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Preventive Effect of Ebelactone B, an Esterase Inhibitor on Rice Sheath Blight Caused by *Rhizoctonia solani*

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Two types of *Rhizoctonia solani* esterases induced by cutin hydrolysate were partially purified by ammonium sulfate precipitation and gel filtration. The esterase I with hydrolyzing activity toward both p-nitrophenyl butyrate and p-nitrophenyl palmitate and the esterase II with hydrolyzing activity toward only p-nitrophenyl butyrate were inhibited by ebelactone B, an esterase inhibitor produced by actinomycetes with IC_{50} values of 0.01 and 0.09 μg/ml, respectively. Spraying on rice seedling with ebelactone B at a concentration of 30 μg/ml completely suppressed infection by *R. solani*. Ebelactone B could not protect the wounded rice seedling and did not show any inhibitory effect on the mycelial growth at a concentration of 1 mg/ml. These results indicate that ebelactone B, an esterase inhibitor protects rice plants from infection with *R. solani* by inhibition of penetration, not through fungitoxic or fungicidal effect.

Some of the first lines of defence a plant has against potential pathogen invaders are its external cuticle layer and the walls of its cells. To invade a plant with any success, a pathogen must first pass through these barriers. Some pathogens including fungi use mechanical means to muscle their way through the protective layers, whereas other pathogens are able to enter plants only at sites, such as wounds, where the barriers have already been breached. Still other pathogens have been proposed to gain entry to plant cells by enzymatically degrading cuticle or cell walls. Cuticle degradation requires an esterase that hydrolyzes the ester bonds of polyester cutin, the structural components of cuticle that are composed of hydroxy and epoxy fatty acids containing 16 and 18 carbon atoms, respectively (12). Cutin esterase was detected in culture fluids of several phytopathogenic fungi grown on cutin as the sole carbon source (7, 10).

Several chemically synthesized cutinase inhibitors such as diisopropyl-fluorophosphate, and organophosphorus pesticides, were shown to prevent infection of *Pisum sativum* by *Fusarium solani* sp. (3) and infection of papayas by *Collectotrichum gloeosporioides* (2). Kolattukudy *et al.* described results of a detailed analysis of virulence of the cutinase gene-disrupted mutant on pea stem sections and pea seedling and demonstrated that cutinase gene disruption significantly decreased the virulence, and provided convincing genetic evidence to support the role of cutinase in the penetration of cutin barriers by phytopathogenic fungi (8).

In this paper, we postulate that the inhibition of cutinase produced by *R. solani* prevents rice plants from being infected by this fungus. Therefore the effect of a known esterase inhibitor, ebelactone B, which had previously been isolated from actinomycetes by Umezawa group (11), on the rice sheath blight caused by *R. solani* was evaluated.

MATERIALS AND METHODS

Strain and Cultivation

Rhizoctonia solani used in this experiment was isolated from Korean paddy soil. This strain was cultivated in PDA media and was used for infection of young rice plant and reisolated from the infected plants to preserve its pathogenecity.

Preparation of Cutin Substrates

Apple cutin was prepared from fresh Fuji apple peelings by a four-step procedure similar to that described by Walton and Kolattukudy (12). Apple peelings were immersed and constantly stirred in boiling aqueous 0.4% oxalic acid and 1.6% ammonium oxalate (10 liters/kg peelings). As soon as the cuticles could be separated from the underlying tissue (about 10-15 min), they were collected on a 2.5 mm (10

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mesh) screen and rinsed thoroughly with large amounts of tap water (step 1). Then 50 g units of the 400 g of crude cuticles recovered in step 1 were treated for 18 h at 30°C with 2 liters of 100 mM sodium acetate buffer (pH 4.5) containing 0.5% cellulase and 0.1% pectinase from *Aspergillus niger* (Sigma Co.). The insoluble material remained (cutin plus epicuticular waxes) was collected on a 2.5 mm screen and rinsed thoroughly with tap water (step 2). This material was then extracted with methanol (1 liter/50 g) followed by 2 : 1 chloroform-methanol (1 liter/50 g) at room temperature (step 3). The remaining product (cutin) was subjected to Soxhlet extraction (three cycles/h) with chloroform for 24 h (step 4). The cutin films obtained were then dried under a hood, and repurified twice (step 2-4).

