Phytoalexin Induction in Beans Resistant or Susceptible to Fusarium and Thielaviopsis

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ABSTRACT

Beans responded to inoculation with pathogens and nonpathogens by producing substances, two of which were found to inhibit germination and growth of fungi. One of these substances (phaseollin) previously has been identified. The second substance apparently is phenolic. It absorbs maximally at 288 nanometers in ethanol. Greater quantities of both phytoalexins were induced in resistant than in susceptible bean lines. The fungi induced different quantities of each phytoalexin, and the phytoalexins appeared to be induced independently. The fungi were differentially sensitive to each phytoalexin. It is suggested that resistance in bean to *Fusarium solani* f. sp. *phaseoli* and *Thielaviopsis basicola* is operative through the formation of two phytoalexins which inhibit the growth of the infecting organisms in vivo, thus restricting the size of the lesions produced in resistant bean lines. Phytopathology 61: 322-327.

Additional key words: Phaseolus sp., bioassay, spectrophotometry, chromatography.

Substantial variability has been observed in the severity of infection of beans by *Fusarium solani* f. sp. *phaseoli* and *Thielaviopsis basicola* (1, 2, 12, 24). Histological studies (10, 19) indicated that growth of hyphae of either pathogen was suppressed early in resistant but not in susceptible bean lines. It was shown further that although there was some correlation between periderm formation and resistance, the periderm did not seem to play a major role in the resistance of beans to either of these pathogens (10, 19).

Huber (10) attributed resistance of bean to *F. solani* f. sp. *phaseoli* to a nonspecific wound response characterized by intense enzymatic activity around the infection site. Muller's work (15) clearly indicated that antifungal substances were induced in beans by *Sclerotinia fructicola* and *Phytophthora infestans*. Cruickshank & Perrin (5) and Perrin (18) subsequently isolated and chemically identified an antifungal substance from beans (7-hydroxy-3′, 4′-dimethyl-chromenochromanocoumarin) and called it phaseollin.

Since the phytoalexin concept was proposed (16, 17), several reports have substantiated the idea that many plant species respond to fungal invasion by producing phytoalexins (3). Gäumann & Kern (7) and Gäumann et al. (8) detected orchinol in several species of orchid, and Cruickshank & Perrin (6) found pisatin in 58 cultivars of *Pisum sativum* and other species of *Pisum*.

The phytoalexin concept appeared to be quite compatible with histological observations (19). This study, therefore, was undertaken with two major objectives: (i) to determine if phytoalexins were produced by bean lines in response to invasion by *F. solani* f. sp. *phaseoli*, *T. basicola*, and other fungi; and (ii) to determine the effectiveness of such phytoalexins in inhibiting the growth of fungi.

MATERIALS AND METHODS.—*Phaseolus coccineus* L. (Scarlet Runner), *P. vulgaris* L. (P.I. 203958, also called N203), *P. vulgaris* L. (Red Kidney), and N203 × (*P. vulgaris*^{2} × *P. coccineus*) — F6 (2051-02) were the main bean lines used in this study. In addition, *P. vulgaris* L. (Yellow Eye), and Yellow Eye × (*P. vulgaris*^{2} × *P. coccineus*) — 2114-12 were used to some extent. Red Kidney and Yellow Eye are susceptible to both pathogens, whereas the other bean lines show various degrees of resistance.

The bean pathogens used were *Fusarium solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans. and *Thielaviopsis basicola* (Berk. & Br.) Ferr. Other fungi utilized were *Cladosporium cladosporioides* (Fres.) DeVries and *Monilinia fructicola* (Wint.) Honey. The fungi were cultivated on potato-dextrose agar in the laboratory for 7-14 days prior to use in phytoalexin induction or in bioassays.

