Role of Cutinase in the Penetration of Apple Leaves by *Venturia inaequalis*

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ABSTRACT

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Cutin hydrolysate induced the production of extracellular cutinase by mycelium and germinating conidia of *Venturia inaequalis*. The level of production was dose-dependent up to 0.25 mg ml^{-1} of hydrolysate added. Glucose was found to act as a repressor of cutinase production, but basal levels of the enzyme could be induced at high concentrations of glucose. Induction of cutinase was inhibited by cycloheximide, but not actinomycin D. *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate was shown to act as an almost quantitative inhibitor of cutinase purified from *V. inaequalis*. In the presence of the inhibitor on the surface of apple leaves inoculated with conidia of the pathogen, the formation of subcuticular mycelium beneath appressoria, and thus, cuticle penetration, was prevented. The results suggest that cutinase is induced by cutin monomers liberated from cuticles upon contact with conidia, and that the enzyme is crucially involved in cuticle penetration. The induction of basal levels of cutinase in the presence of glucose indicates a leakiness of repression and might be related to a more permanent role of cutinase during subcuticular growth of the pathogen.

Many plant pathogenic fungi gain access into their hosts by penetration of intact plant surfaces (1). These directly penetrating pathogens encounter the cuticle, a noncellular and hydrophobic layer covering the outer walls of epidermal cells, as the first surface barrier to be breached (24,38). The enzymatic hydrolysis of the polyester cutin by cutinase, an enzyme secreted during the initial step of host invasion, has been identified as a requirement for penetration of plant cuticles and, therefore, as an essential factor of fungal pathogenicity (16,18).

Although cutinase activity has been detected in the culture fluid of numerous plant pathogens when grown on cutin as the sole carbon source, the validity of the concept that cutinase is of general importance in the infection of plants by fungi remains largely circumstantial (18). Conclusive proof for the involvement of cutinase in plant surface penetration has been established for the host-pathogen system pea-*Fusarium solani* (*Nectria haematococca*). In this system, cutin monomers were found to act as potent inducers of cutinase gene transcription (28,31,42); induction of cutinase production by cutin monomers in mycelium of *F. solani* was completely repressed in the presence of glucose (22); the presence of cutinase at the penetration site of the fungus was demonstrated (29); pathogenicity of cutinase-deficient mutants could be restored by either addition of the enzyme (4,20) or transformation of cutinase-less mutants with the cutinase gene (17); and specific inhibitors of cutinase such as antibodies and organophosphorous pesticides acted as antipenetrants (15,19,23). In addition to *F. solani* f. sp. *pisi*, direct evidence for a crucial involvement of cutinase has been presented for *Alternaria alternata* infecting pear leaves (32,33) and *Colletotrichum gloeosporioides* infecting immature papaya fruits (5–7). Furthermore, the transformation of the wound pathogen *Mycosphaerella* with the cutinase gene from *F. solani* rendered pathogenicity on intact papaya fruit surfaces (8).

In contrast to the enzymatic cuticle dissolution, a mechanical mode of penetration has been proposed for *Pyricularla oryzae* (*Magneporthe grisea*) (14), regardless of the presence of a cutinase gene (36). Lack of evidence for an important role of cutinolytic enzymes also has been reported for *Colletotrichum lagenarium* infecting cucumbers (2). Consequently, the involvement of cutinase in cuticle penetration has to be investigated separately for each host-pathogen system under question.

The presence of cutin dissolution in the infection of apple tissue by *Venturia inaequalis* (Cooke) G. Wint., the causal agent of apple scab, has been suggested as early as 1915 (41). This pathogen forms, in contrast to most biotrophic pathogens, a parasitic mycelium between the cuticle and the epidermal cell wall (3,10,27,37,41). Wiltshire (41) concluded that cutin hydrolysis was not only required by the fungus to "eat its way slowly through the cuticle until it arrives between the cuticle and epidermal cell wall," but also during its parasitic phase of subcuticular growth. This observation suggested a more permanent role of cutinase in disease development, in addition to its normal function as a penetration enzyme.

The result of Wiltshire's (41) early ultrastructural study has been largely confirmed and extended by others. Based on the ultrastructural appearance of the cuticle at the site of penetration, enzymatic hydrolysis rather than mechanical force has been suggested as the means of cuticle penetration (30,37). The presence of transient esterase activity around appressoria can be rated as additional evidence for the involvement of hydrolytic enzymes during penetration (26).

