Pisatin Accumulation and Lesion Development in Peas Infected with Aphanomyces euteiches, Fusarium solani f. sp. pisi, or Rhizoctonia solani

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ABSTRACT

Pea seedlings were inoculated with the root and/or epicotyl-rotting pathogens *Aphanomyces euteiches, Fusarium solani* f. sp. *pisi* or *Rhizoctonia solani*. At given time intervals after inoculation, lesions were excised, and their volumes and pisatin contents were determined. *A. euteiches* lesions expanded rapidly for 5 days, although by 36 h after inoculation pisatin concn in infected tissue was eight times greater than that required to completely prevent growth of the pathogen in vitro. Pisatin concn in lesion tissue decreased after 36 h, yet the quantity of pisatin per lesion increased as the lesion enlarged. Although margins of older lesions usually extended beyond the hyphal front, pisatin was almost uniformly distributed throughout colonized and uncolonized tissue. In *Rhizoctonia*-infected tissue, the amount of pisatin per lesion, pisatin concn in the lesions, and lesion volume increased coincidentally, reaching plateaus 6 days after inoculation. Pisatin concn in 2-day-old *Rhizoctonia* lesions was considerably greater than quantities which were inhibitory in vitro, yet the lesions continued to expand. In *F. solani* f. sp. *pisi* lesions, pisatin concns of up to 5 mg/cm^3 were found; however, the pathogen was insensitive to pisatin in vitro.

The presence of substantial quantities of pisatin in the young expanding lesions containing the sensitive pathogen *A. euteiches* has not been reconciled with the concept of pisatin as a disease resistance factor in peas.

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Additional key words: phytoalexins, pterocarpans, isoflavonoids, antifungal compounds.

Müller (20) defined phytoalexins as antibiotics which result from the interaction of the metabolic systems of host and parasite. In the light of more recent research, this concept has been considerably expanded and modified (5, 6, 8, 17). A number of compounds, including the pterocarpan pisatin from pea (*Pisum sativum* L.), have been classified as phytoalexins. Although the role of pisatin as a resistance factor in pea has not been conclusively demonstrated, several lines of evidence have been brought forward to support this contention. In general, fungal pathogens of pea are more tolerant of pisatin than nonpathogens of pea (4, 27). In addition, pisatin is thought to accumulate to fungistatic levels when resistance is expressed but does not accumulate to such levels in susceptible reactions (7).

Only rarely, however, have natural infection sites been quantitatively examined for pisatin content (3, 11) and no correlations of pisatin accumulation with lesion volume increases have been published. Also, there are exceptions to the general pattern of fungal response to pisatin; some pea pathogens appear to be sensitive to pisatin (4, 27). *Aphanomyces euteiches* Drechs., a stem- and root-rotting pathogen of pea, is such an organism (27). Two other fungi, *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F. R. Jones) Snyd. & Hans. and *Rhizoctonia solani* Kühn, which will also infect the stem area of pea, are reported to be tolerant of pisatin (3, 4, 27). The types of lesions that these three organisms cause on pea are different. Both *A. euteiches* and *F. solani* f. sp. *pisi* produce spreading lesions, while *R. solani* forms what appear to be restricted lesions on pea stems.

The objectives of the present research were (i) to reexamine the effect of pisatin on some of the fungi that previously have been bioassayed against pisatin, and (ii) to accurately quantify disease development and pisatin concn in pea stem tissue infected with the above three pea pathogens. Definite patterns of pisatin accumulation, disease development, and pathogen tolerance to pisatin would be expected if pisatin production is the primary factor responsible for disease resistance in peas.

MATERIALS AND METHODS.—*Cultures.*—*Aphanomyces euteiches* Drechs. Race 1 was provided by Dr. J. L. Lockwood, Michigan State University. *Ascochyta pinodella* L. K. Jones and *Mycosphaerella pinodes* (Berk. & Blox.) Vest. were supplied by V. R. Wallen, Cell Biology Research Institute, Central Experiment Farm, Ottawa, Ontario, Canada. *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F. R. Jones) Snyd. & Hans. isolate 87 was furnished by W. T. Schroeder, N.Y. State Agricultural Experiment Station, Geneva. *Botrytis cinerea* Pers. ex Fr., *Colletotrichum graminicolum* (Ces.) G. W. Wils., *Fusarium oxysporum* Schl. f. sp. *lycopersici* (Sacc.) Snyd. & Hans., *Rhizoctonia solani* Kühn, *Thamnidium elegans* Lk. and *Thielaviopsis basicola* (Berk. & Br.) Ferr. were obtained from Cornell University's stock cultures.

Fungi described as pea pathogens were tested for pathogenicity on pea or were pathogenic cultures obtained from other workers. Fungi described as nonpathogens of pea are species not known to be pathogenic to pea or are isolates tested and demonstrated to be nonpathogenic to pea.