Induction of phytoalexins.—The method used to induce phytoalexins was essentially similar to that described by Muller (14). Each of the above-named fungi was utilized as a phytoalexin inducer in all bean lines. Partially mature, green bean pods were obtained from field-grown plants. After washing and surface disinfesting, the pods were split longitudinally and the seeds removed. Split pods were placed in pyrex trays, and the seed cavities inoculated with a spore suspension (400,000-600,000/ml) of one of the inducing fungi. Pods similarly treated with sterile distilled water served as one control (Control A). A second control (Control B) consisted of the supernatant of a centrifuged spore suspension of the particular inducing fungus after incubation in sterile distilled water for 24 hr. Trays containing the inoculated pods were enclosed in polyethylene bags and incubated at 21 C for 24 hr. The diffusates were then removed by suction into sterilized flasks and stored at 4 C prior to utilization in bioassays. Spores and mycelia were removed from the diffusate by centrifugation. When storage for over 24 hr was essential, diffusates were mixed with equivalent quantities of 95% ethanol prior to storage. Diffusates thus stored were extracted with organic solvents after removal of the ethanol in vacuo. After removal of the diffusates, more sterile distilled water generally was added to the inoculated pods, and the whole incubated

for a further 12 hr. Materials collected after the second period of incubation were stored separately and were not utilized in critical bioassays.

Biological assays.—Materials collected after the initial 24-hr incubation were utilized in bioassays against each of the inducing organisms (*F. solani* f. sp. *phaseoli*, *T. basicola*, *C. cladosporioides*, and *M. fructicola*). In addition, *Botrytis cinerea* Pers. and *Fusarium oxysporum* f. sp. *melonis* (Leach & Curr.) Snyd. & Hans. were used to a limited extent. In the bioassays, a standard quantity of the diffusate containing substances induced by a particular fungus was tested against the inducing fungus itself and other fungi. Since tests were carried out under similar conditions, it was possible to broadly compare the response of the test fungi to diffusate from a particular bean line, and to obtain some indication of the levels of phytoalexins induced by each fungus.

An agar-disc method and a nutrient-spore suspension method were used in the bioassays. With the agar-disc method, a dilute spore suspension (20-30 spores/low power field of the microscope) of the fungus to be assayed was prepared in 5 ml sterile distilled water, mixed with 5 ml melted 2% agar containing a semisynthetic nutritive medium minus biotin (13) and poured into a petri dish (9 cm diam). This gave a layer of spore-seeded agar approx 1 mm thick from which discs (5 mm diam) were cut with a flamed cork borer. Each disc then was placed in a small watch glass, to which was added 0.1 ml of the diffusate.

With the nutrient-spore suspension method, measured quantities of the materials to be tested were placed in small watch glasses and evaporated by blowing with warm air in a fume hood. To this was added 0.1 ml of a dilute spore suspension of the test fungus in a broth of the semisynthetic medium.

The small watch glasses were placed in larger petri dishes, to which were added small quantities of water to maintain a high humidity and thus prevent evaporation of the test medium. The large dishes then were enclosed in polyethylene bags also containing water and incubated at 24 C for 12 hr. After incubation, spores were killed by flooding with 3% formaldehyde, and 50 germ tubes subsequently were measured in each of three replicates. The results were statistically evaluated using the randomized complete-block design with more than one observation per experimental unit (22).

Extraction from diffusates.—The phytoalexins so far isolated all possess lipophilic properties, and Cruickshank & Perrin (4) used light petroleum as an extracting solvent for both pisatin and phaseollin. In a preliminary test, phytoalexin-containing diffusate from Scarlet Runner was extracted with petroleum ether (60-70% hexane) or anhydrous diethyl ether (ether).

Bioassay subsequently of the aqueous fractions indicated that the inhibitory factors were completely removed by the ether but only partially removed when petroleum ether (PE) was the extracting solvent. Ether was the main extracting solvent used in this study, but some selective extraction was carried out by partitioning the aqueous fraction first with PE and then with ether or ethyl acetate as described by Pierre & Bateman (20).

Diffusates containing substances induced by fungi or obtained by incubating bean pods with sterile distilled water (Control A) were partitioned twice with ether (1:2, v/v) by mixing in a Waring Blendor for 3 min at low speed. This was followed by vigorous shaking in a separatory funnel and ultimate separation into ether and aqueous fractions. The ether fraction was evaporated to dryness in vacuo at 40 C and the residue redissolved in a volume of 95% ethanol equivalent to 10% the original volume of the particular diffusate. Extracts obtained in this manner were refrigerated and used subsequently for spectrophotometric, chromatographic, and biological assays.

