Protection of *Pisum sativum* from *Fusarium solani* f. sp. *psi* by Inhibition of Cutinase with Organophosphorus Pesticides

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**ABSTRACT**


The phosphoorganic insecticides Paraaxon, O,O-dimethyl-O-(2,4,5-trichlorophenyl)phosphate, Fosphate, O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphate, Parathion, Dowco 214, and Chloropyrifos, and the phosphoorganic fungicides Hinoan, Inezin, Conen, and Kitarin P were shown to be powerful cutinase inhibitors. The *I₅₀* values ranged from about 10⁻¹¹ to 10⁻¹⁰ M. All pesticides prevented infection of *Pisum sativum* by *Fusarium solani* f. sp. *psi* under controlled conditions of a pea stem bioassay on intact cuticles only. Infection was not prevented on wounded pea stems. The effective pesticide concentrations reflected, to a certain degree, the corresponding *I₅₀* values for cutinase inhibition. No inhibition of mycelial growth was observed at these pesticide concentrations. These results suggest that the pesticides prevented penetration, and thus infection, by inhibiting cutinase released by the fungal infection structure.

**Additional key words:** antipenetrants, cutin, cuticle.

Direct penetration by fungal parasites through intact cuticles, the outer hydrophobic barrier of aerial plant surfaces, has been observed in many host-pathogen interactions (13–16). Based on ultrastructural examinations of penetrating infection structures it was suggested that cuticular penetration was accomplished by enzymatic degradation of the cuticle (1,29). Cuticle degradation requires an enzyme that hydrolyzes the ester bonds of the polyester cutin, the structural component of the cuticle that is composed of hydroxy and epoxy fatty acids containing 16 and 18 carbon atoms, respectively (13–16). The detection of this postulated enzyme, cutinase, in culture fluids of several pathogenic fungi grown on cutin as the sole carbon source was, therefore, consistent with this hypothesis (13–16). Direct proof for the involvement of cutinase in penetration of a pathogen into its host was recently provided for the infection of *Pisum sativum* by *Fusarium solani* f. sp. *psi* (19,22,27).

Selective chemical modification of cutinase purified from this fungus revealed that a catalytic triad consisting of one “active serine,” one histidine residue, and one carboxyl group was involved in the enzymatic mechanism of ester hydrolysis (19). This catalytic triad is characteristic of serine hydrolases, a class of enzymes that is inactivated by active serine directed inhibitors like phosphoorganic compounds. Disopropyfluorophosphatase, a powerful cutinase inhibitor of this type, was recently shown to prevent infection of *P. sativum* by *F. solani* f. sp. *psi* (22). On the basis of this observation it was suggested that inhibitors targeted against cutinase might act as “antipenetrants” and thus prevent fungal infection of plants (22).

In this study we report that a number of organophosphorus pesticides (all powerful cutinase inhibitors) at subtoxic concentrations prevent penetration and thereby, infection in a laboratory bioassay.

**MATERIALS AND METHODS**

*Fusarium solani* f. sp. *psi* isolate T-8 was obtained from H. D. van Etten, Cornell University, Ithaca, NY. The culture was maintained on potato-dextrose agar. O,O-Diethyl-O-p-nitrophenylphosphate (Paraaxon), O,O-diethyl-O-p-nitrophenylphosphorothionate (Parathion) and S,S-diethyllephosphorothiolate (Hinoan) were obtained from Bayer AG, Germany. O,O-Dimethyl-O-(3,5,6-trichloro-2-pyridyl)phosphate (Fosphate), O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphate, O,O-dimethyl-O-(2,4,5-trichlorophenyl)phosphate, O,O-dimethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorothionate (Dowco 214) and O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorothionite (Chloropyrifos) were obtained from Dow Chemical Company, United States. S-Benzyl-O-diisopropyphosphorothiolate (Kitarin P), S,benzyl-O-n-butyl-S-ethylphosphorothiolate (Conen) and S-benzyl-O-ethylphosphorothiolate (Inezin) were provided by M. Yamada, National Institute of Agricultural Sciences, Japan. All chemicals were of analytical grade.

