

The Soybean Phytoalexin, Hydroxyphaseollin, Induced by Fungicides

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ABSTRACT

Hypocotyls of soybeans incubated in suspensions of various fungicides contained the soybean phytoalexin, hydroxyphaseollin (HP). Fifteen of the 27 compounds tested induced detectable quantities of HP. The amounts of HP induced by eight of these compounds were quantitated. Suspensions of maneb (1 mmole/liter) induced the most HP (22 μ g HP/g fresh wt of tissue), and maximal production occurred after 48-hr incubation. Two

decomposition products of maneb, ethylenediamine and polyethylene (thiocarbamoyl) monosulfide, also induced HP. Butylamine, the nonfungitoxic portion of the fungicide benomyl, induced HP as effectively as did benomyl. The fungitoxic portion, benzimidazole carbamic acid, methyl ester, did not induce HP.

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Nonaka et al. (9) isolated a single antifungal principle from fungal infected soybeans, and Klarman & Sanford (5) demonstrated that it was a phytoalexin chemically related to phaseollin, the phytoalexin from *Phaseolus vulgaris*. The structure of the soybean phytoalexin molecule was determined by Sims et al. (11); like those phytoalexins from other legumes, it has a pterocarpin skeleton. It differs from phaseollin by having a hydroxyl group in place of a hydrogen atom in the 6a position and, therefore, has been named hydroxyphaseollin (HP) (3).

Phytoalexins can also be induced by many chemicals. Schwochau & Hadwiger (10) found that low concentrations (10^{-6} M) of actinomycin D and certain other protein-synthesis inhibitors stimulated both protein and pisatin synthesis in peas. Cruickshank & Perrin (2) used mercuric chloride, and Uehara (16) used metallic salts and several organic mercury fungicides to induce pisatin in peas.

It is often noted that some fungicides are more effective under field conditions than would be expected from their fungitoxicity in vitro. One possible explanation of this increased effectiveness might be their ability to induce phytoalexins. In this study, nine fungicides, 10 of their decomposition products, and eight other chemicals were tested for their ability to induce HP.

MATERIALS AND METHODS.—Seeds of soybeans, Harasoy 63, were planted in white quartz sand in plastic trays. Plants were maintained at 20 to 30 C under Gro-Lux fluorescent lamps which supplied 300 ft-c of continuous light to the surface of the trays. Hypocotyls from 6-day-old plants were harvested, washed under tap water, and dried with paper towels.

Ethylene (thiocarbamoyl) disulfide (ETD) and polyethylene (thiocarbamoyl) disulfide (PETD) were prepared according to Klopping & van der Kerk (6); and, ethylene (thiocarbamoyl) monosulfide (ETM), polyethylene (thiocarbamoyl) monosulfide (PETM), and ethylene thiourea (ETU) were prepared by the

methods of Thorne & Ludwig (14). Melting point ranges of prepared products agreed with those reported by the above investigators. All other chemicals were obtained in either technical or reagent grade.

Seven-ml portions of aqueous suspensions (1 mmole/liter) of each chemical tested for induction of HP were added to petri dishes containing 4.7 g of hypocotyl tissue. The concentrations of the fungicides used were approximately equal to or lower than those suggested for use on suitable crops under field conditions. The controls contained 7 ml of distilled water. The petri dishes were closed and maintained for 2 days at 22 C under 580 ft-c of continuous light. Tissues, along with their incubation liquids, were blended in a Waring Blendor for 1 min with 10 ml of boiling distilled water/g fresh wt. The slurry was filtered through four layers of cheesecloth, the residue rinsed with boiling distilled water, and the filtrate centrifuged at 28,700 g for 90 min. The supernatant was filtered through Whatman No. 1 paper and extracted twice with half volumes of ethyl ether. The pooled ether extracts were evaporated to dryness under vacuum, and the residue was dissolved in ether equivalent to 1 ml/dish of plant tissue. All experiments were carried out 2 or more times.

Extracts were bioassayed by the thin-layer chromatography (TLC) method of Klarman & Sanford (5) to detect the presence of HP. Hydroxyphaseollin was quantified as the trimethylsilyl derivative by the gas-liquid chromatography (GLC) method of Keen et al. (4).

RESULTS AND DISCUSSION.—Soybean hypocotyls were incubated in each of 27 chemicals, and extracts were bioassayed to determine which of the chemicals induced HP. Fifteen of the chemicals induced HP, and eight of these were compared for their relative abilities to induce the phytoalexin (Table 1). Maneb and butylamine induced significantly more HP than did the other chemicals.

Concentrations of HP were determined for soybean hypocotyls which had been incubated in 1

TABLE 1. Chemicals that induced hydroxyphaseollin (HP) in soybean hypocotyls^a

Chemical	$\mu\text{g/g}$ fresh wt tissue ^b
Maneb	22.0 a ^c
Butylamine	17.5 ab
Benomyl	11.6 b
Ethylenediamine (ED)	10.4 bc
Cupric chloride	10.4 bc
Nabam	10.2 bc
Diethylamine	7.6 c
Polyethylene (thiocarbamoyl) monosulfide (PETM)	5.4 c
Terrazole	e.
Streptomycin B	e.
Neomycin B sulfate	e.
Phenyl mercuric salicylate	e.
Isobutylamine	e.
Propylamine	e.
Hexylamine	e.

^a Aqueous suspensions of 1 mmole/liter; additional chemicals tested that did not induce HP included: benzimidazole carbamic acid, methyl ester (BCM); polyethylene (thiocarbamoyl) disulfide (PETD); ethylene (thiocarbamoyl) monosulfide (ETM); ethylene (thiocarbamoyl) disulfide (ETD); phenylisothiocyanate; ethylene thiourea (ETU); carbon disulfide (CS_2); bacitracin; sulfur; ferbam; triarmol; and zineb.

