Differential Response of Fusarium solani Isolates to Pisatin and Phaseollin

Hans D. VanEtten and Jeffrey I. Stein

Associate Professor and former Research Technician, Department of Plant Pathology, Cornell University, Ithaca, NY 14853.

This research was supported in part by a Brown-Hazen Grant from the Research Corporation and Rockefeller Foundation Grant GA AS 7701.

The authors would like to thank T. C. Kelsey and P. S. Matthews for technical assistance.

Accepted for publication 1 March 1978.

ABSTRACT

VANETTEN, H. D., and J. I. STEIN. 1978. Differential response of Fusarium solani isolates to pisatin and phaseollin. Phytopathology 68: 1276-1283.

One isolate each of *Fusarium solani* f. sp. *pisi*, *F. solani* f. sp. *phaseoli*, and *F. solani* f. sp. *cucurbitae* was treated with pisatin and phaseollin under a variety of growth conditions. *Fusarium solani* f. sp. *cucurbitae* consistently appeared sensitive to both phytoalexins and *F. solani* f. sp. *pisi* consistently appeared tolerant of pisatin. The response of *F. solani* f. sp. *phaseoli* to pisatin and the response of both *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* to phaseollin varied; whether an isolate appeared sensitive or tolerant to the phytoalexins depended on the growth conditions employed. Exposure of *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* in shake culture to a low concentration of phaseollin markedly enhanced their tolerance to a subsequent higher concentration of phaseollin. This type of response suggests the presence of an adaptive tolerance mechanism. *Fusarium solani* f. sp. *pisi* did not metabolize phaseollin but could metabolize pisatin. However, it grew substantially in shake culture in the presence of pisatin prior to significant metabolism of the phytoalexin. Thus, mechanisms in addition to or instead of metabolic detoxification of pisatin or phaseollin must account for the tolerance of *F. solani* f. sp. *pisi* to these phytoalexins.

The phytoalexins pisatin and phaseollin inhibit the in vitro growth of many fungi, but not all fungi appear to be equally sensitive (5, 6, 7, 23, 26). The more tolerant fungi generally are pathogens of the source plant of the phytoalexin, but there are numerous exceptions. Most of the survey studies have relied on bioassays which measure the effect of the phytoalexins on the linear growth of an organism on semisolid medium (5, 7, 23). However, bioassay conditions can markedly influence the response of fungi to phytoalexins (7, 8, 14, 15, 23) and recent results (2) indicate that the linear growth bioassay can give misleading estimates of sensitivity.

Fungi also differ in ability to metabolize pisatin and phaseollin. Some fungi that appear tolerant are able to metabolize these phytoalexins to less inhibitory products (26). Although this would suggest that tolerance is due to metabolic detoxification, metabolism of isoflavonoid phytoalexins may not always be a detoxification mechanism and other tolerance mechanisms may be more important (26).

The purposes of this investigation were: (i) to determine if there are fungi with consistent and characteristic responses to pisatin and phaseollin regardless of the bioassay conditions, (ii) to examine some of the factors which might cause some fungi to vary in their response to pisatin and phaseollin when different bioassay conditions are employed, and (iii) to determine whether tolerance to pisatin or phaseollin can be expressed without concurrent metabolism of these phytoalexins. One isolate each of three *Fusarium solani* formae speciales (*cucurbitae, pisi,* and *phaseoli*) was selected for this study because there is some information on their metabolism of pisatin and phaseollin (4, 9, 10, 20, 21, 27) and previous studies (5, 7, 10, 12, 13, 21, 23, 24, 28) suggest that they differ in their sensitivity to pisatin and phaseollin.

00032-949X/78/000 230$03.00/0

MATERIALS AND METHODS

Isoflavonoids.—Published procedures were used to obtain phaseollin (16), 1a-hydroxyphaseollone (21, 22), pisatin (27), and 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (DMDP) (28). Quantification of the compounds was based on reported molar extinction coefficients in ethanol of: log $\epsilon = 3.98$ at 280 nm for la-hydroxyphaseollone (21), log $\epsilon = 3.86$ at 309 nm for pisatin (11), log $\epsilon = 3.88$ at 309 for DMDP (27), and log $\epsilon = 4.04$ at 280 nm for phaseollin (1).

Cultures.—*Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyd. & Hans. (syn. *F. solani* var. *martii* f. *pisi*) isolate T-8, *F. solani* (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyd. & Hans. isolate T-162, and *F. solani* (Mart.) Sacc. f. sp. *cucurbitae* Snyd. & Hans. isolate T-131 were used in this study. Hereafter only the forma specialis name will be used to refer to these specific isolates. Isolates were stored as 1- to 2-wk-old cultures on V-8 juice agar [#M-29 in (18)] in the dark at 6 C and as lyophilized cultures (19). Fresh transfers of the isolates were normally started from the 6 C cultures. Spores to be used in the bioassays were obtained from V-8 juice agar cultures incubated at 24-27 C under fluorescent lights and/or diffuse sunlight.

Bioassay.—Glucose-asparagine (GA) medium (25) [20 g glucose, 3.0 g Bacto asparagine (Difco Laboratories, Detroit, MI 48232), 0.87 g K_2HPO_4, 0.14 g $MgSO_4 \cdot 7H_2O$, and 1.0 ml Steinberg's microelements (17) diluted to 1 liter with H_2O], was used for all bioassays, unless noted otherwise. Pisatin and phaseollin, dissolved in dimethylsulfoxide (DMSO), were added to the medium for a final DMSO concentration of 0.5% unless stated otherwise. This concentration of DMSO had no noticeable effect on the growth of the isolates when compared to their growth on medium lacking DMSO.

