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EVIDENCE OF CUTINASE ACTIVITY RELEASED BY Ascochyta pinodes and Ascochyta pisi

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ABSTRACT

The cutinolytic activity of *Ascochyta pinodes* and *Ascochyta pisi* was studied. When the fungi were grown in nutrient medium containing cutin as sole source of carbon, cutinase was released in the medium, as measured by microtitration of fatty acids generated from cutin, or by esterase activity. Diisopropyl fluorophosphate (DFP), a cutinase inhibitor, prevented the infection of unwounded pea leaflets, but not that of Carborundum-wounded leaflets, upon inoculation of *A. pisi* or *A. pinodes*. When DFP application was delayed, its protective effect decreased. Our results suggest that cutinase activity is involved in pea leaf penetration and subsequent infection by *A. pinodes* and *A. pisi*.

The role of cutinase in the penetration of plants by pathogenic fungi has focused the attention of researchers (SHAYKH *et al.*, 1977, DICKMAN *et al.*, 1982, 1983, KOLATTUKUDY, 1984, 1985). Cutinase activity was studied, using cutin as substrate, either by measuring the radioactivity released from labelled cutin (PURDY and KOLATTUKUDY, 1975a), or by assaying pH changes due to the liberation of fatty acids (BASHAN *et al.*, 1985). Cutinase, being an esterase (KOLATTUKUDY, 1985), was also evaluated by using para-nitrophenyl esters as substrate (DICKMAN *et al.*, 1982, DANTZIG *et al.*, 1986). Fungal cutinases are severely inhibited by reagents directed towards active serine, such as diisopropyl fluorophosphate (DFP) (PURDY and KOLATTUKUDY, 1975b). *Fusarium solani* f. sp. *pisi* has been the most studied among numerous fungal species (PURDY and KOLATTUKUDY, 1973, 1975a, DANTZIG *et al.*, 1986, PODILA *et al.*, 1988); cutinase from this fungus was purified and characterized chemically and immunologically.

This paper presents evidence of cutinolytic enzymes released by *Mycosphaerella pinodes* (Berk. et Blox.) Vestergr. (*Ascochyta pinodes* Jones), and *Ascochyta pisi* Lib., and establishes the role of cutinase in the penetration of pea leaves by both fungi.

MATERIALS AND METHODS

Organisms, media and growth conditions

Cultures of *A. pinodes* (strain n°6 132/7/78 from the Commonwealth Mycological Institute, England) and *A. pisi* (strain LG kindly donated by Dr. SPIRE, INRA, France) were stored at -20°C and were routinely grown on meal oat agar medium.

Cutin medium, used to induce cutinolytic activity, was a modified Czapeck-Dox's medium (DICKMAN and PATIL, 1986) containing: 3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g KCl, and 0.01 g $FeSO_4$ $7H_2O$ in 1 liter of distilled water, containing 0.2% (w/v) apple cutin ground to a fine powder as the sole source of carbon. The same mineral solution, with or without addition of 0.2% glucose, was used as control medium. All media were autoclaved for 20 min at 120°C. Erlenmeyer flasks containing 50 ml of medium were inoculated with 1 ml of a suspension of *A. pinodes* (10^5 spores/ml) or of *A. pisi* (10^6 spores/ml), both collected from 15-day old cultures. The vials were incubated for 15 days in a rotary shaker at 26°C, with a photoperiod of 16 hr of light.

Cutin was prepared from apples (cv. "Golden Delicious"), as described by BAKER and BATEMAN (1978). Apple peelings were stirred in a boiling aqueous solution of 0.4% oxalic acid and 1.6% ammonium oxalate (10 liters/kg). The separated cuticle was collected and rinsed thoroughly with tap water (step 1). Aliquots of 50 g of the crude cuticle thus recovered were treated for 18 hr at 30°C with 2 liters of 100 mM sodium acetate buffer (pH 4.5) containing 0.5% cellulase (22180, Fluka) and 0.1% pectinase (76290, Fluka). The residual insoluble material was collected and rinsed thoroughly with tap water (step 2). It was then extracted at room temperature successively with methanol (1 liter/50 g) and 2:1 chloroform/methanol (1 liter/50 g) (step 3), followed by Soxhlet extraction with chloroform for 24 hr at room temperature (step 4). The purified cutin thus obtained was dried under a hood, and was repurified twice through steps 2-4.