Bioassays.—The method of pisatin preparation has been reported elsewhere (27). Pisatin in ethanol (final ethanol concn in the medium was 2%) was added to 1-ml aliquots of Martin's peptone-glucose agar (PGA) (19) [10.0 g glucose, 5.0 g peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO{\cdot}7H_2O$, 22 g agar in 1,000 ml H_2O] as previously described (27). Each petri plate was seeded in the center with an agar plug (2.0 mm in diam) of the test fungus and incubated in the dark for 48 h at 24 ± 2 C. Radial growth was determined by averaging two perpendicular diam of replicate colonies after subtracting the diam of the inoculum disks. Variation between replicates was rarely greater than 10%.

The radial growth of *A. euteiches* was also determined using dimethylsulfoxide (DMSO), instead of ethanol, to maintain pisatin in solution (final DMSO concn in the medium was 1.5%). At each pisatin concn *A. euteiches* was bioassayed in quadruplicate and 95% confidence intervals were established.

The effect of pisatin on the growth of *A. euteiches* in liquid culture was determined in the following manner. Mycelial disks, 7.0 mm in diam, were cut with a cork borer from *A. euteiches* colonies growing on the surface of bacterial filters (Triacetate Metricel, 0.2 μm pore size, Gelman Instrument Co., Ann Arbor, Mich.) resting on PGA medium. Four disks were placed in 4.0 ml of modified Yang and Schoulties growth medium (31) [9 g glucose, 8 g Bacto asparagine (Difco Labs, Detroit, Mich.), 0.1 g glutathione, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.7 g KH_2PO_4, 0.4 g K_2HPO_4 in 1,000 ml H_2O] contained in 25-ml Erlenmeyer flasks. The pH was adjusted to 6.4 with 1.0 N KOH or 1.0 N HCl. The flasks were incubated at 24 ± 2 C on a reciprocal shaker operating at 50 strokes/min. After 24 h the dry wt of *A. euteiches* per flask was ca. 3 mg. At this time pisatin in ethanol was added to the flasks (final ethanol concn in the medium was 2%), and the cultures were incubated for an additional 72 h. Mycelium was collected on tared Whatman #50 filter paper by vacuum filtration and mycelial dry wts were recorded after a 24 h exposure to 75 C. Three experiments, of three replications each, were performed. Significant differences were determined by analysis of variance of three separate experiments, each containing three replicates per treatment.

Production of diseased tissue.—Undamaged pea seeds (*Pisum sativum* L. 'Progress No. 9') were rinsed three times in water prior to immersion in a solution of 0.5% sodium hypochlorite and 48% ethanol. After 10 min, the liquid was decanted and the seeds were rinsed three times in water. To produce stem tissue infected with *F. solani* f. sp. *pisi* and *A. euteiches*, 25-ml Erlenmeyer flasks were loosely packed with vermiculite and filled with 18 ml of Hoagland's nutrient solution (15). The flasks were autoclaved for 10 min (120 C, 1.0 atm); one seed was placed into the neck of each flask and was covered with autoclaved vermiculite. The flasks were incubated in the dark at ca. 100% relative humidity (RH) in controlled environment chambers for 5-7 days. Plants which were to be inoculated with *A. euteiches* were maintained at 20 C, while those that were to be inoculated with *F. solani* f. sp. *pisi* were kept at 29 C. When the seedlings protruded ca. 2 cm above the lips of the flasks they were exposed to 21,500 lx of illumination (14 h daylength) and inoculated 24 h later. Inoculum consisted of 2-mm (diam) plugs cut from the advancing edges of cultures maintained on PGA. The

