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Effect of Maize Pith Free Phenols on Larval Growth and Development of *Sesamia nonagrioides* (Lepidoptera: Noctuidae)

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Abstract: The objective of this research was to evaluate the effect of four phenols previously identified at higher concentrations in maize resistant genotypes (*p*-coumaric acid, ferulic acid, *p*-hydroxybenzaldehyde and vanillin) on the growth and development of *S. nonagrioides* larvae. Three different concentrations of each phenolic compound and a mixture of the four were evaluated. Data were recorded on larval and pupal weight, time to pupation and percentage of pupation and mortality. Growth and survival showed no significant differences between the larvae reared on diets with the different treatments and the control. It was concluded that these four compounds, although found in higher concentrations in resistant genotypes, were not toxic to *S. nonagrioides* in free form under laboratory conditions. Possible ways these compounds could be related with the *S. nonagrioides* resistance are discussed.

Key words: *Zea mays*, bioassay, phenolic compounds, survival, antibiosis, resistance

Introduction

Sesamia nonagrioides (Lefèbvre) (Lepidoptera: Noctuidae) is one of the most harmful pests of maize, *Zea mays* L., in Spain and other Mediterranean regions (Cordero *et al*., 1998; Albajes *et al*., 2002; Gianessi *et al*., 2003). In Northwestern Spain this stem borer has two generations per year. The most important is the second generation larvae, which feed on the pith during plant development, reducing plant growth, grain size and causing indirect yield losses as a consequence of lodging (Larue, 1984). Although variability in resistance to this stem borer has been detected among populations and inbred lines of maize (Malvar *et al*., 1993; Cartea *et al*., 1994; Butrón *et al*., 1999), little is understood about the phytochemical nature of this resistance. Laboratory bioassays are an essential component of investigations of the chemical basis of resistance to insects in crop plants. Plant resistance studies have often focused on the isolation and identification of biologically active compounds in agricultural crops that could inhibit growth and development of insects that feed on plant tissues (Harris, 1979; Schoonhoven *et al*., 1998). The defense functions of 2,4-dihydroxy-7-methoxy- (2H)-1,4-benzoxazin-3- (4H)-one (DIMBOA) and phenolic acids in maize plants have been well documented for European corn borer *Ostrinia nubilalis* (Hubner) (Bergvinson, 1993; Bergvinson *et al*., 1994; Klun and Robinson, 1969), southwestern corn borer *Diatraea grandiosella*

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(Dyan), sugarcane borer *Diatraea saccharalis* (Fabricius) (Ramputh, 2002), pink stem borer *S. nonagrioides* (Ortego *et al*., 1998) and the maize weevil *Sitophilus zeamais* Motschulsky (Arnason *et al*., 1997).

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 some lepidopteran pests (Mihm, 1985). Phenolic compounds are present in plant cells in four different forms: insoluble cell wall-bound phenolics, soluble ester-bound phenolics, soluble glycoside-bound phenolics and free phenolics. Most studies on maize resistance mechanisms have been focused on bound phenolics due to their role on cell wall cross-linking, providing a physical barrier to diseases and insect feeding (Bergvinson *et al*., 1994; Ramputh, 2002; Fry *et al*., 2000; Bily *et al*., 2003). On the other hand, free phenolic acids have low molecular weights and are partially soluble in many solvents, including water. These characteristics are important properties of plant toxins because they must pass through cell membranes to be readily available (Rhoades and Cates, 1976). Some phenols which act as toxins to some species can also act as phagostimulants to others. For example, cinnamic acid stimulates feeding by *Scolytus multistriatus* (Marsham) (Norris, 1977) but inhibits pupation in *Agrotis ipsilon* (Hufnagel) (Reese, 1977). *p*-Coumaric and ferulic acid were phagostimulants for the spotted stem borer, *Chilo partellus* (Swinhoe) (Torto *et al*., 1991), while they inhibit feeding of *S. zeamais* (Serratos *et al*., 1987). *p*-Coumaric acid was toxic to the two spotted spider mite, *Tetranychus urticae* Koch (Leszczynski *et al*., 1988), as ferulic acid was toxic to the dried fruit beetle, *Carpophilus hemipterus* (L.) (Dowd, 1990). Chorogenic acid is considered to be allelopathic (Einhellig, 1985), but it stimulates feeding by *Leptinotarsa decemlineata* (Say) (Hsiao and Fraenkel, 1968) and *Lema trilineata* Olivier (Kogan and Goeden, 1971). Furthermore, other free phenols were toxic to different insect species (Campbell *et al*., 1984, Fischer *et al*., 1990).