Unless stated otherwise the procedure for measuring linear growth inhibition was the same as described previously (23) except that GA medium supplemented with 2.2% agar (GA agar) was used as the bioassay medium and the inoculum plugs for the bioassays always were obtained from GA agar cultures 2 days after uniformly seeding the GA agar surface with a spore suspension.

The effects of phaseollin and pisatin on *F. solani* growing in shake culture were evaluated by procedures similar to those used previously (21). Approximately 500 ml of a spore suspension (about 1×10^5/ml) prepared by washing spores from 1- to 2-wk-old V-8 juice agar cultures with GA medium was added to a 1,000-ml Erlenmeyer flask and incubated at 24 ± 2 C on a reciprocal shaker (100 strokes/min) for 22-24 hr. Then the fungal mass was collected on Whatman No. 1 filter paper and resuspended in fresh medium at a concentration of about 1.5 mg (dry wt)/ml. Mycelium composed the bulk of the material collected but some conidiophores and conidia always were present. Four ml of the resuspended material (about 6 mg dry wt) were placed in 25-ml Erlenmeyer flasks and incubated 24 ± 2 C on a reciprocal shaker at 125 strokes/min. Cultures were incubated 15-30 min before treatment with the phytoalexins. The change in dry weight was determined by collecting the contents of each flask on tared filter paper (Whatman No. 50) by vacuum filtration and weighing the samples after drying at 75 C for 24 hr.

To determine the effect of phaseollin and pisatin on spore germination, spores from 2-day-old V-8 juice agar cultures were suspended (3×10^4/ml) in GA medium and 1.0 ml samples were treated. Aliquots (0.1 ml) were removed and added to the center wells of glass depression slides. The slides were placed in covered petri plates containing moistened filter paper and incubated at 24 ± 2 C in the dark for 12-18 hr. The percentage germination was determined by examining ≥ 100 spores per sample at ×100 magnification. A spore was considered germinated if it had a germ tube ≥ half the diameter of the spore.

Extraction and quantitation of phaseollin, pisatin, and their metabolites in shake culture.—Treated cultures were extracted by adding 20.0 ml of 95% ethanol to each flask and filtering the contents through Whatman No. 50 filter paper (21). The material on the filter paper was washed with about 20 ml of ethanol and the ethanol was removed from the combined filtrates by evaporation under reduced pressure. A few milliliters of water were added to the remaining aqueous fraction and this was partitioned two times with 50 ml of chloroform. After the chloroform fraction was taken to dryness, the residue was taken up in a small amount of chloroform and streaked next to isoflavonoid standards on silica-gel (250 μm) thin-layer chromatography (TLC) plates containing a fluorescent indicator (Sil G-25 UV_{245}, Brinkmann Instruments, Westbury, NY 11590). The TLC plates containing extracts of cultures treated with phaseollin were irrigated in benzene + methanol (9 + 1, unsaturated) (28) and the TLC plates containing extracts of cultures treated with pisatin were irrigated in chloroform + methanol (25 + 1, unsaturated) (27). After irrigation of the plates, the standards were located as UV-quenching spots and the silica gel bands opposite the standards were scraped from the plates and eluted with ethanol. Concentrations of the compounds in these eluates were determined by UV spectrophotometry. To correct for the loss of pisatin or phaseollin during the extraction procedure, two types of controls were used. Phytoalexins were added to live cultures of the fungus and immediately extracted. Phytoalexins also were added to autoclaved cultures and extracted after the same time periods as the treated live cultures. The percentage recovery of pisatin (about 50%) and phaseollin (about 60%) always was similar in both types of controls. To assure that there were no interfering compounds in live fungal cultures that would chromatograph coincidently with any of the isoflavonoids, nontreated live cultures were extracted and processed in the same manner as the treated cultures.

Two replicates of each treatment were included in all experiments and generally the values for replicate treatments in an experiment varied less than 10%. All experiments were repeated at least once unless stated otherwise.

RESULTS

Response of Fusarium solani isolates to pisatin and phaseollin in linear growth, liquid shake culture, and spore germination bioassays.—*Linear growth bioassay.*—Only *cucurbitae* was markedly inhibited by both phaseollin and pisatin (Fig. 1). Forma specialis *phaseoli* was strongly inhibited by pisatin (Fig. 1-B) but was inhibited less than 20% at the maximum phaseollin concentration tested (Fig. 1-A); *pisi* was only slightly inhibited (<20%) by either pisatin or phaseollin (Fig. 1).

Liquid shake culture bioassay.—Growth rates in the controls varied some between experiments, but all three

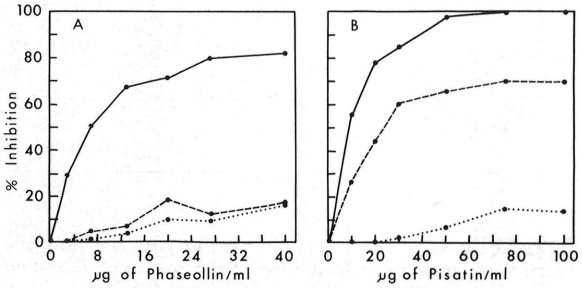

Fig. 1.-(A, B). Effect of **A)** phaseollin and **B)** pisatin on the radial growth of *Fusarium solani* f. sp. *cucurbitae* (•—•), *F. solani* f. sp. *pisi* (• · · · · · •), and *F. solani* f. sp. *phaseoli* (• - - - •). The percent inhibition was determined when the DMSO controls had a net growth of 27 ± 4 mm (4 days for *F. solani* f. sp. *pisi* and *F. solani* f. sp. *cucurbitae* and 8 days for *F. solani* f. sp. *phaseoli*).

isolates grew well in the liquid medium (Fig. 2, 3). The pattern of response of *cucurbitae* to both phaseollin and pisatin and the pattern of responses of *pisi* and *phaseoli* to pisatin were consistent. Growth of *cucurbitae* was affected by all three concentrations of pisatin (Fig. 2-A) and was markedly inhibited by phaseollin even at the lowest concentration (13 μg/ml) tested (Fig. 2-C). The growth of *pisi* and *phaseoli* was only slightly to moderately affected by pisatin (Fig. 2-B, D).

