6a-Hydroxyphaseollin: an Antifungal Chemical Induced in Soybean Hypocotyls by Phytophthora megasperma var. sojae

N. T. Keen, J. J. Sims, D. C. Erwin, E. Rice, and J. E. Partridge

Assistant Professor; Associate Professor; Professors;and Graduate Research Assistant, respectively, Department of Plant Pathology, University of California, Riverside 92502. Professor Rice is associated with the Department of Biochemistry, California State Polytechnic College, Pomona 91166.

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ABSTRACT

6a-hydroxyphaseollin (HP), a new hydroxypterocarpan, was isolated from soybean hypocotyls challenged with *Phytophthora megasperma* var. *sojae*. Hydroxyphaseollin was not detected in extracts from unchallenged hypocotyls, and was the only induced antifungal compound detected in bioassays of hypocotyl extracts from challenged plants. A gas-liquid chromatographic analysis was devised for HP which showed that it accumulated to about 10-fold higher concentrations in inoculated hypocotyls of monogenically resistant soybeans than in a near-isogenic susceptible line. Phytopathology 61:1084-1089.

Additional key words: phytoalexins, resistance, pterocarpans.

Inducible production of antibiotic molecules by higher plants may be a general response to infection by nonpathogenic microorganisms (3, 13). Work of Gray (7) and Klarman & Gerdemann (10, 11) indicates that monogenic resistance in soybeans (*Glycine max* [L.] Merr.) to *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildb. is also expressed by the production of an induced antibiotic material.

Inducibly produced antifungal agents from soybeans have been reported previously by a number of investigators. Cruickshank (4) and Klarman & Sanford (12) obtained evidence indicating that a pterocarpan related to phaseollin (5, 18) was produced by heavy metal or fungus-challenged cotyledons and hypocotyls of soybean plants. This compound had absorption peaks at 288, 293, and 315 nm, and resembled the preparations studied earlier by Uehara (22, 23) and by Nonaka et al. (15) from soybean pods challenged by various fungi.

In early work, Klarman & Gerdemann (11) noted the appearance of a yellow fluorescent substance in extracts from fungus-challenged soybean plants. Paxton and coworkers (2, 6, 16, 17) concluded that the fluorescent material was a major antifungal agent (phytoalexin) accumulating in resistant soybean hypocotyls.

As a prelude to an investigation of mechanisms accounting for monogenic resistance and race specificity in the soybean-*P. megasperma* var. *sojae* system, we examined the nature of inducible production of antifungal chemicals in soybean hypocotyls and devised methods for extraction and quantitation of the antifungal compounds involved.

We report evidence here supporting the conclusion that 6a-hydroxyphaseollin (HP), a new hydroxypterocarpan (J. J. Sims, N. T. Keen, & V. K. Honwad, *unpublished data*), is the induced antifungal chemical produced in monogenically *Phytophthora*-resistant soybean hypocotyls.

MATERIALS AND METHODS.—*Fungus isolates.*—The race 1 isolate of *Phytophthora megasperma* var. *sojae* (P174) was supplied by A. A. Hildebrand, Canadian Department of Agriculture, Harrow, Ontario, Canada, and was maintained on V-8 juice agar. The race 2 isolate employed (P406) was supplied by E. F. Morgan, USDA, Stoneville, Miss. For inoculum production, the fungi were grown for 2-5 days on a pea broth medium (25 g frozen market peas autoclaved in 1 liter water, then filtered) in petri dishes.

Plant material.—Seeds of the near-isogenic soybean cultivars Harosoy (susceptible to *P. megasperma* var. *sojae*) and Harosoy 63 (resistant to races 1 and 2 of *P. megasperma* var. *sojae*) (1) were planted in greenhouse pans containing a mixture of 50% fine sandy loam and 50% peat moss overlaid with vermiculite. Plants were grown in growth chambers at 29-C day, 22-C night temperatures and 14-hr photoperiods under mixed Sylvania VHO fluorescent and incandescent lighting (1,800-2,000 ft-c).

