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THE ACCUMULATION OF CINNAMIC AND BENZOIC ACID DERIVATIVES IN *PTERIDIUM AQUILINUM* AND *ATHYRIUM FELIX-FEMINA**

ANTHONY D. M. GLASS and BRUCE A. BOHM

Department of Botany, University of British Columbia, Vancouver 8, B.C., Canada

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Abstract—Leaves of *Pteridium aquilinum* and *Athyrium felix-femina* were quantitatively examined for the presence of cinnamic and benzoic acid derivatives over a period of 80 days after the emergence of the fiddle heads above ground. In *Pteridium*, *p*-coumaric acid was observed to be the major acid reaching a maximum concentration at 50 days. In *Athyrium*, caffeic acid was the major acid reaching a maximum concentration at 20 days. In both species, the phenolic acids were present as ethanol-soluble as well as ethanol-insoluble components.

INTRODUCTION

RECENTLY, the occurrence of cinnamic and benzoic acid derivatives was surveyed in forty-six species of ferns.[1] The survey established the almost universal occurrence of *p*-coumaric, caffeic, ferulic, *p*-hydroxybenzoic, protocatechuic, and vanillic acids in these plants. The data were qualitative and the analyses were performed upon mature leaves. As such they gave no indication of any dynamic changes in phenolic acid concentration or composition which might occur during the growing season. For this reason it was decided to undertake a quantitative study of phenolic acid concentration as a function of age in two ferns, *Pteridium aquilinum* and *Athyrium felix-femina* and this paper records the results of that study.

RESULTS AND DISCUSSION

The results of the phenolic acid-time study with the two ferns are presented in Tables 1–4. The cinnamic acid and benzoic acid concentrations in the ethanol-soluble fraction of *Pteridium* determined at 10-day intervals are presented in Table 1. Values for acid and base hydrolysates are given. Time zero was defined as the point where a discernible fiddle head was first visible. The values in the table represent averages of four replicates and are expressed as mg of acid per gram dry weight of plant. Table 2 lists the concentrations of phenolic acids released by base hydrolysis of the alcohol-insoluble fraction of *Pteridium*. Four replicate analyses were again employed. Tables 3 and 4 present the data for the ethanol-soluble and ethanol-insoluble fractions of *Athyrium*, respectively, only two replicates were done in the case of the ethanol-insoluble determinations.

It is clear from these data that there is a significant degree of metabolic activity in these two fern species with regard to the simple phenolic acids. In general, the cinnamic acids, *p*-coumaric, caffeic, and ferulic, were present in higher concentration than the correspondingly substituted benzoic acids. Significant fluctuations were also observed in the concentrations

* Part IV in the series "Phenolic Compounds in Ferns".

[1] B. A. BOHM and R. M. TRYON, *Can. J. Botany* **45**, 585 (1967).

TABLE 1. VARIATION IN THE CONCENTRATION OF CINNAMIC ACID AND BENZOIC ACID DERIVATIVES IN THE ETHANOL-SOLUBLE FRACTION OF *Pteridium aquilinum*

Phenolic acid*		Concentration (mg/g dry wt) at							
		10 days	20 days	30 days	40 days	50 days	60 days	70 days	80 days
PCA	Base	0·440*	0·341	0·760	1·063	1·106	0·978	0·913	0·910
	Acid	0·183	0·184	0·117	0·217	0·192	0·155	0·144	0·141
CAF	Base	0·063	0·087	0·293	0·272	0·222	0·235	0·169	0·280
	Acid	0·200	0·176	0·100	0·170	0·132	0·091	0·099	0·093
FER	Base	0·091	0·188	0·158	0·184	0·176	0·160	0·133	0·124
	Acid	0·023	0·120	0·055	0·119	0·104	0·067	0·077	0·073
PHBA	Base	0·105	0·115	0·102	0·139	0·135	0·103	0·145	0·095
	Acid	0·090	0·109	0·046	0·052	0·056	0·059	0·052	0·066
PROTO	Base	0·000	0·030	0·035	0·095	0·163	0·000	0·052	0·000
	Acid	0·076	0·015	0·030	0·101	0·025	0·047	0·049	0·045
VAN	Base	0·068	0·082	0·061	0·066	0·107	0·113	0·108	0·134
	Acid	0·000	0·000	0·025	0·030	0·145	0·055	0·045	0·136