The pattern of response of *pisi* and *phaseoli* to phaseollin varied greatly in repeats of the same experiments. Two examples of the types of variation that were observed are illustrated in Fig. 3. The isolates appeared to be quite tolerant of phaseollin (Fig. 3-A, D) or fairly sensitive (Fig. 3-B, C). At the two lowest concentrations of phaseollin employed (13 and 27 μg/ml) growth always occurred within 24 hr and once growth did occur, the growth rates frequently were comparable to those of the controls.

Spore germination bioassay.—As in the two previous types of bioassays, *cucurbitae* was strongly inhibited by both pisatin and phaseollin and *pisi* was only slightly affected by pisatin (Table 1). The response of *pisi* and *phaseoli* to phaseollin varied considerably between individual experiments, as it had in the liquid shake cultures, but both organisms often were strongly inhibited by the higher concentrations. In most experiments *phaseoli* was only slightly affected by pisatin. At the maximum phytoalexin concentrations at which spore germination occurred, the germ tubes of all isolates were shorter than those of comparable controls.

Effect of selected variables on the responses of Fusarium solani isolates to phaseollin and pisatin.—To determine whether the consistent responses of *cucurbitae* and *pisi* to certain phytoalexins observed in the preceding bioassays could be altered, a selected number of variables which sometimes influence bioassays were studied. In addition, the fungicidal versus fungistatic activity of the phytoalexins and the possibility of adaptive tolerance by some isolates were examined to determine if these properties might explain why some isolates respond in a consistent manner and others in a variable manner.

Effect of age of inocula on the linear growth bioassay.—Conditions were as described for the standard linear growth bioassay except that inoculum was from 1, 4, or 7 day old cultures. Phytoalexin concentrations were 98 μg of pisatin and 40 μg of phaseollin for *pisi* and 20 μg and 13 μg/ml, respectively for *cucurbitae*. Growth was measured twice daily. Growth rates were determined from the linear portion of growth curves and the lag periods were determined by extrapolation to zero growth (2).

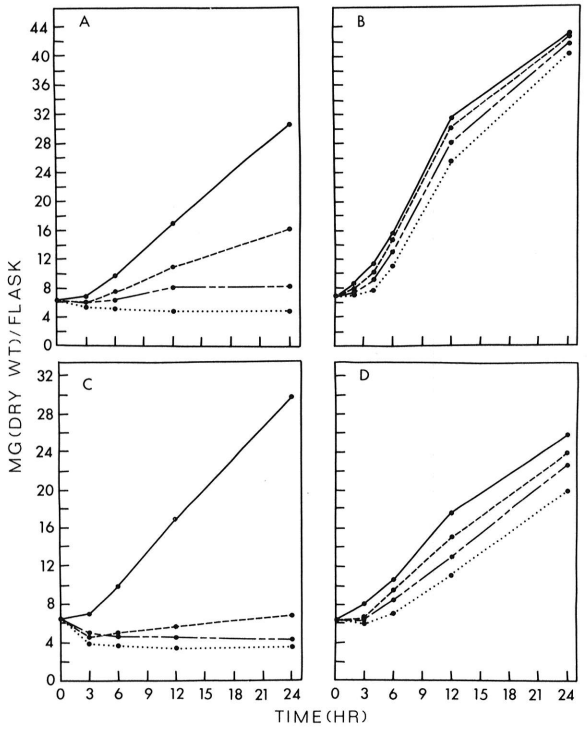

Fig. 2.(A to D). Effect of **A)** pisatin on the growth of *fusarium solani* f. sp. *cucurbitae*, **B)** pisatin on the growth of *Fusarium solani* f. sp. *pisi*, **C)** phaseollin on the growth of *Fusarium solani* f. sp. *cucurbitae*, and **D)** pisatin on the growth of *Fusarium solani* f. sp. *phaseoli* in liquid shake culture. In all Fig. • — • represents the DMSO controls and in A, B, and D • - - - - •, • — - - •, and • · · · · · · • represent 31, 63, and 94 μg of pisatin/ml, respectively, while in C these notations represent 13, 27, and 40 μg of phaseollin/ml, respectively. Each datum point is the average of four samples from two experiments.

