Reduction in Pisatin Sensitivity of *Aphanomyces euteiches* by Polar Lipid Extracts

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ABSTRACT

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Incorporation of a polar lipid extract from pea into growth medium decreased the pisatin sensitivity of *Aphanomyces euteiches* in both semisolid and liquid culture bioassays. This extract also decreased sensitivity to the phytoalexins maackiain and phaseollin. Lipids extracted from squash and bean were also effective. Pea lipids decreased the pisatin sensitivity of *A. e.* f. sp. *phaseoli*, a fungus not pathogenic on pea, but did not decrease the pisatin sensitivity of *Fusarium solani* f. sp. *cucurbitae* or *Neurospora crassa*. Commercially obtained phosphatidylcholine decreased the pisatin sensitivity of all four fungi tested. Experiments on the mechanism of the increased pisatin tolerance in *A. euteiches* suggested that phosphatidylcholine decreased the uptake of pisatin from the medium. The pea lipid extract did not have this effect. Neither phosphatidylcholine nor the pea extract stimulated pisatin demethylation. Attempts to purify the active component from the pea extract indicated that a mixture of compounds was required for the tolerance-enhancing effect.

In his interpretation of the phytoalexin theory, Cruickshank (3) proposed that in incompatible interactions phytoalexin accumulation halts pathogen growth, thereby conferring resistance to the plant. In compatible interactions the pathogen apparently either tolerates the accumulated phytoalexins or avoids eliciting phytoalexin production. The results of most studies on phytoalexin involvement in host-pathogen interactions have been consistent with these hypotheses. However, the *Aphanomyces euteiches* Drechs.–*Pisum sativum* L. interaction is an exception. The phytoalexin pisatin reaches high levels in infected pea tissue during the susceptible interaction with *A. euteiches*, yet this fungus exhibits exceptional sensitivity to pisatin in in vitro bioassays (11,12,15). Pueppke and VanEtten (12) attempted to resolve this apparent anomaly. They concluded that the fungus did indeed encounter high pisatin concentrations in infected tissue, casting doubt on the argument that the anomaly could be explained by a physical separation of the fungus and the phytoalexin in this disease interaction. Also, they indicated that the fungus could not metabolize pisatin, nor could it become tolerant to pisatin by prior exposure to low concentrations of the phytoalexin. Further, they failed to find enhanced pisatin tolerance in *A. euteiches* when low molecular weight extracts from healthy or infected plant tissue were added to bioassay media. This last experiment was performed to evaluate the possibility that a molecule other than pisatin might induce pisatin tolerance in *A. euteiches*.

In preliminary experiments we found that addition of autoclaved pea tissue to bioassay medium did enhance the pisatin tolerance of *A. euteiches*. In further experiments, addition of ethanol extracts of pea tissue to bioassay medium also produced partial relief of the pisatin sensitivity of *A. euteiches*. The purpose of this present study was to purify the factor in the ethanol extracts that caused increased tolerance to pisatin.

MATERIALS AND METHODS

Fungal cultures. Cultures were obtained from the following sources: *Aphanomyces euteiches* and *A. e.* Drechs. f. sp. *phaseoli* Pfend. & Hag. (9) (Dr. D. Hagedorn, University of Wisconsin, Madison); *Neurospora crassa* Shear & Dodge (Dr. A. Srb, Cornell University, Ithaca, NY); *Fusarium solani* f. sp. *cucurbitae* Snyder and Hansen (our lab stocks, isolate T-145). All cultures were maintained and grown as inoculum for radial growth bioassays on potato-dextrose agar (PDA) at 24 C in the dark.