**Cutinase.** Cutinase was purified from the extracellular fluid of *F. solani* f. sp. *psi* grown on cutin as the sole carbon source by the procedure previously described (17,18). Esterase activity was measured with p-nitrophenylbutyrate as a model substrate (17).

**Determination of I₅₀ values.** The inhibitory potency of the pesticides is expressed by the *I₅₀* values. *I₅₀* is the molar inhibitor concentration of pesticide required to cause 50% inhibition of the enzyme activity after 1 h of incubation at 25 C. Cutinase (5 nM) was incubated in 50 mM sodium phosphate buffer (pH 7.5) containing 0.5% (w/v) Tween-20 or in 100 mM HEPES (pH 7.5) containing 0.2 mM sodium dodecyl sulphate (SDS) in the presence of pesticides. Stock solutions of pesticides were made in acetone (final acetone concentration, 5%). After 1 h of incubation esterase activity was measured with the model substrate p-nitrophenylbutyrate as described above. The *I₅₀* values were derived from semilogarithmic plots of pesticide concentration against remaining enzyme activity. To follow the time course of inactivation, cutinase (50 nM) was incubated at 25 C in Tween-20 or SDS buffer as described above. The inactivation was started by addition of the pesticide from a stock solution in acetone. After appropriate time intervals, esterase activity was assayed immediately after dilution of the incubation mixture (1,000-fold) into the assay buffer. All experiments were repeated at least twice with identical results.

**Assay for protective activity.** Protection against the fungus was measured by the “pea stem bioassay” previously described (18,22). Epicotyl segments (40 per test chemical) from 7-day-old etiolated
Seedlings of *P. sativum* 'Perfection' were inoculated with 5-μl droplets of a conidial suspension of *F. solani* f. sp. *pisum*. The infection droplets containing the pesticides were placed on the surface of either intact or wounded (needle pricked) stem sections. Dark-brown lesions formed after 3 days of incubation in the dark were taken as a measure of infection. The pesticides in the droplet represented a 10,000-fold dilution of an emulsifiable concentrate (xylene containing 5% each of Atlox 3403F and Atlox 3404F). In all experiments chemicals contained in the droplets did not cause any lesions. All experiments were repeated at least twice with similar results.

**Assay for fungitoxic activities.** Pesticides were diluted into hot potato-dextrose agar (Difco) from emulsifiable concentrates as described above. Spores of *F. solani* f. sp. *pisum* were mixed with potato-dextrose agar at 45°C, and the mixture was poured into a petri dish. The final spore concentration was 10^6 spores per milliliter. After solidification of the agar, disks (5 mm diameter) were placed in the center of petri dishes containing pesticides in potato-dextrose agar. The diameter of the fungal colony was measured after incubation for 3 days at 25°C.

### RESULTS

**Inhibition of cutinase by organophosphorus pesticides.** All phosphoorganic esters shown in Table 1, developed or used as insecticides, were powerful inhibitors of cutinase. The inhibitory potency, expressed by their IC50 values, was strongly dependent on both the conditions of incubation and the chemical structure of the compounds. All IC50 values were decreased by a factor of 10 to 30 when Tween-20, a nonionic detergent, was substituted by SDS, an anionic detergent, at a low concentration. Binding of SDS at this concentration changes the conformation of the active site of cutinase and increases the catalytic power of this enzyme (*unpublished*). Small alterations of the chemical structure made a substantial difference in the inhibitory power of the insecticides tested. Thus, a substitution of methyl groups in Fosilate by ethyl groups leading to O,O-diethyl O-[3,5,6-trichloro-2-pyridyl] phosphate lowered the IC50 value by a factor of about 500 (Table 1). The IC50 value of 3 × 10^-4 M determined in the presence of SDS represents almost a stoichiometric reaction of cutinase (3 × 10^-4 M) with this inhibitor.

