The Relation Between Pisatin and the Development of Aphanomyces euteiches in Diseased Pisum sativum

Steven G. Pueppke and Hans D. VanEtten

Former Graduate Research Assistant and Assistant Professor, Plant Pathology Department, Cornell University, Ithaca, NY 14853. Present address of the senior author is Department of Biology, University of Missouri-St. Louis, 8001 Natural Bridge Road, St. Louis 63121.

Portions of this work dealing with light and electron microscopy were made possible in part by Cooperative State Research Service (CSRS) Grant No. 316-15-53 awarded to J. R. Aist and H. W. Israel.

This research is part of a Ph.D. thesis submitted by the senior author to Cornell University.

Accepted for publication 5 April 1976.

ABSTRACT

PUEPPKE, S. G., and H. D. VAN ETTEN. 1976. The relation between pisatin and the development of Aphanomyces euteiches in diseased Pisum sativum. Phytopathology 66: 1174-1185.

When wounded *Pisum sativum* epicotyls were inoculated with the fungal pathogen, *Aphanomyces euteiches*, water-soaked lesions appeared within 1.5 days. In young lesions (1.5 to 2 days after inoculation) fungal growth was intercellular and restricted to macroscopically visible lesions. *Aphanomyces euteiches* was isolated from the site of inoculation and from margins of the lesions. The pathogen grew primarily in the cortex, infrequently in the stele, but never in epidermal cells. Although pisatin concentration in subepidermal tissues of young lesions was 20-40 times that which prevents mycelial growth in vitro ($ED_{100} \leqslant 100\ \mu g/ml$), lesions continued to expand rapidly. Pisatin was induced in epicotyls by ultraviolet irradiation. When irradiated epicotyls containing approximately the ED_{100} concentration of pisatin were inoculated, lesion development was not affected appreciably. Differential centrifugation of homogenates of irradiated tissues indicated that pisatin is not associated with particulate cell fractions. When protoplasts were prepared from irradiated leaflets by enzymatic degradation of the cell walls, pisatin was recovered from the enzyme solutions, but not from intact protoplasts. The ED_{100} value of pisatin for *A. euteiches* was $\leqslant 100\ \mu g/ml$, whether pisatin was solubilized with ethanol or not. In four different liquid growth media, dry weight increase of *A. euteiches* was suppressed by 31 μg pisatin/ml medium. The daily addition of pisatin (31 μg/ml/day) to liquid cultures of *A. euteiches* did not result in adaptation of the organism to the accumulating phytoalexin. Dosage-response curves (radial growth versus pisatin concentration) of isolates recovered from *A. euteiches*-infected tissue were identical to that of the initial culture. Pisatin was not metabolically altered after incubation up to 4 days in liquid cultures of the pathogen. Low-molecular-weight components from healthy or infected epicotyls did not affect the sensitivity of *A. euteiches* to pisatin. Thus, although in vitro growth of *A. euteiches* always was prevented by pisatin at $\leqslant 100\ \mu g/ml$, much higher pisatin concentrations did not restrict the pathogen in vivo. The data appear to be inconsistent with concepts of pisatin as a primary resistance factor in pea.

Additional key words: pterocarpans, isoflavonoids, antifungal compounds.

Phytoalexins have been defined as "antibiotics which are the result of an interaction of two different metabolic systems, the host and the parasite" (22). Although the evidence is correlative, phytoalexins may function as a resistance mechanism against potentially pathogenic microorganisms (37). Several theories have been advanced to explain how a successful pathogen overcomes this presumed resistance mechanism (6, 29, 37). These include the capacity of compatible pathogens to tolerate phytoalexins and/or metabolically detoxify the compounds, to prevent the synthesis of the compounds, or in some way to escape from a phytoalexin-containing environment.

Pisum sativum L. (garden pea) produces at least five pterocarpan phytoalexins (23, 27, 33). Of these compounds, pisatin (6a-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan), has been the most extensively studied, and is the only pea phytoalexin which has been proven to be entirely of host origin. *Aphanomyces euteiches* Drechs. is a destructive root- and epicotyl-rotting pathogen of *P. sativum* (16). In an earlier publication (26), we reported that this pathogen was sensitive to pisatin in a variety of in vitro bioassays. Although pisatin is the only known phytoalexin that accumulates in this interaction, its level in young, rapidly-expanding lesions was much greater than that which prevents pathogen growth in vitro. Thus, the compatible interaction of *A. euteiches* and *P. sativum* appeared to be inconsistent with concepts of the phytoalexin theory (26).

The present investigation was undertaken to explain the apparent inconsistency of *A. euteiches* successfully colonizing pea epicotyls containing theoretically inhibitory quantities of pisatin. Several specific questions were addressed. (i) Is viable mycelium of *A. euteiches* present throughout the lesions? (ii) Is the distribution of pisatin in epicotyl tissues or cells such that the pathogen successfully avoids the phytoalexin in vivo? (iii) Do preinoculation treatments which cause pisatin to be present at the time of inoculation alter disease development? (iv) Is *A. euteiches* capable of metabolizing pisatin, perhaps only in localized areas surrounding the hyphae? (v) Is the in vitro sensitivity of *A. euteiches* to pisatin retained if organic solvents are not provided to

solubilize the phytoalexin in the bioassay medium? (vi) Are compounds from healthy and/or infected pea epicotyls able to modify the in vitro sensitivity of *A. euteiches* to pisatin?

MATERIALS AND METHODS

Production of diseased tissue.—Pea seeds (*Pisum sativum* L. 'Progress No. 9') were planted in 25-ml Erlenmeyer flasks as described previously (26). The flasks were held at 100% relative humidity at room temperature (24-27 C) in covered pans which excluded light. When the seedlings protruded 3-5 cm above the lips of the flasks, the pan covers were replaced with clear plastic sheets, and the pans of flasks were transferred to a controlled-environment chamber maintained at 30,000 lx (14-hour photoperiod), 24 C day and 17 C night temperature. After 24 hours, the epidermis of each epicotyl was lightly punctured with the tip of a sterile dissecting needle and inoculated with a 2-mm (diameter) plug from the advancing margin of an *A. euteiches* race 1 culture growing on Martin's peptone-glucose agar (PGA) (21). The plants were maintained in pans covered with clear plastic at the same light and temperature regime as above. Wounded plants inoculated with fungus-free plugs of PGA and harvested 5 days after the inoculation date served as controls.

In some experiments seedlings were pre-irradiated with ultraviolet (UV) light (10) prior to inoculation. When the seedlings were 3-5 cm high, they were placed horizontally 10-15 cm from a Mineralight Model P5 source lamp (Mineralight Co., San Gabriel, California) emitting primarily at 254 nm. The measured incident dose rate was 2,000 to 9,000 ergs/cm^2-second. After a 15-minute irradiation period, the flasks were marked to indicate the sides of the plants that had been irradiated. The plants then were placed in pans with clear plastic covers in the controlled environment chamber. After 1 day, they were inoculated on the irradiated sides and subsequently maintained under the same conditions as nonirradiated plants. Control plants were irradiated and wounded, but not inoculated.

Volume determination of harvested tissues.—A single lesion per plant developed when seedlings (pre-irradiated or not) were inoculated as described. Plants were harvested 1.5, 2, 3, 4, and 5 days after the inoculation date. Although lesions did not develop on irradiated noninoculated plants, areas comparable in size to lesions were removed from the irradiated sides of these plants at each harvest date.

Epidermis and subepidermal tissues were excised separately with a scalpel and forceps. The tissues were transferred to tared weighing vials, and then frozen and lyophilized. After lyophilization the dry weight of tissues in each vial was determined. Sample volumes were calculated by means of an equation described elsewhere (26).

Cytological and histological techniques.—Two separate samples of lesions were prepared by a paraffin embedding procedure for microscopic examination. Each sample contained six 1.5-day-old and six 2-day-old lesions. The tissues were fixed in Craf II (30), dehydrated in a tertiary butyl alcohol series, and embedded in Tissuemat (melting point 52.5 C). Longitudinal serial sections 30-μm thick were cut with a rotary microtome. The sections were stained with safranin-fast green (30) and observed under bright-field or phase-interference optics.