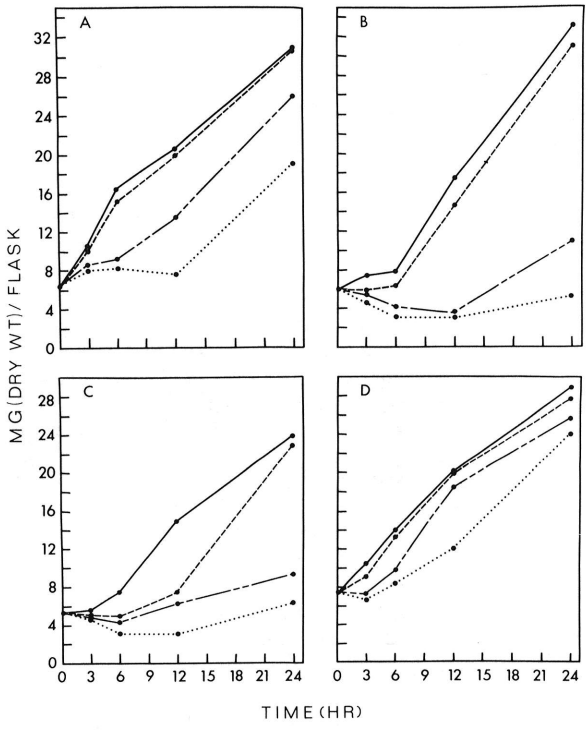

Fig. 3.-(A to D). Effect of phaseollin on the growth of *Fusarium solani* f. sp. *pisi* in liquid shake culture in two separate experiments (A and B) and of phaseollin on the growth of *Fusarium solani* f. sp. *phaseoli* in liquid shake culture in two separate experiments (C and D). Each datum point is the average of two samples. In all figures, •—• represents the DMSO control and • - - - - •, •— - -•, and • · · · · · • represents 13, 27, and 40 μg of phaseollin/ml, respectively.

With these conditions, Bailey et al. (2) demonstrated that the apparent sensitivity of *Colletotrichum lindemuthianum* decreased as the age of inoculum increased. Young inoculum either failed to grow or growth occurred only after a long lag period whereas old inoculum resumed growth after little or no lag. Growth after the lag period was comparable to the controls. Forma specialis *pisi* on phaseollin exhibited a similar pattern (Table 2). However, altering the age of *pisi* inoculum did not alter its high tolerance of pisatin (Table 2). Forma specialis *cucurbitae* remained highly sensitive to pisatin and phaseollin regardless of the age of inoculum used. The growth rates after the lag period did not approach that of the controls even though low levels of phytoalexins were employed (Table 2).

Effect of bioassay medium.—Previous studies (8, 14, 15, 23) have demonstrated that the composition of the bioassay media used can affect the apparent sensitivity of an organism to a phytoalexin. To test whether the consistent response of *pisi* to pisatin and *cucurbitae* to pisatin and phaseollin could be altered by the growth media, the standard linear growth bioassay was employed. The semi-defined media used were V-8 juice agar medium and pea-glucose agar medium. The latter is a modified version of potato-glucose agar medium [M-19 in (18)] in which pea seeds were substituted for potato tuber tissue. Glucose-supplemented *Neurospora* minimal medium (3) without biotin, and *Ustilago* minimal medium [M-100 in (18)] with glucose, malate, or acetate as the sole carbon sources were the defined media used.

TABLE 1. Effect of pisatin and phaseollin on spore germination of *Fusarium solani* formae speciales[a]

Fungus	Concentration required for 50% inhibition[b]		Germination at maximum concentration tested[c]	
	Pisatin (μg/ml)	Phaseollin (μg/ml)	Pisatin (100 μg/ml) (%)	Phaseollin (40 μg/ml) (%)
F. solani f. sp. *cucurbitae*	40 ± 7	11 ± 2	0 (0)	0 (0)
F. solani f. sp. *phaseoli*	> 100	31 ± 14	79 (24-100)	23 (0-64)
F. solani f. sp. *pisi*	> 100	35 ± 4[d]	96 (92-100)	39 (8-98)

[a]Spores (3×10^4/ml) were treated with 10, 20, 30, 50, 75, and 100 μg/ml of pisatin and with 3, 7, 13, 20, 27, and 40 μg/ml of phaseollin. The data for the effect of phaseollin on *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* is from four experiments and the data for the effect of pisatin on *F. solani* f. sp. *pisi* is from five experiments. All other reported data is from three experiments. The percent germination in the DMSO (0.5%) controls was 100% except in one test of *F. solani* f. sp. *phaseoli* against phaseollin in which germination was 97%.

[b]The average values and standard deviations were calculated from the dosage response curves.

[c]Average values are given and ranges are given in parentheses. No germination of *F. solani* f. sp. *cucurbitae* spores occurred at ≥ 75 μg of pisatin/ml and at ≥ 20 μg of phasoellin/ml.

[d]In one experiment less than 50% inhibition was obtained at the maximum phaseollin concentration (40 μg/ml) tested. This concentration was used as the 50% value in summarizing the data.

TABLE 2. Effect of age of inoculum on lag period and growth rate of *Fusarium solani* f. sp. *cucurbitae* and *F. solani* f. sp. *pisi* in linear growth bioassays[a]

Age of inoculum (days)	Treatment	Fungus			
		F. solani f. sp. *cucurbitae*[b]		*F. solani* f. sp. *pisi*[c]	
		Lag (hr)	Rate (mm/hr)	Lag (hr)	Rate (mm/hr)
1	DMSO	9 ± 2	0.33 ± .07	9 ± 1	0.33 ± .04
4		14 ± 4	0.34 ± .05	13 ± 3	0.34 ± .03
7		13 ± 6	0.32 ± .05	11 ± 2	0.35 ± .02
1	Pisatin	18 ± 6	0.22 ± .04	16 ± 6	0.31 ± .02
4		13 ± 4	0.20 ± .03	16 ± 3	0.31 ± .02
7		15 ± 6	0.19 ± .03	15 ± 0	0.31 ± .04
1	Phaseollin	47 ± 2	0.14 ± .03	48 ± 14	0.28 ± .03
4		29 ± 6	0.17 ± .03	24 ± 2	0.29 ± .03
7		37 ± 7	0.15 ± .01	20 ± 8	0.28 ± .02

[a]Plugs (4-mm diam) were removed from inoculum cultures grown for the indicated periods and placed on glucose-asparagine agar amended with dimethylsulfoxide (DMSO) or phytoalexin in DMSO. The final DMSO concentration in the phytoalexin amended media and DMSO control treatment was 0.5%. Data are the averages and standard deviations from two or more experiments.

