Antagonism of *Trichoderma harzianum* ETS 323 on *Botrytis cinerea* Mycelium in Culture Conditions

Chi-Hua Cheng, Chia-Ann Yang, and Kou-Cheng Peng

First and third authors: Institute of Biotechnology, National Dong-Hwa University, Hualien 97401, Taiwan, R.O.C.; and second author: Institute of Medical Science, Tzu Chi University, Hualien 97004, Taiwan, R.O.C.
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ABSTRACT

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Previous studies have shown that the extracellular proteins of *Trichoderma harzianum* ETS 323 grown in the presence of deactivated *Botrytis cinerea* in culture include a putative L-amino acid oxidase and have suggested the involvement of this enzyme in the antagonistic mechanism. Here, we hypothesized that the mycoparasitic process of *Trichoderma* spp. against *B. cinerea* involves two steps; that is, an initial hyphal coiling stage and a subsequent hyphal coiling stage, with different coiling rates. The two-step antagonism of *T. harzianum* ETS 323 against *B. cinerea* during the mycoparasitic process in culture was evaluated

using a biexponential equation. In addition, an L-amino acid oxidase (Th-L-AAO) was identified from *T. harzianum* ETS 323. The secretion of Th-L-AAO was increased when *T. harzianum* ETS 323 was grown with deactivated hyphae of *B. cinerea*. Moreover, in vitro assays indicated that Th-L-AAO effectively inhibited *B. cinerea* hyphal growth, caused cytosolic vacuolization in the hyphae, and led to hyphal lysis. Th-L-AAO also showed disease control against the development of *B. cinerea* on post-harvest apple fruit and tobacco leaves. Furthermore, an apoptosis-like response, including the generation of reactive oxygen species, was observed in *B. cinerea* after treatment with Th-L-AAO, suggesting that Th-L-AAO triggers programmed cell death in *B. cinerea*. This may be associated with the two-step antagonism of *T. harzianum* ETS 323 against *B. cinerea*.

Botrytis cinerea is a necrotrophic fungus associated with >200 host plants (37) and is the causal agent of gray mold disease, which can affect fruit (9), vegetable, and ornamental crops (41). Because of its impact on such a broad range of hosts, *B. cinerea* is one of the most important plant pathogens worldwide (47). Previous studies have reported that *B. cinerea* under culture conditions kills host cells by producing toxins (e.g., oxalic acid and phytotoxic metabolites) and plant cell-wall-degrading enzymes. *B. cinerea* has also been reported to penetrate the host tissue by either hyphae or appressoria through stomata or wounds (14,19,20,38) but the appressorium is not considered capable of penetrating plant tissue by physical pressure alone (31,37).

Fungal strains of the genus *Trichoderma* are important biocontrol agents used as biopesticides worldwide (4), and are attractive biocontrol agents for phytopathogens such as *B. cinerea*, *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*, and *Sclerotium rolfsii* (8,34). *Trichoderma* spp. use several biocontrol mechanisms, including mycoparasitism through the dense coiling of hyphae around the host pathogen, the secretion of antibiotics such as enzymes and secondary metabolites, rhizosphere modification, competition for resources and space, and promotion of plant growth and defense mechanisms (4,7,36,48).

L-Amino acid oxidases (L-amino acid:oxygen oxidoreductase [deaminating], EC 1.4.3.2; L-AAOs) catalyze the oxidative deamination of L-amino acids and produce the corresponding α -keto acids, as well as ammonia and hydrogen peroxide (H_2O_2) (13,30). L-AAO occurs widely in snakes (39,40), bacteria (22), and algae

(46). Because of the cytotoxic effects of this enzyme on various cells, including cancer cells (1), platelets (44), and microorganisms (49), L-AAO has become the focus of research in the fields of biochemistry, physiology, and medicine. However, a previous proteomic study revealed the presence of a putative L-AAO in the secreted proteins of *Trichoderma harzianum* ETS 323 grown in a medium containing deactivated *B. cinerea* hyphae (50).

A more detailed understanding of the mycoparasitic mechanisms of *T. harzianum*, as well as the effect of L-AAO secretion on the antagonism of *T. harzianum* against *B. cinerea*, would produce benefits in the application of *Trichoderma* spp. as a biocontrol agent. In the mycoparasitic process of *Trichoderma* spp., the hyphae of the *Trichoderma* sp. encircle and coil around the hyphae of the host pathogen (a physical process defined as the “hyphal coiling” of *Trichoderma* spp.) (24). Once the host is encircled, *Trichoderma* spp. secrete lytic enzymes such as glucanase and chitinase to penetrate the host and then use the host cellular contents as a source of nutrients (11,25). Here, we assume that the host pathogen may have a defense response to the presence of the initial hyphal coiling of *Trichoderma* spp. in the early stages of the mycoparasitic process. Thus, an antagonism between these two fungi may occur through the secretion of extracellular proteins. Moreover, the process of hyphal coiling in *Trichoderma* spp. may become impeded because of the resistance response of the host pathogen. Therefore, we suggest that the hyphal coiling of *Trichoderma* spp. may undergo a slow phase at a later stage in the continuing mycoparasitic interaction with the host, as the defense response of the host pathogen is attenuated by the secreted proteins of *Trichoderma* spp. Considering these possibilities, we believe that the hyphal coiling process of *Trichoderma* spp. may involve an initial coiling stage and a subsequent coiling stage via different rates of hyphal coiling and suggest that the mycoparasitic process of *Trichoderma* spp. may be split into more than one antagonistic step. Therefore, the aim of this work was to ascertain the effect of *T. harzianum*

Corresponding author: K. C. Peng; E-mail address: kcpeng@mail.ndhu.edu.tw

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L-AAO on the hyphal growth of *B. cinerea* and to present a conceptual framework linking this effect to the mycoparasitic activity of the fungus.