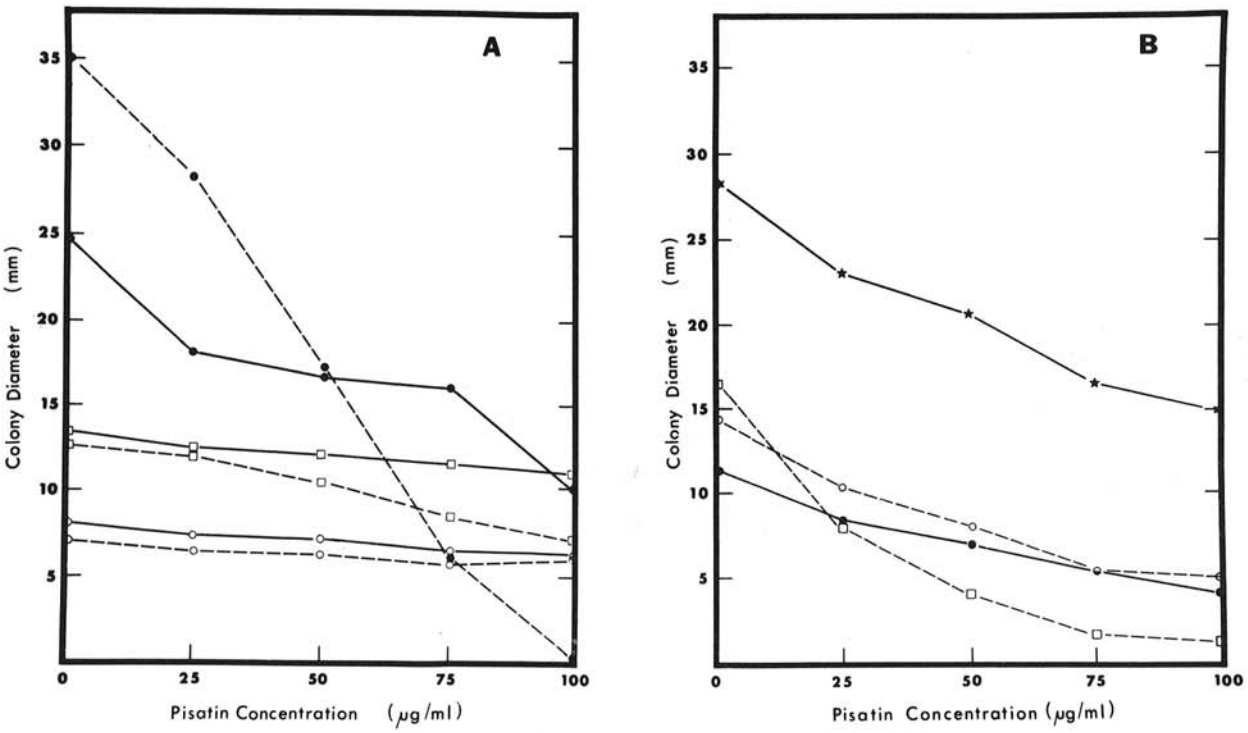

Fig. 1-(A, B). Effect of pisatin on the radial mycelial growth of pathogens and nonpathogens of pea. Measurements were made 48 h after a 2.0-mm (diam) agar plug containing the test fungus was placed on 1.0 ml of Martin's peptone-glucose agar to which pisatin had been added. The final ethanol concn in all treatments was 2.0%. Growth was calculated by measuring two perpendicular diam of each of three replicate colonies, and subtracting the diam of the inoculum plugs. Approximate ED_{50} values (μg/ml) were calculated from the dosage-response curves. **A)** Pathogens: (●- - -●) *Aphanomyces euteiches* (ED_{50} ~ 40); (□- - -□) *Ascochyta pinodella* (ED_{50} ~ 100); (□——□) *Fusarium solani* f. sp. *pisi* (ED_{50} > 100); (○——○) *Mycosphaerella pinodes* (ED_{50} > 100); (●——●) *Rhizoctonia solani* (ED_{50} ~ 90); (○ - - ○) *Theilaviopsis basicola* (ED_{50} > 100). **B)** Nonpathogens: (* ——*) *Botrytis cinerea* (ED_{50} ~ 100); (○- - - -○) *Colletotricum graminicolum* (ED_{50} ~ 50); (●——●) *Fusarium oxysporum* f. sp. *lycopersici* (ED_{50} ~ 75); (□- -□) *Thamnidium elegans* (ED_{50} ~ 25).

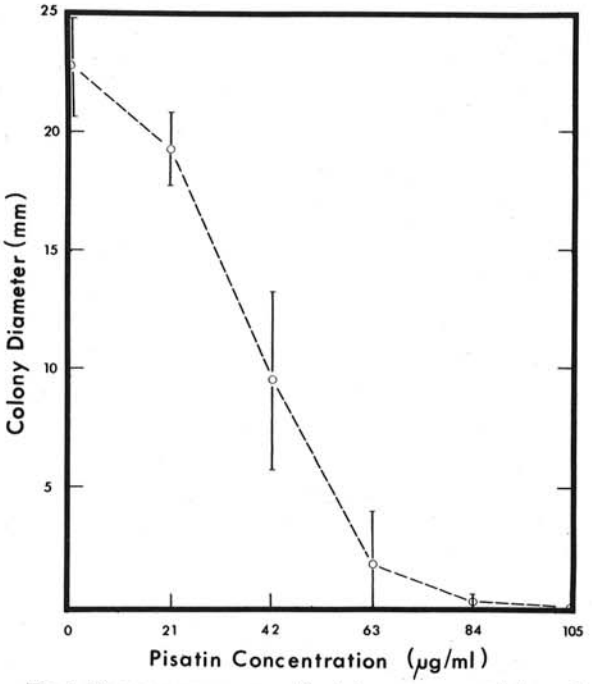

Fig. 2. Dosage-response curve for *Aphanomyces euteiches* and pisatin. Pisatin was dissolved in dimethylsulfoxide (DMSO) and added to Martin's peptone-glucose agar to give a final concn of 1.5% DMSO. Controls contained 1.5% DMSO. Measurements were made 48 h after a 2.0 mm (diam) agar plug containing *A. euteiches* was placed on the medium. Growth was calculated by measuring two perpendicular diam of each of four replicate colonies and subtracting the diam of the inoculum plugs. Confidence intervals ($\alpha = 0.05$) are indicated by vertical bars.

epidermis of each seedling epicotyl (ca. 1.5 cm above the lip of the flask) was punctured with a sterile dissecting needle and an inoculum plug was placed on the wound. The plants were then maintained under the same light, RH, and temp regimes as above.

