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Putative Role of Pith Cell Wall Phenylpropanoids in *Sesamia nonagrioides* (Lepidoptera: Noctuidae) Resistance

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The stem borer *Sesamia nonagrioides* (Lefèbvre) is the most important insect pest that attacks maize, *Zea mays* L., in northwestern Spain. Host plant resistance to this borer was investigated in relation to the cell wall phenylpropanoids content in the pith. Eight inbred lines that differ in resistance were analyzed. Three major simple phenolic acids, *p*-coumaric, *trans*-ferulic, and *cis*-ferulic acids, and three isomers of diferulic acid, 8-5′, 8-O-4′, and 8-5′b (benzofuran form), were identified. The amount of all these compounds was correlated with the resistance level in the genotypes, with the resistant inbreds having the highest concentrations. The role of these compounds in cell wall fortification and lignification is well-documented, suggesting their possible intervention in *S. nonagrioides* resistance. Future studies that focus on these compounds could be useful to enhance *S. nonagroides* resistance.

KEYWORDS: ***Zea mays*; *Sesamia nonagrioides*; resistance; phenylpropanoids; pith**

INTRODUCTION

Sesamia nonagrioides (Lefèbvre) (Lepidoptera: Noctuidae) has become a serious pest of corn, *Zea mays* L., during recent years in the Mediterranean regions (*1*−*3*). In Spain, it has two generations per year. First generation larvae affect plant growth by leaf feeding during the whorl stage, causing direct yield losses, although the main importance is on the second generation larvae that feed on the pith stem during plant development, reducing plant growth, grain size, and causing indirect yield losses as a consequence of lodging (*4*).

Host-plant resistance has become the center of attention in integrated pest management programs. Understanding the mechanism of host-plant resistance will aid in the identification of genes that confer resistance and in the development of resistant varieties through classical breeding or biotechnology. Plant resistance studies have often focused on the identification of biologically active compounds as possible defense mechanisms. In maize, there is considerable scientific documentation on the antibiotic effect of 3,4-dihydro-2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA) and its degradation products, 3,4-dihydro-2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3-one and 6-methoxybenzoxazolinone, on *Ostrinia nubilalis* (Hübner), *Diatraea grandiosella* (Dyar), *Spodoptera frugiperda* (J. E. Smith), and *S. nonagrioides* (*5*−*8*). However, the DIMBOA concentration decreases as the plant grows, so it fails to protect the plants from attack of second generation borers, as well as some other lepidopteran pests of corn (*9*).

There has been a significant increase in the research interest directed toward the hydroxycinnamic acids in maize. Hydroxycinnamic acids and their derivatives are ubiquitous in the plant kingdom in both soluble and bound forms and have roles in cell wall structure and insect defense (*10*). Free *p*-coumaric (**1**) and ferulic (**2**) acids have been reported to be phagostimulants for the spotted stem borer, *Chilo partellus* (Swinhoe) (*11*), but inhibit feeding of other insects such as *Sitophilus zeamais* (Motschulsky) (*12*) (**Figure 1**). Compound **1** was toxic to the two-spotted spider mite *Tetranychus urticae* Koch (*13*), and **2** was toxic in some situations to the sap beetle, *Carpophilus hemipterus* (L.) (*14*). Recent studies have suggested that higher quantities of free **1** in the pith of resistant maize inbreds could contribute in the resistance to *S. nonagroides* (*15*). In the same study, it is suggested that **1** and **2** may comprise a pool from which other various hydroxycinnamic acids and their esters could be formed, providing mechanical resistance through cell wall fortification.

