Kernel Concentrations of 4-Acetylbenzoxazolin-2-one and Diferuloylputrescine in Maize Genotypes and Gibberella Ear Rot

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Kernel concentrations of 4-acetylbenzoxazolin-2-one (4-ABOA) and diferuloylputrescine were measured in a set of *Zea mays* genotypes of differing susceptibility to gibberella ear rot as well as populations from crosses of the least susceptible with the most susceptible. 4-ABOA is a potent inhibiter of the biosynthesis of the phytotoxic mycotoxin deoxynivalenol. Diferuloylputrescine, a chemical associated with insect resistance, varied from 8 to 560 μg/g of dry weight (dw) in the kernels of the segregating genotypes. Concentrations of 4-ABOA varied from 19 to 100 μg/g of dw, and the ratio of F4 lines with high, similar, and low kernel concentrations was 2:10:1. In tests of small groups of genotypes with moderate ear rot tolerance and high susceptibility, mean 4-ABOA concentrations were significantly higher in the former group. This suggests that the presence of adequate kernel concentrations of 4-ABOA conjugate may play a role in resistance to gibberella ear rot.

Keywords: *Gibberella zea; corn; 4-acetylbenzoxazolin-2-one; diferuloylputrescine; deoxynivalenol*

INTRODUCTION

Gibberella zea (Scw.) Petch (asexual stage *Fusarium graminearum* Schwabe) and related species cause gibberella ear rot of corn. This reduces yield, and the infected crop contains deoxynivalenol (DON) and zearalenone, which have major effects on feed consumption and fertility, especially in swine (Prelusky et al., 1994). Although there is evidence for resistance (or reduced susceptibility) in certain genotypes to gibberella ear rot infections from spores growing down the silk channel (Reid et al., 1992), the major difference in the performance of different hybrids lies in their resistance to the spread of the fungus in the cobs and the kernels (Miller, 1994). Analysis of natural and experimental infections demonstrates that *F. graminearum* can be present on the kernels of tolerant hybrids without further growth (Schaafsma et al., 1993).

From data collected during two epidemics of gibberella ear rot in Ontario, as well as extensive experimental inoculation trials, two hybrids, Funks G-4106 and Funks G-4010 (Ciba Seeds Ltd.), were found to have a low incidence of gibberella ear rot (Miller, 1994). Although there are many differences between resistant and susceptible hybrids, one important difference is the factors that affect the phytotoxicity of the principal toxin of *F. graminearum*, DON.

Several studies have shown that DON is a virulence factor in the pathology of *F. graminearum* diseases of wheat and corn (Cossette and Miller, 1995). Deletion mutants of this species lacking the ability to produce DON were shown to be considerably less aggressive in infecting wheat than the wild-type strains (Desjardins et al., 1996; Proctor et al., 1995). DON damages membranes and inhibits protein synthesis needed for the production of new proteins required for resistance (Miller, 1989; Snijders and Kretching, 1992).

Wheat and corn genotypes that are resistant to *F. graminearum* have been shown to possess a number of properties that limit the damage caused by DON. These include DON degradation (Miller and Arnison, 1986; Sewald et al., 1992), a DON-tolerant peptidyl transferase (Miller, 1989; Miller and Ewen, 1997), and increased resistance to the membrane-damaging effects of DON (Cossette and Miller, 1995; Snijders and Kretching, 1992). Kernels from some corn genotypes contain a glycosylated derivative which when hydrolyzed and following rearrangement produces 4-ABOA (Fielder et al., 1994). This chemical inhibits the production of 3-acetyl-DON (3-ADON) by *Fusarium culmorum* without a concomitant reduction of fungal growth. A 50% inhibition of toxin production occurred at ~4 μM 4-ABOA (Miller et al., 1996). All of these properties appear to contribute to the resistance or tolerance of corn genotypes to gibberella ear rot.

Many studies have suggested that phenolics contribute resistance to fungal diseases and insects (Niemeyer 1988), including gibberella ear rot. Assabgui et al. (1993) reported that increased (*E*)-ferulic acid is related to increased tolerance to this disease. The phenolic diferuloylputrescine (DFP) was isolated from extracts of Funks G-4106 and Funks G-4010. DFP did not inhibit 3-ADON biosynthesis (Miller et al., 1996). DFP was found to be present in high concentrations in corn kernel pericarp tissue where it may play a role in resistance to fungal pathogens and insect pests (Sen et al., 1994).