Bioassays. Radial growth bioassays were a modification of the method of Pueppke and VanEtten (12). These bioassays were performed in 35- × 10-mm plastic petri dishes containing 1 ml of glucose-peptone (GP) agar (10 g of peptone, 5 g of glucose, 20 g of agar per liter of 0.04 M potassium phosphate buffer, pH 6.5). Phytoalexins in dimethylsulfoxide (DMSO) were added to the petri dishes before the addition of molten agar. The final DMSO concentration was 0.5% except where noted. A 5-mm agar plug, cut 1 cm behind the edge of a mycelial colony grown on PDA, was placed on the medium in the center of each bioassay plate. The inoculated plates were kept at 24 C in the dark. Fungal growth was determined periodically by measuring and averaging two perpendicular colony diameters and then subtracting the diameter of the inoculum plug. For *N. crassa*, the mycelial plug was placed at the edge of the petri dish, and colony radius instead of diameter was determined. The data reported for the radial growth bioassays are the means of two plates. Variation between these plates rarely exceeded 10%. All experiments shown were replicated.

Growth of *A. euteiches* in liquid media was measured as described previously (11). Briefly, 8-mm mycelial disks were cut from colonies grown on bacterial filters (Gelman Metricel membranes, Ann Arbor, MI) overlaid on GP agar. Four mycelial disks were placed in 4 ml of liquid GP medium contained in a 25-ml Erlenmeyer flask. Pisatin dissolved in DMSO was added to the medium to give a final DMSO concentration of 0.5%. The flasks were incubated with rotary shaking (50 rpm) at 24 C. After 72 hr, the entire contents of the flasks were harvested by vacuum filtration and the dry weight determined as described previously (12). To determine the ability of *A. euteiches* to metabolize pisatin, mycelial disks were added to GP medium as described above except that the medium contained 30 μg of ^{14}C-pisatin (50 μCi/mmole) per milliliter. After 3 days, a 1-ml aliquot was removed and added to 4 ml of scintillation fluid (0.55% 2,5-diphenyloxazole in toluene). In this two-phase system, the radioactive pisatin partitions into the organic phase and is efficiently counted (17).

To study the distribution of pisatin between the medium and the mycelium, a mycelial disk was added to 1 ml of GP medium containing 90 μg of ^{14}C-pisatin (77 μCi/mmole) and 0.5% DMSO. The cultures were shaken at 100 rpm and after 30 min an aliquot of mycelium-free medium was added to 14 ml of Aquasol (New England Nuclear, Boston, MA). The intact mycelial disk was then removed, drained momentarily on a paper towel, and added to 14 ml of scintillation fluid.

Growth and extraction of plant tissue. Seeds were obtained from the following sources: *Pisum sativum* (cultivar Alaska, from Asgrow, Kalamazoo, MI); *Cucurbita maxima* Duchesne (cultivar Pink Banana, from Gurney, Yankton, SD); and *Phaseolus vulgaris* L. (cultivar Top Crop, from Herbst Brothers, Brewster, NY). Seedlings were grown in the dark at room temperature in trays of

vermiculite supplemented with Hoagland's solution (5). Etiolated epicotyls (pea) or hypocotyls (squash or green bean) from 9–10-day-old seedlings were harvested and homogenized with a blender for 1 min in 95% ethanol (4 ml per gram of tissue). The plant debris was removed by filtration after 2–4 hr at room temperature. The ethanol was then removed from the filtrate under vacuum. The resulting aqueous suspension was diluted with H_2O to equal 0.5 ml per gram of original tissue and adjusted to pH 2.5 with concentrated HCl. A precipitate formed within 15 min and was collected by centrifugation (3,000 *g* for 10 min). The pellet was dissolved in a small volume of methanol:chloroform:H_2O (2:1:0.8). This solution was transferred to a separatory funnel and more of the same solvent mixture was added until the final volume was equal to 0.75 ml of methanol per gram of original tissue. Chloroform and then H_2O were added so that the final solvent ratio was 2:2:1.8 (methanol:chloroform:H_2O). This partitioning procedure resulted in a two-phase system with the chloroform phase containing lipids (1). The chloroform phase was taken to dryness. The residue was dissolved in a small volume of hexane and then partitioned between *n*-hexane and H_2O (1:1, one volume equal to 1.5 ml per gram of original tissue). The aqueous phase was discarded. Polar lipids were extracted from the hexane with 60% ethanol (3 ml per gram of original tissue). (All references to lipids or polar lipids in this report refer to this 60% ethanol fraction.) The ethanol phase was taken to dryness and the residue dissolved in a few milliliters of chloroform. This chloroform solution was stored at −20 C in a sealed, argon-purged vial. The chloroform solution was added to hot liquid or agar medium that was subsequently held at 65 C for 1 hr before use in bioassays. This treatment eliminated the chloroform and the resulting medium was slightly cloudy but free of precipitates.

