Do Plant Phenolics Confer Resistance to Specialist and Generalist Insect Herbivores?

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The impact of phenolics on a specialist herbivore, *Manduca sexta*, and a generalist herbivore, *Heliothis virescens*, was investigated using transgenic tobacco with differential expression of phenylalanine ammonia-lyase. Foliar phenolics such as chlorogenic acid, rutin, and total flavonoids differentially accumulated in the respective transgenic tobacco lines; the amount of chlorogenic acid ranged from 201 to 2202 μg g^{-1} of fresh leaf, that of total flavonoids from 211 to 500 μg g^{-1} of fresh leaf, and that of rutin from 73 to 172 μg g^{-1} of fresh leaf. However, the levels of the phenolics and larval growth of *M. sexta* or *H. virescens* were not significantly correlated. Likewise, phenolic levels were not correlated with larval survival of *M. sexta*. These results suggest that plant phenolics are not a determining factor for host plant resistance against these insects in this system.

Keywords: *Phenylalanine ammonia-lyase; phenolics; host plant resistance; Manduca sexta; Heliothis virescens*

INTRODUCTION

Phenolic compounds may play important roles in plant physiological processes such as protection against environmental stresses (e.g., herbivory, infection, or ultraviolet radiation); signal molecules in plant−pathogen interactions; structural constituents of cell walls (i.e., lignin or suberin); and flower pigments (Harborne, 1985; Hahlbrock and Scheel, 1989). The rate-limiting step in the synthesis of most phenolics is dependent upon the enzyme phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), which catalyzes the deamination of phenylalanine to form cinnamic acid (Bate et al., 1994). Despite the overwhelming array of structural diversity found among phenolics (e.g., phenolic acids, coumarins, lignans, lignins, and flavonoids), all are derived from phenylalanine. In the case of flavonoids, one aromatic ring and the C3 side chain arise from phenylalanine, but the other is derived from acetyl-CoA via the polyketide pathway (Dixon and Paiva, 1995).

The role of phenolics in plant antiherbivore defense has been a particularly intense area of study during the past several decades (Appel, 1993). The allocation of phenolics for defense against herbivores is a central tenet of several plant defense theories [e.g., Loomis (1932), Feeny (1976), Bryant et al. (1983), and Coley et al. (1985)]. Furthermore, herbivory has been proposed as a primary selective agent for phenolics during plant evolution (Harborne, 1979). The putative role of phenolics as components of plant defense is based upon numerous studies demonstrating their toxicity to herbivores when incorporated into artificial diets [e.g., Elliger et al. (1981)] or upon more holistic approaches involving the correlation of phenolic content in plants with herbivory or herbivore performance [e.g., Dudt and Shure (1994)]. Further support for their defensive role against herbivores comes from studies showing that herbivory often induces higher PAL activity [e.g., Hartley and Firn (1989)]. However, in some cases, phenolics (e.g., caffeic acid and protocatechuic acid) are known to stimulate feeding and/or growth in certain insect species (Bernays and Woodhead, 1982).

The cinnamic acid derivative, chlorogenic acid, and the flavonoid glycoside, rutin, represent model phenolics in the study of plant antiherbivore defense due to their ubiquitous occurrence among terrestrial plants and well-documented toxicity to insect herbivores (Sondheimer, 1964; Harborne, 1979, 1991; Isman and Duffey, 1983). Numerous laboratories have shown that rutin and chlorogenic acid slow the development of lepidopterans such as *Helicoverpa zea*, *Heliothis virescens*, *Pectinophora gossypiella*, *Manduca sexta*, *Spodoptera litura*, *Spodoptera eridania*, and *Spodoptera exigua* when added to artificial diets (Shaver and Lukefahr, 1969; Duffey and Isman, 1981; Elliger et al., 1981; Isman and Duffey, 1982; Lindroth and Peterson, 1988; Stamp, 1990; Horwath and Stamp, 1993; Stevenson, 1993; Stamp and Yang, 1996).