Preparation of Cutin Hydrolysate (5)

Cutin powder (60 mesh) was suspended in 95% ethanol (30 ml) containing 10% (wt/vol) KOH, and the suspension was refluxed for 16 h in a nitrogen atmosphere. The resulting mixture was acidified with concentrated HCl, and the products were extracted repeatedly with chloroform. The combined chloroform extract was evaporated to dryness under vacuum. A suspension of the hydrolysate (200 mg in 20 ml of water) was then subjected to ultrasonic treatment (about 15×30 s) with cooling after extraction.

Conditions for Enzyme Production

The mineral medium used to grow the fungus contained 2.0 g of L-asparagine, 1.4 g of KH_2PO_4, 1.0 g of $MgSO_4 \cdot 7H_2O$, 4.4 mg of $ZnSO_4 \cdot 7H_2O$, 1.0 mg of $FeSO_4 \cdot 7H_2O$, 86 μg of $MnCl_2 \cdot 4H_2O$, 79 μg of $CuSO_4 \cdot 5H_2O$, and 53 μg of $Na_2MoO_4 \cdot 2H_2O$ per liter of distilled water. The indicated amounts of cutin hydrolysate were added. The pH of the media was then adjusted with concentrated HCl to 5.1. Erlenmeyer flasks(500 ml) containing 150 ml of the production media were inoculated with agar disks of actively growing mycelium of *R. solani*. The inoculated flasks were incubated at 27°C for 7 days.

Partial Purification of Enzyme

The culture fluid was separated by filtration through Whatman No. 1 filter paper. The cutinase was precipitated by addition of $(NH_4)_2SO_4$ upto 70% saturation. The precipitate was dissolved in small amounts of distilled water and applied to a Sephadex G-20 column. The desalted active fractions were freeze-dried, and the dried material was dissolved in small amount of distilled water and applied to a Sephadex G-100 column. The column was eluted with a 0.05 M acetate buffer (pH 5.6). The pooled active fractions were then freeze dried.

Enzyme Assay

Hydrolysis of ρ-nitrophenyl esters of fatty acids was measured spectrophotometrically at 405 nm as described earlier (6) after optimizing assay conditions for the enzyme. The reaction was run in 180 μl of 0.1 M sodium phosphate buffer (pH 8.0) containing 1.6 mM ρ-nitrophenyl butyrate, 0.4% Triton X-100, and 20 μl of the enzyme solution at 25°C for 5 min. During the reaction time linear increases in A_{405} were observed. The liberated ρ-nitrophenol was measured by microplate reader (Bio-RAD). For the measurement of inhibitory activity, the reaction mixture containing 20 μl of enzyme solution and 20 μl of inhibitor solution was preincubated for 3 min, and then 160 μl of substrate solution was added to the preincubated mixture. One unit of enzyme was defined as μ moles of the liberated ρ-nitrophenol/min/ml.

Assay of Antifungal Activity

According to the paper disc method, paper disc soaked with inhibitor solutions of various concentrations (1,000, 500, 250, 120, 60, 30, 15, 10, 5 ppm) were placed on the test plates, which consisted of the PDA agar plates overlayed with the mixture solution (9 : 1) of PDB containing 0.5% molten agar and partially disrupted mycelium of *R. solani*. The disruption of mycelium was carried out by addition of the ceramic balls into the 250 ml-Erlenmeyer flask, which contained cultivated mycelium of the fungus on the PDB, accompanied by subsequent shaking on the rotary shaker. The test plate was incubated at 25°C for 3 days. Activity was evaluated by measuring the inhibitory zone forming around the paper disk.

