Effects of Organophosphorous Pesticides on Cutinase Activity and Infection of Papayas by *Colletotrichum gloeosporioides*

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ABSTRACT

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Thirteen pesticides, predominantly organophosphates, were shown to be potent inhibitors, in vitro, of purified cutinase of *Colletotrichum gloeosporioides*, the causal agent of papaya anthracnose. The I_{50} values (concentration at which the enzyme activity is inhibited 50%) of these inhibitors ranged from 10^{-3} to 10^{-9} M. Four of these inhibitors were further studied to determine their K_i values for the cutinase. *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate, Hinosan, chlorpyrifos, and Inezin had K_i values of 10^{-8} M, 2.3×10^{-4} M, 2.6×10^{-4} M, and 1.2×10^{-4} M, respectively, which generally correlated with their I_{50} values. The same four compounds and *O,O*-dimethyl-*O*-(2,4,5-trichlorophenyl)phosphate at micromolar or lower concentrations also effectively prevented infection of papaya in a laboratory fruit bioassay. None of the compounds was fungitoxic at these concentrations. An anionic detergent, sodium dodecyl sulfate (SDS), was shown to be a reversible inhibitor (K_i, 2.9×10^{-4} M) of cutinase produced by *C. gloeosporioides*. SDS also prevented infection of papaya tissues; at 0.02, 0.2, and 2.0 mM SDS reduced infection by 53, 71, and 82%, respectively.

Additional key words: antipenetrants, *Carica papaya* L., organophosphorous pesticides, postharvest physiology.

The results of two recent studies provide clear experimental evidence that phytopathogenic fungi can penetrate plant cuticles enzymatically. The first of these concerned infection of pea stems by *Fusarium solani* f. sp. *pisi* (15). The second involved the infection of papayas by *Colletotrichum gloeosporioides* (2). In both cases, several lines of evidence showed that a cutin-degrading enzyme produced by the pathogens breached the cuticular barrier of their hosts. Although these are the only sources of direct experimental evidence for the role of cutinases, considerable ultrastructural and other evidence (10,14), including the fact that a number of fungal pathogens produce cutinases on media in which cutin is the sole source of carbon (1,9), indicated that involvement of cutinases of pathogenic fungi in the infection of plant tissues may be a more common phenomenon than hitherto realized.

Selective chemical modification of cutinase from *F. solani* f. sp. *pisi* showed that a catalytic triad consisting of one "active serine," one histidine residue, and one carboxyl group are involved in the

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enzymatic mechanisms of ester hydrolysis (8). This enzyme is severely inhibited by diisopropylfluorophosphate (DFP) and a variety of organophosphates (6). These organophosphates also prevent pathogenic ingress in pea tissues without affecting fungal growth (6).

Since DFP is also a potent inhibitor of the cutinase from *C. gloeosporioides*, it was of interest to determine whether or not other organophosphorous compounds also inhibit this cutinase, and whether such compounds prevent infection of papayas by the pathogen. Here we report data on the in vitro inhibition of the cutinase of *C. gloeosporioides* by several organophosphates, and the effect of such inhibitors on the infection of papaya by the pathogen in a laboratory fruit bioassay. Also reported is a novel finding that sodium dodecyl sulfate (SDS), an anionic detergent that was previously shown to reduce the I_{50} values (micromolar concentrations of inhibitor that reduces enzyme activity 50% after incubation of the reactants at 24 C for 1 hr) of organophosphates against the Fusarium cutinase (6), acts instead as a competitive inhibitor of the cutinase of *C. gloeosporioides*.

