TRICHOLIN, A NEW ANTIFUNGAL AGENT FROM *Trichoderma viride*, AND ITS ACTION IN BIOLOGICAL CONTROL OF *Rhizoctonia solani*

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Tricholin, a ribosome-inactivating protein isolated from the culture broth of *Trichoderma viride*, has been shown to exert fungicidal effects on *Rhizoctonia solani* through a multi-hit kinetic interaction. Tricholin causes a parallel cessation of growth, uptake of amino acids, and protein biosynthesis. The *in vivo* mode of action of tricholin on protein synthesis and cell growth appears to be attributed to the diminishing of the polysome formation in *R. solani* through damage to large ribosomal subunits. These results concur with previous data and prove that tricholin is an effective inhibitor of protein synthesis. The efficacy of tricholin as an antibiotic agent was estimated to have a duration of approximately 42 hours.

In recent years, fungi have been used frequently to control plant diseases caused by other fungi. In most of these cases control of phytopathogens is effected through mycoparasitism[1~3]. The expression of mycoparasitism is either by penetration and formation of hyphae within the host hyphae, or by antibiotic effects exerted as a result of contact[1].

Trichoderma species are antagonists of many soil-borne phytopathogenic fungi[4~6] and have significantly decreased infection and disease through mycoparasitism. Successful control of phytopathogens including *Rhizoctonia solani*, and other fungi has been achieved[7,8]. The molecular mechanism of this antagonism is not yet well understood.

Recently we have isolated an extracellular protein, tricholin, from a strain of *T. viride*, and the molecular action of tricholin was elucidated *in vitro*[9]. In this study we evaluated the antibiotic effect of tricholin on phytopathogenic *R. solani*, and the *in vivo* mode of action of tricholin vs. the host cell was examined. The results implied that tricholin is a potential fungicide for use in agriculture and medicine.

Materials and Methods

Cell Culture and Tricholin

Lyophilized culture of *R. solani* (CCRC 31252) obtained from The Culture Collection and Research Center (Hsinchu, Taiwan) was soaked with sterile water for 30 minutes before transferred to potato dextrose agar plate (Acumedia Inc., Baltimore, U.S.A.), then incubated at 30°C for 24 hours. Single colony of *R. solani* was picked and inoculated into 50 ml of potato dextrose broth (PDB) (Difco Lab., Detroit, U.S.A.) in 250-ml flask. The cells were maintained at 30°C, with constant agitation.

Tricholin used in all experiments was isolated from the culture broth of *T. viride* (CCRC 32654) according to procedures of LIN *et al.*[9].

Fungicidal Effect

Quantitative measurement of fungicidal activity was done by viable counts. *R. solani* cells were incubated in PDB medium as described above with selected concentrations of tricholin. During the course of the test, samples of 0.5 ml were taken at time intervals and were plated on drug-free potato dextrose agar. After incubation, viable fungal colony counts were determined. The number of target sites per cell

were assessed by analysis of the survival-curves.

Protein Synthesis Assay

The labeled precursor L-[^{14}C]leucine (120 Ci/mmol, Amersham, U.S.A.) was added to cell cultures ($OD_{650}=0.3$) at the final concentration of 3 μCi per ml (the experimental volume was 5 ml in 50-ml flask). After 9 hours of incubation, when cell cultures attained 0.5 OD_{650} units (approximately 0.6×10^6 cells/ml), various concentrations of tricholin or blank (sterile water) were added. Duplicate samples (0.5 ml) were taken at various time intervals and diluted immediately in 1 ml of 5% ice-cold trichloroacetic acid (TCA). Precipitates containing labeled leucine were collected on Whatman GF/C filters and washed with 20 ml of 5% ice-cold TCA. The filters were dried, placed into a scintillation fluid, and counted.

