Polyphenolic Pattern in Apple Tree Leaves in Relation to Scab Resistance. A Preliminary Study

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Polyphenols were analyzed by HPLC in apple tree leaves of different scab-resistant and -susceptible cultivars to compare their phenolic contents in relation to their resistance to the disease. Quantitative and slight qualitative differences among cultivars were found. Dihydrochalcones (phloridzin and phloretin) and flavonols were the main phenolic compounds determined in all of the cultivars studied. In the younger stage, it would be possible to differentiate sensitive from resistant apple varieties on the basis of their flavanols contents and phloridzin/flavanol ratio. Likewise, higher levels of two *p*-coumaric acid derivatives were found in cultivars with the polygenic resistance character.

Keywords: *Polyphenols; flavanols; apple leaves; scab resistance*

INTRODUCTION

Scab, caused by *Venturia inaequalis*, is still one of the most difficult and serious diseases of apple to control. Asturias, in the north of Spain, is a relatively important cider apple producing region. To avoid the use of pesticides, the development of scab-resistant varieties appears as the best means of control.

Polyphenols have long been associated with resistance mechanisms of plants. Generally, phenolic metabolism is stimulated by fungal infection; in this sense, the rise of the phenylalanine ammonia-lyase (PAL) activity has been demonstrated in several species of plants (Ward, 1986); also, the increase of 4-hydroxycinnamic acid:CoA ligase activity was observed in affected tissues (Lyons et al., 1990). These enzymes play a central role in the biosynthesis of many phenols, namely flavonoids, lignin, and other wall-bound phenols. Benzoic compounds, namely 4-hydroxybenzaldehyde and vanillin, originated in many plants from cinnamic esters, can be recognized as phytoalexins of the cell wall (Matern and Grimmig, 1993). On the other hand, the accumulation of benzoic acid in apple fruit inoculated with *Nectria galligena* has been observed (Noble and Drysdale, 1983). Treutter and Feucht (1990a) and Feucht et al. (1992) observed a dramatic increase of catechins and their polymers in the boundary zones around the infection of *V. inaequalis* in apple leaves.

Some authors have suggested the importance of polyphenol oxidation products in disease resistance. Holowczak et al. (1962) indicated that oxidation products of phloridzin are very important in the scab resistance process; however, Noveroske et al. (1964) suggested that oxidation products of phloretin rather than phloridzin are the principal inhibitors of the fungus. The importance of those mechanisms of disease resistance could be shown if the toxic oxidation products occur in the resistant but not in the susceptible host (Williams and Kuć, 1969).

Finally, the role of some kind of preformed polyphenols in plants against pathogens has been described in different cases. Treutter and Feucht (1990b) reported higher levels of flavan-3-ols in apple leaf tissues of Vf-resistant cultivars in relation to the susceptible ones.

Table 1. Apple Cultivar Characteristics

cultivar	classification	cultivar	classification
Prima[a]	resistant (gen Vf)	Golden Delicious (GD)[a]	susceptible
Florina[a]	resistant (gen Vf)		
Perico	resistant (polygenic)	Teórica	susceptible
Riega	resistant (polygenic)	Ricu	very susceptible
Perezosa	mildly resistant	Baturra	very susceptible

[a] Dessert apple.

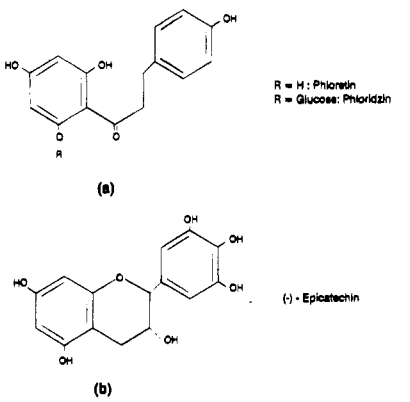

Figure 1. Structures of some of the flavonoids quantified in apple tree leaves: (a) dihydrochalcones; (b) (−)-epicatechin.