The purpose of this paper is to outline data on the variation of 4-ABOA and DFP in crosses of ear rot resistant and susceptible genotypes and report data on the relationship of these chemicals to ear rot resistance.

MATERIALS AND METHODS

The two compounds 4-ABOA and DFP were isolated from Funks G-4106 and G-4010, hybrids of inbreds A, B, and C. The parental inbreds of these hybrids were crossed with gibberella ear rot susceptible genotypes and 62 F-4 lines resulting from self-pollination, randomly selected from three

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S0021-8561(97)00395-6 CCC: $14.00 Published 1997 by the American Chemical Society

such populations for analysis of kernel 4-ABOA and DFP. The kernels from the three populations were obtained from material grown at the same site.

A second experiment was conducted on the relationship between gibberella ear rot resistance and 4-ABOA/DFP concentrations. Trials were planted at Bloomington, IL, in 1990, 1991, and 1992 and at Ithica, MI, in 1991 and 1992. Ears were wound inoculated 14 days past silking by drilling a hole in the center of each ear and inserting a pipe cleaner saturated with inoculum. Inoculum was made by grinding a 14 day old plate of *F. graminearum* in 500 mL of sterile distilled water. At harvest, ratings were made on a 1−9 scale, with a rating of 9 indicating infection of the entire ear. Data were combined to provide a mean from the five environments. The genotypes involved were analyzed for 4-ABOA and DFP.

The corn samples were extracted following a cleanup method generally similar to that of Collins (1989). An air-dry ground corn sample (1 g) obtained from several ears was extracted with 80% methanol [5-fold excess (v/w)] and shaken for 1 h. It was then centrifuged for 10 min at 3000*g*, the supernatant was drawn off, and the sample was re-extracted. The supernatants were combined and dried under vacuum. The resulting extract was taken up in 50% 2-propanol/H_2O (2 mL) and subjected to the following cleanup procedure. The extract was put through octyl-Sepharose (OS) 50% 2-propanol/H_2O (20 mL) to remove most neutral lipids. The column was then washed with 3 bed volumes ($k' < 3$) of 50% 2-propanol/H_2O. Neutral lipids were then removed from the column using 3 bed volumes ($k' > 3$) of 2-propanol and discarded. Strongly acidic constituents were removed by putting the resulting OS ($k' < 3$) eluent through an ion-exchange gel Sephadex QAE A-25. The gel was first converted to the acetate form, the extract was loaded into the column, and the cationic, neutral, and weakly acidic components were removed from the column with 3 bed volumes ($k' < 3$) of 50% 2-propanol/H_2O. The strongly anionic constituents were removed by washing the column with 3 bed volumes ($k' > 3$) of 2-propanol/H_2O/formic acid (5:4:1) and discarded. The gel was then converted to the hydroxide form, and the QAE $^-$OAc ($k' < 3$) fraction was loaded onto the QAE $^-$OH column. The column was then washed with 3 bed volumes ($k' < 3$) of 50% 2-propanol/H_2O to remove any neutral components. The weakly acidic components containing DFP and 4-ABOA were removed from the column using 3 bed volumes ($k' > 3$) of 2-propanol/H_2O/formic acid (50:45:5). The QAE $^-$OH ($k' \geq 3$) fraction was dried under vacuum, taken up in 2-propanol/H_2O (1:1; 1.5 mL), and set aside for HPLC analysis. Due to the ability of DFP to readily isomerize, all extracts were stored at −10 °C in the dark until analyzed.

Quantitative analysis of 4-ABOA and DFP was carried out on a Varian Vista 5000 HPLC system equipped with a UV200 detector operating at 327 nm. Chromatographic data were collected by a Varian DS402 data system. The standard and samples were injected on a CSC Nucleosil 100 ODS2 5 μm (0.3 × 25 cm) analytical column using the following solvent program: 0−15 min, 20% MeOH/H_2O isocratic; 15−50 min, 20−80% MeOH/H_2O; 50−52 min, 80−100% MeOH/H_2O; 52−62 min, 100% MeOH; 62−72 min, 100−20% MeOH/H_2O; flow rate of 1 mL/min. 4-ABOA and DFP were synthesized as reported (Fielder and Collins, 1995; Miller et al., 1996). Retention times for 4-ABOA and DFP were 20 and 32 min, respectively. Analysis was for the *EE* isomer of DFP.