MATERIALS AND METHODS

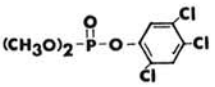
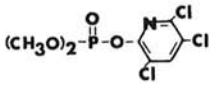
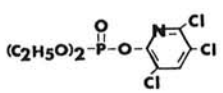
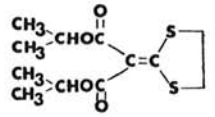
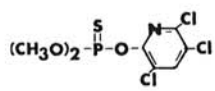
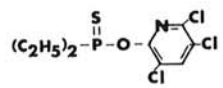
Colletotrichum gloeosporioides Penz. was isolated from papayas (*Carica papaya* L.), and single-spore isolations were made. Cultures were maintained on 10% V-8 juice agar. *O,O*-Dimethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate (Fospirate), *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate, *O,O*-dimethyl-*O*-(2,4,5-trichlorophenyl) phosphate, *O,O*-dimethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothiolate (chlorpyrifos), and phthalimido-*O*, *O*-diethyl ester (phosphonothioic acid) were obtained from Dow Chemical Company (Table 1). *O*-Ethyl-*S,S*-diphenylphosphorodithioate (Hinosan) was obtained from Bayer AG, Germany. *O*-Ethyl-*S*-benzyl-phenylphosphonothiolate (ESBP, Inezin), *O,O*-diisopropyl-*S*-benzylthiophosphate (IBP, Kitazin P), and *O*-butyl-*S*-benzyl-*S*-ethylphosphorodithioate (BEBP, Conen) were provided by M. Yamada, National Institute of Agricultural Sciences, Japan (Table 2). Methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate (benomyl) and 2-(methoxycarbonylamino)-benzimidazole (carbendazim) were obtained from E. I. du Pont de Nemours, USA. The pesticides tetraethyldiphosphate (TEPP), diisopropyl-1,3-dithiolane-2-ylidinemalonate (isoprothiolane), and probenazol were also tested. Sodium dodecyl sulfate was purchased from Sigma Chemical Company. All chemicals were of analytical grade of the active ingredient and did not contain the other formulating ingredients.

Cutinase. As previously described (2), the purified cutinase was obtained from a culture filtrate of *C. gloeosporioides* grown on purified papaya cutin, which was the sole source of carbon. Enzyme activity was measured by spectrophotometric assay with *p*-nitrophenyl butyrate (PNB) as a model substrate (13).

Determination of I_{50} values. The inhibitory activity of the various compounds is expressed in terms of their I_{50} values. Cutinase (3 μg) was incubated in 50 mM sodium phosphate buffer, pH 7.5, containing 0.5% Triton X-100 (w/w) and appropriate concentrations of various inhibitors with a final reaction mixture volume of 3 ml. Acetone stock solutions of pesticides were dispersed in appropriate volumes of water to obtain the desired concentrations of pesticides. The I_{50} values were determined from semilogarithmic plots of pesticide concentration versus enzyme activity by using linear regression analysis. All experiments were repeated at least twice for each chemical with identical results.

Determination of K_i of selected pesticides against cutinase. Four compounds, chlorpyrifos, Hinosan, Inezin, and *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate were chosen for further study. Kinetic parameters of cutinase inhibition by the four inhibitors were determined by following the time course of enzyme inactivation. As in the case of experiments on determination of I_{50} values, 3 μg of cutinase was incubated at 24 C with various concentrations of individual pesticides in 50 mM phosphate buffer, pH 7.5, containing Triton X-100 (3.7 mg/ml). After appropriate time intervals, the incubation mixtures were diluted (1,000-fold)

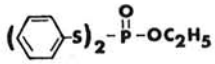
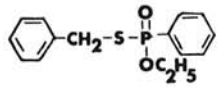
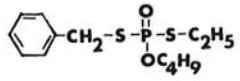
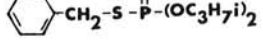
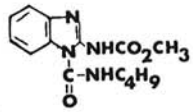
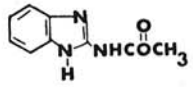
TABLE 1. Cutinase inhibition by organophosphorous insecticides

Chemical structure	Name	I_{50}(μM)[a]
(C_2H_5)-P_2O_7	TEPP[b]	384
	*O,O*-dimethyl-*O*-(2,4,5-trichloro-phenyl)phosphate	0.0039
	Fospirate[b]	10.11
	*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate	0.0011
	Isoprothiolane[b]	603
	Chlorpyrifos-methyl[b]	8.6
	Chlorpyrifos[b]	53.7

[a] I_{50} is defined as the micromolar concentration of inhibitor at which enzyme activity is inhibited by 50% after incubation of the reactants at 24 C for 1 hr.

[b] Generic name.

TABLE 2. Cutinase inhibition by fungicides

Chemical structure	Generic name	I_{50} (μM)
	Hinosan	33.2
	Inezin	187
	Conen	464
	Kitazin P	397
	Benomyl	38.5
	Carbendazim	1000

into the same buffer saturated with PNB, and the cutinase activity was measured. To determine the inhibition kinetics of SDS, 3 μg of cutinase was incubated in 100 mM HEPES buffer, pH 7.5, containing appropriate concentrations of SDS. The SDS stock solutions were in the same buffer. Aliquots from reaction mixtures were diluted in HEPES (100 mM, pH 7.5) containing buffer saturated with PNB (0.42 mM).

