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ABNORMAL METABOLITES OF WHEAT: OCCURRENCE, ISOLATION AND BIOGENESIS OF 2-HYDROXYPUTRESCINE AMIDES*

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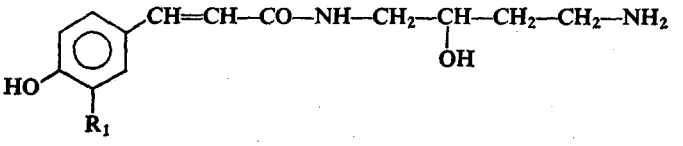
Abstract—2-Hydroxyputrescine amides of ferulic acid and *p*-coumaric acid, first detected in rust-infected, resistant wheat grown at 20°, also occurred in rust-infected, susceptible wheat grown at 25°, and in wheat infected with *Pyrenophora tritici-repentis* or with virulent or avirulent species of *Pseudomonas*. Small amounts of the abnormal metabolites were detected in wheat leaves treated with necrogenic chemicals or with hot water. Leaves of oats and barley, infected with virulent and avirulent pathogens, did not contain these 2-hydroxyputrescine amides. Conditions favoring their production in wheat were determined, and a procedure is given for their isolation. Tracer studies indicated that the aromatic moieties of the abnormal metabolites are derived from shikimic acid via phenylalanine and hydroxycinnamic acids. Tyrosine was not an effective precursor. The origin of the 2-hydroxyputrescine moiety is not known, but it apparently does not arise from free putrescine.

INTRODUCTION

RUST-INFECTED, resistant wheat leaves have been shown to accumulate radioactivity from shikimate-U-^{14}C in fluorescing compounds that were separated from phenylalanine and tyrosine on electropherograms.[1] Corresponding areas on electropherograms prepared with crude extracts from healthy or rust-infected, susceptible leaves did not contain these fluorescing compounds, although some activity was associated with these areas. Two of the fluorescing compounds were subsequently identified[2] as *N*-(*p*-coumaroyl)-2-hydroxyputrescine and *N*-(feruloyl)-2-hydroxyputrescine.



$R_1 = H$ *N*-(*p*-Coumaroyl)-2-hydroxyputrescine
$R_1 = OCH_3$ *N*-(Feruloyl)-2-hydroxyputrescine

Esters of cinnamic acids occur widely in higher plants, but amides of cinnamic acids seem to be rare since only a few have been isolated. These include coumaroylagmatine and the hordatines from barley,[3] and subaphyllin, the putrescine amide of ferulic acid. Since the latter has been isolated from two unrelated species of higher plants,[4,5] we considered that the

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[1] R. ROHRINGER, A. FUCHS, J. LUNDERSTÄDT and D. J. SAMBORSKI, *Can. J. Botany* **45**, 863 (1967).
[2] A. STOESSL, R. ROHRINGER and D. J. SAMBORSKI, *Tetrahedron Letters* **33**, 2807 (1969).
[3] A. STOESSL, *Can. J. Chem.* **45**, 1745 (1967).
[4] A. A. RYABININ and E. M. IL'INA, *Doh. Akad. Nauk. SSSR* **67**, 573 (1949) cited in *Chem. Abs.* **44**, 1455 (1950).
[5] T. A. WHEATON and I. STEWART, *Nature* **206**, 620 (1965).

2-hydroxyputrescine amides might not be restricted to wheat. Other cereals were therefore analyzed for their content of 2-hydroxyputrescine amides. It was also of interest to determine whether formation of these compounds was responsible for rust resistance. Studies were also initiated on the biosynthesis of these abnormal metabolites and a procedure is described for isolating these compounds from wheat.

RESULTS AND DISCUSSION

Occurrence

Wheat leaves, maintained at 20°, contained the abnormal metabolites after infection with avirulent cultures of rust, or with other chlorosis-producing microorganisms, or after other treatments that produced necrosis (Table 1). When maintained at 25°, rust-infected leaves also produced the amides, but leaves maintained in growth chambers at this temperature appeared slightly chlorotic. Thus, production of the amides in wheat was correlated with chlorosis- or necrosis-producing treatments, and the largest amounts were produced under conditions of sustained stress. Since healthy plants, that did not produce the amides, are known to contain ferulate and *p*-coumarate,[1] these tissues either do not produce 2-hydroxyputrescine, or lack the conjugation mechanism necessary for amide formation.