As they argued, the presence of preformed defensive phenols in the resistant cultivars may account for a more efficient response against the pathogen.

In this paper, as part of ongoing research on the development of resistant cider apple varieties, we studied the polyphenolic pattern in apple tree leaves to find a relation between the levels of some phenolic compounds present in leaves and scab resistance that could be useful to screen resistant and susceptible apple varieties.

EXPERIMENTAL PROCEDURES

Materials. Six cider and three dessert apple varieties, with different degrees of scab resistance, were selected for this study from 7-year-old apple trees, rootstock MM106, belonging to the experimental orchard of the Institute in Villaviciosa. Studies on field resistance of apple cultivars were performed following the procedure of Populer et al. (1985), to score the different varieties according to their degree of resistance

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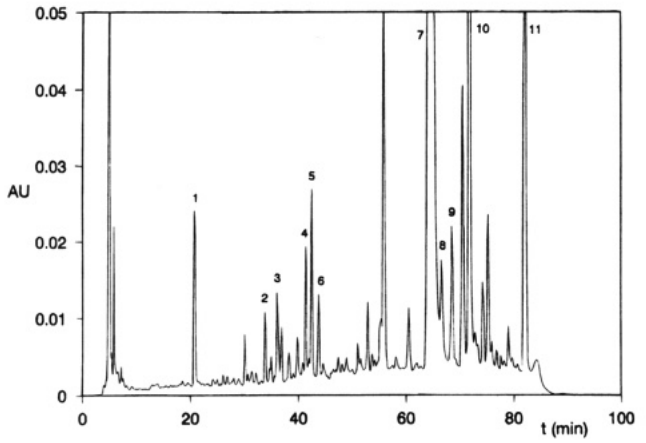

Figure 2. Chromatogram at 280 nm of an apple tree leaf extract. Conditions are described in text. Peaks: (1) benzoic; (2) procyanidin B_2; (3) chlorogenic acid; (4) (−)-epicatechin; (5) *p*-coumaric 1; (6) *p*-coumaric 2; (7) phloridzin; (8) rutin; (9) avicularin; (10) quercitrin; (11) phloretin.

(Dapena and Blázquez, 1994). The results and characteristics of the cultivars are summarized in Table 1. Leaves were collected in July (8 weeks after blooming), at terminal shoot, at three growing stages: basal (adult leaves), medium, and terminal (young leaves).

Sample Preparation. Leaves were freeze-dried and ground in a mill. Around 0.5 g of dry material was extracted with 10 mL of a methanol/water/HCl (80/19.9/0.1) mixture, and 10 mg/g of sodium metabisulfite to avoid oxidation, overnight at 4 °C, without stirring. When methanol had been removed, the extract obtained was washed with hexane (three times) to eliminate the chlorophyll; the aqueous phase was freeze-dried and kept at −20 °C until analysis. This residue was dissolved in 25 mL of the extractant mixture, filtered through a 0.22 μm PVDF filter, and injected onto the HPLC column. Extractions were done in triplicate (relative standard deviation for total polyphenols among replicates was ±5%, data not shown).

Standards and Solvents. All standards (epicatechin, gallic acid, chlorogenic acid, *p*-coumaric acid, phloridzin, phloretin, quercitrin, and rutin) were purchased from Sigma Chemical Co. (St. Louis, MO), except avicularin, which is from Extrasynthèse S.A. (Genay, France). Procyanidin B_2 was kindly provided by Dr. A. G. H. Lea, Reading, U.K. HPLC grade solvents were from Romil Chemicals and were employed as supplied.