Several different extraction and partitioning methods were tested. The procedure described above gave the greatest activity (i.e., the most relief of radial growth inhibition of *A. euteiches* on medium supplemented with the extract and pisatin/dry weight of extract residue). The experiments reported in this paper were performed with two separate preparations that were made using this procedure. Both preparations showed similar activity. The yield of polar lipids using the above procedure was approximately 0.5 mg per gram of original tissue.

Chemicals. Pisatin (18), [3-0-methyl-^{14}C]pisatin (17), (-)-maackiain (16, the crystallization method), and phaseollin (6) were purified by published procedures. Compounds were quantified in ethanol using published molar extinction coefficients: logϵ = 3.86 at 309 nm for pisatin (8), logϵ = 3.90 at 310 nm for maackiain (16), logϵ = 4.04 at 280 nm for phaseollin (14). Phosphatidylcholine (Type III-S, from soybean, 99%) was purchased from Sigma Chemical Company, St. Louis, MO.

Pisatin was also prepared by the following method. This procedure consistently yielded more pisatin with less labor than the previously mentioned method (18). One hundred grams of pea seeds (Alaska) was surface sterilized with 500 ml of ethanol:commercial bleach:water (75:5:20) for 15 min. The seeds were then rinsed with sterile water and transferred aseptically to a sterile enamel pan (40 × 23 × 7 cm) containing 400 ml of water and a single layer of cheesecloth. The pan was covered with aluminum foil and the peas were maintained aseptically at 18 C. After 4 days the peas were covered with 10 mM $CuCl_2$ for 1 hr. The $CuCl_2$ was drained off and the peas were incubated in the dark under aseptic conditions at 18 C for seven more days. The peas were then transferred to a large flask, covered with hexane (analytical grade), and extracted for 12–18 hr with slow (100 rpm) rotary shaking. The hexane was taken to dryness and the residue solubilized in 100–200 ml of methanol. The methanol was then held at −20 C overnight and a cloudy white precipitate formed, which was removed by filtration. The methanol was then taken to dryness and the residue solubilized in 200 ml of 2 N NaOH in MeOH. This solution was held at 50 C for 1 hr. $CHCl_3$ and water were then added so that the final ratios were 1:0.8:1 (MeOH:H O:$CHCl_3$). A two-phase system resulted with the pisatin partitioning into the $CHCl_3$ phase. After these steps the $CHCl_3$ phase was colorless and with a single chromatography step (e.g., thin-layer chromatography [TLC] on silica gel using dichloromethane:methylethylketone [30:1] as a solvent system) pisatin with a purity of 99% was obtained.

RESULTS

Effect of pea polar lipids on pisatin inhibition of *A. euteiches*. As previously reported (12), pisatin concentrations near 100 μg/ml almost completely inhibited the growth of *A. euteiches* on GP medium, whether growth was measured by colony diameter on agar-containing medium (Fig. 1) or by mycelial weight in liquid medium (Table 1). On GP agar, growth that did occur in the presence of 90 μg of pisatin per milliliter was in the form of a sparse colony.

When polar lipids extracted from pea tissue were incorporated into GP agar, radial growth of *A. euteiches* was slightly slower than when the lipids were not included, but the colony appeared denser than on media without the lipids. In liquid cultures, addition of the lipid extract resulted in a 20% increase in dry weight accumulation. In addition to these effects, the lipids also decreased the sensitivity of the fungus to pisatin. In the presence of pisatin (90 μg/ml), growth on lipid-containing agar medium was faster than on pisatin-containing medium that lacked the lipids (Fig. 1) and was also more dense. The relief of pisatin inhibition was less complete in liquid medium, but nonetheless represented an increase in dry weight accumulation by more than 10-fold (Table 1).