[b]Agar contained 20 μg of pisatin/ml or 13 μg of phaseollin/ml.

[c]Agar contained 98 μg of pisatin/ml or 40 μg of phaseollin/ml.

Media were supplemented with 98 μg of pisatin/ml or with 40 μg of phaseollin/ml. *Neurospora* minimal medium did not support the growth of *cucurbitae* and therefore was not used for bioassay of that organism.

The organisms were bioassayed once. Forma specialis *cucurbitae* was consistently sensitive to both pisatin (96 to 100% inhibition) and phaseollin (78 to 100% inhibition); *pisi* consistently appeared tolerant of pisatin (−4 to 9% inhibition) regardless of the media used.

Effects of a phytoalexin pretreatment.—Activation of a mechanism which renders a microorganism less sensitive to the phytoalexins could explain why the growth rate of some fungi in the phytoalexin treatments was comparable to that of the controls after an initial lag period (Fig. 2, 3; Table 2). The liquid shake culture bioassay was used to test for the presence of such a mechanism. Shake cultures (0 hr) were exposed to a low noninhibitory concentration of phytoalexins for 1.5 hr before being exposed to higher inhibitory phytoalexin concentrations. Dry weights were determined at 0 hr and after 13.5 hr. Cultures which received two additions of phytoalexins were compared: (i) with cultures which were pretreated with DMSO (0.5%) alone for 1.5 hr and then treated with the higher phytoalexin concentration; (ii) with cultures which were exposed to the noninhibitory phytoalexin concentration for 1.5 hr and then treated with DMSO alone; and (iii) with cultures which received two consecutive DMSO treatments.

Exposure to 3 μg phaseollin/ml had little effect on the growth of all three isolates, but a 1.5-hr exposure greatly increased the tolerance of *pisi* and *phaseoli* to subsequent treatments with 27 μg phaseollin/ml (Table 3). Exposure to 3 μg phaseollin/ml did not render *cucurbitae* tolerant (Table 3). No indication of enhanced tolerance of *cucurbitae* to phaseollin was evident even when a less inhibitory concentration of phaseollin (10 μg/ml) was used as the second treatment (authors, *unpublished*).

Each isolate also was treated with a low noninhibitory concentration of pisatin (6 μg/ml) to determine if tolerance to pisatin could be enhanced. Cultures subsequently were treated with pisatin concentrations which had been shown to be inhibitory. Formae speciales *cucurbitae* and *phaseoli* were treated with 98 μg/ml and *pisi* with 155 μg/ml. (Trial experiments with concentrations of pisatin up to 300 μg/ml showed that *pisi* could be almost completely inhibited by concentrations above 200 μg of pisatin/ml even though substantial amounts of the pisatin were present as a precipitate in the media.) Pretreatment of the isolates with pisatin did not enhance the tolerance of any of the isolates to pisatin (Table 4). By 13.5 hr growth had occurred in all treatments. The cultures pretreated with 6 μg of pisatin/ml did not demonstrate enhanced growth over those that had not been pretreated. Since there was substantial growth of *pisi* in all treatments, an additional experiment was done with this isolate in which the completely inhibitory concentration of 200 μg of pisatin/ml was used after the pretreatment. No increase over the starting dry weight occurred in the 13.5 hr period regardless of whether or not the cultures had received a pisatin pretreatment.

Effect of the length of exposure time.—A modification of the spore germination bioassay was used with *cucurbitae* to test the effect of the length of exposure to pisatin or phaseollin on the fungicidal or fungistatic nature of these compounds. Aliquots (2.5 ml) of spore suspension prepared in the standard manner were placed in 10-ml conical centrifuge tubes and treated with phytoalexins. At given time intervals the contents were mixed with a 4.5 ml of GA medium and the tubes were

TABLE 3. Effect of a pretreatment with a low concentration of phaseollin on the response of *Fusarium solani* f. sp. *cucurbitae*, *F. solani* f. sp. *pisi*, and *F. solani* f. sp. *phaseoli* to a subsequent treatment with a higher concentration of phaseollin in shake culture[a]

Treatment	Time assayed (hr)	Dry weight of spores and mycelium (mg/flask)		
		F. solani f. sp. *cucurbitae*	*F. solani* f. sp. *pisi*	*F. solani* f. sp. *phaseoli*
None	0	5.6	5.4	5.9
DMSO at 0 hr and DMSO at 1.5 hr	13.5	23.6	19.9	26.4
3 μg phaseollin/ml at 0 hr and DMSO at 1.5 hr	13.5	22.2	19.5	25.8
DMSO at 0 hr and 27 μg phaseollin/ml at 1.5 hr	13.5	5.9	5.1	12.4
3μg phaseollin/ml at 0 hr and 27 μg phaseollin/ml at 1.5 hr	13.5	5.7	12.3	22.7

[a]Approximately 6 mg (dry wt) of the fungi were suspended in 4.0 ml of glucose-asparagine medium contained in 25 ml flasks. Samples were treated with phaseollin (3 μg/ml of medium) in dimethylsulfoxide (DMSO) or a comparable volume of DMSO and incubated at 24 ± 2 C on a reciprocal shaker (125 strokes/min) for 1.5 hr. Some samples then were treated with additional phaseollin (27 μg/ml medium) or DMSO. The final DMSO concentration for each treatment was 0.5%. Data are the averages of two replicates per treatment.

centrifuged for 5 min at about 1,100 *g*. The supernatant was decanted, 7.0 ml of GA medium was mixed with the pellet, and the tubes were recentrifuged. This process was repeated once more, and 1.0 ml of GA medium then was mixed with the pellet. A 0.1-ml aliquot was placed in the wells of depression slides, and percentage spore germination was recorded after about 18 hr at 24 ± 2 C.