Hypocotyls of 5- to 14-day-old plants were inoculated by the method of Klarman & Gerdemann (11) (method 1) or by the following procedure (method 2). A 1- to 2-cm slash wound was made on hypocotyls with a No. 20 gauge hypodermic needle and ca. 0.05 ml minced mycelium of *P. megasperma* var. *sojae* (3 to 5 sec at full speed in a Sorvall Omni-Mixer cup; diluted to an absorbance of ca. 1.0 at 400 nm) was simultaneously introduced into the wounds from a syringe. Plants were maintained at ca. 100% relative humidity (method 1) or ca. 75% relative humidity (method 2) after inoculation. Hypocotyls were harvested at 24-96 hr after inoculation and extracted fresh or after lyophilization.

Tissue extractions.—In the first extraction method, hypocotyls were ground in a Sorvall Omni-Mixer cup at full speed for 1 min in 40 ml/g dry wt of 80% ethanol or 4 ml/g fresh wt of 95% ethanol. The extracts were filtered through fluted filter paper and the residues washed with 80% ethanol. The residue was discarded or, in some experiments, re-extracted with water (1.0 ml/ml ethanol originally used) on a steam

bath for 2 hr. The ethanol extracts were reduced in volume on a rotary evaporator at ca. 45 C. The concentrate was extracted once with hexane, then 3 times with ethyl acetate. The ethyl acetate and hexane fractions were dehydrated with sodium sulfate and dried at 45 C. The water re-extracts and the water fractions of ethanol extracts were reduced in volume at 45 C in vacuo. All extracts were then brought to 1.0 ml/g dry wt or 0.1 ml/g fresh wt tissue extracted and were referred to as "crude extracts".

In the second extraction method, lyophilized tissues were extracted with 50 ml of 90% ethyl acetate in methanol/g dry wt for 30 sec at full speed in the Omni-Mixer. After filtration and evaporation to dryness, the extracts were redissolved in ethyl acetate as above.

Thin-layer chromatography.—Extracts were spotted on thin-layer chromatography (TLC) plates (250 or 500 μ) of Silica Gel GF_{254} (E. Merck, Darmstadt, Germany) or Silica Gel G (Adsorbosil 1, Applied Science Laboratories, State College, Pa.). The plates were developed with one of the solvent systems listed in Table 1.

Some preparations were also chromatographed by reversed-phase partition TLC (Table 1). Adsorbosil 1 plates (500 μ) were dipped into 5% solutions of Dow-Corning electronic silicon fluid in toluene for 2 min, then air-dried.

Polyamide thin-layer plates (250 μ, Macherey Nagel and Co., Düren, Germany) were developed with 70% acetone.

Hydroxyphaseollin was detected on TLC plates by its absorbance under 254 nm light or with the following spray reagents: (i) fast blue salt B (21); (ii) anisaldehyde:sulfuric acid (21); (iii) 50% sulfuric acid and heating at 110 C for 0-10 min; (iv) *p*-nitroaniline (21); (v) antimony trichloride (21); (vi) iodine vapors.

Purification of hydroxyphaseollin.—Harosoy-63 hypocotyls inoculated by method 1 or Harosoy hypocotyls inoculated by method 2 were extracted with aqueous ethanol, and the concentrated extracts were extracted with hexane to remove most of the pigments. The aqueous phase was then extracted 3 times with ethyl acetate. The pooled organic layers were dried, concentrated, and applied to a 5- × 20-cm nylon tubing column packed with dry silica gel (for dry-column chromatography, M. Woelm, Eschwege, Germany) following the procedure of Loev & Goodman (14). The column was eluted with TLC solvent (i) (Table 1) until the solvent front reached the bottom of the column. The column was viewed under ultraviolet light, and the absorbing HP band (ca. R_F 0.4-0.5) sectioned with a razor blade (14). Hydroxyphaseollin was obtained in about 80% purity after elution from the silica gel with ethyl acetate. The purification was continued by preparative TLC using solvents (iii) or (iv) (Table 1) with 0.5 or 1.0 mm Silica Gel G or Silica Gel GF_{254} plates. Edges of the plates were sprayed with detection reagents i or ii or viewed under 254-nm light to detect the HP band, and the corresponding unsprayed areas were removed from the plates. Hydroxyphaseollin was eluted from the silica gel with acetone, and the silica gel removed by centrifugation. The final eluate was dried, dissolved in anhydrous ether, filtered through Whatman No. 50 filter paper and dried overnight under vacuum in darkness.