Attempts to obtain reproducible stem lesions with *R. solani* using the above procedure were unsuccessful, so an alternate method was used. Surface sterilized seeds were planted in autoclaved pans of vermiculite (24 × 40 × 5 cm deep), irrigated with Hoagland's nutrient solution, and maintained at 29 C for 5-7 days before inoculating. Inoculum was prepared by growing *R. solani* in 125-ml Erlenmeyer flasks containing 25 ml of a medium composed of 20 g glucose, 4.6 g casein hydrolysate, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 10 mg thiamine hydrochloride, 1.0 ml of Steinberg's microelement solution (23), and 1,000 ml H_2O. After 7 days, the medium was decanted, and the mycelial mats were homogenized in distilled water for 30 sec using a Waring Blendor. The resulting suspension was diluted to 125 ml/mat and added to the pans of seedlings at the rate of 250 ml/pan. The plants were maintained at 29 C.

Extraction and quantification of pisatin.—Under the above conditions, one discrete lesion per plant formed at the inoculation site when the plant was challenged with *A. euteiches* or *F. solani* f. sp. *pisi*. Pea plants infected with *R. solani* contained one to eight lesions per plant. However, only one *R. solani* lesion per plant was analyzed.

The lesion proper, and an area approximately 2 mm in width surrounding each lesion, were separately excised with a scalpel at various time intervals, placed in tared weighing vials, frozen, lyophilized, and weighed. Uninoculated healthy or wounded epicotyl tissue was treated in a similar manner. Each sample containing nine to 59 lesions (2.3 - 232 mg dry wt) or its equivalent in healthy tissue was suspended in 10.0 ml of ethyl acetate + methanol (9:1, v/v) contained in a 15-ml glass vial. The suspension was agitated for 24 h on a rotary shaker (180 rpm). The insoluble plant debris was collected by vacuum filtration, resuspended in 10.0 ml of solvent and shaken for an additional 24 h. After the second extraction, the suspension was again filtered. The plant debris was resuspended in a small (<1.0 ml) volume of solvent, pulverized with a mortar and pestle, and filtered. This filtrate was pooled with the two previous filtrates and taken to dryness in vacuo. The residue was dissolved in 2.0 ml of 95% ethanol and stored at −16 C until analyzed. Known quantities of pure pisatin were added to lyophilized healthy epicotyls and extracted in the above manner to test the efficiency of the extraction procedure.

Pisatin content of the samples was determined by thin-layer chromatography (TLC). Samples were streaked on 20 × 20 cm analytical plates, which had been precoated with a silica gel layer (0.25-mm thick) containing a fluorescence indicator (Polygram Sil G/UV_{254}, Brinkmann Instruments, or Silica Gel with fluorescence indicator 13181, Eastman Kodak Company). Known quantities of a pisatin standard were spotted on each plate. These aided in locating pisatin in the samples and served as a means to correct for the efficiency of the TLC procedure. Plates streaked with extracts of *A. euteiches* or *R. solani*-infected tissue were developed in hexane + ethyl acetate + methanol (60:40:1, v/v).

TABLE 1. Effect of pisatin on the growth of *Aphanomyces euteiches* in liquid medium

Treatment[a]	Dry weight (mg/flask)[b]		
	Experiment 1	Experiment 2	Experiment 3
Time 0	2.3	3.5	3.3
50 μg pisatin/ml	4.6	3.1	2.5
100 μg pisatin/ml	1.2	1.5	3.2
Control	10.4**	10.9**	11.2**

[a]Dry weights of the Time 0 cultures were taken after incubating at 50 strokes per min for 24 h at 25 C in 4.0 ml of modified Yang and Schoulties (31) growth medium. All other cultures were incubated for an additional 72 h in the presence of pisatin + 2% ethanol or 2% ethanol (control).

[b]Each value is the mean of three replicates. ** indicates significant difference [LSD ($P = 0.01$) = 3.7].

Extracts of *F. solani* f. sp. *pisi*-infected tissue contained several compounds that migrated coincident with pisatin (R_f = 0.43) in the above solvent system. Pisatin in these extracts was separated from such compounds by employing benzene + ethyl acetate + isopropanol (90:10:1, v/v) as the initial solvent and rechromatographing the pisatin area (R_f = 0.68) in toluene + ethyl acetate (9:1, v/v), (R_f of pisatin = 0.47).

After TLC, pisatin-containing areas were located with short wavelength ultraviolet illumination as quench areas at the R_f values adjacent to the pisatin standards. The silica gel in these areas was scraped from the plates and eluted with 15 ml of chloroform. Silica gel was removed by filtration through glass wool. The samples were taken to dryness in vacuo and redissolved in 2 ml of 95% ethanol. Pisatin concn was determined by ultraviolet spectrophotometry using a Bausch and Lomb Spectronic UV200 double-beam recording spectrophotometer and the reported molar extinction coefficient of pisatin (log ϵ = 3.86 at 309 nm in ethanol) (22). Extracts of unstreaked control plates were analyzed to determine the absorbance due to other substances from the silica gel, and the pisatin values were corrected for this absorbance.

