Sensitivity of *Phytomonas davidi* to Antimicrobial Substances

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ABSTRACT

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Phytomonas davidi, a plant-infecting protozoan, was tested for sensitivity to 14 antimicrobial agents in liquid growth media. Cycloheximide, crystal violet, and a proprietary compound TC 1474 were inhibitory to growth at concentrations ≤1 μg/ml of medium. The antitrypanosomals, homidium bromide and berenil, were inhibitory at concentrations as low as 37 and 111 μg/ml, respectively, whereas quinine-HCl, trypanomycin, and sulfaquinoxaline had little or no effect at 1 mg/ml, the highest concentration tested. Three antibacterial and two antifungal compounds had little effect on growth of *P. davidi*, indicating their possible value in formulating selective media. *P. davidi*, collected from naturally infected *Chamaesyce hypericifolia* (spurge) and placed in dilutions of the growth-inhibiting compounds, reacted similarly except to cycloheximide, which showed little or no direct toxicity to either wild or cultured *P. davidi*. None of the tested compounds had activity against *P. davidi* in *C. hypericifolia* plants treated by root immersion.

Additional key word: Trypanosomatidae

Phytomonas davidi LaFont (Fig. 1) is a uniflagellate protozoan inhabiting the latex vessels of euphorbiaceous plants (4). Although *P. davidi* is not known to be pathogenic (3,7), the similar organisms *P. leptovasorum* Stahel (12,13) and *P. staheli* McGhee & McGhee (5,7,11) are associated with lethal diseases of coffee and palms in South America. Little is known about the biology or control of the putatively phytopathogenic phytomonads, although two strains of *P. davidi* have been isolated in pure culture (2,9). *P. davidi* is being studied as a model in an effort to gain further understanding of this genus. This paper details the results of in vitro and in vivo screening of materials inhibitory to *P. davidi*.

MATERIALS AND METHODS

Growth inhibition. Threefold serial dilutions (1,000–0.017 μg/ml) of 14 antimicrobial compounds were made in aPA medium inoculated with a 48-hr culture of *P. davidi* (ATCC 30287) and dispensed into Dynatech microtiter plates at 250 μl/well. The aPA medium (100 ml) consisted of 1.2 g of PPLO broth base (Difco, Detroit, MI), 10 ml of yeastolate (Gibco, Grand Island, NY), 6.0 g of sucrose, 50 μl of pyruvic acid, 1.5 g of HEPES buffer (Sigma, St. Louis, MO), 0.5 ml of phenol-red (0.2% solution, w/v), 10 ml of fetal bovine serum, and 74 ml of water at pH 7.4. Fresh stock solutions of the test compounds were prepared for each experiment. All stocks were 5 mg/ml of water except TC 1474, which was 500 μg/ml in 10% aqueous ethanol and 5% dimethylsulfoxide. The benomyl and pentachloronitrobenzene stocks were aqueous suspensions. Cultures were incubated 2 days at 35 C. Growth was indicated by acid production and development of a pellet of sedimented cells at the bottom of the wells. Additionally, cell counts and effects on cell motility and morphology were made with phase contrast optics at ×400. Each treatment was replicated four times in each of the two tests.

Toxicity to naturally occurring *P. davidi*. Compounds that inhibited growth of cultured *P. davidi* were tested on *P. davidi* collected from the latex of naturally infected *Chamaesyce* (*Euphorbia*) *hypericifolia* (L.) Millsp. Approximately 10 drops of latex were placed in 2 ml of aPA medium to give a population of $2–3 \times 10^5$ cells per milliliter. Inoculated medium was immediately dispensed, 100 μl/well, into microtiter plates. Threefold dilutions of the test compounds were made in the wells, and the rates of cell mortality were determined by examining 10 microscope fields at ×400 with phase contrast optics. Three replicates were made. Additionally, the toxicity of cycloheximide to the cultivated strain of *P. davidi* was assessed in the same manner.

In vivo activity. Naturally infected *C. hypericifolia* seedlings 8–12 cm in height were collected in the field and the roots placed in perlite under intermittent mist in a screenhouse for 1 wk. Seedlings were then treated by root immersion (6) by placing the roots in 10 ml of the growth-inhibiting compounds (25 or 100 μg/ml) in 50-ml flasks. The solutions were replaced every other day, and samples of latex were checked for *P. davidi* at the start of the test, after 2 days, and after 1 wk.

