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Inhibition of Phytoalexin Synthesis in Arachidonic Acid-Stressed Potato Tissue by Inhibitors of Lipoxygenase and Cyanide-Resistant Respiration[1]

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ABSTRACT

Arachidonic acid-stressed potato tuber discs synthesized the phytoalexin rishitin. This synthesis was inhibited by salicylhydroxamic acid (SHAM), and to a lesser extent by tetraethylthiuram disulfide (disulfiram). Disulfiram was less effective apparently because it was inactivated in the tuber discs. Disulfiram and SHAM both inhibited cyanide-resistant respiration of whole potato discs and lipoxygenase extracted from these discs. When low disulfiram concentrations were used, the lipoxygenase inhibition was quickly overcome, again because the disulfiram apparently was inactivated by oxidation.

Phytophthora infestans (Mont.) de Bary causes the rapid accumulation of sesquiterpenoid stress metabolites when it invades hypersensitively resistant potato tuber tissue. These stress metabolites, called 'phytoalexins,' are fungistatic and may be determinants of disease resistance (2). It is therefore important to determine the mechanisms of their synthesis.

Phytoalexins are elicited when cell-free extracts of *P. infestans* are added to potato slices. Recently Bostock *et al.* (2) reported that the major fungal chemicals responsible are 5, 8, 11, 14, 17-eicosapentaenoic and arachidonic acids.

Alves *et al.* (1) presented evidence that potato tissue does not synthesize phytoalexins if the tuber slices are preincubated with SHAM[2], a known inhibitor of cyanide-resistant respiration. However, SHAM is also a potent inhibitor of lipoxygenase (6), an enzyme that catalyzes the free radical incorporation of molecular oxygen into fatty acids containing *cis,cis*-1,4-pentadiene systems. It is possible that highly unsaturated fatty acids elicit phytoalexins, but only if the fatty acids are activated *in situ* by lipoxygenase, a reaction that would be prevented by SHAM. The recent finding that disulfiram inhibits cyanide-resistant respiration, but not lipoxygenase (5) seemed to provide a convenient method of testing this hypothesis.

This report summarizes some of our research concerning inhibition of phytoalexin synthesis by inhibitors of lipoxygenase and cyanide-resistant respiration.

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[2] Abbreviations: SHAM, Salicylhydroxamic acid; disulfiram, tetraethylthiuram disulfide.

MATERIALS AND METHODS

Plant Materials. Tubers of white potatoes (*Solanum tuberosum* L. cv Kennebec) were obtained from field plantings at a West Virginia University Agricultural Experiment Station farm. They were stored at 4°C for approximately 9 months before use. Discs (2 mm × 1 cm) were cut from the pith area of these tubers, rinsed in distilled H_2O and incubated in Petri dishes at 20° C for 24 h.

Chemicals. SHAM (99%) was purchased from Aldrich Chemical Co., Inc. Disulfiram, linoleic acid, and arachidonic acid (all 99%) were purchased from Sigma Chemical Co. Stock solutions (50 mg/ml) of arachidonic and linoleic acids in methanol were prepared and stored under N_2 at −20 °C.

Elicitor Assays. A sufficient volume of arachidonic acid stock solution was added to 0.05 M Mes (pH 5.5) to give more than 100 μg/ml. This mixture was shaken on a Vortex mixer and centrifuged at 10,000*g* for 30 min. The droplets of fatty acid floating on the surface were removed by aspiration and the actual arachidonic acid concentration was determined by titration of a 5-ml aliquot. Ethanol was added to completely dissolve the fatty acid prior to the titration. The arachidonic acid suspension was then diluted to 100 μg/ml with Mes. When used, SHAM and disulfiram in methanol were added to the arachidonic acid suspension before it was diluted. The total methanol concentration was 2% (v/v) in all elicitor assays.

Potato discs were immersed for 20 min in 2% methanol in Mes, in Mes-containing arachidonic acid, or in Mes-containing arachidonic acid and SHAM or disulfiram. The treated discs were incubated at 20°C for 72 h, the entire discs were then homogenized, and the phytoalexin rishitin was assayed by the method of Henfling and Kuc (4).