An additional sample of 1.5- and 2-day-old lesions (six of each age) was prepared for embedding in plastic. Tissue was cut into small (<3 mm long) pieces in a fixing solution containing 5% glutaraldehyde in 0.08 M NaOH-PIPES [1,4-piperazinebis-(ethanesulfonic acid)] buffer (pH 8.0). After incubation overnight, the samples were postfixed for 3 hours in 2% OsO_4 in 0.18 M NaOH-PIPES buffer (pH 6.8). After dehydration in a graded acetone series and rinses in propylene oxide, the samples were embedded in Spurr's (32) low viscosity mix E. Several cross sections (25 μm thick) were cut from each block with a razor blade mounted on a reciprocal microtome. The remaining uncut portions of the blocks then were sawed and remounted such that longitudinal sections (35-μm thick) could be prepared from the same tissue pieces. The sections were mounted in immersion oil and viewed with interference contrast optics on a Zeiss Photomicroscope II.

Isolation of Aphanomyces euteiches from infected epicotyls.—The isolation procedure is modified after that of Sundheim and Wiggen (35). Diseased epicotyls were harvested from 12 plants 2 days after inoculation. Each epicotyl was cut into three segments: the portion which included the lower margin of the lesion, a central portion which included the inoculation site, and the portion which included the upper margin of the lesion. Segments from the centers and margins of individual lesions were processed separately. The segments were sliced into 1- to 2-mm diameter disks, and these were placed in petri plates of sterile water for approximately 30 minutes. Next, the disks were transferred to water agar and incubated for several days at 24 ± 2 C. Hyphae growing from each disk were observed with a dissecting microscope, and *A. euteiches* was identified by its sparse, arachnoid growth habit. Random hyphal tips identified as *A. euteiches* were isolated from contaminating organisms on corn meal agar (Difco Laboratories, Detroit, Michigan). Clean cultures were later transferred to potato-dextrose agar (PDA) slants and stored at 24 ± 2 C. All isolates which were identified as *A. euteiches* produced typical lesions on pea epicotyls.

Extraction and quantitation of pisatin from epicotyls.—A technique for extraction and quantitation of pisatin from pea epicotyls has been described (26). In the present experiments, this technique was utilized with a single modification; the thin-layer chromatography (TLC) solvent was a mixture of benzene, ethyl acetate, and isopropanol (90:10:1, v/v).

Differential centrifugation studies.—Pea seeds were planted in a layer of vermiculite in 33 × 25 × 15-cm-deep pans. The vermiculite was irrigated with Hoagland's (15) nutrient solution, covered to exclude all light, and maintained at room temperature. When the etiolated seedlings were approximately 12 cm high, they were illuminated for 18 hours at approximately 4,300 lx from a fluorescent source. The plants then were removed from the vermiculite and washed. Cotyledons were removed, and the seedlings were placed on moist towels 12 cm from a UV source (Spectroline Model C3-F, Westbury, N.Y.) emitting primarily at 254 nm. Irradiation was for 20

minutes at a measured dose of 750 ergs/cm^2-second. The irradiated plants immediately were transferred to a moist dark chamber.

Subcellular fractions were prepared 2 days after irradiation. Epicotyls were excised and rinsed for approximately 15 minutes in cold running tap water. All later operations were performed at 4 C. The epicotyls (80-91 g) were placed in one volume by weight of a stabilizing medium composed of 0.5 M sucrose in 0.02 M potassium glycylglycine buffer (pH 7.5) (40), and chopped into bits (<1 mm long) with razor blades. The resulting slurry was filtered through four layers of cheesecloth, and the sap was centrifuged at 100 *g* for 20 minutes. After removal of the supernatant, the pellet was washed in stabilizing medium, repelleted, suspended in 10.0 ml of fresh stabilizing medium, and stored at 4 C. The 100-*g* supernatant was centrifuged at 6,000 *g* for 20 minutes. The resulting pellet was washed and stored as above. The 6,000-*g* supernatant was centrifuged at 37,000 *g* for 20 minutes, and the pellet was treated as above. A 15.0-ml aliquot of the 37,000-*g* supernatant was further centrifuged at 258,000 *g* in a Spinco SW-65 rotor for 1 hour. This pellet was resuspended in 10.0 ml of stabilizing medium, but not washed. The aliquots of stabilizing medium used to wash the 100-*g*, 6,000-*g*, and 37,000-*g* pellets were pooled at the end of the experiment.

Each resuspended pellet was subdivided as follows: 1 ml was removed for analysis of protein and chlorophyll, a 4-ml aliquot was fixed with 3% glutaraldehyde, and the remaining 5 ml was retained for pisatin analysis. An aliquot of the 258,000 -*g* supernatant was analyzed in the same way as the resuspended pellets. Protein determination was by the method of Lowry et al. (19), using crystalline bovine serum albumin as a standard. Total chlorophyll was estimated spectrophotometrically according to the method of Strain et al. (34). The aliquots for pisatin analysis were partioned twice with $CHCl_3$ (four volumes per extraction) and purified by a published technique (28). Quantitation was by UV spectrophotometry, utilizing the published molar extinction coefficient of the phytoalexin (log ϵ = 3.86 at 309 nm) (23).

Samples fixed in glutaraldehyde were postfixed for 2 hours in 2% OsO_4 in 0.18 M NaOH-PIPES buffer, dehydrated, and embedded in plastic as already described. Thin sections were stained on the grids for 6 minutes with a hot (60 C) solution of 2% uranyl acetate in ethanol and examined with a Philips EM-200 electron microscope.

Additional differential centrifugation experiments were modified in the following manner. The 100-*g* and 6,000-*g* pellets were discarded, and a 100,000-*g* centrifugation step (20 minutes) was inserted. Aliquots of resuspended pellets and of the 37,000-*g*, 100,000-*g*, and 258,000-*g* supernatants were analyzed for pisatin. Samples were not prepared for protein and chlorophyll estimation or fixed for electron microscopy.

Protoplast studies.—Pea plants were grown to anthesis (3-4 weeks) in flats of steamed soil at room temperature under continuous illumination (approximately 4,300 lx). Leaflets from the upper three to four nodes of plants were excised and placed, abaxial epidermis uppermost, on moist towels in shallow pans. To induce pisatin production, the leaflets were exposed to UV light (254 nm) for 5-20 minutes, as described above. The measured incident dosages for various experiments are presented in Table 2. Immediately after irradiation, the leaflets were placed in darkened chambers and incubated for 2 days at room temperature. Control leaflets were treated identically, but were not irradiated with UV light.

The technique for isolation of protoplasts was modified after that of Constabel et al. (4). The eight to 30 leaflets were rinsed for 60 seconds in 70% ethanol and transferred to a solution of 0.53 M mannitol. After 30-60 minutes, the lower epidermis was removed from the leaflets, and they were placed in a 125-ml Erlenmeyer flask containing 20 ml of a filtered (Whatman #50 paper) solution of 0.75% pectinase (Macerozyme, All Japan Biochemicals, Nishinomiya, Japan), 1.5% cellulase (Onozuka P1500, Kanematsu-Goshu, Tokyo, Japan), and 1.5% hemicellulase (Sigma Biochemicals, St. Louis, Mo.) in mineral salts of the B5 medium of Gamborg et al. (9) containing 0.53 M mannitol (pH 4.5). Streptomycin sulfate (30 μg/ml), tetracycline (2 μg/ml), and penicillin G (76 μg/ml) were included to retard bacterial growth. The mixture was incubated at room temperature on a rotary shaker operating at 75 strokes per minute. After a 2-hour period, the enzyme solution was decanted through four layers of cheesecloth to remove large plant debris. This filtered enzyme solution was designated the 0 to 2-hour fraction, and stored at 4 C until analysis. Fresh enzyme solution was added immediately to the leaves remaining in the flask. At approximately 2-hour intervals for up to 8 hours thereafter, enzyme solutions containing protoplasts were decanted, filtered, and replaced with fresh enzyme solution as described above.

Protoplasts in each fraction (except the 0 to 2-hour fraction) were pelleted by centrifugation at 130 *g* for 5 minutes. The enzyme solution was removed, and the protoplasts were resuspended in fresh B5 salts containing 0.53 M mannitol. Protoplast concentration was determined using a hemacytometer. The percent viable protoplasts was estimated by counting intact and disrupted cells in five ×200 microscope fields of droplets resting on slides without coverslips. (Intact cells excluded the vital stain Trypan blue, and swelled when distilled water was added to the medium.) The protoplasts in each fraction were then ruptured by the addition of nine volumes of distilled water.

At the termination of each experiment, all the solutions containing ruptured protoplasts were combined and stored at 4 C until pisatin extraction and analysis. All centrifuged enzyme solutions subsequent to the 0- to 2-hour fraction were also combined and stored until analysis. Pisatin was extracted and quantified by the procedure described for the differential centrifugation studies.

