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QUALITATIVE AND QUANTITATIVE COMPARISONS OF HYDROXYCINNAMIC ACID DERIVATIVES IN PETALS OF THE RED (llHHP^{r}P^r), WHITE (llhhpp) AND PURPLE (LLhhP^{r}P^r) GENOTYPES OF *IMPATIENS BALSAMINA**

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Abstract—Mature petals of the red (llHHP^{r}P^r), white (llhhpp) and purple (LLhhP^{r}P^r) genotypes were examined quantitatively for hydroxycinnamic acid derivatives and qualitatively for other phenolic compounds. *p*-Coumaric and ferulic acids were analyzed and found to occur free as well as in acid and base labile forms. *p*-Coumaric acid (both free and bound) was found in the highest concentration in the red genotype and lowest in the white, while ferulic acid was found in the highest concentration in the purple genotype, but was essentially equal in both the red and white tissue. The possible role of genes H and L on the qualitative and quantitative distribution of these acids is discussed.

INTRODUCTION

IN A SERIES of earlier papers Hagen,[1-3] and Alston and Hagen[4] have described the flavonoid composition and genetic inheritance of the red, white and purple genotypes of *Impatiens balsamina*. In the red genotype (llHHP^{r}P^r), the major anthocyanin pigment is the 3,5-diglucoside of pelargonidin, acylated with *p*-coumaric or ferulic acid. The purple genotype (LLhhP^{r}P^r) possesses the 3,5-diglucoside of malvidin also acylated with *p*-coumaric or ferulic acid. The white genotype (llhhpp) which does not produce anthocyanin nevertheless possesses the enzymatic potential to produce the acyl 3,5-diglucoside, if the proper substrate is applied.[5] This study was undertaken to make a qualitative comparison of each of the three genotypes and to quantitatively ascertain whether the relative amounts of *p*-coumaric and ferulic acid were different for each genotype.

RESULTS

Quantitative Results

Table 1 shows the concentrations of *p*-coumaric and ferulic acid found in each genotype. All three genotypes yielded low levels of free acids from the unhydrolyzed ethanol-soluble material. *p*-Coumaric was highest in the red genotype and lowest in the white; whereas, ferulic acid was highest in the purple and about the same in the red and white tissue. To check whether these acids were possibly being produced during the drying and handling of

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[1] C. W. HAGEN, JR., *Am. J. Botany* **53**, 46 (1966).

[2] C. W. HAGEN, JR., *Am. J. Botany* **53**, 54 (1966).

[3] C. W. HAGEN, JR., *Genetics* **44**, 787 (1959).

[4] R. E. ALSTON and C. W. HAGEN, JR., *Genetics* **43**, 35 (1958).

[5] R. L. MANSELL and C. W. HAGEN, JR., *Am. J. Botany* **53**, 875 (1966).

TABLE 1. CONCENTRATIONS OF PHENOLIC ACIDS IN ETHANOL-SOLUBLE FRACTION

	Phenolic acid concentration* (μg/g dry wt.)	
Tissue†	PCA	FER
Red (U)	118	64
Purple (U)	84	103
White (U)	54	77
Red (A)	1544	274
Purple (A)	849	309
White (A)	175	259
Red (B)	5700	563
Purple (B)	3875	948
White (B)	351	589

* Key: PCA, *p*-coumaric acid; FER, ferulic acid.
† Red, genotype llHHP^{r}P^{r}; purple, genotype LLhhP^{r}P^{r}, white, genotype llhhpp; U, unhydrolyzed fraction; A, acid hydrolyzed fraction; B, base hydrolyzed fraction.

the tissue, fresh petals were extracted immediately after picking and then processed as described in experimental. Each genotype showed small amounts of free acid comparable to that of the dried tissue.

Acid and base hydrolysis of the ethanol solubles produced a marked increase in the amount of each acid [more for *p*-coumaric acid than for ferulic acid (Table 1)]. When compared to each other, the genotypes showed that the *p*-coumaric acid in the red tissue was roughly 10–15 times that of the white and about twice the concentration found in the purple tissue.

Analysis of the ethanol-insoluble material was made after subjecting this material to alkali digestion (see Experimental). The results of this study (Table 2) indicate that residual alkaline labile derivatives persist even after repeated ethanolic extraction, although the values for each acid are quite low when calculated on grams of original material. This is partly explainable by the fact we have observed only about 34 per cent of the dried petal tissue is ethanol insoluble. Nevertheless, base hydrolysis did yield amounts of *p*-coumaric acid which were at least 13 times greater than the white tissue, but both red and purple had about the same concentration. The amount of ferulic acid is roughly the same in each genotype.

Qualitative Results

The presence of phenolic compounds in the leaves of *I. balsamina* has been examined by Bohm and Towers.[6] They found free ferulic and sinapic acids and two naphthoquinones

TABLE 2. CONCENTRATIONS OF PHENOLIC ACIDS IN THE ETHANOL-INSOLUBLE RESIDUE*

	Phenolic acid concentration (g/g dry wt.)	
Tissue	PCA	FER
Red	85	12
Purple	99	19
White	6	7

* Values based on g dry wt. of original material.

present in the unhydrolyzed extracts. Acid and base hydrolysates revealed a wide variety of both benzoic and hydroxycinnamic acids, namely *p*-hydroxybenzoic, gentisic, ferulic, *p*-coumaric and caffeic. Sinapic acid was absent in the hydrolysates.