Gas chromatography.—Hydroxyphaseollin in purified and crude ethyl acetate extracts was quantitated as the trimethylsilyl ether (TMS) derivative using gas-liquid chromatography (GLC). Crude ethyl acetate extracts could be analyzed directly, but cleaner chromatograms were obtained if the following procedure was used. The crude extract was dried at 45 C, dissolved in ca. 1 ml of dry chloroform (reagent grade), and applied to a 0.5- × 5.0-cm column of silica gel (J. T. Baker Chemical Co., Phillipsburg, N.J., No. 3405) equilibrated with chloroform. The column was washed with 3-5 ml of chloroform, and the effluent discarded. Hydroxyphaseollin was then eluted with 3 ml of ethyl acetate, and the column was regenerated with methanol and re-equilibrated with chloroform. The ethyl acetate column

TABLE 1. R_F values of hydroxyphaseollin and some related pterocarpans in various thin-layer chromatographic systems

Solvent[a]	Adsorbent	Hydroxyphaseollin
i Hexane:ethyl acetate:methanol(60:40:1)	Silica gel[b]	0.30 (Phaseollin = 0.50; dehydrophaseollin = 0.50; pisatin = 0.29).
ii Ethyl acetate:benzene:methanol:water(100:80:20:20)[c]	Silica gel[b]	0.80
iii Chloroform:acetone:28% NH_3 (65:35:1)[d]	Silica gel[b]	0.35
iv Chloroform:acetone:acetic acid(90:10:0.5)[d]	Silica gel[b]	0.50
v *n*-Butanol:acetic acid:water(4:1:5, epiphase)	Silica gel[b]	0.90 (Partial decomposition to blue fluorescent material).
vi Hexane:ethyl acetate:acetic acid(80:20:4)	Silica gel[b]	0.10
vii Chloroform:isopropanol(90:10)	Silica gel[b]	0.45
viii 70% aqueous acetone	Polyamide	0.65-0.70
ix Methanol:acetic acid:water(5:1:5)	Reversed phase on silica gel	0.60

[a] All v/v; all runs were made at room temperature.

[b] Silica Gel G plates (500 μ) were routinely employed, but R_F values differed only slightly, if at all, on 250 μ plates or on Silica Gel GF_{254} plates.

[c] From Klarman & Sanford (12).

[d] It was called to the attention of the authors that combinations of these solvents may be explosive (9); their use therefore warrants caution.

fraction was dried and transferred with anhydrous ether to a 1-dr polypropylene-capped vial, and 100 μg of catechin (K & K Laboratories, Hollywood, Calif.) in ethanol was added as an internal standard. Solvents were expelled in a stream of N_2, and 30 μliters of anhydrous pyridine and 20 μliters of Tri-Sil concentrate (Pierce Chem. Co., Rockford, Ill.) were added, and the vial was capped and allowed to stand at room temperature for at least 30 min before injection. Standards containing 100 μg each of purified hydroxyphaseollin and catechin were prepared similarly and included in each series of unknowns.

The TMS derivatives were chromatographed on a Perkin-Elmer Model 881 gas chromatograph equipped with dual hydrogen flame ionization detectors. One-tenth- to 1.0-μliter volumes of the unknowns or standards containing purified hydroxyphaseollin and catechin were injected onto 1.9 mm- (internal diameter) × 100- or 120-cm stainless steel columns of 3.5% SE-30 on 100-120 mesh Gas Chrom Q (Applied Science). The columns were run isothermally at 215-220 C with a nitrogen flow rate of 15 ml/min. The injector block and detector were maintained at 240 C. Peak areas of hydroxyphaseollin peak 1 (Fig. 3) and catechin were quantitated with a Hewlett-Packard Model 3370A electronic integrator or by peak height measurement. Hydroxyphaseollin concentrations in the crude extracts were then calculated according to the following equation:

$$\mu g \text{ hydroxyphaseollin (HP) per vial} = \frac{\text{Peak area of HP} \times \mu g \text{ catechin internal standard per vial} \times \text{Peak area of catechin in standard vial} \times \mu g \text{ of HP in standard vial}}{\text{Peak area of catechin internal standard} \times \text{Peak area of HP in standard vial} \times \mu g \text{ of catechin in standard vial}}.$$

Data were expressed as μg hydroxyphaseollin per mg dry or fresh weight of tissue extracted. The detection limit for hydroxyphaseollin with this technique was about 0.01 mg/g dry wt tissue extracted.

