Fungistatic Activity of Propyl-N-(γ-dimethylaminopropyl) carbamate on Pythium spp. and its Reversal by Sterols

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ABSTRACT

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In vitro fungistatic activity of the new systemic fungicide propyl-N-(γ-dimethylaminopropyl)carbamate HCl [PDAC, SN66752, propamocarb (proposed)] was studied in solid and liquid synthetic medium (BM-1) against eight *Pythium* spp. The PDAC at concentrations up to 5 μg active ingredient (a.i.)/ml was less fungistatic to *Pythium* spp. than was its S-analog, N-(3-dimethylaminopropyl) thiocarbamic acid Shylester HCl [DTEH, SN41703, prothiocarb (proposed)]. The dosage-response curves for PDAC were linear from 0.5 to 10 μg a.i./ml, with ED_{50} values ranging from 2.5 to 5 μg/ml for all species except *P. myriotylum* which had an ED_{50} value of about 0.1 μg a.i./ml or less. Four *Pythium* spp. (not including *P. myriotylum*) were more sensitive to PDAC at pH of 7.4 than at 6.4 or 5.3. Cholesterol added to BM-1 at 5-30 μg/ml reversed the fungistatic activity of PDAC, but the magnitude of reversal depended on PDAC concentration in BM-1 and on the *Pythium* sp. used. Cholestanol and sitosterol, but not lanosterol, also reversed activity. With *P. myriotylum,* even 30 μg/ml of cholesterol did not reverse activity. Greater amounts of cellular constituents (proteins, carbohydrates, amino acids, and soluble salts) leaked from mycelia of *Pythium* grown in liquid BM-1 supplemented with 0.5 μg a.i./ml PDAC than from mycelia grown in PDAC-free medium. In general, the rate of leakage was constant over a 7-hr period for most cell constituents except soluble salts. Cholesterol added to BM-1 reduced leakage of cell constituents. When added with PDAC, cholesterol reversed the effects of the fungicide on mycelium cell membrane permeability.

Recently, a new fungicide, N-(3-dimethylaminopropyl) thiocarbamic acid S-ethylester HCl [DTEH, SN41703, prothiocarb (proposed)], became available for experimentation with Phycomycetes (21). The fungicide was toxic to at least nine species of *Pythium* (16). When applied to the seed, DTEH reduced Pythium blight of snapbeans (*Phaseolus vulgaris* L.) in the greenhouse and the field, and significantly increased yield in the field (16). In 1977 the oxygen analog of DTEH, propyl-N-(γ-dimethylaminopropyl)carbamate HCl [PDAC, SN66752, propamocarb (proposed)], also became available for experimentation.

Pythiaceous fungi lack the ability to synthesize sterols (8). Exogenous sterols, however, stimulate growth and reproduction in this group of fungi (2, 5, 6, 7, 8, 19) and are incorporated into intracellular membrane systems affecting cell wall permeability (20) and other phenomena (8). Chemicals may be toxic to pathogenic fungi, including Phycomycetes, because they adversely affect cell membrane permeability (4, 10, 11, 22). The experiments reported here were undertaken to determine the fungistatic activity of PDAC to *Pythium* spp. with and without exogenous sterols. A preliminary report has been presented (17).

MATERIALS AND METHODS

In vitro fungistatic activity of PDAC to eight *Pythium* spp. was studied on solid (20 ml per 9-cm diameter petri dish) or liquid (40 ml/250-ml Erlenmeyer flask) synthetic medium (basal medium 1, BM-1) described previously (15). The pH of BM-1 was adjusted with phosphate buffer so that it was 6.9-7.0 after autoclaving. This pH value was chosen because it was reported (21) that DTEH and its analogs were more fungistatic at near neutral or slightly alkaline pH values than at acid pH values.

The PDAC [70% active ingredient (a.i.) in water] was diluted in sterile distilled water and added in appropriate quantities (w/v) to the medium after it was autoclaved and before it was dispensed to flasks or petri dishes. The fungicide was tested in liquid and solid medium at 0.0 (control), 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10, 50, 100, and 300 μg a.i./ml of BM-1. Disks 5 mm in diameter of 5-day-old *Pythium* spp. colonies grown on fungicide-free BM-1 (lacking sterols) were transferred to the center of petri

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dishes or to flasks. Colony radii on solid media were measured at 7 and 14 days and dry weights of mycelial mats of flask cultures were determined after the same intervals. Each treatment was replicated five times and all experiments were performed three times.