MATERIALS AND METHODS

Chemical materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were obtained from Difco (Franklin Lakes, NJ). Number 1 filter paper was obtained from Advantec Toyo Kaisha (Tokyo). Mito-Capture was purchased from BioVision (Palo Alto, CA).

Cultivation of *T. harzianum* ETS 323 and deactivated *B. cinerea*. *T. harzianum* ETS 323 was cultivated following the method described by Yang et al. (50). In brief, *T. harzianum* was grown on PDA-coated plates at 25°C for 7 days. Sterile water (2 ml) was added to flush the plates of *T. harzianum* ETS 323. Conidia ($\approx 10^6$ ml⁻¹) were collected and inoculated in 250 ml of minimal medium nourished with 1% (wt/vol) glucose in a 500-ml Erlenmeyer flask; the minimal medium contained (NH₄)₂SO₄ at 1.4 g liter⁻¹, KH₂PO₄ at 2.0 g liter⁻¹, NaH₂PO₄ at 6.9 g liter⁻¹, MgSO₄ · 7H₂O at 0.3 g liter⁻¹, peptone at 1.0 g liter⁻¹, and urea (pH 5.0) at 0.3 g liter⁻¹. The cultures were grown at 22°C in an incubator with a shaker at 180 rpm for 7 days. The mycelia were then collected by filtration through a cheesecloth, washed three times with sterile Milli-Q (18.2 MΩ) water, and inoculated in fresh minimal medium containing 0.5% (wt/vol) glucose and 0.5% (wt/vol) deactivated *B. cinerea* hyphae. The *B. cinerea* hyphae had been cultivated and then deactivated as previously described (50). These cultures were grown for another 5 days at 22°C in the incubator with the shaker at 180 rpm. The culture medium was collected by filtration through a cheesecloth and number 1 filter paper, and stored at -20°C until use.

Antagonistic assay with *T. harzianum* ETS 323 and *B. cinerea*. A thin layer of PDA was spread on clean, sterile glass microscope slides in the center of sterile culture plates. The medium was inoculated with 4-mm-diameter mycelia of both *T. harzianum* ETS 323 (accession number BCRC 930081; Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan) and *B. cinerea* cut from the growing edges of 5-day-old cultures at the opposing site, and then further incubated for 5 days at 25°C. The glass slides were stained with lactophenol blue (Fluka Chemie, Buchs, Switzerland) to improve the visibility of the hyphae, then observed under a light microscope (IX70; Olympus, Tokyo) with an attached camera (Olympus SC35). The hyphal interactions between the opposing colonies were photographed every 24 h.

Kinetics of the antagonistic effect of *T. harzianum* ETS 323 against *B. cinerea*. *T. harzianum* is a mycoparasite that acts antagonistically by coiling its hyphae around the host hyphae (e.g., *B. cinerea*). The frequency of the hyphal coiling of *T. harzianum* ETS 323 against *B. cinerea* was plotted against time and was defined as the number of coiling *T. harzianum* hyphae per square millimeter on *B. cinerea* hyphae (Fig. 1). Here, we propose that the mycoparasitism of *T. harzianum* against *B. cinerea* involves a two-step antagonism (initial hyphal coiling and then continued hyphal coiling), as described here by equation 1a. Therefore, biphasic kinetics (3,17) may occur in a time course of the hyphal coiling of *T. harzianum* ETS 323 against *B. cinerea*. This biphasic kinetics best fits the biexponential equation 1b. Biphasic kinetics was observed in the exponential growth phase (Fig. 1), with two rates for the hyphal coiling (i.e., $k_{\text{obs},1}$ and $k_{\text{obs},2}$).

$$F(t) = F_1(1 - e^{-k_{\text{obs},1}t}) + F_2(1 - e^{-k_{\text{obs},2}t}) \quad (1b)$$

where T and B are *T. harzianum* ETS 323 and *B. cinerea*, respectively, and TB_1 and TB_2 are the mycoparasitic states of *T. harzianum* against *B. cinerea* via hyphal coiling after steps 1 and 2 of the antagonism, respectively. The designations *coiling* and *more coiling* are the initial and further hyphal coiling of *T. harzianum* ETS 323 against *B. cinerea* for rates $k_{\text{obs},1}$ and $k_{\text{obs},2}$, respectively. We suggest that TB_2 is the state of the final extent of *T. harzianum* coiling against *B. cinerea*. For equation 1b, the independent variable is t , the number of days after hyphal contact. $F(t)$ is the dependent variable and indicates the time-dependent coiling frequency. F_1 and F_2 are the amplitude of the coiling frequency after steps 1 and 2 of the antagonism, respectively. The value of $F_1 + F_2$ is the maximum hyphal coiling frequency of *T. harzianum* against *B. cinerea*. Amplitudes were normalized to the maximum hyphal coiling frequency. The $k_{\text{obs},1}$ and $k_{\text{obs},2}$ are the rate constants of hyphal coiling at steps 1 and 2 of the antagonism, respectively. The kinetic data were fitted to biexponential functions using the software Origin 7.0 (MicroCal, Northampton, MA) implementation of a nonlinear least-squares-fitting algorithm. The initial estimates for F_1 , F_2 , $k_{\text{obs},1}$, and $k_{\text{obs},2}$ were 1 for all parameters.