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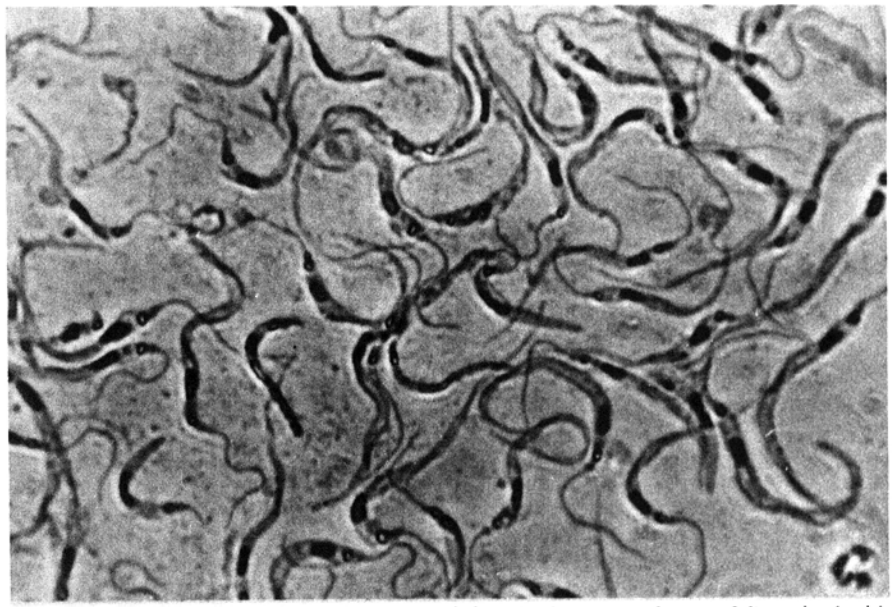

Fig. 1. *Phytomonas davidi*. Latex smear from infected *Chamaesyce hypericifolia* stained with Giemsa (×1,500).

RESULTS

Growth inhibition. The effect of the 14 tested compounds on the growth of *P. davidi* are summarized in Table 1. The antibiotic cycloheximide was the most active compound in these tests, with activity at 0.05 μg/ml. TC 1474 and crystal violet were inhibitory at 1.4 μg/ml, followed by homidium bromide and berenil at 37 and 111 μg/ml. Pentachloronitrobenzene was inhibitory at 1 mg/ml, and quinine-HCl and streptomycin sulfate were somewhat less active. Both quinine-HCl and oxytetracycline-HCl induced spheromastigote (rounded) forms at 1 mg/ml, rather than typical elongate promastigotes of *P. davidi*. Trypanomycin and sulfaquinoxaline had no effect on growth of *P. davidi* at 1 mg/ml, nor did potassium penicillin G, gentamicin sulfate, or benomyl.

Toxicity to naturally occurring *P. davidi*. Toxicities of five compounds to *P. davidi* freshly collected from naturally infected plants were somewhat similar to results from the growth inhibition tests (Table 2). TC 1474 and crystal violet were highly toxic, completely inhibiting motility within minutes at 333 μg/ml and by 50% after 1 hr at 10 and 37 μg/ml, respectively. The 10% ethanol: 5% dimethylsulfoxide solvent used for TC 1474 had no effect on motility of wild *P. davidi* over a 6-hr period. Motility was reduced by 50% after 3.5 hr at 1 mg/ml and after 6 hr at 37 μg/ml for berenil. Homidium bromide reduced motility by 50% after 4 hr at 1 mg/ml and after 6 hr at 333 μg/ml. Cycloheximide showed minimal toxicity to both naturally occurring and cultivated *P. davidi*; many cells were still motile after 24 hr at 1 mg/ml.

In vivo activity. Inhibition of *P. davidi* in naturally infected *C. hypericifolia* seedlings treated with homidium bromide, berenil, TC 1474, or cycloheximide at 25 μg/ml by root immersion was not observed. Concentrations of these compounds at 100 μg/ml were phytotoxic, producing severe foliar burning so that in vivo activity could not be assessed. Although cycloheximide resulted in foliar burning at 25 μg/ml, sufficient latex was obtained after 1 wk to determine the presence of *Phytomonas*. Crystal violet was highly phytotoxic to roots at 25 μg/ml, causing shoots to wilt and precluding in vivo testing of this compound.

