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SHORT COMMUNICATION

CHANGES IN PHENOLIC CONSTITUENTS OF MILDEW-INFECTED HYDRANGEA LEAVES

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Abstract—A glucoside of cyanidin has been isolated and identified from young hydrangea leaves, naturally infected with powdery mildew. Synthesis of phenolic acids and coumarin derivatives decreased markedly in diseased leaves as shown by quantitative and specific radioactivity measurements. The results suggest possible utilization, by the fungus, of phenolic compounds or their precursors.

INTRODUCTION

LEAVES of *Hydrangea macrophylla* Ser. (Saxifragaceae) contain, besides the common substituted cinnamic and benzoic acid derivatives,[1] two coumarins,[2] umbelliferone (7-hydroxycoumarin) and hydrangetin (7-hydroxy-8-methoxycoumarin) and the phenyl-*iso*-coumarin, hydrangenol[3] (3,3'-dihydroxy derivative). It was observed that young hydrangea leaves, naturally infected with the powdery-mildew *Erisyphe cichoracearum* DC.,[4] soon begin to accumulate a red pigment in the palisade cells. At this stage of infection the whitish mycelium with its characteristic spores were mostly confined to the spongy tissue of the leaf. Since host tissues are known to accumulate aromatic compounds following fungal infection,[5] it was of interest to identify the pigment and demonstrate its biosynthesis from phenylalanine-^{14}C in view of the possible changes in other phenolic compounds.

RESULTS AND DISCUSSION

Identification of the Pigment

Using standard chromatographic (paper and TLC) and spectrophotometric methods[6] a pure sample of the pigment was isolated from naturally infected mature leaves. The chromatographic and spectral characteristics of the pigment were similar to those of a glucoside of cyanidin and the hydrolysis products, to an authentic sample of cyanidin (Fluka, Buchs, Switzerland) and glucose. The ratio of glucose and cyanidin in the pigment was determined as 1:1 and its quantity amounted to approximately 30 μM/g fresh material.

Phenolic Synthesis by Healthy and Diseased Leaves

Disks from healthy and diseased young leaves were administered L-phenylalanine-U-^{14}C in the light and their extracts were analyzed by a combination of quantitative fluorometry

[1] R. K. IBRAHIM, *Biosynthesis of Phenolic Acids and Lactones in Higher Plants*, Ph.D. Thesis, McGill University, Montreal (1961).
[2] B. A. BOHM, R. K. IBRAHIM and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **39**, 1389 (1961).
[3] R. K. IBRAHIM and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **38**, 627 (1960).
[4] S. K. GAYED, Personal communication.
[5] I. A. M. CRUICKSHANK and D. R. PERRIN, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 511, Academic Press, London. (1964).
[6] J. B. HARBORNE, in *Comparative Biochemistry of the Flavonoids*, Academic Press, London (1967).

and liquid scintillation. The results (Table 1) show that phenylalanine-^{14}C was converted to *p*-coumaric acid, caffeic acid, umbelliferone, hydrangetin and hydrangenol, all labelled with ^{14}C, in both healthy and diseased leaves. Radioactive cyanidin was synthesized by diseased leaves only and contained about 10 per cent of the radioactivity in the ethanol-soluble fraction. It is apparent that infection of hydrangea leaves with powdery mildew, though it induced the formation of cyanidin glucoside, caused a significant decrease in phenolic synthesis. This was exhibited by the decreased incorporation of label in both alcohol-soluble and insoluble fractions, as well as the decreased amounts and low specific activities of individual phenols.

TABLE 1. QUANTITIES AND SPECIFIC ACTIVITIES OF MAJOR PHENOLIC COMPOUNDS IN HEALTHY AND MILDEW-INFECTED HYDRANGEA LEAF DISCS ADMINISTERED PHENYLALANINE-^{14}C* FOR 8 hr IN LIGHT

Leaf Material	% Uptake of activity	dpm/mg dry material		Quantity† and specific activity‡					
		Alcohol-soluble	Alcohol-insoluble	Cyanidin	*p*-Coumaric	Caffeic	Umbelliferone	Hydrangetin	Hydrangenol
Experiment 1									
Healthy	70·2	19,965	4,829	—	7·01	5·42	11·15	3·23	20·35
				—	(2.15)	(2·17)	(1·92)	(1·07)	(0·12)
Diseased	74·6	12,829	3,792	2·85	5·04	4·03	4·09	0·65	10·81
				(1·68)	(0·78)	(0·98)	(0·55)	(0·26)	(0·05)
Experiment 2									
Healthy	82·1	25,167	5,905	—	8·02	6·44	9·72	3·07	18·94
				—	(2·65)	(2·40)	(2·01)	(0·92)	(0·15)
Diseased	79·3	17,586	4,694	3·07	5·8	4·78	4·87	1·55	11·01
				(2·25)	(1·05)	(0·81)	(0·75)	(0·31)	(0·07)

* 10 μc in 0·5 ml water (15·3 mc/mmol).
† μM/g dry weight, determined fluoremetrically.
‡ μc/mM (values in brackets).