Preparation of low-molecular-weight extracts.—Plants were grown in flats of steamed soil in a glasshouse. When the seedlings were 3-5 cm high (approximately 10 days after planting) soil in half the flats was drenched with an aqueous suspension of fragmented *A. euteiches* mycelium, which was prepared from 3- to 5-day-old liquid cultures of the pathogen incubated in Yang and Schoulties' medium (41) at 25 ± 2 C. The remaining flats were noninoculated and served as healthy controls. Nine days after the inoculation date, batches of healthy and diseased plants were removed gently from the soil and

washed in a stream of running water. The epicotyls were excised from the plants and maintained at −16 C until extraction.

Low-molecular-weight compounds were extracted by grinding tissue samples (22-50 g) with a Waring Blendor in two volumes of 0.02 M potassium glycylglycine buffer (pH 7.5). Each brei was passed through four layers of cheesecloth and centrifuged at 5,000 *g* for 30 minutes. The supernatants were passed through an ultrafiltration membrane (Diaflo UM-2, Amicon Corp., Lexington, Mass.) with an exclusion molecular weight of 1,000.

Liquid culture bioassays.—Bioassays were performed in 25-ml Erlenmeyer flasks. After a 4-ml aliquot of medium was dispensed to each flask, pisatin dissolved in ethanol was added. Control flasks received ethanol only. Mycelial disks (7.0-mm diameter) were cut with a cork borer from the advancing margin of *A. euteiches* mycelium growing on bacterial filters resting on PGA medium, and four disks were placed in each flask. Four mycelial disks also were transferred to each of two tared Whatman #50 filter papers, and time 0 mycelial dry weight was determined after exposure of the papers to 75 C for 24 hours. The inoculated flasks were maintained at 25 C on a reciprocal shaker operating at 50 strokes per minute. After 72 hours, the mycelium from each flask was collected by vacuum filtration on tared filter paper. Mycelial dry weight was determined as for the time 0 disks. Procedural details for specific assays are as follows:

Bioassays of low-molecular-weight extracts.—Dry components of Yang and Schoulties' growth medium were mixed thoroughly and added to the extracts to give the nutrient composition of the medium. The extracts then were filter-sterilized with bacterial filters (Triacetate Metricel, 0.2-μm pore size, Gelman Instrument Co., Ann Arbor, Mich.). After the nutrient-amended extracts were dispensed into flasks, 125 or 250 μg of pisatin in 20 μliters of ethanol was added to each flask. Control flasks received 20 μliters of ethanol only. Flasks containing filter-sterilized Yang and Schoulties' growth medium served as a second type of control.

Bioassays of pisatin not solubilized with organic solvents.—Pisatin (200 or 400 μg in 20 μliters of ethanol) was added directly to empty 25-ml flasks. Control flasks received 20 μliters of ethanol. The flasks were placed in a 75-C forced air drying oven until the ethanol had evaporated. Yang and Schoulties' growth medium and *A. euteiches* were then added to each flask, and the experiment was carried out as described above. Growth in these flasks was compared to growth in flasks to which the ethanolic solutions of pisatin were added directly to the medium containing *A. euteiches*. Two duplicate experiments, each consisting of three replicates per treatment, were performed.

Bioassays with pisatin added daily.—Flasks containing *A. euteiches* in Yang and Schoulties' medium received one of five treatments: (i) 20 μliters of ethanol added at time 0, (ii) 20 μliters of ethanol added at time 0 and at 24 and 48 hours later, (iii) 375 μg pisatin in 20 μliters of ethanol added at time 0, (iv) 125 μg pisatin in 20 μliters of ethanol added at time 0, and (v) 125 μg pisatin in 20 μliters ethanol added at time 0 and at 24 and 48 hours later. Two experiments, each consisting of three replicates per treatment, were performed.

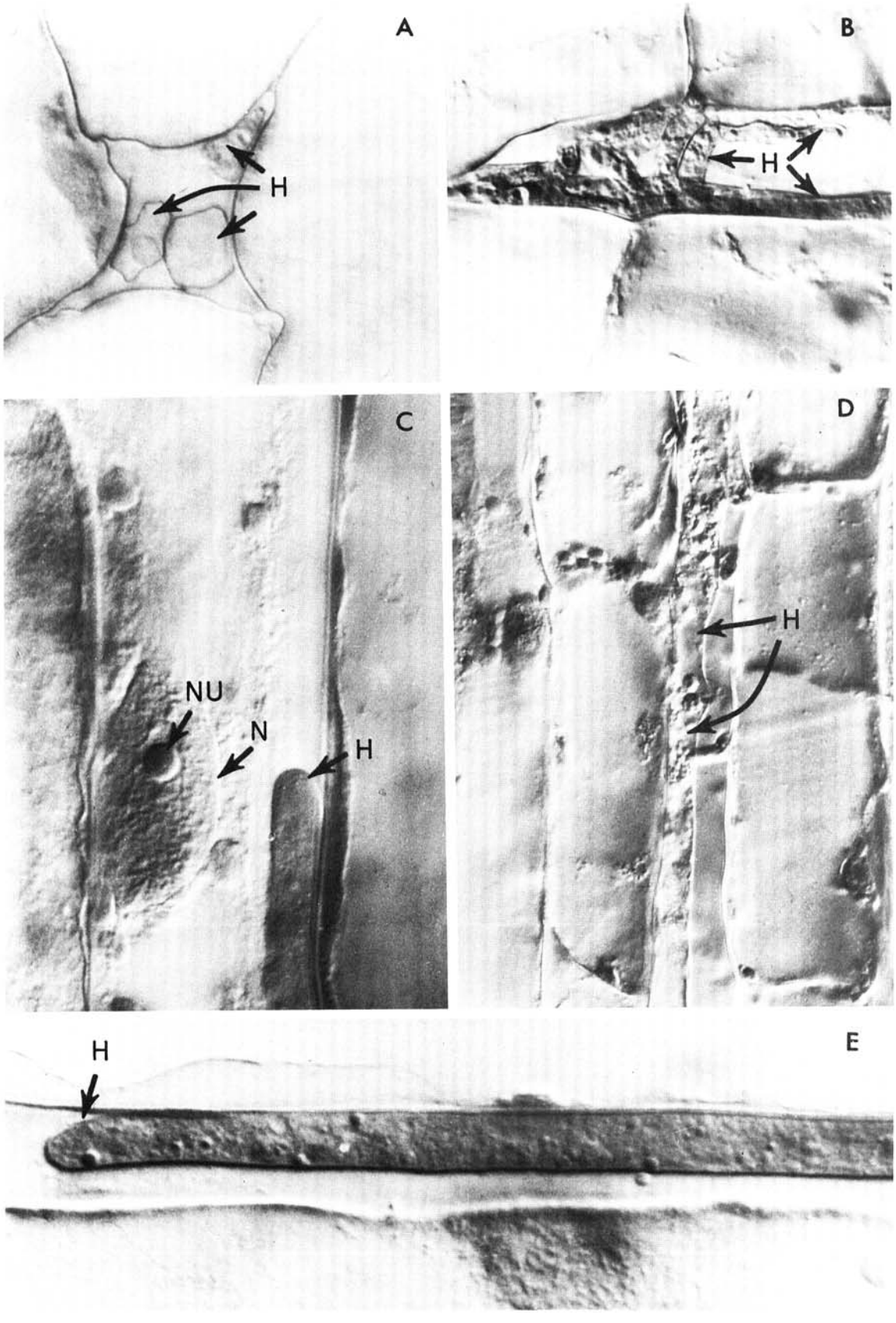
Recovery of pisatin from liquid cultures of Aphanomyces euteiches.—Flasks of *A. euteiches* containing the following media were prepared as described above. The four media utilized were: (i) Yang and Schoulties' medium, (ii) Yang and Schoulties' modified medium (41) (containing 8.0 g asparagine and 9.0 g glucose/liter). (iii) a medium composed of 0.5% glucose and 2% peptone in water, and (iv) the Saprolegnia medium of Powell et al. (25). Pisatin (125 μg) in 20 μliters of ethanol was then added to the flasks. Two types of controls were utilized. Some flasks containing living fungus received 20 μliters of ethanol only; other flasks of autoclaved (10 minutes, 120 C) mycelial disks received pisatin in ethanol. For each medium, two flasks that received pisatin were prepared for analysis at time 0 by the addition of $CHCl_3$. After 2 and 4 days, $CHCl_3$ was added to flasks containing fungus + pisatin and autoclaved fungus + pisatin. At the same times the dry weight of mycelium in flasks containing fungus + pisatin and fungus + ethanol was determined. The extraction and pisatin quantification techniques have been described (28).