Bioassays.—Antifungal materials on TLC plates were bioassayed by a modification of the method devised by Klarman & Sanford (12). Spores of *Cladosporium cucumerinum* Ell. & Arth. in water were sprayed onto the plates, which were then oversprayed with half strength potato-dextrose agar (Difco) at ca. 45 C. Inhibitory zones on the plates appeared after 24-48 hr as white zones on a dark background (12).

Crude extracts and purified preparations of hydroxyphaseollin were also bioassayed with *P. megasperma* var. *sojae* by modification of the method devised by Pierre & Bateman (20). Extracts in organic solvents or water (0-30 μliter) were added to sterile 5.5 cm petri plates, organic solvents were allowed to evaporate, and 0.1 ml of 95% ethanol was added and swirled to dissolve the extracts and sterilize the water solutions. Then 3 ml of molten V-8 juice agar were added to each plate and swirled thoroughly before solidification. Five-mm mycelial discs from V-8 agar plates of races 1 or 2 of *P. megasperma sojae* were placed in the center of the assay plates, which were then incubated at 25 C. Colony diameters were measured at intervals, colony areas computed, and ED_{50} values estimated from the dosage response curves obtained with purified hydroxyphaseollin.

RESULTS.—Little antifungal activity was detected in bioassays of crude extracts from unchallenged hypocotyls and from Harosoy hypocotyls inoculated by method 1, which confirmed the results of Gray et al. (7); considerable activity was detected in similar extracts from challenged Harosoy 63 hypocotyls at 2-3 days after inoculation. When inoculation method 2 was used, Harosoy plants were partially resistant, and considerable antifungal activity was detected in ethanol extracts from inoculated hypocotyls.

The induced antifungal activity in aqueous ethanol extracts of challenged Harosoy-63 hypocotyls partitioned into ethyl acetate from water (Fig. 1), but none was extracted from water by hexane. Little antifungal activity was present in water extracts of tissue previously extracted in ethanol. Less activity was detected in the water fraction of ethanol extracts than in ethyl acetate fractions (Fig. 1), and no significant differences were observed in the antifungal activities of the former extracts from unchallenged or fungus-challenged hypocotyls.

The chromatographic bioassay detected a single inhibitory zone in the ethyl acetate fraction of challenged Harosoy 63 hypocotyls (Fig. 2-A) that was not present in extracts from unchallenged plants and which