Cell wall bound forms of phenolic acids are the major phenylpropanoid components in cereals and consist largely of **1** and **2** hydroxycinnamic acids (*16*). Several reports have established that these phenolics are ester-linked to cell wall polysaccharides (arabinoxylan) and may be dimerized under the

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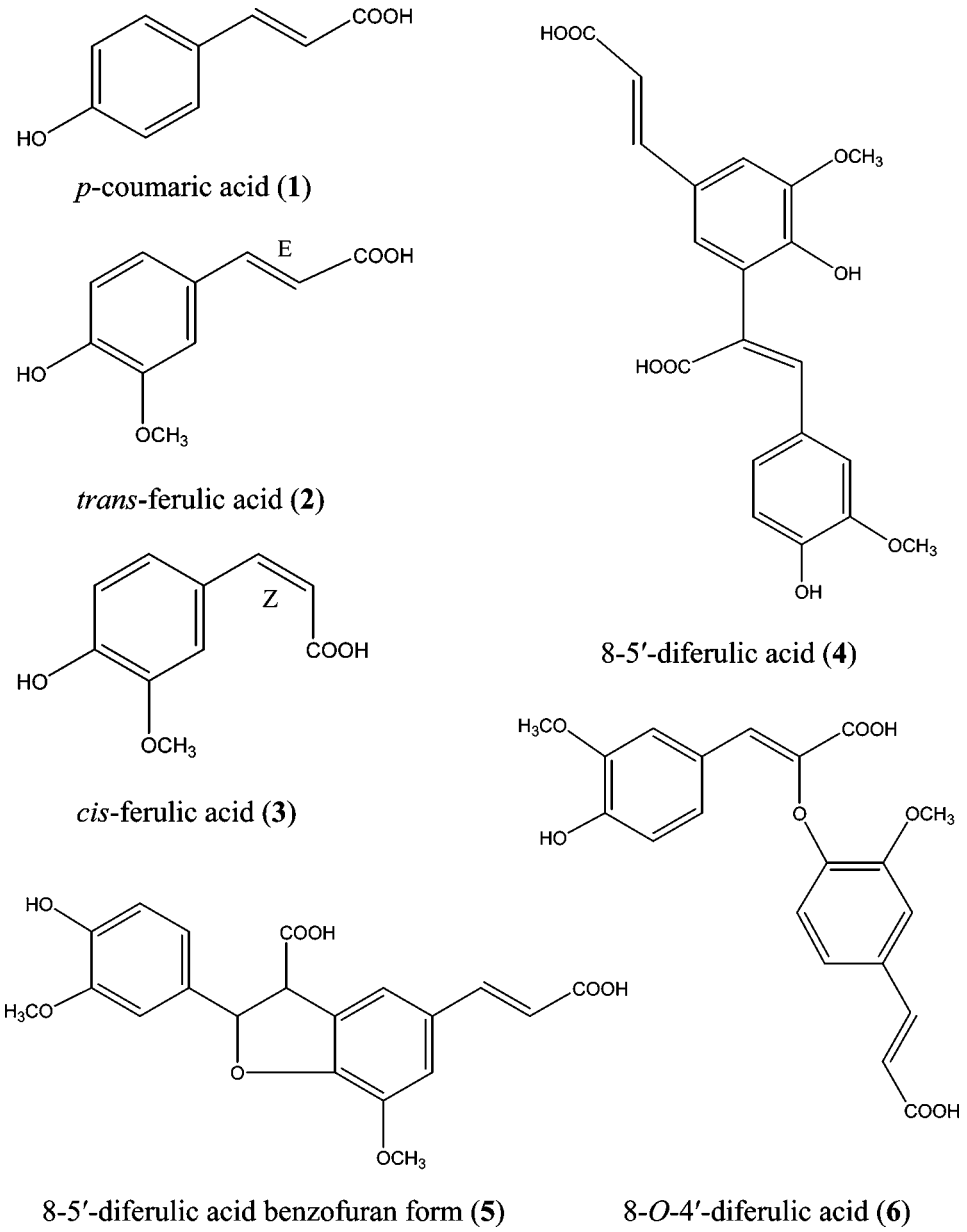

Figure 1. Chemical structures of cell wall phenylpropanoids identified in the pith tissue of eight maize inbred lines.