The toxicity of phenolics such as chlorogenic acid has been frequently attributed to their propensity to be oxidized enzymatically via polyphenol oxidase (PPO), laccase, or peroxidase (POD) or by chemical processes catalyzed by alkaline pH and/or transition metals (Felton et al., 1989; Appel, 1993). This oxidation forms quinones that covalently bind to proteins, thus limiting their bioavailability as nutrients, and may form reactive oxygen species (e.g., superoxide radical and H_2O_2) that damage essential nutrients or integral molecules such as lipids, proteins, and nucleic acids (Felton et al., 1989, 1992; Appel, 1993; Summers and Felton, 1994).

Despite over 30 years of research on the ecological significance of phenolics, there is continuing controversy regarding their ecological importance (Appel, 1993; Matsuki, 1996). Direct evidence for their role in anti-

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herbivore defense remains elusive because of the difficulty in separating the effect of phenolics from those of other plant components (Harborne, 1985; Appel, 1993). These controversies highlight the necessity of utilizing more direct, molecular approaches to resolve the putative role of phenolics in plant−herbivore interactions, rather than relying upon artificial diets that may not accurately reflect their toxicity *in planta*.

Such an approach has been invaluable in assessing the role of phenolics in defense against phytopathogens. A bean *PAL2* transgene gene was introduced in the sense orientation to tobacco, which led to transgenic lines overexpressing or underexpressing PAL activity (Elkind et al., 1990; Howles et al., 1996). Accumulation of phenylpropanoids such as chlorogenic acid, which accounts for 60% of total soluble foliar phenylpropanoid products, and PAL levels in these transgenic plants were positively correlated (Bate et al., 1994). Increased disease susceptibility was found in plants with suppressed levels of the constitutive phenylpropanoid products (Maher et al., 1994). The suppressed PAL plants also failed to develop systemic acquired resistance (Pallas et al., 1996).

The present study was initiated to determine the impact of plant phenolics on insect resistance by using transgenic tobacco in which PAL expression has been specifically altered. The insects used in this system were *M. sexta*, a specialist feeding on plants in Solanaceae, and *H. virescens*, a generalist that feeds on plants from multiple families.

MATERIALS AND METHODS

Plant and Insect Materials. To assess the role of phenolics in plant resistance, tobacco lines that are epigenetically suppressed in PAL expression or that overexpress PAL were used. The generation of the tobacco lines 274-T4, 160-P3, 104-T5, and 10-6T1 from *Nicotiana tabacum* cv. Xanthinc has been described (Elkind et al., 1990; Pallas et al., 1996; Howles et al., 1996). These lines contain the bean *PAL2* gene under the control of its own promoter as well as additional cauliflower mosaic virus 35S enhancer sequences. The 6-16 274-T4 and 160-P3 lines are sense-suppressed lines in which the introduction of the bean transgene caused reduced accumulation of tobacco PAL transcripts (Elkind et al., 1990). The line 104-T5 is a fifth-generation line that partially recovered from the sense suppression and exhibits slightly decreased PAL activity (Bate et al., 1994). The 10-6T1 line changed from a sense-suppressed line to an overexpressing line in a single generation (Howles et al., 1996). The overall PAL activity in 10-6T1 is greater than that of the wild type, and the levels of endogenous PAL transcripts are returned to wild type. The C-17 control lines have lost the bean *PAL2* gene through segregation and are now operationally wild type. The fact that they did have the transgenes and formerly exhibited sense suppression makes them suitable controls.

Plants were grown from stem cuttings of each tobacco line in 2-L plastic pots filled with Redi Earth Peat-Lite soil mixture in a greenhouse. Pots were arranged in a completely randomized experimental design. Plants were watered every day and fertilized with Osmocote (N/P/K = 14:14:14) monthly. Greenhouse conditions were (1) 14-h photophase, using a high-pressure sodium light (1000 W) and (2) day temperature 27 ± 2 °C and night temperature 19 ± 2 °C. Tobacco plants at the preflower stage were used in the experiments.

