JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phenolics in Maize Genotypes Differing in Susceptibility to *Gibberella* Stalk Rot (*Fusarium graminearum* Schwabe)

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The relationship between phenolic compounds and maize pith resistance to *Fusarium graminearum*, the causal agent of *Gibberella* stalk rot, was investigated. The phenolic acid profiles in the stalks of six maize inbred lines of varying susceptibility were evaluated from silking to grain maturity. Four different fractions of phenolic compounds were extracted from inoculated and non-inoculated (control) pith tissues: insoluble cell-wall-bound, free, soluble ester-bound, and soluble glycoside-bound phenolics. Analysis by HPLC revealed that *p*-coumaric acid and ferulic acid were the most abundant compounds in the soluble and cell-wall-bound fractions. The quantity of free, glycoside-bound, and ester-bound phenolics in the pith was lower than the level required for the inhibition of *Fusarium* growth or mycotoxins production; however, significant negative correlations between diferulic acid contents in the cell walls and disease severity ratings 4 days after inoculation were found. The results indicated that future studies should focus on the levels of diferulic acids during the early infection process. Diferulates may play a role in genotypic resistance of maize to *Gibberella* stalk rot as preformed barriers to infection.

KEYWORDS: ***Zea mays; Fusarium graminearum*; stalk rot resistance; phenolic compounds**

INTRODUCTION

Stalk rot of maize (*Zea mays* L.) caused by *Fusarium* species leads to substantial yield losses every growing season around the world (*1*−*3*). These fungal organisms decay pith tissue in the lower stalk internodes, which can often result in poor kernel fill and premature plant death. As the rotting pith pulls away from the stalk rind, the structural integrity of the stalk changes, and the plant may lodge. *Fusarium graminearum* Schwabe [sexual stage: *Gibberella zeae* (Shchwein.) Petch] is only one of many species responsible for stalk rot but is considered to possess the highest pathogenicity (ability to cause disease) and aggressiveness (the amount of disease caused) (*3*, *4*). Distinctive symptoms in the stalk are a tan-to-brown discoloration of the lower internodes and a pink-to-reddish discoloration of the pith tissue. *Fusarium graminearum* produces the phytotoxic non-macrocyclic trichothecenes deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON) (*5*). The toxic effects of these mycotoxins are well documented in animals and humans (*6*, *7*). DON is water-soluble, and translocation in the phloem as a bulk flow of solution is assumed to occur (*3*). DON produced in the stalk or ear of maize can be found in tissues not invaded by *F. graminearum* (*8*, *9*).

Cultural practices can help reduce the incidence and severity of *Fusarium*, but the most practical approach for disease control is to plant resistant hybrids. Resistance against the spread of the pathogen within the host was identified after the artificial inoculation of silks, kernels, and stalks (*11*, *12*). Several resistance mechanisms have been proposed for silks and kernels (*13*−*16*); however, the mechanisms responsible for resistance in the stalk are neither established nor understood.

Plant secondary metabolites, especially phenolic compounds, have often been implicated in disease resistance by a number of researchers (*17*−*20*). Some phenolics occur constitutively and function as preformed resistance barriers, but others are formed in response to the entrance of a pathogen as part of an active defense response (*19*). Phenolic compounds have been shown to inhibit the in vitro growth of several fungal genera (*21*, *22*); significant inhibitory effects were reported for *Fusarium* species (*15*, *23*, *24*). In addition, the evidence strongly suggests that the esterification of phenols to cell-wall materials is a common theme in the expression of resistance (*25*). The presence of phenols in host cell walls is usually associated with an increase in fungal enzymes and acts as a physical barrier against fungal penetration (*20*, *26*). Previous studies on maize indicate that phenolics in grain may be involved in resistance

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10.1021/jf070641e CCC: $37.00
Published on Web 06/05/2007

Table 1. Disease Severity Ratings at Four Harvest Times for Six Inbred Lines after Inoculation of the Stalk with a Conidial Suspension of *Fusarium graminearum* in 2004 and 2005[a]

		days after inoculation[c]			
inbred line	rr[b]	0 days	4 days	28 days	FINAL
CO441	5	1.00a	2.00bc	3.46cd	6.20a
CO400	6	1.00a	3.70a	5.33a	5.86ab
CO348	1	1.00a	1.37c	2.40e	3.27c
73353	2	1.00a	2.47abc	3.13de	3.83c
CO359	4	1.00a	3.20ab	4.97ab	5.04b
CO433	3	1.00a	1.73c	4.23bc	5.70ab
LSD ($P \leq 0.05$)			1.38	0.97	0.85

[a] Means within a column followed by the same letter are not significantly different ($P \leq 0.05$). [b] rr: resistance ranking in previous evaluations. [c] Days after inoculation: 0 days after inoculation = not inoculated; 4 days after inoculation; 28 days after inoculation; FINAL (approximately 70 days after inoculation, at normal grain harvest).

to *Gibberella* ear rot (*14*, *15*), and recent papers reported on the possible role of dehydrodimers of ferulic acid in the genotypic resistance to *F. graminearum* (*27*).