2-Hydroxyputrescine amides were not detected in healthy plants of oats and barley or in any of the following host–parasite complexes (primary leaves examined 7 days after inoculation): barley var. "Montcalm"/*Puccinia hordei*, race 44 (susceptible reaction); barley var. "OAC-21"/*P. hordei*, race 44 (resistant reaction); barley var. "Vantage"/*Pyrenophora teres* (susceptible reaction); barley line CI 5791/*P. teres* (resistant reaction); oat line UKR 1269/*Puccinia coronata*, race 264 (susceptible reaction); oat line UKR 1269/*P. coronata*, race 305 (resistant reaction). It is possible that the formation of 2-hydroxyputrescine amides is restricted to wheat, since conditions favoring amide production in wheat did not elicit amide formation in oats or barley.

Occurrence and formation of the abnormal metabolites fits the pattern reported for production of phytoalexins.[6] To qualify as a phytoalexin, a compound must possess antimicrobial activity. At present it is not known whether the 2-hydroxyputrescine amides have antimicrobial properties, because insufficient amounts were available for testing. However, even if they exhibit these properties, it is unlikely that they are involved in rust resistance, because they were present in normally resistant wheat in which the resistance gene *Sr*6 had been rendered ineffective by increasing the temperature (20–25°) (Table 1).

Several experiments were conducted to determine conditions favoring production of the abnormal metabolites in wheat. Although the abnormal metabolites were formed in wheat leaves as a response to a variety of treatments (Table 1), the largest amounts were produced in rust-infected, resistant-reacting leaves. To determine the amide content of leaves at various times after inoculation, primary leaves of plants of the resistant line of Chinese spring wheat were inoculated with *P. graminis* f. sp. *tritici*, race 56, and samples were taken 2, 4, 6 and 8 days later. The amounts of *N*-(feruloyl)-2-hydroxyputrescine, expressed as μmole ferulic acid equivalents g fr. wt., were 0·06, 0·18, 0·41 and 0·49, respectively. Extracts from samples harvested 8 days after inoculation contained large amounts of substances that were difficult to separate from the 2-hydroxyputrescine amides. Therefore, leaves with 6-day-old rust infections were used in all later studies and for isolation of the abnormal metabolites.

The content of 2-hydroxyputrescine amides in rust-infected, resistant-reacting leaves was not affected by detaching the leaves for periods up to 24 hr before extraction, and similar

[6] I. A. M. CRUICKSHANK, *Ann. Rev. Phytopathol.* **1**, 351 (1963).

TABLE 1. OCCURRENCE OF 2-HYDROXYPUTRESCINE AMIDES IN WHEAT

Plant material*	Treatment or pathogen used	Tissue damage or disease reaction	Relative amount† of 2-hydroxyputrescine amides
Near-isogenic lines‡ of Chinese spring wheat,			
Primary leaves			
S	Untreated	None	−
S	*Puccinia graminis* f. sp. *tritici*, race 56	Susceptible	±§
S (greenhouse)	*Puccinia graminis* f. sp. *tritici*, race 56	Susceptible	−
R	Untreated	None	−
R	*Puccinia graminis* f. sp. *tritici*, race 56	Resistant	+++
S (25°)	Untreated	None	−
S (25°)	*Puccinia graminis* f. sp. *tritici*, race 56	Susceptible	+++
R (25°)	Untreated	None	−
R (25°)	*Puccinia graminis* f. sp. *tritici*, race 56	Susceptible	+++
S and *R*	*Pseudomonas atrofaciens*	Hypersensitive	++
S and *R*	*Pseudomonas* spp.	Susceptible	+++
R (greenhouse)	Leaves shaded to simulate senescence	Etiolated	−
S and *R*	Leaves flooded with H_2O (at 20°)	None	−
S and *R*	Leaves flooded with H_2O (at 90°)	Necrotic	+
S and *R*	Leaves flooded with 0·2% NH_4OH	Necrotic	++
S and *R*	Leaves flooded with 2·1% H_2O_2	Necrotic	+
Variety "Selkirk"			
Primary leaves	Untreated	None	−
Primary leaves	*Puccinia recondita*, race 15	Resistant	+++
Primary leaves	Leaves rubbed with carborundum	No apparent damage	−
Secondary leaves	Untreated	None	−
Secondary leaves	*Pyrenophora tritici—repentis*	Chlorotic, necrotic	++
Variety "Noroeste 66"			
Flag leaves (field)	Natural rust epidemic	Resistant	++++

* Growth chambers at 20°, unless indicated otherwise.

