4-Hydroxybenzaldehyde and Vanillin as Toxins Formed in Leaf Wound Sap of Phaseolus lunatus

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ABSTRACT

On the basis of R_f values, chromogenic reactions, and ultraviolet-absorption spectra in ethanol and sodium ethoxide, the fungitoxic aldehydes found in leaf wound sap of *Phaseolus lunatus* were identified as 4-hydroxybenzaldehyde and 3-methoxy-4-hydroxybenzaldehyde (vanillin). A molecular concentration of hydrocyanic acid equivalent to the combined concentration of the aldehydes was evolved concurrent with aldehyde formation. Two glycosides separated by paper chromatography yielded hydrocyanic and 4-hydroxybenzaldehyde when treated with β-glucosidase (from almonds). The authentic aldehydes, as well as protocatechuic aldehyde, were more toxic to germination of uredospores of *Uromyces phaseoli* and *Puccinia sorghi,* and teliospores of *Ustilago avenae* and *U. nuda,* than corresponding benzoic or cinnamic acid derivatives. Cyanohydrins derived from the aldehydes were more than 10 times as toxic as the aldehydes. Biochemical reactions are proposed to explain the accumulation of the aldehydes. Phytopathology 60:161-165.

Three fungitoxic components have been isolated from wound sap of *Phaseolus lunatus* L. 'Thaxter' (2). Two of the components, unknown B and C, occurred as major constituents of leaf wound sap and minor constituents of stem wound sap. These compounds appeared to be phenolic aldehydes.

This work was undertaken to characterize and identify unknowns B and C.

MATERIALS AND METHODS.—*Preparation of unknown aldehydes.*—Plants were grown, tissue comminuates were prepared, and fungitoxins were isolated as described previously (2). Diethyl-ether extracts containing wound accumulation products from about 4 kg of leaf tissue from 4-week-old plants were extracted three times with 1/4 volumes of 2% sodium bisulfite, and the combined bisulfite extracts were washed several times with small volumes of ether. After adding 1/6 volumes of concentration HCl, the bisulfite fractions were held in vacuo for 30 min at 20°C to remove residual sulfur dioxide, and finally extracted three times with 1/2 volumes of ether. These procedures were repeated three times to give an aldehyde preparation in ether. Unknowns B and C next were separated by column chromatography (2). Fractions from column chromatography, containing unknowns B and C, were then combined, dried in vacuo at 20°C, and dissolved in methanol for further separation by paper chromatography.

Characterization of aldehydes.—The R_F values of unknowns B and C and aromatic aldehydes (obtained from various commercial sources) were compared by paper chromatography using the following solvent systems (v/v): ligroin:methanol:water (L:M:W, 4:3:1); benzene:ligroin:methanol:water (Bz:L:M:W, 50:50:1:50); chloroform:ligroin:methanol:water (C:L:M:W, 2:7:1:5); ligroin:1-butyl ether:water (L:BE:W, 6:1:1 and 6:2:2); benzene:acetic acid:water (Bz:A:W, 125:72:3 and 6:7:3); petroleum ether:ethyl acetate:water (PE:EA:W, 6:1:1); chloroform: 2% formic acid (C:F, 1:1); chloroform:acetic acid:water (C:A:W, 2:1:1); 2% formic acid (F), 33% methanol in water (M:W); and 1-butanol:3% ammonium hydroxide (B:NH_3, 1:1). When two-phase solvent systems were used, the papers were sprayed lightly (avoiding saturation) with the aqueous phase after the preparations to be chromatographed were applied. The papers were then allowed to equilibrate for about 5 min, and developed with the organic phase. Unknowns B and C and authentic aromatic aldehydes were chromatographed alone and in combination (cochromatography).