In our analysis of the petals of this plant we observed some of the same compounds which were found in leaf tissue. For qualitative comparisons of each genotype and extract, a standard volume was chromatographed.

Spot 1 which was present in all extracts gave R_f values and color reactions characteristic of the 2-hydroxy-1,4-naphthoquinone which has previously been reported in this flower tissue.[7] Spot 2 was also found in most extracts and has subsequently been identified by co-chromatography and spectral analysis as being *p*-hydroxybenzoic acid. This was absent from unhydrolysed fresh extracted material. Spot 4 occurred in greatest abundance in the base hydrolyzed extracts. It resembles caffeic acid under u.v. and ammonia. There is also close correlation during co-chromatography with authentic material, but sufficient quantities have not been available for spectral analysis.

Spots 9–12 appear to be *cis–trans* isomers of phenolic esters. Spots 9, 10 yielded ferulic acid under both acid and base hydrolysis, while spots 11, 12 yielded *p*-coumaric acid. Both sets of spots disappear completely during base hydrolysis. The fluorescence of these spots is identical with that of the glucose esters of these compounds as reported by Harborne and Corner,[8] however, complete conformation of their identity has not yet been obtained. Spots 3, and 5–8 are unidentified.

DISCUSSION

The presence of anthocyanins, acylated with *p*-coumaric or ferulic acid, in the red and the purple genotypes of *Impatiens balsamina* and the demonstration that the white genotype possesses the potential to acylate exogenous anthocyanins has led to a quantitative study of these acylating moieties. In all three genotypes each acid was found in low concentration as the free acids. The existence of free hydroxycinnamic acids in biological tissue is somewhat rare, but Asen and Emsweller have reported the presence of free ferulic acid in an inter-specific hybrid of *Lillium*.[9] The existence of low levels of free *p*-coumaric and ferulic acids might well be expected in the colored genotypes of *I. balsamina* since degradation of the acylated anthocyanins appears to occur readily.[1,10]

The data obtained from both acid and base hydrolysis show clearly that derivatives of the hydroxycinnamic acids were the main forms present in the tissue. The quantity of *p*-coumaric acid was highest in the red genotype in the acid, base and unhydrolyzed fractions. The purple genotype was intermediate between the other two. In the case of ferulic acid, however, the purple genotype was found to possess a greater amount than either of the other two genotypes. The ferulic acid content of both the red and white tissue was essentially the same in each.

Alston and Hagen have presented genetic evidence for the inheritance of flower color in *I. balsamina*.[4] They showed that the genes at the *p* locus apparently regulate a general reaction in anthocyanin synthesis; whereas, genes H and L control specific substitutions on the ring

[6] B. A. Bohm and G. H. N. Towers, *Can. J. Botany* **40**, 677 (1962).
[7] S. Clevenger, *Arch. Biochem. Biophys.* **76**, 131 (1958).
[8] J. B. Harborne and J. J. Corner, *Biochem. J.* **81**, 242 (1961).
[9] S. Asen and S. L. Emsweller, *Phytochem.* **1**, 169 (1962).
[10] K. S. Dodds and D. H. Long, *J. Genet.* **53**, 136 (1955).

structure.[11-13] The results of our study while not resolving this question do show that gene H besides producing pelargonidin, also produces a higher ratio of *p*-coumaric to ferulic acid than gene L which gives malvidin. Gene L, on the other hand, shows just the reverse. Thus methylation activity must be greater in the purple genotype than in either the red or the white, since ferulic acid is highest in this tissue and also since malvidin is the anthocyanin produced.

The nearly equal concentration of ferulic acid in both the red and the white genotypes is interesting since its concentration does not apparently increase in the presence of anthocyanin. *p*-Coumaric acid, however, shows a very large increased concentration in both colored genotypes compared to the white tissue.

The results from the ethanol-soluble material show that small amounts of acid persist after extraction but can be released by alkaline hydrolysis. This pool of compounds might be comparable to the situation in wheat where it has also been shown that quantities of hydroxycinnamic acids occur in the ethanol-insoluble forms but can be liberated by mild alkaline hydrolysis.[14]

The qualitative studies have revealed the presence of a compound resembling caffeic acid. It might be expected that caffeic acid would be present since biosynthetic studies have shown caffeic acid to be a potential intermediate in the synthesis of ferulic acid from *p*-coumaric.[15] It also appears more abundant in the base hydrolysates which suggests that other ester derivatives are present.

The reason for production of *p*-hydroxybenzoic acid during tissue preparation is somewhat obscure at present, however, one likely possibility is that this compound exists as a glycoside which is hydrolyzed during drying by an aryl glucosidase known to be present in this tissue.