Early studies suggested the possibility that some natural acids with phenolic structure could be related with resistance to *S. nonagrioides*; at the same time, they showed inhibition of the fungal genera *Fusarium* (Anglade and Molot, 1967). Furthermore, recent studies put forward a possible role of free phenols in *S. nonagrioides* resistance due to the higher level of these compounds in the pith of genotypes resistant to this pest (Santiago *et al*., 2005). To determine if these compounds have an antibiotic effect, insect growth and development must be assessed. The goal of this study was to determine the possible effects of selected phenols on *S. nonagrioides* larval growth and development.

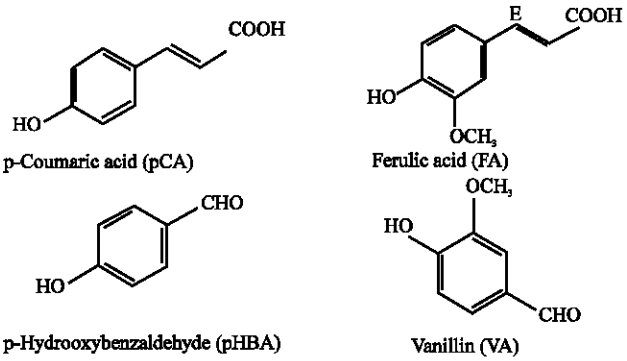
Materials and Methods

Selected Compounds

Four phenolic compounds identified and quantified in previous studies in the pith of maize were selected for laboratory feeding trials (Fig. 1). *p*-Coumaric (pCA) and ferulic acid (FA) were the most abundant free phenols found in resistant genotypes in extensively studies with thirteen maize inbred lines (Santiago *et al*., 2005). Early studies showed *p*-hydroxybenzaldehyde (pHBA) and vanillin (VA) at low, but higher concentrations in resistant genotypes during some trials, so these compounds were also incorporated into meridic diets. Levels detected in the pith tissue and higher and lower concentrations were tested.

Laboratory Bioassays

Laboratory tests were conducted to investigate the antibiotic effect of the four phenols on the growth and development of *S. nonagrioides* larvae. Commercial phenolics purchased from Sigma-Aldrich Química, SA (Madrid, Spain) were dissolved in water with a sonicator and added to the artificial diet (Eizaguirre, 1989). Sixteen treatments were tested. Twelve consisted of diets containing



	Concentration (µg/g dry weight)		
Phenols	High	Medium	Low
p-Coumaric acid (pCA)	100	60	20
Ferulic acid (FA)	20	12	4
p-Hydrooxybenzaldehyde (pHBA)	10	6	2
Vanillin (VA)	10	6	2
Mixture of phenols (Mix)	140	84	28

Fig. 1: Chemical structure and concentrations of the different phenols added to meridic diets in the laboratory bioassays

just one of the four phenols at low, medium or high concentration (Fig. 1). Besides the effect of a particular compound, it is also important to consider the combinations and interactions among compounds, which can be responsible for the appearance of a toxic effect (Cates, 1996; Nelson and Kursar, 1999). A mixture (Mix) of the four compounds at low, medium and high concentration was also tested, adding three treatments to the experiment (Fig. 1). Finally, artificial diet with distilled water was used as control (Control).

All treatments and control were evaluated in tests of ten replications each, arranged in a randomized complete block design. Approximately 10 g portions of diet per treatment and replication were placed into 9 cm-diameter Petri dishes. A 10-day-old larvae was placed into each dish, the dishes were sealed with parafilm and placed in a environmental controlled room at 26°C with a photoperiod of 16:8 (L:D). Fresh diet was provided every week so that all larvae ate as much as they wanted. Larval weight and the number of dead and missing larvae (larvae that escape from the dishes), were recorded weekly until pupation. Pupal weight and time to pupation were also registered.

Statistical Analysis

Repeated measures analysis was used to analyze the weekly weight measurements of larvae. A growth curve of weight on time was estimated for each treatment and homogeneity of linear and quadratic coefficients were tested for each pair of treatments. The analysis was made using the MIXED procedure of SAS (SAS Institute, 2000). All factors were considered random except for treatment which was considered fixed. The covariance was calculated following Littell *et al*. (1996). Additionally, analysis of variance and comparisons of means among treatments were calculated for larval weight. Larval weight means was adjusted by initial larval weight by analysis of covariance (SAS Institute, 2000). An analysis of variance and comparison of means among treatments were also calculated to analyze larval survival (proportion of total larval mortality, mortality during the first week and proportion of larvae that reach pupae stage).

