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Diferulate Content of Maize Sheaths Is Associated with Resistance to the Mediterranean Corn Borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae)

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The leaf sheaths of selected inbred lines of maize (*Zea mays* L.) with variable levels of stem resistance to the Mediterranean corn borer *Sesamia nonagrioides* (Lefèvbre) were evaluated for antibiotic effect on insect development. Phytochemical analyses of leaf sheaths were conducted for cell wall phenylpropanoid content to gain a better understanding of maize-resistance mechanisms. Laboratory bioassays established that sheath tissues from different genotypes significantly affected the growth of neonate larvae. Three hydroxycinnamates, *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. Significant negative correlations were found between larvae weight and diferulic acid content for six genotypes. These results are in agreement with previous studies concerning the role of cell wall structural components in stem borer resistance.

KEYWORDS: ***Zea mays*; *Sesamia nonagrioides*; sheath; feeding bioassay; resistance; phenylpropanoids**

INTRODUCTION

Maize, *Zea mays* L., grown in northwestern Spain and the Mediterranean area is commonly exposed to two distinct Mediterranean corn borer *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae) generations each growing season (*1*, *2*). Infestation commences at an early phenological stage of plant growth. After completing the first generation, stem borers of the second generation attack maize at its reproductive stage (*3*). Second-generation egg masses are deposited between the leaf sheath and the stem, usually on the internodes below the primary ear. After hatching, emerging larvae move toward the lower part of the internode while they feed on the leaf sheath for 10−14 days (*4*, *5*). Later, second-generation larvae feed on the pith tissue, reducing plant growth and yield and increasing plant lodging.

Variability in resistance to this stem borer has been detected among populations and inbred lines (*6*−*8*), and a considerable amount of research has been focused on the maize pith as the tissue responsible for that resistance (*4*, *9*, *10*). However, these studies showed that although antibiosis of maize pith plays a role in the resistance of some genotypes, other resistance mechanisms may be involved.

Because newly emerged larvae are often limited in their dispersal abilities, another factor to consider is the initial feeding in the leaf sheath. The establishment of newly hatched *S. nonagrioides* larvae on a maize plant is a biological occurrence the success of which depends on a precarious balance among a number of factors operating for or against larval survival. Different physical factors could be responsible for high mortality, such as drowning, dislodging, desiccation, and predation. In addition, plant-borne factors could contribute to the resistance of borer establishment. Laboratory bioassays, where insect development is evaluated without the confounding effects of environmental factors, may facilitate the identification of detrimental effects due to feeding on specific tissues, whereas chemical analyses of these tissues could help in the identification of the resistance factors. In this sense, phenylpropanoid compounds (**Figure 1**) have been studied as a line of defense against corn insect pests feeding on different tissues. In kernels, it has been demonstrated that cell wall bound phenolics are involved in the resistance to the maize weevil *Sitophilus zeamais* Motschulsky (*11*, *12*), and Gibberella ear rot [*Fusarium graminearum* (Schwabe)] (*13*). In the leaves, higher levels of cell wall phenolics have been found in genotypes resistant to the European corn borer [*Ostrinia nubilalis* (Hübner)] (*14*) and

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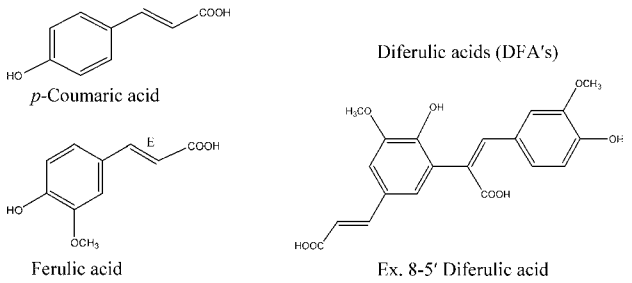

Figure 1. Structures of cell wall phenylpropanoids present in maize tissues.

the tropical borers *Diatraea grandiosella* (Dyan) and *Diatraea saccharalis* (Fabricius) (*15*). Furthermore, recent studies with the maize pith have reported higher quantities of cell wall phenylpropanoids in genotypes resistant to *S. nonagrioides* (*16*). The specific objectives of the current study were (*1*) to study the influence of the leaf sheaths from different maize genotypes on the initial development of second-generation larvae of *S. nonagrioides*, particularly the effect on larval weight gain and survival, and (*2*) to determine the relationship between the concentration of cell wall phenylpropanoids and the level of resistance in the leaf sheaths.