Purification of putative Th-L-AAO. The extracellular protein extract from the culture medium was concentrated with an Amicon Ultra-4 centrifugal filter unit (3-kDa molecular mass cut-off; Millipore, Billerica, MA) and subsequently applied to the two steps of purification by ÄKTAprime plus 100 FPLC (Healthcare Bio-Sciences, Piscataway, NJ). The protein-containing extract was loaded onto a ConA-Sepharose column (5 ml; GE Healthcare Bio-Sciences), which was washed with a buffer containing 1 mM CaCl₂, 1 mM MnCl₂, 0.5 M NaCl, and 0.02 M Tris-HCl (pH 7.4),

Fig. 1. Hyphal coiling frequency of *Trichoderma harzianum* ETS 323 against *Botrytis cinerea* was evaluated by microscopy in the interaction zone between *T. harzianum* and *B. cinerea*. Coiling frequency was defined as coiling number per square millimeter. The asterisk indicates hyphal lysis of *B. cinerea*. For allowing a direct comparison of the percentage of the amount of hyphal coiling, the value representing the final extent of the coiling frequency has been normalized to 1. Hyphal coiling kinetics was fitted to the biexponential equation 1b. The nonlinear fit curve with R^2 was 0.99. Broken lines reflect the theoretical decomposition of the time-dependent coiling of *T. harzianum* against *B. cinerea* into two phases (fast phase and slow phase). F_1 and F_2 are the amplitude of the coiling frequency after steps 1 and 2, respectively, of the antagonism of *T. harzianum* against *B. cinerea* (see Materials and Methods). Values are the means of three independent experiments. Bars represent the standard error from the mean ($n = 3$).

then eluted with a linear gradient of methyl- α -D-mannopyranoside (0 to 0.5 M) in 0.02 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl at a flow rate of 1 ml min⁻¹. Glycoprotein fractions were collected and concentrated by ultrafiltration, and subjected to a Superdex HR 75 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden). This glycoprotein fraction column was eluted with 20 mM sodium phosphate buffer (pH 7.4) at a flow rate of 0.2 ml min⁻¹. The following protein standards were used for calibration: myoglobin (17 kDa), bovine serum albumin (67 kDa), and human immunoglobulin (150 kDa). All chromatographic steps were monitored at 280 nm and performed at 25°C.

Analysis of Th-L-AAO secretion by *T. harzianum* ETS 323.

The activity of Th-L-AAO was determined by enzyme-coupled assay (2,27,32,45). In brief, L-Phe and D-Phe were used as substrates for Th-L-AAO, and were added to a 200- μ l reaction mixture containing 4.7 U of horseradish peroxidase, 25 mM sodium phosphate buffer (pH 7.5), *o*-dianisidine at 0.5 mg ml⁻¹, 2% Triton X-100, and a known amount of purified Th-L-AAO (6 μ M) or crude extract. After 15 h at 37°C, H₂O₂ generated by the reaction was measured by reading the absorbance at 450 nm on an enzyme-linked immunosorbent assay plate reader (Model 680; Bio-Rad, Hercules, CA).

The K_m and the maximum reaction rate (V_{max}) for the substrate were determined using a nonlinear least square fit of the experimental data to the Michaelis-Menten equation, using Origin 7. All experiments were performed at least three times.

Analysis of the effect of Th-L-AAO on *B. cinerea* hyphal growth. Two discs (2 mm in diameter) of fungal *B. cinerea* hyphae were cut from the edges of 5-day-old fungal cultures grown in PDA at 25°C and inoculated on the opposite sides of 9-cm-diameter PDA-coated plates. A sterile filter paper disc (6 mm) impregnated with the appropriate concentration of Th-L-AAO at 3, 6, or 12 μ g disc⁻¹ was placed on the advancing fungal hyphae on one side, with a control disc containing sterile distilled water on the other side. The plates were further incubated at 22°C for 5 days. The hyphal growth was photographed and the diameters were recorded daily. The percentage of hyphal growth reduction was calculated using the formula hyphal growth reduction (%) = $\{[D_{(c)} - D_{(t)}]/D_{(c)}\} \times 100$ %, where $D_{(c)}$ is the diameter of the growth area of *B. cinerea* hyphae on the control plate and $D_{(t)}$ is the diameter of the growth area of *B. cinerea* hyphae on the Th-L-AAO-containing plate. The control treatment with the highest growth was compared with other Th-L-AAO treatments in a one-way analysis of variance (ANOVA). Means were separated using Tukey's honestly significant difference (HSD) test and to identify significant groupings. Statistical analyses were performed using the Statistical Software Package for the Social Sciences (version 12.0.1 for Windows; SPSS Inc., Chicago).

