Toxicity of Pyroxychlor to *Pythium aphanidermatum*

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ABSTRACT

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A dosage-response curve, based upon inhibition of mycelial growth of *Pythium aphanidermatum* on pyroxychlor-treated potato-dextrose agar (PDA), gave ED_{50} and ED_{100} of 3 μg/ml and 9 μg/ml, respectively. In liquid medium 10 to 100 μg pyroxychlor per milliliter was equally effective in inhibiting growth of *P. aphanidermatum*. Mycelia exposed 24 or 96 hr to 20 μg pyroxychlor per milliliter and transferred to PDA overcame the inhibitory effect of the fungicide and grew similarly to the control. Above 50 μg pyroxychlor per milliliter recovery of *P. aphanidermatum* was incomplete and the fungitoxic effects increased with concentration and exposure time. After exposure for 4 hr to 50 or 100 μg pyroxychlor per milliliter or 6 hr to 20 μg pyroxychlor per milliliter, the rate of thymidine-^{3}H incorporation into DNA was inhibited 100, 100, and 85% of the control, respectively. After 6 hr of exposure to 100 μg pyroxychlor per milliliter incorporation of uridine-^{3}H into RNA and L-leucine-^{3}H into protein of *P. aphanidermatum* was 49 and 51%, respectively, of the control. Exposure for 8 hr to 20, 50, or 100 μg pyroxychlor per milliliter inhibited respiration of *P. aphanidermatum* 8, 25, and 48%, respectively, of the control. The primary site of action by pyroxychlor appears to involve inhibition of DNA synthesis.

Additional key words: Fungicide, DNA synthesis.

Pyroxychlor [2-chloro-6-methoxy-4-(trichloromethyl)pyridine] (Dow Chemical Company, Midland, MI 48640) is a basipetally translocated fungicide that controls phycomycetous soilborne pathogens (4) when applied in a foliar spray (3), in a soil drench (2), or in transplanting water (3). The effects of pyroxychlor on various cellular processes of *Pythium aphanidermatum* are reported.

MATERIALS AND METHODS

Culture method. *Pythium aphanidermatum* (Edson) Fitzpatrick (American Type Culture Collection 16994) was grown on modified Eckert's medium (5) in stationary cultures at 30 C. Four-day-old mycelial mats, washed three times with distilled water, were cut into 6-mm-diameter disks and blotted with filter paper before being suspended in test solutions. Pyroxychlor was suspended in ethanol for delivery to cells, and controls received similar amounts of ethanol without pyroxychlor.

Toxicity studies. Potato-dextrose agar (PDA) was autoclaved (15 min, 121 C) and cooled to 50 C before adding pyroxychlor to provide final concentrations of 0, 2, 3, 4, 5, 7, 9, and 20 μg/ml. The media were solidified in 10-cm-diameter sterile petri dishes. Immediately after transfer of *P. aphanidermatum* to the media, the dishes were sealed with masking tape and incubated at 30 C. When the diameter of control reached 75 mm (~48 hr), the percentage inhibition was calculated, a dosage-response curve was constructed using a lot-probit transformation, and the ED_{50} value was derived. Each treatment level was replicated 10 times.

Fungistatic and fungitoxic effects of pyroxychlor were evaluated by growing *P. aphanidermatum* in modified Eckert's medium (100 ml per 250-ml Erlenmeyer flask) on a gyrotory shaker at 30 C. After 2 days pyroxychlor was added to give final concentrations of 0, 10, 20, 50, 100, and 1,000 μg/ml. Twenty-four and 96 hr after the toxicant was added, mycelial fragments were aseptically removed, washed with sterile distilled water, and plated on PDA. Cultures were incubated at 30 C and colony diameters were measured daily for 7 days. Each treatment level was replicated 12 times.

The effect of 10, 20, 50, and 100 μg pyroxychlor per milliliter upon mycelial dry-weight increase was determined by growing *P. aphanidermatum* in modified Eckert's medium as previously