In the Dendroid method, a small piece of potato dextrose agar which was already grown with *R. solani* was placed on the center of water agar plates containing inhibitors of various concentrations. The spreading of mycelium from the agar piece placed on the center of the water agar plate was measured.

Pot Assay

Preparation of Test Plants: Rice seedlings were grown in polystyrene pots(5 cm ϕ×5 cm) containing standardized soil in a green house for 20-25 days.

Preparation of Inoculum: *R. solani* grown on potato dextrose agar was inoculated into 1 liter-autoclaved Erlenmeyer flask containing wheat bran, rice hull and water (2 : 1 : 1 in volume) and cultivated for 3 weeks at 27°C. The resulting culture material was dried at room temperature and used for inoculum.

Ebelactone B Application: Ebelactone B was dissolved in a small amount of methanol and diluted with 500 ppm Tween 20 solution to the required concentration. The solution was sprayed with a sprayer onto foliage and stem of the 3rd foliage formed rice seedling.

Inoculation: Ten grams of inoculum were placed on the soil in plant pots which had been sprayed with ebelactone B solution 24 h previously.

Disease Development: Inoculated plants were incubated in a humid chamber for 4 days to establish infection and to encourage the development of pathological symptoms. At the end of incubation, preventive activity was expressed as the percentage of the uninfected area of rice plant.

RESULTS AND DISCUSSION

Induction of Esterase by Cutin Hydrolysate

To test whether cutin hydrolysis products induce esterase, *R. solani* was grown in a media containing 0.1% glucose and 0.005% chemically prepared hydrolysate of cutin. Growth of the fungus did not appear to be affected by the presence of cutin hydrolysate. The extracellular fluid was examined for esterase activity using a model substrate, p-nitrophenyl butyrate (PNB) which was known to be a good substrate for purified cutinase from *Fusarium solani* f. sp. *pisi* (6, 7).

At the first day of cultivation, PNB hydrolytic activity could be detected in the medium. The extracellular activity increased almost linearly for the next 3 days, and slow further increases were observed thereafter (Fig. 1B). Even though equally vigorous growth of the fungus occurred in the glucose medium containing no hydrolysate, a little PNB hydrolase activity was detected in the extracellular fluid.

The effect of the amount of cutin hydrolysate added to the medium on the production of extracellular cutinase was also examined (Fig. 1B). Since the amount of glucose added might affect the rate of cutinase production, equal quantities of glucose (0.1%) were used in all cultures. With this level of glucose, PNB hydrolase production began from day 1 at all concentrations of cutin hydrolysate. However, the rate of production of extracellular cutinase was increased with increasing the amount of hydrolysate up to about 100 μg/ml; further increase in the concentration of cutin hydrolysate did not result in any further increase in the total amount of extracellular hydrolase activity. Thus, the rate of production and the total amount of hydrolase produced were dependent on the concentration of cutin hydrolysate, and a saturation pattern of response was observed with respect to the amount of hydrolysate added. When the cutin hydrolysate was added to glucose media at the second day of cultivation, a two fold increase in the esterase production was observed (Fig. 1A). This result indicates that glucose in the media represses the induction of esterase by cutin hydrolysate.

Partial Purification and Characterization of Esterase Induced by Cutin Hydrolysate

Ammonium sulfate saturation (70%) of the extracellular fluid obtained by the filtration of the culture broth on the celite resulted in the precipitation of the bulk of the proteins which represented esterase activity. The precipitated protein was fractionated by gel filtration on a Sephadex G-100 column (Fig. 2).