Cutinase from *V. inaequalis* grown on cutin as the sole carbon source was purified and characterized recently (21). The enzymatic properties were different from those reported for cutinase produced by the stem-base pathogen *F. solani*, and indications exist that these differences are a reflection of the different tissue specificities (35). The goal of the present study was to investigate the basic features of cutinase induction and the role in infection for a typical leaf pathogen. This included the analysis of effects of *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate on the initial steps of leaf infection. The active site-directed cutinase inhibitor, which originated from an attempt to design more potent cutinase-based antipenetrants, was previously found to protect apple leaves from infection by *V. inaequalis* (25).

MATERIALS AND METHODS

Materials and enzyme assays. The preparation of apple cutin and tritiated grapefruit cutin followed described procedures (21). *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate was a generous gift from Bayer AG, Leverkusen, Germany. [1,3-^{3}H]-diisopropylfluorophosphate (163 GBq mmol^{-1}) was obtained from

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NEN Research Products, Boston. All other chemicals were from Sigma Chemical Company, St. Louis. Cutin hydrolysate from purified apple cutin was prepared according to the procedure described by Lin and Kolattukudy (22).

Cutinase activity was determined with *p*-nitrophenyl butyrate and tritiated grapefruit cutin as substrates (21). The I_{50} value for inhibition of cutinase by *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate was determined according to a procedure described before (19). Cutinase (2 nM) purified from *V. inaequalis* (21) was incubated in phosphate buffer (200 mM, pH 8; 0.6% [v/v] Triton × 100) in the presence of various concentrations of the inhibitor. Enzyme activity was determined after 1 h of incubation at 20 C.

Culture conditions. Maintenance and culturing of *V. inaequalis* (strain Maine 8 (+)) on cutin was done as described before (21). In experiments with cultures amended with cutin hydrolysate, cutin was omitted from the medium. Instead, glucose and cutin hydrolysate were added at concentrations as indicated. Stock solutions of cutin hydrolysate (1 mg ml^{-1}) in water were prepared by sonification for 15 min (30 s intervals of sonification and cooling) using a Branson Sonifier 250 (Danbury, CT) at setting 6. The stock solutions were diluted with culture medium to final hydrolysate concentrations. For experiments with actinomycin D and cycloheximide, cultures were scaled down to 10 ml of medium and were incubated in glass petri dishes. Conidia were prepared in "wick" cultures (27), using Roux bottles and 100 ml of culture medium.

Microscopy. Leaf material used in this study was the terminal leaves from apple seedlings grown for 3 wk (20 C) from pregerminated seeds collected from open-pollinated McIntosh fruits. Four infection droplets (8 μl of 0.1% [v/v] Tween 20) containing conidia of *V. inaequalis* ($5–8 \times 10^5$ ml^{-1}) were placed on the adaxial surface of a detached apple leaf. Conidia were derived from diseased leaves collected from McIntosh trees and stored in paper towels and wax paper at 2 C. The infection droplets contained either *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate (0.15 μM, diluted from an acetone stock solution to a final acetone concentration of 0.1%) or acetone (0.1%) alone. Inoculated leaves were incubated in petri dishes lined with moist filter paper at 20 C.

The development of conidia on leaves was monitored using the epi-UV fluorescence equipment of a Zeiss PhotoMicroscope III (50-watt mercury lamp). The portions of leaves inoculated with infection droplets were excised and placed on a microscope slide. One drop of Calcofluor (1 mg ml^{-1}) and fluorescein diacetate (0.01 mg ml^{-1}) dissolved in 10 mM potassium phosphate buffer, pH 7.3, was added to each leaf piece. Three replicates excised from different leaves were used to determine the germination rate, appressoria formation, and development of subcuticular mycelium. Three replicates and a total of 100 conidia per replicate were evaluated for each treatment. Calcofluor was used to stain nonviable conidia. Fluorescein diacetate stained viable germ tubes, appressoria, and subcuticular mycelium. Calcofluor and fluorescein diacetate fluorescence was accomplished with the following filter combination: excitation at 365 nm, dichroic reflectance (400–440 nm) at 395 and 460 nm, and emission at 420 and 470 nm.

Other methods. Glucose was determined with a glucose diagnostic kit (Sigma no. 115) according to the procedure outlined by the manufacturer. Conditions for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, labeling of serine esterases with diisopropyl fluorophosphate, and fluorography have been described elsewhere (21,35).