RESULTS

Histological development of Aphanomyces euteiches in pea epicotyls.—Unrestricted epicotyl lesions 1.5 to 2 days after inoculation were oblong, sunken, tawny areas with discrete margins. Microscopic study of sectioned diseased epicotyl tissues prepared either by the paraffin- or by the plastic embedding procedure confirmed that the pathogen had grown from the inoculum plugs into the wounds. Hyphal strands were observed throughout cortical tissues of lesions, but only once was a hypha observed in advance of a visible lesion margin. The pathogen was not observed in epidermal tissues, and although xylem cells were dark and sometimes occluded, hyphae invaded stelar tissues infrequently. Growth of the pathogen was primarily parallel to the axes of the epicotyls. Although it was difficult to determine if hyphae in longitudinal sections were within or between cells, examination of cross sections from the same tissue blocks revealed that the pathogen was strictly intercellular (Fig. 1), except at the inoculation site. In cross sections, spaces between cortical cells often were occupied by several parallel hyphal strands (Fig. 1-A).

Fungal morphology in the diseased tissue was highly variable. In cross section, some hyphae appeared to be entirely vacuolate, whereas others were partially or entirely filled with cytoplasm (Fig. 1-A). Variation in hyphal contents also was revealed in longitudinal sections; some hyphae were vacuolate and others possessed dark homogeneous protoplasm (Fig. 1-B, C, D, E). Contorted bundles and fasciculate areas of hyphae often were observed in colonized tissues (Fig. 1-B), but mycelial strands proximal to advancing tips usually were linear (Fig. 1-C, E). The microscopic appearance of plant cells in colonized cortical tissues was not conspicuously different from that of noninoculated control tissues.

Location and concentration of pisatin in diseased epicotyls.—Because pathogen development in 1.5- and 2-day-old infected pea epicotyls is intercellular and in subepidermal tissues only, experiments were performed to determine if pisatin concentration in subepidermal lesion tissues was of the magnitude that prevents


A
H
B
H
C
NU
N
H
D
H
E
H

pathogen growth in vitro ($ED_{100} \leqslant 100$ μg/ml). The data from these studies are in Fig. 2. In both epidermal and subepidermal lesion tissues, pisatin concentration is greatest 1.5-2.0 days after inoculation. Thereafter, pisatin levels in both tissue types diminish, but never descend to values less than four times the ED_{100} concentration. The maximum pisatin concentrations (sometimes as much as 40 times the in vitro ED_{100} concentration) were in subepidermal tissues of young lesions. Yet in spite of the substantial quantities of pisatin, lesion volume increased rapidly.

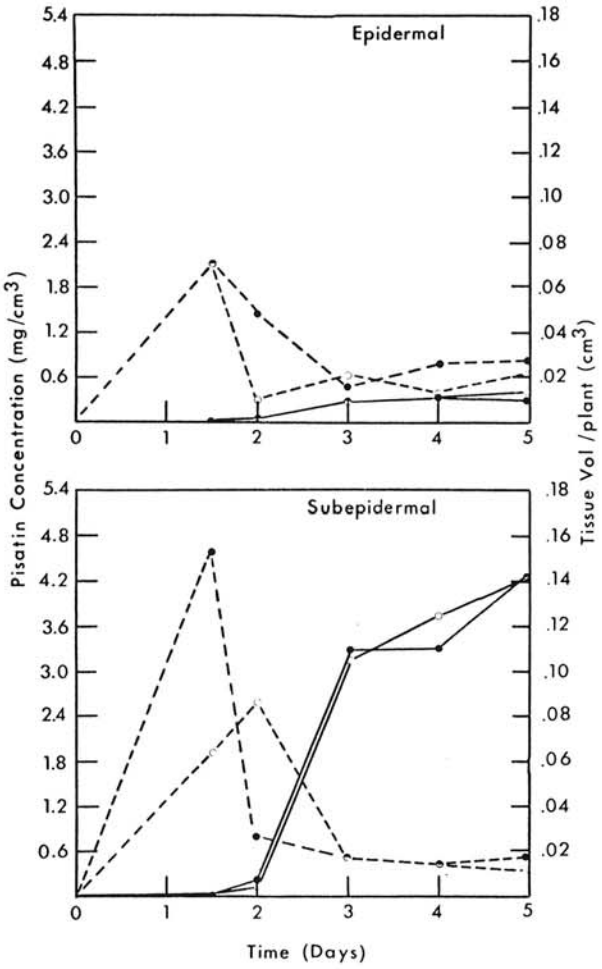


Fig. 2. Pisatin concentration in and volume of epidermal and subepidermal lesion tissues of pea epicotyls infected with *Aphanomyces euteiches*. Solid lines indicate tissue volume, and dashed lines indicate pisatin concentration. o = Experiment 1; • = Experiment 2. Plants were inoculated at time 0, and sample size varied from six to 16 plants.

When epicotyls were pre-irradiated with UV light, pisatin levels 1 day later (Fig. 3) were more than 900 μg/cm³ of epidermal tissue. In subepidermal tissues, the values for the two experiments were 90 and 108 μg/cm³; i.e., approximately the ED_{100} concentration. When these irradiated plants were wounded (but not inoculated) and incubated for an additional 5 days, pisatin concentration in epidermal and subepidermal tissues of harvested lesion-sized areas was greatest until the second or third day after inoculation, and then gradually declined.

Pisatin concentration in subepidermal tissues was greatest from 1.5-3.0 days after the inoculation date (Fig. 3). Surprisingly, these concentrations generally were

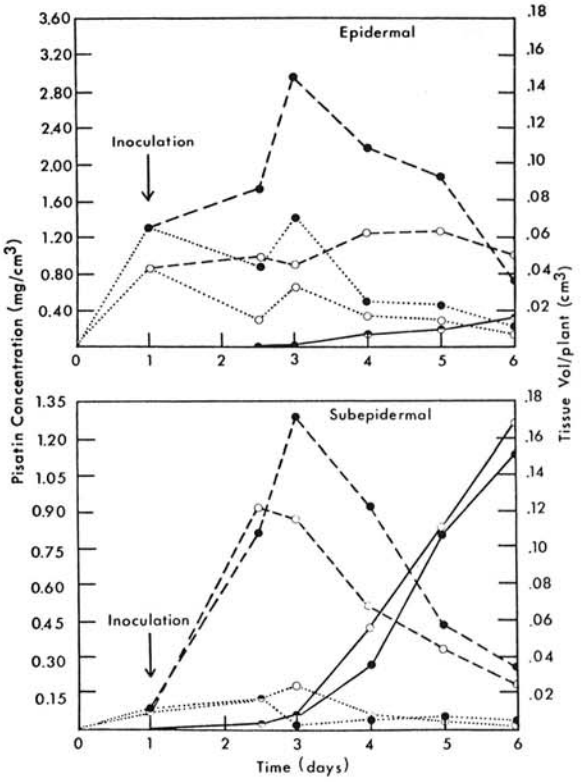


Fig. 3. Pisatin concentration in and volume of pre-irradiated epidermal and subepidermal tissues of pea epicotyls during infection by *Aphanomyces euteiches*. Epicotyls were irradiated with ultraviolet light (1.8 to 8.1 × 10^6 ergs/cm², λ_{max} 254 nm), incubated for 1 day, wounded, and inoculated with the pathogen. Controls were irradiated and wounded, but not inoculated. Solid lines indicate lesion tissue volume of inoculated plants. Dotted lines indicate pisatin concentration in noninoculated control tissues, and dashed lines indicate pisatin concentration in lesions of inoculated plants. o = Experiment 1; • = Experiment 2. Sample size varied from six to 16 plants.