For study of the effect of pH on the growth of five *Pythium* spp., BM-1 medium was autoclaved, divided into three portions, and adjusted with sterile NaOH or HCl solutions to pH values of 5.3, 6.4, and 7.4. The media were buffered at these values with phosphate buffer and the PDAC was added to the media at 5 μg a.i./ml. Batches of BM-1 adjusted to the three values without PDAC were the controls.

The effects of sterols on the fungistatic activity of PDAC also were studied. In one experiment cholesterol, sitosterol, lanosterol, and cholestanol were dissolved in ethanol and added to autoclaved liquid BM-1 (20 μg sterol/ml of BM-1). In another experiment cholesterol was added to BM-1 at 0, 5, and 30 μg/ml and the fungicide at 0, 10, and 50 μg a.i./ml.

To determine whether toxicity of PDAC to *Pythium* spp. was due to leakage of cell constituents from mycelium, *P. ultimum* Trow or *P. myriotylum* Drechsler was grown in liquid BM-1 supplemented with cholesterol (20 μg/ml), PDAC (0.5 μg/ml for *P. myriotylum* or 1 μg a.i./ml for *P. ultimum*), or both. Mycelia were harvested during the exponential growth phase (after 6 days of incubation), pooled, washed with sterile distilled water, resuspended in 100 ml of distilled water, and maintained at 25 C. After 1, 2.5, 4, and 7 hr mycelia were filtered from the solutions for dry weight determinations and the aqueous solutions were analyzed for soluble protein, carbohydrates, amino acids, and inorganic salts. All data were analyzed statistically.

Protein was determined by the Lowry method (12). Carbohydrate and amino acids were determined as anthrone (+) materials and ninhydrin (+) materials by the methods of Morris (13) and Rosen (18), respectively, and the amounts are given as mg released/g dry weight of mycelium. Total soluble inorganic salts were measured with a conductivity bridge and specific conductance was given as μmhos/g dry weight of mycelium (9). Phosphate-P ($PO_4^{\equiv}$-P) was determined with the vanadomolybdophosphoric method (9).

RESULTS

Fungistatic activity of PDAC in vitro.—At fungicide concentrations up to 5 μg a.i./ml of solid BM-1, the PDAC was less toxic to *Pythium* spp. than DTEH (16). On solid BM-1 the ED_{50} values for *P. aphanidermatum* (Edson) Fitzpatrick, *P. debaryanum* Hesse, and *P. splendens* Braun were from 2.5 to 5 μg PDAC/ml (Fig. 1-A, whereas ED_{50} values for DTEH were 0.5 μg a.i./ml or less. At the lower concentrations used, *P. myriotylum* was the most sensitive species tested. In liquid BM-1, *P. aphanidermatum, P. debaryanum,* and *P. splendens* were very sensitive to PDAC at concentrations ranging from 0.5 to 10 μg/ml (Fig. 1-B). Maximum inhibition of growth occurred at 10 μg/ml and above. Maximum inhibition of growth of *P. myriotylum* occurred at 0.25 μg PDAC/ml. Response curves of *P. aphanidermatum* to dosages were similar to those of *P. arrhenomanes* Drechsler, *P. butleri* Subramanian, *P. irregulare* Buisman, and *P. ultimum.*

Effect of pH of the growth medium.—In-vitro inhibition of growth of *P. aphanidermatum, P. arrhenomanes, P. splendens,* and *P. ultimum* by PDAC was pH-dependent, with growth most suppressed at pH 7.4 and the least suppressed at pH 5.3 (Fig. 2). The degree of pH-dependent suppression, however, was determined by the *Pythium* sp. used. *Pythium myriotylum* was just as sensitive to PDAC (5 μg a.i./ml) at pH 5.3 as at 7.4.