RESULTS AND DISCUSSION

In a previous paper, 4-ABOA isolated from two gibberella ear rot tolerant genotypes was found to potently inhibit the biosynthesis of 3-ADON, the precursor of deoxynivalenol (Miller et al., 1996). Data on the occurrence of this chemical in segregating material from crosses of the three Funks inbreds concerned (inbreds A−C) with two Funks inbreds that are susceptible to gibberella ear rot are presented in Table 1. A smaller data set from a cross of Funks inbred A by Pioneer 3953 (highly susceptible to ear rot) is also

Table 1. Kernel 4-ABOA and DFP for F4 Lines from Three Crosses

		kernel concn (μg/g)	
project	line	4-ABOA	DFP
A × E	1	69.0	22.6
	2	30.6	122.8
	3	60.9	16.7
	4	76.0	11.8
	5	39.0	8.7
	6	88.7	15.7
	7	61.2	7.5
	8	40.4	22.2
	9	19.6	15.9
	10	44.2	39.7
	11	36.7	45.8
	12	101.5	53.3
	13	95.9	66.4
	14	33.9	30.9
	15	50.6	558.2
	16	30.7	165.4
	17	46.4	11.3
	18	58.0	33.7
	19	24.2	11.3
	20	16.5	41.7
	21	78.7	263.8
	22	34.2	19.5
	23		110.8
	24	10.4	12.0
	25	5.4	237.3
		48.0 ± 26.6[ab]	77.8 ± 122.2[d]
C × P3953	1	68.1	26.2
	2	60.6	101.7
	3	82.3	151.3
	4	14.3	394.8
	5	44.6	94.8
	6	54.4	102.2
	7	49.5	19.1
	8	21.4	165.9
	9	31.2	100.7
	10	42.9	32.2
	11	28.9	236.6
		45.2 ± 20.5[b]	129.5 ± 109.3[d]
B × F	1	23.9	95.9
	2	33.9	21.3
	3	28.5	104.5
	4	45.8	27.5
	5	62.8	141.0
	6	24.2	21.0
	7	34.2	30.1
	8	19.4	101.1
	9	43.4	145.3
	10	22.8	212.2
	11	17.9	254.7
	12	30.4	38.3
	13	29.3	83.4
	14		126.0
	15	21.6	18.8
	16	32.8	
	17		58.8
	18	35.1	
	19	38.9	289.2
	20		10.2
	21	100.0	299.8
	22	16.9	70.8
	23	29.1	131.0
	24	37.8	89.3
	25	44.6	41.0
	26	26.9	76.6
		34.9 ± 17.3[bc]	103.0 ± 83.0[d]

[a] Kernels were collected from several ears. Concentrations of 4-ABOA and DFP represent duplicate extractions. Values marked by "a" and "c" are significantly different at $p < 0.040$. Values in the same column marked by the same letter are not significantly different.

Table 2. Kernel 4-ABOA and DFP and Ear Rot Tolerance for 14 Inbreds

	kernel concn (μg/g)	
gibberella ear rot[a]	4-ABOA	DFP
5.5	57.2	70.5
7.5	54.4	145.5
6.0	46.4	29.1
6.5	22.1	167.9
6.5	18.5	71.1
6.4 ± 0.7^{a}	39.7 ± 18.2^{c}	96.8 ± 57.7^{e}
5.0	77.9	80.2
5.0	77.5	102.0
3.5	74.9	110.8
4.0	64.6	27.5
4.0	57.5	43.5
3.0	52.0	64.9
5.0	51.1	95.2
4.5	41.1	21.6
3.5	38.5	118.8
4.1 ± 0.7^{b}	59.2 ± 15.1^{d}	62.7 ± 38.8^{e}

[a] Ear rot ratings (scale of 1−9, 9 being completly infected) represent 5 station years of data, LSD = 1.8. Concentrations of 4-ABOA and DFP represent duplicate extractions. Values marked by "a" and "b" are significantly different at $p < 0.001$; those by "c" and "d" at $p < 0.037$.

presented. The object of these tests was to determine whether kernel 4-ABOA concentrations varied in such crosses.