Fig. 1-(A to E). Histology and cytology of *Aphanomyces euteiches* in cortical tissues of pea epicotyls. Sections were stained with 2% OsO_4 for 3 hours, and all photographs are by interference contrast microscopy. **A)** Three intercellular hyphae (H) in cross section. The outlines of the hyphae are irregular, and some cells are highly vacuolate. × 1,980. **B)** Longitudinal section illustrating the intercellular proliferation of hyphae (H) in colonized tissue. ×930. **C)** Longitudinal section revealing a dark intercellular phyhal tip (H) extending along an apparently healthy cortical cell. The host cell nucleus (N) with prominent nucleolus (NU) is beside the tip. × 1,600. **D)** Longitudinal section showing an intercellular hypha (H) with vacuolate protoplasm. × 690. **E)** Intercellular hyphal tip (H). The fungal protoplasm appears homogeneous in this longitudinal section. × 1,900. Photomicrographs B) and E) are of tissue fixed 1.5 days after inoculation. Photomicrographs A), C), and D) are of tissue fixed 2 days after inoculation.

lower than corresponding values from tissue which was not pre-irradiated (Fig. 2), but nevertheless they were at least eight times the ED_{100} concentration. When kinetics of lesion expansion of pre-irradiated and nonirradiated plants are compared, it is apparent that lesion growth rates are similar (Fig. 2, 3). However, at 3 days after inoculation, lesions from irradiated plants consistently were smaller than those from nonirradiated plants.

Cellular and subcellular localization of pisatin.—Although pisatin was present in 1.5- and 2-day-old lesions (Fig. 2) it was plausible that the phytoalexin was localized within cells and not contacted by the intercellular pathogen. Therefore we examined the subcellular localization of the phytoalexin by differential centrifugation. Complications inherent in the analysis of fungus-containing tissues were avoided by inducing pisatin with UV light, a treatment which causes no visible damage. Results of a typical experiment are presented in Table 1. Pisatin was not recovered from particulate subcellular fractions composed of structurally intact organelles (as determined from electron micrographs). The phytoalexin was detected in the final supernatant solution. In later experiments, a 100,000-*g* centrifugation step was inserted to preclude the possibility that vesicles were ruptured by centrifugation at 258,000 *g* directly following the 37,000-*g* step (H. W. Israel, *personal communication*). Pisatin in these experiments was not associated with particulate fractions, but was recovered from aliquots of the 37,000-*g* 100,000-*g*, and 258,000-*g* supernatants.

The above data indicate that the phytoalexin is soluble either within cells or in extracellular spaces. To separate intracellular and extracellular components, protoplasts from UV-irradiated pea leaflets and enzyme solutions used for protoplast preparation were analyzed for pisatin. Total pisatin recovery was 1.6-14 μg per leaflet, whereas each leaflet produced 140,000 to 600,000 protoplasts. Pisatin distribution in a number of experiments is presented in Table 2. Pisatin invariably was present in the 0- to 2-hour fractions, which were composed of the initial enzyme solutions, many broken cells, and a few intact protoplasts. The phytoalexin also was detected in the enzyme solutions from which protoplasts had been collected, but little or no pisatin was recovered from the pelleted protoplasts.

Viability of plant and fungal cells in young lesions.—Experiments to examine pisatin localization were performed with apparently healthy cells, which presumably retained their selective permeability properties. To determine if cells in young lesions were also viable, epicotyls containing 1.5- and 2-day-old lesions were excised and immersed in a 0.5% aqueous solution of Trypan blue, which is excluded from living cells (18). After 3 hours in the stain, 1.5-day-old lesions, but not adjacent tissues, were colored darkly. Although cells near the inoculation point of 2-day-old lesions were stained, cells near the lesion margins remained viable and did not accumulate the stain. Only injured cells of wounded, noninoculated controls accumulated the stain.

Whether the mycelium throughout the young lesions is viable is also critical in understanding the relationship of *A. euteiches* to pisatin. Fungal isolation was made from samples of 2-day-old lesion tissues proximal to the inoculation point and adjacent to lesion margins. Thirty-two margin segments and 68 proximal segments from 12 plants were analyzed. Viable *A. euteiches* was recovered from 81% of the margin segments and 78% of the segments proximal to the inoculation point.

TABLE 1. Distribution of pisatin in and components of subcellular fractions of ultraviolet-irradiated pea epicotyls[a]

Fraction	Components	Total pisatin (μg)	Total protein (mg)	Total chlorophyll (mg)
100-*g* pellet	cell fragments, chloroplasts	0	1.5	<15
6,000-*g* pellet	mitochondria, chloroplasts	0	7.7	268
37,000-*g* pellet	endoplasmic reticulum, ribosomes	0	5.5	<15
258,000-*g* pellet	microsomes	0	10.1	0
258,000-*g* supernatant	. . .	67	176.5	0
Wash solutions	. . .	14	. . .	. . .

[a]The tissue preparation was filtered through cheesecloth prior to differential centrifugation. Tissue debris collected on the cheesecloth contained 504 μg pisatin. The filtrate (108 ml) contained 81 μg of pisatin. Aliquots of each fraction were analyzed. The reported pisatin, protein, and chlorophyll values were adjusted to represent the total composition of each fraction. Total recovery of pisatin was 6.5 μg/g fresh weight of tissue. Ultraviolet irradiation was at 750 ergs/cm^2-second primarily at 254 nm for 20 minutes. Nonirradiated tissue produced 3.5 μg pisatin/g fresh weight of tissue.

TABLE 2. Pisatin distribution in protoplast preparations from ultraviolet(UV)-irradiated pea leaflets

Experiment	UV dosage (ergs/cm^2)	Total protoplasts ($\times 10^6$)	Protoplasts not disrupted (%)	Pisatin (μg)		
				0-2 hour fraction	Combined enzyme solutions	Combined protoplast suspensions
1	9.0×10^5	2.6	65	15	10	9
2	9.0×10^5	3.1	67	52	43	0
3	9.0×10^5	18.1	71	35	13	0
4	4.2×10^6	5.4	~25	145	56	0
5	4.2×10^6	7.9	35	35	65	<4

Sensitivity of Aphanomyces euteiches to pisatin.—Results of the localization experiments did not support the hypothesis that physical separation of fungus and phytoalexin might explain the insensitivity of *A. euteiches* to pisatin in vivo. Consequently, a series of experiments was designed to reexamine the sensitivity of *A. euteiches* to pisatin. In earlier studies, bioassays of pisatin against *A. euteiches* were performed with media in which the phytoalexin was solubilized with organic solvents (26, 36). To test whether pisatin is active in nonamended aqueous media, the pathogen was added to flasks of medium containing the phytoalexin, with or without organic solvent. The inhibitory activity of pisatin is similar in both cases, and the ED_{100} concentration was $\leqslant$ 100 μg/ml (Table 3).

Following inoculation of epicotyls with *A. euteiches*, pisatin increases from undetectable levels to high concentrations in a 1.5-day period (Fig. 2). Thus, the pathogen is given an opportunity to adapt to the phytoalexin during the period when pisatin concentration is increasing. In liquid growth bioassays, this adaptation period was simulated by exposing *A. euteiches* to increasing concentrations of pisatin over a 3-day period (the initial concentration permitted growth equivalent to approximately 15% of controls). The pathogen did not adapt to pisatin; growth was inhibited similarly in treatments receiving 375 μg pisatin in a single dose or in three 125-μg doses over the 3-day period (Table 4).

If *A. euteiches* and pisatin interact in vivo, they do so in the complex chemical environment of a diseased plant. In contrast, the in vitro bioassay environment is relatively simple and chemically defined. Hypothetically, in vivo a compound(s) from diseased tissue could react with pisatin and/or the pathogen to render the phytoalexin nontoxic

TABLE 3. Effect of pisatin, with and without ethanol as a solubilizing agent, on growth of *Aphanomyces euteiches* in liquid medium[a]

	Net fungal dry weight (mg/flask)[b]			
	No ethanol		Ethanol	
Treatment	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Control	8.4	6.3	7.9	5.9
50 μg pisatin/ml	2.9	−0.5	0.2	0.2
100 μg pisatin/ml	−0.4	−0.6	−0.7	−0.7

[a]Pisatin was added to each flask in 20 μliters of ethanol. Controls received 20 μliters of ethanol only. In "no ethanol" treatments, ethanol was removed by exposing the flasks to 75 C until they were dry. Four milliliters of Yang and Schoulties' (41) growth medium and four disks from the advancing margins of *A. euteiches* colonies growing on bacterial filters resting on PGA (21) were then added. For "ethanol" treatments, pisatin in ethanol was added directly to flasks containing mycelial disks in medium. Incubation was on a reciprocal shaker at 50 strokes per minute and 25 C for 3 days.

[b]Fungal dry weight at time 0 was 1.2 and 1.0 mg/flask for Experiments 1 and 2, respectively. To calculate net dry weight, time 0 dry weight was subtracted from the mean of the recorded weights (three replicates per treatment).