The relief of pisatin inhibition by the lipids (1 mg/ml) decreased substantially at higher pisatin concentrations (> 135 g/ml). Lipid concentrations higher than 1 mg/ml only slightly increased tolerance to pisatin (90 μg/ml), but at lipid concentrations lower

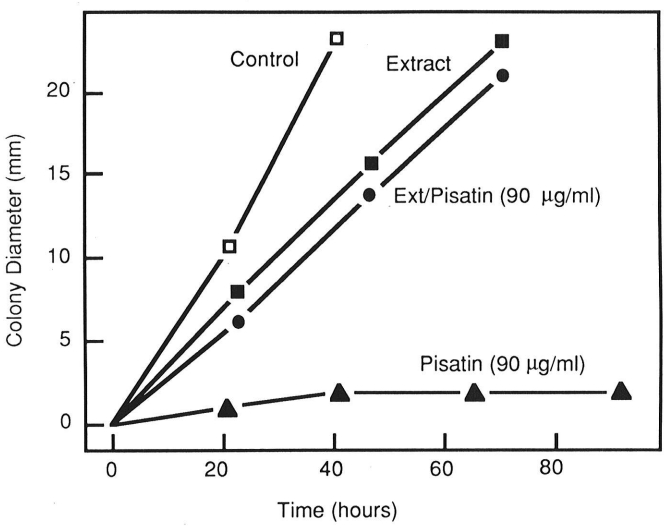

Fig. 1. Effect of pea lipid extract on the pisatin sensitivity of *Aphanomyces euteiches*. Radial growth on glucose-peptone medium amended with lipids from pea (1 mg/ml) and/or pisatin (90 μg/ml). EXT = pea lipid extract.

TABLE 1. Effect of a lipid extract from pea and phosphatidylcholine (PC) on growth of *A. euteiches* in liquid medium[a]

Medium	Net fungal dry weight (mg/flask)	
	Control	Pisatin (90 μg/ml)
Glucose-peptone	18.4 ± 1.6	0.2 ± 0.5
Glucose-peptone + lipid extract (1 mg/ml)	22.7 ± 0.6	7.5 ± 0.9
Glucose-peptone + phosphatidylcholine (1 mg/ml)	21.1 ± 1.1	21.3 ± 1.4

[a] Flasks contained 4 ml of glucose-peptone medium and four *A. euteiches* mycelial disks. Flasks were grown at 24 C with rotary shaking and harvested after 3 days. All cultures contained 0.5% dimethylsulfoxide. Net fungal dry weight equals the final weight minus the weight of the original mycelial disks. Three replicates per treatment were used, and the values represent the average with the standard deviation.

than 0.5 mg/ml, pisatin tolerance decreased significantly (data not shown). Therefore the pisatin and lipid extract concentrations used for the remaining studies were 90 μg/ml and 1 mg/ml, respectively.

Effect of pea polar lipids on the sensitivity of other fungi to pisatin. Three additional fungi were used to test the specificity of the polar lipid extract. *N. crassa* and *F. s.* f. sp. *cucurbitae* did not show enhanced pisatin tolerance when grown on medium supplemented with polar lipids from pea (Figs. 2 and 3). *A. e.* f. sp. *phaseoli*, however, did show enhanced pisatin tolerance (Fig. 4).

Effect of pea polar lipids on inhibition by other phytoalexins. Phaseollin and maackiain, like pisatin, are pterocarpan phytoalexins and thus have the same benzofuran-benzopyran fused ring structure. The presence of 18 μg of phaseollin per milliliter or 40 μg of maackiain per milliliter produced nearly complete inhibition of *A. euteiches* radial growth, comparable to the effect produced by 90 μg of pisatin per milliliter. On a medium supplemented with polar lipids from pea (1 mg/ml), the sensitivity of *A. euteiches* to phaseollin and maackiain decreased. The decrease in sensitivity to phaseollin was similar to that seen for pisatin at 90 μg/ml on lipid-amended vs. control medium. The decrease in sensitivity to maackiain on lipid-amended medium was of a lesser magnitude.