* Key: PCA, *p*-coumaric acid; CAF, caffeic acid; FER, ferulic acid; PHBA, *p*-hydroxybenzoic acid; PROTO, protocatechuic acid; VAN, vanillic acid; Base, alkaline hydrolytic procedure; Acid, acidic hydrolytic procedure.

TABLE 2. VARIATION IN THE CONCENTRATION OF CINNAMIC ACID AND BENZOIC ACID DERIVATIVES IN THE ETHANOL-INSOLUBLE FRACTION OF *Pteridium aquilinum*

Phenolic acid*	Concentration (mg/g dry wt) at							
	10 days	20 days	30 days	40 days	50 days	60 days	70 days	80 days
PCA	0·078*	0·158	0·460	0·550	1·071	0·467	0·612	0·634
CAF	0·045	0·064	0·085	0·192	0·331	0·219	0·217	0·247
FER	0·017	0·054	0·137	0·178	0·216	0·125	0·208	0·222
PHBA	0·032	0·019	0·023	0·118	0·075	0·159	0·000	0·107
PROTO	0·024	0·152	0·020	0·269	0·000	0·260	0·000	0·436
VAN	0·000	0·037	0·000	0·000	0·000	0·000	0·000	0·234

* For Key, see Table 1.

TABLE 3. VARIATION IN THE CONCENTRATION OF CINNAMIC ACID AND BENZOIC ACID DERIVATIVES IN THE ETHANOL-SOLUBLE FRACTION OF *Athyrium felix-femina*

Phenolic acid		Concentration (mg/g dry wt) at							
		10 days	20 days	30 days	40 days	50 days	60 days	70 days	80 days
PCA	Base	0·604	0·388	0·391	0·368	0·371	0·228	0·253	0·304
	Acid	0·101	0·181	0·172	0·070	0·056	0·000	0·169	0·000
CAF	Base	0·703	1·333	0·490	0·476	0·867	0·149	0·558	0·573
	Acid	0·197	0·854	0·371	0·276	0·235	0·100	0·208	0·144
FER	Base	0·000	0·051	0·094	0·184	0·185	0·152	0·153	0·128
	Acid	0·000	0·016	0·064	0·161	0·000	0·000	0·061	0·098
PHBA	Base	0·100	0·060	0·079	0·130	0·161	0·118	0·074	0·104
	Acid	0·056	0·057	0·088	0·000	0·032	0·000	0·051	0·000
PROTO	Base	0·050	0·163	0·169	0·181	0·285	0·051	0·118	0·156
	Acid	0·049	0·044	0·063	0·115	0·076	0·000	0·214	0·243
VAN	Base	0·015	0·024	0·144	0·234	0·250	0·225	0·258	0·261
	Acid	0·000	0·000	0·109	0·317	0·256	0·095	0·277	0·393

of the cinnamic acids during the period of study reported in this paper. Fluctuations in the concentrations of the benzoic acids were also observed but these were not of the magnitude observed with the cinnamic acid derivatives.

The two species showed a distinctive difference in the identity of their major cinnamic acid derivative. Even in the very young fiddle heads of *Pteridium* there was present a relatively high concentration of *p*-coumaric acid. The concentration of this compound showed a

TABLE 4. VARIATION IN THE CONCENTRATION OF CINNAMIC ACID AND BENZOIC ACID DERIVATIVES IN THE ETHANOL-INSOLUBLE FRACTION OF *Athyrium felix-femina*