Polyphenol Analyses. Analyses were performed with a Waters Associates system, equipped with a 712 automatic injector, M510 pumps, a Millenium v. 2.0 software data module, and a 996 diode array detector, monitoring at 280, 320, and 360 nm. The extracts were analyzed on a reversed-phase Supelcosil LC-18 column (25 × 0.46 cm; 5 μm, Supelco, Inc., Bellefonte, PA), using as solvents 2% acetic acid in water (solvent A) and methanol (solvent B). Gradient elution profile consisted of a series of three linear steps, starting with 2% B, to reach 42% B in 50 min, from 42% B to 50% B in 10 min, and isocratic elution at 50% B from 60 to 77 min. Flow rate was 0.6 mL/min, and temperature for analyses was kept at 25 °C.

Table 2. Dihydrochalcone Contents in Apple Tree Leaves (Milligrams per Gram of Dry Weight)[a]

		phloridzin			phloretin		
cultivar	resistance grade	T	M	B	T	M	B
Prima	R	140.0 ± 4.2	130.7 ± 3.9	123.2 ± 1.3	10.9 ± 9.9	1.27 ± 4.7	0.33 ± 6.9
Florina	R	71.2 ± 10.2	72.8 ± 5.9	94.3 ± 6.8	5.43 ± 12.3	1.03 ± 11.9	0.31 ± 10.8
Perico	R	96.4 ± 1.8	68.2 ± 2.5	81.9 ± 1.7	5.97 ± 1.5	4.71 ± 4.0	6.54 ± 1.5
Riega	R	129.4 ± 6.1	110.7 ± 3.6	114.5 ± 2.4	3.79 ± 4.3	3.89 ± 0.5	5.13 ± 3.6
Perezosa	MR	120.9 ± 11.8	56.9 ± 8.1	63.6 ± 0.9	1.10 ± 3.6	1.86 ± 6.5	3.29 ± 8.7
Teórica	S	89.3 ± 4.0	89.2 ± 7.6	102.1 ± 5.8	6.80 ± 4.2	5.12 ± 10.5	0.57 ± 3.5
GD	S	115.2 ± 1.2	131.4 ± 1.6	128.9 ± 3.7	8.18 ± 1.9	2.17 ± 4.6	1.06 ± 9.4
Ricu	VS	131.2 ± 2.1	95.1 ± 5.8	130.8 ± 4.3	3.60 ± 7.4	9.45 ± 1.5	8.68 ± 5.0
Baturra	VS	54.1 ± 2.0	73.3 ± 0.6	81.5 ± 3.2	3.45 ± 4.8	7.41 ± 3.8	0.66 ± 18.5

[a] T, terminal; M, medium; B, basal. Legends for resistance grade: R, resistant; MR, mildly resistant; S, susceptible; VS, very susceptible.