Mean concentrations of 4-ABOA of the two larger sets of segregating genotypes (A × E and B × F) were 48.0 and 34.9 μg/g, respectively. These values were significantly different ($p < 0.04$). Considering values that were significantly higher, similar, or lower than the mean (±1 SD), the ratios of both data sets were similar at 2:10:1. There are few data on the inheritance of analogous compounds in corn. In the case of the hydroxamic acid DIMBOA, no dominant genes have been observed and perhaps five loci control its biosynthesis (Niemeyer, 1988). The present data suggest a similar pattern. Both the differences in mean and the segregation into low- and high-4−ABOA lines demonstrate that this metabolite varies in segregating populations derived from crosses of ear rot resistant and susceptible inbreds. Although the number of segregants tested was small, the Funks C inbred × Pioneer 3953 showed a similar mean 4-ABOA value and segregation pattern (Table 1).

The phenolic DFP showed much greater variation in kernel concentrations than 4-ABOA in these segregating populations. The mean kernel DFP values of the two larger populations based on Funks germplasm were not significantly different. However, the distribution of values in the A × E cross was lower than in the second (B × F): median values were 33 and 89 μg/g, respectively. This suggests that there were differences between the two populations in kernel DFP concentrations. In the more limited Funks C × Pioneer 3953, the median kernel DFP value was 101.7 μg/g. In all three segregating populations, kernel DFP concentrations were similar to those reported in some *Sitophilis zeamais* resistant land race material (Sen et al., 1994).

The genotypes in the limited field trial fell into two categories: susceptible (rating 5.5−7.5) and moderately tolerant (3−5; Table 2) separable on the LSD of the data. The mean ratings of the two groups were significantly different ($p < 0.001$). Mean kernel 4-ABOA concentrations were higher in the more tolerant group ($p < 0.037$). The median value kernel 4-ABOA concentration was also higher in the ear rot moderately tolerant group. The mean and median kernel concentrations of the phenolic DFP were similar in both groupings.

4-ABOA is a potent inhibiter of 3-ADON biosynthesis above 4 μM; more inhibitor does not result in more inhibition. In the plant, 4-ABOA is most likely present as a glycoside or other conjugate as are other similar compounds (Fielder et al., 1994). Such compounds are hydrolyzed to the active forms during the process of fungal invasion (Niemeyer, 1988). In such cases, on the basis of current knowledge, it is impossible to relate a kernel 4-ABOA concentration with the effective concentration near the invading fungal mycelium. Nonetheless, it is intuitive that the process of inhibiting the biosynthesis of the phytotoxin is less likely in germplasm with lower concentrations of 4-ABOA. These preliminary data suggest that gibberella ear rot tolerance involves (in part) the presence of effective concentrations of 4-ABOA.

This conclusion is consistent with data from other toxigenic plant systems. Kuti et al. (1989) showed that there were unknown chemicals present in extracts of muskmelons resistant to *Myrothecium roridum* that reduced production of the phytotoxin roridin E (a macrocylic trichothecene) in vitro. The specific productivity (micrograms of toxin per milligram of mycelium) was roughly halved when the fungus was grown on extracts of a resistant cultivar compared to similar extracts of a susceptible muskmelon cultivar, or, a defined medium. The phenolics ferulic, *p*-coumaric, and vanillic acids from peanuts inhibited aflatoxin production by *Aspergillus flavus* at 1 mM (Fajardo et al., 1995). Glyceollin isolated from soybeans inhibited aflatoxin biosynthesis at ~0.2 nM (Song and Karr, 1993). In corn embryos, the existence of labile factors that inhibit aflatoxin production have been demonstrated (Brown et al., 1993).

The significance of the present data is being investigated in greater detail through the use of specific crosses designed to measure the effect of adequate concentrations of 4-ABOA on gibberella ear rot.

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Received for review May 14, 1997. Revised manuscript received August 5, 1997. Accepted August 28, 1997.[⊗] This research was partially supported by the Agricultural Research Institute of Ontario, in a project administered by Mr. Ken Hough of the Ontario Corn Producers' Association, and by Dr. Ken Campbell of Ciba-Seeds Canada. CFAR Contribution 2432.

JF970395E

[⊗] Abstract published in *Advance ACS Abstracts*, October 15, 1997.