Eggs of *H. virescens* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on artificial diet as formulated by Chippendale (1970) until used in the experiments. Eggs of *M. sexta* were purchased from Carolina Biological Supply Co. (Burlington, NC).

Effect of Variable PAL Expression on Accumulation of Foliar Phenolics and PPO and POD Activities. To determine the effect of variable PAL expression on phenolic levels, three plants at preflower stage of each tobacco line were used. Five hundred milligrams of terminal foliage from each plant was extracted in 10 mL of 50% methanol at 60 °C for 24 h. The extract was filtered through a 0.45 μm Whatman polypropylene filter. The filtrate from each sample was immediately used for analysis of the following phenolic compounds.

Total flavonoids were quantified according to the procedure as described by Hedin et al. (1992). A 0.5 mL of filtrate was added to 1 mL of 1% diphenylboric acid in absolute methanol (v/v). The absorbance was read at 440 nm with rutin (Sigma Chemical Co.) as a standard. The assay was replicated three times for each sample.

To specifically analyze for chlorogenic acid and rutin, 10 μL of filtrate was used for each injection in reversed-phase high-performance liquid chromatography (RP-HPLC) using a Nova-Pak C_{18}, 3.8 × 150 mm, stainless steel column (Waters Associates). The separation solvents included (initial) butanol/methanol/acetic acid/distilled deionized (dd) water (0.25:1.25:2:96.5) and (final) butanol/methanol/acetic acid/dd water (5:25:2:68). Both of the solvents contained 18 mM ammonium acetate (Murphy and Stutte, 1978). The separation was programmed with a linear gradient for 30 min from the initial solvent to 50% of the final solvent, followed by 20 min linearly from 50% to 100% of the final solvent (Bi et al., 1997). Mobile phase flow was 1 mL min^{-1}. Absorbance at 254 and 280 nm was monitored by a multiwavelength detector. Peak area, peak height, and retention time were determined on a CR501 Chromatopac (Shimadzu). The sample peaks for chlorogenic acid and rutin were identified by comparison of retention times and 254:280 nm absorbance ratios with the respective standards. Concentrations were determined from standard curves of chlorogenic acid and rutin and were expressed on a per fresh weight basis. The analysis was replicated four times.

To determine the effect of variable PAL expression on activities of foliar enzymes of the POD and PPO, three preflowering-stage plants from each tobacco line were used. A terminal expanding leaf from each plant was excised. Preparation of foliar tissue for enzyme assays was as described by Bi and Felton (1995). The POD assay used guaiacol as the hydrogen donor according to the procedure of Ridge and Osborne (1970). Fifty microliters of enzyme solution was mixed with 1.0 mL of substrate containing 1 mM H_2O_2 and 2 mM guaiacol in 0.1 M potassium phosphate buffer, pH 7.0. POD activity was measured at 470 nm.

The PPO activity was determined by following the procedure of Ryan et al. (1982). A 50 μL aliquot of foliar homogenate was assayed with 1 mL of 3.0 mM chlorogenic acid in 0.1 M potassium phosphate buffer, pH 7.0. The increase in absorbance was monitored at 470 nm.

Effect of Variable PAL Expression on Insect Resistance. To determine the impact of various phenolic levels on *M. sexta* resistance, five tobacco plants of each line were used. At the preflower stage, 15 neonates were placed on each plant contained in a screen bag. After 8 days, the larvae were collected and weighed individually.

To determine the impact of various phenolic levels on larval growth of *H. virescens*, five tobacco plants of each line were used. At the preflower stage, four fourth-instar *H. virescens* were placed on each plant contained in a screen bag to prevent larval escape. After 84 h, the larvae were collected and weighed individually. The weight gain and relative growth rate (RGR) were then calculated. There were no significant differences among the initial weights used in the different treatments.

Statistics. Least significant difference (LSD) tests in one-way completely randomized ANOVA and PROC REG (SAS Institute Inc., 1988) were used to analyze the data in this study.