Extraction from tissues.—Bean hypocotyls (healthy or infected with *F. solani* f. sp. *phaseoli* or *T. basicola*) were triturated in 70% ethanol (1:4, v/v) in a Waring Blendor. The triturated material was strained through cheesecloth and centrifuged (1,000 *g* for 10 min). After centrifugation, distilled water equivalent to the original volume of ethanol was added and the ethanol removed in vacuo at 40 C. This was partitioned and treated as outlined for diffusates.

Chromatographic examination and spectrophotometric assays.—Materials to be examined were spotted or streaked on Whatman No. 1 or No. 3 paper and chromatographed at 19-21 C with an ascending solvent consisting of *n*-propanol and distilled water (20:80, v/v). Two-way chromatograms were prepared with 2% acetic acid as the second solvent. Chromatograms of the various extracts were examined in ultraviolet light with or without ammonia, and also after treatment with various reagents: sodium hydroxide, hydrochloric acid, or aluminum chloride (9, 21, 23). Particular areas of chromatograms were eluted with ethanol for spectrophotometric and biological assays. Chromatograms were dipped in or sprayed with various reagents for detection of particular groups of compounds (21).

RESULTS.—*Bioassay of diffusates.*—The results of the bioassays are summarized in Fig. 1. One apparent anomaly merits explanation. In the first experiment (Fig. 1, 1-A–1-E), the phytoalexin-containing diffusate induced in Scarlet Runner (SR) failed to significantly inhibit the test organisms. In addition, growth of *C. cladosporioides* in the diffusate from control pods was approx half that obtained with the other bean lines. These features were attributed to the possible dilution of both the inhibitory factors and nutrients contained in the diffusates, owing to the comparatively large pod size of SR and failure to standardize the quantity of the spore suspension in the initial experiment. When these features were rectified and bioassays repeated, the phytoalexin-containing diffusate from SR, N203, and 2051-02 totally inhibited *C. cladosporioides*, whereas that from Red Kidney (RK) inhibited this fungus 51.2%. With *F. solani* f. sp. *phaseoli* as the test organism, inhibition was 68.9, 66.7, 47.8, and 31.2%, with phytoalexin-containing diffusates from SR, 2051-02, N203, and RK, respectively.