TABLE 4. Effect of low levels of pisatin added daily on the growth of *Aphanomyces euteiches* in liquid culture

Treatment	Times when treatment was administered (days)	Net fungal dry weight (mg/flask)[a]	
		Experiment 1	Experiment 2
20 μliter ethanol	0	12.1	11.7
20 μliter ethanol	0, 1, and 2	9.9	12.1
375 μg pisatin in 20 μliters of ethanol	0	−0.4	. . .
125 μg pisatin in 20 μliters of ethanol	0	. . .	1.6
125 μg pisatin in 20 μliters of ethanol	0, 1, and 2	0.2	0.1

[a]Fungal dry weight at time 0 was 0.7 and 0.9 mg/flask for Experiments 1 and 2, respectively. Net dry weight was calculated by subtracting the time 0 weight from the mean of the recorded weights (three replicates per treatment).

TABLE 5. Effect of low-molecular-weight extracts from healthy and diseased pea epicotyls on the sensitivity of *Aphanomyces euteiches* in liquid culture to pisatin

Treatment	Net fungal dry weight (mg/flask)[a]
Healthy tissue extract control	16.5
Healthy tissue extract + 125 μg pisatin	2.3
Healthy tissue extract + 250 μg pisatin	0.1
Diseased tissue extract control	16.6
Diseased tissue extract + 125 μg pisatin	1.8
Diseased tissue extract + 250 μg pisatin	0.6
Yang and Schoulties' medium control	9.8
Yang and Schoulties' medium + 125 μg pisatin	1.1
Yang and Schoulties' medium + 250 μg pisatin	0.2

[a]Fungal dry weight at time 0 was 0.6 mg per flask. Net fungal dry weight was calculated by subtracting the time 0 weight from the mean of the recorded weights (two replicates per treatment).

or the fungus insensitive to the phytoalexin. Thus, *A. euteiches* would be sensitive in vitro because the hypothetical chemical is absent. Attempts to bioassay pisatin against *A. euteiches* in crude aqueous extracts from ground pea tissue were unsuccessful, because the extract was too viscous to sterilize by passage through bacterial filters.

Therefore, a crude fraction of low-molecular-weight compounds that are obtained easily from epicotyls was chosen for bioassay. Results of the assay (Table 5) indicate that the extracts did not affect the sensitivity of *A. euteiches* to pisatin. The same results were obtained when protein extracts from diseased or healthy epicotyls were tested (S. G. Pueppke, *unpublished*).

Cultures of *A. euteiches* were isolated from diseased epicotyls and bioassayed to determine if they exhibited a range of sensitivities to pisatin. Pea epicotyls were inoculated with a stock *A. euteiches* culture, and two new isolates (α and β) were retrieved from the tissue by the standard isolation technique. Epicotyls were then inoculated with isolates α and β, and the corresponding reisolates, α' and β', were recovered. Recovered isolates were assayed within 1 month of isolation from tissue.

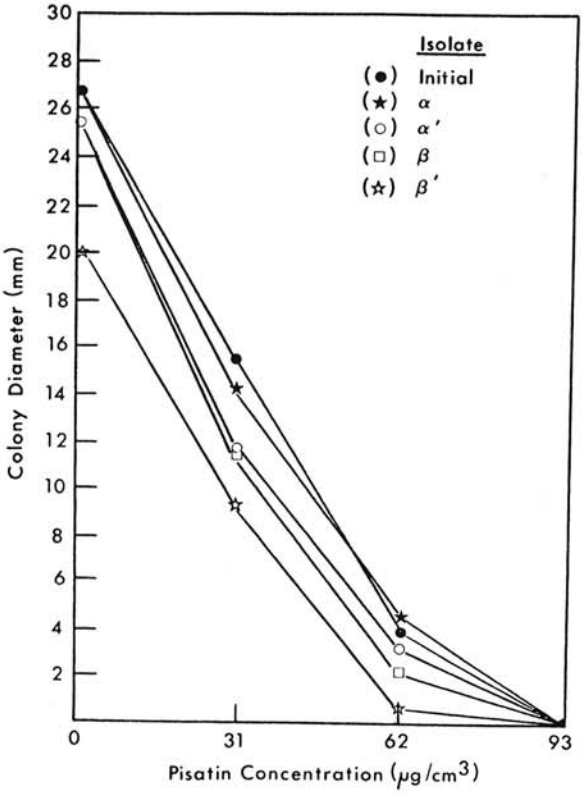


Fig. 4. Dosage-response curves for *Aphanomyces euteiches* isolates and pisatin. Pisatin was dissolved in ethanol and added to PGA (21) to give a final concentration of 0.5% ethanol. Controls received 0.5% ethanol only. Radial growth measurements were made 48 hours after a 4.0-mm (diameter) agar plug containing *A. euteiches* was placed on the medium. Growth was calculated by measuring two perpendicular diameters of each of three replicate colonies and subtracting the diameters of the inoculum plugs.

Dosage-response curves of pisatin versus the five isolates were determined by a published bioassay technique (28), utilizing radial growth measurements made after incubation of assay dishes for 48 hours at 24 ± 2 C. The data are in Fig. 4. Growth rates of the isolates differ, but isolates recovered from epicotyls were not more tolerant of pisatin than was the initial stock culture.

Metabolic transformation of pisatin by Aphanomyces euteiches.—A hypothetical mechanism for the observed insensitivity of *A. euteiches* to pisatin in epicotyls is degradation of pisatin to noninhibitory metabolites at the hyphal surfaces. Thus, pisatin would be absent in localized areas around the hyphae. To test the hypothesis, liquid cultures of *A. euteiches* (living or autoclaved), were supplied with a low concentration of pisatin. Pathogen growth and pisatin concentration were monitored for 4 days in each of four media (Fig. 5). Pathogen growth and its inhibition by pisatin depended on both the medium and the incubation time. For each medium, the rate of pisatin loss in living cultures and in those containing autoclaved fungus was similar, and no new degradation products were detected.

DISCUSSION

We have summarized a portion of the evidence for and

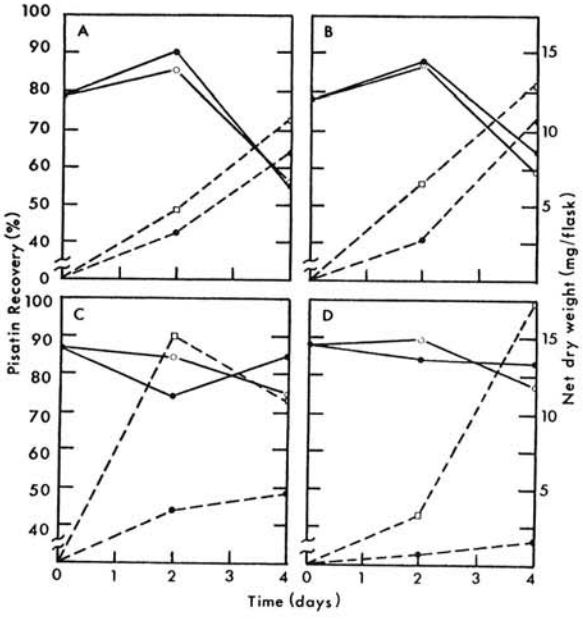


Fig. 5-(A to D). Recovery of pisatin from autoclaved and nonautoclaved liquid cultures of *Aphanomyces euteiches* and the effect of pisatin on growth of *A. euteiches* in liquid culture. (●——●) = pisatin recovery from live fungus; (○——○) = pisatin recovery from autoclaved fungus. **A)** Yang and Schoulties' (41) medium. **B)** Yang and Schoulties' modified medium containing 8.0 g asparagine and 9.0 g glucose/liter. **C)** 0.5% glucose and 2% peptone in water. **D)** Saprolegnia medium of Powell et al. (25). Net dry weight was calculated by subtracting the time 0 weight (1.6 mg/flask) from the mean of the recorded weights of replicate flasks. (□----□) = fungal dry weight in controls receiving 20 μliters ethanol only. (●----●) = fungal dry weight in treatments receiving pisatin.

against the existence of various compatibility and incompatibility mechanisms based on phytoalexins (37). The *A. euteiches-P. sativum* interaction is compatible, and prima facie, this could reflect the occurrence of several phenomena. *Aphanomyces euteiches* could be tolerant of pisatin, or prevent the accumulation of the phytoalexin in vivo. Alternatively, the pathogen may avoid tissues containing pisatin, or the phytoalexin could be enclosed in some subcellular compartment, and thus separated from the pathogen.