Demethylpterocarpin, an antifungal pterocarpan with spectrometric properties similar to those of pisatin, has been extracted from *Monilinia fructicola*-infected pea pods (24). To determine if demethylpterocarpin contaminated our pisatin samples, randomly-selected lesion extracts from all three pathogen-suscept systems were analyzed by TLC using hexane + diethyl ether (1:1, v/v) as a solvent system. A demethylpterocarpin sample was kindly supplied by V. Higgins. Silica gel at R_f values adjacent to demethylpterocarpin standards (R_f demethylpterocarpin = 0.38, R_f pisatin = 0.31) did not contain detectable amounts of demethylpterocarpin, as determined by ultraviolet spectrometry (1). When randomly-selected extracts were subjected to gas-liquid chromatography, using the system described by Keen et al. (16), peaks were not observed at the retention time (R_t) of the demethylpterocarpin standard (R= 0.34 relative to catechin).

Volume determination of lesion samples.—A standard equation was prepared to allow rapid calculation of the volume of lesion samples from dry wt data. The volume of 11 separate samples of healthy epicotyls (0.10 to 0.75 cm^3 each) was determined by water displacement. The samples were transferred to tared weighing vials and frozen. After lyophilization, the samples were weighed and a linear regression analysis of the volume to dry wt data was performed. The relationship was Volume (cm^3) = $-0.0133 + 0.0139 \times$ dry wt (mg); this regression was significant at $\alpha = 0.001$.

RESULTS.—*Effect of pisatin in vitro on pathogens and nonpathogens of pea.*—The effect of pisatin on the radial growth of six pathogens and four nonpathogens of pea is shown in Fig. 1. The concn required for 50% inhibition of growth (ED_{50}) was calculated from the dosage-response curves. For nonpathogens, the ED_{50} values ranged from 25 μg/ml of medium for *Thamnidium elegans* to ca. 100 μg/ml for *Botrytis cinerea*. Pathogens also varied in their sensitivity, but with the exception of *A. euteiches*, ED_{50} values for pathogens were 90 μg/ml or greater. Growth of *Mycosphaerella pinodes*, *F. solani* f. sp. *pisi*, and *T. basicola*, was unaffected, even at the highest pisatin concn. Although the ED_{50} value of *A. euteiches* is about 40 μg/ml, it was the only organism completely inhibited by 100 μg/ml. Similar results with *A. euteiches* were obtained when DMSO was substituted for ethanol as a solubilizing agent (Fig. 2). Under these conditions, radial growth inhibition was greater than 90% at only 63 μg of pisatin per ml and no growth occurred at 105 μg/ml.

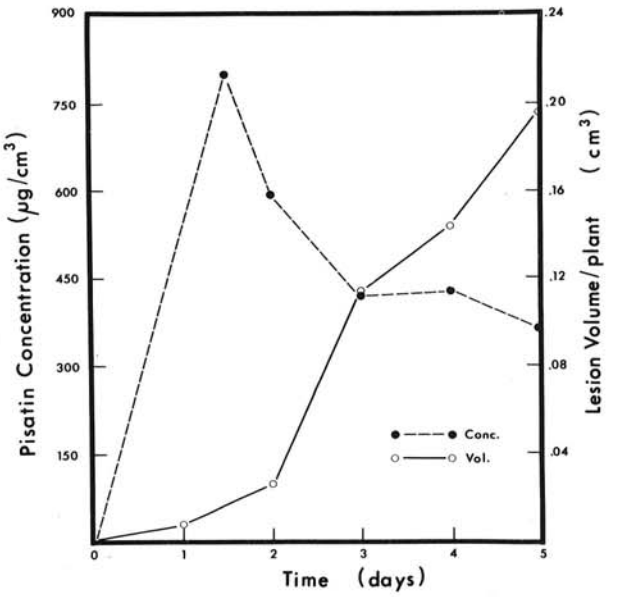

Fig. 3. Pisatin concn in the lesions and lesion volume per plant in pea epicotyls inoculated with *Aphanomyces euteiches*. Sample size varied from 9 to 41 plants, and each point is the mean of the values from two separate experiments.

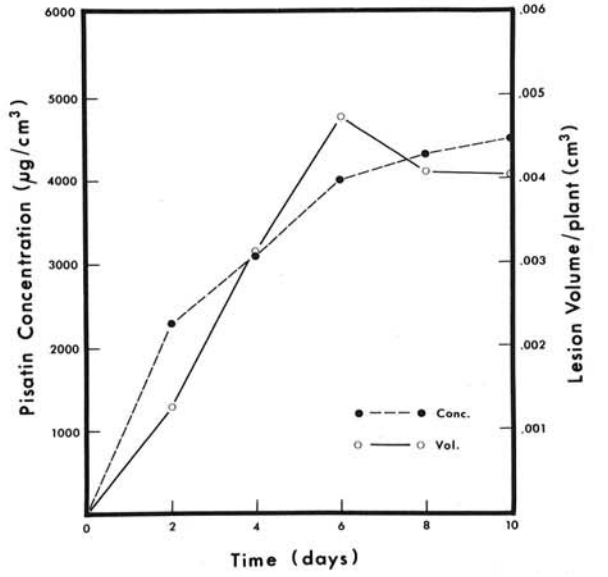

Fig. 4. Lesion volume per plant and pisatin concn per lesion in pea epicotyls inoculated with *Rhizoctonia solani*. Sample size varied from 26 to 59 plants and each point is the mean of the values from two separate experiments.