In the present study, the levels of different fractions of phenolic compounds were monitored in field-grown inbred lines of maize after inoculation with *F. graminearum* or sterile water (control). The specific objectives of the study were (1) to identify soluble and cell-wall phenolic compounds in the pith of maize, (2) to determine if a relationship exists between phenolic compounds and resistance to stalk infection by *F. graminearum*, and (3) to monitor phenolic levels in response to fungal attack.

MATERIALS AND METHODS

Plant Materials and Experimental Design. Five Agriculture and Agri-Food Canada (AAFC) inbred lines (CO441, CO433, CO400, CO348, and CO359) and one inbred line (73353) from Cornell University were used in this study. On the basis of previous evaluation at the Central Experimental Farm (CEF) of AAFC, Ottawa, Ontario, Canada, the ranking of these inbreds from most to least resistant is CO348, 73353, CO433, CO359, CO441, and CO400 (**Table 1**). The six inbreds were planted in 2004 and 2005 at the CEF in a split−split-plot design with three replicates. The main plot unit consisted of genotype, the subplot unit consisted of two inoculation treatments (inoculation with a conidial suspension and inoculation with sterile water as a control), and the sub-subplot unit consisted of four harvest times (0, 4, and 28 days after inoculation and a final harvest approximately 70 days after inoculation at normal grain-harvesting time). Each sub-subplot was a 3.5-m-long row separated by 0.7 m and sown with 15 seeds per row.

Inoculum production followed the protocol developed by Reid et al. (*11*) using three local DON-producing isolates (DAOM 180378, DAOM 194276, and DAOM 212678) obtained from the National AAFC Culture Collection. The isolates were cultured separately and then mixed in equal proportions just prior to inoculation. To determine each inbred's silking date accurately, plots were monitored until 50% of the plants had exposed silks. Inoculations were conducted 4−7 days after silk emergence using an automatic vaccinator. Two milliliters of a conidial suspension (2.5×10^5 spores/mL) of *F. graminearum* were injected at a 45° angle downward into the middle of the first internode above the uppermost aerial root node. The center 10 plants of each 15-plant row were inoculated. At each harvest time, five stalks were randomly split in each row for each treatment and genotype, and the severity of stalk rot infection was estimated using a rating scale where 1 = no visible spread of the pathogen from the inoculation point; 2 = 1−25%; 3 = 26−50%; 4 = 51−75%; 5 = >75% of inoculated internode symptomatic; 6 = symptoms have spread to one adjacent internode; and 7 = symptoms have spread to two or more adjacent internodes (*12*). After evaluation, the inoculated internode was harvested, and the pith tissue was obtained by removing the rind manually. Pith samples were stored at −20 °C until further processing.

Extraction of Different Phenolic Fractions. Four different fractions of phenolic compounds were extracted from the pith tissue samples: insoluble cell-wall-bound phenolics and free, soluble ester-bound, and soluble glycoside-bound phenolics. Extraction was based on a procedure previously described (*28*) with some minor modifications. Freeze-dried pith samples were ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA) with a 0.75 mm screen, and then 1 g of material was extracted with 30 mL of 80% methanol. The suspension was homogenized for 30−60 s with a Polytron mixer (Brinkman Instruments, Westbury, NY) before being centrifuged at 1000*g* for 10 min. After centrifugation, the supernatant was collected, and the remaining precipitate was rehomogenized and centrifuged as above. The pellets contain the insoluble cell-wall-bound phenolics, and the supernatants contain the soluble phenolics.

The supernatants for soluble phenolic acids were combined and concentrated in a Speed Vac (Savant Instruments, Holbrook, NY) to 20 mL. These aqueous solutions were acidified using 6 N HCl to a pH of 2.0 before extraction with 20 mL of ethyl acetate. The ethyl acetate extract was reduced to dryness in a Speed Vac at medium settings without a radiant cover, and the resulting precipitate was resuspended in 3 mL of HPLC-grade methanol. This solution was used to determine free phenolic compounds.

The remaining aqueous solution was divided into two parts. The fraction for soluble glycoside-bound phenolic determination was hydrolyzed in 10 mL of 2 N HCl for 1 h at 4 °C; whereas alkaline hydrolysis for 3 h in the dark and under a nitrogen atmosphere was used to release the soluble ester-bound phenolic compounds. After both digestions, the pH of the solutions was adjusted to 2.0, and the phenolics were extracted with 30 mL of ethyl acetate. The ethyl acetate extracts were reduced to dryness in a Speed Vac system, and the resulting precipitates were resuspended in 3 mL of HPLC methanol.

The pellet containing ester-bound phenols incorporated into the cell wall was then shaken in 20 mL of 2 N NaOH under nitrogen flow in the dark for 4 h. Digested samples were neutralized with 6 N HCl, and the pH was lowered to 2.0. After centrifugation, the supernatant was collected, and the pellet was washed twice with distilled water (10 mL each). Supernatants were pooled and then extracted twice with ethyl acetate (40 mL each). Collected organic fractions were combined and reduced to dryness using a Speed Vac for 4 h. The final extract was dissolved in 3 mL of HPLC methanol. All of the extracts were stored at −20 °C prior to HPLC analysis.