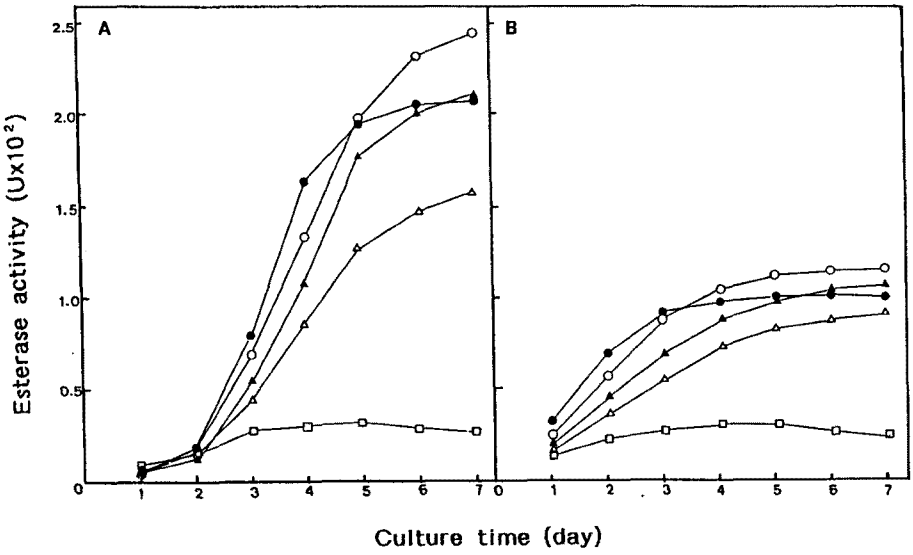

Fig. 1. Time course of production of extracellular esterase by *Rhizoctonia solani*.
The mineral medium (100 ml each) contained 0.1% glucose and the indicated amounts of cutin hydrolysate at the time of inoculation (B). After 2 days of cultivation without cutin hydrolysate, the indicated amount of cutin hydrolysate was added (A). Cutinase activity of the extracellular fluid was measured with PNB. □—□ : No addition, △—△: 25 μg/ml, ▲—▲: 50 μg/ml, ○—○ : 100 μg/ml, ●—● : 150 μg/ml.

Spectrophotometric assay of the column fractions with p-nitrophenyl butyrate yielded two peaks of esterase activity. When p-nitrophenyl palmitate was used as substrate, only one peak of activity was observed. Purdy *et al.* (6) have observed that cutinase from *F. solani pisi* hydrolyzed p-nitrophenyl butyrate, but not p-nitrophenyl palmitate whereas the nonspecific esterase from this organism hydrolyzed both of these esters.

The esterase activity (esterase I fraction) in fractions from 30 to 40 may be nonspecific since the enzyme fractions degrade both p-nitrophenyl butyrate and p-nitrophenyl palmitate. The protein with esterase activity (esterase II) in the fractions from 60 to 70 hydrolyzed only p-nitrophenyl butyrate.

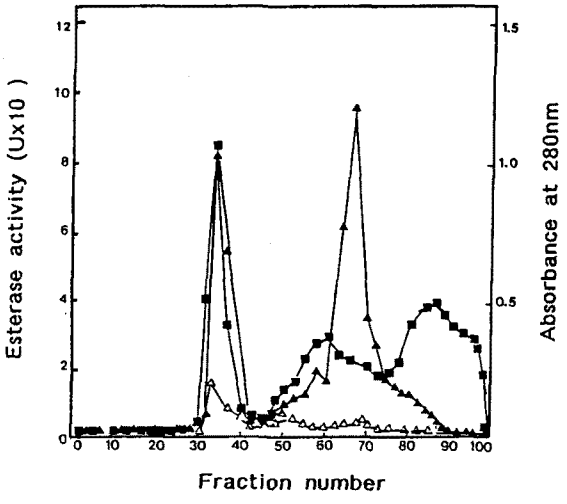

Fig. 2. Sephadex G-100 gel filtration of the extracellular fluid from *R. solani* induced by cutin hydrolysate to generate cutinases.
■—■: Protein, ▲—▲: PNBase (hydrolyzing activity p-nitrophenyl-butyrate), △—△: PNPase (hydrolyzing activity p-nitrophenyl-palmitate).

The optimum temperature of esterase I fraction was 45°C whereas that of the esterase II fraction was in the range of 50-60°C (Fig. 3). Aliquots (1 ml) of the active fraction was incubated at different temperatures from 30 to 70°C for 1 h. After incubation the remaining enzyme activity was determined spectrophotometrically. Both esterase fractions were unstable above 45°C and lost more than 80% of their activity in 1 h at 60°C (Fig. 3).