Paper chromatograms were examined under short and long wavelength ultraviolet lamps (254 nm or 365 nm maximum output) for absorption or fluorescence. Parts of chromatograms were then sprayed with each of the following chromogenic reagents: (i) a fresh mixture of 1% ferric chloride and 1% potassium ferricyanide; (ii) sulfanilic reagent (11); (iii) nitroaniline reagent I (11); (iv) nitrosonaphthol reagent (11); (v) a saturated solution of 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl; and (vi) 1% phloroglucinol in 4 N HCl. Color development was noted after a few sec, 5 min, and 1 hr.

The visible absorption spectra of compounds formed with reagents v and vi were determined by adding 2 ml of each reagent to 1 ml of the aldehydes in ethanol. After 30 min, the reaction mixture from reagent v was diluted to 10 ml with 2 N HCl, and the precipitate was collected, washed with 2 N HCl, and dissolved in chloroform for spectrophotometry. The reaction mixture from reagent vi was diluted to 10 ml with ethanol and used directly for spectrophotometry.

Unknown aldehydes were prepared for spectrophotometry by repeated chromatography on Whatman No. 40 paper, using B:NH_3 as the solvent. Unknowns B or C were then eluted with ethanol, dried in vacuo at 20°C, and dissolved in 2% sodium bisulfite. Aldehydes were recovered from their bisulfite complexes as described. Their ultraviolet absorbances were determined in both 100% ethanol and 100% ethanol containing 0.02 M NaOH.

Measurements of aldehydes and hydrocyanic acid (HCN).—The production of HCN was determined qualitatively by its reaction with picric acid (10).

Concurrent formation of aldehydes and HCN were measured quantitatively. Comminuates were placed in the first of a series of three sealed vessels interconnected by aeration tubes; the second vessel contained distilled water, and the third 0.1 M NaOH. After desired incubation periods, 1 ml of 2 N HCl for each 4 g of tissue was added to the comminuates. The HCN was trapped in the NaOH solution by flushing the system with nitrogen for 2 hr at 28°C. HCN content was then determined by titration with silver nitrate (1).

Aldehydes were isolated and chromatographed with $B:NH_3$ as described previously. Ethanolic eluates containing aldehydes from the chromatograms were evaporated to dryness in vacuo at 20°C. Residues were then dissolved in 0.1 N NaOH (1 ml/g fresh wt tissue) to determine absorbance at 332 nm for 4-hydroxybenzaldehyde and at 350 nm for vanillin; when necessary, further dilutions were made to give absorbance readings of 0.200 to 1.000. Reference curves for quantitative interpulation were prepared by treating known amounts of authentic aldehydes in the same manner. Results are expressed as the average of four replicates.

Identification and isolation of cyanohydrins and cyanogenic glycosides.—Cyanohydrins were determined qualitatively by their rapid breakdown in 0.1 M potassium phosphate buffer (pH 7.0) at 30°C yielding HCN and a benzaldehyde derivative. Cyanogenic glycosides were identified by their reaction with β-glucosidase (from almonds) to yield HCN, benzaldehyde derivative, and sugar.

Cyanohydrins were separated from aldehydes by two-dimensional chromatography, using C:A:W and Bz:A:W (6:7:3) as solvents. The presence of cyanohydrins and aldehydes was also determined by two-dimensional chromatography, using C:A:W and $B:NH_3$ as solvents. Cyanohydrins decomposed to aldehydes in $B:NH_3$; thus, two spots representing vanillin and 4-hydroxybenzaldehyde were observed in this system.

Cyanogenic glycosides were extracted by refluxing 100 g leaves in 900 ml 95% ethanol for 20 min. Ethanolic extracts were evaporated to 10-20 ml in vacuo, 100 ml of water was added, and the solution was filtered. The filtrate was washed 3 times with 1/4 volumes of ether (which was discarded) and then extracted 3 times with 1/4 volumes of 1-butanol. The combined butanol extracts were washed with 1/4 volumes of water, and evaporated to dryness in vacuo at 40°C. Residues were dissolved in 70% ethanol and applied to Whatman No. 40 paper for chromatography, using 33% methanol, 2% formic acid, 75% methanol, 75% ethanol, butanol: acetic acid: water (B:A:W, 67:10:23; v/v), butanol:pyridine:water (B:P:W, 6:4:3), and butanol:methanol:water:benzene (B:Bz:M:W, 3:1:1:1). Cyanogenic glycosides were located on the dried chromatograms by spraying with β-glucosidase, incubating for 1 hr in a moist chamber, and spraying with 2,4-DNPH reagent.