RESULTS

Induction of cutinase by cutin monomers. Mycelium of *V. inaequalis* grown on polymeric cutin as the sole carbon source produced cutinase as the exclusive serine esterase released into the extracellular fluid (21). Thus, *p*-nitrophenyl butyrate could be employed as a model substrate in assays of cutinase activities. Cutin monomers derived from chemical hydrolysis of cutin induced the production of cutinase in mycelium of *V. inaequalis* when incubated in the presence of 0.1% (w/v) glucose (Fig. 1). Enzyme production was initiated after a lag phase of 1 wk and was dependent on the concentration of cutin monomers, with a sharp rise from 0.1 to 0.25 mg ml^{-1}. The concentration of 0.25 mg ml^{-1} had no inhibitory effect on mycelial growth of *V. inaequalis* and was used for all subsequent induction experiments.

Under the conditions employed in our study, mycelial growth of *V. inaequalis* was slow, and glucose was only depleted after 3 wk of incubation. However, considerable quantities of cutinase had already been produced at this time (Fig. 1), and glucose repression of cutinase induction appeared less stringent than previously reported for *F. solani* (22). This aspect was investigated in a separate experiment and with various levels of glucose (Table 1). Highest enzyme levels were reached in the complete absence of glucose, indicating that induction of cutinase by cutin monomers was repressed. However, glucose repression was not complete, and a basal level of activity was excreted at high glucose concentrations (Table 1). The production of this low level of cutinase was clearly induced by cutin monomers, since extracellular esterase activity remained below detectable levels for 6 wk in the presence of glucose but absence of cutin monomers.

The fatty acids unique to cutin are distinguished by hydroxy groups in ω and midchain positions, and both of these structures were shown to be essential for cutinase induction in *F. solani* (22,28,42). A comparative study with cutin hydrolysate and palmitic acid indicated that this specificity also was valid for *V. inaequalis* (Fig. 2). No enzyme activity was induced in medium amended with palmitic acid even after a prolonged period of incubation.

Induction of extracellular esterase in mycelium of *V. inaequalis* was inhibited by 70% in the presence of cycloheximide (1 μg ml^{-1}) when induced cultures were tested after 12 days of incubation. Actinomycin D (50 μg ml^{-1}) as an inhibitor of RNA synthesis had no effect on the induction of cutinase. Esterase

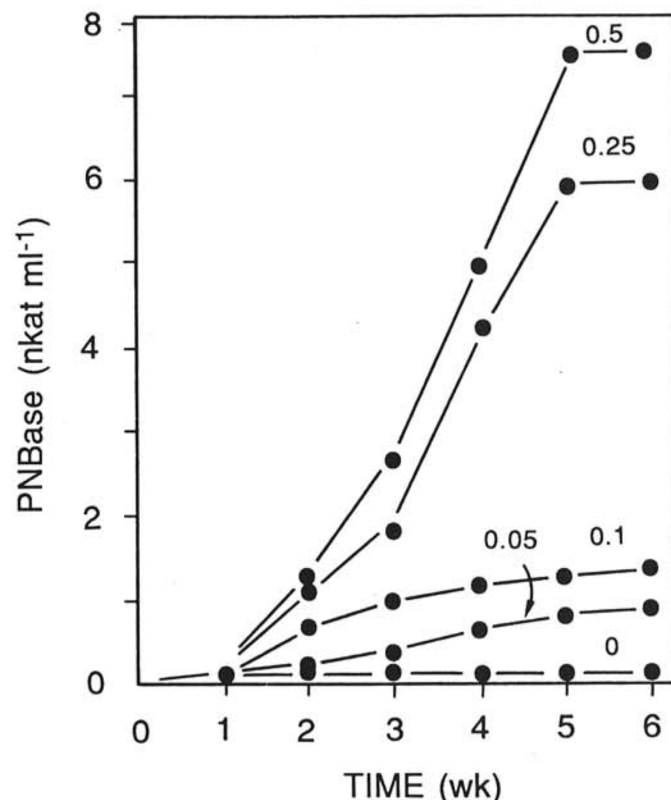

Fig. 1. Induction of cutinase by cutin hydrolysate. Mycelium of *Venturia inaequalis* was transferred to growth medium containing glucose (1 mg ml^{-1}) and cutin hydrolysate (mg ml^{-1}) as indicated. Small samples were withdrawn from the cultures and assayed for enzyme activity with *p*-nitrophenyl butyrate as the substrate (PNBase).

activity also could be induced in germinating conidia of *V. inaequalis*, in addition to the mycelial stage. The basal level of activity released by 4×10^6 conidia in the absence of cutin monomers was 0.13 nkat after germination for 24 h (60% germination rate). The enzyme level was increased to 2.5 nkat when cutin hydrolysate was added.