The accumulation of flavonoids has been reported in diseased tissues[7] and considered a symptom of early senescence as a result of fungal infection. Therefore, cyanidin formation may account for the decreased synthesis and low specific activity of *p*-coumaric and/or caffeic acids, since both compounds are formed from a common precursor, phenylalanine or cinnamic acid, which is required for cyanidin synthesis. However, the marked drop in the synthesis of coumarins cannot be explained in view of the findings by many workers on the accumulation of phenolics in host tissues following fungal infection.[5]

Examination of autoradiographs of the diseased leaf extracts revealed small amounts of radioactivity in a number of unidentified metabolites; two of which correspond to *p*-hydroxybenzoic and protocatechuic acids that were not labelled in healthy leaves. Although the nature of the other metabolites has not been investigated they may be breakdown products resulting from fungal metabolism of the host phenolic constituents. *p*-Hydroxybenzoic and protocatechuic acids have been reported among the degradation products of phenylalanine and tyrosine by saprophytic basidiomycetes.[8] Although the metabolism of phenolic com-

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[8] K. MOORE, P. V. SUBBA RAO and G. H. N. TOWERS, *Biochem. J.* **106**, 507 (1968).

pounds by obligate parasites is far from understood, however, β-oxidation of cinnamyl compounds in a manner analogous to fatty acid oxidation,[9] or ring cleavage of aromatic nuclei to CO_2, may be possible.

EXPERIMENTAL

Isolation of the Pigment

20 g of naturally infected mature leaves, selected from several diseased plants, were blended in hot 1% MeOH–HCl and the alcohol evaporated *in vacuo*. The residue was streaked on several sheets of Whatman No. 3 paper and chromatographed in BAW and 1% HCl as developing solvents. The pigment was purified with cellulose-TLC using the same solvents.

Identification of Cyanidin and its Glucoside

R_f values of the isolated pigment were 0·40 in *n*-BuOH–HOAc–H_2O (4:1:2·2), 0·18 in 1% HCl and 0·24 in *n*-BuOH–2 N HCl (1:1, upper layer); $\lambda_{max}^{MeOH-HCl}$ 272, 522 nm; $\lambda_{max}^{MeOH-AlCl_3}$ 275, 556 nm. Hydrolysis of the pigment with 1 N HCl for 30 min at 100° gave cyanidin with R_f 0·70 in BAW and 0·50 in Forestal; $\lambda_{max}^{MeOH-HCl}$ 276, 455^{sh}, 535 nm; $\lambda_{max}^{MeOH-AlCl_3}$ 280, 460^{sh}, 560 nm; and glucose which was identified by cochromatography with authentic sample. The glucose/cyanidin ratio was determined by the method of Somogyi[10] for the former and spectrophotometrically at 535 nm for the latter (molar extinction coefficient 25,000).

Administration of Phenylalanine-C^{14}

Twenty leaf disks (five to six leaves) from healthy or diseased plants (early stage of infection) were vacuum infiltrated and floated on 0·5 ml (10 μc) of L-phenylalanine-U-^{14}C (15·3 mc/mmole, Nuclear-Chicago Corp.) in a covered Petri dish in the light (*ca.* 6600 lux) for 8 hr. Leaf disks were then rinsed with water and extracted with 1% MeOH-HCl and the residue dried. Measured aliquots were assayed for radioactivity and the rest of the extract was evaporated to an aqueous residue which was subjected to alkaline and acid hydrolysis, then extracted with ether. The ether extract was blown to dryness, taken in alcohol and quantitatively spotted on cellulose–silica gel TLC plates and chromatographed in benzene–HOAc–H_2O (2:2:1) followed by 2% HOAc, for phenolic acids and coumarins. Cyanidin was recovered from the aqueous layer of the hydrolyzate by extraction with *n*-AmOH and chromatography in BAW.

Quantitative and Radioactivity Determinations

Individual phenolic compounds were quantitated by direct *in situ* fluorometry[11] on TLC plates and cyanidin concentration was measured spectrophotometrically at 535 nm. Their radioactivity was determined by liquid scintillation counting. The activity of the alcohol-insoluble residue was determined by dry combustion of 5–10-mg samples in presence of O_2 using the Schöniger flask method. A mixture (6 ml) of phenylethylamine–absolute methanol–toluene (1:2:1) was used to absorb $C^{14}O_2$, and 1 ml samples were counted for radioactivity.

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