Exposure to 27 μg of phaseollin/ml or greater for 1 min or more resulted in 100% mortality. Spores which had not germinated 18 hr after these treatments did not germinate after an additional 24-48 hr. The protoplasm of such spores appeared granulated and disorganized. In contrast, short-term exposures to pisatin (75 and 100 μg/ml) had little effect on spore viability. Spores swelled to about 1.5 times their original size after continuous exposure to pisatin for ≥24 hr, but they did not germinate. If spores exposed to pisatin for as long as 3 days then were washed as described above, most of the spores germinated within the next 18 hr.

Phytoalexin tolerance and metabolism of pisatin or phaseollin by Fusarium solani.—It has been demonstrated that *F. solani* f. sp. *pisi* can demethylate pisatin to DMDP (27) and that *F. solani* f. sp. *phaseoli* can oxidize phaseollin to la-hydroxyphaseollone (20, 21, 22). To test the ability of the *F. solani* isolates used in this study to metabolize phaseollin or pisatin, shake cultures of the fungi were prepared in the standard way and treated with a slightly inhibitory concentration of phytoalexins (31, 62, and 100 μg of pisatin/ml and 7, 27, and 27 μg of phaseollin/ml for *cucurbitae, phaseoli,* and *pisi,* respectively). The cultures were incubated for 24 hr, extracted and assayed for phytoalexin content. For each treatment two cultures were used to measure dry weight changes and two cultures to determine phytoalexin content.

After 24 hr no phaseollin was detected in the phaseollin-treated cultures of *phaseoli* and only a small amount of pisatin was detected in the pisatin-treated cultures of *pisi,* confirming previous reports (20, 21, 22, 27) that these formae speciales can metabolize phaseollin and pisatin, respectively. No metabolism of either phytoalexin was observed with the other combinations even though all cultures grew in the presence of the phytoalexins (dry weights at 24 hr ≥ 4 times the initial dry weight). Also, no metabolism of phaseollin by *pisi* was detected even when the fungus was pretreated by the procedure illustrated in Table 3 to enhance its tolerance to phaseollin.

The relative rates of phytoalexin metabolism and fungal growth might indicate whether the tolerance of *pisi* to pisatin and *phaseoli* to phaseollin depends on the ability of these isolates to metabolize these compounds. To determine whether fungal growth could occur before phytoalexin metabolism, cultures of these isolates were treated with pisatin or phaseollin and assayed for the phytoalexins and their metabolic products at a short interval after treatment. Dry weights were measured at the same time on identically treated cultures. Because a

TABLE 4. Effect of a pretreatment with a low concentration of pisatin on the response of *Fusarium solani* f. sp. *cucurbitae, F. solani* f. sp. *pisi,* and *F. solani* f. sp. *phaseoli* to a subsequent treatment with a higher concentration of pisatin in shake culture[a]

Treatment	Time assayed (hr)	Dry weight (mg/flask)		
		F. solani f. sp. *cucurbitae*	*F. solani* f. sp. *pisi*	*F. solani* f. sp. *phaseoli*
None	0	5.6	6.7	5.9
DMSO at 0 hr and DMSO at 1.5 hr	13.5	23.6	27.1	26.4
6 μg pisatin/ml at 0 hr and DMSO at 1.5 hr	13.5	21.1	26.6	25.6
DMSO at 0 hr and at 1.5 hr: 98 μg of pisatin/ml	13.5	9.3		19.0
or 155 μg of pisatin/ml	13.5		22.4	
6 μg pisatin/ml at 0 hr and at 1.5 hr: 98 μg of pisatin/ml	13.5	8.6		18.4
or 155 μg of pisatin/ml	13.5		21.0	

[a]Approximately 6 mg (dry wt) of the fungi were suspended in 4.0 ml of glucose-asparagine medium contained in 25-ml flasks. Samples were treated with pisatin (6 μg/ml of medium) in dimethylsulfoxide (DMSO) or a comparable volume of DMSO and incubated at 24 ± 2 C on a reciprocal shaker (125 strokes/min) for 1.5 hr. Some samples then were treated with additional pisatin (98 or 155 μg/ml of medium) in DMSO or DMSO. The final DMSO concentration for each treatment was 0.5% except for the 1.5-hr treatments of *F. solani* f. sp. *pisi* in which it was 2%. Data are the average of two replicates per treatment.

pretreatment with a low concentration of phaseollin enhanced the tolerance of *phaseoli* to higher concentrations of that compound (Table 3), the effect of such pretreatment on the metabolism of phaseollin also was measured. Growth of *pisi* occurred before much pisatin was metabolized (Table 5). However, no measurable increase in dry wt of *phaseoli* occurred in this assay before phaseollin was completely metabolized, whether or not the culture was pretreated with phaseollin (Table 5).

DISCUSSION

Several bioassay conditions were used in this study, yet the relatively high degree of sensitivity of *cucurbitae* to pisatin and phaseollin and the relatively high degree of tolerance of *pisi* to pisatin remained constant. Minor differences in the absolute amount of inhibition did occur occasionally, but because the differential response was observed regardless of the physical and chemical nature of the medium, growth stage tested, or phytoalexin exposure regime used, the difference appears to reflect intrinsic properties of the organisms and not just in vitro artifacts. Very high or low concentrations of the phytoalexins may obscure these differences, and it is probable that there is some bioassay condition that can mask the differential effect. At the highest concentrations of pisatin normally used (100 μg/ml), the growth of *pisi* was slightly inhibited and at concentrations greater than 200 μg/ml the growth of the organism in shake culture could be completely inhibited for 24 hr. This implies that a pisatin sensitive site (or sites) exists in this organism.