HPLC Analysis. All standards and samples were filtered through a 2 μm pore size poly(tetrafluoroethylene) filter (Chromatographic Specialties, Brockville, ON, Canada) before analysis. Analyses were performed using a Hewlett-Packard ChemStation series 1100 chromatograph with a Waters YMC ODS-AM (Waters, Milford, MA) narrow bore column (100 × 2 mm i.d.; 3 μM particle size). Elution conditions with a mobile phase system of acetotrinile (solvent A) and trifluoroacetic acid (0.05%) in water (pH 3.4) (solvent B) were as follows: initial conditions 10:90 (A/B), changing to 30:70 in 3.5 min, then to 32:68 in 6.5 min, then to 100:0 in 4 min, and then to isocratic elution with 100:0 for 4.5 min, finally returning to the initial conditions in 3 min. The mobile phase flow rate was 0.3 mL/min, and the total analysis time was 21.5 min. The sample injection volume was 4 μL, and the elution profiles were monitored online by UV absorbance at 325, 280, and 254 nm. Retention times were compared with those of freshly prepared standard solutions. Standards of the most common phenolics (caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and vanillic acid) were purchased from Sigma (St. Louis, MO). The identities of diferulates were confirmed by comparison with the authentic 5-5′ standard and/or retention time and UV spectra previously published (*29*). In selected samples, the presence of pseudomolecular ion $[M + H]^+$ at 387.2 *m*/*z* in positive APCI mass spectra was checked (*30*).

Statistical Analysis. Combined analyses of variance (ANOVA) for disease severity ratings and phenolic contents were computed with the PROC GLM procedure of SAS, version 9.1 (*31*). Year, replication, and their interactions were considered to be random factors. Comparisons of means among genotypes, harvest times, and inoculation

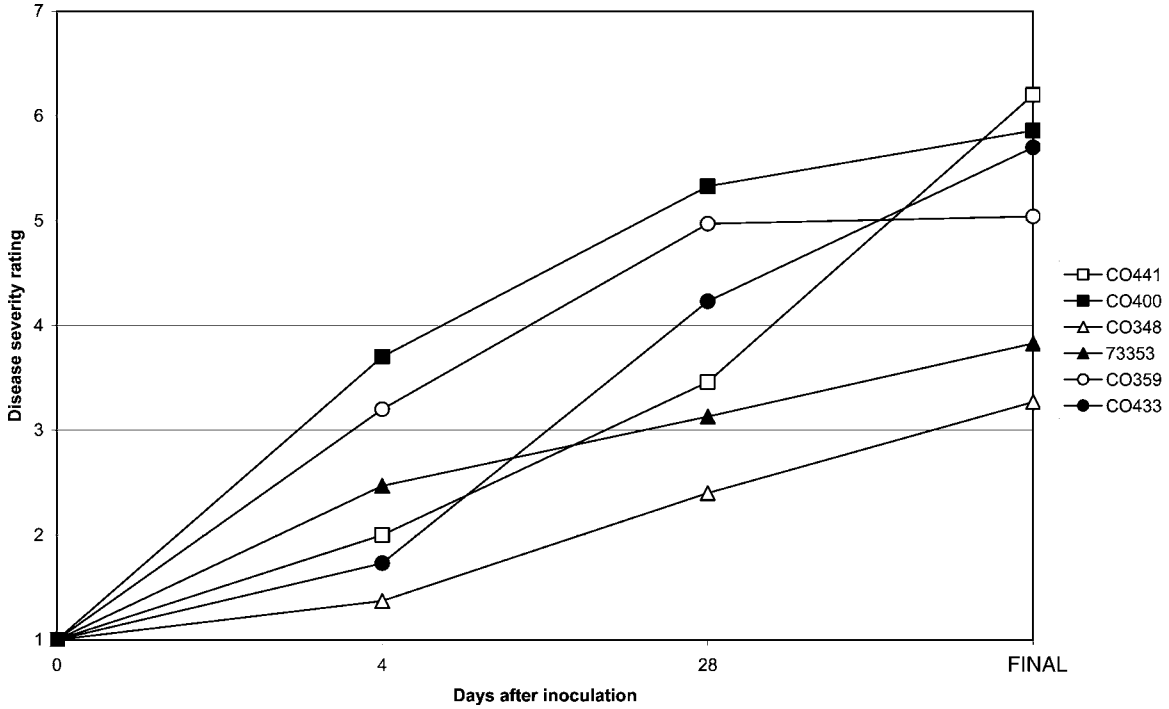

Figure 1. Disease progress for six inbred lines after artificial inoculation of the stalk with *Fusarium graminearum*.

treatments were made by the least significant difference method (LSD). In addition, Pearson correlation analyses between the different compounds and disease ratings were calculated.

RESULTS AND DISCUSSION

Disease Severity Ratings. Significant differences in *Gibberella* stalk rot disease severity between conidia-inoculated treatments (average 3.24) and water-inoculated control treatments (average 2.73) were observed (data not shown). Disease ratings for the water control were higher than expected (data not shown). Natural infection takes place through roots or wounds in the stalk shortly after pollination (*5*); it is quite probable that wounds created by the control inoculations provided an entrance for the naturally occurring isolates. Genotype x treatment interactions were not significant, so results are discussed only for conidia-inoculated samples.