Binding Assay and Proteolytic Digestion

R. solani cells were prepared exactly as they were for protein synthesis assays. Tricholin was radioiodinated with carrier-free iodine (Amersham, Boston, MA) using IODO-GEN iodinating reagent (Pierce, Rockford, IL). The iodinated tricholin retained its biological activity with a specific activity of 3×10^3 cpm/ng. All binding assays were performed at room temperature (25°C). Cells (2.4×10^4) in a final volume of 200 μl were gently mixed with various concentrations of [^{125}I]tricholin and incubated at room temperature for various time intervals. The cells were centrifuged using Kubota microcentrifuge (Model KM15200) at speed of 5,000 rpm, 5 minutes, at 25°C and washed 3 times with 10 mM Tris-HCl, pH 7.6. The cells were finally resuspended in 10 mM Tris-HCl, pH 7.6, transferred to a separate tube and the radioactivity was determined.

Proteolytic digestion of tricholin treated cells was performed as follows: *R. solani* was grown for 9 hours and was incubated with [^{125}I]tricholin for another 9 hours. The cells were collected and washed with 0.1 M phosphate-buffered saline, pH 7.6 (PBS) vigorously. The treatments of protease were done by resuspending cells with 0.3 ml of PBS then 3 units of protease K (600 units/ml, Boehringer Mannheim GmbH, Germany) were added into cells solution and incubated at 37°C for 30 minutes. The cells were collected and washed with PBS three times then sodium dodecyl sulfate (SDS) at the final concentration of 0.05% was added before cells subjected to French press (16,000 psi). Cell lysates were taken and analyzed in 15% polyacrylamide gel electrophoresis containing SDS[10], and autoradiograph at −70°C.

Analysis of Polysomes

Tricholin treated and non-treated *R. solani* cells were harvested by centrifugation, washed twice with PBS and were broken by a French press (16,000 psi). The extract was centrifuged at $12,000 \times g$ for 20 minutes and the resulting post-mitochondrial supernatant was used immediately for polysome analysis.

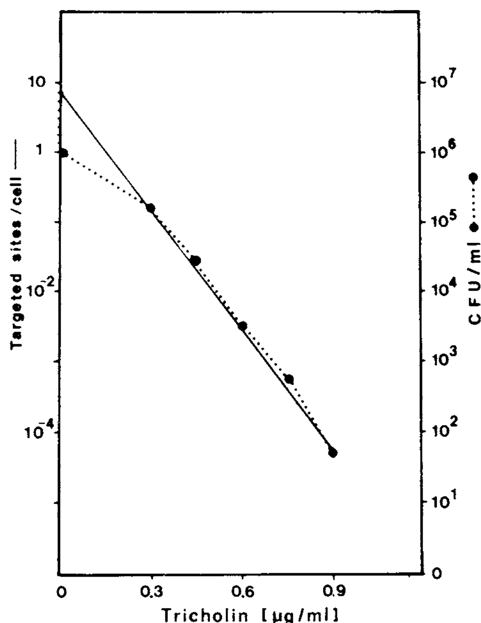
A post-mitochondrial supernatant (0.4 ml) representing about 3×10^4 cells was adjusted to the condition of polysomal buffer containing 20 mM Tris-HCl, pH 7.6; 500 mM KCl, 8 mM $MgCl_2$, and layered on 0.5～1.5 M linear sucrose gradients containing polysomal buffer. The centrifugation was done using a Beckman SW 41 rotor, at 40,000 rpm, for 2.5 hours at 4°C. The gradients were analyzed by an ISCO U.S.A. absorbance monitor.

Results

Fungicidal Action of Tricholin

The effect of tricholin on the growth of *R. solani* cells was examined by cell viability testing (Fig. 1). Tricholin exerted a fungicidal mode of action on *R. solani* which was dependent upon concentration. The correlation of cell growth and *in vivo* protein synthesis was determined. *R. solani* cells were allowed to grow for 12 hours at 30°C, then [^{14}C]leucine and different amounts of tricholin were added into the broth medium while growth continued. At several time intervals (indicated in Fig. 2) aliquots were taken to measure cell density and the incorporation of [^{14}C]leucine. The results showed that cessation of incorporation of [^{14}C]leucine into TCA precipitated proteins was parallel to that of cell viability and to

Fig. 1. Concentration dependence of that fungicidal effect of tricholin on *R. solani*. *R. solani* cells were inoculated into PDB medium.