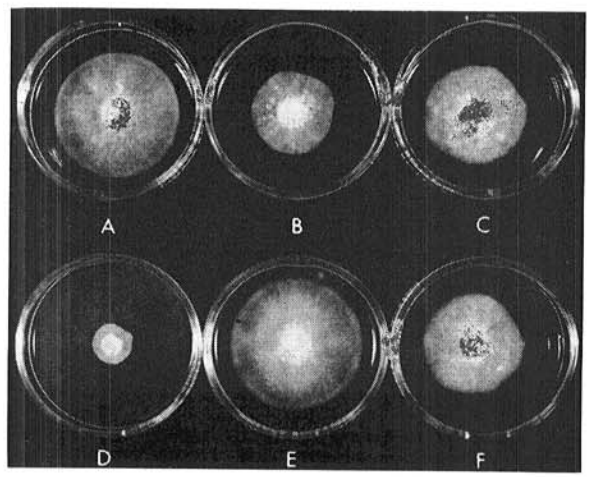

Fig. 1. Growth of *Phytophthora megasperma* var. *sojae* (P174) on 3 ml of V-8 juice agar supplement with various extracts from fungus-challenged Harosoy 63 soybean hypocotyls. **A)** Nonsupplemented control; **B)** control, supplemented with 30 μg/ml purified hydroxyphaseollin; **C)** 30 μliters of H_2O fraction of ethanol extract; **D)** 30 μliters of ethyl acetate fraction of ethanol extract; **E)** 30 μliters hexane fraction of ethanol extract; **F)** 30 μliters of hot water extract from tissue previously extracted with ethanol. The plates were photographed at 5 days after initiation of cultures. All extracts were from Harosoy 63 hypocotyls challenged with race 1 of *P. megasperma* var. *sojae* for 72 hr. All extracts were brought to 0.1 ml/g fresh wt tissue extracted.

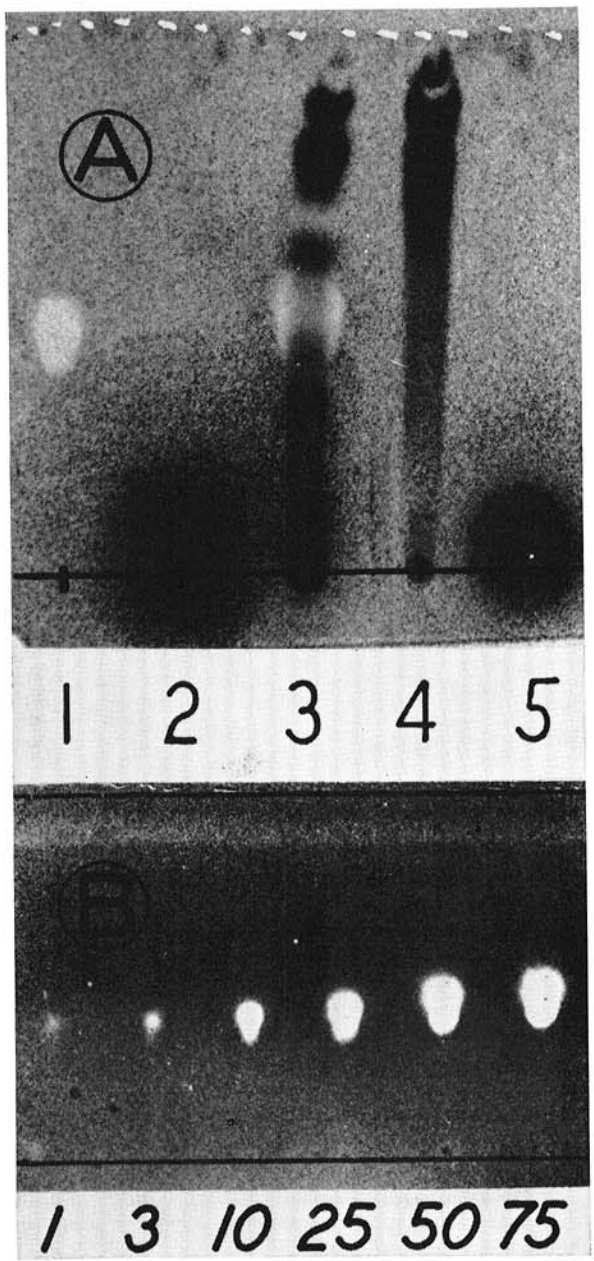

Fig. 2. A) Thin-layer chromatographic bioassay of extracts from Harosoy 63 soybeans challenged with race 1 of *Phytophthora megasperma* var. *sojae* for 48 hr. All extracts were brought to 1.0 ml/g dry wt tissue. Extracts (10 µliters) were spotted and the plates developed with hexane: ethyl acetate:methanol (60:40:1) before spraying with *Cladosporium cucumerinum* spores. Light areas denote antifungal activity; darker areas denote pigments in the extracts or stimulation of fungus growth. 25 µg Purified hydroxyphaseollin (1); H_2O fraction of ethanol extract (2); ethyl acetate fraction of ethanol extract (3); hexane fraction of ethanol extract (4); hot water re-extracted tissue (5). Upper and lower lines denote solvent front and starting lines, respectively. **B)** Thin-layer chromatographic bioassay of various µg amounts of purified hydroxyphaseollin. Chromatographic conditions were as in Fig. 2-A.