activity of cell wall or intracellular peroxidases (*17*). Such dimmers [mainly diferulic acids (DFAs)] (**Figure 1**) can serve as cross-linking agents within the cell wall to increase mechanical strength and render tissue less accessible to herbivores. Phenylpropanoids have been studied as a line of defense against maize insect pests feeding on different tissues. In the whole kernel, the role of cell wall bound phenolics in resistance to *S. zeamais* has been demonstrated (*18*, *19*), and recently, several isomers of DFAs have been identified and characterized within the maize pericarp and aleurone and proposed as structural components of cell wall (*20*) and resistance factors to maize weevil (*S. zeamais*) (*21*) and Fusarium ear rot [*Fusarium graminearum* (Schwabe)] (*22*). In addition, some phenylpropanoid derivatives present in maize kernels have shown inhibitory activity against aflatoxin biosynthesis (*23*). In the leaves, higher levels of cell wall phenolics have been found in resistant genotypes to European corn borer (*Ostrinia nubilalis*) (*24*) and the tropical borers, *Diatraea grandiosella* (Dyan) and *Diatraea saccharalis* (Fabricius) (*25*). The current study extends previous research on phenolic compounds in the grain and leaves of maize by determining if resistance to *S. nonagrioides* can be related with cell wall fortification in the pith of maize stalks. Our goal was to determine the relationship between the concentration of cell wall phenylpropanoids and the level of resistance for the stem and the pith.

MATERIALS AND METHODS

Experimental Design. Eight maize inbred lines previously evaluated for stem and pith resistance were used in this study (**Table 1**). Stem resistance was based mainly on reduced gallery length after artificial infestation with *S. nonagrioides* in the field (*26*), while pith resistance was based on laboratory bioassays, in which larval growth was measured after feeding the larvae with maize pith tissue (*27*). Inbred lines were grown at Pontevedra, a location in northwestern Spain (42° 25′ N, 8° 38′ W and 20 m above sea level) in 2002 and 2003. The experimental design was a randomized complete block design with three replicates. Each plot had two rows spaced 0.80 m apart, and each row consisted of 25 two-kernel hills spaced 0.21 m apart. After plants were thinned to one per hill, the plant density was approximately 60000 plants ha $^{-1}$. The soil type was acid sandy loam. Trials were irrigated once, and cultural operations, fertilization, and weed control were carried out according to local practices.

Table 1. Pedigree, Stem Resistance, and Pith Resistance for the Eight Maize Inbred Lines Used in This Study

inbred line	pedigree	stem resistance[a]	pith resistance[b]
A509	A78 × A109	resistant	resistant
CM151	Mt42 × WF9[2]	resistant	resistant
CO125	Pfister 44	resistant	resistant
EP39	Fino	resistant	resistant
EP42	Tomiño	susceptible	susceptible
EP47	(EP4 × A239) EP4[2]	susceptible	susceptible
F473	Doré de Gomer	resistant	susceptible
PB130	Rojo vinoso de Aragón	resistant	susceptible

[a] Stem resistance classification based on reduced gallery length after artificial infestation in the field (*26*). [b] Pith resistance classification based on the growth of the larvae reared on the pith of each inbred line (*27*).

To accurately define the inbred's silking date, plots were checked until 50% of plants had exposed silks. At silking time and 15 and 30 days after silking, the fourth above-ground internode was hand-harvested. Five to eight plants were collected depending on the amount of tissue. The pith tissue was obtained by manually removing the rind and immediately frozen (−20 °C).

Cell Wall Phenylpropanoids Extraction. Extraction of cell wall phenolics was based on a procedure previously described (*28*) with some minor modifications. The dry pith material was ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA) with a 0.75 mm screen, and then, 1 g of sample was extracted in 30 mL of 80% methanol and mixed with a Polytron mixer (Brinkman Instruments, Westbury, NY). Samples were extracted for 1 h and next centrifuged for 10 min at 1000*g*. The supernatant was discarded, and the remaining pellet containing the cell wall bound material was then shaken in 20 mL of 2 N NaOH under nitrogen flow 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 (Savant Instruments, Holbrook, NY) for 2 h at medium settings without a radiant cover. The final extract was dissolved in 3 mL of high-performance liquid chromatography (HPLC) grade methanol and stored at −20 °C prior to HPLC analysis.