Lipoxygenase Assays. Lipoxygenase was fractionated with $(NH_4)_2SO_4$ and dialyzed by the method of Sekiya *et al.* (7) and assayed (6) using linoleic acid as the substrate. The reaction mixture contained 5% (v/v) methanol or this amount of methanol containing SHAM or disulfiram.

Respiration Assays. Five potato discs and 4 ml of 0.05 M Mes (pH 5.5) were placed in each flask of a Gilson differential respirometer. The Mes contained 0.1 mM KCN when this inhibitor was included as a treatment. SHAM or disulfiram in ethylene glycol monomethyl ether or ethanol, respectively, or one of these solvents, was added to each flask. The organic solvent concentration was 2% (v/v) in each flask. The center wells contained 0.2 ml of 10% (w/v) NaOH and fluted filter paper. Respiratory measurements were carried out over a 2-h period with readings taken every 15 min.

RESULTS

Potato tuber discs became necrotic and accumulated high concentrations of rishitin when treated with arachidonic acid and then incubated at 20°C for 72 h. SHAM, and to a lesser extent disulfiram, inhibited this synthesis (Fig. 1).

Both SHAM and disulfiram inhibited cyanide-resistant respiration (Fig. 2). The maximum inhibition was approximately the same for each of these chemicals, but the concentrations that caused 50% inhibition were 0.4 and 2.4 mM for disulfiram and SHAM, respectively.

SHAM and disulfiram also both inhibited potato tuber lipoxygenase and again the maximum inhibition was approximately the same for these two chemicals. The concentration of SHAM required for 50% inhibition was approximately 1.9 mM, almost the same as the concentration that caused 50% inhibition of the cyanide-resistant respiration. In contrast, disulfiram inhibited 50 to 60% of the lipoxygenase (Fig. 3), even when as low as 3 μM disulfiram was tested (Fig. 4). At low disulfiram concentrations, it became apparent that the inhibition of lipoxygenase was temporary (Fig. 4). Figure 4 also shows that the time required for reversal of the inhibition by disulfiram was at least approximately proportional to the disulfiram concentration.

DISCUSSION

Alves *et al.* (1) reported that pretreatment of potato slices with SHAM prevents phytoalexin synthesis when the slices are subsequently incubated with homogenates of *P. infestans*. They concluded that cyanide-resistant respiration is required for potato phytoalexin synthesis, a logical deduction because SHAM is known to be an inhibitor of this type of respiration (3). Since then, Bostock *et al.* (2) have shown that highly unsaturated fatty acids are the chemicals in *P. infestans* homogenates responsible for eliciting potato phytoalexins. We have now shown that SHAM inhibits elicitation of the phytoalexin rishitin by commercial arachidonic acid (Fig. 1). We propose that caution is required in interpreting the role of cyanide-resistant respiration in the synthesis of potato phytoalexins. It is possible that highly unsaturated fatty acids in homogenates of *P. infestans* elicit potato phytoalexins only if they are oxidized by lipoxygenase, a reaction that is inhibited by SHAM (Fig. 3).

Disulfiram inhibits cyanide-resistant respiration but not lipoxygenase in soybean mitochrondria preparations (5). We had hoped to use SHAM and disulfiram to discriminate between the need for lipoxygenase and cyanide-resistant respiration for phytoalexin synthesis by potato tuber discs incubated with arachidonic acid.