Bioassays.—Teliospores of *Ustilago avenae* (Pers.) Rostr. and *U. nuda* (Jens.) Rostr. were collected from field-grown plants and used after less than 2 months storage at 5°C; uredospores of *Uromyces phaseoli* (Pers.) Wint. and *Puccinia sorghi* Schw. were collected from inoculated plants in the greenhouse and used immediately.

The hydroxymandelonitriles (cyanohydrins) were synthesized, using Method No. 2 of Buck (5). Other authentic compounds were obtained from commercial sources and were checked for purity by paper chromatography before being used in the bioassays. Twofold dilution series were prepared for each compound adjusted to pH 6.0 with HCl or NaOH; each concentration was mixed with an equal volume of 4% Difco-Bacto water agar at 50°C. Four replications of 2 ml each were placed in 10 × 25 mm bioassay dishes seeded with spores using a settling tower. After incubation at 22°C, the spores were killed by spraying with methylene blue in lactophenol, and ED_{50} (median effective dose) values were determined from probit plots of the data (3). Each compound was tested twice and results are means.

RESULTS.—The R_F values, chromogenic reactions, and ultraviolet absorbance of unknown B (vanillin) and unknown C (4-hydroxybenzaldehyde) are shown in Tables 1 and 2 and Fig. 1, respectively. In each case, characteristics of unknowns B and C were identical to those of vanillin and 4-hydroxybenzaldehyde, respectively.

The formation of the aldehydes in wound sap was correlated with the evolution of a similar amount of HCN from the wound sap (Fig. 2). Ether extracts from sap (expressed at 2°C and clarified rapidly by adjusting to pH 5.0 and centrifuging) yielded small spots

TABLE 1. R_F values (×100) of unknown B (vanillin) and unknown C (4-hydroxybenzaldehyde) in various solvent systems[a]

Solvent system[b]	Unknown B (vanillin) $R_F \times 100$	Unknown C (4-OH BA) $R_F \times 100$
Weakly Polar		
L:M:W (4:3:1)	10	0
Bz:L:M:W (50:50:1:50)	38	6
C:L:M:W (2:7:1:5)	56	5
L:BE:W (6:1:1)	28	13
(6:2:2)	35	22
Bz:A:W (6:7:3)	69	35
(125:72:3)	89	69
PE:EA:W (6:1:1)	76	55
C:F (1:1)	97	45
C:A:W (2:1:1)	93	63
Strongly polar		
F	64	69
M:W (1:2)	69	71
$B:NH_3$ (1:1)	28	42

[a] Since each unknown and the authentic aldehyde in parentheses below it migrated as single spots in cochromatography, a single R_F value is given for both.

[b] Abbreviations are as follows: A = acetic acid; B = 1-butanol; BE = 1-butyl ether; Bz = benzene; C = chloroform; EA = ethyl acetate; F = 2% formic acid in water; L = ligroin; M = methanol; NH_3 = 3% ammonia in water; PE = petroleum ether (30-60°C boiling range); and W = distilled water.