Before we can more clearly understand the significance of the differences in hydroxycinnamic acid content of each genotype, however, we first need to examine in more detail the extent to which each of these acids participate in anthocyanin acylation and other reactions in this tissue. It will also be important to see whether the total amount of hydroxycinnamic acids produced is a function of total anthocyanin content and how the concentrations of these compounds change during bud morphogenesis. These studies are currently under way.

EXPERIMENTAL

Plant material. The plant material employed in this study were the homozygous lines of genotype llhhpp (white), LLhhP^rP^r (purple) and llHHP^rP^r (red) and only freshly-opened, paired petals were used for analysis.[1] The tissue was collected, dried at 70°, ground and stored in a desiccator until analyzed. 1·5 g of the dried tissue was refluxed twice in 150 ml of 80% EtOH for 1·5 hr. The supernatants were removed by filtration through glass fiber filters and combined. The residue was collected, dried at 70°, weighed and stored in a desiccator.

The ethanol-soluble material was concentrated on a flash evaporator to 1/10 original vol. This aqueous material was divided into 3 equal vol. for extraction or hydrolysis followed by extraction. For extraction of free acids one of the unhydrolyzed aqueous fractions was adjusted to pH 1 with HCl and extracted. Acid hydrolysis was done on the second aqueous fraction by adjusting the solution to 2N with HCl and boiling for 3 min, then allowing to cool. Base hydrolysis of the third aqueous fraction was effected by bringing the solution of 2 N with NaOH and boiling for 3 min, then cooling. The base hydrolysis was acidified to pH 1 with HCl before extraction.

[11] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 261, Academic Press, New York (1967).
[12] D. HESS, *Z. Pflanzenphysiol.* **53**, 1 (1965).
[13] R. E. ALSTON, *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 196, Academic Press, London (1964).
[14] S. Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966)
[15] A. C. NEISH, *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 321, Academic Press, London (1964).

The acid and base hydrolysates and the unhydrolyzed fraction were extracted with Et_2O for 9 hr in a continuous liquid–liquid extractor. The Et_2O fractions were taken to dryness on a rotary evaporator and redissolved in 25 ml 95% EtOH. Aliquots of these solutions were spotted on Whatman No. 1 chromatography paper and developed descending in benzene–HOAc–H_2O (10:7:3, upper phase) then ascending in NaCl–HCl–H_2O (15:4:500). For qualitative comparisons the papers were observed in visible and u.v. light (366 and 254 nm) before and after fuming with NH_3 vapor. Papers were also sprayed with diazotized *p*-nitroaniline and oversprayed with 2 N NaOH. *p*-Coumaric and ferulic acids were further verified by co-chromatography with authentic compounds and by comparing their absorption maxima in EtOH and NaOEt with reported values.[9] Several of the unknown compounds listed in Table 3 were also compared spectrophotometrically with known compounds.

TABLE 3. CHARACTERISTICS OF THE MAJOR UNKNOWN COMPOUNDS OBSERVED DURING QUALITATIVE COMPARISONS OF THE EXTRACTS OF THE RED, WHITE AND PURPLE GENOTYPES OF *Impatiens balsamina*

	$R_f \times 100$†		U.V. Fluorescence		Colour reaction	
Spot No.*	BzAW	NaCl	(336 nm)	+NH_3	DPNA‡	+NaOH
1	73	47	—	—	y	y
2	35	57	—	—	y	ro
3	9	0	y	bry	yor	bn
4	6	15	b	bw	bn	—
5	8	40	—	—	y	gr-bn
6	7	72	dk	gr	—	—
7	24	82	y	y	—	—
8	40	86	y	y	bn-pk	gr
9	16	50	b	gn	lt pk	—
10	17	61	b	gn	lt pk	—
11	5	52	—	brb	—	—
12	6	70	—	brb	—	—

* Spots 1–4 were present in unhydrolysed and in both acid and base hydrolysed extracts: spots 9–12 were present in unhydrolysed and base hydrolysed extracts. Spots 5–6 and 7–8 were present only in base and acid hydrolysates respectively. Spots 3 and 4–8 were *not* identified.

† See Experimental for solvent composition.

‡ DPNA, diazotized *p*-nitroaniline; y, yellow; ro, rose; or, orange; bn, brown; w, white; b, blue; gr, gray; pk, pink; gn, green; dk, dark; lt, light; br, bright.

1·5 g of the ethanol-insoluble material was digested in 50 ml of 1 M NaOH for 2 hr at room temp. The mixture was centrifuged and the supernatant adjusted to pH 1 with HCl before being extracted continuously with Et_2O. The Et_2O soluble materials were dissolved in 5 ml of 95% EtOH and chromatographed as described above.

Quantitative values were obtained by eluting the phenolic acids from the chromatograms with 1·5 ml of 95% EtOH. 0·05 ml 2 N NaOH was added and the absorption was measured at the following wavelengths: *p*-coumaric—333 nm and ferulic—345 nm, in a Beckman DB-G recording spectrophotometer. Calculations were based on standards taken through the same procedures. Values are expressed in μg/g dry weight of petal tissue.

Each acid was analyzed from at least 3 replicate tissue samples and 5–6 determinations were made for each acid from each sample. The ethanol-insoluble analysis was done only on duplicate samples.

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