RESULTS

Effect of Variable PAL Expression on Foliar Phenolic Accumulation and PPO and POD Levels. Foliar levels of chlorogenic acid are strongly correlated

Table 1. Foliar Phenolic Levels in Transgenic Tobacco Lines[a]

line	chlorogenic acid (μg g^{-1} of fresh leaf)	total flavonoids (μg g^{-1} of fresh leaf)	rutin (μg g^{-1} of fresh leaf)
10-6T1 OX-10	2202.3 (66.5) a	463.8 (103.5) ab	161.5 (2.5) b
10-6T1 OX-11	1509.8 (102.2) b	379.5 (38.1) abc	108.0 (1.7) d
10-6T1 OX-18	1998.2 (118.9) a	500.0 (45.1) a	164.6 (2.7) b
C17-1	961.6 (44.5) cd	381.2 (29.7) abc	172.4 (1.9) a
C17-2	885.8 (29.7) ed	391.6 (16.1) abc	159.6 (1.9) b
C17-3	672.3 (116.9) ef	270.2 (17.2) cd	89.7 (0.6) e
104-T5	1184.1 (144.9) c	373.5 (68.7) bc	134.9 (0.5) c
6-16 274-T4-1	211.1 (20.9) h	228.1 (22.6) d	72.7 (0.0) g
6-16 274-T4-2	542.4 (87.0) gf	217.8 (13.8) d	82.2 (5.5) f
160-P3-1	411.9 (3.2) gh	315.0 (18.2) cd	131.7 (0.4) c
160-P3-2	201.4 (17.2) h	210.9 (26.9) d	72.7 (0.0) g
160 P3-3	660.5 (90.7) ef	231.5 (13.0) d	72.7 (0.0) g

[a] Means in the same column followed by a different letter were significantly different at $LSD_{0.05}$. Numbers in parentheses are standard errors.

Table 2. Foliar POD and PPO Activities in Transgenic Tobacco Lines[a]

line	POD (OD min^{-1} g^{-1})	PPO (OD min^{-1} g^{-1})
10-6T1 OX-10	15.44 (1.09) a	11.681 (0.019) cd
10-6T1 OX-11	14.91 (0.69) ab	12.506 (0.097) b
10-6T1 OX-18	14.53 (0.25) ab	13.166 (0.084) a
C17-1	15.51 (0.42) a	11.99 (0.102) c
C17-2	14.04 (0.29) ab	11.935 (0.154) c
C17-3	14.35 (0.81) ab	11.096 (0.168) e
104-T5	14.53 (1.20) ab	10.908 (0.379) e
6-16 274-T4-1	14.84 (0.15) ab	11.303 (0.186) de
6-16 274-T4-2	13.51 (0.43) bc	11.131 (0.147) e
160-P3-1	11.83 (0.60) c	11.303 (0.175) de
160-P3-2	14.32 (0.50) ab	11.344 (0.192) de
160-P3-3	13.27 (0.77) cb	12.375 (0.231) bc

[a] Means in the same column followed by a different letter were significantly different at $LSD_{0.05}$. Numbers in parentheses are standard errors.

with the level of PAL activity in the transgenic plants (Table 1). The concentration of chlorogenic acid is 1.8−2.6-fold higher in the PAL overexpressed line (10-6T1 OX-10, 10-6TI OX-11, and 10-6TI OX-18) and 1.4-fold higher in the 104-T5 line compared to the average concentration in the control line (C17-1−3). In contrast, the chlorogenic acid level is 1.3−4.2-fold lower in suppressed lines (6-16 274-T4-1, 6-16 274-T4-2, 160-P3-1−3) in comparison to the average level in controls.

Total flavonoid levels in PAL partially suppressing and PAL overexpressing tobacco lines are 7−44% higher than in the controls, whereas the concentrations in PAL suppressing lines are 9−40% lower than in the controls (Table 1). Rutin levels in PAL overexpressing or partially suppressing lines are similar to those in controls, and the levels in the suppressing lines are up to 48% lower than in the controls (Table 1). The differential expression of PAL only slightly changed foliar POD and PPO levels (Table 2).