TABLE 2. Chromogenic reactions of unknown B (vanillin) and unknown C (p-hydroxybenzaldehyde)[a]

Spot test	Unknown B (vanillin)	Unknown C (4-OH BA)
Ultraviolet (254 nm)	Purple absorbance	Purple absorbance
2, 4 Dinitrophenyl hydrazine	Orange	Rust
Phloroglucinol	Orange-pink	Orange-yellow
Sulfanilic	Pale orange	Pale yellow
Nitroaniline	Pale violet	Pale pink
Ferric chloride-cyanide	Blue (slow)	Blue (slow)
Nitrosonaphthol	Orange	Pale pink

[a] Since each unknown and the authentic aldehyde in parentheses below it gave identical reactions, a single color is listed for both.

corresponding to the cyanohydrins of 4-hydroxybenzaldehyde and vanillin when chromatographed using C:A:W × Bz:A:W (6:7:3) as solvents (Fig. 3). These spots released HCN when placed in 0.1 M potassium phosphate buffer at pH 7.0. Likewise, when these preparations were chromatographed using 33% methanol as a solvent, the band at R_F .70-.85 released HCN when placed in the pH 7.0 buffer.

Two cyanogenic glycosides (R_F .84 and .70 with 75% methanol, .70 and .60 with 75% ethanol, and .70 and .48 with B:Bz:M:W), formed 4-hydroxybenzaldehyde and HCN in the presence of β-glucosidase. The released sugar (or one of the released sugars) appeared to be glucose; however, positive identification was not made.