occurred at the same R_F as purified hydroxyphaseollin. As was found in the *Phytophthora* bioassay, the TLC bioassay showed that little or no antifungal activity was present in the water or hexane fractions of extracts from challenged plants (Fig. 2-A). Stimulatory compounds were present near the origin when water extracts were chromatographed. Other inhibitory spots could be detected in all crude ethyl acetate fractions when greater amounts were applied to the TLC plates, but they occurred in equal intensity from unchallenged and challenged hypocotyls of the two varieties. Essentially equal concentrations of the induced antifungal material were extracted by the aqueous ethanol method 1 from fresh or lyophilized tissues, and by the nonaqueous method 2, based on observed inhibition in the two bioassays.

The induced antifungal material isolated from ethyl acetate crude extracts of fungus-challenged plants was a colorless, nonfluorescing viscous liquid that absorbed light at 254 but not 360 nm on TLC plates. These preparations were homogeneous on all TLC systems (Table 1) when detection by 254 nm light or detection reagents were used. However, the active principle could not be crystallized, appeared to give at least two overlapping spots in the TLC bioassay (Fig. 2-B), and gave three GLC peaks (Fig. 3). Peak 1 was successfully isolated by preparative TLC on Adsorbosil-1 plates using solvents i or vi (Table 1) with multiple development, but GLC peaks 2 and 3 were not isolated by TLC. Isolation of these peaks by preparative GLC has thus far failed because of excessive decomposition when silylated derivatives were collected from the outlet ports of a thermal conductivity chromatograph.

The soybean antifungal agent has been identified as 6a-hydroxyphaseollin (I, Fig. 3) on the basis of chem-

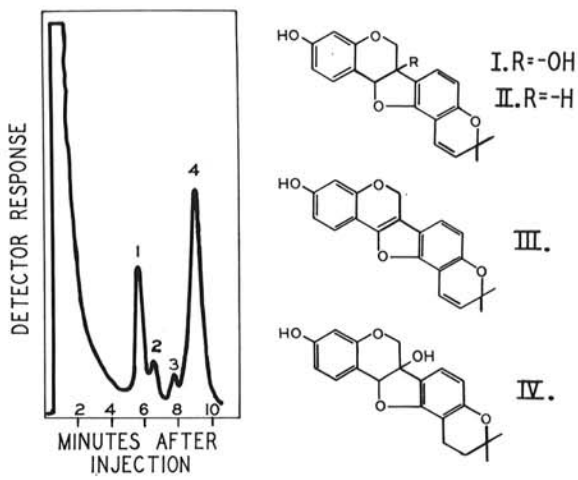

Fig. 3. (Left) Gas chromatogram of ca. 3 µg of the trimethylsilyl derivative of purified hydroxyphaseollin (peaks 1, 2, & 3) and ca. 3 µg TMS-catechin internal standard (peak 4). Attenuation = ×20; 1.9-mm × 100-cm column of 3.5% SE-30 on Gas-Chrom Q was run isothermally at 215 C; other conditions were as in MATERIALS AND METHODS. (Right) I. Proposed structure of 6a-hydroxyphaseollin (I); Phaseollin (II); Dehydrophaseollin (III); Dihydro, hydroxyphaseollin (IV).

ical and spectral data (J. J. Sims, N. T. Keen, & V. K. Honwad, *unpublished data*). Combined gas chromatography-mass spectrometry has shown that the three GLC peaks are all isomers of the disilyl derivative of hydroxyphaseollin (all have $M+ = 482$), although their configurations have not as yet been firmly established (N. T. Keen & J. J. Sims, *unpublished data*).

Similar to pisatin (19) and phaseollin (5), purified hydroxyphaseollin is soluble in water to about 40 μg/ml. It is very soluble in methanol, ethanol, and intermediate polarity organic solvents, but is virtually insoluble in hexane. Like the related hydroxypterocarpan pisatin (19), hydroxyphaseollin is labile in strong acid, dehydrating to form dehydrophaseollin (III, Fig. 3) (J. J. Sims, N. T. Keen, & V. K. Honwad, *unpublished data*). It tends to break down slowly in organic solvents over a period of months to give dehydrophaseollin and other unidentified fluorescent products.