For light microscopy, a thin layer of PDA was spread on sterile microscope slides in the center of sterile culture plates. One disc (2 mm in diameter) of fungal *B. cinerea* hyphae was cut from the growing edge of 5-day-old fungal cultures and transferred to the center of the PDA-coated slides. A sterile filter paper disc impregnated with 6 μ g of Th-L-AAO was placed on the fungal hyphae. The glass slides were stained with lactophenol blue and observed daily under a light microscope for 3 to 4 days.

Effects of Th-L-AAO on *B. cinerea*-infected apple fruit and tobacco leaves. Th-L-AAO was tested for its effect on the development of *B. cinerea* on inoculated apple fruit and tobacco leaves. The postharvest apple fruit was wounded with a 7-mm-diameter cork borer, then inoculated with a 6-mm-diameter hyphal plug from a 5-day-old PDA plate of *B. cinerea* (28). For determining the effect of Th-L-AAO, Th-L-AAO at 30 μ g ml⁻¹ was applied at the infection site prior to the inoculation of *B. cinerea*; Th-L-AAO was not applied at the infection site in the case of control treatment. The inoculated apple fruit were stored at 25°C in the dark. After 6 days, the lesions spreading on the

surface of the apple were photographed, and the radii of the expansion zones were recorded. All experiments were performed in triplicate. One-way ANOVA was used to compare treatment groups. The α level of 0.05 was considered statistically significant. Tukey's HSD test was used to identify significant groupings. Statistical analyses were performed using the Statistical Software Package for the Social Sciences (version 12.0.1; SPSS Inc.) (also see experiments pertaining to tobacco leaves below).

Tobacco leaves were detached from 12-week-old plants grown in the greenhouse and inoculated with a hyphal plug of *B. cinerea* from a 5-day-old PDA-containing plate in order to examine the effects to the fungus (5). For testing the effect of Th-L-AAO on *B. cinerea* fungal pathogenesis, a sterile filter paper disc impregnated with Th-L-AAO at a concentration of 6 μ g disc⁻¹ was applied at the infection site, while a control disc impregnated with sterile distilled water was applied at the infection site. Afterward, the inoculated tobacco leaves were placed in petri plates with moist filter paper discs at 25°C in the dark. After 14 days of incubation, the development of *B. cinerea* was photographed. The radii of the spreading lesions were recorded and then used to evaluate disease effect in Th-L-AAO-treated leaves and untreated leaves. All experiments were performed in triplicate.

Detection of reactive oxygen species generation. To determine the apoptotic response in *B. cinerea*, we measured reactive oxygen species (ROS) generation in Th-L-AAO-treated *B. cinerea*. For determining ROS generation in cells, *B. cinerea* hyphae were loaded with 2',7'-dichlorofluorescein diacetate (H₂DCF-DA) (21). After 4 days of incubation with or without Th-L-AAO (6 μ g disc⁻¹) treatment, the hyphal portion was immersed in 1 μ M H₂DCF-DA for 15 min after a brief rinse with distilled water, and continuously incubated for 12 h on a PDA plate. DCF fluorescence was visualized with a fluorescence microscope (Olympus IX70) and detected at an excitation wavelength of 485 nm and emission wavelength of 535 nm using a spectrofluorometer.

Estimation of lipid peroxidation activity. Lipid peroxidation is a consequence of ROS action on membrane structure (6) and was examined in Th-L-AAO-treated *B. cinerea* as a measure of ROS-related damage. Malondialdehyde (MDA), a major product of lipid peroxidation, was quantified to estimate the extent of lipid peroxidation in this fungus. In brief, *B. cinerea* was treated with Th-L-AAO (6 μ g disc⁻¹) for 4 days. *B. cinerea* was not treated with the Th-L-AAO in the case of control treatment. The hyphae were collected and their MDA concentrations were determined. The reaction mixture consisted of 0.125 ml of KCl, 0.05 ml of 8.1% sodium dodecyl sulfate (SDS), 0.375 ml of 20% acetic acid (pH 3.5), 0.375 ml of a 0.8% aqueous solution of thiobarbituric acid (TBA), and 0.125 ml of hyphal homogenate. The mixture was heated at 95°C for 60 min and then cooled on ice for 15 min. Afterward, 1 ml of *n*-butanol and pyridine (15:1, vol/vol) was added, and the mixture was centrifuged at 4°C for 10 min at 4,000 $\times$ g. The MDA concentration in the cell homogenate was determined using the TBA assay (33). The organic layer was separated, and its absorbance was measured at 535 nm using a UV-visible spectrophotometer. The mean values for three independent experiments were calculated.

DNA extraction and analysis. DNA fragmentation is a key feature of programmed cell death (18). Here, we examined the effect of Th-L-AAO on DNA damage in *B. cinerea*. After 5 days of incubation with or without Th-L-AAO (6 μ g disc⁻¹) treatment, the *B. cinerea* hyphae (0.3 g) were frozen in liquid nitrogen and ground into a fine powder. Each hyphal sample was resuspended with 700 μ l of lysis buffer (50 mM Tris-HCl [pH 7.2], 1% mercaptoethanol, and 3% SDS) and then incubated at 65°C for 60 min with shaking every 15 min. Next, the samples were mixed with an equal volume of chloroform and isoamyl alcohol (24:1, vol/vol). After gentle shaking for 5 min, the mixture was centrifuged for 10 min at 12,000 $\times$ g. Total DNA was precipitated by the addition of 50 μ l of 3 M NaOAc and 500 μ l of 2-propanol.