† — (-) Not detected, (+, ++, +++, ++++) relative amounts.

‡ The resistant line (*R*) contains the temperature-sensitive gene *Sr*6 that conditions resistance to race 56 of *P. graminis* f. sp. *tritici* at temperatures ≤20°; *S* = susceptible line.

§ 2-Hydroxyputrescine amides were sometimes detected in susceptible-reacting leaves grown in growth chambers under high light intensity. Under these conditions non-infected leaf areas appeared slightly chlorotic.

amounts of the amides were present in plants grown in the greenhouse and in plants grown in growth chambers (9600 lx; 16 hr light period; 19°). More of the abnormal metabolites were produced in heavily inoculated leaves (>200 infections/leaf) than in lightly inoculated leaves (approximately 50 infections/leaf), and leaves harvested at the end of a 4 hr light period (9600 lx; 19°) contained more of the amides than similar leaves harvested at the end of a dark period. Feeding of 0·15 μmoles of shikimate/g fr. wt. of leaves for 22 hr after detachment also increased the amount of abnormal metabolites.

Biogenesis

After feeding of ^{14}C-labelled hydroxycinnamic acids and some of their known precursors, activity in the abnormal metabolites was determined (Table 2). Although no degradations were performed, it is assumed that the activity resided in the aromatic moieties and not in 2-hydroxyputrescine. Tyrosine-U-^{14}C did not give rise to activity in either of the abnormal metabolites. The reason for this is not known, but it may be related to the relatively inefficient conversion of tyrosine to bound cinnamic acid esters in primary leaves of wheat.[7] In previous experiments,[7] phenylalanine-U-^{14}C gave rise to more activity in esters of ferulate than in those of *p*-coumarate. In the present experiment, more activity from phenylalanine-U-^{14}C was recovered in the 2-hydroxyputrescine amide of *p*-coumaric acid than in that of ferulic acid. This may indicate that the aromatic moieties of the abnormal metabolites are derived from a different pool of cinnamic acids than that used for the synthesis of cinnamic acid esters. The amount of activity in each of the two abnormal metabolites after feeding of labelled hydroxycinnamic acids is consistent with current views[8,9] on the origin of phenylpropanoid constituents in plants.

There are no reports on the occurrence of free 2-hydroxyputrescine in natural sources, and we have not detected the free base in any of our extracts. The origin of the 2-hydroxyputrescine moiety of the abnormal metabolites is therefore of interest. We carried out preliminary experiments to determine whether the 2-hydroxyputrescine moiety is derived via pathways analogous to those of putrescine formation in other plants.[10,11] In rust-infected, resistant wheat leaves that contained the 2-hydroxyputrescine amides, activity from arginine-U-^{14}C, ornithine-5-^{14}C, and proline-U-^{14}C was incorporated with very low efficiency into the abnormal metabolites. Putrescine-1-^{14}C, fed to rust-infected resistant wheat leaves, was metabolized, but not incorporated into the abnormal metabolites. This may indicate that putrescine is not a precursor of the 2-hydroxyputrescine amides. Further studies on the origin and possible metabolic function of the abnormal metabolites are in progress.

EXPERIMENTAL

Plant Material and Treatments

Conditions of growth and inoculation with rusts were as previously described.[1] In most cases, plants were inoculated 7 days after seeding and harvested 6 days later. Standard procedures were used for preparation of inoculum and for inoculation with *Pyrenophora*[12] and *Pseudomonas*.[13] Treatment of leaves with necrogenic chemicals was performed by flooding[13] and the treated leaves were harvested 2 or 3 days later when symptoms had developed. When radioactive tracers were fed, leaves were detached, allowed to take up the isotope solution through their cut ends and maintained for 22 hr at 21° in the light.[1]

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[8] S. Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).
[9] S. YOSHIDA, *Ann. Rev. Plant Physiol.* **20**, 41 (1969).
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[13] W. A. F. HAGBORG, *Proc. Can. Phytopath. Soc.* **35**, 17 (1968).

