Dehydrodimers of Ferulic Acid in Maize Grain Pericarp and Aleurone: Resistance Factors to *Fusarium graminearum*

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ABSTRACT

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The relationship between the primary cell wall phenolic acids, dehydrodimers of ferulic acid, and maize grain resistance to *Fusarium graminearum*, the causal agent of gibberella ear rot, was investigated. Concentrations of dehydrodimers of ferulic acid were determined in the pericarp and aleurone tissues of five inbreds and two hybrids of varying susceptibility and in a segregating population from a cross between a resistant and susceptible inbred. Significant negative correlations were

found between disease severity and diferulic acid content. Even stronger correlations were observed between diferulic acid and the fungal steroid ergosterol, which is an indicator of fungal biomass in infected plant tissue. These results were consistent over two consecutive field seasons, which differed significantly for temperature and rainfall during pollination, the most susceptible stage of ear development. No correlation was found between the levels of these phenolics and deoxynivalenol levels. This is the first report of in vivo evidence that the dehydrodimers of ferulic acid content in pericarp and aleurone tissues may play a role in genotypic resistance of maize to gibberella ear rot.

Additional keywords: cell wall cross-linking, diferulate, HPLC, kernel, phenolics, segregating population.

Fusarium graminearum Schwabe, (teleomorph *Gibberella zeae* (Schwein.) Petch), is responsible for one of the major fungal diseases occurring sporadically in cool and temperate maize (*Zea mays* L.) cultivation areas (32). This disease known as gibberella ear rot, is a cause of great concern because of the ability of this mold to produce mycotoxins, such as trichothecenes and zearalenone that affect grain quality and also represent a potential risk for livestock and human health (19). One of the trichothecenes produced by *F. graminearum* is deoxynivalenol (DON, vomitoxin), which causes feed refusal and decreased weight gain in swine (22). In the infected host plant, this fungal secondary metabolite is believed to destabilize biological membranes (5) and inhibit protein biosynthesis (6) in plant tissues.

Chemical treatments and good cultural practices can help reduce *Fusarium* spp. incidence and severity but cannot solve the problem alone. The more reliable method for management of *F. graminearum* is the use of maize varieties that have been bred for improved resistance (25). Knowledge of resistance mechanisms makes it easier to select for characteristics that confer resistance (26). Resistance mechanisms of maize to ear rot include physical factors, such as pericarp thickness (14) and husk covering (36), or biochemical factors. Plant secondary metabolites, especially phenolic compounds, have often been implicated in mechanisms of

plant resistance (7). Previous studies on maize indicate that phenolic compounds such as inducible flavones in silk (26) and structural phenolics in grain (1,17) may be involved in maize resistance to ear rot. Phenolics in grain have been implicated in resistance to insects of maize, such as maize weevils (4). The majority of grain phenolics are located in the outer layers of the grain, the pericarp and aleurone layers, and in the germ (30). Ferulic acid (FA) is the predominant phenolic compound of maize tissue, and is mainly found bound to cell wall polysaccharides as a complex: FAXX (*O*-{5-*O*-[(*E*)-feruloyl]- α -L-arabinofuranosyl}-(1-3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylose) (8). *p*-Coumaric acid (*p*CA) is the second most frequent phenolic acid in maize cell walls and is generally bound to lignin. Bound forms of FA can be dimerized by peroxidases located in Golgi (9) or in the cell wall (23) to form cross-links between arabinoxylans chains that fortify the cell wall. Up to eight forms of dehydrodimers of ferulic acid (DFAs) have been discovered (12) since the first isomer, the 5-5' form (Fig. 1, for structure), was first reported in the cell wall of *Triticum aestivum* (16). The DFAs have been suspected to lower cell wall extension (8). Four forms of DFA are present in maize grain (Fig. 1).

The objective of the current study was to determine the role of phenolic compounds, especially dimers, in resistance of some Canadian maize genotypes to kernel infection by *F. graminearum*.