As expected, the inbreds exhibited significant differences in disease severity ratings when inoculated with the conidial suspension of *F. graminearum* (**Table 1**). The two inbreds with the highest resistance (73353 and CO348) had the lowest disease severity ratings at grain harvest (FINAL), followed by CO359 and CO433 and the two susceptible genotypes, CO400 and CO441 (**Table 1**, **Figure 1**). The intermediate and susceptible genotypes behaved as expected, although a shift in the ranking order from previous results was observed. In addition, the inbred CO433 did not differ significantly from the most susceptible group (**Table 1**).

Figure 1 shows the progress of disease severity ratings at four harvest times after the silking date. The variation in the resistance ranking along harvest times is interesting. Normally, screening for resistance by evaluating disease severity takes place at grain harvest. The previous classification of the inbreds from most resistant to most susceptible was CO348, 73353, CO433, CO359, CO441, and CO400. At early (4 days) and intermediate (28 days) stages of infection, inbred CO400 was the most susceptible, and the inbred CO348 was the most resistant. However, inbred CO441 was classified as the most susceptible at final harvest but could be included in a resistant group in preceding evaluations (**Table 1**, **Figure 1**). It is important to note the differences between the two susceptible inbreds: CO400 was a susceptible inbred during all evaluations whereas inbred CO441 was classified as susceptible only at the final harvest. Senescence or cell death of pith tissue precedes stalk rot, apparently beginning at the top of the plant and progressing downward (*32*). Living pith tissue resists pathogens, but dead tissue does not. Apparently, the synthesis of cellular resistance substances decreases with senescence (*33*). In inbred CO441, earlier cellular senescence could be responsible for the increase in susceptibility. However, both resistant genotypes, CO348 and 73353, showed a resistant trend for all disease severity evaluations (**Figure 1**).

Phenolic Contents of Pith Tissues. For the purpose of the study, only the concentrations of the E isomers of phenolic acids were calculated. Efforts were made in the extraction to minimize the photoisomerization reactions to which phenolic acids are susceptible (*34*, *35*).

According to the HPLC analysis, *p*-coumaric (**1**) and ferulic acid (**2**) were the most abundant compounds in both soluble and cell-wall-bound phenolic acids extracted from the pith of the genotypes studied (**Tables 2** and **3**). Compound **1** was present in higher amounts than **2** in the four fractions evaluated. Other soluble phenolics could not be identified with the standards that we used.

Soluble Phenolics. The lowest concentrations of free **1** (33.15 μg/g dry weight) and free **2** (13.72 μg/g dry weight) were quantified at the beginning of the evaluation (0 days) in inbreds CO359 and CO348, respectively, whereas the highest concentrations of free **1** (260 μg/g dry weight) and free **2** (37.76 μg/g dry weight) were quantified at the last harvest time (FINAL) in inbreds CO441 and 73353, respectively (**Table 2**). However, no significant differences were found among inbreds for free **1**, whereas significant differences were observed 4 days post-inoculation for free **2**. The lowest (16.68 μg/g dry weight) and highest (29.19 μg/g dry weight) concentrations of free **2** at 4 days were present in resistant inbreds CO348 and 73353, respectively.

Table 2. Mean Concentrations (μg/g Dry Weight) at Four Harvest Times of the Major Soluble Phenolics Identified in the Inoculated Pith of Six Inbred Lines in 2004 and 2005[a]