MATERIALS AND METHODS

Plant Materials and Field Plots. Eight inbred lines with different levels of stem resistance to *S. nonagrioides* were used in this study. Inbreds A509, CM151, CO125, EP39, F473, and PB130 have reduced gallery length under field artificial infestation, whereas inbreds EP42 and EP47 are highly susceptible (*8*). 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, using common agronomic practices. Two planting dates were tested in 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 seedlings had been thinned to one plant per hill, plant density was approximately 60000 plants ha^{-1}.

Leaf Sheath Bioassays. *S. nonagrioides* larvae reared in the laboratory for one generation were fed on leaf sheaths from seven inbred lines under controlled conditions. Plant emergence for the inbred F473 failed in two of the three trials; consequently, this inbred was harvested for phytochemical analysis only. Three bioassays were arranged according to the field plots. Single neonate larvae were weighed using a microbalance (accurate to 0.001 mg) to record initial weights (average weight = 2 mg) and thereafter individually transferred to 50-mL plastic cups containing leaf sheath sections (±4 cm^2). All sheaths were harvested from the fourth internode above-ground starting at silking time (period with the highest natural infestation). Sixty larvae were fed on each inbred line in each test. The cups were then placed in a controlled environment room for 12 days at 26 °C with a photoperiod of 14:10 (L/D) h. Fresh leaf sheaths were replenished every 4 days, and larval weight and survival were recorded. Leaf sheaths were replaced to avoid deterioration; the tissue (±4 cm^2) was not completely eaten at any time or for any genotype.

Phenylpropanoid Analysis. In the 2002 trial and the late planting of the 2003 trial, five to eight sheaths from each inbred line and replication were collected from the fourth above-ground internode and immediately frozen (−20 °C). Different periods were assessed to check possible changes in the concentration (silking time and 15 and 30 days after silking). Extraction of cell wall phenolics was based on a procedure previously described with some minor modifications (*16*). One gram of dry ground material 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 then centrifuged for 20 min at 1500*g*. The pellet was shaken in 20 mL of 2 N NaOH under nitrogen flow for 4 h, and after that the pH was adjusted to 2.0. After centrifugation, the supernatant was collected and the pellet washed twice with distilled water (10 mL each time). Supernatants were pooled and then extracted twice with ethyl acetate (40 mL each time). Collected organic fractions were combined and reduced to dryness. 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.

High-Pressure Liquid Chromatography. Analyses were performed using a Hewlett-Packard ChemStation series 1100 chromatograph with a YMC ODS-AM (Waters, Milford, MA) narrow-bore column (100 × 2 mm i.d.; 3 μm particle size). A binary gradient with acetonitrile (A) and trifluoroacetic acid (0.05%) in water (pH 3.4) (B) at a flow rate of 0.3 mL/min was used. 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 254 nm. Retention times were compared with freshly prepared standard solutions of *p*-coumaric and ferulic acids (Sigma, St. Louis, MO). The absorption UV spectra of diferulates (DFAs) were compared with published spectra (*17*).

Statistical Analysis. Combined analyses of variance (ANOVA) for larval weight, larval survival, and cell wall phenylpropanoid content were computed with the PROC GLM procedure of SAS (*18*). Year and replication were considered to be random. Comparisons of means among inbreds were made by the least significant difference method (LSD). Pearson correlation analysis between the different compounds (involving two trials) and larval weight at 12 days was calculated; in addition, linear regression analyses were carried out (dependent variable: weight). Finally, the log rank statistic was used to compare survival distributions (*9*, *19*). The statistic determined whether differences between survival functions of larvae reared on different genotypes were significant at the 0.05 probability level [LIFETEST procedure of SAS (*18*)].

Table 1. *S. nonagrioides* Larval Weight and Survival after Initial Development on Leaf Sheaths of Seven Inbred Lines of Maize Grown in Pontevedra in 2002 and 2003 across Three Bioassays[a]

	larval weight after feeding (mg)				larval survival (%)		
inbred line	0 days	4 days	8 days	12 days	4 days	8 days	12 days
A509	2.14a	8.65bc	27.98bc	66.72b	87.8a	75.6a	66.9a
CM151	2.17a	7.23c	23.52c	51.26c	84.4a	71.7a	60.6a
CO125	2.28a	8.57bc	27.62bc	70.61b	86.1a	67.2a	58.9a
EP39	2.15a	10.20ab	33.89ab	74.21b	83.3a	74.4a	63.9a
EP42	2.39a	10.79a	37.26a	98.34a	83.3a	72.2a	66.7a
EP47	2.39a	9.83ab	31.67ab	67.54b	90.0a	74.4a	67.2a
PB130	2.38a	9.99ab	34.19ab	75.76b	88.3a	75.6a	67.8a
LSD ($P \leq 0.05$)		2.08	7.54	14.82			

[a] Means within a column followed by the same letter are not significantly different.