MATERIALS AND METHODS

Germ plasm. Two groups of germ plasm were used in two separate experiments. In the first experiment, five Agriculture and

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Agri-Food Canada (AAFC) inbred lines (CO433, CO432, CO325, CO359, and CO344) and two commercial hybrids (Pioneer 3905 and Funks G-4106) were used. CO433 and CO432 were developed with improved resistance to gibberella ear rot. CO325 is an inbred with moderate resistance to infection through kernel wounds. CO359 and CO344 were used as susceptible checks. Pioneer 3905 is a susceptible hybrid while Funks G-4106 has very good resistance to kernel infection by *F. graminearum*. In the second experiment, a recombinant inbred F₆ population produced from another AAFC inbred developed with improved resistance (CO387) and a susceptible parent CG62 (21) was used.

Fungal inoculum preparation. Three isolates of *F. graminearum* (DAOM180376, DAOM194276, and DAOM212678) obtained from the AAFC fungal culture collection were used for inoculation. All three isolates are equally aggressive and produce DON upon infection of maize ears. Inoculum was produced using a

modified form of Bilay's liquid medium as per Reid et al (25). A 1-cm² plug of potato dextrose agar culture of a single isolate of the pathogen was added to 200 ml of autoclaved medium in each of a series of 500-ml Erlenmeyer flasks. The flasks were placed on a shaker under natural light supplemented with cool white fluorescent light (50 W/m²) and shaken for 1 h at 4 h intervals for 8 days. Macroconidial counts in these flasks reached 2×10^6 ml⁻¹. Just prior to inoculation, spore suspensions were filtered through four layers of cheesecloth, and a cocktail of the three isolates was prepared by mixing equal quantities of conidial suspension of each isolate.

Experimental design. For the first experiment, the five inbreds and two hybrids were planted in 2000 and 2001 at the Central Experimental Farm of AAFC, Ottawa, Ontario, Canada. The experimental design consisted of a split-plot design with three replicates. The main plot unit consisted of genotype and the sub-

Fig. 1. Chromatogram of a typical pericarp-aleurone sample and chemical structures of the main phenylpropanoids found in the cell wall of maize (34).

plot unit consisted of two inoculation treatments (inoculation with conidial suspension and inoculation with sterile water as a control). Inoculation procedures followed the protocol of kernel inoculation developed by Reid et al. (25). Inoculations were conducted 12 days after mid-silk emergence with an automatic pipetor injecting 1 ml of 10^6 macroconidia per ml suspension of *F. graminearum*. At harvest time, approximately 60 days after inoculations, three ears were randomly harvested in each plot for each genotype, and the severity of ear rot infection was estimated using a rating scale where: 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 exhibit visible symptoms of ear rot such as pinkish mycelial growth (25). After symptom assessment, the kernels were removed from the central portion of the cob (rachis) using a scalpel, bulked within each row, and a 30-g sample for mycotoxin

and ergosterol analysis was collected. Grains of control plants were also collected for the analysis of the cell wall phenylpropanoid content. Any grain material was freeze-dried and stored in cold room at 7°C before processing

Hourly temperatures and daily precipitation were recorded at the research centre standard weather site within 0.5 km of the experiment.

High throughput cell-wall phenylpropanoids extraction. The freeze-dried grain samples were soaked overnight in water at 4°C after which the combined pericarp-aleurone layer was separated from the endosperm using a pearl mill (Strong Scott, Winnipeg, Canada). This equipment was found to be the most convenient for an efficient separation of the endosperm (reduced to flour) from the outer layers (pericarp and aleurone) and the germ that generally stayed intact after 10 s of milling. The final separation of the

Fig. 2. Correlations between disease severity or ergosterol content and dehydromers of ferulic acid (DFA) content in the pericarp-aleurone layers of five inbreds and two commercial hybrids inoculated with *F. graminearum* in 2000 and 2001. Values are the mean of three replicates except for CO344 in 2000 with two replicates. Correlations type Pearson and confidence intervals at 95% are represented. Content of DFA is expressed in $\mu\text{g/g}$ of pericarp and aleurone dry weight (DW).

pericarp-aleurone fraction was performed manually before being freeze-dried. Pericarp-aleurone samples (0.1g) were digested with 3 ml of 2N NaOH for 3 h in darkness. Samples were homogenized for 15 s after 2 h of digestion with a Polytron mixer (Brinkman Instruments, Westbury, NY). The homogenizer was washed with 1 ml of fresh 2N NaOH (pooled with sample). Samples were acidified to pH 2.0 with 790 μ l of concentrated 12N HCl, vigorously mixed, and extracted twice with 5 ml of ethylacetate. Collected organic fractions were combined and reduced to dryness using a Speed Vac (Savant Instruments, Holbrook, NY) for 90 min at medium settings without a radiant cover. The final extract was dissolved in 1 ml of high-performance liquid chromatography (HPLC) grade methanol and stored at 18°C until HPLC analysis.