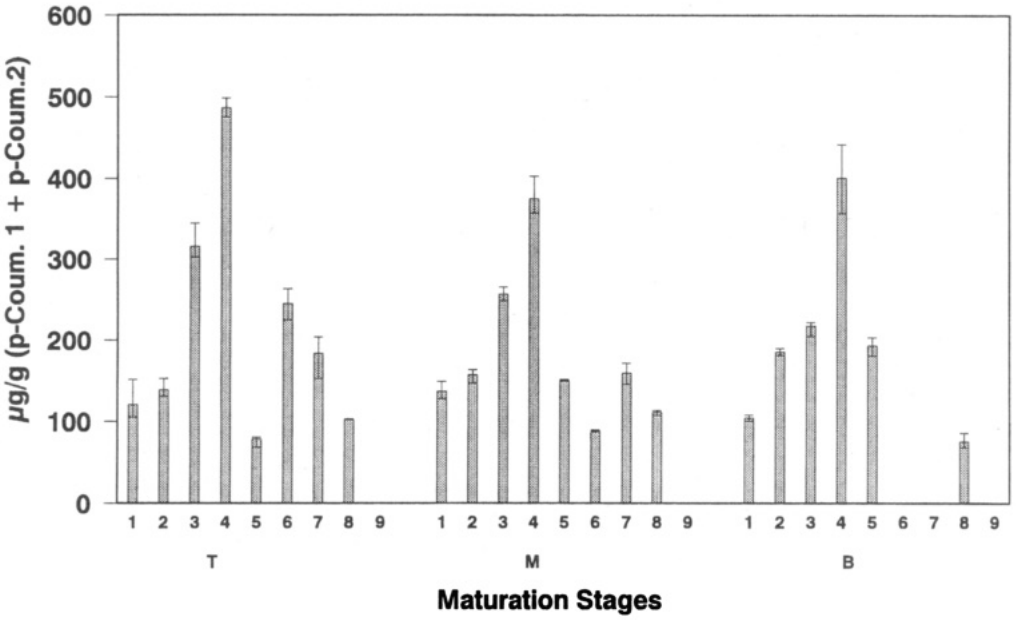

Figure 3. *p*-Coumaric 1 plus *p*-coumaric 2 contents in apple tree leaves during maturation. Maturation stage: T, terminal; M, medium; B, basal stage. Legend for cultivars: (1) Prima; (2) Florina; (3) Perico; (4) Riega; (5) Perezosa; (6) Teórica; (7) Golden Delicious; (8) Ricu; (9) Baturra.

Table 3. Phenolic Acid Contents in Apple Leaves during Maturation (Micrograms per Gram of Dry Weight)[a]

cultivar	resistance grade	benzoic			chlorogenic			*p*-coumaric 1			*p*-coumaric 2		
		T	M	B	T	M	B	T	M	B	T	M	B
Prima	R	97.8 ± 0.8	338.2 ± 1.7	709.5 ± 2.3	175.8 ± 4.5	233.9 ± 7.3	344.0 ± 2.7	ND[b]	ND	ND	105.1 ± 0.4	137.2 ± 7.2	104.5 ± 3.4
Florina	R	114.2 ± 0.1	209.7 ± 6.2	436.4 ± 7.6	286.3 ± 4.5	309.2 ± 8.5	196.5 ± 9.9	69.2 ± 4.1	77.2 ± 4.6	88.0 ± 2.5	93.4 ± 2.6	80.2 ± 6.1	97.5 ± 3.7
Perico	R	181.2 ± 4.1	329.0 ± 2.5	410.5 ± 0.7	493.7 ± 5.8	357.3 ± 14.0	321.4 ± 3.7	138.7 ± 8.7	123.1 ± 5.7	113.4 ± 4.2	180.5 ± 6.2	136.9 ± 2.2	104.6 ± 7.4
Riega	R	184.8 ± 6.3	421.1 ± 3.4	673.4 ± 1.9	371.4 ± 3.0	327.8 ± 5.6	453.8 ± 5.0	316.9 ± 5.2	230.5 ± 6.9	250.0 ± 8.5	165.7 ± 2.9	144.6 ± 4.6	160.0 ± 9.9
Perezosa	MR	413.0 ± 4.8	339.4 ± 8.8	168.9 ± 2.9	194.0 ± 1.7	215.0 ± 5.8	229.5 ± 3.9	78.8 ± 15.3	75.3 ± 2.9	99.9 ± 7.4	ND	76.6 ± 4.0	92.2 ± 11.0
Teórica	S	265.9 ± 2.8	388.3 ± 7.4	492.6 ± 5.4	333.6 ± 5.0	113.3 ± 1.6	ND	65.8 ± 12.4	ND	ND	179.3 ± 5.3	88.9 ± 1.8	ND
GD	S	202.5 ± 1.6	300.9 ± 3.7	387.8 ± 2.5	299.8 ± 3.2	238.1 ± 13.7	197.5 ± 0.5	97.8 ± 2.4	73.9 ± 8.5	ND	95.1 ± 11.0	85.6 ± 6.5	ND
Ricu	VS	171.8 ± 1.5	317.0 ± 5.8	529.1 ± 3.0	261.9 ± 16.3	925.2 ± 6.5	903.2 ± 13.7	102.9 ± 1.0	111.5 ± 3.0	ND	ND	ND	76.9 ± 11.9
Baturra	VS	234.3 ± 1.8	307.1 ± 16.3	367.5 ± 8.3	1139.3 ± 4.1	339.3 ± 14.8	275.2 ± 11.6	ND	ND	ND	ND	ND	ND

[a] See Table 2 for abbreviations. [b] Not determined.