Assay for measuring prevention of infection. The protective effect of various cutinase inhibitors was measured with the papaya fruit bioassay described before (2). Conidial suspensions (25 μl) containing approximately 25,000 spores of *C. gloeosporioides* and appropriate concentrations of inhibitors plus a nonionic/anionic emulsifer (Atlox 3403F and Atlox 3404F) were applied to papaya fruits (cultivar Kapoho Solo) with either intact or mechanically wounded cuticles and incubated under conditions previously described (2). Control fruits were inoculated with spore suspensions and the emulsifier only. All fruits were colorbreak to ¼ ripe. The percentage of treated fruits showing infection after 7 days was compared to the controls.

Assay for fungitoxic activities. The inhibitors were incorporated at various concentrations into a basal medium of 10% V-8 juice agar by diluting them into the liquid (45 C) media prior to pouring into petri dishes. To the edge of these dishes were transferred filter paper disks (1.5 mm in diameter). The inoculum that had been grown separately on V-8 juice agar consisted primarily of mycelium, although a few spore masses were observed. The plates were incubated in the dark at 25 C for 5 days at which time radial growth of the fungal mycelium was measured and compared to fungal growth in control plates containing disks of inoculum on V-8 juice agar only.

RESULTS

Inhibition of cutinase. The results on the inactivation of *C. gloeosporioides* cutinase by various inhibitors are presented in Tables 1–3. Table 1 presents data on inhibition of cutinase by phosphoorganic esters, which are used as insecticides. All of these compounds proved to be powerful inhibitors of cutinase with I_{50} values ranging from 10^{-4} to 10^{-9} M.

Table 2 presents I_{50} values of four organophosphorous fungicides, benomyl and its primary breakdown product, carbendazim, which are carbamates. The I_{50} values of the organophosphates against cutinase again show their potent inhibitory effect on the enzyme. The I_{50} values of the fungicides ranged from 10^{-3} to 10^{-5} M.

In previous studies we observed that inhibition of *C. gloeosporioides* cutinase by DFP was time dependent, suggesting an irreversible inhibition. Treatment of the *C. gloeosporioides* cutinase with [^{3}H]DFP followed by SDS-polyacrylamide gel electrophoresis also showed that the diisopropylphosphoryl group was covalently attached to the enzyme (2). To determine the kinetics of inhibition of *C. gloeosporioides* cutinase, we employed the equations developed by Meloche (11) for studying the inactivation kinetics of irreversible enzyme inhibitors. According to his model, a noncovalent, reversible enzyme-inhibitor complex is formed by the irreversible inhibitor with the enzyme prior to covalent bond formation between the two. The inactivation in such cases would proceed according to equation 1,

$$E + I \rightleftarrows EI \rightarrow EI_I \qquad (1)$$

in which E represents the enzyme; I, the inhibitor; EI, the Michaelis-Menten type enzyme-inhibitor complex; and EI_I, the covalently bound enzyme-inhibitor complex. From this equation, the following equation can be derived,

$$t_{½} = (T_{½}\ K_i/[I]) + T_{½} \qquad (2)$$

in which $t_{½}$ is the inactivation half time; $T_{½}$, the inactivation half time at substrate saturation; and K_i, the inhibitor binding constant. Thus a plot of $t_{½}$ versus the reciprocal of inhibitor concentration should give a straight line with a slope of $T_{½}K_i$ and an intercept at $T_{½}$. When half times of inactivation are plotted as a function of the reciprocals of inhibitor concentration, a straight line with a slope of $T_{½}K_i$ and an intercept at $T_{½}$ are obtained.

The time course of inhibition of cutinase produced by *C. gloeosporioides* at three concentrations of chlorpyrifos, 1, 50, and 100 μM, was done. The data were plotted with the y-axis expressed on a semilogarithmic scale (Fig. 1). Values of $t_{½}$ obtained in this way were plotted against the reciprocal of the concentration of the inhibitor to obtain Fig. 2, which gave $T_{½}$ of 1.0 minute and a K_i of 2.6×10^{-8} M. Similar plots were constructed from time course inhibition data for *O, O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphate, Hinosan, and Inezin (Table 3).