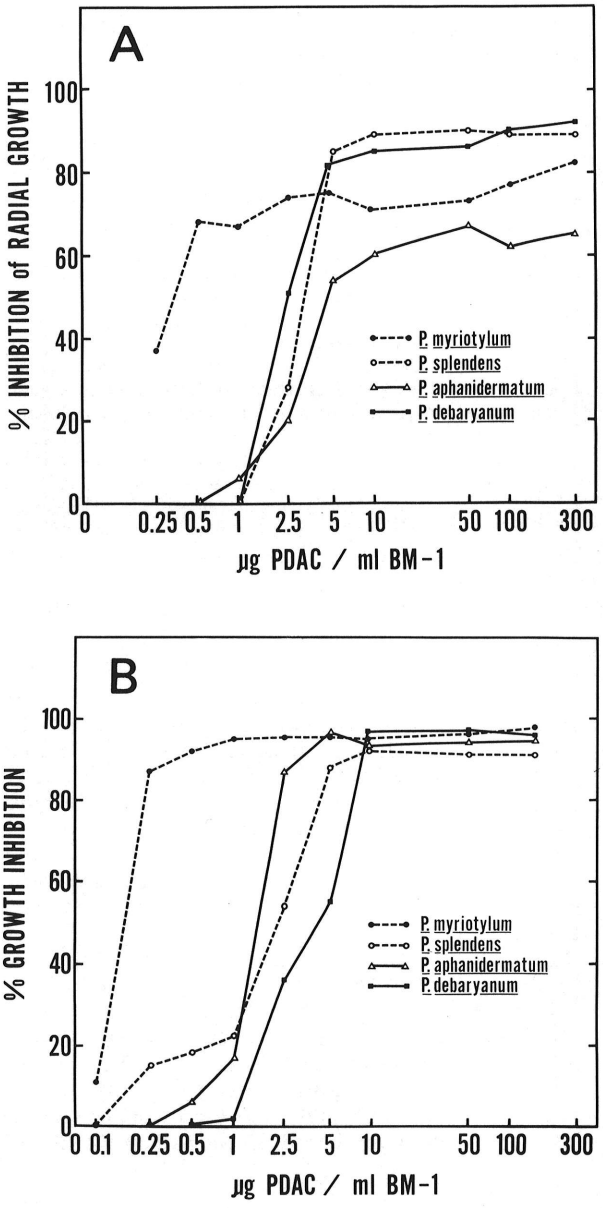

Fig. 1-(A,B). Rate-response curves of four representative *Pythium* spp. on basal medium (BM-1) amended with propyl-N-(γ-dimethylaminopropyl) carbamate HCl (PDAC). **A)** Effect of PDAC on radial growth on solid BM-1. **B)** Effect of PDAC on growth as measured by dry weight of mycelium in liquid BM-1.

Effect of sterols on PDAC activity.—Cholesterol, cholestanol, and sitosterol, but not lanosterol, at 20 μg/ml of BM-1 reduced or annulled the inhibition by PDAC (10 μg a.i./ml) of growth of *P. ultimum* but not that of *P. myriotylum* (Fig. 3). The magnitude of the reversal of toxicity depended on the concentration of PDAC in BM-1 and on the *Pythium* isolate used (Fig. 4). With *P. myriotylum* even 30 μg/ml of cholesterol did not reduce the fungistatic activity of PDAC. With *P. irregulare* 5 μg/ml of cholesterol completely annulled activity of PDAC, even at 50 μg a.i./ml of the fungicide.

Effect of PDAC on mycelial cell permeability and reversibility of the PDAC effect by sterols.—In *P. ultimum,* leakage of all materials assayed (soluble salts, anthrone(+) substances, ninhydrin(+) substances, $PO_4^{\equiv}$-P, and protein) was greater from mycelia grown in liquid BM-1 supplemented with PDAC (0.5 μg a.i./ml) than from mycelia grown in nonsupplemented medium (Table 1). In *P. myriotylum,* there were no differences in leakage of anthrone(+) substances and soluble protein from its mycelia grown in supplemented or nonsupplemented medium. Significant differences, however, were observed in the leakage of ninhydrin(+) substances, $PO_4^{\equiv}$-P, and inorganic salts between *P. myriotylum* mycelia and those of *P. ultimum.* In general, cholesterol added alone to BM-1 reduced leakage of mycelial constituents from both *Pythium* spp. compared with leakage from the nonsupplemented controls. In all instances, cholesterol added to the growth medium at 20 μg/ml reversed the effect of PDAC on leakage of cell constituents.