Effect of polar lipids from other plants. Polar lipid extracts from etiolated squash and bean hypocotyls, prepared in the same way as those from peas, also increased the pisatin tolerance of *A. euteiches*. When lipids from these plants were incorporated at 1 mg/ml into GP agar containing 90 μg of pisatin per milliliter, the radial growth rate and mycelial density were similar to those observed with pea lipids.

Comparison of pea lipids with phosphatidylcholine. Bull (2) found that fungal tolerance to phytoalexins could be increased by phospholipids, a possible component of the active extract from peas. Therefore, the effect of a purified phosphatidylcholine preparation on the antifungal activity of pisatin was tested. This phospholipid produced a high degree of pisatin tolerance in *A. euteiches* in both radial growth (Fig. 5) and dry weight (Table 1) bioassays. Unlike the pea extract, however, there was no differential effect on fungi as it greatly increased the pisatin tolerance of *F. s.* f. sp. *cucurbitae* and *N. crassa*, as well as *A. e.* f. sp. *phaseoli* (data not shown).

Chemical nature of the tolerance-enhancing factor from pea. The pisatin tolerance-enhancing activity measured in the initial ethanol-soluble fraction of pea epicotyls was recovered in good yield by the partial purification procedure scheme described in Materials and Methods; discarded fractions contained little or no activity. Thus the chemical properties of the active components were those of a relatively polar lipid material.

Attempts at further purification of the active factor included TLC on silica gel, alumina, and cellulose and high-pressure liquid chromatography on C-18 silica gel, each in several solvent systems. Several major chemically detectable components were resolved by these procedures, but none of the fractions contained a significant yield of the original tolerance-enhancing activity. These results suggested that either the active principle was degraded during chromatography or that biological activity was produced by a mixture of compounds.

To distinguish between these two possibilities a sample of the lipid extract was separated by TLC, and the silica gel from the whole plate was eluted as a single pool. This chromatographed, recombined material relieved pisatin inhibition of *A. euteiches* approximately as effectively as a separate sample that had not been chromatographed (Fig. 6). When the TLC plate was divided into four zones, which together included all of the material, little or no relief of inhibition was obtained with any of the fractions (Fig. 6). Therefore, the tolerance-enhancing activity of the pea lipid extract appears to be due to at least two chromatographically separable components. A single attempt was made to recombine the fractions in all possible pairs. None of these combinations produced significant relief of pisatin inhibition. Further evidence for the multiple component nature of the tolerance-enhancing activity came from an attempt to improve the final 60% ethanol:hexane partitioning. When the partitioning was between 90% MeOH and

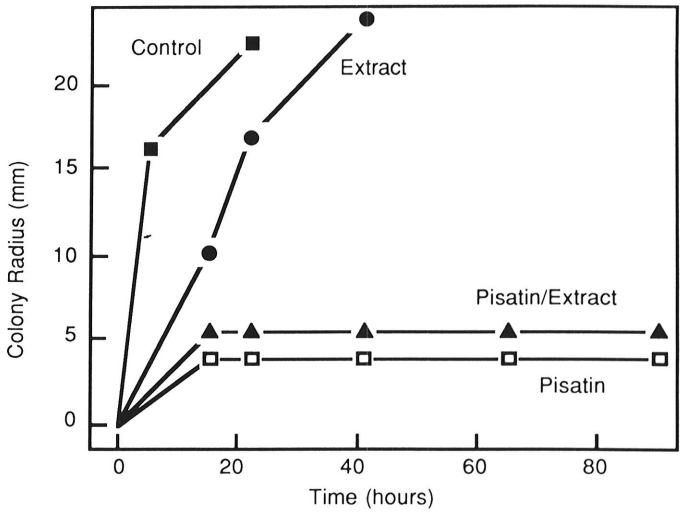

Fig. 2. Effect of pea lipid extract on the pisatin sensitivity of *Neurospora crassa*. Radial growth on glucose-peptone medium amended with lipids from pea (1 mg/ml) and/or pisatin (90 μg/ml).