RESULTS AND DISCUSSION

S. nonagrioides larval weight and survival were affected in different ways by the inbred lines evaluated in the current study (**Table 1**). Although larvae reared on leaf sheaths of the inbred CO125 tended to have lower survival (percent), there were no significant differences among genotypes. Furthermore, survival distributions of larvae fed on different genotypes of maize were equivalent because differences among log-rank values were not significant (data not shown). In contrast, significant differences in larval weight were apparent 4, 8, and 12 days after the larvae were placed on the various inbred leaf sheaths. Larvae fed on EP42 were significantly heavier than larvae fed on CM151, A509, and CO125 at all days evaluated. The weight of larvae fed on inbreds CM151 and EP42 were significantly different from every other inbred after 12 days, with CM151 having the smallest larvae and EP42 the heaviest.

Table 2. Mean Concentrations (Micrograms per Gram)[a] for Cell Wall Phenylpropanoids Identified in the Leaf Sheaths of Eight Inbred Lines of Maize Grown in Pontevedra in 2002 and 2003[b]

	simple hydroxycinnamates			diferulates		
inbred line	**1**	**2**	**3**	**4**	**5**	**6**
A509	5001a	2204b	253cd	93.2ab	112.8abc	95.8abc
CM151	3495cde	1930c	216d	77.0b	92.7c	87.2bc
CO125	3814bcd	2488a	314ab	99.7a	129.3a	102.1ab
EP39	3123e	2211b	208d	90.7ab	122.4ab	94.9abc
EP42	3845bc	2503a	363a	80.0b	90.8c	79.6c
EP47	3234de	2549a	339ab	102.2a	121.4ab	107.7a
F473	4368b	2209b	300bc	87.8ab	98.0cb	87.2bc
PB130	3934bc	2032bc	243d	83.2b	89.3c	82.4c
LSD ($P \leq 0.05$)	591	252	56	16.4	28.6	19.5

[a] Dry weight concentration. [b] Means within a column followed by the same letter are not significantly different. [c] Simple hydroxycinnamates: **1**, *p*-coumaric acid; **2**, *trans*-ferulic acid; **3**, *cis*-ferulic acid. Diferulates: **4**, 8-5′ DFA; **5**, 8-O-4′ DFA; **6**, 8-5′ DFA benzofuran form.

A previous study reported differences in the weight and survival of *S. nonagrioides* larvae when fed on pith tissue from the same genotypes; inbred lines CM151, CO125, and EP39 showed pith resistance, whereas lines A509, EP42, EP47, and PB130 were highly susceptible (*9*). However, it was suggested that pith antibiosis alone was not sufficient to supply enough field resistance in some cases. According to this conclusion, in the current study we have checked a possible role of the sheath tissues in the overall resistance of some genotypes. Larvae reared on inbreds PB130, CO125, EP39, EP47, and A509 exhibited intermediate growth; the inbred CM151 had an antibiotic sheath that reduced the larvae weight, whereas higher larval development was found in the inbred EP42. Larger differences are shown in these results, in contrast with preceding studies that reported low sheath antibiosis to *S. nonagrioides* for the maize composite EPS12, except for the resistant Bt-hybrid checks (*5*).

Among the possible resistance components related to differences in larval development are antibiotic factors, such as 3,4-dihydro-2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA), or structural components that reduce the quality of or accessibility to nutrients (*14*, *20*, *21*). DIMBOA is found in maize as a glycoside at high levels during the early stages of plant development; however, DIMBOA concentration decreases as the plant grows, so this compound fails to protect the plants from the attack of the second generation of various lepidopteran pests (*22*). High fiber content alone may increase the bulk density of the tissue, but in the presence of phenylpropanoid−carbohydrate complexes, the strength may increase, thereby providing tougher physical barriers that restrict insect penetration into sheath tissues (*23*).