All four fungicides (Table 2) tested were cutinase inhibitors. SDS again increased the inhibitory power of all four compounds. The IC50

### TABLE 1. Cutinase inhibition by organophosphorus insecticides

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Name</th>
<th>IC50 (μM)</th>
<th>Tween-20</th>
<th>SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C2H5)2PO(O-C6H4)NO2</td>
<td>Paraoxon</td>
<td>1.6</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>(C4H9)2PO(O-C6H4)Cl2</td>
<td>O,O-Dimethyl-O-(2,4,5-trichlorophenyl) phosphate</td>
<td>0.18</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>(CH3)2PO(O-C6H4)Cl2</td>
<td>Fosilate</td>
<td>13.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>(C2H5)2PO(O-Cl)3</td>
<td>O,O-Diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphate</td>
<td>0.027</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>(C2H5)2PO-Cl2</td>
<td>Chloropyrifos</td>
<td>23.0</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>*</sup>Common name.

### TABLE 2. Cutinase inhibition by organophosphorus fungicides

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Common name</th>
<th>IC50 (μM)</th>
<th>Tween-20</th>
<th>SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)2P-OC2H5</td>
<td>Hinosan</td>
<td>39</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>CH2-S-PO-OC2H5</td>
<td>Inezin</td>
<td>1.000</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>CH2-S-P-S-C6H5</td>
<td>Conen</td>
<td>&gt;1,000</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>CH2-S-P-OC3H72</td>
<td>Kitazin P</td>
<td>&gt;1,000</td>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

### Fig. 1. Time course of inhibition of cutinase by Hinosan. Cutinase was incubated with indicated concentrations of the inhibitor in the presence of Tween-20 or sodium dodecyl sulphate (SDS). The experimental conditions are described under Materials and Methods.
values reflect the structure activity relationship established for the inhibition of acetylcholinesterase. S-Arylisothiocyanates were generally more active than S-alkylthiophenols (Hinosan vs Conen), and phosphonate esters were more reactive and more biologically active than phosphate esters (Inezin vs Conen) (6). Kitazin P was found to be relatively weak cutinase inhibitor when compared to the insecticides listed in Table 1. Phosphothiophenolates, like Hinosan, caused a time-dependent inactivation of the enzyme. This inactivation proceeded in a pseudo first order reaction with a second-order rate constant of 12 s⁻¹ M⁻¹ in the presence of Tween-20 and 70 s⁻¹ M⁻¹ in the presence of SDS (Fig. 1). The activation of cutinase by SDS is reflected in the decrease of the I₅₀ value observed in the presence of SDS. The time-dependency of the reaction indicates that phosphothiophenolates, like Hinosan, inactivate cutinase by an irreversible phosphorylation of the "active serine" residue.

Prevention of infection by phophoorganic pesticides. A specially designed bioassay served as the basis to investigate the possibility as to whether the above inhibitors of cutinase might prevent enzymatic breaching of the cuticle and thus function as antipermeants. Pea stem segments were inoculated with a suspension of microconidia and macroconidia of F. solani l. sp. pisi (isolate T-8). The inoculation droplet contained an emulsifier and the pesticides at different concentrations, with a medium concentration close to the I₅₀ value determined for cutinase in the presence of SDS. An infection was manifested after 3 days of incubation by a dark brown infection area. Inoculum droplets containing only emulsifier usually caused about 95% infection. The percent of segments showing infection in the presence of pesticides was compared to this control. To show that any inhibition of infection was caused by inhibition of penetration, and not by a fungitoxic effect, the infection droplets were also placed on a wounded surface. A fungitoxic mode of action should prevent infection even when a wound is provided for entry of the pathogen into its host. Possible growth inhibition caused by the pesticides was, in addition, investigated by radial growth experiments. A representative example of this set of experiments is shown in Fig. 2.