HPLC Analysis of Cell Wall Phenylpropanoids. Each sample was filtered through a 0.2 μm pore poly(tetrafluoroethylene) filter (Chromatographic Specialties, Brockville, ON) before analysis. All analyses were performed using a Hewlett-Packard ChemStation series 1100 chromatograph with a YMC ODS-AM (Waters, Milford, MA) narrow bore column (100 mm × 2 mm i.d.; 3 μM particle size). The solvent system consisted of acetonitrile (A) and trifluoroacetic acid (0.05%) in water (pH 3.4) (B) at a flow rate of 0.3 mL/min. The elution conditions 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, then isocratic elution with 100:0 for 4.5 min, finally returning to the initial conditions in 3 min. The sample injection volume was 4 μL, and the elution profiles were monitored by UV absorbance at 325 and 280 nm. Retention times were compared with freshly prepared standard solutions of *p*-coumaric and ferulic acids (Sigma, St. Louis, MO) and of 5-5′-DFA synthesized by the group of N. Towers (University of British Columbia, Vancouver, Canada). The absorption UV spectra of other diferulates were compared with published spectra (*29*). To confirm the presence and identities of different DFAs, a liquid chromatography−mass spectrometry procedure with atmospheric pressure chemical ionization (APCI) in positive mode was used on selected samples (*28*).

Statistical Analysis. Combined analyses of variance (ANOVA) for the cell wall phenylpropanoids identified were computed with the PROC GLM procedure of SAS (*30*). Year and replication were considered random. Comparisons of means among inbreds were made by Fisher protected least significant difference (LSD) method (*31*). We also carried out contrast among groups of resistant and susceptible inbreds based on stem and pith resistance. We grouped the inbreds in resistant (R), susceptible (S), and variable (V). Inbred lines stem resistant and pith susceptible, or vice versa, were classified as variable (V).

Table 2. Mean Concentration for Cell Wall Phenylpropanoids Identified in the Pith of Eight Maize Inbred Lines Grown in Pontevedra in 2002 and 2003[a]

	μg/g[b]					
	simple hydroxycinnamates[c]			diferulates[c]		
inbred line	**1**	**2**	**3**	**4**	**5**	**6**
A509	6437 b	1795 b	86.8 b	77.6 b	100.9 ab	78.0 b
CM151	4950 c	1784 b	71.6 c	71.7 b	81.6 bc	68.9 b
CO125	8486 a	2144 a	118.7 a	96.7 a	124.6 a	98.8 a
EP39	3953 d	1859 b	77.1 bc	72.1 b	95.2 bc	73.2 b
EP42	4874 c	1769 b	72.2 c	62.2 bc	73.7 cd	60.4 b
EP47	4280 cd	1885 b	59.7 d	68.3 b	75.7 bc	62.5 b
F473	4280 cd	1284 c	49.2 de	44.8 c	44.9 e	38.8 c
PB130	3964 d	1038 c	42.2 e	46.3 c	49.4 de	41.4 c
LSD ($P \leq 0.05$)	788	250	11.1	17.6	16.8	17.9

[a] Means within a column followed by the same letter are not significantly different ($P \leq 0.05$). [b] μg/g dry weight concentration. [c] Simple hydroxycinnamates: **1**, *p*-coumaric acid; **2**, *trans*-ferulic acid; and **3**, *cis*-ferulic acid. Diferulates: **4**, 8-5′-DFA; **5**, 8-*O*-4′-DFA; and **6**, 8-5′-DFA benzofuran form.

RESULTS AND DISCUSSION

The maize inbreds used in this study were selected to represent a wide spectrum of susceptibility to *S. nonagrioides* (**Table 1**), and as expected, we observed a significant variation in the cell wall phenylpropanoid contents of their pith tissue (**Table 2**). Data for three major simple phenolic acids, *p*-coumaric (**1**), *trans*-ferulic (**2**), and *cis*-ferulic (**3**) acids, and three isomers of DFA, 8-5′-DFA (**4**), 8-*O*-4′-DFA (**5**), and 8-5′-b-DFA (benzofuran form) (**6**), identified and quantified by HPLC are shown (**Figure 1** and **Table 2**). The identified compounds were grouped into simple hydroxycinnamates (**1**−**3**) and diferulates (**4**−**6**).