TABLE 3. Inactivation kinetics of cutinase inhibitors

Compound	I_{50}(μM)	$T_{½}$(min.)	K_i(μM)
O,O-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphate	0.0011	0.7	0.01
Hinosan	33.2	1.3	225
Chlorpyrifos	53.7	1.0	260
Inezin	186.8	2.7	118
Sodium dodecyl sulfate (SDS)	107.2	-	(285)

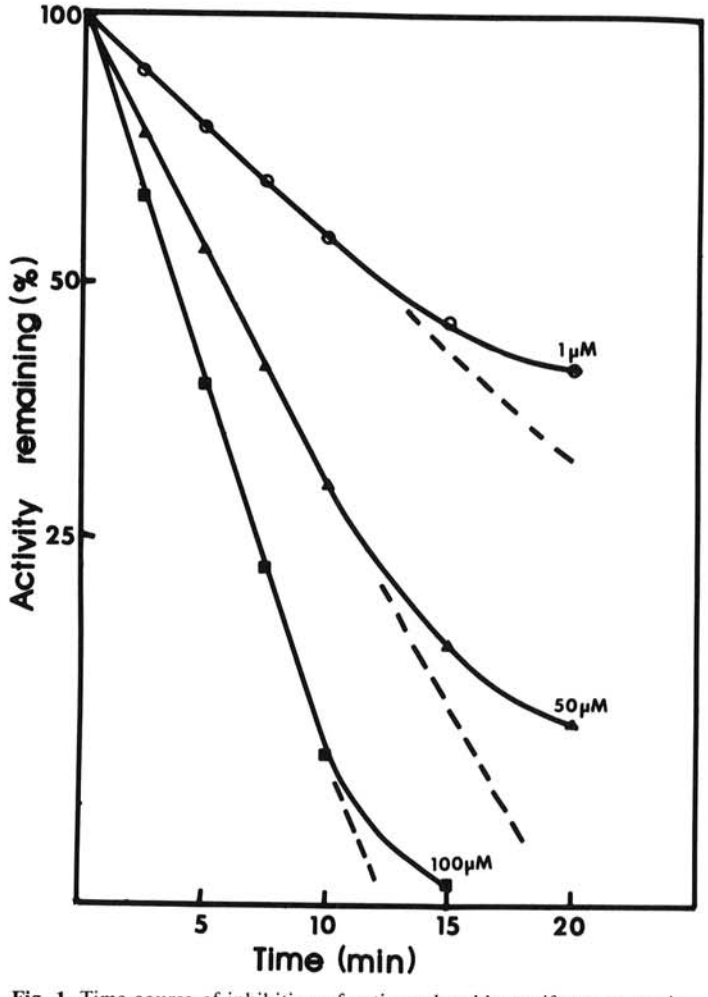


Fig. 1. Time course of inhibition of cutinase by chlorpyrifos on a semilogarithmic plot. Cutinase was incubated with the following concentrations of inhibitor: ○, 1 μM; ▲, 50 μM; and ■, 100 μM. Phosphate buffer with Triton X-100 was used. Aliquots of the emulsifiers (Atlox 3403F and Atlox 3404F), dispersed in xylene (final concentration, 0.0005%), were used with the inhibitor and with the control treatment. The dashed lines represent the pseudo-first-order phase.

It has been reported that SDS lowers the I_{50} values of organophosphates against the *Fusarium solani* f. sp. *pisi* cutinase (6); these values were decreased by a factor of 10 to 30 when SDS was used along with the inhibitors. Therefore, it was of interest to test the effect of SDS on the inhibition of *C. gloeosporioides* cutinase by organophosphates. Surprisingly, it was found that SDS was by itself a potent inhibitor of the enzyme, and this inhibition was not time dependent. A study of the kinetics of inhibition showed that SDS is a competitive inhibitor of the cutinase of *Colletotrichum* (Fig. 3). The I_{50} value for SDS was 107 μM and its K_i was 2.9×10^{-4} M. An interesting feature of this inhibition is that it is easily reversed by the addition of Triton X-100 (*unpublished*).

Prevention of papaya fruit infection by cutinase inhibitors. It was recently established that rabbit antibodies prepared against cutinase prevented fungal infection of papayas with intact cuticular surfaces. If these barriers were mechanically breached with a needle prick prior to inoculation, the rabbit antibodies did not prevent infection (2). Furthermore, DFP, a cutinase inhibitor, was equally effective in preventing infection (2). These results raised the possibility that the organophosphates and other inhibitors described here might prevent papaya anthracnose by impeding fungal penetration of the cuticle.