At log phase, various concentrations of tricholin were added to the culture. Growth was continued for 24 hours when samples were taken and diluted serially and then were spread on PDA plates. The survival curve and the minimal fungicidal concentration were determined by quantitation of colony-forming units (CFU). The number of targeted sites per cell were estimates made by the analysis of the intercept of the slope of the survival curve shown on the right hand vertical axis. These data represent the mean ± S.D. of three experiments performed in duplicate.

the growth of cells (estimated from the optical density) (Fig. 2, A, B and C).

Although our preparation of tricholin was electrophoretically and immunologically pure[9], we were aware that the action of tricholin might be affected by contamination with other agents. Hence, a precautionary step was added. The experimental conditions were essentially the same as in Fig. 2C. As the tricholin treated cells became inhibited (8 hours after administering tricholin), a 3 fold excess of anti-tricholin serum was added to the broth medium, and growth was allowed to continue. Reversible inhibition of the antibiotic effect of tricholin was observed (Fig. 3). This clearly ruled out the possibility of contamination by other antimicrobial agents, and indicated that tricholin was the primary factor that caused the inhibition of the cell growth.

Time and concentration effects of tricholin to host cells were examined. The duration of the efficacy of tricholin as an antibiotic agent vs. *R. solani* was *ca.* 42 hours as summarized in Table 1. Tricholin inhibited protein synthesis in *R. solani* at both low and high concentrations. However, at low dosage levels the effect lasted only 3 to 4 hours; the cells then resumed their growth. This was possible because a certain fraction of the cell population could escape antibiotic action and survive. At high concentrations of tricholin protein synthesis in *R. solani* was completely inhibited. It was noted that the rate of uptake of leucine by cells was not affected by the concentration of tricholin. The time lag for the incorporation of leucine into cells was about 2 hours. Incorporation of amino acids is necessary before cells can initiate protein biosynthesis. The antibiotic effect of tricholin lasted approximately 42 hours after its administration to *R. solani* cells in broth medium. A correlation between the cell number and the effective concentration of tricholin was calculated. The estimated value of killing concentration was 0.7 μg per 10^6 cells.

In vivo Action of Tricholin in *R. solani* Cells

Our preliminary binding data indicated that tricholin was able to bind to host cells (time for maximum binding is 50 minutes), and there appeared to be at least one type of specific receptor binding observed from the competition experiment (data not shown). When cells were incubated with [^{125}I]tricholin and washed vigorously before treatment with protease K, [^{125}I]tricholin was found in cell lysates. The autoradiography indicated that the intensities of radioactivity were the same in cell lysates of [^{125}I]tricholin

Fig. 2. The antibiotic effect of tricholin on *R. solani*.

The effect coordinately expressed by (A) the number of viable cells (by CFU). (B) the OD_{650} units (growth); and (C) the incorporation of L-[^{14}C]leucine. The incubation time was counted at the time of adding tricholin after the growth of *R. Solani* cells reached at 0.5 OD_{650} units. The concentrations of tricholin used were 0.04 μg (1 ×) and 0.4 μg (10 ×) per 1 ml of culture. Controls (○), tricholin treated (●).