Enzyme assays

Cutinase was estimated by measuring esterase activity, using para-nitrophenol acetate (PNPA) as the substrate and para-nitrophenol (PNP) as end product (PARKKINEN *et al.*, 1978). The enzymatic reaction was carried out in 7 ml of 30 mM phosphate buffer (pH 7) mixed with 1 ml enzymatic preparation (the fungal culture filtrate) and 2 ml of 0.2 mM PNPA (obtained from a solution of O.1 M PNPA in methanol), supplemented or not with DFP. Incubation was at 35°C for 2 hr. PNP was quantified by measuring absorbance at 400 nm, and the esterase activity unit (U) was expressed as 1µM PNP/min.

Cutinase activity was also measured by NaOH microtitration of fatty acids released from the cutin substrate. Culture filtrates (50 ml) were dialysed for 48 hr and lyophilized. The lyophilized filtrates thus recovered (3 mg from *A. pinodes* and 5 mg from *A. pisi)* were incubated for 24 hr at 35°C in the medium of BASHAN *et al.* (1985) (the reaction medium), made of 2 ml of 1 mM phosphate buffer pH 7.5, 1 ml of cutin suspension at 10 mg/ml, and 1 drop of toluene. The reaction medium was supplemented or not with 10 mM of DFP.

Fatty acids were extracted from 3 ml reaction medium with 6 ml of a mixture containing isopropanol, heptane and N-sulfuric acid (4:1:1, v/v/v) (DOLE, 1956). Microtitration of 0.5 ml of the upper phase (heptane phase) was performed with NaOH (0.0025 N), after addition of 0.5 ml ethanol, under nitrogen flux, using 0.067 mM palmitic acid as a standard. The released fatty acids were expressed as palmitic acid equivalent.

Controls were performed without lyophilized filtrate, or using culture filtrate boiled for 15 min before its lyophilization.

Biological assays

The third leaflet of 15-day old pea plants *Pisum sativum* L., cv. "Maxi", was detached and placed on water agar medium in Petri dishes. Leaflets were inoculated by placing a drop (supplemented or not with DFP) of fungal suspension (50 µl of 10^5 spores/ml for *A. pinodes* or 100 µl of 5×10^6 spores/ml for *A. pisi*) containing 0.01% Tween 20, 0.1% glycerol and 2-(N-morpholino) ethane-sulfonic acid buffer either at 0.1% for *A. pinodes* or 0.05% for *A. pisi*. Petri dishes were incubated at 26°C under 2000 lux with a photoperiod of 16 hr light. Symptom intensity of unwounded or Carborundum-wounded leaflets, was evaluated at 48 hr (*A. pinodes*) or 96 hr (*A. pisi*) after inoculation (three replicates of 10 leaflets each). The following scale was used to evaluate symptom intensity on the leaf surface under the drop of inoculum:

0 - no spots
1 - few small brown necrotic spots
2 - numerous large brown necrotic spots
3 - generalized necrosis extending under the whole drop.

RESULTS

Esterase activity estimated by using PNPA as substrate

With both *A. pinodes* and *A pisi*, esterase activity was found in the cutin culture medium, but was absent in the medium without cutin, or in medium containing glucose as carbon source. When culture filtrates were boiled for 15 min, esterase activity was abolished (Table 1). Furthermore, esterase activity was almost entirely inhibited by addition of DFP in the enzymatic reaction medium.

TABLE 1

Esterase activity* in filtrates from liquid culture medium of *A. pinodes* or *A. pisi* after 15 days

Culture medium	Treatment of filtrate	*A. pinodes*	*A. pisi*
Mineral medium + cutin	-	0.37	0.48
Mineral medium + glucose	-	0.01	0.02
Mineral medium	-	0.00	0.00
Mineral medium + cutin	boiled	0.00	0.00
Mineral medium + glucose	boiled	0.00	0.00
Mineral medium	boiled	0.00	0.01

* Esterase activity expressed as U/ml, using p-nitrophenyl acetate as substrate

The regression line 1/V = f (1/[S]) established from enzymatic reaction speed (V) and substrate concentration ([S]), permitted the estimation of the K_m, which was 8.40 x 10^{-2} mM for *A. pinodes* (with a coefficient of variation of 53.5%) and 9.92 x 10^{-2} mM for *A. pisi* (with a coefficient of variation of 43.6%).

Microtitration of fatty acids released from cutin

Table 2 shows the release of fatty acids from cutin by the lyophilized culture filtrates of either *A. pinodes* or *A. pisi*, previously grown on cutin culture medium.

When the enzyme reaction medium did not contain lyophilized filtrate, or was prepared with lyophilized boiled filtrate, or was supplemented with DFP (10 mM), the release of fatty acids was reduced by 90%, as compared to the medium containing lyophilized filtrate.