All the inhibitors tested in the fruit bioassay reduced infection of papaya fruit with intact cuticles (Figs. 4 and 5). To test whether prevention of infection was due to inhibition of fungal penetration and not due to the toxicity of the compounds to the pathogen, wound inoculated controls were employed. If lack of infection was due to inhibition of fungal growth, then wounding would have no effect on the protection afforded by the chemical. The results clearly showed that when cuticular barriers were breached by wounding prior to spore inoculation, there was no significant protection when compared to the controls, which had about 95% infection.

The radial growth experiments on the effect of the cutinase inhibition on the pathogen itself corroborated the above findings that the inhibitors were not fungitoxic or fungistatic at the concentrations used in the protection experiments. Conen and Kitazin P caused some mycelial growth retardation in the petri dishes at the higher concentrations (10-fold greater than their respective I_{50} values). This was also true of benomyl and carbendazim. SDS caused no inhibition of mycelial growth. All compounds were effective in suppressing infection, and generally, those with lower I_{50} values were more effective at low concentrations than those with higher I_{50} values.

DISCUSSION

All insecticides tested in this study inhibited the *C. gloeosporioides* cutinase, though to varying degrees (Table 1). As reported in the case of *F. solani* f. sp. *pisi*, which is a serine hydrolase (13), the mechanism of inhibition in the present case appears to be the same as that shown for inhibition of cholinesterase by organophosphorous compounds (4). Modification of chemical structure of a phosphoorganic compound can result in a marked change in its potency against a serine hydrolase (4). For example, by substituting ethyl groups for the methyl groups in Fospirate, the I_{50} value was lowered by about 10,000 (Table 1).

The organophosphates shown in Table 2 were developed as systemic fungicides against the rice blast fungus, *Pyricularia oryzae* (4,5,12). The results on the effect of organophosphorous fungicides (Table 2) also follow the structure activity relationships seen in the inactivation of cholinesterase by these compounds (4). Phosphorate esters are generally more inhibitory than their corresponding phosphate esters (Inezin versus Conen). Similarly arylthiolates are usually more inhibitory than *S*-alkylthiolates (Hinosan versus Conen). Again, these results are similar to those reported by Köller et al (6), although the absolute I_{50} values for various insecticides and fungicides for the enzyme of *Colletotrichum* are not the same as those reported for the enzyme of *F. solani*.

Of the two nonorganophosphorous fungicides, benomyl and carbendazim, benomyl is a more potent inhibitor of *C. gloeosporioides* cutinase (Table 2) than is carbendazim. Carbendazim is considered to be the "active" breakdown component of benomyl. However, our data suggest that the other breakdown product, *n*-butyl-isocyanate, which is a potent inhibitor