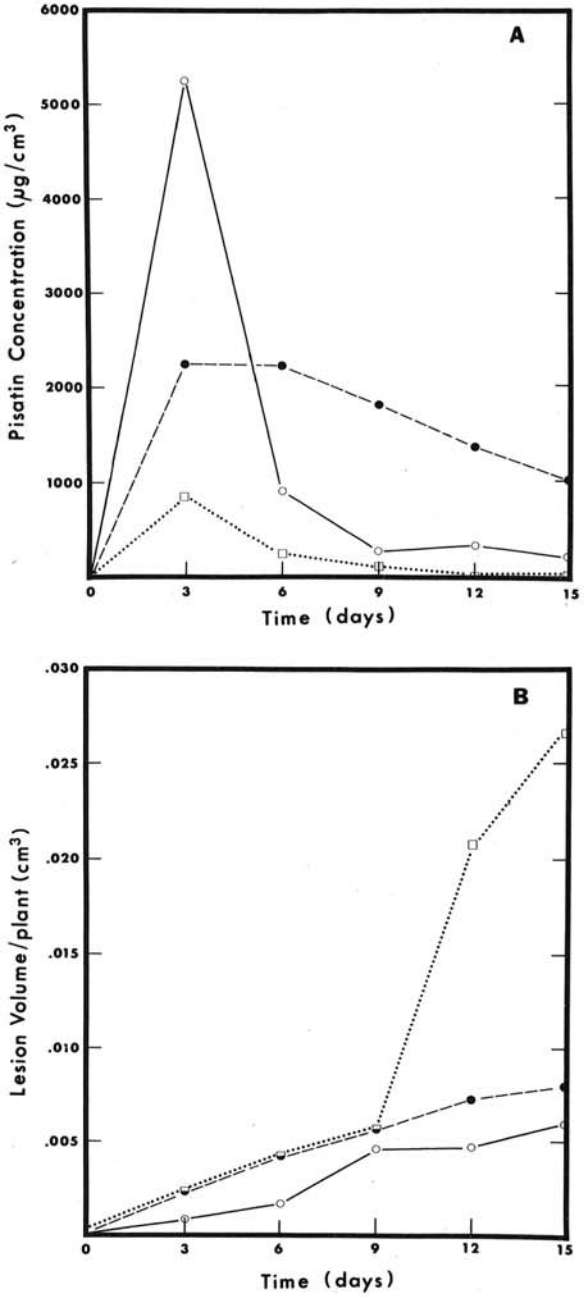

Fig. 5-(A, B). **A)** Pisatin concn in the lesions, and **B)** lesion volume per plant in pea epicotyls inoculated with *Fusarium solani* f. sp. *pisi*. The results of three replicate experiments are plotted as dashed, dotted and solid lines. Each line indicates data from a single experiment. Sample size varied from 10 to 22 plants.

The effect of pisatin on the dry wt of *A. euteiches* growing in liquid medium is shown in Table 1. Dry wt decreases were consistently recorded 3 days after exposure to 100 μg of pisatin/ml. Although at 50 μg/ml either slight losses or slight gains in dry wt were observed, statistical analysis indicates no significant growth differences between the time 0 controls and any of the pisatin treatments [LSD (P = 0.01) = 3.7]. All of the bioassay data are in agreement that the organism does not grow in concns of pisatin greater than 100 μg/ml of medium.

Pisatin concentration in pea epicotyls during lesion development.—The changes with time in *A. euteiches* lesion volume per plant and pisatin concn in these lesions are represented in Fig. 3. Pisatin concn reached a maximum of nearly 800 μg/cm³ of lesion 36 h after inoculation, when the lesions were small, slightly shrunken areas. As the epicotyl was enveloped, lesion volume increased dramatically to a maximum of about 0.2 cm³ per plant. Although a decrease in pisatin concn accompanied lesion volume increase, pisatin concn remained well above that which completely prevents in vitro growth of *A. euteiches*. The 2 mm area of tissue surrounding young lesions yielded only small quantities of pisatin, but those of 4- and 5-day-old lesions produced up to 236 μg/cm³.

Mycelial growth of *A. euteiches* in diseased epicotyls was determined by examining tissue sections (25- to 40-μm thick) prepared with a freezing or fresh-tissue microtome, and stained with cotton blue. At 36 h after inoculation the lesions appeared as distinct areas of discolored cortical tissue, which were colonized by the pathogen. After another 12 h, the mycelium was ramified in the degenerating tissue and often crowded into host cells. Tissue colonization did not keep pace with lesion expansion. Within 5 days after inoculation, single homogeneous-appearing lesions encompassed the area from the lowest leaves to the cotyledons, yet only the tissue extending from the point of inoculation to about half-way to the lesion margins was extensively colonized. However, hyphae occasionally were present in the area of the epidermis near the advancing edge of 4- to 5-day-old lesions. Since each plant contained a single lesion, younger and older halves of 5-day-old lesions could be separated and independently assayed for pisatin. A concn of 406 μg of pisatin/cm³ occurred in the extensively colonized tissue as compared to 283 μg/cm³ in the younger lesion areas. Pisatin accumulation in epicotyl lesions did not prevent *A. euteiches* from growing into the roots. By 10 days after inoculation, roots were extensively colonized and many oospores were observed in the root tips.