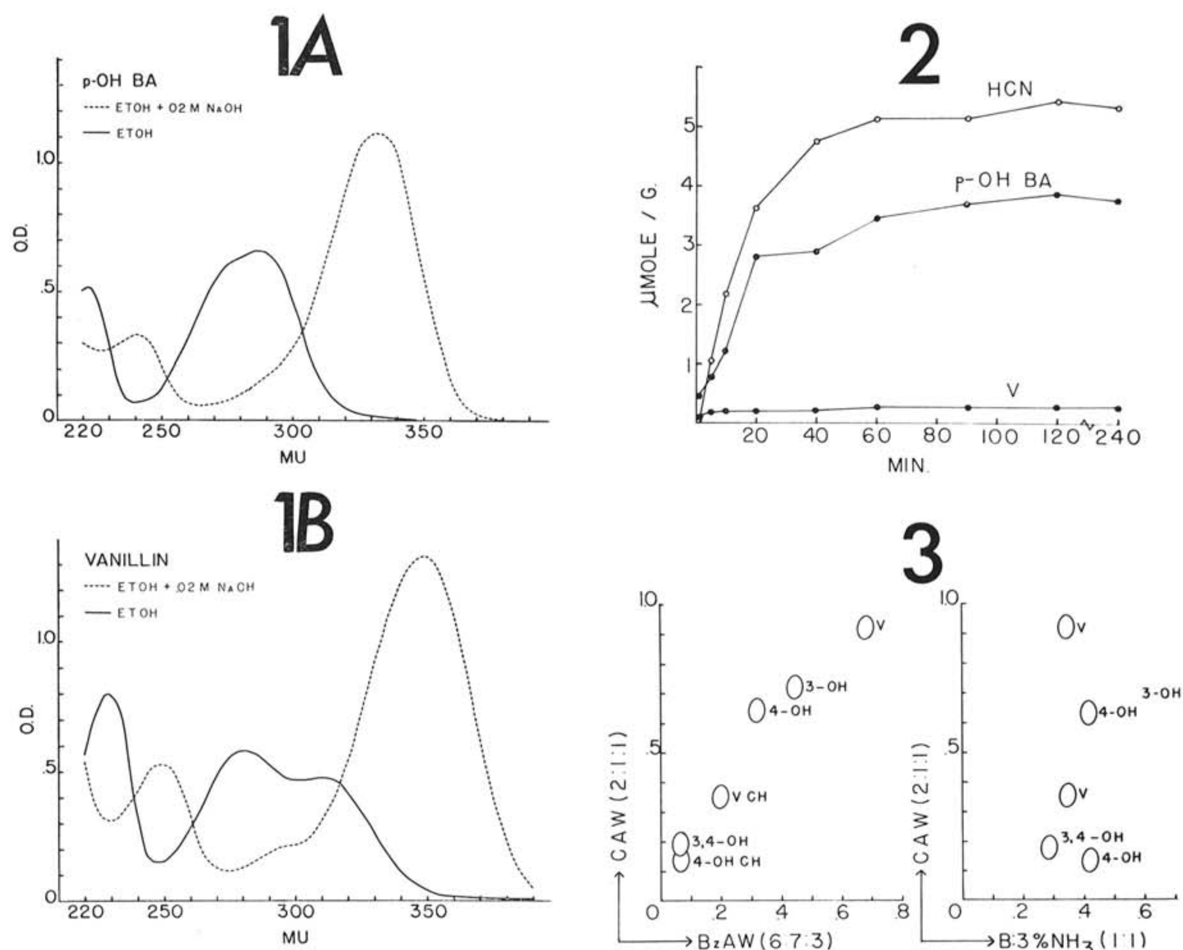

Fig. 1-3. **1)** The absorbance (optical density, OD) of 50 nM solutions (or identical µg/ml solutions of unknowns) of **A)** 4-hydroxybenzaldehyde (unknown C) and **B)** vanillin (unknown B), in 100% ethanol and 100% ethanol containing 0.02 M NaOH. **2)** Quantities of hydrocyanic acid (HCN), p-hydroxybenzaldehyde (p-OH BA), and vanillin (V) produced in comminuates of primary leaves of *Phaseolus lunatus* 'Thaxter' after various incubation intervals. **3)** Separations of 4-hydroxybenzaldehyde (4-OH), 3-hydroxybenzaldehyde (3-OH), protocatechuic aldehyde (3,4-OH), vanillin (V), 4-hydroxybenzaldehyde cyanohydrin (4-OH CH), and vanillin cyanohydrin (V CH) on chromatograms developed with chloroform:acetic acid:water (C:A:W) × benzene: acetic acid:water or CAW × butanol:3% ammonium hydroxide ($B:NH_3$). V CH and 4-OH CH break down to V and 4-OH in alkaline solution; thus, two spots of each of the latter occur when $B:NH_3$ is used as a second solvent for solutions containing both the aldehydes and the mandelonitriles.

TABLE 3. ED_{50} (median effective dose) values for phenolic compounds against uredospore germination of *Uromyces phaseoli* and *Puccinia sorghi* and teliospore germination of *Ustilago nuda* and *U. avenae*

Compound	Bioassay organism[a]			
	U. phaseoli	*P. sorghi*	*U. avenae*	*U. nuda*
Benzaldehydes		*μg/ml*		
4-OH	160	80	25	100
3,4-di-OH	180	195	45	580
4-OH, 3-OCH_3	130	150	25	115
Cyanohydrins				
4-OH	3	10	15	17
4-OH, 3-OCH_3	11	17	42	65
Benzoic acids				
4-OH	350	NI[b]	NI	NI
3,4-di-OH	NI	520	NI	NI
4-OH, 3-OCH_3	NI	NI	NI	NI
Cinnamic acids				
4-OH	380	170	280	NI
3,4-di-OH	350	480	NI	NI
4-OH, 3-OCH_3	80	185	NI	NI

[a] *U. phaseoli* and *P. sorghi* uredospores, and *U. avenae* and *U. nuda* teliospores were incubated at 22°C for 5 and 24 hr, respectively.

[b] NI = no significant (LSD = .05) inhibition at 1,000 μg/ml.

The toxicities of the aldehydes and related compounds to spore germination are shown in Table 3. Aldehydes were much more fungitoxic than corresponding benzoic or cinnamic acid derivatives. The cyanohydrins were about 10 times as toxic as the aldehydes.

DISCUSSION.—The primary fungitoxic aldehydes isolated from leaf wound sap of *P. lunatus* appeared to be 4-hydroxybenzaldehyde and vanillin; the former was about 90% of the total aldehyde fraction. Volatile aldehydes may have been present in wound sap, but would have been lost in the isolation procedures. Likewise, methyl ethyl ketone and acetone, breakdown products of the cyanogenic glycosides, lotaustralin and linamarin, respectively, may also have been lost from the wound sap. The parent glycosides of the latter are reported to occur in seeds of *Phaseolus lunatus* (6).