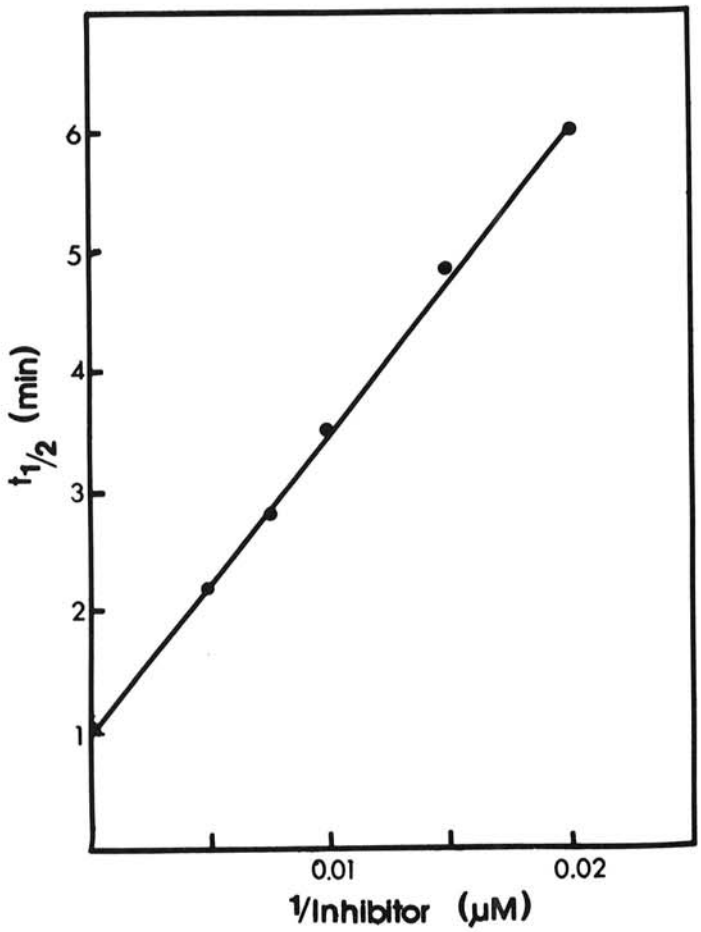


Fig. 2. Half time ($t_{1/2}$) of cutinase inhibition as a function of the inverse of inhibitor concentration. The $t_{1/2}$ values were obtained by extrapolation from Fig. 2.

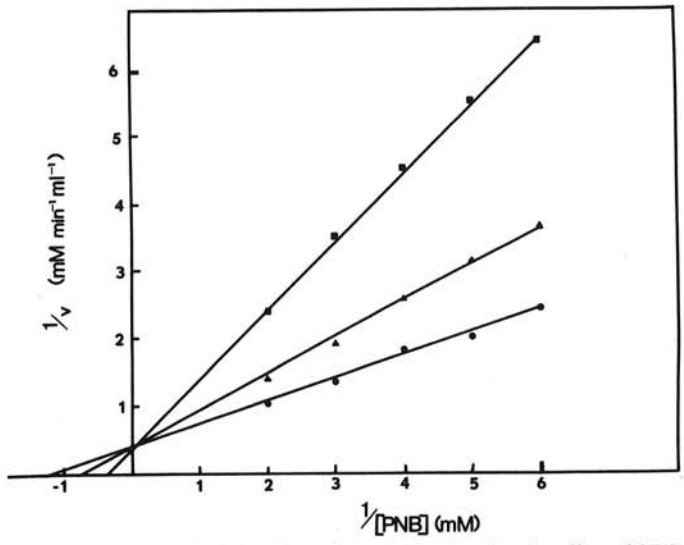


Fig. 3. Lineweaver-Burk double reciprocal plot showing the effect of SDS on the hydrolysis of *p*-nitrophenyl butyrate by the cutinase of *Colletotrichum gloeosporioides*. The following concentrations of SDS were used: •, 25 μM; ▲, 100 μM; and ■, 200 μM.

of the Fusarium cutinase (7), may be responsible for the very low I_{50} value of benomyl. It has been demonstrated that under aqueous conditions, benomyl spontaneously hydrolyzes to form carbendazim and *n*-butyl-isocyanate, and the latter covalently modifies active serine (7).

Although the inhibitory potency of pesticides is often expressed in terms of their I_{50} values, it is more precisely given by the inhibitor binding constant, K_i, and the half time of inhibition at saturating inhibitor concentration, $T_{½}$. This is because I_{50} values are not absolute and can vary depending on the enzyme concentration and incubation time. To examine the relationship between I_{50} and K_i values, four organophosphorous pesticides (two fungicides and two insecticides) with the lowest I_{50} values were selected for determining their K_i and $T_{½}$ against the Colletotrichum cutinase (Table 3).

O, O-Diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate, which had the lowest I_{50} value of the four pesticides tested (Table 3), also had the lowest K_i. It is interesting to note that the $T_{½}$ value of this insecticide is not much different from the $T_{½}$ values of the other three pesticides. This means that all four compounds react with the cutinase at about the same rate. However, *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate has a very much higher affinity for cutinase than do the other three compounds. Inezin, which has a very high I_{50} relative to the other compounds, actually has a much lower K_i than Hinosan and chlorpyrifos, thus differing from the other three pesticides. It appears that because Inezin reacts more slowly ($T_{½}$, 2.7 min), its I_{50} value is high even though it has a higher affinity (lower K_i) for the cutinase than Hinosan and chlorpyrifos.