Lesion volume and pisatin concn increased similarly in *Rhizoctonia*-infected tissue (Fig. 4). Leveling off of lesion volume was associated with a change in the lesions from a yellow, translucent, hydrotic state to a dried, sunken, brown condition. Pisatin concn reached a plateau six days after inoculation and constituted 6-7% of the dry wt of 6-, 8-, and 10-day-old lesions. Although the maximum pisatin concn corresponded to both restriction of lesion growth and changes in the appearance of infected tissue, pisatin levels 2 and 4 days after inoculation were considerably greater than the concn required to cause 50% inhibition of radial growth in vitro. Pisatin concn in the 2 mm area of tissue surrounding the lesions was never more than 15% of that found in the lesions.

F. solani f. sp. *pisi* lesion volume increased, and pisatin concn generally decreased, from 3 to 15 days after inoculation (Fig. 5). Since variation in the data points from the three replicate experiments was often substantial, results of the individual experiments are

plotted. Only trace amounts of pisatin were detected in the 2-mm-wide area of tissue surrounding the lesions.

The major contaminating compound in the pisatin extracts from *F. solani* f. sp. *pisi*-infected tissue migrated to $R_f = 0.40$ in the toluene + ethyl acetate TLC system. The ultraviolet absorption spectrum of this compound exhibited a single peak at 286 nm with a shoulder at 291 nm. Four test fungi were exposed to the compound for 48 h at a concn of 0.9 absorbance units at 286 nm per ml of medium. The compound inhibited the radial growth of *A. euteiches* 35%, *F. oxysporum* f. sp. *lycopersici* 38%, *F. solani* f. sp. *pisi* 16%, and *R. solani* 19%. It was detected only in *Fusarium*-infected tissue. Structure determination and biological activity studies are in progress.

The data from each pathogen-suscept system were subjected to least squares regression analysis to determine whether the total quantity of pisatin per lesion, rather than the pisatin concn in the lesions, changed with time. A linear increase with time in pisatin per *A. euteiches* lesion, and a quadratic increase in *R. solani* lesions was observed (Table 2). For *F. solani* f. sp. *pisi* there were no significant changes from 3 to 15 days after inoculation.

TABLE 2. Accumulation of pisatin in lesions on pea epicotyls inoculated with *Aphanomyces euteiches, Fusarium solani* f. sp. *pisi,* or *Rhizoctonia solani*[a]

A. euteiches[b]		*F. solani* f. sp. *pisi*[c]		*R. solani*[b]	
Time (days)	Pisatin/lesion[d] (μg)	Time (days)	Pisatin/lesion (μg)	Time (days)	Pisatin/lesion[d] (μg)
1.5	10.6	3	4.1	2	3.1
2	13.9	6	3.8	4	10.1
3	47.8	9	4.7	6	18.5
4	62.7	12	4.1	8	17.5
5	70.4	15	3.4	10	18.8

[a]Pisatin was not recovered from healthy or wounded epicotyls at day 0.

[b]Values represent the mean of two experiments of 9-59 plants each.

[c]Values represent the mean of three experiments of 10-22 plants each.

[d]Values represent a significant increase of pisatin with time.

DISCUSSION.—The linear growth bioassays indicate that, in general, pathogens are less sensitive to pisatin than nonpathogens, as reported previously (4, 27). However, the pea pathogen *A. euteiches* was the most sensitive organism tested, while the nonpathogen *B. cinerea* was relatively insensitive.

Cruickshank (4) bioassayed 50 different fungal isolates against pisatin and found a good correlation between tolerance to pisatin and pathogenicity on pea. In his study he did find one exception to this pattern. *Septoria pisi* West., a pea pathogen, was sensitive to pisatin. In a subsequent study (7), he determined the ability of *S. pisi* and 18 other fungi to induce the accumulation of pisatin in drops of spore suspensions added to seed cavities of detached pea pods. The concn of pisatin that accumulated ranged from 10 μg/ml to 116 μg/ml of "diffusate" solution. The lowest concn occurred when *S. pisi* was used as the inoculum. Thus, the interaction between the pisatin-sensitive pea pathogen and pea tissue resulted in the accumulation of a low level of pisatin. The results of these studies led Cruickshank (7) to conclude . . . . . "that the situation required for a resistant reaction is a host in which infection stimulates production of a phytoalexin at a concentration above the threshold which inhibits the fungus. Susceptibility may be due to the inability of the infecting fungus to stimulate the formation of the phytoalexin characteristic of the host or to the capacity of the fungal pathogen to be tolerant of the phytoalexin produced." In our study only the interaction between *F. solani* f. sp. *pisi* and pea clearly fits this pattern. Pisatin occurs in high concns in *F. solani* f. sp. *pisi* lesions on pea, but the pathogen is highly tolerant of pisatin.