TABLE 2

Microtitration of fatty acids released from cutin by lyophilized culture filtrates of *A. pinodes* or *A. pisi* previously grown in cutin medium

Reaction medium*	Mean (µg)**	Coeff. of variation	Corrected*** mean (µg)
Control (without lyophilized filtrate) = background	14.3	3.2%	0
Control (without lyophilized filtrate) + DFP	15.0	9.2%	0.7
A. pinodes			
Lyophilized boiled filtrate	14.7	11.0%	0.4
Lyophilized boiled filtrate + DFP	16.3	16.3%	2.0
Lyophilized filtrate + DFP	16.9	13.7%	2.6
Lyophilized filtrate	39.8	16.3%	25.5
A. pisi			
Lyophilized boiled filtrate	16.6	16.6%	2.3
Lyophilized boiled filtrate + DFP	15.9	8.4%	1.6
Lyophilized filtrate + DFP	17.5	8.9%	3.2
Lyophilized filtrate	45.7	17.7%	31.4

* With addition of cutin substrate according to BASHAN *et al.* (1985)
** Mean result of three replicates, expressed as equivalent of palmitic acid
*** Obtained after substracting the mean value of control without lyophilized filtrate.

Effect of DFP on symptom intensity on detached pea leaflets

Necrotic spots induced by *A. pinodes* appeared on pea leaflets 48 hr after inoculation. With *A. pisi*, clear necrotic spots were observed after 96 hr.

Increasing DFP concentration in the inoculum resulted in a correlative decrease of symptom intensity. The DFP concentration needed for complete symptom inhibition was 7.5 mM for *A. pinodes* (10^5 spores/ml) and 5 mM for *A. pisi* (5×10^6 spores/ml).

Light microscopy observations of the inoculation sites showed that spore germination of *A. pinodes* was completed within 24 hr, when the first microscopic lesions appeared. In the presence of DPF (below 10 mM), germination was almost normal, but no lesions were formed.

For both fungal species, the addition of DFP to the inoculum prevented symptom appearence on unwounded leaflets (10-20 mM for *A. pinodes* and 5-10 mM for *A. pisi*), but did not inhibit lesion formation on Carborundum-wounded leaflets (Table 3). With *A. pinodes* (10^5 spores/ml), DFP (7.5 mM) prevented symptom appearance when added up to 12 hr after inoculation. Addition of DFP between 12 to 36 hr after inoculation gradually lost its protective effect (Figure 1). A comparable decreased inhibition by DFP (5 mM) was also observed between 18 and 54 hr for *A. pisi* (5×10^6 spores/ml) (Figure 2).

TABLE 3

Effect of DFP on symptom intensity* of detached pea leaflets inoculated with *A. pinodes* or *A. pisi*

DFP (mM)	*A. pinodes* (10^5 spores/ml)		*A. pisi* (5×10^6 spores/ml)	
	Unwounded leaflets	Wounded leaflets	Unwounded leaflets	Wounded leaflets
0	2.8	3.0	1.9	2.0
5	0.1	3.0	0.0	2.0
10	0.0	3.0	0.0	2.0
15	0.0	3.0	0.0	0.0
20	0.0	2.0	-	-
25	0.0	0.0	-	-

*Symptoms observed after 48 h (*A. pinodes*; 0-3 scale) or 96h (*A. pisi*; 0-2 scale), after inoculation of pea leaflets, either unwounded or wounded with Carborundum.

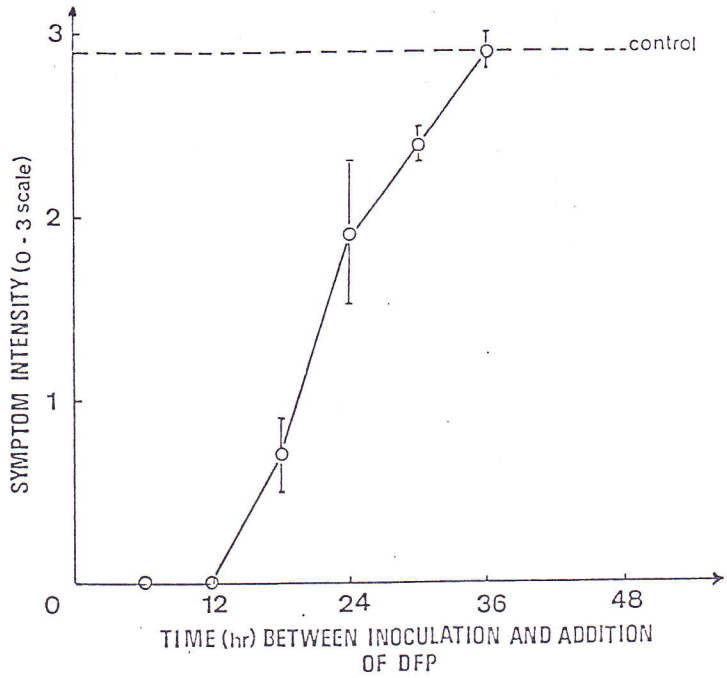

Fig. 1: Effect of adding DFP at intervals after inoculation, on symptom intensity induced by *A. pinodes* on detached pea leaflets.