Although there was some variability in the results of

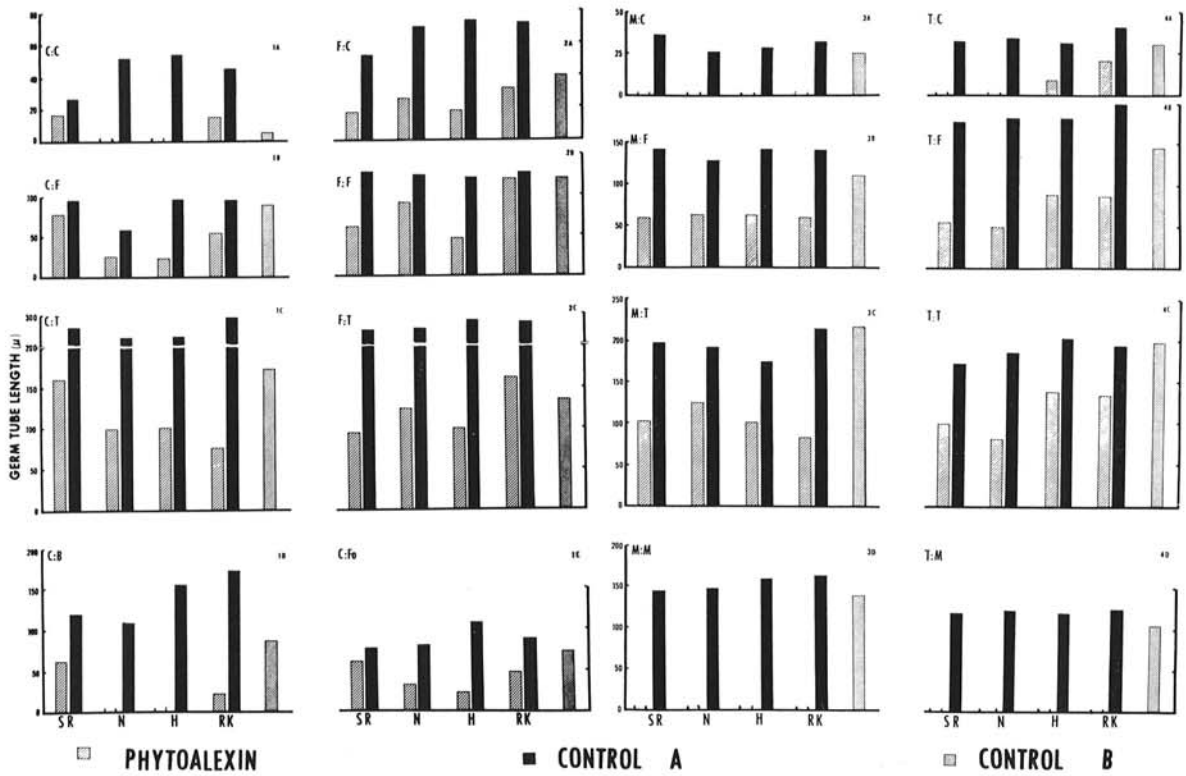

Fig. 1. Biological assays of diffusates induced by *Cladosporium cladosporioides, Fusarium solani* f. sp. *phaseoli, Monilinia fructicola,* or *Thielaviopsis basicola* and diffusates produced in the absence of fungi, tested principally against each of the inducing fungi. Histograms show average growth of 3 × 50 germtubes measured after 12 hr in test material. Letters at the top left of each group of histograms refer to the inducing fungus (first letter) and the test fungus (second letter). The fungi are indicated as follows: B = *Botrytis cinerea;* C = *C. cladosporioides;* F = *F. solani* f. sp. *phoseoli;* Fo = *F. oxysporum* f. sp. *melonis;* M = *Monilinia fructicola;* T = *T. basicola.* The bean lines are indicated as follows: H = 2051-02; N = P.I. 203958; RK = Red Kidney; SR = Scarlet Runner. Control A consisted of diffusate from pods treated with distilled water, whereas Control B consisted of the supernatant of a centrifuged spore suspension of the particular inducing fungus.

these bioassays, in general, the following points were indicated: (i) The phytoalexin-containing diffusates from each bean line inhibited growth of the test organisms to some extent; (ii) diffusates from resistant lines caused greater inhibition than that from the susceptible cultivar Red Kidney; (iii) *M. fructicola* and *C. cladosporioides* appeared to induce greater quantities of phytoalexins than did the bean pathogens *F. solani* f. sp. *phaseoli* and *T. basicola;* and (iv) *M. fructicola* and *C. cladosporioides* were more sensitive to the phytoalexins than were *F. solani* f. sp. *phaseoli* and *T. basicola.*

Chromatographic, spectrophotometric and biological assays of extracts—The spectrophotometric assays revealed that all phytoalexin-containing diffusates absorbed in the ultraviolet range, whereas those from the control treatments did not. These extracts absorbed maximally from 260 nm through 288 nm. This broad spectral pattern was produced by the combined absorbance of three substances which had max absorbance at 260, 279, and 288 nm, respectively. There was some variability in the absorbance of diffusates induced by a particular fungus in each bean line, but SR appeared to contain the greatest concn of absorbing substances and RK the least; N203 and 2051-02 were intermediate.

Chromatographic examination also indicated that the phytoalexin-containing extracts from all bean lines apparently contained qualitatively similar compounds. Extracts were streaked, and developed to about 9 inches from the source. The paper then was dried and divided into 1-inch strips parallel to the source of application. Each strip was eluted in 95% ethanol and tested spectrophotometrically and biologically. The results of a typical bioassay are shown in Table 1. These tests repeatedly showed that inhibitory properties were associated with the area from 5-9 inches, with greatest inhibition at 7 and 8 inches. This area also possessed substances which absorbed in the ultraviolet range and reacted positively to ferric ferricyanide, vanillin, tetrazotized benzidine, and sulphanilic acid reagents, all of which are used to detect phenolic compounds (21). Similar reactions were not obtained with extracts of diffusates from the control treatments.