TABLE 2. BIOSYNTHESIS OF 2-HYDROXYPUTRESCINE AMIDES FROM CARBON-14 LABELLED COMPOUNDS IN RUST-INFECTED RESISTANT WHEAT LEAVES*

Expt.	Compound fed (specific activity)	Activity fed (μc)	Activity† in 2-hydroxyputrescine amides		Activity‡ residing in 2-hydroxyputrescine amides of	
			mμc	% Of total fed	*p*-Coumarate	Ferulate
I	Shikimate-U-^{14}C (7·95 mc/mM)	7·50	140	1·87	yes	yes
II	Quinate-U-^{14}C (5 mc/mM)	1·46	7·05	0·48	yes	yes
	Phenylalanine-U-^{14}C (504 mc/mM)	1·47	6·48	0·44	yes	trace
III	Phenylalanine-U-^{14}C (504 mc/mM)	0·44	1·93	0·44	yes	trace
	Tyrosine-U-^{14}C (334 mc/mM)	0·44	background	0·00	no	no
	p-Coumarate-U-^{14}C (0·95 mc/mM)	1·00	5·20	0·52	yes	yes
	Ferulate-U-^{14}C (0·63 mc/mM)	1·00	2·31	0·23	no	yes
	Caffeate-U-^{14}C (0·85 mc/mM)	1·00	0·96	0·10	no	yes

* Experiment I was carried out with plants of the resistant line of Chinese spring wheat, infected with *Puccinia graminis* f. sp. *tritici*, race 56, and experiments II and III with plants of the resistant wheat variety "Selkirk" infected with *P. recondita*, race 15. Primary leaves, detached 6 days after inoculation, were fed the radioactive compounds and extracted after a metabolic period of 22 hr in the light (21°; 11,000 lx).

† Residing in mixture of *N*-(*p*-coumaroyl)- and *N*-(feruloyl)-2-hydroxyputrescine, unresolved on thin-layer chromatograms.

‡ Visual observation of autoradiograms prepared from thin-layer electropherograms. On these electropherograms both amides were separated from each other, and the *cis*- and *trans*-forms of each amide migrated at different rates.

Isolation

Milligram amounts of both abnormal metabolites were isolated from wheat (var. "Selkirk") infected with an avirulent strain of *Puccinia recondita*, race 15, or from plants of the resistant line of Chinese spring wheat infected with *P. graminis* f. sp. *tritici*, race 56. Several such isolations were made and the following describes a typical isolation procedure.

Step 1. *Extraction.* Approximately 25,000 seedlings, grown in the greenhouse, were heavily inoculated with rust 7 days after seeding, and the primary leaves (1·68 kg) were harvested 6 days later. Harvesting began after the plants had received at least 4 hr of light. Approximately half of the leaves were detached, allowed to stand overnight in a 4 mM solution of ammonium shikimate (pH 6) in tap water and extracted. The remaining leaves were extracted immediately. All leaves were cut to 1 cm length and boiled with 96% ethanol (1 l./100 g fr. wt.). After filtration, batches of leaf pieces were homogenized in a blendor with 90% MeOH (total of 10 l.), the suspensions were filtered, and the filter cakes percolated with 80% EtOH (5 l.) and acetone (2·5 l.). All extracts were combined and dried *in vacuo* in 400-ml batches, each containing 2 g Celite analytical filter aid (Canadian Johns-Manville Co., Port Credit, Ontario).

Step 2. *Fractionation on Celite.* The Celite was suspended in 1 l. H_2O, heated to 50°, and filtered through Celite and Whatman No. 5 filter paper. The filter cake was washed with 500 ml of hot H_2O, resuspended in H_2O, and extracted further with 2·5 l. of hot H_2O. All aqueous extracts were combined, concentrated *in vacuo*, and made to 2 l. with H_2O.

Step 3. *Fractionation on Amberlite IR*-120. The aqueous extract (250 ml) was passed through a column containing 50 ml of Amberlite IR-120 (H^+). Each column was washed with 250 ml H_2O and the effluents were discarded. The resin was adjusted to pH 9 with 5 N NH_4OH in an ice-bath, and the columns were then repacked and each was eluted with 400 ml of 5 N NH_4OH. The columns were further eluted, during two successive days, with several batches of 5 N NH_4OH, totalling 150 ml/column. The combined eluates were dried *in vacuo*.

Step 4. *Fractionation on Amberlite IRC*-50. The residue was dissolved in 300 ml water, the extract divided into six equal portions, and each portion was passed through a column containing 25 ml Amberlite IRC-50 (H^+). Each column was washed with 250 ml H_2O and the effluents were discarded. Each column was eluted with 200 ml 2 N acetic acid. The eluates were combined and dried *in vacuo*.

Step 5. *Fractionation on Rexyn* 201. The residue was dissolved in H_2O and equal portions of the extract were passed through six columns, each containing 30 ml of Rexyn-201 (OH^-) (Fisher Scientific Co.). Each column was washed with 250 ml H_2O, the effluents were discarded, and the eluates (from 270 ml 2 N acetic acid/column) were dried *in vacuo*.