Table 1. Mean minimum inhibitory concentration (MIC) of compounds tested against growth of *Phytomonas davidi*

Compound	Source	MIC (μg/ml)[a]
Cycloheximide	The Upjohn Co. Kalamazoo, MI	0.05
TC 1474	Jeersannidhi Anderson Institute Walnut Creek, CA	0.9
Crystal violet	Sigma Chemical Co. Saint Louis, MO	1.4
Homidium bromide	Sigma Chemical Co.	37
Berenil	Sigma Chemical Co.	111
Pentachloronitrobenzene	Niagra Chemical Co. Buffalo, NY	>1,000
Benomyl	Dupont Chemical Co. Wilmington, DE	>1,000
Trypanomycin	Diamond Shamrock Chemical Co. Cincinnati, OH	>1,000
Quinine-HCl	Sigma Chemical Co.	>1,000
Sulfaquinoxaline	Merck and Co. Rahway, NJ	>1,000
Potassium penicillin G	Sigma Chemical Co.	>1,000
Oxytetracycline-HCl	Pfizer Chemical Co. New York, NY	>1,000
Streptomycin sulfate	Pfizer Chemical Co.	>1,000
Gentamicin sulfate	Schering Chemical Co. Baltimore, MD	>1,000

[a] Minimum concentration inhibitory to growth of *P. davidi* in aPA medium.

Table 2. Toxicity of five compounds to *Phytomonas davidi* collected from naturally infected *Chamaesyce hypericifolia*

Compound	Motility loss (%)	Minimal time (hr)[a] resulting in motility loss at various concentrations (μg/ml)[b] 1,000	333	111	37	13	4.4
Crystal violet	50	...	...	<0.1	1.0	4.0	>7.0
	100	<0.1	<0.1	0.3	4.0	>7.0	...
TC 1474	50	nd[c]	nd	0.2	1.0	2.5	6.5
	100	nd	nd	<0.2	4.0	6.5	7.0
TC 1474 solvent[d]	50	>7.0	...	...	...	...	...
	100	>24.0	...	...	...	...	...
Berenil	50	3.5	4.0	5.0	6.0	>7.0	...
	100	>7.0	...	...	...	...	...
Homidium bromide	50	4.0	7.0	>7.0	...	...	...
	100	>7.0	>7.0	...	...	...	...
Cycloheximide	50	>7.0	...	...	...	...	...
	100	>24	...	...	...	...	...
Control (aPA medium)	50	>7.0	...	...	...	...	...
	100	>24.0	...	...	...	...	...

[a] Mean of three replicates.
[b] In aPA medium.
[c] Not determined.
[d] 10% ethanol and 5% dimethylsulfoxide in water.

DISCUSSION

Several compounds were found to have a high degree of activity against *P. davidi* in in vitro tests. These included the antifungal antibiotic cycloheximide, an experimental compound TC 1474, and crystal violet, which is often used as a standard of comparison in antiprotozoal testing (7). Homidium bromide and berenil were marginally active against *P. davidi* in both growth inhibition and direct toxicity tests. Cycloheximide, the most potent growth inhibitor, was not directly toxic to either wild or cultivated cells of *P. davidi*, thus indicating a limitation to direct toxicity testing. Toxicity tests have been performed for *P. staheli* cells squeezed from diseased palm tissue (1,10), but inhibition was seen for only two compounds at concentrations of 100 μg/ml or higher. Cultures of *P. staheli* would obviously provide a more sensitive means of testing compounds for antiprotozoal activity. The potential use of penicillin, streptomycin, oxytetracycline, gentamicin, and benomyl in preparing selective media for *Phytomonas* is indicated by the lack of growth-inhibiting activity of these compounds.

Failure to inhibit *P. davidi* in infected *C. hypericifolia* indicates that either the compounds were not translocated, were inactivated within the plant, or did not accumulate in the laticiferous system to which the protozoa are limited. Because the phytotoxicity symptoms of all the tested compounds except crystal violet consisted of foliar burning, it is assumed that translocation to the foliage occurred via the xylem. This suggests the latter two of the three possibilities for lack of activity. The plants treated with crystal violet wilted and only the roots were stained, indicating lack of translocation and wilting because of lack of water due to root malfunction.

Research on the nutrition of *P. davidi* now in progress will concentrate on the culture of *P. staheli*. Research on the translocatability in plants of compounds

having activity against *Phytomonas* would shed light on potential means of control. Although *P. davidi* is not in itself a plant pathogen, information on this organism should provide insight into the biology and potential approaches to control of the apparently phytopathogenic phytomonads.

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