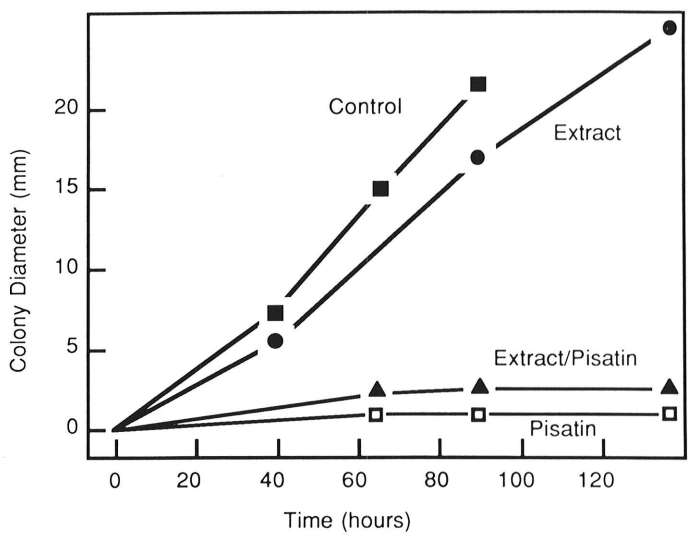

Fig. 3. Effect of pea lipid extract on the pisatin sensitivity of *Fusarium solani* f. sp. *cucurbitae*. Radial growth on glucose-peptone medium amended with lipids from pea (1 mg/ml) and/or pisatin (90 μg/ml).

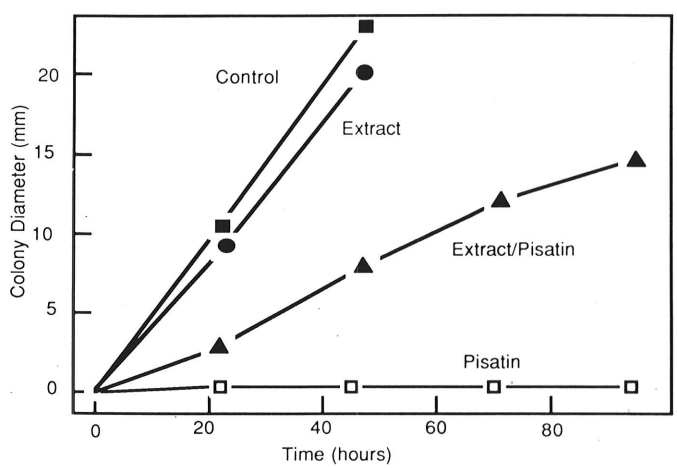

Fig. 4. Effect of pea lipid extract on the pisatin sensitivity of *Aphanomyces euteiches* f. sp. *phaseoli*. Radial growth on glucose-peptone medium amended with lipids from pea (1 mg/ml) and/or pisatin (90 μg/ml).