Impact of Variable PAL Expression on Insect Resistance. Significant differences were found among the *M. sexta* weights and survivals when larvae fed for 8 days on tobacco lines with differential PAL expression, but the differences were not in correspondence with differential levels of chlorogenic acid, rutin, or total flavonoids (Table 3). There were no significant differences in third-instar *H. virescens* weight gains or growth rates when the larvae fed for 84 h on PAL overexpressed or suppressed plants compared to the controls (Table 4).

Although PAL regulation is well-correlated with some phenolics, the correlation between those compounds and insect growth was not significant on the basis of the respective values of the correlation coefficient (R^2) and probability (P) (Table 5). Larval growth of *M. sexta* was not negatively correlated with the foliar chlorogenic acid, rutin, and total flavonoids as indicated by the respective R^2 and P of 0.0596, 0.4445; 0.0373, 0.5475; and 0.0756, 0.3869 (Table 5). The growth rate of third-instar *H. virescens* was also not negatively correlated

Table 3. *M. sexta* Growth and Survival on Transgenic Tobacco Lines[a]

line	larval wt (mg)	larval survival (%)
10-6T1 OX-10	250.87 (15.25) ab	48 ab
10-6T1 OX-11	217.87 (11.07) b	48 ab
10-6T1 OX-18	249.35 (25.83) ab	41 ab
C17-1	145.04 (17.33) c	32 b
C17-2	209.39 (18.11) bc	39 ab
C17-3	182.19 (17.53) bc	45 a
104 T5	280.27 (24.59) a	47 ab
6-16 274 T4-1	255.02 (20.17) ab	54 a
6-16 274 T4-2	254.15 (12.97) ab	44 ab
160 P3-1	292.76 (34.99) a	45 ab
160 P3-2	131.82 (17.58) c	47 ab
160 P3-3	177.00 (16.68) bc	51 ab

[a] Means in the same column followed by a different letter were significantly different at $LSD_{0.05}$. Numbers in parentheses are standard errors. Neonate *M. sexta* fed on pre-flower-stage tobacco for 8 days.

Table 4. Third-Instar *H. virescens* Growth on Transgenic Tobacco Lines[a]

line	wt gain (mg)	RGR[b] (mg day^{-1} mg^{-1})
10-6T1 OX-10	79.47 (8.13) a	1.48 (0.15) ab
10-6T1 OX-11	73.50 (9.65) ab	1.44 (0.19) abc
10-6T1 OX-18	69.67 (2.90) ab	1.38 (0.06) abc
C17-1	63.03 (6.05) ab	1.14 (0.11) bc
C17-2	72.80 (3.91) ab	1.45 (0.08) abc
C17-3	71.47 (9.71) ab	1.46 (0.20) ab
104 T5	52.61 (5.12) b	1.03 (0.21) c
6-16 274 T4-1	79.38 (8.06) a	1.56 (0.16) a
6-16 274 T4-2	64.55 (10.33) ab	1.22 (0.20) abc
160 P3-1	80.47 (6.97) a	1.59 (0.14) a
160 P3-2	57.63 (6.61) ab	1.16 (0.13) abc
160 P3-3	65.64 (7.55) ab	1.24 (0.14) abc

[a] Means in the same column followed by a different letter were significantly different at $LSD_{0.05}$. Numbers in parentheses are standard errors. Third-instar *H. virescens* fed on pre-flowering tobacco plant for 84 h. [b] RGR, relative growth rate.