After 20 min, the samples were centrifuged for 20 min at $12,000 \times g$ and washed with 200 μ l of 70% ice-cold ethanol. The *B. cinerea* hyphal DNA was then recovered by centrifugation for 5 min at $12,000 \times g$, and the sample was dried under vacuum for 20 min. The total DNA was dissolved in 50 μ l of Tris-EDTA buffer (10 mM Tris-HCl and 0.1 mM EDTA). For detecting DNA laddering, the samples were run on a 1% (wt/vol) agarose gel, and the DNA in the gel was visualized and photographed under ultraviolet light after staining with 0.5% (vol/vol) ethidium bromide. All experiments were performed in triplicate.

Determination of mitochondrial membrane potential. Mitochondrial transmembrane potential reduction is an early event in apoptosis (29) and mediates ROS generation in a cell. Thus, we examined the effect of Th-L-AAO treatment on the mitochondrial membrane potential in *B. cinerea*. The mitochondrial membrane potential was determined by the MitoCapture apoptosis detection kit. In normal cells, MitoCapture accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. However, in apoptotic cells, MitoCapture cannot aggregate in the mitochondria because of the altered mitochondrial transmembrane potential; therefore, it remains in the cytoplasm in its monomer

Fig. 2. Light microscopy images of the antagonism of *Trichoderma harzianum* ETS 323 and *Botrytis cinerea* co-cultured for **A**, 2; **B**, 3; **C**, 4; **D**, 5; and **E and F**, 6 days are shown. **A**, Arrows indicate *T. harzianum* ETS 323 hyphae contacted with *B. cinerea* hyphae. The *T. harzianum* ETS 323 hyphae (T) are thin, and *B. cinerea* hyphae (B) are rough. **B**, **C**, and **D**, Arrows indicate intensive coiling on *B. cinerea*. **E and F**, Arrows indicate *B. cinerea* hyphal lysis and death at 6 days. All images are at the same magnification.

form, fluorescing green. After 3 days of incubation in medium with or without Th-L-AAO ($6 \mu\text{g disc}^{-1}$) treatment, the *B. cinerea* samples were incubated in the incubation buffer and MitoCapture reagent for 30 min at 37°C . The cells were then collected, and the fluorescence was analyzed using a spectrophotofluorometer (Olympus IX70) at an excitation wavelength of 488 nm and an emission wavelength of 590 nm.

Fig. 3. A, Analytical Superdex HR 75 10/30 column gel filtration showed that Th-L-AAO elutes as a single peak; the arrow indicates the Th-L-AAO-containing fraction. B, Molecular mass of Th-L-AAO was determined to be 67 kDa. BSA = bovine serum albumen. C, Purified Th-L-AAO (indicated by the arrow) from *Trichoderma harzianum* ETS 323 after the 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel was stained with Coomassie R-250. Lane M: molecular mass marker; lane 1: crude extract filtrate; lane 2: preparation after ConA-Sepharose column chromatography; lane 3: purified Th-L-AAO (67 kDa).

RESULTS

Antagonism between *T. harzianum* ETS 323 and *B. cinerea*.

While growing near the *B. cinerea* hyphae, *T. harzianum* ETS 323 produced hyphal branches oriented toward the pathogenic colony (Fig. 2). After 2–3 days of co-culture, *T. harzianum* ETS 323 hyphae contacted and coiled with the *B. cinerea* hyphae (Fig. 2A and B). After the fourth and fifth days of incubation, more coiling was observed, causing the cessation of *B. cinerea* hyphal growth (Fig. 2C and D). On the sixth day of incubation, hyphal lysis of *B. cinerea* was observed (Fig. 2E and F). The time-dependent coiling behavior of *T. harzianum* ETS 323 is shown in Figure 1.

Kinetics of coiling frequency of *T. harzianum* ETS 323 on *B. cinerea* hyphae. The kinetics of *T. harzianum* ETS 323 coiling frequency on *B. cinerea* hyphae were evaluated by biexponential equation 1b. Details of the biexponential equation are defined in the Supplemental Material provided. Here, a biphasic rate constant ($k_{\text{obs},1}$ and $k_{\text{obs},2}$) for *T. harzianum* hyphal coiling was evaluated, suggesting that a two-step antagonism was involved in the mycoparasitism of *T. harzianum* against *B. cinerea*. In the first step of the antagonism, the rate constant of the hyphal coiling (fast phase, $k_{\text{obs},1}$) was found to be 1 day^{-1} . The amount of hyphal coiling frequency reached saturation at 27% of maximum hyphal coiling, with $F_1 = 0.27$ at 15 h. In the second step of the antagonism, the rate constant of the hyphal coiling (slow phase, $k_{\text{obs},2}$) was observed to be 0.4 day^{-1} . The amount of hyphal coiling frequency was 73% of maximum hyphal coiling, with $F_2 = 0.73$. These data indicated that the rate-determining step in the mycoparasitism of *T. harzianum* ETS 323 against *B. cinerea* was step 2, because $k_{\text{obs},1}$ was 2.5-fold faster than $k_{\text{obs},2}$, and the mycoparasitic process of *T. harzianum* at the initial hyphal coiling stage of TB_1 reached $\approx 30\%$ of maximum hyphal coiling.