		inbred lines						LSD
phenolic compound[b]	time[c]	CO441	CO400	CO348	73353	CO359	CO433	($P \leq 0.05$)
				Free Phenolics				
1	0d	93.77a	34.15a/B	46.34a	59.44a	33.15a	56.93a/B	
	4d	88.69a	41.26a/B	60.89a	103.39a	46.74a	95.40a/B	
	28d	111.76a	54.45a/B	74.60a	80.09a	72.78a	123.00a/B	
	FINAL	260.11a	95.22a/A	71.52a	122.27a	70.60a	218.30a/A	
	LSD ($P \leq 0.05$)		40.05				84.72	
2	0d	22.79a	15.04a/D	13.72a/B	16.77a/C	15.13a	18.26a	
	4d	24.21b	18.01c/C	16.68c/B	29.19a/B	19.52c	26.83ab	3.98
	28d	32.96a	35.96a/A	32.28a/A	33.44a/AB	36.79a	42.23a	
	FINAL	33.18a	27.80a/B	33.30a/A	37.76a/A	32.16a	29.05a	
	LSD ($P \leq 0.05$)		2.27	8.62	7.47			
				Glycoside-Bound Phenolics				
1	0d	16.56a	15.01a	13.47a	17.12a	12.78a	20.60a	
	4d	15.27a	11.72a	13.46a	17.20a	11.95a	15.41a	
	28d	17.17a	15.31a	12.07a	16.49a	13.46a	16.68a	
	FINAL	19.76a	15.93a	13.10a	20.67a	11.41a	41.04a	
	LSD ($P \leq 0.05$)							
2	0d	10.58a	11.79a	9.18a	9.64a/B	9.49a	12.91a	
	4d	10.74a	8.60a	8.90a	10.77a/AB	9.53a	10.23a	
	28d	10.71a	12.17a	9.27a	11.00a/A	11.93a	11.43a	
	FINAL	9.21a	10.48a	8.98a	11.97a/A	10.22a	12.15a	
	LSD ($P \leq 0.05$)				1.28			
				Ester-Bound Phenolics				
1	0d	44.52a	23.61c	26.85bc	44.83a	37.56abc	38.27ab	14.25
	4d	42.26a	26.13b	27.09b	44.16a	36.46ab	50.69a	14.99
	28d	53.25a	19.94a	25.47a	41.92a	41.80a	64.36a	
	FINAL	34.15a	42.99a	37.74a	60.12a	43.83a	65.69a	
	LSD ($P \leq 0.05$)							
2	0d	31.34a	15.28a/B	18.58a/C	20.14a/B	21.03a	21.10a	
	4d	25.72a	20.97c/B	22.59a/BC	31.38a/B	28.13a	30.57a	
	28d	63.56a	28.88a/B	36.20a/B	65.42a/AB	73.23a	64.16a	
	FINAL	26.86c	68.45abc/A	81.75ab/A	108.36a/A	109.64a	35.14bc	49.10
	LSD ($P \leq 0.05$)		27.89	17.48	48.61			

[a] Means within a row followed by the same lowercase letter are not significantly different ($P \leq 0.05$). Uppercase letters were used for the columns when significant. [b] Simple hydroxycinnamic acids: **1**, *p*-coumaric acid; **2**, *trans*-ferulic acid. [c] Time: harvest time (days).

HCl extraction yielded low levels of glycoside-bound phenolic compounds whose concentrations were not significantly different among genotypes (**Table 2**). However, NaOH extraction yielded higher concentrations for ester-bound phenolics, and greater variation was observed among the different genotypes (**Table 2**). Significant differences were found 0 and 4 days after inoculation for ester-bound **1** and at the final harvest for ester-bound **2**. The lowest concentrations of ester-bound **1** at 0 days (23.61 μg/g dry weight) and 4 days (26.13 μg/g dry weight) were quantified in susceptible inbred CO400, whereas the highest concentrations at 0 days (44.83 μg/g dry weight) and 4 days (50.69 μg/g dry weight) were present in resistant inbred 73353 and intermediate inbred CO433, respectively. The lowest (26.86 μg/g dry weight) and highest (109.64 μg/g dry weight) concentrations of ester-bound **2** at the final harvest time (FINAL) were present in susceptible inbred CO441 and intermediate inbred CO359, respectively (**Table 2**).

Earlier studies suggested that some natural acids with phenolic structure inhibited the fungal genera *Fusarium* (*36*). Moreover, Molot (*37*) reported a significant correlation between the levels of phenolic compounds in the stalks at fertilization and the percentage of stalks lodging as a result of stalk rot. The significant in vitro inhibitory effect of phenolic acids on *Fusarium* species suggests that these compounds could contribute to disease resistance. Assabgui et al. (*15*) reported that 647 μg of **2**/g of fungal growth media retarded mycelium growth of *F. graminearum* in vitro by 50%. The addition of **1**, **2**, and their combination to PDA significantly ($P \leq 0.05$) reduced the growth of *F. graminearum* and *F. culmorum* in vitro at concentrations between 316 and 793 μg/g for **1** and 329 and 668 μg/g of growth media for **2** (*24*). Combinations of **1** and **2** showed synergistic activity in reducing mycelial growth. Several studies have also indicated an inhibitory effect of phenolic compounds on mycotoxin production (*38*−*40*). The mycotoxin inhibition property of the soluble ester-bound extracts tested in liquid culture was over 150 μg/g for **2** (*28*). The quantity of free, glycoside-bound, and ester-bound phenolics that we found in the pith of maize is lower than the threshold of inhibition for *Fusarium* growth or mycotoxin production. A very limited quantity of **2** that is soluble as free form or conjugated to short sugar chains was previously quantified in maize flour (*41*). This fraction is more likely to react with *Fusarium.* Because most phenylpropanoids are esterified to the cell wall, it is possible that they have a limited direct biochemical-mediated inhibition of the infecting fungus.