Phenolic acid	Concentration (mg/g dry wt) at							
	10 days	20 days	30 days	40 days	50 days	60 days	70 days	80 days
PCA	0·113	0·096	0·117	0·077	0·076	0·073	0·063	0·061
CAF	0·252	0·245	0·391	0·188	0·108	0·149	0·111	0·122
FER	0·000	0·000	0·029	0·059	0·000	0·061	0·044	0·075
PHBA	0·090	0·044	0·034	0·000	0·047	0·066	0·063	0·109
PROTO	0·144	0·100	0·095	0·123	0·118	0·243	0·149	0·370
VAN	0·000	0·000	0·000	0·000	0·107	0·135	0·150	0·214

TABLE 5. CHARACTERISTICS OF UNKNOWN COMPOUNDS OBSERVED IN *Pteridium* AND *Athyrium*

Unknown compound*	Source†	Fluorescence at 3660 Å	Fluorescence with NH_3	Colour reaction with DPNA‡
1	P (a, b)	Yellow	Intensified	Mauve
2	P, A (a, b)	None	None	Green
3	P, A (a)	None	None	Mauve
4	P, A (b)	None	None	Blue to mauve
5	P, A (b)	None	Blue	Orange
6	P, A (a)	None	None	Mauve
7	A (a)	None	None	Grey
8	P, A (a)	Blue	Intensified	None
9	P, A (a)	None	Blue	Mauve
10	A (b)	None	None	Blue
11	P (a)	None	Blue	Violet

* Numbers correspond to unknown in diagram (Fig. 1).

† P, *Pteridium;* A, *Athyrium;* a, acid, hydrolysate of ethanol-soluble fraction; b, alkaline hydrolysate of ethanol-soluble fraction.

‡ Diazotized *p*-nitroaniline oversprayed with dilute sodium hydroxide.

remarkable increase during the period of the plant's most rapid growth, reaching a maximum at 50 days (Fig. 1). This pattern is similar to that observed by Kuc and Nelson[2] in their study of wheat plants. Their kinetic studies showed that the quantity of *p*-coumaric acid per unit weight of lignin reached a maximum at 60 days and thereafter declined. In *Athyrium*, caffeic acid replaced *p*-coumaric acid as the major acid and reached a maximum concentration at 20 days (Fig. 2). After this time, despite some considerable fluctuations, the concentration of

[2] J. KUC and O. E. NELSON, *Arch. Biochem. Biophys.* **105,** 103 (1964).

this acid declined. By contrast, vanillic acid was initially absent or present in very small quantity in both species. For as long as the study was continued the quantity of this compound showed a steady increase.

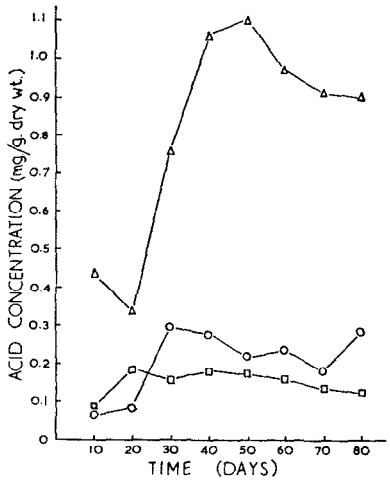

FIG. 1. VARIATION IN CINNAMIC ACID CONCENTRATION IN THE BASE HYDROLYSATE OF THE ETHANOL-SOLUBLE FRACTION OF *Pteridium*.

△, *p*-coumaric acid; ○, caffeic acid; □, ferulic acid.

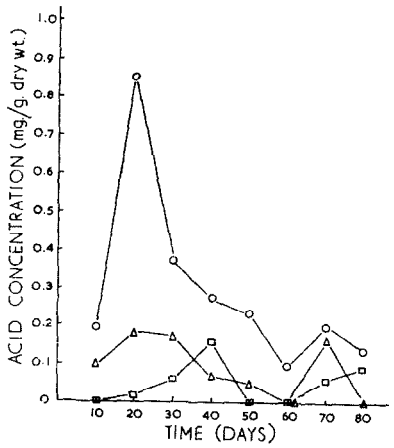

FIG. 2. VARIATION IN CINNAMIC ACID CONCENTRATION IN THE ACID HYDROLYSATE OF THE ETHANOL-SOLUBLE FRACTION OF *Athyrium*.