The response of *phaseoli* to phaseollin and pisatin and the response of *pisi* to phaseollin varied depending on the bioassay method used. Forma specialis *phaseoli* appears sensitive to pisatin in radial growth bioassays (Fig. 1) but highly tolerant in shake culture and spore germination bioassays (Fig. 2 and Table 1). The response of *phaseoli* and *pisi* to phaseollin was influenced by whether the organisms had been pretreated with a low concentration of phaseollin (Table 3). In addition, when the spore germination bioassay or the liquid shake culture bioassay was employed, results for these isolates differed in replicate experiments (Fig. 3 and Table 1).

The reason for the variation in responses is not completely known, but differences in both the need for a period of adaptation for development of tolerance and the type of antifungal activity (fungicidal vs. fungistatic) may play a role. Phaseollin can exert a rapid fungicidal effect [(14, 24) and this study]. Both *pisi* and *phaseoli* have an apparent adaptive tolerance of phaseollin. This suggestion is based primarily on the fact that a short pretreatment with a low level of phaseollin markedly enhanced their tolerance to higher phaseollin concentrations (Table 3). Bioassaying an organism with adaptive tolerance under conditions which do not allow enough cells (because of biomass or physiological state) to survive the early effects of phaseollin could delay development of tolerance and make the organism appear sensitive to the phytoalexin (14).

The tolerance of *pisi* to pisatin may be a constitutive property of the organism because attempts to enhance its high level of tolerance to pisatin were not successful (Table 4). A constitutive tolerance and/or the observation that pisatin was not markedly fungicidal may explain the consistency of the response of this isolate to pisatin in various bioassay conditions. However, we cannot explain the variability in response of *phaseoli* to

TABLE 5. Metabolism of phaseollin by *Fusarium solani* f. sp. *phaseoli* and metabolism of pisatin by *F. solani* f. sp. *pisi* in relation to growth[a]

Fungus	Phytoalexin treatment	Time assayed (hr)	Dry weight[b] (mg/flask)	Phytoalexin recovered corrected for extraction efficiency[c] (%)	Phytoalexin metabolite (μg/ml)
F. solani f. sp. *pisi*	98 μg of pisatin/ml at 0 hr	0	6.4		
		6	9.0 (11.4)	95 (63)	1[d]
F. solani f. sp. *phaseoli*	13 μg of phaseollin/ml at 0 hr	0	5.8		
		1.5	5.1 (5.9)	0 (49)	0[e]
F. solani f. sp. *phaseoli*	4 μg of phaseollin/ml at 0 hr, 27 μg of phaseollin/ml at 1.5 hr	0	6.6		
		3.0	6.9 (8.0)	0 (52)	15[e]

[a]Approximately 6 mg (dry wt) of the fungi were suspended in 4.0 ml of glucose-asparagine medium in 25-ml flasks. Phytoalexins in dimethylsulfoxide (DMSO) were added to the cultures (final DMSO = 0.5%) and samples incubated at 24 ± 2 C on a reciprocal shaker (125 strokes/min). Two flasks per treatment were used to measure dry weight changes and two were used to determine phytoalexin content. Data are the averages of two samples.

[b]Values in parentheses are the dry weights of control flasks containing 0.5% DMSO.

[c]Values in parentheses are the average of the percent phytoalexin recovered in the efficiency controls.

[d]Phytoalexin metabolite was 3,6a-dihydroxy-8,9-methylenedioxypterocarpan.

[e]Phytoalexin metabolite was la-hydroxyphaseollone.

pisatin. There are obviously factors other than those mentioned above which influence the response of these fungi to pisatin and phaseollin in vitro.

Results of this study support the suggestion (26, 27) that metabolism of phytoalexins does not alone explain tolerance in some fungi. In shake culture, substantial growth of *pisi* occurred before a large amount of the pisatin was metabolized to DMDP (Table 5). Forma specialis *phaseoli* was tolerant of pisatin in shake culture but did not metabolize pisatin and *pisi* did not metabolize phaseollin, yet prior exposure to a low concentration of phaseollin resulted in increased tolerance to a higher concentration applied later. Metabolism of phaseollin by *F. solani* f. sp. *phaseoli* can be enhanced by a phaseollin pretreatment (21), but our data (Table 5) do not clarify whether enhanced tolerance depends on enhanced metabolism.

Even though only one representative isolate of each *forma specialis* of *F. solani* was used in this study, the results clearly indicate that some fungi can tolerate different levels of phytoalexins in vitro. If the underlying physiological bases for tolerance (or sensitivity) and its regulation were known, they undoubtedly would help explain why bioassay conditions can influence the sensitivity of fungi to phytoalexins. Such information also could help determine whether this tolerance is expressed during and/or is needed for pathogenesis.