Step 6. *Trituration with absolute ethanol.* The combined residues were triturated with 5 ml EtOH. The EtOH-soluble material containing the amides was removed, and the EtOH-insoluble material dissolved in H_2O, dried *in vacuo*, and re-triturated. The trituration cycle was repeated seven times. All EtOH-soluble fractions were combined, filtered, and dried.

Step 7. *Preparative thin-layer chromatography.* Camag microcrystalline cellulose was washed with 0·1% EDTA, H_2O, ammoniacal MeOH and H_2O, and 0·5 mm thick layers (20 × 20 cm) were prepared. Before chromatography, the plates were washed to the top with the organic phase of solvent I [95% EtOH–Et_2O–H_2O–conc. NH_4OH (40:50:50:5)]. The EtOH-soluble material from step 6 was dissolved in 2·5 ml 80% EtOH, and 3 × 40 µl were applied as a streak (Camag "Chromatocharger") on the origin of each of twenty TLC plates. The 2-hydroxyputrescine amides were separated by developing with the organic phase of solvent I. The amides were located with u.v. and, on guide strips, with diazotized sulfanilic acid and layers containing each amide were scraped off and eluted with ammoniacal methanol. Each eluate was then re-chromatographed separately on similar layers washed and developed with the organic phase of solvent II [BuOH–HoAc–H_2O (4:1:5)]. The amides were located as above, and eluted from scrapings with MeOH containing a few drops of HOAc.

Final yield of each amide was approximately 15 mg. High voltage TLC electrophoresis and chromatography in several systems showed that the isolated amides were sufficiently pure for structural studies.[2] The amides can be purified further by acetylation (3:2 mixture of pyridine–acetic anhydride, overnight at room temperature) and chromatography of the products with Et_2O–MeOH–HOAc (90:10:2) on layers of silica gel GF 254.

Extraction and Fractionation in Small Samples

The leaves were cut into small pieces, homogenized, extracted with alcohol and acetone, and aqueous fractions were prepared as described before.[1] All extracts were then fractionated on Amberlite IR-120 (H^+-form). In experiments with leaves extracted immediately after detachment, Amberlite eluates (2 N NH_4OH) were analyzed directly by TLC or electrophoresis. In isotope experiments, where leaves were maintained for 22 hr after detachment, it was necessary to further purify the Amberlite IR-120 eluates on Amberlite IRC-50.[14] Eluates (4 N acetic acid) from these columns were then analyzed by TLC or electrophoresis.

[14] J. AWAPARA, V. E. DAVIS and O. GRAHAM, *J. Chromatog.* 3, 11 (1960).

To separate the abnormal metabolites from other materials, the column eluates were chromatographed on TLC (silica gel H) with $CHCl_3$–MeOH–conc. NH_4OH (2:2:1) as solvent (tank with paper liner; 150-mm run). To separate the abnormal metabolites from each other, TLC high-voltage electrophoresis on MN cellulose 300 was used (Desaga apparatus, 100 V(cm, 18 min) with either of two buffers: pH 2 (57 ml acetic acid and 17 ml formic acid, made to 1 l. with water), pH 6·1 (0·02 M acetic acid, adjusted with conc. NH_4OH). Both electrophoresis systems separated *cis*- and *trans*-forms of the abnormal metabolites.

Detection and Estimation of Amides

On TLC, the abnormal metabolites gave a purple reaction with ninhydrin. They also reacted with reagents usually used for detecting phenolic compounds, and the colors produced were characteristic for *p*-coumarate or ferulate. Fluorescence in u.v. (365 nm) was the most sensitive method for detection, and amide content was estimated routinely by visual comparison of the size and intensity of fluorescing spots. For quantitative estimation of the 2-hydroxyputrescine amide of ferulic acid, amide-containing silica gel H layers were removed from TLC plates, and extracted with 25% aqueous MeOH. Amide content was determined fluorometrically in 0·2 N NH_4OH (Zeiss spectrophotometer PMQII, with fluorimeter attachment ZFM4; activation and fluorescence maxima at 365 nm and 466 nm, respectively). Since the fluorescence spectrum of the amide is similar to that of ferulate, ferulic acid was used as a standard and results were expressed as ferulic acid equivalents.

Determination of Radioactivity

TLC supports containing the amides were scraped off the plates, suspended in liquid scintillator[15,16] and counted.

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