All o xo-anaols of the insecticides shown in Table 1 prevented infection on pea stem with intact cuticular barrier only (Fig. 3). Substantial decrease of infection was not observed when the pea stem surface was wounded prior to inoculation with spores. Consistent with this finding none of the compounds showed inhibition of mycelial growth at these concentrations. These compounds at the concentrations used in the present experiments showed no discernible inhibition of spore germination when directly tested in an aqueous medium. The effectiveness of these organophosphorus insecticides reflected, to a certain degree, their cutinase inhibitory activity (Table 1). O,O-Dimethyl-O-(2,4,5-trichlorophenyl)phosphate and O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphate, the most powerful cutinase inhibitors, were highly protective at concentrations as low as 10⁻⁶ M. The thiocyanates listed in Table 1 were not included in this study because they might be converted into the corresponding o xo-anaols during the period of incubation (6).

The organophosphorus fungicides listed in Table 2 gave results similar to those obtained with the insecticides (Fig. 4). Again, all compounds prevented infection on pea stems with intact surfaces only. Conen and Kitazin P showed slight infection inhibition on wounded pea stem at the highest concentration applied. At these concentrations, however, a slight growth inhibition was also observed. The effective concentrations reflected again the cutinase inhibitory activity expressed by the I₅₀ values (Table 2).

Fig. 2. Photograph of pea epicotyl segments and fungal colonies 72 hr after inoculation. A conidial suspension of Fusarium solani l. sp. pisi was used to inoculate intact (IS) or wounded (WS) stem segments or to start mycelial growth (MG) on potato-dextrose agar. The spore suspension and the potato-dextrose agar contained 0, 1, 10, or 100 μM Hinosan.
DISCUSSION

The inhibitory action of organophosphorus esters against cholinesterases, attributed to the irreversible phosphorylation of an "active serine residue," is the biochemical basis for insecticidal properties of this class of pesticides (6). It was recently shown that diisopropylfluorophosphate inactivated cutinase by the same mode of action (19). In addition to these insecticides a number of organophosphorus fungicides have been developed (7,8,20). All compounds listed in Table 2 are used as systemic fungicides against the rice blast fungus, *Pyricularia oryzae* (7,8,20,25). From a mechanistic point of view it appeared possible that these compounds might also inhibit cutinase. In fact, all of the organophosphates mentioned above were found to be potent inhibitors of cutinase. Among them, compounds containing a thiophosphoryl group were generally less active as acetylcholinesterase inhibitors than the corresponding oxo-analogs (6). This general rule holds true for the inhibition of cutinase as well (Table 1).

Rabbit antiserum prepared against cutinase was recently shown to prevent infection of pea stem segments by *F. solani* f. sp. *pisii* (22). This protective action, however, was observed only on stems with intact cuticular barriers. Specific inhibition of cutinase with antibodies had no effect when the defensive barriers were mechanically breached (18,22). This finding raised the possibility that the phosphoorganic inhibitors of cutinase described above might prevent the enzymatic breaching of the cuticle and thus might function as "antipenetrants." The results reported in the present paper show that the inhibition of cutinase by the organophosphates does in fact protect *P. sativum* against *F. solani* f. sp. *pisii*, at least under the controlled conditions of the bioassay used.

All phosphoorganic pesticides tested, insecticides as well as fungicides, were shown to be cutinase inhibitors in vitro. All of these chemicals effectively prevented the infection of pea stems by *F. solani* f. sp. *pisii*, but only when applied on surfaces with intact defensive barriers. They were without any protective effect on wounded surfaces, and the effective concentrations reflected, to a certain degree, their cutinase inhibitory power. Furthermore, all pesticides showed no inhibitory effect on fungal growth at these concentrations. Taken as a whole the results strongly indicate that the protective effect was due to the inhibition of penetration of the infection structure, most likely mediated by inhibition of cutinase excreted during the early phase of spore germination. Cutinase released during this early stage of infection has been shown to be essential in the compatible interaction of *F. solani* f. sp. *pisii* with peas (18,22,27) and *Colletotrichum gloeosporioides* with papaya fruits (5).