LITERATURE CITED

1. BAILEY, J. A., and R. S. BURDEN. 1973. Biochemical changes and phytoalexin accumulation in Phaseolus vulgaris following cellular browning caused by tobacco necrosis virus. Physiol. Plant Pathol. 3:171-177.
2. BAILEY, J. A., G. A. CARTER, and R. A. SKIPP. 1976. The use and interpretation of bioassays for fungitoxicity of phytoalexins in agar media. Physiol. Plant Pathol. 8:189-194.
3. BEADLE, G. W., and E. L. TATUM. 1945. Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Bot. 32:678-686.
4. CHRISTENSON, J. A., and L. A. HADWIGER. 1973. Induction of pisatin formation in pea foot region by pathogenic and nonpathogenic clones of Fusarium solani. Phytopathology 63:784-790.
5. CRUICKSHANK, I. A. M. 1962. Studies on phytoalexins. IV. The antimicrobial spectrum of pisatin. Aust. J. Biol. Sci. 15:147-159.
6. CRUICKSHANK, I. A. M. 1965. Phytoalexins in the leguminosae with special reference to their selective toxicity. Tagungsber. Dtsch. Akad. Landwirtschaft-swiss. Berlin 74:313-332.
7. CRUICKSHANK, I. A. M., and D. R. PERRIN. 1971. Studies on phytoalexins. XI. The induction, antimicrobial spectrum and chemical assay of phaseollin. Phytopathol. Z. 70:209-229.
8. DEVERALL, B. J., and P. M. ROGERS. 1972. The effect of pH and composition of test solutions on the inhibitory activity of wyerone acid towards germination of fungal spores. Ann. Appl. Biol. 72:301-305.
9. DE WIT-ELSHOVE, A., and A. FUCHS. 1971. The influence of the carbohydrate source on pisatin breadkown by fungi pathogenic to pea (Pisum sativum). Physiol. Plant Pathol. 1:17-24.
10. NONAKA, F. 1967. Inactivation of pisatin by pathogenic fungi. Agric. Bull. Saga. Univ. (Japn.) 24:109-121.
11. PERRIN, D. R., and W. BOTTOMLEY. 1962. Studies on phytoalexins. V. The structure of pisatin from Pisum sativum L. J. Am. Chem. Soc. 84:1919-1922.
12. PUEPPKE, S. G., and H. D. VANETTEN. 1974. Pisatin accumulation and lesion development in peas infected with Aphanomyces euteiches, Fusarium solani f. sp. pisi, or Rhizoctonia solani. Phytopathology 64:1433-1440.
13. PUEPPKE, S. G., and H. D. VANETTEN. 1976. Accumulation of pisatin and three additional antifungal pterocarpans in Fusarium solani-infected tissues of Pisum sativum. Physiol. Plant Pathol. 8:51-61.
14. SKIPP, R. A., and J. A. BAILEY. 1976. The effect of phaseollin on the growth of Colletotrichum lindemuthianum in bioassays designed to measure fungitoxicity Physiol. Plant Pathol. 9:253-263.
15. SKIPP, R. A., and J. A. BAILEY. 1977. The fungitoxicity of isoflavanoid phytoalexins measured using different types of bioassay. Physiol. Plant Pathol. 11:101-113.
16. SMITH, D. A., H. D. VANETTEN, and D. F. BATEMAN. 1975. Accumulation of phytoalexins in Phaseolus vulgaris hypocotyls following infections by Rhizoctonia solani. Physiol. Plant Pathol. 5:51-64.
17. STEINBERG, R. A. 1950. Growth on synthetic nutrient solution of some fungi pathogenic to tobacco. Am. J. Bot. 37:711-714.
18. STEVENS, R. B., ed. 1974. Mycology guidebook. University of Washington Press, Seattle and London, 703 p.
19. TOUSSOUN, T. A., and P. E. NELSON. 1976. A pictorial guide to the identification of Fusarium species. 2nd edition. Pennsylvania State University Press, University Park and London, 43 p.
20. VAN DEN HEUVEL, J., and J. A. GLAZENER. 1975. Comparative abilities of fungi pathogenic and nonpathogenic to bean (Phaseolus vulgaris) to metabolize phaseollin. Neth. J. Plant Pathol. 81:125-137.
21. VAN DEN HEUVEL, J., and H. D. VANETTEN. 1973. Detoxification of phaseollin by Fusarium solani f. sp. phaseoli. Physiol. Plant Pathol. 3:327-339.
22. VAN DEN HEUVEL, J., H. D. VANETTEN, J. W. SERUM, D. L. COFFEN, and T. H. WILLIAMS. 1974. Identification of la-hydroxyphaseollone, a phaseollin metabolite produced by Fusarium solani. Phytochemistry 13:1129-1131.
23. VANETTEN, H. D. 1973. Differential sensitivity of fungi to pisatin and to phaseollin. Phytopathology 63:1477-1482.
24. VANETTEN, H. D. 1976. Antifungal activity of pterocarpans and other selected isoflavonoids. Phytochemistry 15:655-659.
25. VANETTEN, H. D., and D. F. BATEMAN. 1971. Studies on the mode of action of the phytoalexin phaseollin. Phytopatholgy 61:1363-1372.
26. VANETTEN, H. D., and S. G. PUEPPKE. 1976. Isoflavonoid phytoalexins. Pages 239-289 *in* J. Friend and D. R. Threlfall, eds. Biochemical aspects of plant parasitic relationships. Annu. Proc. Phytochem. Soc. 13:239-289.
27. VANETTEN, H. D., S. G. PUEPPKE, and T. C. KELSEY. 1975. 3,6a-Dihydroxy-8,9-methylenedioxypterocarpan as a metabolite of pisatin produced by Fusarium solani f. sp. pisi. Phytochemistry 14:1103-1105.
28. VANETTEN, H. D., and D. A. SMITH. 1975. Accumulation of antifungal isoflavonoids and la-hydroxyphaseollone, a phaseollin metabolite, in bean tissue infected with Fusarium solani f. sp. phaseoli. Physiol. Plant Pathol. 5:225-237.