96	2.3	77.3	15.6	18.0	82.7
Regression analysis[c]					
$F =$	3.7 NS	12.76**		15.86**	15.98**
$r^2 =$	0.29	0.59		0.64	0.64
		$y = -34.6 + 1.1x$		$y = 71.7 - 0.55x$	$y = 19.2 + 0.62x$
df =	1,9	1,9		1,9	1,9
x = wet period (hr)					
y = dependent variable					

[a] Length of time (hr) inoculated plants were covered with plastic bags after inoculation.
[b] DON (μg) per gram weight of kernels.
[c] Regressions do not include the controls. NS = not significant; * $P = 0.05$, ** $P = 0.01$. Values are averages from three replications except for 24 hr which had two replications. Analyses are of pooled data from inoculations of heads at the water, milk, and early dough stages of development.

TABLE 3. Deoxynivalenol (DON) content of seven wheat cultivars inoculated in the field at different stages of maturity with *Gibberella zeae*

Developmental stage of grain at inoculation[b]	DON (μg/g) of grain[a]							
	Abe	Arthur	Augusta	Frankenmuth	Ionia	Tecumseh	Yorkstar	Average
Early milk	14.0	4.6	9.4	19.6	1.9	2.4	12.4	9.2
Milk	4.7	4.5	7.7	2.9	9.4	9.5	9.1	6.8
Early dough	1.0	2.1	3.6	6.4	7.0	3.1	8.9	4.6
Mid-dough	0	5.6	0	4.8	0	3.3	0	2.0
Average	4.9	4.2	5.2	8.4	4.6	4.6	7.6	

Regression analysis (days from inoculation to harvest by levels of DON)

$F = 13.16$
$r^2 = 0.34$
$y = -8.2 + 0.3x$
df = 1,26
y = deoxynivalenol
x = days to harvest after inoculation

Analysis of variance

Source of variation	df	SS	MS	F
Cultivars	6	67.19	11.20	0.61
Developmental stage	3	199.46	66.49	3.65*
Error	18	327.80	18.21	
Total	27	594.45	220.17	

[a] Controls were of uninoculated heads; no DON was detected. Values are averages of four replications.
[b] Heads were covered for 72 hr with plastic bags immediately after inoculation.

for each replication were similar within a treatment, one replication in each of the 72- and 96-hr treatments weighed less than 1 g, and both contained about four times more deoxynivalenol than each of the other replications within the respective treatment. The r^2 for deoxynivalenol concentration and hours of head wetness was 0.35 for all replications, and 0.73 without the two high samples. The deoxynivalenol content and the percent of deoxynivalenol as described above were 0, 0, 1.2 (9.4%), 13.6 (32.8%), and 37 (47.9%) when the two high replications were omitted. Regressions were significant with or without these two replications ($P = 0.01$).

Field experiments. *1982.* Concentrations of deoxynivalenol decreased significantly ($P = 0.01$), when averaged over all cultivars, the later heads were inoculated (Table 3). The correlation coefficient between days from inoculation to harvest and deoxynivalenol levels was positive but low ($r = 0.58$). Overall, there was little difference in levels of deoxynivalenol among cultivars, and these differences were not significant. However, four cultivars (Abe, Augusta, Ionia, and Yorkstar) did not have detectable levels of deoxynivalenol when the heads were inoculated at the mid-dough stage. Rainfall after the time heads were inoculated until harvest may have influenced deoxynivalenol formation, since heads inoculated later received less rain; 19.0, 18.4, 14.9, and 14.1 cm for heads inoculated at the early milk, milk, early dough, and mid-dough stages, respectively.

1983. The dry weight of 100 kernels from inoculated heads decreased ($P = 0.01$) as time of coverage with plastic bags increased from 3 to 96 hr. Kernels from covered controls were not affected (Table 4). Deoxynivalenol concentrations increased in heads covered with plastic bags from 3 to 72 hr after inoculation; and 15-acetyl-deoxynivalenol was not identified in any of the field samples. All replications and all treatments of grain from inoculated heads contained deoxynivalenol, but no grain from control heads had deoxynivalenol. There was 6.7 cm of recorded rainfall between the time of inoculation and harvest. In the 2 days immediately before inoculation there was 7.2 cm of rain, and 0.3 cm fell between 36 and 72 hr after inoculation.