Table 4. Flavonol Contents in Apple Leaves during Maturation[a]

cultivar	resistance grade	rutin			quercitrin			avicularin		
		T	M	B	T	M	B	T	M	B
Prima	R	4292.2 ± 9.5	6140.1 ± 6.0	8749.6 ± 5.6	9013.5 ± 2.4	2987.8 ± 1.4	2552.7 ± 1.3	717.1 ± 2.7	710.1 ± 1.1	649.1 ± 1.8
Florina	R	1586.3 ± 6.3	1613.1 ± 8.1	2350.1 ± 5.2	3696.7 ± 9.7	3373.0 ± 6.6	4497.8 ± 6.4	492.2 ± 6.8	677.7 ± 4.1	548.5 ± 5.5
Perico	R	855.8 ± 15.5	3209.7 ± 4.6	3266.7 ± 12.2	2292.0 ± 1.0	2433.5 ± 3.2	3020.6 ± 1.3	288.2 ± 1.8	556.2 ± 5.1	696.2 ± 6.4
Riega	R	ND[b]	ND	ND	4736.1 ± 5.1	4650.7 ± 2.7	5802.7 ± 2.4	515.3 ± 7.3	451.9 ± 11.4	498.3 ± 0.9
Perezosa	MR	210.3 ± 12.7	122.0 ± 12.7	ND	2473.0 ± 11.9	2528.6 ± 5.8	2297.0 ± 2.0	ND	ND	ND
Teórica	S	4391.9 ± 5.4	4551.9 ± 3.0	3537.1 ± 4.4	4969.5 ± 2.7	5080.5 ± 7.5	1956.6 ± 7.3	1427.5 ± 6.6	1031.1 ± 11.2	569.6 ± 7.0
GD	S	6843.1 ± 0.9	8854.5 ± 1.1	8406.1 ± 2.8	2071.8 ± 1.3	2481.3 ± 0.7	7078.5 ± 2.5	360.3 ± 1.6	430.0 ± 1.7	356.8 ± 2.6
Ricu	VS	142.5 ± 10.1	ND	ND	2199.1 ± 1.6	2317.6 ± 5.0	3183.2 ± 4.1	141.5 ± 5.4	ND	ND
Baturra	VS	ND	ND	ND	607.9 ± 10.2	720.2 ± 3.4	3149.4 ± 9.7	195.7 ± 8.7	263.3 ± 2.7	1455.7 ± 9.4

[a] See Table 2 for abbreviations. [b] Not determined.