△, *p*-coumaric acid; ○, caffeic acid; □, ferulic acid.

In both species larger quantitites of phenolic acids were present as base sensitive ethanol-soluble conjugates than acid-sensitive forms. In *Pteridium* the acid labile conjugates showed little variation during the life of the leaves. It would appear unlikely that acid labile forms represent significant quantities of active metabolites in this plant. *Athyrium*, in contrast, showed as much variation in its acid labile conjugates as in its base labile forms. The ethanol-insoluble component, in *Pteridium*, commenced at a lower level of concentration than the

ethanol-soluble component. During the period of the study there was considerably more variation in this ethanol-insoluble fraction than in the ethanol-soluble counterpart. There was, however, a more decided trend toward an overall increased accumulation with time in the former. Ester linkage to cell-wall material may represent a resting place for phenolic acids prior to becoming involved in further metabolism. In *Athyrium* only small quantities of phenolic acids were found as ethanol-insoluble forms. Furthermore, these forms showed little variation during the season.

The most striking observation in this study is the dynamic status of the cinnamic acids, particularly *p*-coumaric acid in *Pteridium* and caffeic acid in *Athyrium*. Both of these compounds increased rapidly initially and later declined in concentration. In a study of lignin biosynthesis in wheat plants Stone *et al.*[3] found that the greatest increase in the production

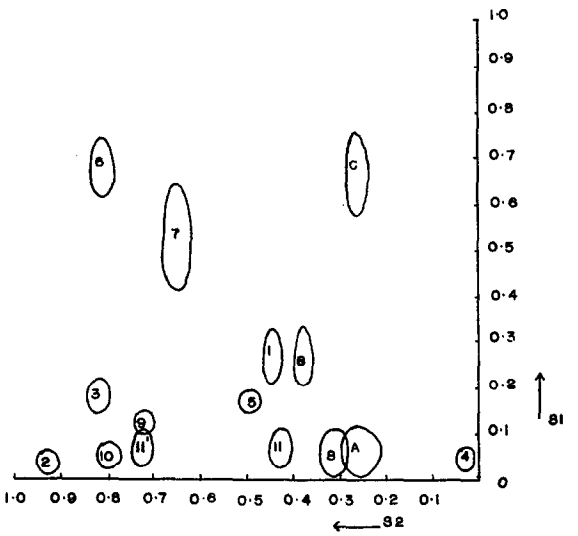

FIG. 3. DIAGRAM OF UNKNOWN COMPOUNDS OBSERVED IN *Pteridium* AND *Athyrium*.

S_1 was the organic phase of benzene:acetic acid:water (10:7:3, v/v). S_2 was 2% formic acid. The figures along the axes represent R_f values. The spots labelled A, B, and C are caffeic acid, *p*-coumaric acid, and ferulic acid, respectively, and are included as standard markers.

of lignin occurred 45 to 70 days after seeding. Higuchi and Brown[4] also observed a slower rate of lignification in younger wheat plants. Their observations suggest that the slower rate is due to the cumulative effect of slower reactions at several stages rather than relative inactivity of an enzyme system at any one stage of the biosynthetic pathway. It may well be that during the early stages of growth, when photosynthetic activity is usually pronounced, large quantities of potential lignin precursors are set aside in the form of esters, glycosides, etc. to enter the main lignin pathway at a later time. The maximum concentration of caffeic acid in *Athyrium* was reached at age 20 days. At this age the leaves were about 30 cm tall, pale green in colour, and still partly circinate. These young leaves emerge between fully grown leaves of the same rhizome and are thus considerably shaded at this particular age. Considering the biosynthetic origin of phenylpropanoid molecules from carbohydrate[5] it is intriguing

[3] J. E. STONE, M. J. BLUNDELL and K. G. TANNER, *Can. J. Chem.* **29**, 734 (1951).
[4] T. HIGUCHI and S. A. BROWN, *Can. J. Biochem. Physiol.* **41**, 65 (1963).
[5] A. C. NEISH, *Ann. Rev. Plant Physiol.* **11**, 55 (1960).

that caffeic acid should reach its maximum concentration at a time when the photosynthetic potential is still far from being realized. Perhaps phenylpropanoid molecules synthesized in the older leaves are transported to the younger leaves in preparation for the lignification process. Alternatively, the hydrolysis of food reserves located in the rhizome may provide the necessary raw materials.