HPLC analysis of cell wall phenylpropanoids. Each sample was filtered through a 0.2- μ m pore polytetrafluoroethylene (PTFE) filter before analysis. All the analyses were performed on an Agilent HP Chemstation 1100, with Diode Array Detector and Mass Spectrometer detector model VL (Agilent, Palo Alto, CA). The reverse phase HPLC procedure was developed previously (A. C. Bily, *unpublished data*). Briefly, a binary gradient with acetonitrile and 0.05% trifluoroacetic acid (TFA) in water was applied to a short and narrow column YMC ODS-AM 2 mm $\times$ 100 mm, 3 μ m in diameter (Waters Corporation, Milford, MA). This method allowed us to complete 50 analyses per day with less than 500 ml of solvent. To confirm the identities of DFA, an LC-MS procedure with Atmospheric Pressure Chemical Ionization (APCI) in positive mode (3) was used on selected samples.

Ergosterol and deoxynivalenol analysis. Whole grain material was collected from the central part of the infected ear and freeze-dried as previously described. Ergosterol, a fungal cell wall component that remains in plant tissues after infection (29) and is often used to measure fungal biomass in infected plant tissues, was extracted using the microwave-assisted procedure developed previously (37). Quantification was achieved using an HPLC procedure based on an isocratic gradient with acetonitrile (80%) and methanol (20%) for 10 min and at a flow rate of 0.6 ml per min and detection at 282 nm. Five microliters were injected in a column Zorbax Eclipse XDB-C8 4.6 $\times$ 150 mm, 5 μ m (Agilent, Palo Alto, CA) maintained at 50°C.

DON was extracted using the method described by Trenholm et al. (34) with the following modifications. After extraction of grain powder (1 g) with 5 ml of acetonitrile/water (84:16) for 1 h, 1 ml of the supernatant was purified twice using a 0.5 g mixture of charcoal/aluminum oxide (1:1), each time for 10 s and centrifuged at 3,000 $\times$ g for 3 min. The extract was filtered with a 0.05 mm syringe filter. The sample was dried under nitrogen flux (30 min at 60°C). For the gas chromatography (GC) analysis of DON, samples were derivatized using 100 μ l of Sigma-Sil-A (Sigma, St. Louis, MO) according to the protocol described by the manufacturer. GC analyses were carried out with a Hewlett-Packard model 5890 gas chromatographer equipped with ECD and autosampler. One microliter was injected in a column J&W DB-5, 1.5- μ m film thickness, 0.53 mm i.d. and 30 m in length. The temperature gradient used was as follows: at initial conditions, 120°C for 6 min, ramp at 10°C per min to 180°C, and then isothermal for 1 min. The injection and detector temperature were kept at 350 and 270°C, respectively, and the column flow rate was 1 ml per min.

Statistical analysis. A one way analysis of variance (ANOVA) followed by Duncan's test was used to compare means of monomers and DON contents. Tests were considered as significant at $P > 0.05$. Pearson correlations and P values were also calculated using Systat statistical software (version 8.0 for Windows).

RESULTS AND DISCUSSION

In the first experiment, different Canadian maize genotypes known to represent a wide spectrum of susceptibility to *Gibberella* ear rot were investigated for a relationship between their primary

cell wall phenylpropanoid content of the outer layers of the grain (Fig. 1) and their resistance to *Gibberella* ear rot. Disease severity and ergosterol content were found to be negatively correlated with the content of DFA in the pericarp and aleurone layers for both years of the experiment (Fig. 2). As expected, the two inbreds with improved resistance (CO432 and CO433) had the lowest disease severity ratings followed by CO325 and the two susceptible inbreds, CO344 and CO359 (Fig. 2). The commercial hybrids also behaved as expected, with Funks G-4106 having lower disease severity ratings than Pioneer 3905, although the spread was not as great as it was between susceptible and resistant inbreds. Significant positive correlations were found between ergosterol and disease severity (Fig. 3), this finding confirms the reliability of ergosterol as infection marker (29). Overall, disease severity was higher in 2000 than in 2001 (Figs. 2 and 3). Temperatures were significantly lower in 2000 especially during the