Significant differences among harvest times were found only for ester-bound **2**. Concerning individual genotypes, a significant increase was observed for free **1** in inbreds CO400 and CO433 and for the free and ester-bound **2** in inbreds CO400, CO348, and 73353 (**Table 2**). Furthermore, there was a significant increase along harvest time for glycoside-bound **2** in inbred 73353. Although these increases could suggest a liberation of phenolics during the degradation of tissues by fungal hydrolases and esterases (*37*, *42*), the same trend for most of these

Table 3. Mean Concentrations (μg/g Dry Weight) at Four Harvest Times of the Major Cell-Wall-Bound Phenolics Identified in the Inoculated Pith of Six Inbred Lines in 2004 and 2005

		inbred lines						LSD
phenolic compound[b]	time[c]	CO441	CO400	CO348	73353	CO359	CO433	($P \leq 0.05$)
1	0d	7259.2a	3487.8c	4848.2bc	5817.5ab	4514.1bc	8049.9a	2236.1
	4d	7603.8a	3209.6c	4485.7b	5306.5b	4244.2bc	6590.8a	1119.3
	28d	8902.6a	4813.4a	5960.7a	51393a	5144.0a	8347.2a	
	FINAL	8976.4a	5805.4bc	4828.3c	4934.2c	4951.2c	8346.1ab	2612.4
	LSD ($P \leq 0.05$)							
2	0d	2835.5a	1281.2c/C	1683.9bc	1794.9b	1984.7b	2927.2a	445.7
	4d	2910.7a	1421.8d/BC	1856.2cd	2191.0bc	2095.0c	2569.9ab	447.8
	28d	3233.8a	2524.9a/A	2156.1a	2583.4a	2502.8a	3034.9a	
	FINAL	2223.5cd	2090.4d/AB	2160.8cd	2670.6b	3048.6a	2435.8bc	334.6
	LSD ($P \leq 0.05$)		743.3					
3	0d	64.64ab/A	32.16d	60.67ab	42.19cd	52.31bc	69.15a	12.46
	4d	62.98a/A	37.60b	55.14a	49.72ab	52.23a	62.27a	14.26
	28d	62.38a/A	66.01a	46.47a	51.44a	52.98a	69.72a	
	FINAL	29.66a/B	51.52a	49.56a	46.85a	62.52a	43.54a	
	LSD ($P \leq 0.05$)	16.07						
4	0d	81.68a	40.88c	73.17ab	50.66c	66.38b	83.74a	13.75
	4d	80.50a	43.72b	69.85a	63.48a	70.89a	75.77a	17.23
	28d	84.82a	76.95a	64.50a	66.36a	61.70a	74.03a	
	FINAL	70.67b	99.98a	66.51b	62.35b	78.57b	68.28b	18.54
	LSD ($P \leq 0.05$)							
5	0d	74.85a/A	30.09d	68.92ab	46.94cd	57.38bc	78.45a	14.78
	4d	74.72a/A	40.41c	65.75ab	57.94b	61.17ab	73.80ab	16.27
	28d	72.98a/A	62.48a	54.85a	57.43a	50.57a	58.89a	
	FINAL	30.06a/B	63.40a	47.53a	47.74a	59.66a	37.26a	
	LSD ($P \leq 0.05$)	13.78						
DFAt[d]	0d	211.18a/A	109.14d	202.76ab	139.79cd	176.07bc	231.34a	39.69
	4d	218.19a/A	121.73b	190.74a	171.15a	184.29a	211.84a	47.32
	28d	220.17a/A	205.44a	165.81a	175.23a	165.26a	202.64a	
	FINAL	130.39c/B	214.89a	163.60abc	156.94abc	201.05ab	149.07bc	
	LSD ($P \leq 0.05$)	43.45						

[a] Means within a row followed by the same lowercase letter are not significantly different ($P \leq 0.05$). Uppercase letters were used for the columns when significant. [b] Simple hydroxycinnamates: **1**, *p*-coumaric acid; **2**, *trans*-ferulic acid. Diferulates: **3**, 8-5′ DFA; **4**, 8-O-4′ DFA; **5**, 8–5′ DFA benzofuran form. [c] Time: harvest time (days). [d] DFAt: total diferulate content.

genotypes was observed in the water-inoculated control, and there were no significant differences among treatments (data not shown). In addition, resistant and susceptible genotypes showed the same tendency. Therefore, we concluded that soluble phenolic compounds seem not to be related to a direct response to *F. graminearum* infection.

Cell-Wall-Bound Phenolics. Unsurprisingly, the highest levels of phenolic compounds were released from alkaline treatment of the cell-wall-bound phenolic fractions (**Table 3**). A representative chromatogram of the maize pith extract is shown in **Figure 2**. Arabinoxylan chains reinforced with ester-linked phenolics are major components of cereal walls (*43*). Moreover, during cell-wall deposition, xylans are cross linked by the peroxidase-mediated coupling of ferulate monomers into a complex array of dimers (DFAs) and trimers (*44*).

As with disease severity ratings, the contents of monomers of **1** and **2** were significantly different between genotypes. In agreement with previous studies (*45*−*46*), **1** was found to be the predominant species of the cell wall phenolics in the pith of the genotypes tested. The lowest concentrations of cell-wall-bound **1** (3209.6 μg/g dry weight) and cell-wall-bound **2** (1281.2 μg/g dry weight) were quantified at the beginning of the evaluation, at 4 and 0 days, respectively, in susceptible inbred CO400, whereas the highest concentrations of cell-wall-bound **1** (8976.4 μg/g dry weight) and cell-wall-bound **2** (3233.8 μg/g dry weight) were quantified at the final harvest time (FINAL) and at 28 days, respectively, in susceptible inbred CO441 (**Table 3**). Low contents of cell-wall-bound **1** and **2** in the inbred CO400 suggest a possible role of these compounds in the susceptibility of this genotype along the evaluations; however, resistant and intermediate genotypes did not differ significantly from these. In addition, no significant differences among inoculation treatments were found (data not shown). Monomers of phenolic acids have often been reported as factors in plant−pathogen interactions (*47*); however, the levels of these phenylpropanoids did not show correlations with the resistance classification or disease severity ratings during the 2 years of evaluation in the current study. Our results are in accordance with previous studies focused on maize grain and *F. graminearum* resistance regarding the contents of these monomers (*27*, *28*).