In an earlier study we reported that *A. euteiches* is sensitive to pisatin in several in vitro bioassays ($ED_{100} \leqslant 100$ μg/ml). Furthermore, pisatin concentrations in excess of those which theoretically prevent pathogen growth accumulate in the unrestricted lesions produced by *A. euteiches* on pea epicotyls (26). Thus, the basis for compatibility in this interaction appeared not to be lack of phytoalexin induction or tolerance of pisatin by the pathogen. The current studies represent an attempt to determine, within the framework of the phytoalexin theory, the reason(s) for susceptibility in the compatible *A. euteiches-P. sativum* interaction.

Growth of *A. euteiches* in pea tissues is reportedly both intracellular and intercellular (7, 16). In the present experiments, pathogen growth until at least 2 days after inoculation was strictly intercellular and in cortical tissues of lesions (Fig. 1). If pisatin was localized in epidermal cells of lesions, the pathogen and phytoalexin would not be expected to contact one another. When pisatin concentrations in epidermal and subepidermal lesion tissues were monitored, pisatin levels greatly in excess of the ED_{100} concentration were present in both tissues (Fig. 2). Nevertheless, these data do not constitute proof that the pathogen and the phytoalexin interact in subepidermal tissues. Pisatin might be in the same tissues as the pathogen, but physically sequestered in specialized cells or organelles. It is known that certain flavonoids are localized in plant cells; e.g., chloroplasts of *Avena sativa* contain high concentrations of flavonoids (39). In general, flavonoids may be concentrated in cell vacuoles (11), but no studies of physical compartmentalization of isoflavonoid phytoalexins have appeared.

We studied the subcellular distribution of pisatin by fractionation of pisatin-containing epicotyls and analysis of the components by differential centrifugation. Pisatin never was associated with particulate cell fractions (Table 1). Since integrity of the subcellular components was confirmed by electron microscopy, it is unlikely that pisatin was originally compartmentalized in particulate cell fractions, but subsequently released during the isolation procedure.

The differential centrifugation studies did not permit distinction between pisatin from intercellular spaces or from cell protoplasm. One method to differentiate between intra- and extracellular pisatin is the preparation of protoplasts from pea tissues containing pisatin. Removal of protoplasts from bathing enzyme solutions would effect separation of components contained within the plasmalemma from those outside. The protoplast studies (Table 2) indicate that virtually all of the recovered pisatin was in either the 0-2 hour fraction, which contained the first enzyme solution and many broken cell fragments, or in the later supernatant enzyme solutions. The most straightforward interpretation is that pisatin occupied intercellular spaces in these tissues. Alternately, it is conceivable that pisatin initially was within cells, but was pumped out during the isolation procedure. A third possibility is that the threshold UV dose for pisatin induction is greater than that which damages cells, and thereby precludes isolation of intact protoplasts. Pisatin originally contained in these damaged cells from surface layers could explain recovery of the phytoalexin from supernatants, but yet not require that pisatin was initially in intercellular spaces. Thus, cells which formed viable protoplasts may not have received a UV dose sufficient to induce pisatin synthesis. However, the facts remain that pisatin was not bound to any particulate cell fraction (Table 1) and could not be recovered from within the plasmalemma (Table 2).

Additional data intimate that pisatin is very likely not compartmentalized within suscept cells in vivo. In the present study, many cells in colonized tissues lost the ability to exclude the vital stain Trypan blue. Other workers reported that pisatin diffuses into aqueous drops of spore suspensions (5, 6) or solutions of chemicals (24) placed in contact with pea cells. Pea roots similarly exude pisatin into nonsterile aqueous media (3), and callus cultures of pea tissues release pisatin into growth media (1). Furthermore, physiological concentrations of pisatin disrupt the plasmalemma of pea cells (31).

Phytoalexins are by definition antimicrobial compounds. It follows that bioassays of antifungal activity play a critical role in determining if compounds are phytoalexins. Antifungal activity of phytoalexins is assessed by criteria such as radial mycelial growth, spore germination, or germ-tube growth inhibition, and by suppression of fungal dry weight increase in liquid cultures. Bioassay results are affected by the type of assay, as well as medium composition and environment (37). Many isoflavonoid phytoalexins are sparingly soluble in aqueous media, and therefore the compounds usually are dispensed into the assay media with solubilizers (often ethanol or dimethylsulfoxide). Use of solubilizers in bioassays has been criticized (8), since these same compounds undoubtedly are unavailable in vivo. However, solubilization of pisatin with organic solvents is not necessary for activity of the phytoalexin against *A. euteiches* (Table 3). The ED_{100} value is $\leqslant 100$ μg/ml, whether pisatin is solubilized with organic solvents or not.

A second criticism of bioassays is the discrepancy in the composition of different bioassay media and plant tissue. Indeed, exposure of *A. euteiches* growing in four different media to concentrations of pisatin below the ED_{100} value leads to pronounced differences in observed growth inhibition (Fig. 5). Mansfield and Deverall (20) demonstrated that the phytoalexin wyerone acid prevents germination of spores of the leaf pathogen, *Botrytis cinerea*. Addition of pollen extract renders spores totally insensitive to the phytoalexin. Thus, addition of plant components to the media may strikingly influence bioassay results. In the present study, amendment of bioassay media with tissue extracts did not affect the sensitivity of *A. euteiches* to pisatin (Table 5). Nevertheless, the possibility remains that other types of extracts or extracts prepared by alternate procedures could modulate the in vitro response of *A. euteiches* to pisatin.

Some fungi are capable of metabolizing their suscepts'

phytoalexins to noninhibitory products in vitro. For example, *Ascochyta pisi* demethylates pisatin to 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (17), and *Fusarium solani* f. sp. *phaseoli* oxidizes the phytoalexin phaseollin to 1α-hydroxyphaseollone (14). In each case the metabolite was also detected in infected tissues (17, 38). Since the parent phytoalexins can also be recovered from tissue infected with these pathogens (12, 38), phytoalexin degradation in vivo is apparently a localized phenomenon occurring only in areas near mycelium. Furthermore, high phaseollin concentrations (>30 μg/ml) are inhibitory to liquid cultures of *F. solani* f. sp. *phaseoli* if the pathogen is not exposed to the phytoalexin for a period of adaptation (13).

An analogous situation perhaps could occur with *A. euteiches;* i.e., the pathogen degrades pisatin in localized areas near mycelium in vivo, but is nevertheless sensitive to high concentrations of pisatin in vitro. This contention was tested by exposing liquid cultures of *A. euteiches* to low pisatin concentrations and assaying for the appearance of metabolites and a decrease in phytoalexin concentration. Although growth of the fungus was not prevented by the phytoalexin, no metabolites were detected after incubation for 4 days, and loss of pisatin from living cultures parallelled that from autoclaved controls (Fig. 5). Thus, the data indicate that *A. euteiches* lacks the capacity to degrade pisatin. Moreover, *A. euteiches* did not adapt to pisatin when quantities of the phytoalexin which did not prevent growth were supplied daily to liquid cultures (Table 4). Results of a separate experiment (Fig. 4) indicate that the initial *A. euteiches* isolate and isolates recovered from lesions exhibit similar dosage-response curves with pisatin. Thus, in vivo there is apparently no selection for pisatin-tolerant mycelium.

The data in Fig. 3 are portentous as they apply to the role of pisatin in *A. euteiches*-infected peas. Epicotyls were irradiated with UV light and inoculated later, when the ED_{100} concentration of pisatin had accumulated in subepidermal tissue (epidermal tissues contained even greater levels of pisatin). Lesion formation was not prevented (Fig. 3). In fact, lesion development was similar to that in nonirradiated plants; 3-day-old lesions in irradiated plants occupied less volume than their counterparts in nonirradiated plants, but lesion volume per plant after 5 days was identical. Thus, even theoretically inhibitory concentrations of preformed pisatin do not significantly alter lesion development. Analogous UV induction experiments have been performed with soybeans susceptible to *Phytophthora megasperma* var. *sojae* (2). In contrast to the present study, pre-irradiation of soybean hypocotyls induced the soybean phytoalexin, and rendered a large proportion of the plants resistant to the pathogen.