Changes in the absorption spectra of eluates from different areas of the chromatograms indicated the presence of three ultraviolet-absorbing substances in the ether extracts. A distinct tailing effect was evident, with consequent overlap of the absorption spectra.

TABLE 1. Influence of eluates from chromatographed[a] extracts of phytoalexin-containing diffusates from *Phaseolus coccineus* on germination and growth of *Fusarium solani* f. sp. *phaseoli*

Area on chromatogram (inches)	Germ tube[b] length (μ)	Growth as % control
Source	237.7	79.4
1	229.4	76.6
2	256.2	85.6
3	261.2	87.3
4	252.4	84.3
5	117.9	39.4
6	48.8	16.3
7	0.0	0.0
8	0.0	0.0
9	113.8	38.0
Control[c]	299.3	100.0

[a] Extracts were chromatographed by ascending solvent (*n*-propanol:water). Solvent front 8.5 inches.
[b] Average of 50 spores/treatment.
[c] Equivalent area of paper similarly chromatographed without added material.

This tailing was reduced somewhat when materials were chromatographed on paper that was washed in acid and base (11). The substances subsequently were separated chromatographically and are referred to as Substances I, II, and III. These three substances were present in varying amounts in the phytoalexin-containing diffusates from all bean lines.

Bioassays indicated that only Substances I and II possessed antifungal properties. In ethanol, Substance I had absorption maxima at 279, 286, and 315 nm. For reasons stated elsewhere (20), this substance is considered to be similar to phaseollin (5, 18). Substance II had a relatively symmetrical spectrum and absorbed maximally at 288 nm, whereas Substance III absorbed maximally at 260 nm (20). The R_F in *n*-propanol:water (1:4 v/v) was 0.71 and 0.85 for Substances I and II, respectively.

Quantitative estimation of each inhibitory substance in diffusates induced in bean lines by different fungi.—Substance I (phaseollin) can be selectively removed from the diffusates by partitioning with petroleum ether (PE). The second inhibitory material then can be extracted from the aqueous fraction by partitioning with ether or ethyl acetate (EA) (20). Five ml of phytoalexin-containing diffusate from various fungus-host combinations were partitioned twice by shaking (3 min) in a separatory funnel, first with PE (1:4 v/v) and then with EA (1:2 v/v). The PE and EA fractions were evaporated to dryness separately. The residue from each fraction was redissolved in 5 ml 95% ethanol and examined spectrophotometrically. Further quantitative dilutions were made where necessary.

The results, which are shown in Table 2, indicated that (i) different fungi induced different quantities of each phytoalexin in a particular bean line; (ii) a particular fungus induced different amounts of each substance in different bean lines; and (iii) Substances I and II were induced independently.

Dosage-response curves of test organisms to phaseollin and Substance II.—Serial quantities of standardized extracts containing phaseollin or Substance II were used in bioassays against test fungi. Materials were standardized spectrophotometrically by determining the absorbance of a given volume contained in 3.0 ml ethanolic solution.

The results of these bioassays clearly indicated the differential sensitivity of the test organisms to the inhibitory substances. With phaseollin, *T. basicola*, *F. solani*, *M. fructicola*, and *C. cladosporioides* had ED_{50} values at concn that absorbed at 0.56, 0.31, 0.27, and 0.15 (equivalent to 59.6, 33.0, 28.7, and 15.9 μg), respectively, at 279 nm. At a concn which absorbed at 1.0 (equivalent to 106.4 μg) the same fungi were inhibited 58, 76, 80, and 93%, respectively. With Substance II, *F. solani*, *M. fructicola*, and *T. basicola* had ED_{50} values at concn that absorbed at 0.36, 0.28, and 0.27 at 288 nm, respectively. At higher concn, *M. fructicola* was more adversely affected. This fungus was inhibited totally at a concn which absorbed between 0.4 and 0.8, whereas at an absorbance level of 1.0, *F. solani* and *T. basicola* were inhibited 85 and 93%, respectively. Of the bean pathogens, therefore, *F. solani* was more sensitive to phaseollin than *T. basicola*, whereas the opposite was true with Substance II.