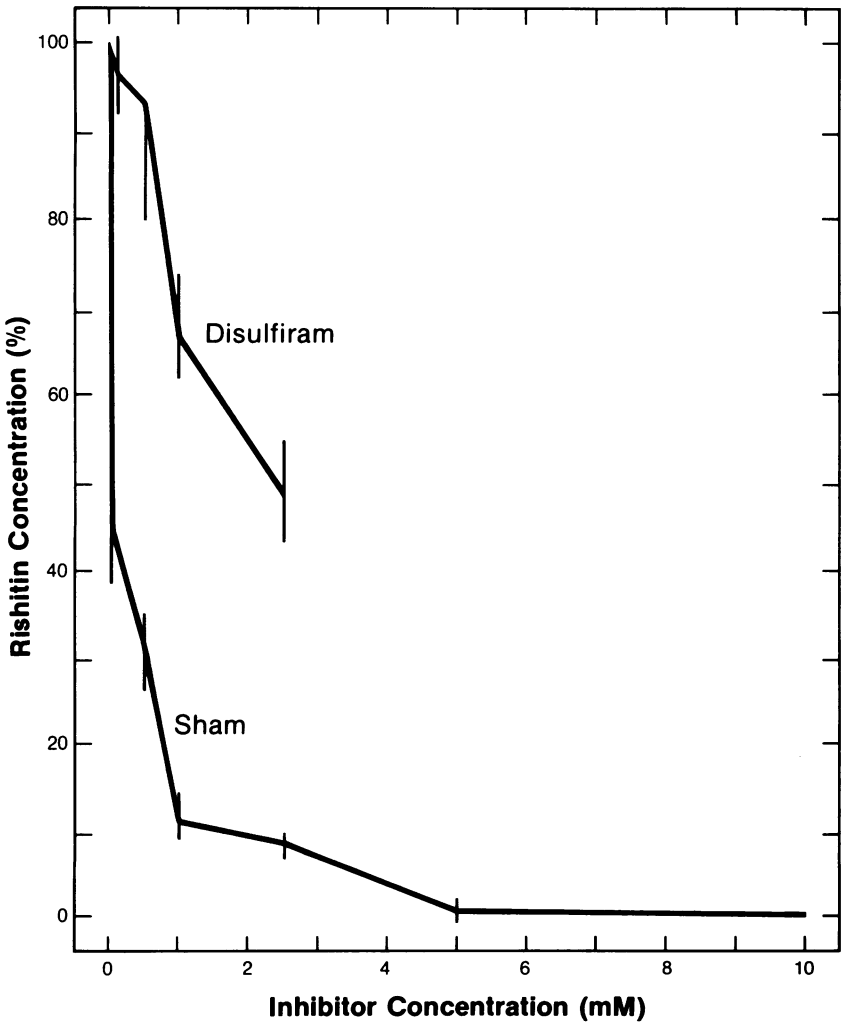

FIG. 1. Synthesis of rishitin by Kennebec potato discs that were incubated in 20°C air for 24 h and then immersed for 20 min in Mes containing 100 μg/ml arachidonic acid and various concentrations of SHAM or disulfiram. Each point is the average of four replications and the vertical bars are the SE. In the absence of either inhibitor, the rishitin level was 28.1 ± 2.0 μg/g fresh weight.