Finally, to analyze larval survival and time to pupation, the Kaplan-Meier estimates of the survival and time to pupation functions were calculated for each treatment and curves were compared using the log rank test (Ordás *et al*., 2002; Cantor, 1997). Survival functions were significantly different

if they deviated from the expected values of the null hypothesis (that survival functions are equivalent in all treatments). The statistic determines whether differences between survival functions are significantly different at any probability level, thus indicating that larvae have significantly different survival in some treatments than in others (LIFETEST procedure of SAS). Missing larvae were censured for larval survival analysis, which means that the analysis considered that larvae lived at least until they disappeared. When larvae reached pupal stage, the larvae survival was scored as reaching 42 days. Missing and dead larvae were removed before analyzing time to pupation data.

Results

The growth of the larvae reared on each treatment fitted a parabolic function (Table 1). The linear parameters were positive and higher than the quadratic ones, the latter being negative. This means that the weight of larvae increased quickly in the first days, but larval weight remained almost invariable for many days thereafter. The coefficient of determination (r^2) varied from 0.62 to 0.75 (data not shown).

Larvae fed on diets containing pCA and FA showed similar growth patterns. Larvae reared on diet containing low concentrations of both phenols had a linear growth, while the quadratic coefficient was not significant (Table 1). The linear coefficient for diet containing the lowest concentration of FA was significantly smaller than the linear coefficient for the highest concentration. However, the highest concentration showed a significant negative quadratic coefficient. In general, for each week, weight did not show significant differences among diets containing pCA or FA (Table 1).

The linear and quadratic coefficients of larval growth curves for pHBA at different concentrations were homogeneous. However, larval weights at 7 and 14 days were lower on diets with high than with low pHBA concentrations (Table 1) and larval weight at 21 days on diets with the highest pHBA concentration was significantly lower than on diets with the lowest pHBA concentration. However, weight of larvae fed on this compound was not significantly different from the control.

Table 1: Larval weight at five dates adjusted by initial larval weight and linear and quadratic coefficients±SD of larval weight curves on time (mg) for each phenol at different concentrations

		Larvae weight (mg/larva)						
Phenols	C†	7 days	14 days	21 days	28 days	35 days	Linear coefficient	Quadratic coefficient
pCA	H	34.9	158.1	203.0	221.0	294.1	12.19±1.28a-d*	-0.128±0.034a-d*
	M	30.9	136.7	247.3	228.9	193.1	12.96±1.39a-d*	-0.176±0.039b-d*
	L	32.9	134.6	213.4	343.9	337.1	10.09±1.60a*	-0.010±0.051a
FA	H	30.8	139.9	238.6	245.9	247.7	13.81±1.44b-d*	-0.185±0.042cd*
	M	35.3	155.9	246.2	239.5	259.5	13.65±1.42a-d*	-0.175±0.041b-d*
	L	25.8	115.5	228.7	271.2	304.9	9.64±1.45a*	-0.024±0.043a
pHBA	H	27.9	148.8	206.8	236.8	287.9	10.20±1.54a-c*	-0.079±0.043a-c
	M	28.7	126.2	266.8	268.1	282.8	10.78±1.54ab*	-0.055±0.047ab
	L	30.4	168.4	295.8	260.8	266.8	11.21±1.27a-c*	-0.056 ± 0.031a
VA	H	36.2	171.8	286.0	288.5	275.1	14.09±1.59b-d*	-0.152±0.050a-d*
	M	30.4	144.3	324.1	299.9	278.1	13.32±1.61b-d*	-0.125±0.052a-d*
	L	21.5	119.7	195.5	260.1	261.5	11.15±1.79a-c*	-0.085±0.043a-d
Mix	H	39.5	144.6	232.7	241.9	253.0	11.74±1.36a-d*	-0.101±0.038a-d*
	M	44.1	178.4	246.0	262.5	225.8	15.28±1.36d*	-0.222±0.039d*
	L	39.1	158.1	262.2	287.8	280.2	14.91±1.44cd*	-0.179±0.043b-d*
Control		29.8	155.6	233.9	262.7	256.1	12.20±1.46a-d*	-0.130±0.043a-d*
LSD ($p \leq 0.05$)		14.5	54.60	83.80	65.90	75.90		

†, High (H), Medium (M) and Low (L) concentrations (Fig. 1), Coefficients within a column followed by the same letter are homogeneous ($p \leq 0.05$), *, Coefficients are significantly different from zero ($p \leq 0.05$), Phenols: *p*-coumaric (pCA), ferulic acid (FA), *p*-hydroxybenzaldehyde (pHBA) and vanillin (VA)

Although regression coefficients were homogeneous for the three VA concentrations on diets, larvae fed on them showed the opposite trend to larvae reared on pHBA diets (Table 1). Larval weight was less for the lowest concentration than for the higher ones. The larval weight at 21 and 28 days were significantly smaller for the low concentration than for any other concentration (Table 1).