Leaf sheaths of the corn genotypes tested showed significant differences in their cell wall phenylpropanoid contents (**Table 2**). Three major simple hydroxycinnamates, *p*-coumaric (**1**), *trans*-ferulic (**2**), and *cis*-ferulic (**3**) acids, were identified and quantified by HPLC (**Table 2**). In addition, bound forms of **2** can be dimerized by peroxidases to form cross-links between arabinoxylans chains (*24*, *25*) (**Figure 2**). Three isomers of diferulic acid (DFA), 8-5′-DFA (**4**), 8-O-4′-DFA (**5**), and 8-5′b-DFA (**6**) (benzofuran form), were present in the leaf sheaths (**Table 2**). **1** usually forms few cross-linked structures by radical coupling mechanisms; however, it can undergo photocatalyzed cyclodimerization to form truxillic and truxinic acids (*26*). Nevertheless, these cyclobutane dimers were not detected in the samples.

Significant differences among inbreds were found for the content of hydroxycinnamates in the combined ANOVA (**Table 2**). The genotypes A509 and EP39 showed the highest and lowest concentrations for **1**, respectively, whereas the inbred EP42 showed the highest concentration for **2** and CM151 the lowest. However, larvae fed on EP42 were the heaviest and larvae fed on CM151 the lightest (**Table 1**). This result was unexpected, in that such an extensive body of evidence has been published indicating that these two monomers (**1** and **2**) act as resistance factors in insect−plant interactions (*12*, *23*, *27*). Resistant lines to *D. grandiosella* and *D. saccharalis* showed higher levels of both compounds in the leaves, with 19% more **1** and 23% more **2** (*15*). Furthermore, the amount of these compounds was negatively correlated to leaf-feeding damage by different borer species (*15*, *27*). In addition, recent studies with the pith of maize showed significantly larger concentrations of **1** and **2** in genotypes resistant to *S. nonagrioides* (*16*).

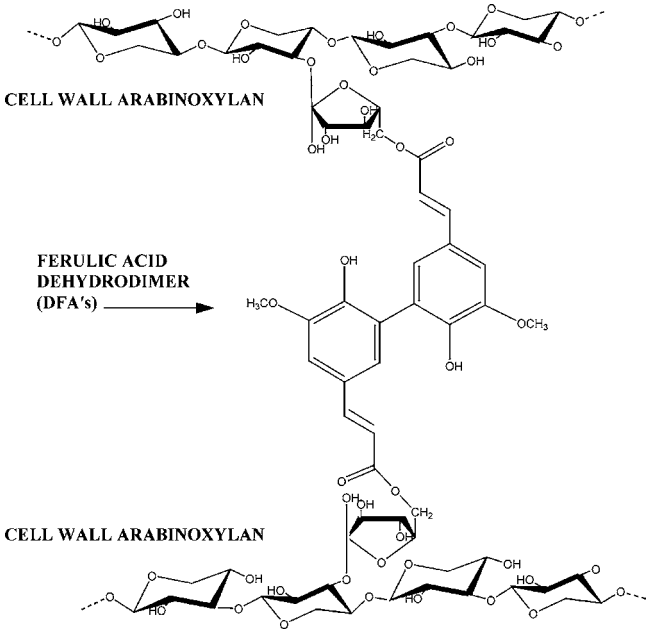

Figure 2. Cell wall cross-linking between two arabinoxylan chains mediated by diferulic acids (DFAs).

Nevertheless, checking individual inbreds, the genotypes EP42 and CM151 showed comparable concentrations for both monomers in the pith (*16*). It is possible that each inbred possesses a particular and different mechanism of defense or a combination of several mechanisms which interact to determine the level of resistance (*28*); thus, a genotype with one particular mechanism of defense needs different amounts of other resistance compounds, altering in this way the actual relationship of these compounds with *S. nonagrioides* resistance (*9*, *10*). The sheath resistance of inbred CM151 could be due to an antibiotic or structural component not evaluated, showing in this way low contents of **1** and **2**. Field resistance of inbred CM151 to Mediterranean corn borer could be due to antibiotic substances present in the pith (*9*), and these factors could be also present in the leaf sheath. On the other hand, higher weights in larvae reared on EP42 could be due to the action of nutritional components more critical than a higher concentration of **2**. Among the nutritional components most important to insects, nitrogen is a key element determining insect survival, growth, and development (*29*−*31*). Although previous studies showed no significant variation on the protein leaf content (nitrogen) in a recurrent selection for *O. nubilalis* resistance (*23*), future studies regarding this new topic need to be addressed for *S. nonagrioides* resistance.