In the case of esterase I fractions, as the pH was raised from 5.0 to 9.0 there was a sharp increase in the hydrolysis rate and a further increase in pH resulted in a sharp decline in the hydrolysis rate (Fig. 4). The optimum pH of the esterase II fraction was 8.0. Both of the esterase fractions were stable at a pH range of 6 to 9. Esterase I fraction was more stable at pH 10 than esterase II fraction.

The effect of ebelactone B on the two fractions of esterase produced by *R. solani* was investigated. Both esterase fractions were inhibited by ebelactone B (Fig. 5). However, differences between the two enzyme fractions were apparent. Ebelactone B was more inhibitory toward the esterase I than esterase II by a factor of 9. The IC_{50} value of ebelactone B against the esterase I fraction is 0.01 μg/ml and that against the esterase II fraction is 0.09 μg/ml. Köller *et al.* (4) reported that the cutinases from *Venturia inaqualis* and *R. solani* were inhibited by ebelactone B with IC_{50} values of 0.67 and 0.013 μg/ml respectively. These different patterns of cutinase inhibition by ebelactone

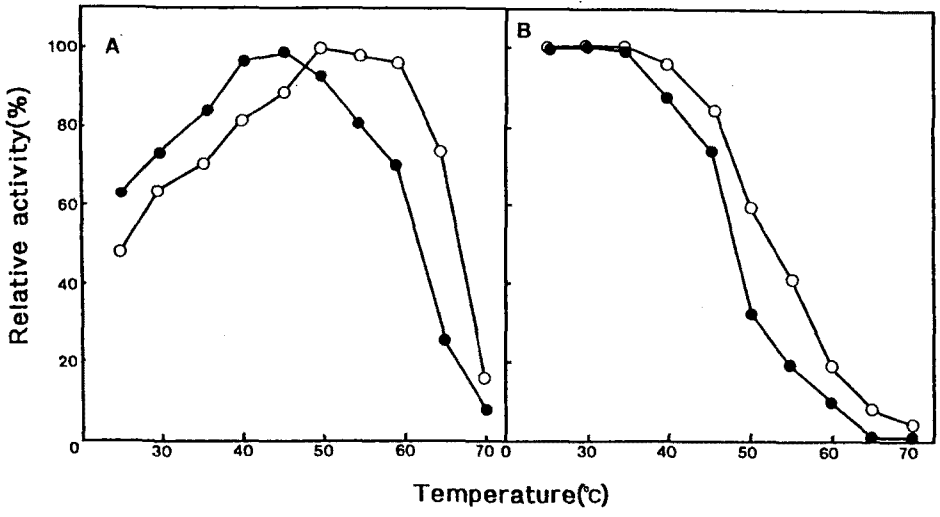

Fig. 3. Effect of temperature on the enzyme activity (A) and enzyme stability (B).
○—○: Esterase I, ●—●: Esterase II. (B): Aliquots (1 ml) of active fraction was incubated at different temperature from 30 to 70°C for 1 h. After incubation the remaining activity was determined spectrophotometrically.