Hillis and Swain[6] analysed leaves of *Prunus domestica* for total phenols, leucoanthocyanins and flavonols at intervals during the growing season and found that the amounts increased rapidly until the leaves reached maximum size and then decreased. Their results suggest that in the older leaves the phenolic compounds are either metabolized or translocated to other parts of the plant.

During the course of the examinations of these ferns several "unknown" compounds were observed. These are illustrated in the diagram (Fig. 3). The behaviour of these compounds under u.v. light (3660 Å) and their colour reaction to diazotized *p*-nitroaniline are recorded in Table 4. One compound in particular (No. 1 in Fig. 3) proved to be interesting in that its occurrence (restricted to *Pteridium*) was limited to leaves between the ages of 20–30 days. At its maximum it appeared in reasonably high concentration, the concentration dropped rapidly thereafter, and the molecule did not reappear throughout the remainder of the plant's life. Attempts to isolate the compound for structural analysis have so far failed, due to its instability.

MATERIALS AND METHODS

All ferns were collected from the University grounds where they were growing wild. At approximately 10 days after their emergence above ground about forty fiddle heads of each species were labelled; thereafter samples were taken every 10 days for analysis. Individual leaves were cut into small pieces and blended with a Waring Blendor. The ethanol-soluble material was then extracted by repeated boiling of the ground material in 80% EtOH. The ethanolic solution was separated from the insoluble material by filtration and then evaporated to dryness. The weight of this dried residue was recorded. The ethanol-insoluble material was oven-dried and weighed. A total dry weight estimate for each leaf was obtained by summation of the ethanol-soluble and insoluble dry weights.

The ethanol-soluble residue was filtered through celite and the filtrate was divided into two portions. Acid hydrolysis was performed upon one portion by adjusting the solution to 1 N with HCl, bringing the solution to boiling and then allowing it to cool to room temperature. Acid hydrolysates were extracted directly with ether in a continuous extractor for 9 hr. The second solution was subjected to basic hydrolysis by adjusting it to 1 N with NaOH, heating the solution to boiling and then allowing it to cool to room temperature. Basic hydrolysates were acidified to pH 2 with HCl prior to ether extraction. Ether extracts were evaporated to dryness and made up to standard solutions using 95% ethanol. Aliquots of these solutions were spotted onto each of two sheets of Whatman No. 1 chromatography paper. Each sheet was developed, by descending chromatography, first in benzene:acetic acid:water (10:7:3; organic phase) and then in 2% formic acid for the second dimension. The locations of the phenolic acids were determined by examination of the chromatograms under u.v. light (3660 Å) before and after fuming with ammonium hydroxide, and after spraying with diazotized *p*-nitroaniline followed by 2 N NaOH. One chromatogram of each pair was not sprayed. The phenolic acids were eluted from this chromatogram with 95%

[6] W. E. HILLIS and T. SWAIN, *J. Sci. Food Agric.* **10**, 135 (1959).

ethanol and each solution made up to a known volume with the same solvent. The absorbance of each solution was determined using a Beckman DU Spectrophotometer at the wavelength of maximum absorption of the compound concerned. The quantity of each acid was then obtained from standard calibration curves and extrapolated to an expression of the weight in mg per g dry weight of leaf.

3 g of the dried ethanol-insoluble material was treated with 100 ml of 1 N NaOH for 2 hr at room temperature. The filtrate was acidified to pH 2 with HCl and extracted continuously with ether. Thereafter analyses were performed in the same manner as described for the ethanol-soluble fraction.

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