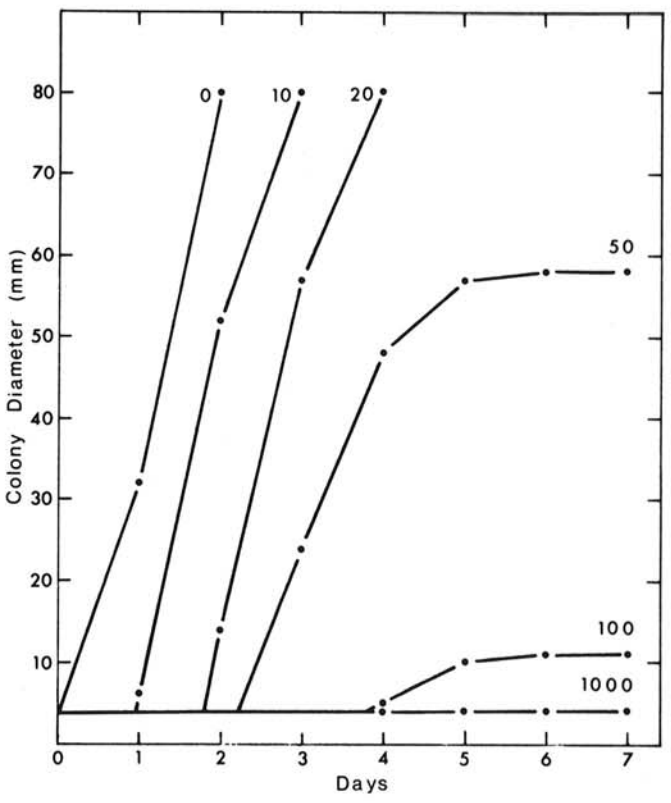

Fig. 1. Fungistatic and fungitoxic effects of 0, 20, 50, 100, and 1,000 μg pyroxychlor per milliliter against a 2-day-old shake culture of *Pythium aphanidermatum*. Values plotted are mean diameters of the colonies that developed from mycelial fragments after 96 hr of exposure, washing with sterile distilled water, and plating on PDA.

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described. Mycelial dry weights of the fungal cultures were determined when the toxicant was added (0 hr) and after 24 and 96 hr. Each treatment level was replicated eight times.

Respiration measurements. Effect of pyroxychlor on respiration of *P. aphanidermatum* was measured manometrically with a Gilson Differential Respirometer (Gilson Medical Electronics Inc., Middleton, WI 53562) by standard manometric techniques (6). Mycelial disks (5.0 mg dry weight per milliliter) were suspended in 3-ml of a solution containing 10.0 g glucose, 4.0 g KH_2PO_4, 3.0 g $K_2HPO_4 \cdot 3\,H_2O$, 0.5 g K_2SO_4, 0.6 g $MgCl_2$, and 1,000 ml water (pH 6.4), and incubated at 30 C. Each treatment level was replicated six times.

L-leucine, uridine, and thymidine incorporation. Effects of pyroxychlor on protein and on RNA and DNA syntheses were studied by suspending mycelial disks in a reaction medium (10.0 g glucose, 1.5 g L-asparagine, 0.1 g thiamine HCl, 4.0 g KH_2PO_4, 3.0 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g K_2SO_4, 0.6 g $MgCl_2$, and 1,000 ml water (pH 6.4) containing 0.1 mM L-leucine-4, 5-^{3}H (sp act 1 mCi/mmole), 0.1 mM uridine-5-^{3}H (sp act 5 mCi/mmole), or 0.1 mM thymidine-(methyl)-^{3}H (sp act 5 mCi/mmole) (Schwarz-Mann, Orangebury, NY 10962). Disks were removed at 2-hr intervals and radioactivities in protein, RNA, and DNA fractions were determined (1). Each treatment level was replicated six times.

RESULTS

Toxicity studies. A dosage-response curve for pyroxychlor against *P. aphanidermatum* was linear with a slope of 1.5 and ED_{50} of 3 μg pyroxychlor per milliliter. At that time concentrations of pyroxychlor above 9 μg/ml inhibited growth completely. However, between 72 and 134 hr, irregularly-shaped colonies appeared on medium containing 20 μg pyroxychlor per milliliter, the highest concentration tested. To determine if *P. aphanidermatum* had developed tolerance to pyroxychlor, mycelial plugs were transferred from the 20 μg pyroxychlor per milliliter of medium to freshly prepared PDA containing 0 and 20 μg pyroxychlor per milliliter. Mycelial plugs from a *P. aphanidermatum* culture not exposed previously to pyroxychlor were used as controls. No difference in growth rate or habit was observed among isolates after 48 hr on medium without pyroxychlor; however, on medium containing fungicide the isolate from 20 μg pyroxychlor per milliliter had a colony diameter of 45 mm compared to no growth in the control. Growth was observed on the control only after 96 hr. The isolate from 20 μg pyroxychlor per milliliter retained the ability to grow on medium containing the fungicide after two successive transfers on medium without pyroxychlor.