The mixture of phenols showed the highest linear coefficients at medium concentration, having the highest increase on the larval weight just during the first week (Table 1).

Larval mortality did not significantly differ between treatments (Table 2). The range varied from 15-17% of dead larvae when fed on the mixture of phenols (Low: 28 μg g^{-1} dry weight) and FA diets (High: 20 μg g^{-1} dry weight) to 37-39% of dead larvae on the highest concentrations of pHBA (High: 10 μg g^{-1} dry weight) and 1 diets (Low: 20 μg g^{-1} dry weight). A total larval mortality is an important trait to analyze, however most of the deaths took place during the first week, when the larvae are most susceptible to toxic compounds. During this period, there was higher mortality when they fed on pHBA diets than when they fed on FA or Mixed diets, especially for the highest pHBA concentration (Table 2), although non significant differences were observed.

Survival data for larvae fed on different treatments were not equivalent. Table 2 shows the values of the log rank statistic in each treatment as well as the comparisons between treatments. The positive values of the statistic indicate a number of dead larvae larger than the number expected under the null hypotheses of equivalent survival distributions, while negative values indicate there were fewer deaths than expected under the null hypothesis. For example, there would be about 4 to 7 deaths above expected among larvae fed on pHBA diets, if survival functions were homogeneous. In conversely, there were around 2 to 5 deaths below that expected among larvae reared on Mix and FA diets (Table 2). Finally, in the comparisons between treatments the highest value of the log rank statistic was obtained for larvae reared on diets with the highest concentration of pHBA.

Time to pupation was also studied using a survival analysis. Survival data often consists of a variable response that measures the length of time until a specified event occurs, in this case the event was pupation. Although the lowest value of time to pupation was obtained in larvae reared on diets with 100 μg g^{-1} dry weight of pCA, the lowest values of log rank statistic at any concentration were shown in larvae fed on pHBA diets. In contrast, the highest values were in larvae reared on VA diets (Table 2). However, even though we could detect some possible trends in the survival and time to pupation log rank data, these values were not significantly different from the control.

Table 2: Proportion of total larval mortality, mortality during the first week and larvae that reach pupae state (±SE) and values of the log-rank statistic for homogeneity of survival and time to pupation curves for each phenol at different concentrations

Phenols	C †	Total larval mortality (%)	Mortality in the 1st week (%)	Pupae (%)	Log-rank survival	Log-rank time to pupation
pCA	H	20.9±8.8	16.0±6.7	60.5±12.5	-1.92ab	-5.90a
	M	26.9±8.8	12.0±6.7	46.5±12.5	-1.13a-c	0.89a-c
	L	38.9±8.8	16.0±6.7	48.5±12.5	1.16 bc	2.92a-c
FA	H	16.9±8.8	10.0±6.7	62.5±12.5	-5.46a	-0.32a-c
	M	22.9±8.8	6.00±6.7	50.5±12.5	-3.00ab	1.88a-c
	L	32.9±8.8	14.0±6.7	54.5±12.5	1.88a-c	-1.68a-c
pHBA	H	36.9±8.8	32.0±6.7	36.5±12.5	7.96c	-2.66a-c
	M	32.9±8.8	22.0±6.7	52.5±12.5	4.11bc	-5.00ab
	L	20.9±8.8	18.0±6.7	44.5±12.5	-0.60a-c	-3.12a-c
VA	H	28.9±8.8	20.0±6.7	52.5±12.5	2.08a-c	2.51a-c
	M	30.9±8.8	18.0±6.7	56.5±12.5	2.71a-c	2.39a-c
	L	24.9±8.8	16.0±6.7	44.5±12.5	0.72a-c	4.87a-c
Mix	H	20.9±8.8	18.0±6.7	64.5±12.5	-2.06ab	-0.06a-c
	M	20.9±8.8	10.0±6.7	62.5±12.5	-3.76ab	3.76bc
	L	14.9±8.8	12.0±6.7	64.5±12.5	-5.20a	-0.06a-c
Control		27.0±8.1	20.0±6.7	56.0±11.8	2.53a-c	-0.42a-c