Purified hydroxyphaseollin produced an inhibitory spot in the TLC bioassay at 1 μg or more (Fig. 2-B). The ED_{50} value for inhibition of mycelial growth of *P. megasperma* var. *sojae* was 7×10^{-5} M HP, and no significant difference was noted in the sensitivity of the two races. Various derivatives of hydroxyphaseollin, including dehydrophaseollin (III, Fig. 3), dihydro, 6a-hydroxyphaseollin (IV, Fig. 3), and the monoacetate and disilyl derivatives of 6a-hydroxyphaseollin were all active in the TLC bioassay.

We have been unable to isolate hydroxyphaseollin from unchallenged hypocotyls of the two soybean cultivars, or to detect HP in extracts from unchallenged plants by GLC. Analyses by GLC showed that hydroxyphaseollin accumulated up to 3.0 mg/g dry wt hypocotyls at 48 hr after inoculation of Harosoy 63 by method 1, but reached only about 0.3 mg/g dry wt in challenged Harosoy tissues. Additional data on the kinetics of hydroxyphaseollin accumulation in incompatible and compatible soybean-*P. megasperma* var. *sojae* combinations are published elsewhere (8).

The yellow fluorescent substance discovered by Klarman & Gerdemann (11) and worked with extensively by Frank & Paxton (6) and Paxton & Chamberlain (17) was detected in the water fractions of ethanol extracts from challenged tissues and in direct aqueous tissue extracts. The material was partially purified by acidic butanol extraction (J. D. Paxton, *personal communication*) and preparative TLC using solvent v (Table 1) and isopropanol:ethyl acetate:water (8:2:2, v/v). Growth of *P. megasperma* var. *sojae* was not inhibited in the petri dish bioassay when up to 500 μg of preparations of the fluorescent material were added/plate. Similarly, this material did not produce inhibition zones in the TLC bioassay when up to 200 μg were spotted and the plates developed with various solvent systems before bioassaying.

DISCUSSION.—Our data confirm those of Klarman & Sanford (12) indicating that a single induced antifungal compound accumulates in *Phytophthora*-challenged soybean hypocotyls. The active principle has been identified as 6a-hydroxyphaseollin (J. J. Sims, N. T. Keen, & V. K. Honwad, *unpublished* data), and meets the usual criteria offered to define phytoalexins (8). Considerable evidence indicates that hydroxyphaseollin is mechanistically involved in monogenic *Phytophthora* resistance in soybeans, the most convincing fact being that it is produced faster by at least an order of magnitude in the hypocotyls of soybeans carrying a resistance allele than in near-isogenic lines lacking a resistance allele (7, 8).

Hydroxyphaseollin was probably detected chromatographically in the original work of Klarman & Gerdemann (11), based on identical R_F values (0.89-0.90) in solvent system v (Table 1), and on the partial decomposition of hydroxyphaseollin which occurs in this solvent. Chamberlain & Paxton (2) reported that this 0.8-0.9 R_F chromatographic fraction protected susceptible soybean plants against *P. megasperma* var. *sojae*. Hydroxyphaseollin also appears identical to the material isolated by Klarman & Sanford (12), based on similarity of spectral data (12; J. J. Sims, N. T. Keen, & V. K. Honwad, *unpublished data*) and identical migration on TLC plates (R_F 0.80) with solvent iii (Table 1).

Although our extracts from *Phytophthora*-challenged plants contained the yellow fluorescent compound described by Klarman & Gerdemann (11), the partially purified material exhibited no antifungal properties in either of the bioassays used. While the fluorescent substance remained in the aqueous phase of water-ethyl acetate partitions, all the induced antifungal activity from fungus-challenged soybean hypocotyls partitioned into the organic phase. In view of these findings and other anomalies such as peculiar production kinetics (6, 17), the unidentified fluorescent material does not seem to be directly involved in the *Phytophthora*-resistance response in soybeans, and should not properly be considered a phytoalexin.

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