Significant differences ($P \leq 0.05$) among inbreds were found for the content of monomers of **1**−**3** in the combined ANOVA (**Table 2**). Compound **1** was found to be the predominant phytochemical species of the cell wall phenolics in the pith of all genotypes studied. Compound **2** is 70% less concentrated. Compounds **1** and **2** are the most prevalent phenylpropanoids of maize stems (*32*, *33*). The cis stereoisomer **3** was only a minor constituent (less than 5% of the total ferulic acid) and may be partly the result of a stereoisomerization during the extraction process. This study is different from previous studies with grain and leaves, which reported a greater amount of **2** than **1** (*21*, *25*), but our results are in accordance with previous studies focused on the pith, where the **1** was the most abundant phenolic compound (*34*). The inbred line CO125 had the highest concentrations for both compounds, while the EP39 and PB130 had the lowest concentrations for **1** and **2**, respectively. The individual inbreds EP42 (susceptible) and CM151 (resistant) possessed similar concentrations for both monomers (**Table 2**). However, in contrast analyses (between all resistant and all susceptible inbreds), there were significantly larger concentrations of **1** in all resistant groups and higher amounts of **2** in the pith resistant group (**Table 3**). In individual inbreds, different mechanisms may operate, such as the presence of an antixenotic compound that could act as an insect repellent or higher hardness of tissues that decrease the feeding facilities, altering this way the actual relation of these compounds with the *S. nonagrioides* resistance (*15*, *27*).

Other studies reported that **1** and **2** act as factors in host-plant interactions (*21*, *22*, *34*, *35*). Populations of maize with

Table 3. Contrast for Cell Wall Phenylpropanoids Identified in the Pith of Eight Maize Inbred Lines Grown in Pontevedra in 2002 and 2003[a]

	μg/g[b]					
	simple hydroxycinnamates[c]			diferulates[c]		
resistance group	**1**	**2**	**3**	**4**	**5**	**6**
			stem resistance			
resistant	5351 a	1634 a	73.9 a	67.5 a	81.9 a	65.8 a
susceptible	4537 b	1834 a	66.0 a	65.5 a	75.3 a	61.8 a
LSD ($P \leq 0.05$)	782					
			pith resistance			
resistant	5987 a	1899 a	89.6 a	80.0 a	101.7 a	80.4 a
susceptible	4349 b	1494 b	55.8 b	55.4 b	60.9 a	50.8 b
LSD ($P \leq 0.05$)	595	162	8.4	6.4		7.0
			stem and pith resistance			
resistant	5888 a	1931 a	91.1 a	81.0 a	102.1 a	81.4 a
susceptible	4537 b	1834 a	66.0 b	65.5 b	75.3 b	61.8 b
variable	4864 b	1364 b	58.9 b	55.8 c	64.4 b	52.3 b
LSD ($P \leq 0.05$)	822	199	12.0	9.3	14.4	10.2

[a] Means within a column and resistance group followed by the same letter are not significantly different ($P \leq 0.05$). [b] μg/g dry weight concentration. [c] Simple hydroxycinnamates: **1**, *p*-coumaric acid; **2**, *trans*-ferulic acid; and **3**, *cis*-ferulic acid. Diferulates: **4**, 8-5′-DFA; **5**, 8-*O*-4′-DFA; and **6**, 8-5′-DFA benzofuran form.

high phenolic acid content in the grain were more resistant to attack by *S. zeamais* than those with low phenolic acid content (*36*). Both cell wall bound **1** and **2** showed higher levels in leaves of resistant lines to *D. grandiosella* and *D. saccharalis*, with 19% more **1** and 23% more **2** (*25*). In addition, the concentrations of these compounds were significantly and negatively correlated to leaf feeding damage by different borer species (*25*, *35*), and further investigations showed a negative relationship between damage parameters for the pith tissue and **1** content (*34*). Concerning the possible way that these compounds could contribute to the resistance, **1** monomers are primarily esterified to syringil units in lignins (*37*−*39*), so its concentration is probably related to cell wall lignification (*40*), while **2** is esterified to arabinose subunits of arabinoxylan chains and jointly with its dimers (DFAs) could cross-link arabinoxylans chains to each other and to lignin enhancing the cell wall strengthening and stiffening (*40*−*42*). In this way, both compounds could be related with insect resistance due to an increase of the tissues mechanical strength that could decrease larvae penetration and feeding, a mechanism already proposed for some other authors (*21*, *25*, *34*, *43*).