Pythium ultimum was grown in BM-1 with and without sterol to determine whether PDAC damaged *Pythium* cell membranes. After 6 days of growth in PDAC-free BM-1 the mycelium was harvested, washed with sterile distilled water, and suspended in distilled water containing various concentrations of PDAC up to 20 μg/ml. Even at 20 μg/ml PDAC did not affect cell wall permeability and leakage from mycelia whether previously grown in the presence or absence of sterol.

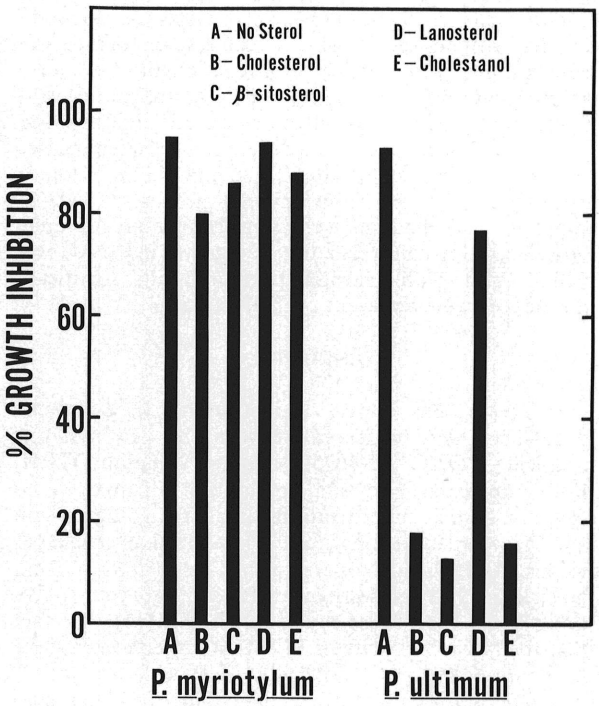

Fig. 3. Effect of sterols added to liquid basal medium 1 (BM-1) at 20 μg sterol/ml on growth inhibition of *Pythium myriotylum* and *P. ultimum* by propyl-N-(γ-dimethylaminopropyl) carbamate HCl (PDAC) (10 μg PDAC a.i./ml).

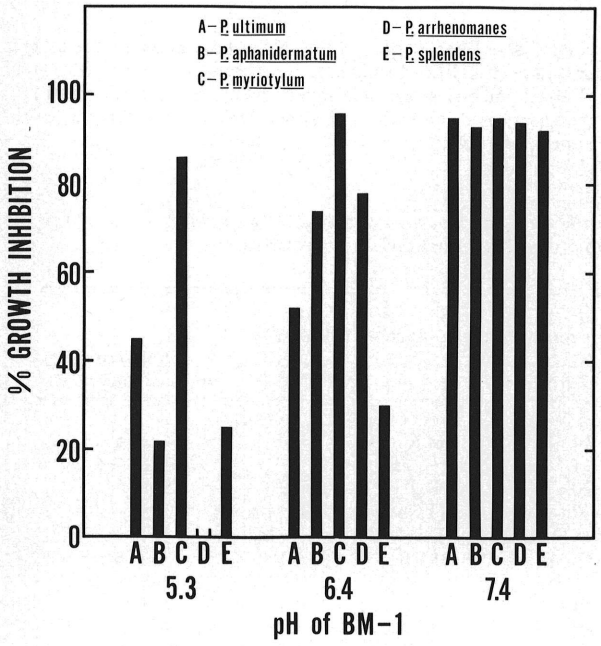

Fig. 2. Effect of pH of liquid basal medium 1 (BM-1) on growth inhibition of five *Pythium* spp. by propyl-N-(γ-dimethylaminopropyl) carbamate HCl (PDAC) (5 μg PDAC a.i./ml).