Purification of Th-L-AAO from the culture medium of *T. harzianum* ETS 323. The putative L-AAO from the extracellular protein extracts of *T. harzianum* ETS 323 grown in a medium containing deactivated *B. cinerea* was purified by two steps of column chromatography (Fig. 3A and B). The protein extract was first retained on the ConA-Sepharose column and recovered in the

Fig. 4. For examining the effect of deactivated *Botrytis cinerea* hyphae on Th-L-AAO secretion, the amount of Th-L-AAO secreted in 250 ml of the culture medium over a period of 5 days was determined using 30 mM L-phenylalanine as the substrate. $\circ$ = *T. harzianum* ETS 323 grown in the presence of deactivated *B. cinerea* hyphae (medium II). $\blacklozenge$ = *T. harzianum* ETS 323 grown in absence of deactivated *B. cinerea* hyphae (medium I). Values represent the means of three independent experiments, and the error bars represent the standard deviation from the mean ($n = 3$).

mannopyranoside-eluted fraction (Fig. 3C, lane 2). Purified Th-L-AAO showed a peak at the retention volume of 8.2 ml for the culture medium in gel filtration. The SDS polyacrylamide gel electrophoresis showed that purified Th-L-AAO has a molecular mass of 67 kDa (Fig. 3B and C, lane 3).

Substrate activity. Th-L-AAO substrate activity was tested against 17 L-amino acids, and the highest specific activity was detected with L-phenylalanine (L-Phe). The activity of purified Th-L-AAO against L-Phe and D-Phe was investigated. Th-L-AAO exhibited high activity against L-Phe as the substrate but had no detectable activity against D-Phe as the substrate. The kinetic parameters of L-Phe determined by the nonlinear least-squares fit method were $K_m = 10.7$ mM and $V_{max} = 477.4$ $\mu\text{M min}^{-1}$.

Effect of deactivated *B. cinerea* hyphae on Th-L-AAO secretion by *T. harzianum* ETS 323. The enzyme-coupled assay was used to determine the amount of Th-L-AAO secreted by *T. harzianum* ETS 323 in the presence or absence of deactivated *B. cinerea* hyphae. The amount of Th-L-AAO secreted in the culture medium was recorded over a period of 5 days (Fig. 4). During the period between days 2 and 3, the Th-L-AAO secretion was greatly elevated when *T. harzianum* ETS 323 was grown in the presence of deactivated *B. cinerea* hyphae (medium II), as compared with when *T. harzianum* ETS 323 was grown in the absence of deactivated *B. cinerea* hyphae (medium I). After 5 days of incubation, *T. harzianum* ETS 323 exposed to the deactivated *B. cinerea* hyphae secreted a threefold amount of Th-L-AAO compared with the control, which was incubated without *B. cinerea* hyphae.

These results indicated that the amount of Th-L-AAO secreted was increased in the presence of *B. cinerea* hyphae.

Effect of Th-L-AAO on *B. cinerea* hyphal growth. The Th-L-AAO-treated *B. cinerea* samples showed hyphal growth inhibition on PDA plates (Fig. 5). On the fourth day of incubation (Fig. 5A), lysis of the *B. cinerea* hyphae was observed after Th-L-AAO treatment, in contrast to the normal hyphal growth of control-treated *B. cinerea*. After the fifth day of incubation (Fig. 5B), the Th-L-AAO on the treated disc resulted in the thinning of the hyphae on the control disc, further confirming the antagonistic activity of Th-L-AAO. Various concentrations of Th-L-AAO (3, 6, and 12 $\mu\text{g disc}^{-1}$) were applied to the *B. cinerea* samples for 5 days and effectively inhibited *B. cinerea*, with up to 83.3% hyphal growth reduction on the fifth day of treatment (Fig. 5C). The hyphal growth of control-treated *B. cinerea* was threefold and sixfold higher than that of Th-L-AAO-treated *B. cinerea* on the fourth and fifth days of treatment, respectively.

The concentration of Th-L-AAO at 6 $\mu\text{g disc}^{-1}$ resulted in a 75% reduction in *B. cinerea* hyphal growth and, thus, was chosen for subsequent experiments. As shown by light microscopy, the *B. cinerea* samples treated with this concentration of Th-L-AAO showed abnormal characteristics, such as the development of cytosolic vacuoles, which were not observed in the control group (Fig. 6A). Following vacuole formation (Fig. 6B), the hyphal cell wall was disrupted (Fig. 6C). Finally, lysis occurred in the abnormal *B. cinerea* hyphae. By contrast, the control samples showed smooth hyphal walls and intact cytosols (Fig. 6A).

Fig. 5. Paper disc method results for the effect of Th-L-AAO on *Botrytis cinerea* hyphal growth: growth of *B. cinerea* on potato dextrose agar media with Th-L-AAO (L) or without Th-L-AAO (C). *B. cinerea* treated with Th-L-AAO (6 $\mu\text{g disc}^{-1}$) for A, 4 and B, 5 days. A, Left arrow indicates the large amount of hyphal lysis and right arrow indicates the regular hyphal growth. B, Arrow indicates the thinning hyphae. C, *B. cinerea* hyphal growth at three different concentrations of Th-L-AAO; * indicates *B. cinerea* hyphal lysis. Mean values with different letters indicate significant differences among treatments ($\alpha = 0.05$) at 3, 4, and 5 days of incubation according to Tukey's honestly significant difference test. Values are the means of three independent experiments and bars represent the standard deviation from the mean ($n = 3$).