Fig. 3. Correlation between disease severity and ergosterol content in whole grain at harvested time. Correlations type Pearson and confidence intervals at 95% are represented.

silking stage between 10 July and 10 August when plants are more susceptible to ear rot (Fig. 4). In addition, rainfall was higher in 2000 than in 2001 creating much more humid conditions during pollination, thus better conditions for mycelial development during the early stage of infection (32). The slopes of the regression curves are steeper in 2001 than in 2000 (Fig. 2). This could indicate that DFA acts more efficiently when conditions are less favorable for disease development.

As with disease severity, the content of monomers of *p*CA, *E*-ferulic acid (*E*-FA) and *Z*-ferulic acid (*Z*-FA) were significantly

different between genotypes (Table 1). In agreement with previous studies (1), *E*-FA was found to be the predominant species of the cell wall phenolics in all the genotypes studied (Fig. 1). The Trans stereoisomer *Z*-FA was only a minor constituent (less than 10% of the total FA) and may be partly the results of a stereoisomerisation during the extraction process. Monomers of phenolic acids have been often reported as factors in plant-pathogen interactions (1,7) however, levels of these phenylpropanoids did not show a clear correlation with disease in 2000 and 2001 (Table1). FA, in its free unconjugated form, is known to be fungistatic to *F. graminearum*

Fig. 4. Minimum and maximum daily temperatures and total rainfall for 2000 and 2001 from 10 July (silking) to 10 October (harvest) at Ottawa, Ontario, Canada.

TABLE 1. Concentrations of cell wall bound monomers of phenolic acids and deoxynivalenol in five inbred lines and two commercial hybrids of maize in 2000 and 2001^z

	2000				2001			
	<i>p</i> CA mg/g	<i>E</i> -FA mg/g	<i>Z</i> -FA mg/g	DON µg/g	<i>p</i> CA mg/g	<i>E</i> -FA mg/g	<i>Z</i> -FA mg/g	DON µg/g
Inbred lines								
CO433	0.94 a	17.81 a	1.65 a	45 a	0.88 a	14.58 a	1.32 a	55 a
CO432	1.22 b	17.24 a	1.29 b	96 b	0.54 b	16.59 b	1.25 a	68 a
CO325	1.20 b	18.56 a	1.45 a	102 b	1.56 b	13.87 a	1.16 a	95 b
CO359	1.22 b	17.99 a	0.93 c	175 c	0.90 a	17.89 b	0.65 b	175 c
CO344	1.60 c	19.21 a	1.55 a	102 b	2.05 c	19.87 b	1.25 a	113 b
Hybrids								
Funks G-4106	1.05 a	20.63 b	1.33 b	37 a	1.25 b	18.29 b	0.86 b	52 a
PIONEER 3905	1.16 b	17.96 a	1.15 b	64 b	1.26 b	16.25 b	1.05 a	84 b

^z Means in the same column are not significantly different when followed by the same letters (one-way analysis of variance and then Duncan's test with a critical *P* = 0.05). *p*CA = *p* coumaric acid; *E*-FA = *E*-ferulic acid; *Z*-FA = *Z*-ferulic acid; and DON = deoxynivalenol.

Fig. 5. Correlations between disease severity and pericarp-aleurone content of total dehydromers of ferulic acid (DFAs) and individual isomers of DFAs in harvested grains of a segregating population of CG62 $\times$ CO387. $n = 75$. Pearson correlations and confidence intervals at 95% are represented. The two parents CO387 (resistant), and CG62 (susceptible), are also localized. Content of DFA is expressed in $\mu\text{g/g}$ of pericarp and aleurone dry weight (DW).

at an EC₅₀ of 650 ppm (1). A very limited quantity of FA is actually soluble as free form or conjugated to short sugar chains (31). This fraction is more likely to react with *Fusarium*. Since most phenylpropanoids are esterified to the cell wall, it is possible that they have a limited direct biochemical mediated inhibition of the infecting fungus.