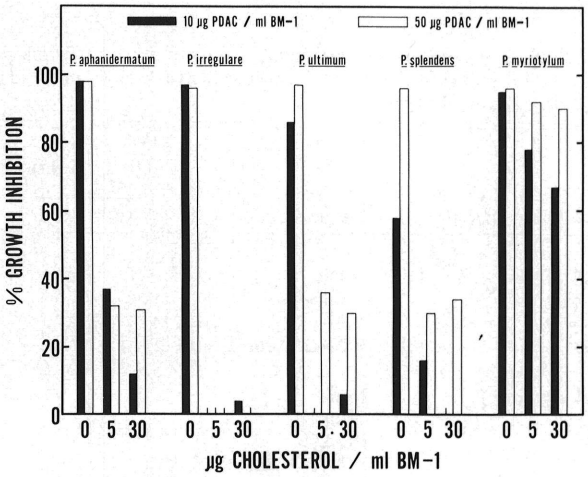

Fig. 4. Effect of cholesterol added to liquid basal medium 1 (BM-1) at 0, 5, and 30 μg/ml on growth inhibition of five *Pythium* spp. by propyl-N-(γ-dimethylaminopropyl) carbamate HCl (PDAC) (10 and 50 μg PDAC a.i./ml).

Pythium ultimum was grown in liquid BM-1 without PDAC and in BM-1supplemented with PDAC at 1 μg a.i./ml to determine rate of leakage from mycelia. After 6 days of growth, the mycelia were removed, washed in distilled water, and suspended in distilled water for the assays. To determine rate of leakage, we withdrew the mycelium for dry weight determination for each time period and assayed the suspension liquid from separate containers for assay of soluble constituents 1, 2.5, 4, and 7 hr after suspension. In general, the rate of leakage was constant over a 7-hr period for all cell constituents except soluble salts (Fig. 5). The rate of release of soluble salts from the mycelium was only constant for an initial 4-hr period. At 7 hr there were no differences between the two curves for soluble salts. After 2.5, 4, and 7 hr the amounts of materials leaked from mycelia grown in PDAC-supplemented medium were significantly greater than those leaked from mycelia that had grown in PDAC-free BM-1. With soluble carbohydrates and salts, significant differences were apparent even after 1 hr.

DISCUSSION

In the present studies several similarities and some differences were observed between the new systemic fungicide PDAC (SN66752) and its S-analog DTEH. Both fungicides had similar patterns of toxicity to *Pythium* spp. at concentrations higher than 5 μg a.i./ml; and they were more fungistatic at alkaline than at acid pH values. At fungicide concentrations below 5 μg a.i./ml, however, the toxicity values for PDAC were four to five times lower than those found for DTEH (16). The fact that the in vitro toxicity of PDAC at low concentrations to *Pythium* spp., and especially to *P. ultimum* and *P. aphanidermatum,* was lower than that of DTEH may explain why PDAC was less effective than DTEH in reducing Pythium blight of bean in the field in early plantings caused by these two species (G. C. Papavizas, and J. A. Lewis, *unpublished*).

Our observation that sterols prevented or reduced the fungistatic activity of PDAC (Fig. 3, 4) is not new. Sijpesteijn et al (21) found that cholesterol added at concentrations as low as 1 μg a.i./ml to the growth medium appreciably reduced activity of DTEH against *P. irregulare*. As with DTEH, the fungistatic activity of PDAC was prevented or appreciably reduced by cholesterol, cholestanol, and sitosterol, but not by lanosterol. This generalization, however, was not

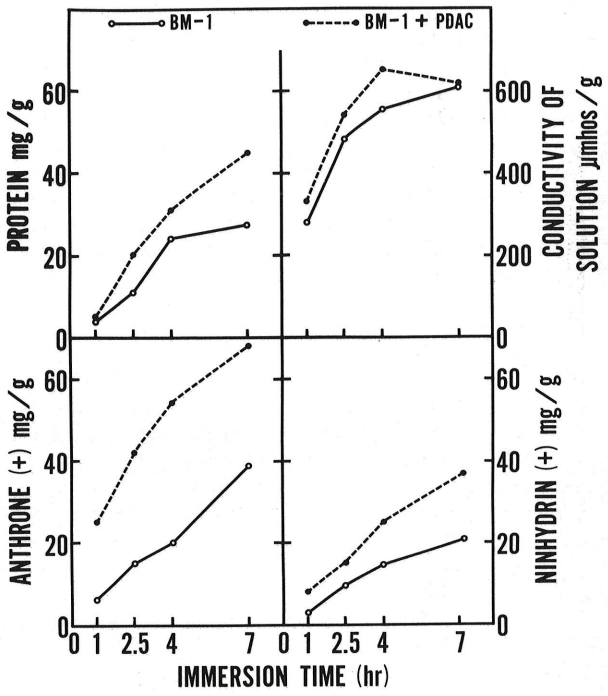

Fig. 5. Effect of propyl-N-(γ-dimethylaminopropyl) carbamate HCl (PDAC) added to liquid basal medium 1 (BM-1) at 1 μg a.i./ml on leakage of inorganic salts, protein, anthrone(+) substances, and ninhydrin(+) substances from mycelium of *Pythium ultimum*.