There are numerous reports of in vitro metabolism of phytoalexins by fungi (2, 10, 12, 13, 14, 18, 21, 25, 26, 28, 29, 30) and there are several reports of metabolism of pisatin by *F. solani* f. sp. *pisi* (2, 21, 29, 30). Hadwiger et al. (9) found less than expected recovery of pisatin from *F. solani* f. sp. *pisi*-challenged pod tissue and interpreted this to mean that in vivo metabolism was occurring, although the validity of their conclusion has been questioned (6). In the present study, the pisatin concn in *F. solani* f. sp. *pisi* lesions began to decrease 3 days after inoculation. Christenson and Hadwiger (3) recently reported a similar decrease of pisatin concn in *F. solani* f. sp. *pisi*-infected pea tissue. Our data indicate that there is no statistically significant change in total pisatin content per lesion from 3 to 15 days after inoculation, even though lesion volume per plant increases (Table 2, Fig. 5). This implies that pisatin production had stopped, or that pisatin production and in vivo metabolism by the pathogen were in equilibrium. Alternatively, pisatin turnover by the plant may have been stimulated. The decrease in pisatin concn in lesion tissue may simply reflect the continuing increase in lesion volume after cessation of pisatin accumulation.

The outcome of the *R. solani*-pea interaction is less clear-cut. This pathogen showed some sensitivity to pisatin, but even at the highest concn of pisatin assayed (100 μg/ml) it was inhibited only 60% (Fig. 1). It is also the only pathogen-pea interaction of the three that we examined in which lesion development was restricted. Lesion restriction and attainment of maximum pisatin concn in the lesions occurred coincidentally, but pisatin had much earlier accumulated to a concn which should have been inhibitory to *R. solani.*

The results obtained with the *A. euteiches*-pea interaction are definitely not compatible with the concept that susceptibility is based on tolerance of the pathogen to pisatin, or on failure of the pathogen to induce pisatin. Hyphae of the sensitive pathogen, *A. euteiches,* produced larger lesions and colonized pea tissue much more rapidly than those of the insensitive pathogen, *F. solani* f. sp. *pisi.* By 36 h after inoculation, pisatin concn in the lesions had reached eight times that which entirely prevented growth of *A. euteiches* in vitro (Fig. 3). Although the pisatin concn decreased after 36 h, it remained higher than that which completely inhibited growth of *A. euteiches* in vitro; yet the lesion continued to expand.

In pea leaflets infected with *Ascochyta pisi,* Heath and Wood (11) found pisatin concns 2-3 times those which caused greater than 70% inhibition of germ tube growth in bioassays. Germ tube growth was not completely

prevented, even at 1,000 μg of pisatin/ml of medium. However, *A. pisi* mycelium both tolerates and degrades pisatin, and the behavior of this pathogen is thus not the same as that of *A. euteiches* (2, 29, 30). Although the observed sensitivity of fungi to phytoalexins is dependent on factors such as the growth parameter being measured (27), incubation time (4, 10), and composition of the medium (27, 30), the sensitivity of *A. euteiches* to pisatin has remained relatively constant over variations in these factors (Fig. 1-2, Table 1 and *unpublished*).

Fungi that readily metabolize a phytoalexin generally are very tolerant of that phytoalexin in vitro (13, 18, 21, 29, 30). The sensitivity of *A. euteiches* to pisatin in vitro suggests that it does not have the ability to readily detoxify pisatin. The total amount of pisatin per *A. euteiches* lesion increases linearly until at least 5 days after inoculation (Table 2). Thus, the decrease in pisatin concn in older lesion tissue (Fig. 3) does not appear to result from the metabolism of pre-existing pisatin or the cessation of pisatin production. Proportionately greater increase in lesion volume per plant than in total pisatin per lesion could account for the observed decrease in pisatin concn in older lesions. If metabolism of pisatin by *A. euteiches* or the plant occurs in this infected tissue, it is more than offset by continuing pisatin production.

The possibility does exist, however, that the in situ sensitivity of *A. euteiches* is different than in vitro bioassays indicate. There is no evidence that pisatin is physically separated from mycelium of *A. euteiches* in vivo, and although the pathogen appears to exist primarily in more mature areas of 5-day-old lesions, pisatin accumulates throughout the infected tissue and up to 2 mm beyond the advancing lesion margins. Lesion expansion and in vivo growth of the pathogen is not, however, restricted. By 10 days after epicotyl inoculation, mycelium of *A. euteiches* extensively colonizes the suscept root system. Oospores are produced in profusion in the root tips 15-20 cm from the point of epicotyl inoculation.

Further investigation of the *A. euteiches*-pea interaction may explain the apparent contradiction. The fact that pisatin concn in expanding *A. euteiches* lesions is greatly in excess of in vitro tolerance levels of the pathogen suggests that the role of pisatin as the primary resistance factor in peas should be reexamined.

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