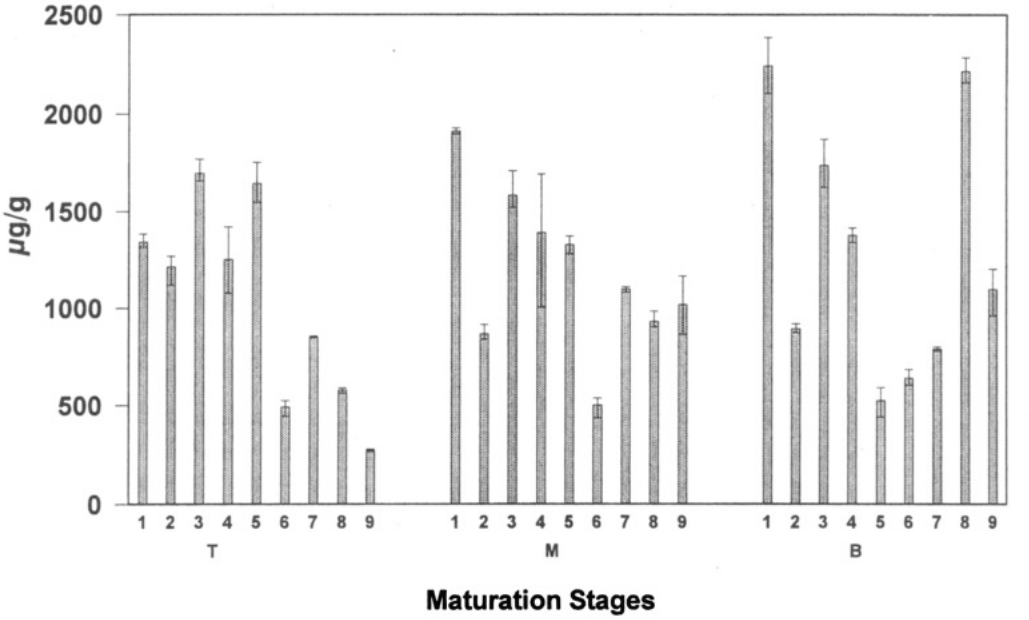

Figure 4. Flavan-3-ols (procyanidin B_2 plus epicatechin) content in apple tree leaves during maturation. Legends for cultivars and maturation stages are as in Figure 3.

RESULTS AND DISCUSSION

Compounds mentioned in this work were chosen on the basis of their interest as main components or their possible involvement in resistance mechanisms. They were identified by co-injection with the correspondent standards, when available, by both retention time and spectra. Among unknown compounds, only "benzoic" (absorption maxima at 264 nm) and the *p*-coumaric derivatives 1 and 2 (absorption maxima at 312 nm) were included since they could be assigned with high-purity detector criteria. Some of the compounds analyzed in apple leaves are shown in Figure 1.

Quantification of phenolic compounds was done by the external standard method. The compounds referred to as "benzoic" and "*p*-coumarics 1 and 2" were determined using gallic acid and *p*-coumaric acid as standards, respectively. Results are reported as micro- or milligrams per gram of dry weight. A typical chromatogram of a polyphenolic extract of apple leaves at 280 nm is shown in Figure 2.

Dihydrochalcones, phloridzin, and its aglycon, phloretin, were the main components in all of the samples studied. Phloridzin represented between 5.4% and 14% dry weight of leaves, according to the cultivar and the maturity stage considered. Phloretin concentrations exhibited greater variations than phloridzin among cultivars and leaf ages (e.g. 0.03−1.1% dry weight of leaves in cv. Prima), as shown in Table 2. Hunter and Hull (1993) found lower levels of phloridzin and phloretin in their samples, which may be related to the effect of cultural conditions. Thus, the accumulation of phloridzin in apple roots and leaves under conditions of moderate nitrogen fertilization has been described elsewhere (Hutchinson et al., 1959).

Generally, a decrease of phloridzin during maturation was observed in the case of resistant varieties, except cv. Florina, while the sensitive cultivars showed an increase in their phloridzin concentration as leaf matured. The hypothesis that resistant varieties have the ability (under genetic control) to induce a specific metabolic environment leading to fungus inhibitors could be considered. Phloridzin is oxidized to 3-hydroxyphloridzin and further to the corresponding o-quinone (Williams and Kuć, 1969), which can exhibit toxicity to the fungus (Misaghi, 1982).

Derivatives of hydroxycinnamic acids have been associated as precursors for compounds to expression of resistance. Chlorogenic was the main cinnamic acid in all of the samples studied (0.01−0.12% dry weight) (Table 3). No relation was found between chlorogenic acid levels and scab resistance, as reported by Amiot (1990) in apple fruits. However, we found higher levels of two derivatives of *p*-coumaric acid, called *p*-coumaric 1 and *p*-coumaric 2, in leaves of polygenic resistant varieties, as shown in Figure 3. It is remarkable that not one of these cinnamics was found in cv. Baturra in any of the samples analyzed.