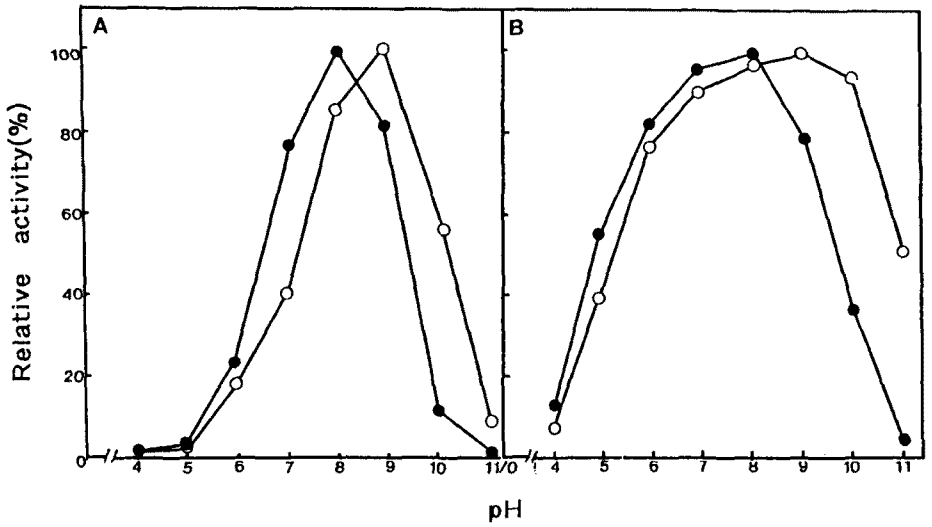

Fig. 4. Effect of pH on the enzyme activity (A) and stability (B).
○—○ : Esterase I, ●—● : Esterase II.

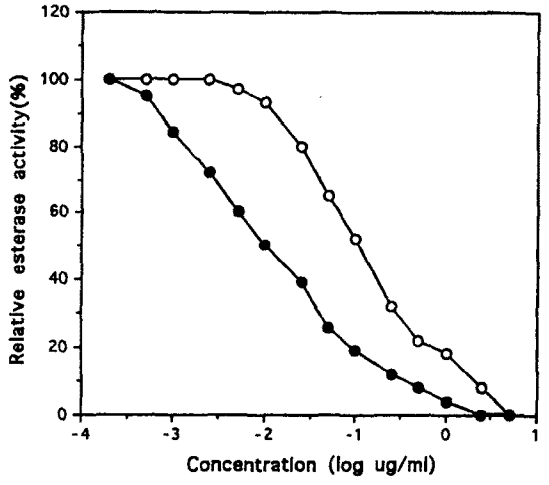

Fig. 5. Inhibition of Rhizoctonia solani esterases by ebelactone B.
○—○ : Esterase I, ●—● : Esterase II.

B substantiate the enzymatic diversity of cutinase produced by fungal plant pathogens. Organophosphate esters, like the ebelactone inhibitor of serine esterase, were shown to inhibit fungal cutinase and thus, to protect plants from disease by acting as antipenetrants (2, 3). The potency of ebelactone B as an inhibitor of fungal cutinase might open a novel field of application as plant protectants in agriculture. However, the *in vivo* effect of ebelactone B on the infection of fungal plant pathogen was not investigated.

Effect of Ebelactone B on Rice Sheath Blight

To test whether the infection of rice seedlings could be prevented by ebelactone B, a pot assay was performed. When ebelactone B solution at the concentration of 15 μg/ml was applied to the rice plants, 90% protection of rice plants against *R. solani* was observed. Validamycin A (4-O-β-D-glucopyranosyl-validoxylamine A), a commercially available microbial antibiotic for the prevention of rice sheath blight, has been reported to suppress growth of *R. solani* in rice plants under protective conditions at a concentration of 15 ppm (1). The effective concentration of both compounds was at a similar level, but the mode of action was quite different. The inhibition of trehalose metabolism by validamycin and its intracellular hydrolysis product, validoxylamine results in the retardation of fungal growth (9). At the concentration of 30 μg/ml, ebelactone B completely protect rice plants from infection with *R. solani* in pot assay (Table 1). To show that inhibition of infection was caused by inhibition of penetration, and not by a fungitoxic effect, the ebelactone B solution (30 μg/ml)

Table 1. Preventive effect of ebelactone B on rice sheath blight.