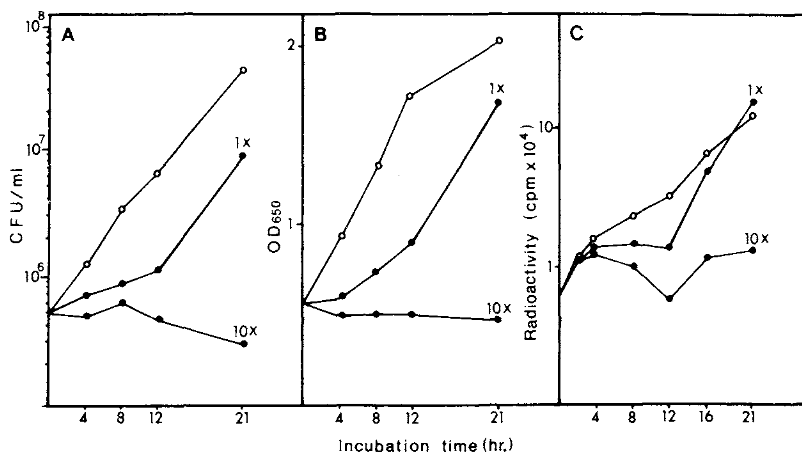
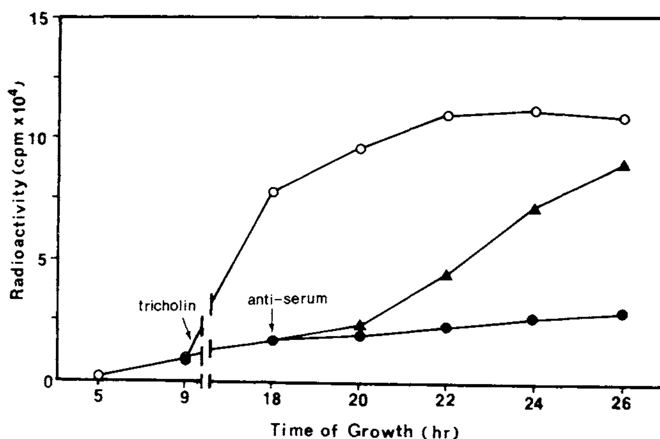


Fig. 3. Reversible inhibition of protein synthesis by tricholin is reversible.

R. solani cells were grown with L-[^{14}C]leucine (120 Ci/mmol) for 9 hours at 30°C (cell density OD_{650}=0.5), then 0.4 μg (per 1 ml of culture) of tricholin were added. When tricholin-treated cultures became effected (9 hours after administering tricholin), then a 3 fold excess of anti-tricholin serum was added to the broth medium (indicated by arrow) and growth was allowed to proceed (▲). Controls were done by incubating cells with (●) and without tricholin (○). Aliquots of duplicate samples cells were taken at the time indicated and the incorporation of [^{14}C]leucine was measured as described in the text.



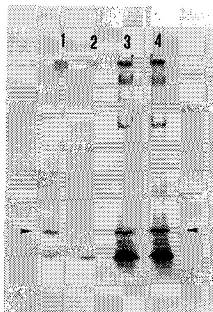
treated cells regardless of protease K treatment (Fig. 4). The results of proteolytic digestion indicated that tricholin did get into the host cells intact. Hence, the *in vivo* action of tricholin on *R. solani* cells was investigated further, and the polysomal profiles of *R. solani* cells were examined. *R. solani* cells were grown for 9 hours before treatment with 0.35 μg (per 1 ml of culture) of tricholin, a dosage that is known to

Table 1. The efficacy of tricholin against *R. solani* cells.

Dosage of Tricholin (μg in 1 ml)	Percentage of inhibition					
	Uptake of leucine			Cell density		
	Time of growth (hours)			Time of growth (hours)		
	18	27	42	18	27	42
Control	0	0	0	0	0	0
0.04	59	46	0	54	54	0
0.2	81	73	0	45	69	0
0.4	82	70	0	81	52	0
0.8	94	86	29	75	76	48

Cells in exponential phases were incubated separately with indicated amounts of tricholin. At time intervals, aliquots were removed, [^{14}C]leucine incorporation and cell growth were measured. The values were the summary of several experiments with duplicate samples.

Fig. 4. Proteolytic digestion of tricholin treated cells.



R. solani cells were grown for 9 hours and then incubated with [^{125}I]tricholin for another 9 hours. The cells were washed vigorously and broken by French press (16,000 psi). The cell lysates were analyzed by polyacrylamide gel electrophoresis containing SDS and autoradiography. Lane 1 shows the intact [^{125}I]-tricholin; lane 2 shows the intact [^{125}I]-tricholin being treated with protease K; lane 3 represents the total cell lysate from [^{125}I]-tricholin treated cells; and lane 4 is the same as lane 3 but with protease K treatment under the same conditions as lane 2. The intact form of tricholin is indicated by arrow.