After 24 hr exposure to 10, 20, 50, or 100 μg pyroxychlor per milliliter, mycelial dry weight at all treatment levels was about 58% less than were starting weights (0 hr), and 70% less than controls. Mycelial dry weight continued to decline in treatments receiving pyroxychlor and by 96 hr was 70% less than the starting weights and 90% less than the controls.

Fungistatic and fungitoxic effects of pyroxychlor were investigated by liquid-shake culture. Mycelium exposed 24 or 96 hr to 20 μg pyroxychlor per milliliter or less completely overcame the effect of the fungicide when washed and plated on PDA. Growth rates were similar to those of controls (Fig. 1). However, exposure to pyroxychlor delayed initiation of growth, and this delay increased progressively as the fungicide concentration was increased. Only 75% of the mycelial fragments exposed 96 hr to 50 μg pyroxychlor per milliliter and plated on PDA developed visible mycelial growth during 7 days of observation. None of the mycelial fragments exposed 24 or 96 hr to 1,000 μg pyroxychlor per milliliter developed visible mycelial growth after being removed from the toxicant; at 100 μg pyroxychlor per milliliter only 33 and 13% of the mycelial fragments produced visible colonies after exposure for 24 and 96 hr, respectively. Colonies developing after treatment with 50

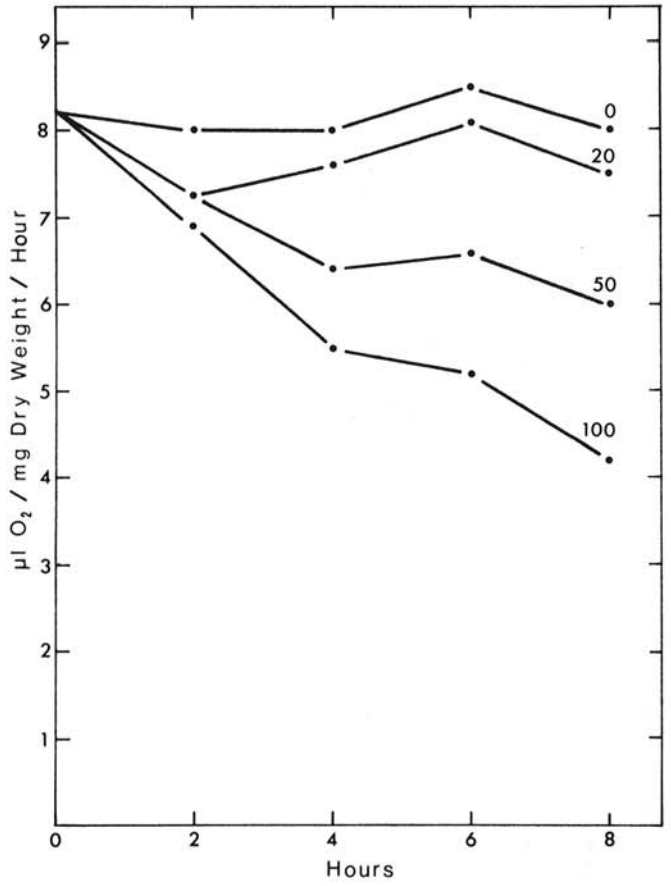

Fig. 2. Effects of 0, 20, 50, and 100 μg pyroxychlor per milliliter on respiration of *Pythium aphanidermatum* mycelia.