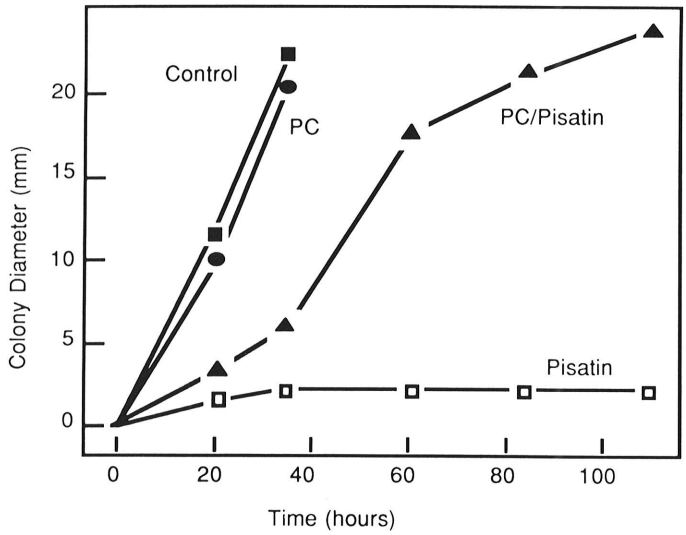

Fig. 5. Effect of phosphatidylcholine on the pisatin sensitivity of *Aphanomyces euteiches*. Glucose peptone medium was amended with phosphatidylcholine (1 mg/ml) and/or pisatin (90 μg/ml). PC = phosphatidylcholine.

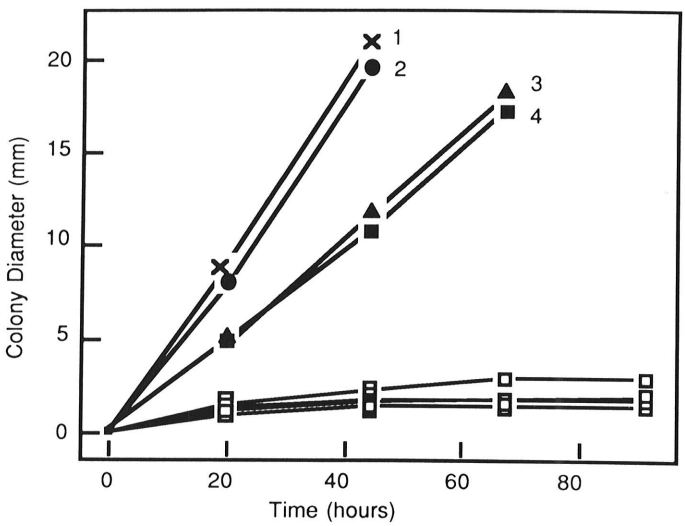

Fig. 6. Effect of chromatography on the activity of the pea lipid extract towards *Aphanomyces euteiches*. The polar lipids (1 mg) were chromatographed on thin-layer chromatography plates (silica gel, 250 μm; solvent system $CHCl_3$:MeOH, 4:1). Two plates were run in parallel. The silica gel was removed from the plates and eluted with MeOH. The MeOH was then taken to dryness and the residue dissolved in molten glucose-peptone medium. With one plate the silica gel from the entire plate was removed, eluted, and the residue used in bioassays with **(3)** and without **(2)** 90 μg of pisatin per milliliter. The other plate was divided into four horizontal bands. The silica gel was removed from these areas, eluted, and the residues used individually in bioassays with 90 μg of pisatin per milliliter (the lower four lines on the graph). (Controls of these bands are not shown. Growth of these controls was similar to (1) below.) Additional controls were polar lipids (1 mg/ml) that had never been chromatographed, with **(4)** and without **(1)** 90 μg of pisatin per milliliter.

hexane little activity was recovered from either fraction.

Possible mechanisms of tolerance enhancement by polar lipids. Two possible mechanisms of action of the pea lipids or phosphatidylcholine are: stimulation of pisatin degradation and decreased uptake of pisatin by the mycelium in lipid-containing medium.

A. euteiches has been reported to initiate pisatin metabolism by demethylating pisatin at the 3-0-methyl position (10). However, disappearance of [3-0-methyl-^{14}C]-pisatin from *A. euteiches* cultures was slow (29–25% after 3 days) and was approximately equal in pea lipid-, phosphatidylcholine-, and nonamended GP medium. In a separate experiment nonradioactive pisatin was used. No known degradation products of pisatin were found after extraction of the cultures and TLC of the extracts.

Table 2 presents data from an experiment that evaluated the possibility that lipids affect the absorption of pisatin from the medium. Liquid culture conditions were equivalent to the initial conditions in the dry weight and pisatin degradation experiments. One half hour after addition of ^{14}C-pisatin, the mycelium incubated in phosphatidylcholine-amended medium contained only 30% as much pisatin as mycelium incubated in either pea polar lipid- or nonamended medium (Table 2). In two additional experiments (data not shown), the method of inoculum production differed slightly from the experiment shown in Table 2, and more mycelium was used in the assay. All experiments clearly showed that addition of phosphatidylcholine reduced the amount of pisatin found in the mycelium. Incorporation of pea polar lipids did not have this effect.