Formation of phytoalexins at infection sites.—Ether extracts from the equivalent of 0.1 g *Fusarium*-infected tissues of SR inhibited germ tube growth of *F. solani* f. sp. *phaseoli* and *T. basicola* 76.1 and 60.4%, respec-

TABLE 2. Spectrophotometric estimation of each phytoalexin induced by different fungi in bean lines. Absorbance was measured for phaseollin (I) and Substance II (II) at 279 and 288 nm, respectively, in 3.0 ml ethanolic mixture. Final figures were obtained using the following formula:

$$\frac{\text{Absorbance} \times \text{volume of original extract}}{\text{Volume of extract used}}$$

	Monilinia fructicola		*Thielaviopsis basicola*		*Fusarium solani*	
Bean line	I	II	I	II	I	II
Scarlet Runner	2.5	16.5	2.2	17.2	1.2	25.0
N203	2.4	13.4	1.7	10.9	0.9	26.4
2051-02	2.5	9.4	2.1	9.0	1.0	17.5
2114-12	2.0	9.1	1.5	9.1	0.9	16.2
Red Kidney	1.8	4.0	1.1	3.9	0.9	8.6
Yellow Eye					0.4	0.9

tively, whereas similar extracts from *Fusarium*-infected RK caused 14.9 and 26.5% inhibition of these fungi in comparison with equivalent extracts from healthy tissues. Extracts from *Fusarium*- or *Thielaviopsis*-infected hypocotyls from SR, 2051-02, N203, and RK revealed the presence of substances with absorption spectra similar to those of the phytoalexins which were induced in the pods of these bean lines. Chromatographic examination indicated that the inhibitory substances in tissue extracts also had similar R_F and reacted positively to ferric ferricyanide, diazotized sulphanilic acid, and tetrazotized benzidine.

DISCUSSION.—Bioassays clearly demonstrated the fungistatic nature of diffusates which contained materials induced by the interaction of fungi, both pathogens and nonpathogens, in various bean lines. Although several substances were formed in each host-fungus interaction, only two substances were shown to possess fungistatic properties. One substance appears to be similar to phaseollin (5, 18, 20). The second substance still is unidentified, but indications are that it is also a phenolic compound.

It is apparent from the present study that at least two species of bean (*P. coccineus* and *P. vulgaris*) and their hybrids are capable of producing the inhibitory substances, but the extent to which either substance is produced by a particular bean line is determined by both the inherent capability of the plant and the particular inducing organism. The fungi used in this investigation induced greater quantities of phytoalexins in resistant lines than in susceptible lines. This is indicated by both the bioassays and spectrophotometric examination of the phytoalexins when selectively extracted in petroleum ether and ethyl acetate.

It has been shown also that different organisms induce different quantities of each phytoalexin in a particular bean line. Since the substances apparently are induced independently, any one substance may predominate in a particular situation. Furthermore, the dosage-response curves revealed that whereas *F. solani* was more sensitive to phaseollin than *T. basicola,* the situation was reversed with Substance II.

This differential induction of the phytoalexins coupled with differential sensitivity of the fungi to each phytoalexin may be among the main contributory factors to the variable response of a particular cultivar to different pathogens or strains of a particular pathogen.

Bean lines vary in their response to infection by *F. solani* f. sp. *phaseoli* and *T. basicola.* This variability is evident mainly as a reduction in the number and size of lesions on resistant lines. Histological examination has revealed that although a wound periderm is formed especially in resistant lines, the periderm is not the primary operative factor in the resistance of beans to these pathogens (19). In fact, the nature of the response suggested the involvement of fungistatic materials which effectively restricted the spread of hyphae within the tissues of resistant lines. Pierre & Bateman (20) have reported the production of phaseollin and Substance II by Red Kidney bean in response to infection by *Rhizoctonia solani,* and calculated that a single lesion contained 6-8 times the concn of fungistatic substances required to inhibit *R. solani* in vitro. In this study, it has not been possible to determine the quantities of phytoalexins per se, but the presence and fungistatic nature of the phytoalexins have been clearly demonstrated both in pod diffusates and infected tissues. It is suggested that these phytoalexins may be operative in the resistance of beans to *F. solani* f. sp. *phaseoli* and *T. basicola* by creating within the lesion a chemical environment which is incompatible with growth of these fungi.

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