In addition, recent studies have noted diferulates as an example of secondary metabolites with functions in conferring structural reinforcement and hence resistance (*21*, *25*). Up to eight forms of dehydrodimers of ferulic acid (DFAs) have been discovered (*44*) since the first isomer, the 5-5′ form, was first reported in the cell wall of *Triticum aestivum* (*44*). Four isomers were previously identified in grain and leaves of maize (*21*, *25*). In this study, we clearly observed the presence of three of them in the pith tissue: **4**−**6** (**Table 2**). The signal in the UV detector was not clear for definitive identification of 5-5′-DFA due to its trace amounts and the coelution with some other compounds. Therefore, it was not included in the study. The 5-5′-DFA represents only a small and variable portion of the total diferulates in cell grass walls (*45*).

Significant differences ($P \leq 0.05$) among inbreds were found for the main diferulates, with the resistant inbred CO125 having the highest values and the variable inbreds F473 and PB130 the lowest (**Table 2**). Furthermore, in the stem and pith classification, the resistant genotypes showed the highest amounts of diferulates, and the variable genotypes F473 and PB130, classified as susceptible in pith resistance, showed the lowest (**Table 3**). This reinforces the possible role of phenylpropanoids in *S. nonagroides* resistance. Cross-linking of polysaccharides by DFAs is considered particularly important in the fortification of the cell walls (*40*), and these dimers have been correlated with tissue toughness and pest resistance in previous studies (*21*, *25*). Additional evidence for the possible role of DFAs was provided by the observation that DFAs increased in cycles of selection for borer resistance in the maize synthetic BS9 (*34*). On the basis of this report and on the results obtained in this study, three cycles of recurrent selection to improve stalk resistance to *S. nonagrioides* in the maize synthetic EPS12 are currently in evaluation for the phenylpropanoids content in the pith.

Table 4. Mean Concentration of Cell Wall Phenylpropanoids Identified in the Pith of Eight Maize Inbred Lines Grown in Pontevedra in 2002 and 2003 during Three Sampling Dates[a]

	μg/g[b]					
	simple hydroxycinnamates			diferulates		
sampling	**1**	**2**	**3**	**4**	**5**	**6**
at silking	4307 a	1395 b	55.6 a	55.8 a	59.6 a	50.3 a
15 days after silking	4683 a	1520 b	64.4 a	57.9 a	73.1 a	57.5 a
30 days after silking	6495 a	2180 a	96.3 a	88.1 a	108.7 a	87.3 a
LSD ($P \leq 0.05$)		153				

[a] Means within a column and resistance group followed by the same letter are not significantly different ($P \leq 0.05$). [b] μg/g dry weight concentration. [c] Simple hydroxycinnamates: **1**, *p*-coumaric acid; **2**, *trans*-ferulic acid; and **3**, *cis*-ferulic acid. Diferulates: **4**, 8-5′-DFA; **5**, 8-*O*-4′-DFA; and **6**, 8-5′-DFA benzofuran form.

In relation with the changes in the concentration during maturation, an increase of **2** along the three harvest times was observed (**Table 4**). This agrees with recent observations that showed a continued accumulation of **2** in the secondary cell walls, long after internode elongation had ceased (*33*). An increase along harvest times was also noted for the other compounds, although the whole period checked was not long enough to confirm significant differences (**Table 4**).

In summary, the cell wall phenylpropanoids in the pith tissue of maize stalks appear to be important in conferring resistance to *S. nonagrioides* attack. Their role in the cell wall fortification has been noted in the literature, and this structural resistance could be more difficult to overcome than a toxic defense. In the future, quantitative traits locus mapping for these cell wall components could be a useful tool for enhancing resistance to *S. nonagrioides*.

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