TABLE 1. Leakage of constituents[x] from mycelium of *Pythium myriotylum* and *P. ultimum* in distilled water after the fungi were grown for 5 days in liquid basal medium 1 (BM-1) supplemented with propyl-N-(γ-dimethylaminopropyl) carbamate HCl (PDAC, SN66752) and cholesterol

Pythium species	Supplement[y]	Substance in solution (mg/g mycelial dry wt)				Conductivity (μmhos/g dry wt)
		Anthrone (+)	Ninhydrin (+)	Protein	$PO_4^{\equiv}$-P	
P. myriotylum	None	19 a[z]	14 bc	24 a	1.3 b	421 b
	Cholesterol	7 b	11 cd	9 bc	0.6 e	311 cd
	PDAC	22 a	20 a	30 a	1.6 a	507 a
	PDAC + cholesterol	5 b	7 d	11 bc	0.8 cd	253 d
P. ultimum	None	24 b	17 b	18 b	2.0 b	353 b
	Cholesterol	33 b	7 c	11 d	1.3 c	215 d
	PDAC	59 a	29 a	38 a	4.0 a	403 a
	PDAC + cholesterol	28 b	9 c	15 c	1.8 bc	290 c

[x]Constituents were determined by techniques specified in the methods section 4 hr after suspension of mycelium in distilled water.
[y]Cholesterol was added in ethanol solution at 20 μg/ml to BM-1 and PDAC was added as aqueous solution at 0.5 μg/ml for *P. myriotylum* and 1 μg/ml for *P. ultimum*.
[z]Numbers followed by the same letter do not differ significantly ($P = 0.05$) by Duncan's multiple range test.

applicable to all *Pythium* spp. tested. Fungicide toxicity was reduced more by sterols in *P. irregulare, P. ultimum, P. splendens,* and *P. aphanidermatum* (in that order) than in *P. myriotylum*. In fact, there was very little reduction of the toxicity of PDAC to *P. myriotylum,* even at 30μg/ml sterol. It may be argued here that the concentrations of PDAC used in these experiments (10 and 50 μg a.i./ml) were too high to obtain a reversal of the fungistatic activity in *P. myriotylum*. However, it was found in subsequent experiments on lipid synthesis that sterols could not annul activity of PDAC to *P. myriotylum* even at 0.25 μg a.i./ml (G. A. Bean, and G. C. Papavizas, *unpublished*). Also, it is possible that the mechanism of action of PDAC differs slightly from species to species.

Our research on membrane permeability suggested that small amounts of PDAC (0.5–1.0 μg a.i./ml) can cause a rapid loss of intracellular material from *Pythium* mycelium. When present during growth, PDAC adversely affected membrane permeability as shown by leakage out of cells of four classes of cellular constituents (Table 1, Fig. 5). When placed in distilled water, PDAC-damaged mycelium began losing constituents almost immediately. The quantities of all leaked constituents increased for 4 hr and of most constituents increased for 7 hr. It is interesting to note that PDAC caused leakage only when it was in contact with the fungus during growth. It was not effective after the mycelium had developed.

In the present studies, leakage of cell constituents was less from mycelium of *P. ultimum* grown on BM-1 supplemented with cholesterol than on BM-1 without cholesterol. Similar results were obtained by Sietsma and Haskins (20) and by others (8). However, more important for our understanding of the mechanism of action of PDAC is the ability of sterol to nullify or reduce the effect of PDAC on cell membrane permeability in mycelium of *P. ultimum*. The mechanism of action of PDAC on *Pythium* spp. is possibly related to the cell membrane function of *Pythium*. Polyene antibiotics reportedly may alter cell membrane permeability and result in loss of vital cell components (4, 10, 11). The antibiotics may cause membrane damage by binding to sterols in the plasma membrane (11). In our experiments, leakage in *Pythium* spp. caused by PDAC was reversed by sterol, possibly by formation of PDAC-sterol complexes as has been suggested in similar systems (1, 3, 14, 22).

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