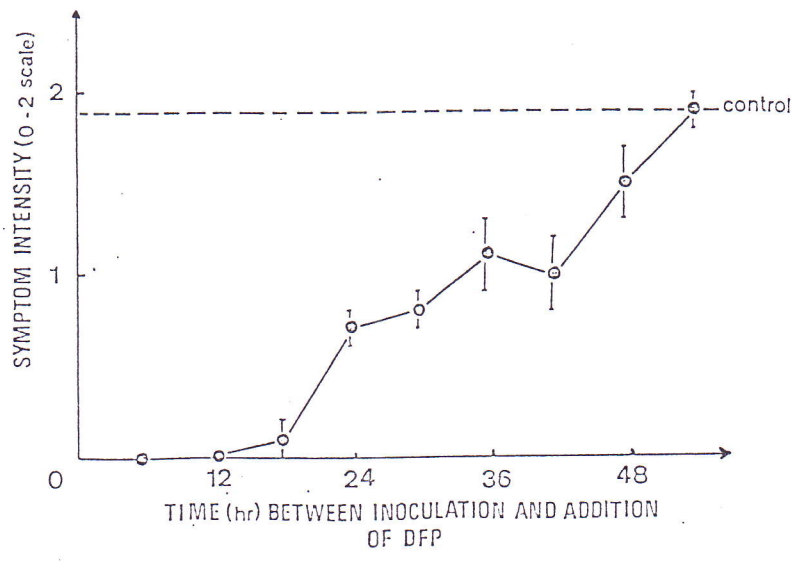

Fig. 2: Effect of adding DFP at intervals after inoculation, on symptom intensity induced by *A. pisi* on detached pea leaflets.

DISCUSSION

Fungal cutinases have been generally evaluated by their esterase activity, using para-nitrophenyl esters of short chain fatty acids (DICKMAN *et al.*, 1982, DANTZIG *et al.*, 1986), because this model substrate is used in a very convenient spectrophotometric assay to detect cutinase activity.

However, cutinolytic enzymes, devoid of esterase activity when using para-nitrophenyl esters as substrate, have been identified in *Colletotrichum lagenarium* (BONNEN and HAMMERSCHIMIDT, 1989), thus making it necessary to use comparatively a more specific enzyme assay to quantify cutinolytic activity.

Such activity has been measured by a direct enzyme assay, using radioactive cutin as substrate (KOLATTUKUDY, 1981), or indirectly by titrating liberated fatty acids with NaOH, using phenolphthalein as an indicator (BASHAN *et al.*, 1985). We developped a technique of microtitration of the liberated fatty acids directly after extraction from the reaction medium by heptane. Indeed, in our experimental conditions, the titration of fatty acids directly in the reaction medium by the technique of BASHAN *et al.* (1985) was not feasible due to the low concentration of such fatty acids and the buffering capacity of this medium.

Our results on cutinase assays, obtained by measuring esterase activity, or by fatty acid microtitration, were consistent with the hypothesis that cutinase is released specifically in the extracellular fluid of 15-day old cultures of *A. pinodes* or *A. pisi*, when grown on a cutin medium. No esterase activity of the filtrate was measured when either fungus was inoculated to mineral medium supplemented or not with glucose as source of carbon.

As expected within the hypothesis of cutinase production by *A. pinodes* and *A. pisi* when grown on cutin culture medium, addition of DFP in the enzyme reaction medium inhibited both the esterase activity and the release of fatty acids.

Our study indicate that DFP protected unwounded pea leaflets against infection by *A. pinodes* or *A. pisi* (on the basis of symptom intensity), although this reagent did not affect spore germination significantly. Moreover, inhibition by DFP was only effective if the inhibitor was added less than 12 hr after inoculation, before penetration of the cuticle by the pathogens. DFP prevented infection of pea by *A. pinodes* or *A. pisi* only when the cuticle was intact, and not when the epidermis was mechanically abraded with Carborundum.

This inhibition of penetration and subsequent infection by a cutinase inhibitor, together with our overall results, suggest that a cutinase-mediated process of penetration across the cuticle is involved in the successful infection of pea leaves by *A. pinodes* or *A. pisi*.

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