The DON content did not correlate well with either parameter measured even if generally lower levels of DON were found in resistant genotypes (Table 1). This result emphasizes the fact that in vivo trichothecene biosynthesis is difficult to correlate with symptoms (27). Environmental factors controlling disease expression and mycotoxin accumulation are still not fully understood. Some very aggressive isolates of *F. graminearum* are known to produce low levels of ergosterol and DON (10). Moreover, *F. graminearum* mutants unable to produce trichothecenes can still cause disease in maize (13). These findings indicate that other pathogenicity components, such as hydrolytic enzymes, have a role in disease.

Two genotypes (CO433 and CO432) with a very different genetic background exhibited high levels of DFAs and high levels of resistance for both years. It is reasonable to suggest that the resistance of these genotypes is partially due to their higher levels of cross-links in the cell wall of their pericarp and aleurone cells. The dehydromers of ferulic acid originate from the oxidative coupling of two residues of ferulic acid esterified to an arabinosyl part of the arabinoxylan chain, main polysaccharide component of the primary cell wall of the cereal (8). This cross-linking occurs under the control the peroxidases and its cofactor H₂O₂, in situ in the cell wall or in intracellularly during the biosynthesis of the cell wall (9).

To further investigate the significance of these correlations, a more genetically uniform population of 75 individuals, created from a cross of a resistant and susceptible parent, was also investigated for DFAs and resistance. Once again a significant negative correlation was observed between levels of DFAs and disease ratings (Fig. 5). This experiment allowed examination of individual isomers of DFA (Fig. 5). The strongest correlation was observed with total DFA (Fig. 5), which could indicate that at the same concentration, individual species of DFA (5-5', 8-O-4', 8-5', 8-5'b) participate equally in resistance. A large proportion of the total DFAs content is made up of the two species 8-O-4' and 5-5' in the genotypes studied, which suggests that these two forms are primarily responsible for the resistance observed.

Varieties with high levels of DFAs in the two experiments showed greater resistance to *F. graminearum* spread on the ear because symptoms and ergosterol were lower. The current theory based on work of Bergvinson et al. (2) is that DFAs strengthen the physical integrity of the cell wall of young tissues by linking hemicellulose chains together. DFAs are known to stabilize the wall at the early stage of plant development (20). It is also known that DFAs impede in vitro cell-wall degradation by fungal hydrolases, such as *Aspergillus* esterases (11). *Fusarium graminearum*, as with many pathogenic fungi, has a large spectrum of extracellular enzymatic activities (15,33). In the current experiments, initial penetration in the ears was mechanically facilitated, but mycelial progression on intact neighboring grains was reduced in maize genotypes with high levels in DFAs. *F. graminearum* is necrotrophic, and it invades the plant tissues aggressively, killing the host cells to obtain nutrients, as opposed to biotrophic pathogens that establish an intricate feeding relationship with living host cells. A recent study with a GFP (Green Fluorescent Protein) transformed strain of *F. graminearum*, indicated that the mycelium remains in the apoplast, surrounding the cells at the early stage of kernel infection (18). These cell-wall components seem to be an important part of the early barrier against this fungal pathogen. Special attention should therefore be given to levels of DFAs during the early infection process.

This study is the first report of evidence that DFAs may play an important role in maize resistance to ear rot. These factors are

stable and do not seem to be influenced by climatic variation. Moreover, DFAs are well known to be important maize resistance factors to various insects such as the European corn borer, *Ostrinia nubilalis* (2), and several tropical maize borers (24). By analogy with the different components of the resistance to *F. graminearum* in wheat described by Schroeder and Christensen (28), DFA would represent a type I resistance (resistance to initial penetration, for example, through insect wounds) and a type II resistance (resistance to propagation due to a lower degradability of the cell wall). Ancient Mexican landraces, such as Chapalote, are known to be rich in DFA content, and exhibit high levels of resistance to insect pests. Thus, this resistance factor is a very good candidate to be included in Integrated Pest Management programs. Modern crop breeding programs where yield is the first goal have generally led to a decrease of some major secondary metabolites such as DFAs. Moreover, the identification of elite inbred lines (such as CO433 and CO432) with competitive yields, as well as DFA content and fungal resistance, indicates that DFAs do not compromise maize yield greatly. Modern tools like marker-assisted selection will enable breeders to restore the presence of these compounds to acceptable levels for better resistance. We are currently mapping these cell wall components on several segregating populations.

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