The cutinase nature of the induced esterase was investigated with freeze-dried extracellular fluid derived from mycelial cultures incubated in the presence of cutin monomers (Fig. 2). A strong protein band that comigrated with cutinase purified from *V. inaequalis* grown on cutin as carbon source comprised the bulk of extracellular protein (Fig. 3). No increase in mycelial dry weight was observed over the entire course of incubation, and the biological activity in the presence of cutin hydrolysate was apparently restricted to the almost exclusive production and secretion of cutinase. The major protein band was the exclusive protein labeled after treatment with $[^3H]$-diisopropyl fluorophosphate used as an active site probe for serine esterases (Fig. 3). The enzyme could be purified by passage of the extracellular fluid through a phenyl Sepharose column and subsequent elution with isopropanol (60%, v/v) similar to the procedure described for the purification of cutinase (21). The homogenous esterase exhibited cutinase activity with an optimum of cutinolytic activity at pH 6.0 (Fig. 2), and the specific cutinase activity was not different from homogenous cutinase purified from *V. inaequalis* grown on cutin. The results indicate that cutinase induced by cutin monomers is identical with the enzyme purified from cutin-grown cultures.

TABLE 1. Effect of glucose on the induction of cutinase. Mycelium of *Venturia inaequalis* was incubated in the presence of cutin hydrolysate (0.25 mg ml^{-1}) and glucose at indicated concentrations

Glucose (mg ml^{-1})		PNBase (nkat ml^{-1})[a]			
		Time (days)			
Start	End[b]	3	7	10	14
0.0	0.0	<0.01	0.49 (0.01)[c]	3.19 (0.31)	5.12 (0.25)
1.0	0.6	<0.01	0.23 (0.04)	1.65 (0.21)	1.63 (0.40)
10.0	9.2	<0.01	0.20 (0.01)	0.56 (0.06)	0.34 (0.16)

[a] Esterase activity was determined with *p*-nitrophenyl butyrate as substrate (PNBase). Figures are the means of three replicate cultures.
[b] Determined after 14 days of incubation.
[c] Standard error in parentheses.

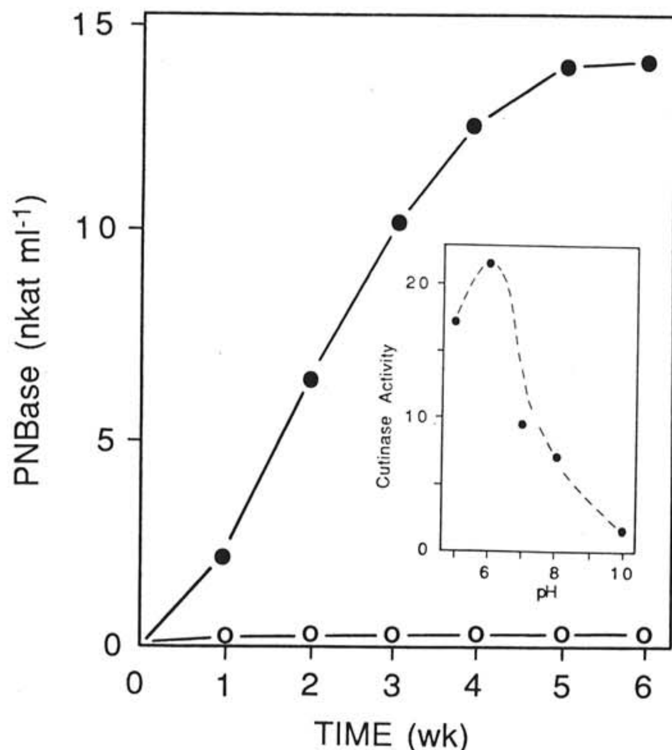

Fig. 2. Effect of fatty acids on the induction of cutinase. Mycelium of *Venturia inaequalis* was transferred to medium containing cutin hydrolysate (●) or palmitic acid (○) at concentrations of 0.25 mg ml^{-1}. Esterase activity was assayed with *p*-nitrophenyl butyrate as the substrate (PNBase). The pH dependency of cutinase activity (Bq released by 1 nkat PNBase activity per hour) of the esterase purified from induced cultures is shown in the insert.