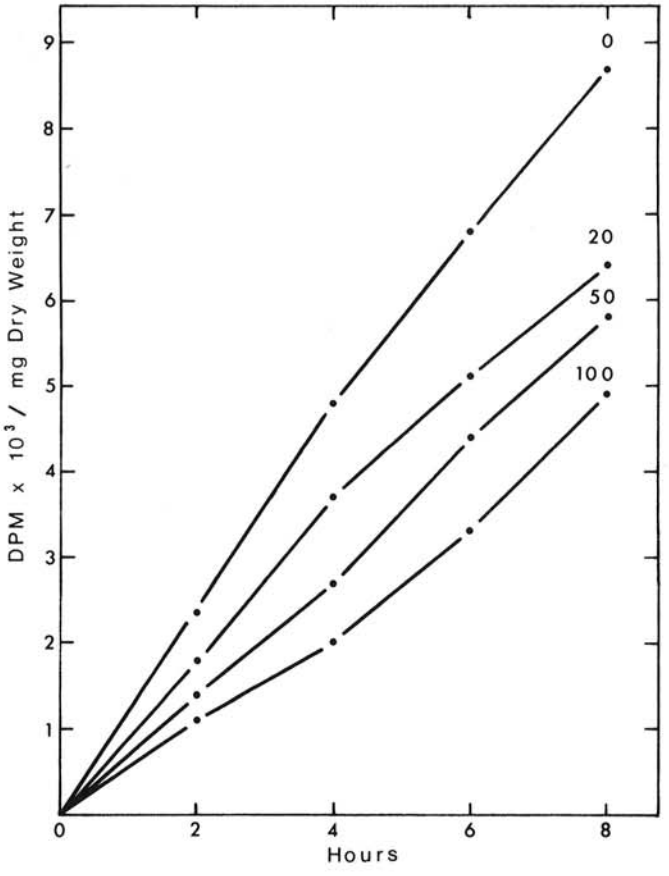

Fig. 3. Effects of 0, 20, 50, and 100 μg pyroxychlor per milliliter on the incorporation of L-leucine-4, 5-^{3}H into the protein fraction of *Pythium aphanidermatum* mycelia.

and 100 μg pyroxychlor per milliliter and subsequent plating on PDA were irregular in shape and less dense than were control colonies. Within 48 hr after initiation of growth on PDA, these colonies ceased expansion and only a few hyphae continued growing. This indicates incomplete recovery of *P. aphanidermatum*.

Respiration. After 8 hr of exposure, respiration of *P. aphanidermatum* was inhibited 8, 25, and 48% of the control by 20, 50, and 100 μg pyroxychlor per milliliter, respectively (Fig. 2).

Incorporation of L-leucine into protein. After the 8-hr exposure, incorporation of L-leucine into pyroxychlor-treated mycelial disks was inhibited 26, 33, and 44% of the control by 20, 50, and 100 μg pyroxychlor per milliliter, respectively (Fig. 3).

Incorporation of uridine into RNA. During the first 6 hr of exposure, 50 and 100 μg pyroxychlor per milliliter inhibited the incorporation of radiolabeled uridine into RNA by 28 and 49%, respectively (Fig. 4). Between 6 and 8 hr after exposure, the rate of uridine incorporation into the untreated mycelial disks was 3× higher than during the first 6 hr. The rate of uridine incorporation into RNA did not increase in the pyroxychlor-treated mycelia between 6 and 8 hr. After 8 hr, total incorporation of uridine into RNA was inhibited 31, 50, and 59% of the control by 20, 50, and 100 μg pyroxychlor per milliliter, respectivley.

Incorporation of thymidine into DNA. After 6 hr, 20 μg pyroxychlor per milliliter inhibited the rate of thymidine incorporation into the DNA fraction of *P. aphanidermatum* by 85% of the control (Fig. 5). Fifty and 100 μg pyroxychlor per milliliter totally inhibited thymidine incorporation into DNA after 4-hr of exposure to the toxicant.

DISCUSSION

Incorporation of radiolabeled thymidine into the DNA fraction of *P. aphanidermatum* mycelia was strongly curtailed by pyroxychlor after 2 hr (Fig. 5). However, pyroxychlor did not appreciably inhibit respiration (Fig. 2), the incorporation of L-leucine into protein (Fig. 3), or uridine into RNA (Fig. 4) after 2 hr. Because thymidine incorporation was curtailed while other cellular processes continued with only moderate inhibition pyroxychlor's primary site of action appears to be more closely associated with DNA synthesis than with other phases of metabolism. Future studies with pyroxychlor should investigate the effects of DNA metabolism and nuclear division between 0 and 2 hr to determine if the inhibition of DNA synthesis is a direct or indirect action.

Pyroxychlor at concentrations below 20 μg/ml acts as a fungistat, since *P. aphanidermaturm* recovers from the inhibitory effects when transferred to PDA lacking pyroxychlor. At concentrations below 20 μg/ml, the primary site of action appears to involve reversible inhibition of DNA synthesis. At concentrations above 50 μg pyroxychlor per milliliter the compound becomes fungitoxic. The fungitoxic effects are enhanced by progressively higher concentrations of pyroxychlor in the medium and increased time of exposure.

A strain of *P. aphanidermatum* was isolated that developed tolerance to pyroxychlor. The nature of the tolerance was not determined but it was stable through two successive transfers on medium without pyroxychlor. Additional studies with pyroxychlor should investigate the nature of tolerance and the potential of other fungi to develop such tolerance.