Köller et al (6) reported that in all cases, substitution of Tween-20 by SDS lowered the I_{50} values of organophosphorous compounds against the Fusarium cutinase by as much as two orders of magnitude. They suggested that SDS caused a conformational change in the cutinase, which made the active site of the enzyme more accessible to or more reactive with the inhibitors. Since the Fusarium and Colletotrichum cutinases share many properties (2) we expected SDS to affect the cutinase produced by *C. gloeosporioides* similarly. Instead, we found that SDS, at the concentration at which it was used in the Fusarium cutinase study (0.2 mM), totally inhibited the Colletotrichum enzyme. At a much lower concentration (100 μM), where it had little inhibitory effect, SDS did not lower the I_{50} value of *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate against Colletotrichum cutinase significantly. Thus, in contrast to the action of this detergent on the *F. solani* f. sp. *pisi* enzyme, SDS does not appear to potentiate the other inhibitors. The kinetics of inhibition of cutinase by SDS showed that in fact, SDS acts as a competitive inhibitor of this enzyme, suggesting that it can bind at the substrate binding site. The apparent difference between the interaction of SDS with the two cutinases is presumably due to the differences in the substrate binding site. This difference is reflected in substrate specificity of

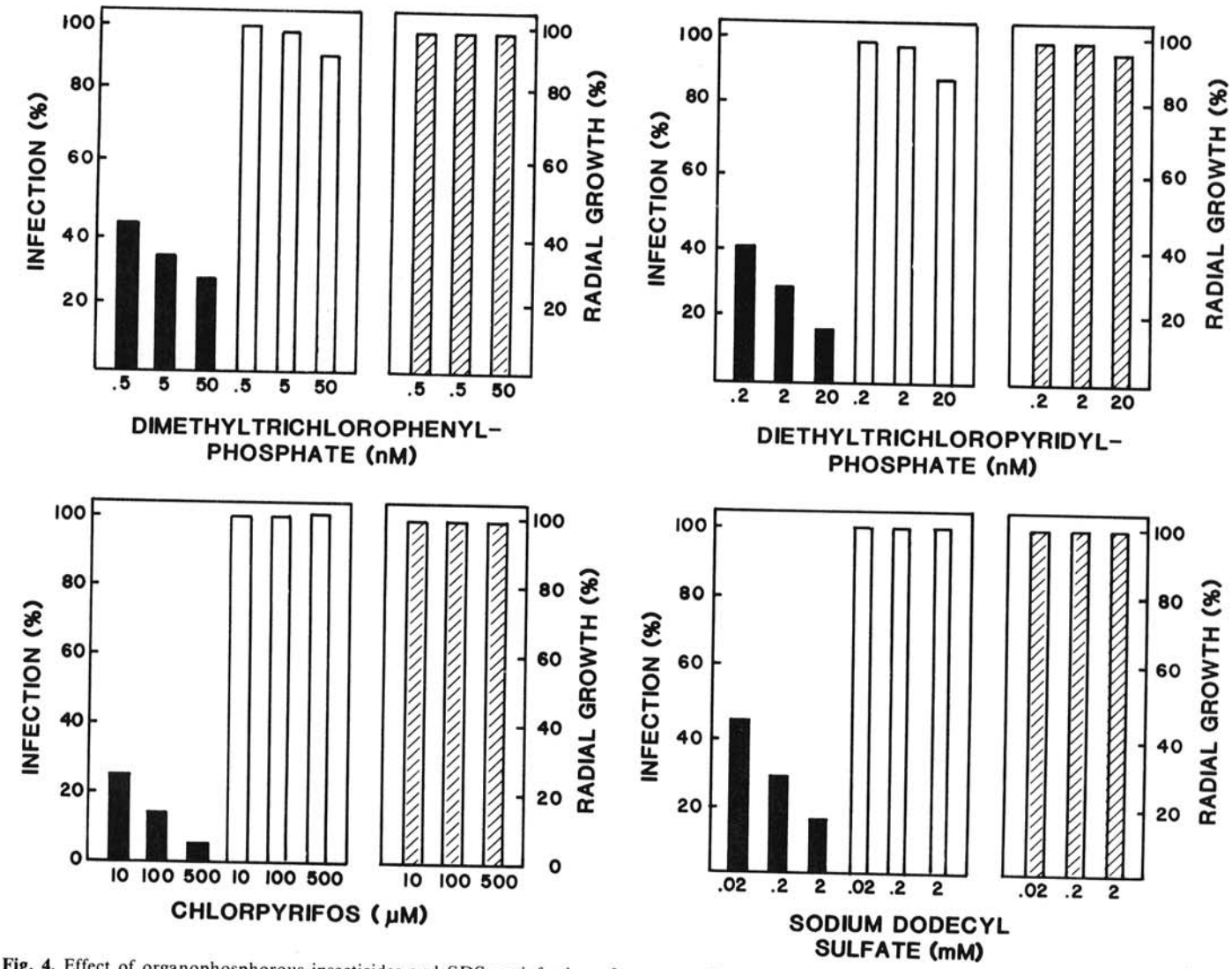


Fig. 4. Effect of organophosphorous insecticides and SDS on infection of papaya and on growth of *Colletotrichum gloeosporioides* in vitro. Spore suspensions containing pesticides at indicated concentrations were used to inoculate intact (■) or mechanically wounded (□) papaya fruit surfaces. Percent infection is expressed as percent of control containing no inhibitor. Mycelial growth measurements (▨) were made from 10% V-8 juice agar plates containing pesticides at the indicated concentrations. Measurements of mycelial growth were made after 5 days of incubation, and compared to controls.