Effect of a specific cutinase inhibitor on penetration. Several active site-directed inhibitors of serine esterases have been employed as specific cutinase inhibitors with antipenetrant activities (7,15,19). The I_{50} value of *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate, an optimized cutinase inhibitor with asymmetric substitution (25), was 2 nM for cutinase purified from *V. inaequalis.* Since the enzyme concentration used in the inhibitor assays was 2 nM, inhibition was almost quantitative.

The development of conidia germinating in the absence and presence of the inhibitor on the surface of young apple leaves was investigated by means of vital staining and fluorescence microscopy. This technique allowed us to evaluate and quantitate germination, appressoria formation, and cuticle penetration. Penetration was defined as the appearance of hyphae beneath the cuticle of susceptible leaf tissue, as suggested by Nusbaum and Keitt (27). The inhibitor was employed at a concentration of 1.5 μM (0.5 μg ml^{-1}); a concentration of 15 μM (5 μg ml^{-1}) was previously shown to protect apple seedlings from scab development (25).

In an initial experiment, the developmental stages of conidia on leaf surfaces were evaluated 72 h after inoculation and thus subsequent to cuticle penetration (27). In the absence of *O*-methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate, subcuticular

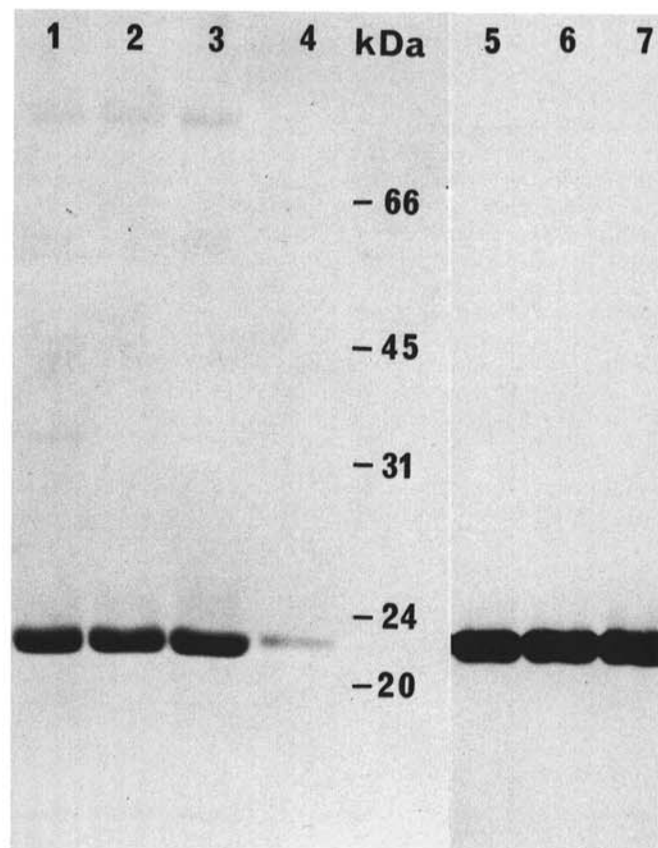

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of extracellular proteins after induction of cutinase with cutin hydrolysate. Lanes 1, 2, and 3, protein pattern after 4, 5, and 6 wk of incubation; lane 4, cutinase purified from *Venturia inaequalis* grown on cutin; lanes 5, 6, and 7, fluorography of lanes 1, 2, and 3 after treatment with diisopropyl fluorophosphate.

mycelium had developed from 47% of the appressoria, whereas similar structures were absent in the presence of the inhibitor. A significant inhibition of appressoria development became apparent as an additional effect of the inhibitor, and the observation interval was extended to 96 h in order to exclude an overall delay of development as the primary mode of inhibitor action (Table 2). As observed before, the frequency of appressorium formation was significantly decreased for the first 72 h. However, developing germ tubes recovered from this effect, and the frequency of germ tubes without appressorium was not significantly different ($P = 0.18$) between the treated and nontreated sample after 96 h of germination. The most dramatic effect of the cutinase inhibitor was the complete and lasting block of stromata formation beneath the cuticle. In nontreated samples, subcuticular stromata with a radius of approximately 10 μm were clearly visible after 24 h of germination, and after 96 h, subcuticular hyphae had elongated to approximately 100 μm. At this stage, no signs of subcuticular structures beneath appressoria were observed for germ tubes developing in the presence of the inhibitor.