TABLE 4. Concentrations of deoxynivalenol (DON) in field harvested ripe grain after enclosing wheat heads inoculated with *Gibberella zeae* (isolate W-8) in plastic bags for various times after inoculation

Treatments times (hr)	DON (μg/g)[a]		Dry wt/100 kernels	
	W-8[b]	Control[c]	W-8[b]	Control[c]
3	0.6	0	3.40	3.25
6	0.8	0	3.23	3.40
24	4.7	0	2.93	3.65
48	4.7	0	2.75	3.70
72	11.0	0	2.20	2.55
96	6.7	0	2.53	3.35
Regression analysis[d]				
$F = 66.9$		$F = 17.7$		NS
$r^2 = 0.74$		$r^2 = 0.45$		
$y = 0 + 0.97x$		$y = 3.28 - .0758x$		
df = 1,22		df = 1,22		

[a] DON (μg/g) per gram dry weight of kernels.
[b] Average of four replications.
[c] Average of two replications.
[d] x = wet periods (hr). y = deoxynivalenol (g/g wheat), or dry wt.

DISCUSSION

The production of deoxynivalenol in wheat inoculated in the greenhouse depended on the duration of head wetness, and occurred independently of the stage of kernel development after the kernels were filled. As has been reported (1,14), yield reductions were greatest when infections occurred prior to the development of fully filled kernels. The production of deoxynivalenol in field experiments (1983) when the heads were kept wet for only 3 and 6 hr may reflect additional periods when the heads were wet due to rainfall, since deoxynivalenol was identified in greenhouse-ripened grain in only one sample when the heads were kept wet for only 24 hr and not in any samples harvested immediately after a 24-hr incubation time. Lower levels of deoxynivalenol in grain collected from heads with kernels inoculated at later developmental stages in the 1982 field experiment may also indicate fewer hours of wetness due to less rainfall.

Deoxynivalenol formation may occur only after the kernels are invaded, and not when mycelia grow over the surface of the kernels prior to infection. The effect of the plastic bags on temperature was not studied, but it is highly probable that the temperature inside the bags was much higher than ambient air temperature, especially in the field experiments. Temperatures above 25 C were less favorable for infection (1) making it unlikely that higher temperatures favored infection in these experiments. However, they may have reduced infection, which could explain differences in the deoxynivalenol content of grain between the greenhouse and the field experiments. Higher temperatures inside the bags did not appear to damage the plant tissue since the control heads developed normally. Even though deoxynivalenol has been identified in all parts of the kernel (7), additional research on the early production of deoxynivalenol in plant tissues is needed.

The 15-acetyl analog of deoxynivalenol was identified only in greenhouse-inoculated grain and only after high levels of deoxynivalenol were produced. This may reflect a different physiology between wheat plants grown in the greenhouse and the

field. However, studies in liquid culture with *G. zeae* indicate that the major trichothecene produced may change over time, depending on the particular isolate, and on the substrate (A. El-Bahrawy, L. P. Hart and J. J. Pestka, *unpublished*).

The very high levels of deoxynivalenol in one sample of grain from each of the 72- and 96-hr moist period treatments in the greenhouse experiments when grain was harvested immediately after the treatment may have resulted from severe infection since the weight of the grain was low. However, the relative effect of fungal mass, extent of kernel invasion, rate of growth, and environment have not been adequately investigated.

It is clear that deoxynivalenol can be produced in wheat when there is adequate moisture for fungal growth, even at later stages of kernel development. This suggests that while various measures, such as fungicide sprays, applied during flowering may reduce yield losses due to head scab, additional attention to wet periods after flowering and grain fill need to be considered to prevent later infections and subsequent contamination with deoxynivalenol.

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