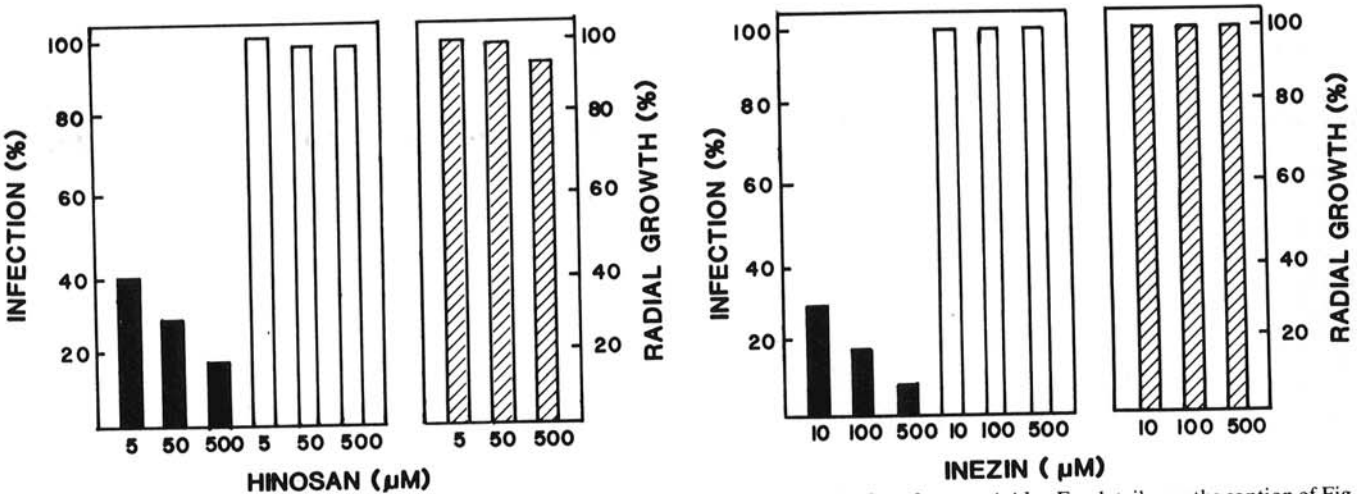


Fig. 5. Effect of organophosphorous fungicides on infection of papaya and on the in vitro growth of *C. gloeosporioides*. For details, see the caption of Fig. 4.

the two enzymes; the Colletotrichum enzyme can hydrolyze model substrates with hydrocarbon chain lengths ranging from C_4 to C_{16} (2) at comparable rates, but the Fusarium cutinase can hydrolyze only the *p*-nitrophenyl ester of the short-chained C_4 acid, PNB, at appreciable rates. The present findings concerning the inhibitory action of organic phosphates and derivatives on cutinase, even though obtained under controlled conditions in the laboratory, provide a rational basis for designing strategies to control the pathogen in the field.

It has previously been shown that *C. gloeosporioides* penetrates papaya fruit by dissolving the cuticular barrier of papayas with a cutinase (2). The present results show that inhibitors of *C. gloeosporioides* cutinase are effective in reducing infection of papayas by the pathogen at least under controlled laboratory conditions. Therefore, it may be feasible to use the cutinase inhibitors under field conditions as well. It has been recently demonstrated (3) that infection of papayas in the field occurs at all stages during fruit development, but the fungus remains latent until after fruit ripening. Thus, in order to reduce losses caused by anthracnose, inhibitors of cutinase will have to be tested under field conditions, and not as a postharvest treatment, to determine whether they are effective under such conditions. Field experiments are currently in progress.

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