Shiraishi et al. (31) reported recently that pisatin ruptures the plasmalemma of protoplasts from pea leaflets and causes cells previously loaded with the vital dye neutral red to lose the stain. Pisatin concentration in *A. euteiches* lesions is much greater than 300 μg per ml (Fig. 2). Therefore, the phytoalexin may actually contribute to the death of suscept cells in tissues infected with *A. euteiches*.

High pisatin concentrations, if they constitute a resistance response, are ineffective in the *A. euteiches-P. sativum* interaction. Moreover, data from studies of this interaction do not lend experimental support to available theories which offer mechanisms whereby compatible pathogens overcome phytoalexins, and therefore, an additional possibility should be emphasized. It may not be essential for a compatible pathogen to prevent the synthesis of the suscept's phytoalexin, to physically avoid the phytoalexin in vivo, or to be tolerant of the phytoalexin (as determined by bioassays). Hence phytoalexins may bear no decisive role in the establishment of compatible pathogen-suscept interactions.

LITERATURE CITED

1. BAILEY, J. A. 1970. Pisatin production by callus cultures of Pisum sativum L. J. Gen. Microbiol. 61:409-415.
2. BRIDGE, M. A., and W. L. KLARMAN. 1973. Soybean phytoalexin, hydroxyphaseollin, induced by ultraviolet irradiation. Phytopathology 63:606-609.
3. BURDEN, R. S., P. M. ROGERS, and R. L. WAIN. 1974. Investigations on fungicides. XVI. Natural resistance of plant roots to fungal pathogens. Ann. Appl. Biol. 78:59-63.
4. CONSTABEL, F., J. W. KIRKPATRICK, and O. L. GAMBORG. 1973. Callus formation from mesophyll protoplasts of Pisum sativum. Can. J. Bot. 51:2105-2106.
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., and D. R. PERRIN. 1963. Studies on phytoalexins. VI. Pisatin: the effect of some factors on its formation in Pisum sativum L., and the significance of pisatin in disease resistance. Aust. J. Biol. Sci. 16:111-128.
7. CUNNINGHAM, J. L., and D. J. HAGEDORN. 1962. Penetration and infection of pea roots by zoospores of Aphanomyces euteiches. Phytopathology 52:827-834.
8. DALY, J. M. 1972. The use of near-isogenic lines in biochemical studies of the resistance of wheat to stem rust. Phytopathology 62:392-400.
9. GAMBORG, O. L., R. A. MILLER, and K. OJIMA. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.
10. HADWIGER, L. A., and M. E. SCHWOCHAU. 1971. Ultraviolet light-induced formation of pisatin and phenylalanine ammonia lyase. Plant Physiol. 47:588-590.
11. HARBORNE, J. B. 1973. Flavonoids. Pages 344-380 *in* L. P. Miller, ed. Phytochemistry, Vol. 2. Van Nostrand-Reinhold, New York. 445 p.
12. HEATH, M. C., and R. K. S. WOOD. 1971. Role of inhibitors of fungal growth in the limitation of leaf spots caused by Ascochyta pisi and Mycosphaerella pinodes. Ann. Bot. (Lond.) 35:475-491.
13. HEUVEL, J. VAN DEN, and H. D. VAN ETTEN. 1973. Detoxification of phaseollin by Fusarium solani f. sp. phaseoli. Physiol. Plant Pathol. 3:327-339.
14. HEUVEL, J. VAN DEN, H. D. VAN ETTEN, J. W. SERUM, D. L. COFFEN, and T. H. WILLIAMS. 1974. Identification of 1α-hydroxyphaseollone, a phaseollin metabolite produced by Fusarium solani. Phytochemistry 13:1129-1131.
15. HOAGLAND, D. R., and D. I. ARNON. 1938. The water-culture method for growing plants without soil. Univ. Calif. Agric. Exp. Stn. Circ. 347. 39 p.
16. JONES, F. R., and C. DRECHSLER. 1925. Root rot of peas in the United States caused by Aphanomyces euteiches (n. sp.). J. Agric. Res. 30:293-325.
17. LAND, B. G. VAN'T, E. D. WIERSMA-VAN DUIN, and

A. FUCHS. 1975. In vitro and in vivo conversion of pisatin by Ascochyta pisi. Acta Bot. Neerl. 24:256 (Abstr.).
18. LILLIE, R. D. 1969. H. J. Conn's Biological Stains. Williams and Wilkins, Baltimore, Maryland. 498 p.
19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
20. MANSFIELD, J. W., and B. J. DEVERALL. 1971. Mode of action of pollen in breaking resistance of Vicia faba to Botrytis cinerea. Nature (Lond.) 232:339.
21. MARTIN, J. P. 1950. Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. Soil Sci. 69:215-232.
22. MÜLLER, K. O. 1961. The phytoalexin concept and its methodological significance. Recent Adv. Bot. 1:396-400.
23. 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.
24. PERRIN, D. R., and I. A. M. CRUICKSHANK. 1965. Studies on phytoalexins. VII. Chemical stimulation of pisatin formation in Pisum sativum L. Aust. J. Biol. Sci. 18:803-816.
25. POWELL, J. R., W. W. SCOTT, and N. R. KRIEG. 1972. Physiological parameters of growth in Saprolegnia parasitica Coker. Mycopathol. Mycol. Appl. 47:1-40.
26. PUEPPKE, S. G., and H. D. VAN ETTEN. 1974. Pisatin accumulation and lesion development in peas infected with Aphanomyces euteiches, Fusarium solani f. sp. pisi, or Rhizoctonia solani. Phytopathology 64:1433-1440.
27. PUEPPKE, S. G., and H. D. VAN ETTEN. 1975. Identification of three new pterocarpans (6a,11a-dihydro-6*H*-benzofuro[3,2-c][1]-benzopyrans) from Pisum sativum infected with Fusarium solani f. sp. pisi. J. Chem. Soc. Perkin Trans. I. 946-948.
28. PUEPPKE, S. G., and H. D. VAN ETTEN. 1976. Accumulation of pisatin and three additional antifungal pterocarpans in Fusarium solani-infected tissues of Pisum sativum. Physiol. Plant Pathol. 8:51-61.
29. RAHE, J. E., and R. M. ARNOLD. 1975. Injury-related phaseollin accumulation in Phaseolus vulgaris and its implications with regard to specificity of host-parasite interaction. Can. J. Bot. 53:921-928.
30. SASS, J. 1958. Botanical microtechnique. Iowa State Univ. Press, Ames. 228 p.
31. SHIRAISHI, T., H. OKU, M. ISONO, and S. OUCHI. 1975. The injurious effect of pisatin on the plasma membrane of pea. Plant Cell Physiol. 16:939-942.
32. SPURR, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
33. STOESSL, A. 1972. Inermin associated with pisatin in peas inoculated with the fungus Monilinia fructicola. Can. J. Biochem. 50:107-108.
34. STRAIN, H. H., B. T. COPE, and W. A. SVEC. 1971. Analytical procedures for the isolation, identification, estimation, and investigation of the chlorophylls. Meth. Enzymol. 23:452-476.
35. SUNDHEIM, L., and K. WIGGEN. 1972. Aphanomyces euteiches on peas in Norway. Isolation technique, physiologic races, and soil indexing. Sci. Rep. Agric., Univ. Norway 51:1-17.
36. VAN ETTEN, H. D. 1973. Differential sensitivity of fungi to pisatin and to phaseollin. Phytopathology 63:1477-1482.
37. VAN ETTEN, H. D., and S. G. PUEPPKE. 1976. Isoflavonoid phytoalexins. Proc. Phytochem. Soc. 12:(In press).
38. VAN ETTEN, H. D., and D. A. SMITH. 1975. Accumulation of antifungal isoflavonoids and 1α-hydroxyphaseollone, a phaseollin metabolite, in bean tissue infected with Fusarium solani f. sp. phaseoli. Physiol. Plant Pathol. 5:225-237.
39. WEISSENBÖCK, G., and V. SCHNEIDER. 1974. Experiments on the localization of flavonoids in plastids. III. Flavonoid content of chloroplasts from Avena sativa L. and the problem of contamination of plastids by flavonoids during isolation. Z. Pflanzenphysiol. 72:23-35.
40. YAMAZAKI, R. K., and N. E. TOLBERT. 1969. Malate dehydrogenase in leaf peroxysomes. Biochim. Biophys. Acta 178:11-20.
41. YANG, C. Y., and C. L. SCHOULTIES. 1972. A simple chemically, defined medium for the growth of Aphanomyces euteiches and some other Oomycetes. Mycopathol. Mycol. Appl. 46:5-15.