^b Mean value of HP from three replications.

^c Treatment means followed by the same letter are not significantly different at the 1% level according to the Hartley variation of the Q-Method (12).

^d Quantities of HP not determined.

mmole/liter suspensions of maneb for 12, 24, 36, 48, 72, and 96 hr (Fig. 1). To determine whether the continuous presence of maneb was necessary for HP production, the liquid was removed from a similar group of hypocotyls after 48-hr incubation. The hypocotyls were rinsed in distilled water and

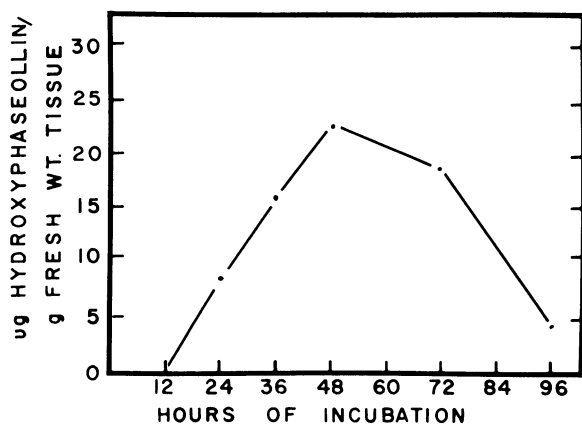


Fig. 1. Changes in hydroxyphaseollin concentration in soybean hypocotyls with time of incubation in a 1 mmole/liter suspension of maneb.

incubated for an additional 48 hr in distilled water. The hypocotyls were blended along with the distilled water and centrifuged. The supernatant was extracted and the ether extracts were examined by GLC; HP was not detected.

Three of the four dithiocarbamate fungicides, zineb, nabam, and maneb, are monoalkyldithiocarbamates which yield similar decomposition products and possibly undergo conversion to isothiocyanates (15). Nabam and maneb induced readily detectable amounts of HP, but zineb did not. Of the nine decomposition products of maneb tested, only ethylenediamine and PETM induced HP. Because of zineb's low solubility in water (<10 ppm) (7), ethylenediamine and PETM probably were not released in high enough concentrations to induce HP. Suspensions of zineb to which had been added 1% methanol or pyridine to increase solubility also failed to induce HP, and concentrations higher than 1% could not be used because of phytotoxicity. The low solubility of ferbam in water may also explain its inability to induce HP.

Aqueous suspensions of maneb (1 mmole/liter) were aerated and, at various intervals (1, 2, 8, 13, 20, 24, 27, 30, and 32 days), 25- μ liter portions were spotted on TLC-plates which were developed in *n*-butanol:ethanol:water (120:33:57, v/v) (8), air-dried, and bioassayed. Fresh maneb produced two spots inhibitory to *Cladosporium cucumerinum* Ellis & Arth. at R_F values of 0.4 and 0.82. One-day-old maneb produced four inhibitory spots, and the size and number of spots decreased over a period of 30 days, after which no inhibitory spots were observed. The R_F value of 0.82 corresponded to that of prepared ETM. No ETM could be detected in solutions of maneb after 28 days. The ETM, which did not induce HP, polymerizes to PETM (8) which induced HP. As could be expected, when there was no more ETM to polymerize to PETM, HP production ceased.

The 3,5-dinitrobenzamide (DNP) derivatives of aged maneb were compared by chromatography to the DNP derivative of reagent grade ethylenediamine using Fast Blue Salt B as a spray reagent (13). By this method, ethylenediamine, which induced large quantities of HP, could be detected in maneb suspensions for at least 21 days. Therefore, both ethylenediamine and PETM probably are responsible for induction of HP by aged maneb suspensions. The ultimate fate of the intermediate decomposition products of maneb is not fully understood. Perhaps PETM completely decomposes or complexes with other compounds; it was absent, however, in concentrations high enough to induce HP after ca. 30 days. A decomposition product of maneb, H_2S , was not examined for its ability to induce HP.

Benomyl, a systemic fungicide, also induced HP. In aqueous solutions, benomyl decomposes into benzimidazole carbamic acid, methyl ester (BCM) (1), and butylamine with the release of CO_2 . The BCM is as fungitoxic as benomyl towards most fungi (1);

however, BCM did not induce HP (Table 1). Germination of conidia of *C. cucumerinum* was not affected by 12-hr incubation in a 10^{-3} M solution of butylamine, but a similar concentration induced sufficient HP to completely inhibit germination. Quantities of 1 μ g of HP can be detected by the TLC-bioassay, and a concentration of 7×10^{-5} M inhibits mycelial growth of *Phytophthora megasperma* var. *soj*ae, a serious pathogen of soybeans (4).

Most investigators agree that benomyl may penetrate plants more readily than BCM, but that BCM is the fungitoxic moiety (1). In our studies, 3-day-old soybean seedlings were treated on each of 4 days with 500 ml of 1 mmole/liter suspension of benomyl, and care was taken not to wet the foliage. Exposure of soybean roots to benomyl in this manner did not result in the induction of HP in any of the aboveground portions, suggesting that neither benomyl nor butylamine was present. The fungitoxic compound recovered from all aboveground portions of treated plants corresponded to technical grade BCM when compared by TLC; it was thus assumed to be BCM rather than benomyl. The two additional short-chain amines (6 or fewer carbons) also induced HP, but none more effectively than did butylamine (Table 1).

All compounds that induced HP, except phenyl mercuric salicylate and cupric chloride, are amines or release an amine on decomposition. This suggests that short-chain amines in concentrations neither fungitoxic nor phytotoxic may aid in disease control by stimulating the natural defense mechanisms of plants.

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