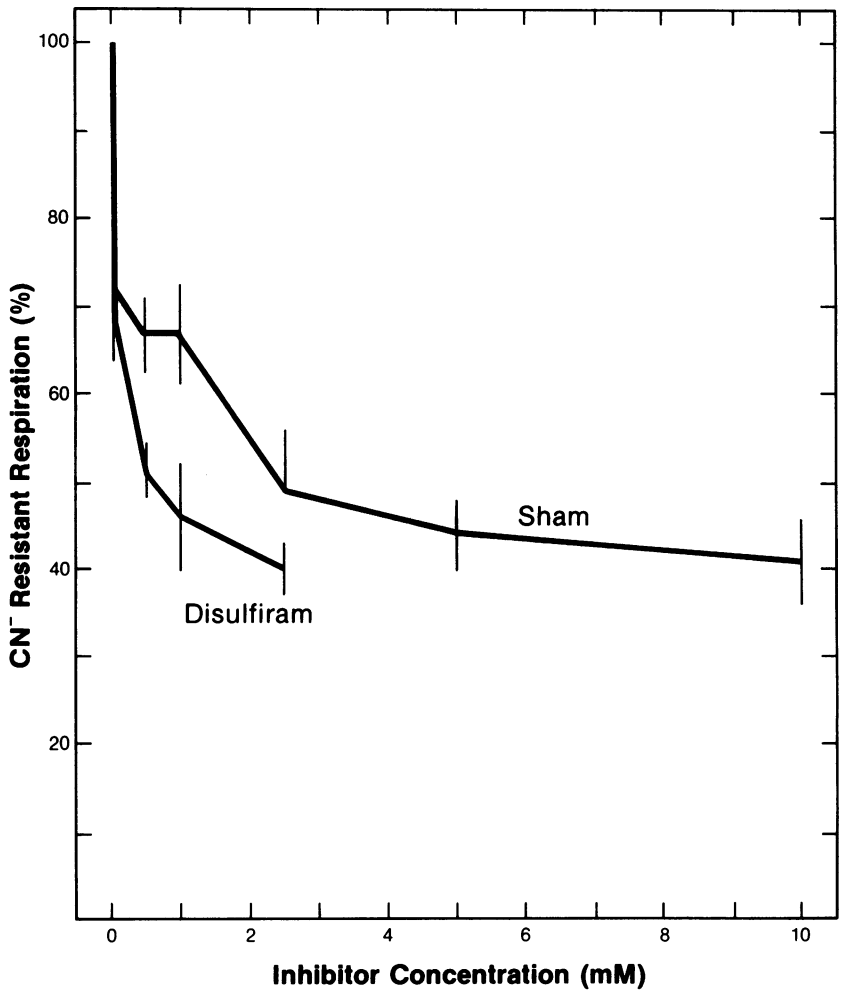

FIG. 2. Cyanide-resistant respiration of 24-h aged Kennebec potato discs in the presence of various concentrations of SHAM or disulfiram. Each point is the average of five replications and the vertical bars are the SE. In the absence of either inhibitor, but in the presence of the organic solvents used with SHAM and disulfiram, the cyanide-resistant respiration was 56.6 ± 1.5 and 47.1 ± 3.3 μl O_2/h·g fresh weight.

Our work, however, showed that disulfiram is at least as effective as SHAM in inhibiting both cyanide-resistant respiration and lipoxygenase (Figs. 2 and 3). In contrast, disulfiram causes less inhibition of phytoalexin synthesis than does SHAM (Fig. 1). We believe that this is because disulfiram is inactivated in some manner during the lipoxygenase reaction (Fig. 4). We only demonstrated this *in vitro*, and we do not know how much disulfiram was taken up by the potato cells involved in rishitin synthesis. However, potato tuber is rich in lipoxygenase and the tuber discs are incubated for 3 d between the time they are placed into disulfiram and arachidonic acid and the time the phytoalexins are assayed. It is easily conceivable that this is enough time for the disulfiram to be inactivated *in vivo*. We do not know the mechanism of the inactivation of disulfiram, but we suspect that it is oxidized by a lipoxygenase oxidation product.

There is essentially complete inhibition of phytoalexin synthesis above 2 mM SHAM (Fig. 1) whereas a substantial portion of cyanide-resistant respiration (Fig. 2) and lipoxygenase (Fig. 3) remain even in the presence of 10 mM SHAM. It is conceivable that SHAM is inhibiting phytoalexin synthesis via a mechanism other than the two we discussed.

Grover and Laties (3) reported that disulfiram was an inhibitor of cyanide-resistant respiration of potato mitochondria but not of intact potato slices. In contrast, we found that disulfiram inhibits cyanide-resistant respiration of intact slices with our experimental conditions (Fig. 2). This difference may be because we age our tissue for 24 h in air before measuring the respiration, whereas Grover and Laties (3) induced cyanide-resistant respiration by incubating whole tubers with ethylene or by aging potato slices in aerated solutions. It is also possible that the difference is due to different potato cultivars being used in the two studies.

We were not able to achieve our original goal, to distinguish between a need for cyanide-resistant respiration or lipoxygenase for potato phytoalexin synthesis. However, we did show that it is premature to assume that this synthesis requires cyanide-resistant respiration. The data also show that disulfiram does inhibit cyanide-resistant respiration of intact potato slices under some experimental conditions. Finally, we demonstrated that caution is required when attempting to use disulfiram to determine if oxidation is due to lipoxygenase or cyanide-resistant respiration.