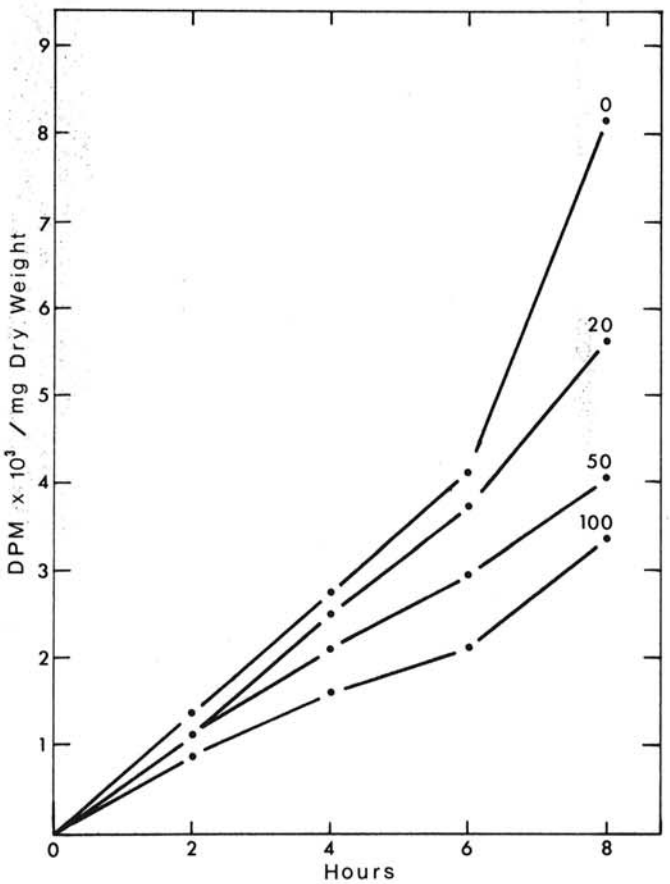

Fig. 4. Effects of 0, 20, 50, and 100 μg pyroxychlor per milliliter on the incorporation of uridine-5-^{3}H into the RNA fraction of *Pythium aphanidermatum* mycelia.

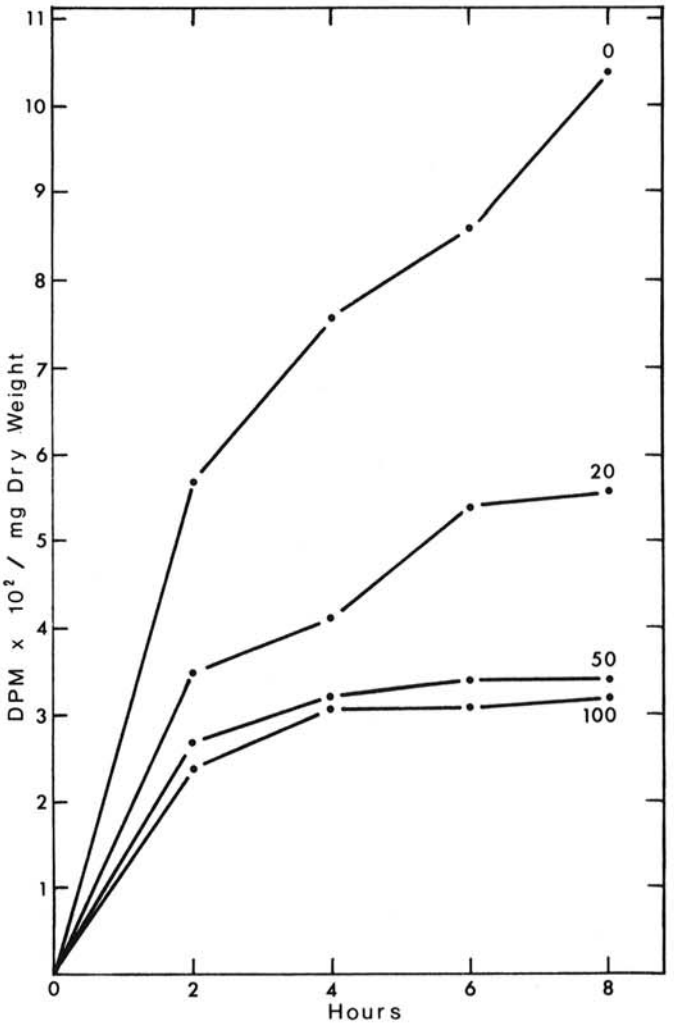

Fig. 5. Effects of 0, 20, 50, and 100 μg pyroxychlor per milliliter on the incorporation of thymidine-(methyl)-^{3}H into the DNA fraction of *Pythium aphanidermatum* mycelia.

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