TABLE 2. Effect of a lipid extract from peas and phosphatidylcholine on the partitioning of pisatin between mycelium and medium[a]

	^{14}C-pisatin (cpm)	
Medium	Medium	Mycelium
Glucose-peptone (GP)	36,100 ± 1,700	5,200 ± 500
GP + lipid extract (1 mg/ml)	37,500 ± 1,800	5,100 ± 1,100
GP + phosphatidylcholine (1 mg/ml)	40,500± 1,100	1,500 ± 400

[a] One *Aphanomyces euteiches* mycelial disk was incubated in 1 ml of glucose-peptone (GP) that contained ^{14}C-pisatin (90 μg/ml, 77 μCi/mmol) in 0.5% dimethylsulfoxide. After 30 min the medium and the intact mycelial disks were removed, added to scintillation fluid, and counted. Six replicates per treatment were used. The values are the average with the standard deviation.

DISCUSSION

Pisatin inhibition of *A. euteiches* has two notable features: the exceptional pisatin sensitivity of the fungus and the inconsistency of bioassay results with the phytoalexin theory (11). The data presented here demonstrate that the sensitivity of *A. euteiches* to pisatin can be reduced by incorporating polar lipids into the growth medium. Certainly the decreased pisatin sensitivity produced by pea lipids is not highly specific because 1) lipids from other plant sources are also effective, 2) tolerance is produced to other phytoalexins, and 3) tolerance is enhanced in a fungus, *A. e.* f. sp. *phaseoli*, that is not a pea pathogen (9, J. Sweigard, *unpublished results*).

Modification of the growth medium has also altered the toxicity of phytoalexins to other fungi (13). Especially relevant to our data, Bull (2) found that phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine relieved the inhibition of *Rhizoctonia solani* by phaseollin and kievitone. The mechanism for this tolerance was not determined. The results of our study suggest that phosphatidylcholine reduces the uptake of pisatin by fungi, perhaps in a nonspecific way because this compound increased the pisatin tolerance of all fungi tested. The activity of the pea lipid extract appeared to involve some other mechanism because it affected these fungi differentially and because no change in pisatin uptake was detected.

Other mechanisms that may account for the activity of the extract include: 1) induction of a nondegradative tolerance mechanism (4), 2) inactivation or masking of a potential pisatin binding site(s), or 3) use of the lipids by the fungus to alter cell membranes or to repair cell membrane damage caused by pisatin. The last possibility seems especially plausible because membranes appear to be one site of phytoalexin action (13).

In vitro bioassays are used to assess the toxicity of phytoalexins. These assays obviously simplify the complex environment of the disease interaction. The data presented here and those of Bull (2)

show that certain lipids or lipid extracts can significantly alter phytoalexin toxicity. Incorporation of relatively large quantities of lipids into bioassay media in these studies no doubt does not reflect the actual disease situation. Nonetheless, lipids represent a major molecular class and their relevance should be addressed in future phytoalexin toxicity studies.

Still unanswered is whether the enhanced pisatin tolerance of *A. euteiches* can explain the inconsistency between the in vitro bioassays and the apparent disease situation (11). Two cubic centimeters of pea tissue are required to produce 1 mg of polar lipids. When this amount of polar lipids was added to 1 ml of medium only moderate tolerance to a relatively low pisatin concentration (90 μg/ml) occurred, especially in liquid culture bioassays (Table 1). Pisatin concentrations up to 4 mg/ml have been measured in *A. euteiches* lesions (11). Nevertheless, the actual effective phytoalexin concentration that a pathogen encounters in the plant has never been determined (7). The lipid concentration in a lesion has not been measured either. To argue that polar lipids do or do not play a role in the interaction between *A. euteiches* and pisatin in the plant therefore seems premature.

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