The relationship between *IC₅₀* and effectiveness of protection is complicated by the fact that the two parameters are measured under drastically different experimental conditions. For example, the inhibition of the enzyme is measured after treatment of the pure...
enzyme with the chemical for a relatively short period of time whereas under the bioassay conditions the inhibitor must stay in the drop in an active form and react with the enzyme, which would be released by the fungus over a period of time. Thus, if the inhibitor is relatively unstable but highly reactive with the enzyme such an inhibitor could show an extremely low I50 value, but not a high degree of protection. A relatively long lasting inhibitor with high inhibitory capacity would be a desirable antipenetrant.

The organophosphorus fungicides Hinosan and Kitazin P were shown to inhibit the growth of P. oryzae, a rice plant pathogen (25). The biochemical mode of action was reported to be inhibition of cell wall biosynthesis (21,23) or, more recently, inhibition of phosphatidylcholine biosynthesis (2,4,11,12). Both fungicides were inhibitors of cutinase purified from F. solani f. sp. pisi and both prevented penetration of this fungus into its host P. sativum at subtoxic levels. Both chemicals were reported to inhibit fungal penetration into rice as well. In this case, however, the inhibition of spore germination seemed to be responsible for this mode of fungicidal action (25). Recently developed fungicides against P. oryzae like Rabeide and Fuji-one prevented penetration of the fungus without any obvious effect on spore germination (25). Rabeide was reported to prevent appressorium formation (3). The specific effect of Fuji-one on penetration was confirmed, but the biochemical mode of action of this protective activity remains unknown (24). Both of these fungicides were tested in the present study, but showed no cutinase inhibitory activity. However, the breaching of the defensive plant barriers is only one event during the early stages of infection. This infection process proceeds regularly from spore germination through formation of infection structure and penetration. The specific chemical inhibition of any one of these steps should prevent penetration and thus infection. It was suggested that inhibition of cell penetration is a general nonhost defense mechanism of plants to infection and that this general resistance is exhibited in this early stage of infection (10,28). Inhibitors targeted against a prepenetration step would, therefore, mimic this natural plant defense system.

Cutinase was shown to be essential in one of these prepenetration steps (18,22,28). We showed in this study that inhibition of this enzyme indeed prevented penetration and consequently infection of the plant. The possibility that the pesticides tested also affected some other process essential for infection cannot be completely ruled out. Much further work is needed to decide whether it is practically feasible to use antipenentrants as protective agents in the field. If such an approach is feasible, it is conceivable that systemic protectants could be used. The structure of such chemicals might be designed in such a way that they would distribute themselves in the cuticle at an effective concentration. It might be possible to design a potent cutinase inhibitor that could be attached to another molecule to provide the desired systemic properties. Once the material reaches aerial parts of the plant the attachment might be broken and the inhibitor might diffuse into the cuticle. It might also be possible to combine a fungicidal activity with an antipenetrant activity so that the chemical could prevent penetration and inhibit growth of the organisms, which might already have penetrated. For

![Fig. 4. Inhibition of infection by phosphoorganic fungicides. For details see legend to Fig. 3.](image-url)
example, new phosphoorganic fungicides are usually developed on
the basis of their fungitoxic properties (eg. 9,26). In such cases,
investigation of their cutinase inhibitory activity in addition to
toxic properties at this stage of development might result in
fungicides with protective as well as curative properties. Obviously
many critical parameters related to the structural requirements and
effective concentrations would have to be determined. In any case,
our findings open the possibility of designing new or improved
agents to protect plants against fungal attack by using cutinase as
a target enzyme.

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