†, High (H), Medium (M) and Low (L) concentrations (Fig. 1), Log-rank statistic within a column followed by the same letter means that curves are homogeneous ($p \leq 0.05$), Phenols: *p*-coumaric (pCA), ferulic acid (FA), *p*-hydroxybenzaldehyde (pHBA) and vanillin (VA)

Pupal weight did not show significant differences among larvae reared on the different diets (data not shown). Pupation occurs when larvae reach around 300 mg independently of larval feeding. Proportion of larvae that reach the pupae stage was lower for the larvae fed on diets with the highest concentration of pHBA than larvae fed on diets with the highest concentration of pCA or FA; or any concentration of the Mix (Table 2).

Discussion

Todd *et al*. (1971) found many phenolic acids present in higher plants to be toxic to the greenbug, *Schizaphis graminum* (Rodani) and many of them were present in resistant barley strains. Lesczynski *et al*. (1988) confirmed a toxic effect of pCA to the spider mite, *T. urticae* and later, Lege *et al*. (1995) found higher concentrations of this compound in cotton resistant genotypes to this pest. In maize, Serratos *et al*. (1987), studying the feeding response of the maize weevil, *S. zeamais*, clearly demonstrated that pure phenolic substances have significant antifeedant properties at a concentration close to the phenolic content in the grain. In this study, although a negative trend could be suggested for the highest concentration of pHBA, present findings indicate that none of the phenolics or mixtures incorporated into artificial diets had any statistically significant effect on growth, final weights or survival, compared to control.

These results are surprising, in that such an extensive body of evidence has been accumulated indicating that these compounds are toxic when incorporated into artificial diets (Dreyer *et al*., 1981; Serratos *et al*., 1987; Arnason *et al*., 1992), as well as previous studies showed higher concentration of free phenols in maize resistant genotypes (Santiago *et al*., 2005).

It is possible that the phenolic toxicity could be highly susceptible to modification by other dietary constituents. Different studies have shown that the presence of other plant oxidative enzymes, the type of dietary protein and the presence of antioxidants are determinants of phenolic toxicity (Felton *et al*., 1992; Appel, 1993; Summers and Felton, 1994). However, Warnock *et al*. (2001), studying the development of *O. nubilalis* larvae reared on diets containing liophilized maize silk tissues and pCA or FA additions, proved no effect of these compounds on larvae weight, even with higher concentrations than that tested in the present study. These results agree with the lack of an antifeedant effect of free pCA and FA in our laboratory test.

In relation with the higher levels of the four compounds in resistant genotypes, these compounds did not show any effect on *S. nonagriodes* development as free phenolics. Studying the occurrence of phenolics in plants is useful to distinguish between soluble and bound forms of a compound. Distinction is important since its role in resistance could be structural or biochemical. At a structural level the phenolic constituents of the cell wall of graminaceous plants consist largely of pCA and FA (Hartley and Jones, 1978). Both acids take part in mechanical resistance through cell wall fortification and lignification (Akin *et al*., 1992; Bergvinson *et al*., 1994, 1995). In this regard, Santiago *et al*. (2005) suggested that higher quantities of free pCA in resistant inbreds may perform a pool from which the other various hydroxycinnamic acids and their esters were formed (Bate-Smith, 1962). Thus, although these compounds did not show a direct antibiotic effect in the current study, they could still be related to borer resistance. In fact, recent studies with maize pith have pointed out higher quantities of cell wall phenylpropanoids in resistant genotypes to *S. nonagrioides* (Santiago *et al*., 2006).

The major feeding deterrent to greenbug, *S. graminum*, isolated from sorghum leaves was pHBA (Dreyer *et al*., 1981); while VA is known as a chemioatractant (Eveland and Haseeb, 1993), even carrying out a sexual pheromone function (Ubik *et al*., 1975). In the present study these two compounds showed no effects on the growth and development of *S. nonagrioides* larvae. In the same way, the mixture containing a combination of all phenols did not show any kind of synergistic or antagonistic effect at the tested concentrations.

In summary, our findings demonstrate that the free phenols tested do not have an apparent effect on the larval performance of the lepidopteran *S. nonagrioides*. However, this does not rule out the possibility that these compounds may be present in high concentration in resistant genotypes to play a structural role in the resistance to this pest. This issue is currently under further evaluation.

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