Table 5. Correlation between Larval Growth and Foliar Phenolics and Oxidases in Transgenic Tobacco

	H. virescens RGR		*M. sexta* growth	
	R^2	P	R^2	P
chlorogenic acid	0.0031	0.8631	0.0596	0.4445
total flavonoids	0.0159	0.6962	0.0756	0.3869
rutin	0.0009	0.9255	0.0373	0.5475
POD	0.0405	0.5307	0.096	0.3281
PPO	0.1260	0.7282	0.0305	0.5874

with the differential levels of chlorogenic acid, rutin, and total flavonoids (Table 5). Additionally, the growth of both *M. sexta* and *H. virescens* was not correlated with foliar POD or PPO levels in transgenic tobacco (Table 5).

DISCUSSION

Our findings indicate that phenolics such as chlorogenic acid may not play a direct role in resistance against lepidopteran insects in tobacco. These results are surprising, in that such an extensive body of evidence has been accumulated indicating that chlorogenic acid and other phenolics are toxic when incorporated into artificial diets [e.g., Elliger et al. (1981), Summers and Felton (1994), and Yang and Stamp (1995)]. Although our experiment did not examine any potential long-term effects (e.g., pupation rates) of elevated phenolics on insect development, we have successfully reared *M. sexta* on PAL overexpressing lines with no observable detriment to growth or survival. This study provides a precautionary note for plant breeding efforts. Plant breeding programs that focus on increasing the content of chlorogenic acid and other phenolics in agronomic crops for resistance against lepidopteran insects [e.g., Gueldner et al. (1992) and Wiseman et al. (1992)] perhaps require reevaluation.

This study demonstrates the inadequacy of relying upon artificial diets and correlational studies to implicate secondary compounds as mediators of plant defense. Phenolic toxicity, in particular, may be highly susceptible to modification by other dietary constituents. We have previously shown that the presence of oxidative enzymes, the type or "quality" of dietary protein, and the presence of antioxidants are determinants of phenolic toxicity (Felton et al., 1989, 1992; Summers and Felton, 1994). Indeed, foliage contains numerous antioxidants (e.g., carotenoids, glutathione, and antioxidant enzymes) that are absent or at minimal levels in artificial diets. The levels of antioxidants in foliage may be in sufficient quantity and diversity to prevent the prooxidant toxicity of chlorogenic acid [see Summers and Felton (1994)]. Also, the toxicity of phenolics with artificial diets may be greater due to the presence of comparatively large amounts of free transitional metals (i.e., iron, copper, or manganese) that catalyze phenolic oxidation. The transitional metals in plant tissues are generally protein bound and less likely to participate as catalysts.

Environmental conditions also affect phenolic toxicity to insects. Stamp (1994) and Yang and Stamp (1995) reported that fluctuating temperature regimes (e.g., 8−12 °C difference between day and night) were required for rutin in an artificial diet to exert negative effects on the growth rates of larval *M. sexta*. However, our evidence indicates that the growth rates of *M. sexta* were unaffected when the larvae fed on differential levels of phenolics in transgenic tobacco with ca. 10 °C difference in day and night temperature in the greenhouse (Table 3).

Our conclusions require qualification. First, phenolics are a structurally diverse class of phytochemicals and one should not draw inference that all phenolics act similarly on these insects. Second, although the genetic constitution of the plant lines varied by a single gene (i.e., *PAL*), the levels of expression of other metabolic pathways may be modified due to the creation of metabolic sinks (Yao et al., 1995) or the signaling properties of the phenolic compounds. For instance, elevated salicylic acid levels, which are regulated in part by PAL (Pallas et al., 1996), inhibit the expression of protease inhibitors in solanaceous plants such as tomato (Doherty et al., 1988; Doares et al., 1995). It is possible that increased phenolics in the overexpressing lines may negatively affect other defenses such as protease inhibitors, hence the lack of correlation of larval growth with phenolics. This issue is currently under investigation. In summary, our findings demonstrate that, within the genetic background of the tobacco cultivar used here, phenolics do not inhibit the growth and survival of the lepidopterans *M. sexta* and *H. virescens*. Apparently, chlorogenic acid and other phenylpropanoids may play far more critical roles in plant disease resistance (Maher et al., 1994; Yao et al., 1995; Pallas et al., 1996).

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