DISCUSSION

The most elaborate model describing the involvement of cutinase in the invasion of host plants has been developed for the stem-base pathogen *F. solani* f. sp. *pisi* (16,17). Small quantities of cutinase were shown to be excreted by spores in the earliest stage of germination. Cutin monomers released by the action of this "cuticle-sensing" cutinase acted as inducers of bulk cutinase required for cuticle dissolution and penetration. Any inhibitory disturbance of these sequential events, be it by mutational inactivation of the cutinase gene or by specific inhibition of cutinase activity, has been shown to prevent the step of penetration.

Regardless of the different properties of cutinases produced by leaf pathogens (21,35), the general principles of the cutinase model are valid for *V. inaequalis* infecting apple leaves. Small quantities of cutinase, most likely with a similar function in cuticle sensing, were released from conidia germinating in water. Likewise, cutin monomers were potent inducers of de novo cutinase production in both germinating conidia and mycelium of the fungus. Cutinase induction might not only involve de novo protein synthesis as indicated by the inhibitory action of cycloheximide, but also gene transcription, regardless of the failure to inhibit cutinase induction with actinomycin D. A similar apparent lack of actinomycin D activity on mRNA synthesis has been reported for *F. solani* (22), but transcription of the cutinase gene was later shown to precede cutinase synthesis (28,42).

In addition to these similarities, *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate as a highly active cutinase inhibitor with previously reported protective activity against apple scab (25) blocked the development of subcuticular stromata and, thus, penetration. Although a delay of appressoria development became apparent as a side effect of the inhibitor, the complete lack of subcuticular development indicated a crucial role of cutinase in cuticle penetration similar to *F. solani* and other pathogens (15,18).

Regardless of a similar sequence of events in cuticle sensing and involvement of cutinase in penetration, a difference between *F. solani* and *V. inaequalis* was observed in the induction of cutinase by cutin monomers. In the mycelial stage of *F. solani*, induction was fully repressed by glucose (22). A similar repression was observed for *V. inaequalis*, but basal levels of the enzyme could still be induced in the presence of the repressor. The leakiness of glucose repression might be functionally related to the subcuticular phase of the scab fungus. At the junction of plant cuticles with cell walls, the cuticle often is dispersed with microfibrils believed to be cellulosic and pectic in nature (12). In apple leaves, this microfibril-containing region was most abundant within the inner one-third to one-half of the cuticle, but was frequently observed throughout the cuticle layer (11). During biotrophic growth of *V. inaequalis* between the cuticle and epidermal walls, the fungus encounters polymeric carbohydrates but also cutin to be breached by the growing mycelium, and indications of cuticle dissolution during this stage have been described (41). *V. inaequalis* was shown to degrade cellulose and pectin in vitro (9,13), and a crucial role for cell wall-degrading enzymes during subcuticular growth has been suggested (39). Additional cutinase not repressed in the presence of free sugars might be equally crucial for proliferation of mycelium within the subcuticular region of mixed polymers.

Although extensive subcuticular proliferation is rare among plant pathogens, subcuticular mycelial growth prior to the penetration of epidermal cells has been reported for other leaf pathogens such as *Alternaria brassicae* (34), *Septoria nodorum* (43), and *Cochliobolus heterostrophus* (40). A crucial function of cutinase during this subcuticular phase might extend the activity of cutinase inhibitors used in the control of plant diseases. These inhibitors have been discussed as plant protectants with antipenetrant activity and, therefore, would require an application prior to infection. A postinfection activity, however, might be feasible if subcuticular growth would depend on cutinase, and if the inhibitor would penetrate the cuticle.

TABLE 2. Effect of *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate on the infection of apple leaves by *Venturia inaequalis*

	Developmental stages (percentage of germinated conidia)[a]		
Time (h)	A[b]	B[b]	C[b]
Control			
24	18.9 (2.8)[c]	77.3 (4.3)	3.8 (3.2)
48	6.0 (2.3)	46.8 (1.0)	47.2 (2.2)
72	5.2 (0.4)	51.1 (8.2)	43.7 (7.9)
96	8.5 (1.3)	33.2 (5.0)	58.3 (4.9)
Inhibitor[d]			
24	34.4 (3.3)	65.6 (3.3)	0.0 (0.0)
48	45.4 (2.1)	54.6 (2.1)	0.0 (0.0)
72	38.9 (4.5)	61.1 (4.5)	0.0 (0.0)
96	22.3 (8.4)	77.7 (8.4)	0.0 (0.0)

[a] Germination rate was >85% in all cases.
[b] A, germ tubes without appressoria; B, germ tubes with appressoria; C, subcuticular stromata.
[c] Standard error in parentheses.
[d] *O*-Methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate.

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