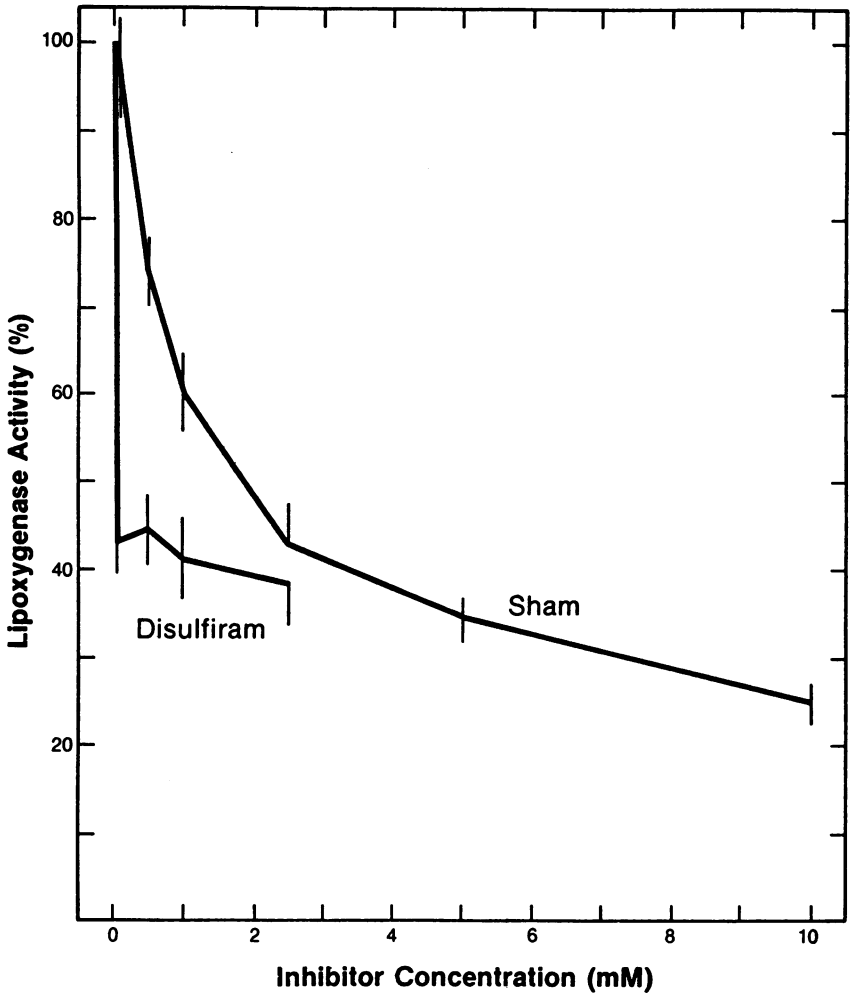

FIG. 3. The lipoxygenase activity in partially purified extracts of 24-h aged Kennebec potato discs assayed in the presence of various concentration of SHAM and disulfiram. Each point is the average of four replications and the vertical bars are the SE. In the absence of either inhibitor, the activity was 3.5 ± 0.8 μmol O_2/ml·min.

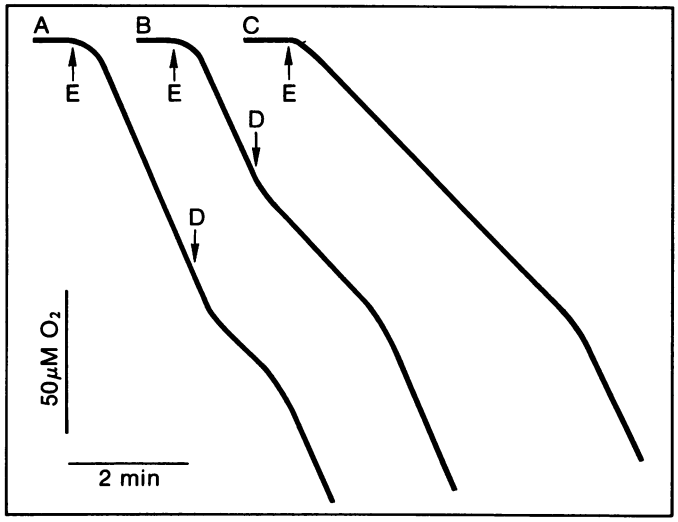

FIG. 4. Lipoxygenase O_2 consumption traces. Disulfiram was added at the time indicated by the arrows (D) to give final concentrations of 3 (A) and 6 μM (B) or prior to enzyme (E) addition to give a final concentration of 12 μM (C).

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