In addition to the **1** and **2** monomers, diferulate isomers 8-5′ DFA (**3**), 8-O-4′ DFA (**4**), and 8-5′ DFA benzofuran (**5**) were found in the insoluble cell-wall-bound fraction (**Table 3**) (structures shown in **Figure 2**). These isomers have been previously identified in the pith and sheath of maize (*45*, *46*). The signal in the UV detector was not clear enough for a definitive identification of 5-5′ DFA because of its trace amount and its co-elution with other compounds.

The highest contents of **3** (69.15 μg/g dry weight), **4** (83.74 μg/g dry weight), and and **5** (78.45 μg/g dry weight) were quantified at the beginning of the evaluation (0 days) in inbred CO433, whereas the lowest concentrations of **3** (32.16 μg/g dry weight), **4** (40.88 μg/g dry weight), and **5** (30.09 μg/g dry weight) were quantified at 0 days in inbred CO400. No significant differences among genotypes were found at 28 days. Significantly higher contents of **4** (99.98 μg/g dry weight) were found in inbred CO400 at grain harvest (FINAL) (**Table 3**). Similarly, the highest concentration of the total content of

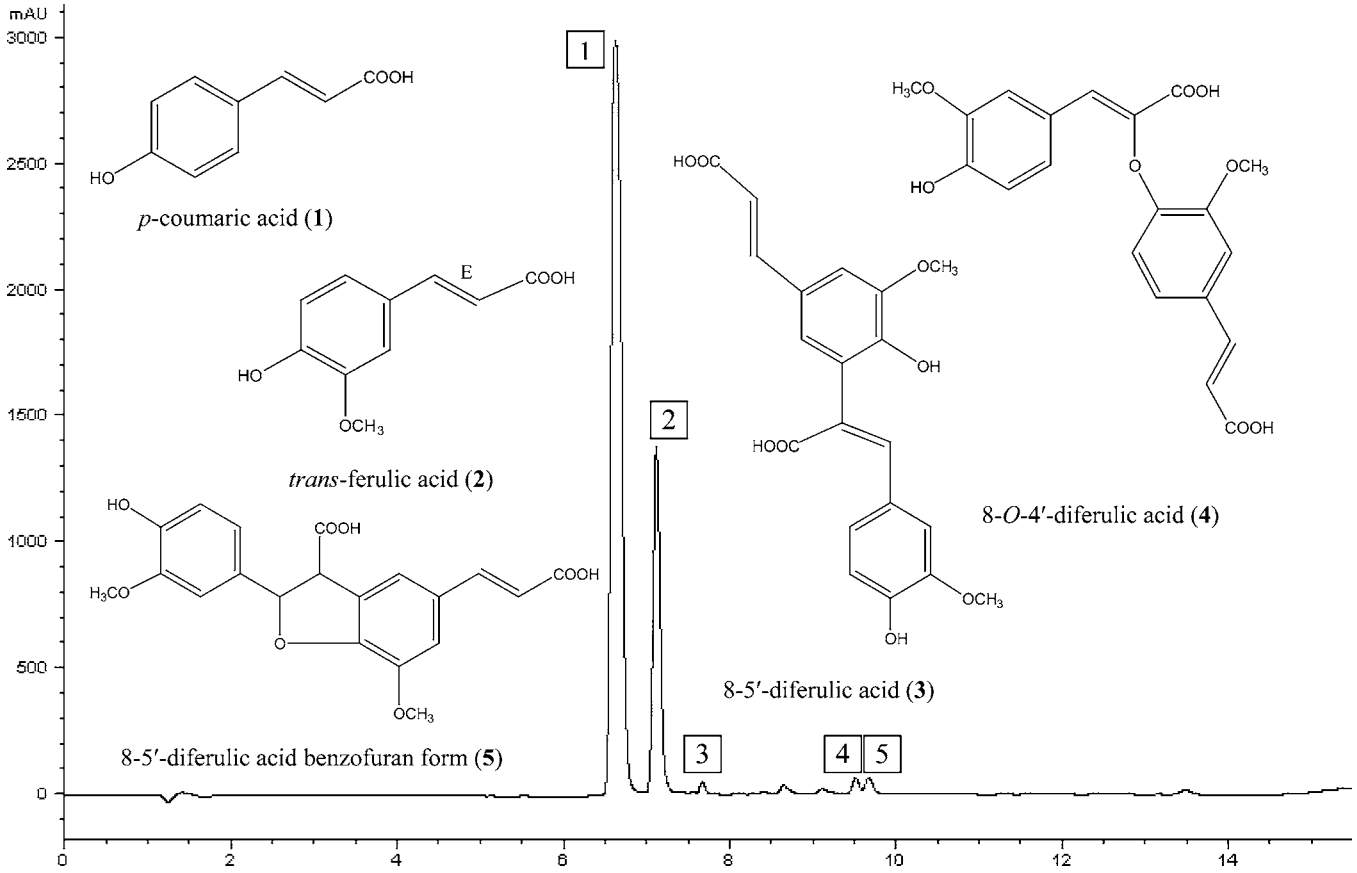

Figure 2. HPLC elution profile at 325 nm of a typical pith sample and chemical structures of the main phenylpropanoids found in the cell wall of maize.