Several observations indicate that the aldehydes, particularly 4-hydroxybenzaldehyde, are formed by a cyanogenic mechanism similar to that reported for 4-hydroxybenzaldehyde in *Sorghum vulgare* (8, 9) and for benzaldehyde in *Prunus* sp. (4): (i) Equimolar concentrations of the aldehydes and HCN were evolved concurrently from injured tissues; (ii) compounds corresponding to the intermediate mandelonitriles were demonstrated by chromatography; (iii) two glycosides which yielded 4-hydroxybenzaldehyde and HCN, when treated with almond β-glucosidase, were demonstrated by paper chromatography; and (iv) clarified sap of *P. lunatus* had β-glucosidase and oxynitrilase activity when it was mixed with the authentic mandelonitrile glucoside, amygdalin. Apparent glycosides of *P. lunatus* and synthesized mandelonitriles of benzaldehydes (4-hydroxybenzaldehyde and vanillin) also were decomposed to HCN and aldehydes by wound sap of *P. lunatus*. These results suggest that the aldehydes are formed as shown in Fig. 4.

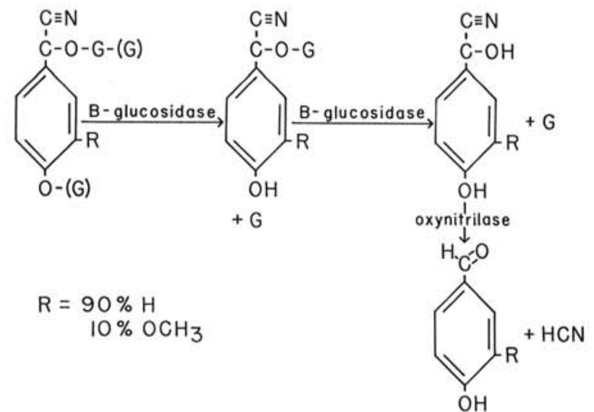

Fig. 4. A proposed biochemical mechanism for aldehyde formation in leaf wound sap of *Phaseolus lunatus* 'Thaxter'. G = unidentified sugar; (G) indicates possible locations of a second sugar.

The biosynthesis of the cyanogenic glycosides was not investigated. However, others (4, 7, 8, 9) have shown that cyanogenic glycosides are synthesized from β-D-glucose and the L-amino acid corresponding to the aglycon; thus L-tyrosine, L-phenylalanine, L-isoleucine, and L-valine are the precursors of dhurrin, prunasin, lotaustralin, and linamarin, respectively. In each case, the carboxyl of the amino acid is lost and the remaining structure remains intact during the glycoside synthesis. Hence, tyrosine would be the suspected precursor of the cyanogenic glycosides that yield 4-hydroxybenzaldehyde in *P. lunatus*. An amino acid corresponding to vanillin, however, has not been reported in *P. lunatus;* vanillin may have been formed by a mechanism other than cyanogenesis, since the parent glycoside was not isolated. The small amount of the vanillin mandelonitrile could have been formed chemically by the reaction of HCN with vanillin in wound sap.

The relative fungitoxicity of the aldehydes and their mandelonitriles suggest that the latter may be the most important fungitoxins in wound sap. Quantitative measurements of the mandelonitriles are very difficult. These compounds break down very rapidly at pH 7.0 or above in aqueous solution. At pH 5-6, they break down more slowly in aqueous solution; however, the rate of breakdown is greatly increased by adding oxynitrilase, heating, or placing in vacuo. Slight differences in the pH of cell sap or the oxynitrilase activity of the sap could have marked influences on the efficacy of these compounds in tissues of different ages or from different varieties. Careful consideration of these facts is necessary before the role of the aldehydes or mandelonitriles in disease resistance can be determined.

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