The compound referred to as benzoic accumulated as leaf maturation progressed in all of the cultivars studied, with the exception of Perezosa (Table 3). This accumulation was greater for cv. Prima and Riega among the resistant varieties, and for cv. Teórica and Ricu among the sensitive ones, and no relation was found between its concentration and resistance to the disease.

Flavonols were found in high concentrations in different samples. Only avicularin, rutin, and quercitrin were assigned and analyzed. As shown in Table 4, quercitrin and rutin were the main flavonols in the apple tree leaves analyzed (rutin 0.01−0.9%; quercitrin 0.06−0.9% dry weight). However, no relation was found between flavonol levels and scab resistance in apples.

Flavanol concentrations [procyanidin B_2 plus (−)-epicatechin] were higher in resistant varieties (0.12−0.18% dry weight) than in the susceptible ones at the younger leaf stage (0.03−0.08% dry weight), as shown in Figure 4. The mean level of flavanols observed in young apple leaves of the scab-resistant group was 0.14% dry weight, which is 2.3 times greater than that showed by the susceptible apple cultivars at the same maturity stage (0.06% dry weight). These results were in agreement with those reported by Treutter and Feucht (1990b). The *t* test and the Levene test for equality of variances were applied to the flavanol contents at the terminal stage. They gave a significant difference between the resistant and the susceptible

Table 5. Flavanol Contents in Apple Leaves during Maturation (Micrograms per Gram of Dry Weight)[a]

		procyanidin B_2			epicatechin		
cultivar	resistance grade	T	M	B	T	M	B
Prima	R	411.5 ± 7.7	417.6 ± 4.9	596.9 ± 11.6	931.4 ± 2.9	1496.7 ± 1.5	1647.0 ± 3.7
Florina	R	278.5 ± 0.4	188.6 ± 5.1	121.0 ± 4.1	908.3 ± 9.3	672.5 ± 4.3	775.7 ± 4.2
Perico	R	967.1 ± 4.6	674.4 ± 1.5	687.7 ± 2.1	716.9 ± 1.0	839.3 ± 1.9	946.5 ± 7.2
Riega	R	705.7 ± 6.8	129.8 ± 8.7	221.8 ± 10.0	615.8 ± 5.5	1460.6 ± 9.0	1149.8 ± 2.9
Perezosa	MR	728.2 ± 12.4	533.5 ± 5.0	94.5 ± 6.4	922.6 ± 3.8	829.5 ± 5.4	431.4 ± 16.3
Teórica	S	106.0 ± 12.3	85.9 ± 19.0	102.3 ± 10.0	415.6 ± 4.1	434.0 ± 6.4	538.0 ± 5.7
GD	S	237.0 ± 1.8	357.0 ± 2.5	ND[b]	615.9 ± 0.9	744.9 ± 1.1	792.1 ± 1.3
Ricu	VS	69.9 ± 13.2	328.3 ± 4.7	1194.3 ± 4.9	507.6 ± 3.4	606.1 ± 4.2	1022.8 ± 5.0
Baturra	VS	ND	ND	ND	273.1 ± 10.0	1041.9 ± 16.4	1126.4 ± 9.5

[a] See Table 2 for abbreviations. [b] Not determined.