If we look at the mean content of these compounds, **1** was

found to be the predominant phytochemical species of the cell wall phenolics in the sheath of all genotypes studied, whereas **2** was 40% less concentrated. Our results are in accordance with previous reports on the concentration of hydroxycinnamates in the leaf sheaths of the maize population BS9 (*23*). However, the content in the leaf sheath of **1** was lower than the content in the pith for the same plants and genotypes, whereas the content of **2** was slightly higher (*16*). The stereoisomer **3** was only a minor constituent (<5% of the total ferulic acid) in both studies. It is interesting to note that the mean content of **1** in the pith of susceptible genotypes was similar to the highest content of this compound in the leaf sheath, suggesting that the effects on the resistance could be functional after a specific amount of this compound in the tissue, further related with the lignin deposition (*32*, *33*).

Heteroxylans are cross-linked by peroxidase-mediated coupling of **2** monomers into a complex array of dimers (*34*), and this oxidative coupling probably contributes to wall stiffening, lignin formation, growth cessation, and pest resistance (*12*, *16*, *35*−*37*) (**Figure 2**). In the current study, three isomers of DFAs were found in the leaf sheath tissue: **4**, **5**, and **6** (**Table 2**). Additionally, as in previous pith analysis, DFA 5-5′ was detected in some samples, although the trace amounts and the coelution with other compounds made its quantification difficult (*16*).

Significant differences among inbreds were found for the main diferulates (**Table 2**). With regard to the hypothesis of a particular resistance mechanism operating in certain genotypes, the inbred CM151 was removed from the data analysis and discussion. This way, we could check that the inbred EP42, with which larvae showed the highest weight after 12 days of feeding, showed the lowest contents for **4** and **6** and was in second place in the group of low contents of **5**. Besides, the genotypes A509 and EP47, which had the smallest larvae following CM151, had higher concentrations of the diverse diferulates. The regression analysis (excluding the inbred CM151) showed a possible cause−effect relationship between variables (**4**, $R^2 = 0.61$, $P \leq 0.06$; **5**, $R^2 = 0.44$, $P \leq 0.14$; **6**, $R^2 = 0.60$, $P \leq 0.07$; and total DFA, $R^2 = 0.56$, $P \leq 0.08$), although the significance was marginal. However, significant negative correlations between DFA content (excluding the inbred CM151) and larval weight after 12 days of feeding (**4**, $r = -0.78$, $P \leq 0.05$; **5**, $r = -0.67$, $P \leq 0.05$; and **6**, $r = -0.78$, $P \leq 0.05$) were found. On the other hand, no significant correlations were found for the simple hydroxycinnamates with or without the inbred CM151 (data not shown). The addition of the inbred CM151 showed no significant correlations, hiding the relationship between the DFA content and the larval development for the other six genotypes. Future studies have been established for further evaluation of inbred CM151. As a final point, significant negative correlation (excluding the inbred CM151) was found between the total DFA content and the larval weight (total DFA, $r = -0.75$, $P \leq 0.05$). According to these results, recent studies have reported significant negative correlations between susceptibility parameters and diferulic acid content (*12*, *13*).

Lower levels of diferulates may have facilitated feeding by making nutrients more accessible to neonate larvae of *S. nonagrioides* feeding on leaf sheath tissues and consequently enhancing susceptibility of the whole plant, a mechanism previously proposed for other stem borers (*15*, *23*). However, as mentioned above, some other factors could be affecting the larvae performance; for example, low amounts of diferulates in the inbred EP42 could contribute to heavier larvae, although a richer nutritional composition could enhance this difference in relation to other genotypes, such as the inbred PB130, which had intermediate larval growth and low diferulate content.

In relation with the changes in the concentration, throughout the period of attack (30 days period after silking) the genotypes showed consistent concentrations (data not shown), although an increase during the whole development could be expected (*38*).

In summary, the leaf sheath of maize plants appears to play a role in the successful development of neonate *S. nonagrioides* larvae. Differences in susceptibility among the inbreds EP42 and CM151 could be established at an early stage of the borer attack. The content of simple hydroxycinnamates in the leaf sheath is not given as an explanation of differences between genotypes; however, in the majority of the inbreds the amount of cross-linking components (DFAs) in the cell wall could be a resistance factor. Nevertheless, the level of resistance of the diverse genotypes evaluated in this study could be mediated by single structural and/or nonstructural components, including several that still need to be evaluated.

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