Ebelactone B (μg/ml)	Pot assay	Paper disc	Dendroid
5	0	-[a]	-
10	62	-	-
15	90	-	-
30	100	-	-
60	100	-	-
120	nt[b]	-	-
250	nt	-	-
500	nt	-	-
1000	nt	-	-

[a]No antifungal activity. [b]Not tested. The preventive activity in pot assay was represented as a percentage of the uninfected area of rice plants.

was also sprayed on wounded surfaces of rice plants. A fungitoxic mode of action should prevent infection even when a wound is provided for entry of the pathogen into its host. This compound could not protect the wounded rice plant. The antifungal activity of ebelactone B against *R. solani* was investigated by the paper disk method and the Dendroid test. The compound did not show any inhibitory effect on the mycelial growth at the concentration of 1 mg/ml. This compound at the concentration of 1 mg/ml showed no discernible inhibition of mycelial growth when directly tested in an aqueous medium.

These results indicate that ebelactone B, an esterase inhibitor, protects rice plants from infection with *R. solani* by inhibition of penetration, not by fungitoxic effect or fungicidal effect. Based upon these results, the screening of specific cutinase inhibitors from natural sources might supply a new method of discovering useful plant protectants for agriculture.

REFERENCES

1. Ando, O., M. Kifune, and M. Nakajima. 1995. Effects of trehazolin, a potent trehalase inhibitor, on *Bonbyx mori* and plant pathogenic fungi. *Biosci. Biotechnol. Biochem.* **59**: 711-712.
2. Dickman, M. B., S. S. Patil, and P. E. Kolattukudy. 1983. Effects of organophosphorous pesticides on cutinase activity and infection of papayas by *Colletotrichum gloeosporioidus*. *Phytopathol.* **72**: 1209-1214.
3. Köller, W., C. R. Allan, and P. E. Kolattukudy. 1982. Protection of *Pisum sativum* from *Fusarium solani* f. sp. *pisi.* by inhibition of cutinase with organophosphorus pesticides. *Phytopathol.* **72**: 1425-1430.
4. Köller, W., F. Trail, and D. M. Parker. Ebelactones inhibit cutinases produced by fungal plant pathogens. *J. Antibiot.* **43**: 734-735.
5. Lin, T. S. and P. E. Kolattukudy. 1978. Induction of a biopolyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani* f. sp. *pisi. J. Bacteriol.* **131**: 942-951.
6. Purdy, R. E. and P. E. Kolattukudy. 1975. Hydrolysis of plant cuticle by plant pathogens: purification, amino acid composition, and molecular weight of two isozymes of cutinase and a nonspecific esterase from *Fusarium solani* f. sp. *pisi. Biochem.* **14**: 2824-2831.
7. Purdy, R. E. and P. E. Kolattukudy. 1975. Hydrolysis of plant cuticle by plant pathogens. Properties of cutinase I, cutinase II, and a nonspecific esterase from *Fusarium solani* f. sp. *pisi. Biochem.* **14**: 2832-2840.
8. Rogers, L. M., M. A. Flaishman, and P. E. Kolattukudy. 1994. Cutinase gene disruption in *Fusarium solani* f. sp. *pisi.* decreases its virulence on Pea. *Plant Cell* **6**: 935-945.
9. Shigemoto, R., T. Okuno, and K. Matsuura. 1989. Effect of validamycin on the activity of trehalase of *Rhizoctonia solani* and several sclerotial fungi. *Annals of the Phytopathology Society of Japan* **55**: 238-241.
10. Soliday, C. L. and P. E. Kolattukudy. 1976. Isolation and characterization of a cutinase from *Fusarium roseum culmorum* and its immunological comparison with cutinase from *Fusarium solani pisi. Arch. Biochem. Biophys.* **176**: 334-343.
11. Umezawa, H., T. Aoyagi, K. Uotani, M. Hamada, T. Takeuchi, and S. Takahashi. 1980. Ebelactone, an inhibitor of esterase, produced by actinomycetes. *J. Antibiot.* (Tokyo) **31**: 639-641.
12. Walton, T. J. and P. E. Kolattukudy. 1972. Determination of the structures of cutin monomers by a novel depolymerization procedure and combined gas chromatography and mass spectrometry. *Biochem.* **11**: 1885-1897.

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