diferulic acids (DFAt) at 0 days (231.34 μg/g dry weight) was found in intermediate inbred CO433, and the lowest concentration of the total content of diferulic acids (DFAt) at 0 days (109.14 μg/g dry weight) was found in susceptible inbred CO400. Four days after inoculation, inbred CO400 showed the lowest concentrations of all of the dimers identified, whereas genotype CO441 showed the highest (**Table 3**).

High levels of diferulates are known to inhibit cell-wall degradation by hydrolases of *Aspergillus* (*26*). *Fusarium graminearum* is known to be an aggressive necrotrophic pathogen; it possesses a large array of extracellular enzymes (e.g., esterases, xylanase, and polygalacturonase) (*48*, *49*) that allow the destruction of the host tissues and consequently a release of nutrients from the dead plant cells. A study with a *F. graminearum* isolate transformed with green fluorescent protein (GFP) suggested that during the first period of the interaction the fungus surrounds the plant cell in the apoplast (*50*). The insolubilization of diferulic compounds in the cell walls can modify its mechanical properties and decrease its extensibility (*43*, *51*, *52*); consequently, the cell wall is less biodegradable (*53*, *54*). The abundance of DFAs in cell walls makes polysaccharides less sensitive to the cell-wall-degrading enzymes of pathogens (*52*, *54*). Therefore, lower contents of DFAs in the cell walls, as found in susceptible inbred CO400 in this study, could facilitate fungal propagation in the early stages of interaction.

Inbreds CO433 and CO441, although susceptible at final harvest, had higher concentrations of DFAs when disease severity ratings were low in the early stages of infection. Furthermore, a significant decrease of **3** and **5** by final harvest time was recorded in CO441, in agreement with the period where the susceptibility of this inbred increased (**Table 1**, **Table 3**). However, there were no significant differences among treatments or genotypes for these compounds at that time (FINAL).

By reinforcing the possible role of DFAs in the early stages of infection, significant negative correlations between DFA contents and disease severity ratings 4 days after inoculation (**3**: $r = -0.79$, $P \leq 0.05$; **4**: $r = -0.72$, $P \leq 0.05$; and **5**: $r = -0.81$, $P \leq 0.05$) were found. No significant correlations were found for other soluble fractions or cell-wall monomers **1** and **2** (data not shown). Moreover, a significant negative correlation was found between the total DFA content and disease severity at 4 days (DFAt: $r = -0.78$, $P \leq 0.05$). These results agree with those in several other studies that have reported significant negative correlations between susceptibility parameters and diferulic acid content (*46*, *55*). Bily et al. (*27*) suggested that the resistance of maize genotypes to *Gibberella* ear rot could be partially due to the higher levels of cross links in the cell wall of kernel pericarp and aleurone cells. In agreement with our results, significant negative correlations were found between disease severity and diferulic acid content.

The increase in the accumulation of the post-infection phenolic contents in cell walls has been reported in several other host parasite interactions, and it was often associated with plant resistance (*52*, *56*−*60*). However, in the present report no significant differences between conidia-inoculated treatment and water-inoculated control were found (data not shown). In this case, the DFAs could function in the pith tissues as preformed resistance barriers prior to infection. Low contents of DFAs in the early stages of infection would leave time for the pathogen to develop abundantly and to produce toxins, breaking a possible resistance response and leading to typical symptoms of disease.

In conclusion, the analysis of a number of maize genotypes did not reveal a clear correlation between resistance/susceptibility to *F. graminearum* and the content of phenolic compounds in different fractions of stalk pith tissue. 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. Nevertheless, disease resistance has multifaceted mechanisms in cereals, and many other components could contribute to a genotype's overall resistance.

ACKNOWLEDGMENT

We thank the corn breeding and pathology crew of ECORC, Agriculture and Agri-Food Canada, and especially T. Woldemariam for preparing fungal inoculum and assisting in sample collection. We thank A. Saleem for HPLC advice. R.S. acknowledges a postdoctoral fellowship from the Autonomous Government of Galicia (Spain). This research was supported by the Natural Sciences and Engineering Research Council (MFA and discovery programs).

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Received for review March 6, 2007. Revised manuscript received May 2, 2007. Accepted May 3, 2007. R.S. acknowledges funding from the Secretaria Xeral de Investigación e Desenvolvemento, Xunta de Galicia, Spain.

JF070641E