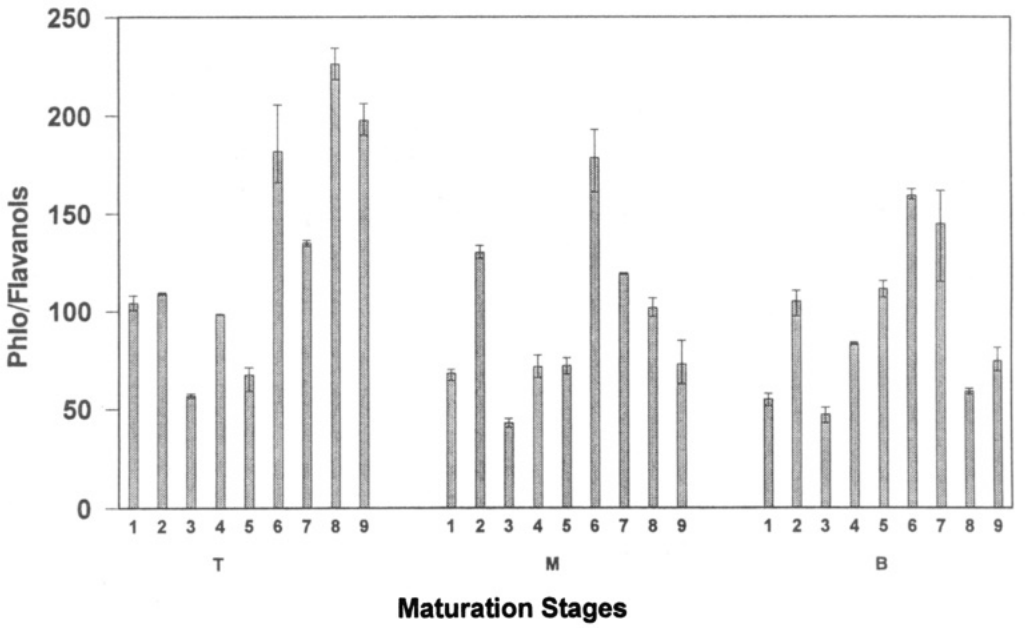

Figure 5. Phloridzin/flavanol content relationship during maturation of apple tree leaves. Legends for cultivars and maturation stages are as in Figure 3.

cultivar groups, at the confidence level of 95% [$t = 7.07$; t_c (critical value) = 1.71].

As leaf maturation progressed, some sensitive varieties showed higher flavanol contents than the resistant ones. Cv. Ricu and Baturra experienced increases in their contents of 280% and 300%, respectively (Table 5), from the younger to the adult age. This fact could be related either to late response to the fungus attack, reported in other cases (Lamb et al., 1989; Nicholson and Hammerschmidt, 1992), or to ontogenic resistance observed in adult apple leaves (Valsangiacomo and Gessler, 1988).

Some differences were found among cultivars in their phloridzin/flavanol ratio, which was higher for all of the susceptible varieties studied [$t = 9.25$; t_c(critical value) = 1.71, confidence level, 95%], at the younger leaf stage (Figure 5). As leaf matured, this ratio became lower for cv. Baturra and Ricu, which is explained by the spectacular increase of flavan-3-ols in these varieties. Therefore, for young leaves, it would be possible to differentiate between sensitive and resistant cultivars on the basis of their flavanol contents and phloridzin/flavanol ratio.

CONCLUSIONS

High-performance liquid chromatography has been used to describe the polyphenolic profiles in apple tree leaves of several cultivars. *p*-Coumaric derivatives 1 and 2 occurred in higher levels in varieties with polygenic resistance. Significant differences were found between susceptible and resistant cultivars at younger leaf age for flavanol contents and phloridzin/flavanol ratio. Therefore, these parameters may be useful to differentiate resistant from susceptible apple varieties. However, more varieties, with strict control of host–fungus interactions, must be studied to reach some correlation between the polyphenolic concentrations in leaves and scab resistance in apple.

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Received for review January 18, 1995. Revised manuscript received May 19, 1995. Accepted May 30, 1995.[⊗] We are indebted to the Comisión Interministerial de Ciencia y Tecnología (CICYT) for a contract (to A.M.P.) (ALI92-1027-CO2-02), to the Instituto Nacional de Investigaciones Agrarias (INIA) of Spain (Project SE93-089), and to the Principado of Asturias for financial support.

JF950035W

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1995.