Fig. 6. *Botrytis cinerea* samples were treated with A, sterile water for 4 days and with Th-L-AAO at 6 $\mu\text{g disc}^{-1}$ for B, 3 and C, 4 days. B, Arrows indicate the cytosolic division of *B. cinerea* after incubation with Th-L-AAO for 3 days. C, Arrow indicates a significant amount of hyphal lysis after 4 days.

Th-L-AAO inhibits *B. cinerea* development on postharvest apple fruit and tobacco leaves. Apple fruit inoculated with *B. cinerea* and treated with Th-L-AAO showed significant inhibition of *B. cinerea*-induced lesions after 6 days (Fig. 7A), whereas untreated apple fruit exhibited development of *B. cinerea* lesions around the site of infection (Fig. 7B). The untreated apple fruit displayed a 12-fold increase in the lesion radius relative to the treated apple fruit ($\alpha = 0.05$) (Table 1).

Th-L-AAO-treated tobacco leaves also showed enhanced inhibition to *B. cinerea* infections (Fig. 7C). The untreated control leaves exhibited spreading lesions around the site of infection with *B. cinerea* (Fig. 7D), whereas the leaves treated with Th-L-AAO showed suppression of the lesions. The untreated tobacco leaves showed a 17-fold higher lesion radius when compared with the treated tobacco leaves ($\alpha = 0.05$) (Table 1).

Th-L-AAO-induced ROS generation in *B. cinerea*. ROS generation was detected by H_2DCF -DA. The green fluorescence

increased in the Th-L-AAO-treated *B. cinerea*, indicating increased ROS generation in these hyphae (Fig. 8). The untreated *B. cinerea* showed no fluorescence throughout the 4 days of incubation (Fig. 8A). In contrast, the fluorescence was appreciable in the Th-L-AAO-treated *B. cinerea* hyphae on the second day of incubation (data not shown) and noticeable in the hyphae after 3 days (Fig. 8B). However, more hyphae were visualized with fluorescence on the third compared with the second day of incubation. On the fourth day, the Th-L-AAO-treated *B. cinerea* hyphae showed many green fluorescent particles, possibly representing lysates of *B. cinerea* (Fig. 8C).

Lipid peroxidation in Th-L-AAO-treated *B. cinerea*. The concentration of MDA, a marker of lipid peroxidation, increased in a time-dependent manner in the hyphae of *B. cinerea* after treatment with Th-L-AAO. MDA concentrations of 0.2, 0.5, and 12.5 μ M were observed on the second, third, and fourth days of incubation, respectively, with Th-L-AAO. Increased MDA con-

Fig. 7. Postharvest treatment of apple fruit and tobacco leaves with *Botrytis cinerea*. **A**, Th-L-AAO-treated apple fruit inoculated with the *B. cinerea*. **B**, Control (untreated) apple fruit inoculated with *B. cinerea*. Tobacco leaves challenged with *B. cinerea* and **C**, treated with Th-L-AAO and **D**, untreated. Arrow B indicates a plug of *B. cinerea* and arrow L indicates one sterile filter paper disc impregnated with Th-L-AAO.

Fig. 8. *Botrytis cinerea* untreated with Th-L-AAO. *B. cinerea* was treated with Th-L-AAO at 6 μ g disc⁻¹ for **B**, 3 and **C**, 4 days. **A**, Arrow indicates the absence of reactive oxygen species (ROS) in *B. cinerea* hyphae. **B** and **C**, Arrows indicate the positions of representative intracellular ROS generation. All scale bars are presented in the same magnification.

centrations were apparent in Th-L-AAO-treated *B. cinerea*, consistent with lipid peroxidation, whereas no MDA was observed in the control treatment (nontreated with Th-L-AAO).

DNA damage in Th-L-AAO-treated *B. cinerea*. Next, we investigated the DNA damage caused by Th-L-AAO-induced ROS stress in *B. cinerea*. DNA fragmentation of *B. cinerea* was seen only after treatment with Th-L-AAO (Fig. 9).

Effect of Th-L-AAO treatment on the mitochondrial membrane potential in *B. cinerea*. The mitochondria of the untreated *B. cinerea* showed red fluorescence in the MitoCapture assay, implying that the mitochondrial membrane potential remained intact (Fig. 10A). However, the green fluorescence seen in the

TABLE 1. Efficacy of the L-amino acid oxidase Th-L-AAO against infection by *Botrytis cinerea* in postharvest apple fruit and tobacco leaves

Host	Radii of the lesions (mm) ^z	
	Untreated with Th-L-AAO	Treated with Th-L-AAO
Apple fruit	12.1 ± 2.3 a	1.1 ± 1.0 b
Tobacco leaf	17.2 ± 3.2 a	0.0 ± 0.0 b

^z Radii of the lesions were measured from the edge of the inoculum plug on the surface of the fruit or leaf. Values are means ± the standard deviation of three independent experiments. Means with the same letter are statistically equivalent according to Tukey's honestly significant difference test.

Fig. 9. DNA fragmentation of Th-L-AAO-treated *Botrytis cinerea*. Lane 1: molecular mass marker; lane 2: *B. cinerea* without 5-day Th-L-AAO treatment; lane 3: *B. cinerea* with 5-day Th-L-AAO treatment.

Fig. 10. For evaluating in *Botrytis cinerea* the effect of Th-L-AAO treatment on mitochondrial membrane potential, *B. cinerea* was treated with Th-L-AAO at 6 µg disc⁻¹ for 3 days. **A**, Fluorescence represents mitochondria with an intact membrane potential; **B**, fluorescence represents disrupted membrane potential. All scale bars are presented in the same magnification.

Th-L-AAO-treated *B. cinerea* samples (Fig. 10B) suggested depolarization of the mitochondrial membrane, as induced by Th-L-AAO.

DISCUSSION

Trichoderma spp. are common biocontrol agents against fungal phytopathogens (4,8,34,51). The mechanisms of their biocontrol have been found to involve various important modes of action, including mycoparasitism, antibiosis, and induction of plant resistance (4,15,35,36,48). Here, we reported that the mycoparasitism of *T. harzianum* ETS 323 against *B. cinerea* occurred in the form of a two-step antagonism (hyphal coiling with a biphasic rate) under culture conditions. A possible explanation for this two-step process may lie in the observation that *T. harzianum* ETS 323 first makes contact with *B. cinerea* via less hyphal coiling ($F_1 = 0.27$) in the fast phase ($k_{obs,1} = 1 \text{ day}^{-1}$), with the mechanism of antagonism between these two fungi being the secretion of extracellular proteins. Afterward, *T. harzianum* ETS 323 shows more extensive coiling ($F_2 = 0.73$) in the slow phase ($k_{obs,2} = 0.4 \text{ day}^{-1}$), when the defense response of *B. cinerea* is blocked by the *T. harzianum* ETS 323-secreted proteins. This may provide an explanation for the involvement of the initial hyphal coiling stage (TB_1) in the mycoparasitism of *T. harzianum* ETS 323 against *B. cinerea*.

The mycoparasitic process of *Trichoderma* spp. has been reported to include two distinct classes of enzymes: cell-wall-degrading enzymes and proteases (10,11,16,24,26,42). The cell-wall-degrading enzymes, such as chitinases (11) and glucanases (24), directly affect the integrity of the pathogen cell wall. The proteases produced by *Trichoderma* spp. can break down enzymes or cell wall proteins produced by certain pathogens (10,16,26,42). The present study allowed for the direct observation of the Th-L-AAO-based *T. harzianum* ETS 323 antagonism against *B. cinerea*. The results showed cytosolic division and cytoplasmic leakage in Th-L-AAO-treated *B. cinerea*. Th-L-AAO treatment also showed direct disease control against *B. cinerea* development on postharvest apple fruit and tobacco leaves. Prior research has suggested that the mitochondria-related apoptosis in cells may cause endogenous ROS generation and result in DNA damage (12,23,43). Our results indicate that Th-L-AAO treatment caused an apoptosis-like response in *B. cinerea*, as seen by depolarization of the mitochondrial membrane potential, ROS generation, DNA fragmentation, and progressive lipid peroxidation in Th-L-AAO-treated *B. cinerea*. These findings suggest

that the effect of Th-L-AAO on *B. cinerea* is very different from that of the cell-wall-degrading enzymes and proteases observed in other mycoparasitic mechanisms. As mentioned above, several lytic enzymes have been suggested to be involved in the antagonism of *Trichoderma* spp. (10,11,16,24,26,50,51) and are capable of breaking down the cell wall components of the host pathogen. However, an L-amino acid oxidase (Th-L-AAO), a new class of enzymes related to the antagonism of *Trichoderma* spp. against plant pathogens (e.g., *B. cinerea*), was presented here and showed antagonistic activity by inducing an apoptosis response in *B. cinerea*.

Previous studies have shown that the secretion of Th-L-AAO was observed when *T. harzianum* ETS 323 was grown in the presence of *B. cinerea* (50). Our results showed that the amount of secreted Th-L-AAO increased threefold with exposure to deactivated *B. cinerea* hyphae, and that an appreciable level of ROS generation occurred in Th-L-AAO-treated *B. cinerea* during the 2 days of incubation. Taken together, these findings suggest that Th-L-AAO was secreted after hyphal contact between *T. harzianum* ETS 323 and *B. cinerea*, leading to the induction of programmed cell death in *B. cinerea*. These indications seem to agree with the model of two-step antagonism: *T. harzianum* ETS 323 first contacts *B. cinerea* through less hyphal coiling in the fast phase while extracellular proteins such as Th-L-AAO are secreted, and then more coiling of *T. harzianum* ETS 323 occurs in the slow phase after the defensive response of *B. cinerea* is blocked by the Th-L-AAO-induced apoptotic response. This suggests that the Th-L-AAO-induced apoptotic response might be associated with the two-step antagonism of mycoparasitism against *B. cinerea*.

In conclusion, we have reported the two-step antagonism of *T. harzianum* ETS 323 against *B. cinerea* in the process of mycoparasitism in culture. In addition, an L-amino acid oxidase (Th-L-AAO) was identified as a protein secreted by *T. harzianum* ETS 323 when grown in the presence of deactivated *B. cinerea* hyphae. This purified Th-L-AAO effectively prevented *B. cinerea* hyphal growth by inducing an apoptotic-like response, and this programmed cell death in *B. cinerea* seemed to correlate with the two-step antagonism of *T. harzianum* ETS 323 in mycoparasitic mechanisms.

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