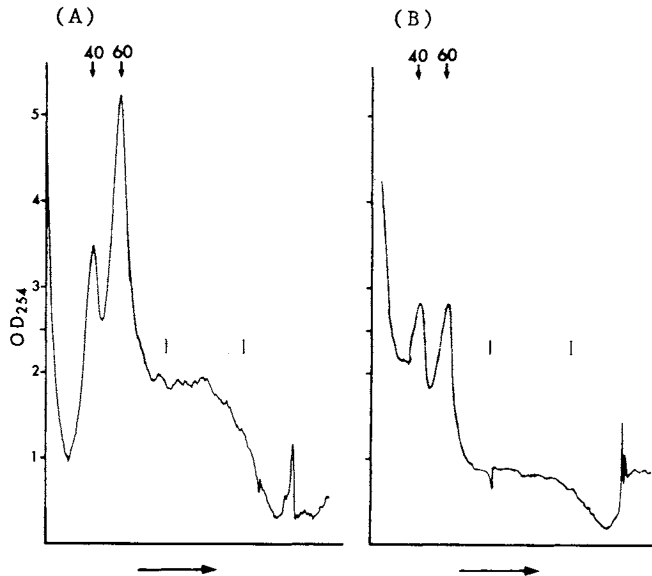
inhibit 50% of cell growth (from data of Fig. 1), then the cells were grown continuously for 9 hours and harvested for polysome analysis. At the same time, the untreated cells were grown for 12 hours and used as the control. The polysome profile of tricholin-treated cells was diminished as compared to control cells when the same OD_{650} unit equivalent of cells was used (Fig. 5). In addition, the ratio of 40S and 60S subunits was drastically changed in the tricholin treated cells while, the peak high of 40S ribosomal subunits between control and treated cells remained the same (Fig. 5). This is a good indication that large ribosome subunits were the target of tricholin *in vivo*. The decrease in large ribosomal subunits in tricholin treated cells would accounts for the action of tricholin on *R. solani*. The remaining 60S subunits in the polysome profile may come from surviving cells, since the measurement of OD_{650} quantitates either live or dead cells. The results concur with our previous *in vitro* data[9)].

Discussion

In this study we showed that tricholin exerted a fungicidal effect against *R. solani* by mechanisms involving inhibition of growth and amino acid incorporation. Other antimicrobial peptides from various microorganisms have been reported. In general they are relatively small in molecular size[11~16)], contain some unusual amino acids, and are, in most cases, formed *via* non-ribosomal mechanism of biosynthesis[14)] or from the post-translational modification[15,16)]. The chemical properties of tricholin have been characterized[9)]; it has a relative large molecular weight (*ca.* 14 kDa) with no traces of modified amino acids. Thus, the fungicidal action of tricholin is of considerable interest in understanding the mechanism of antigonism. In addition, several species of *Trichoderma* produce antifungal metabolites such as alkyl pyrones[17,18)] and Trichotoxin A-50[19)] which are antagonistic to fungi. However, possible contamination

Fig. 5. Analysis of polysomal profiles of cytoplasmic extracts from *R. solani* cells.

R. solani cells were grown and processed as described in the text. Then, the amount of cells from control (0.385 OD_{650} units/ml) (A) and tricholin treated cells (0.412 OD_{650} units/ml) (B) were analyzed on 0.5~1.5 M linear sucrose gradients in a buffer system containing 20 mM Tris-HCl, pH 7.6; 500 mM KCl, 8 mM $MgCl_2$. The migration of 40 S and 60 S ribosomal subunits is indicated. The bar marks equivalent positions of the polysome regions. The scales give relative optical density (monitored at OD_{254}). The direction of gradient is left to right as indicated by arrow.



by these antimicrobial agents in our preparation has been ruled out by the use of anti-tricholin serum (Fig. 3). This result clearly indicated that tricholin was the primary factor that caused the cessation of the growth of *R. solani.* Previously, we have reported that the mechanism of tricholin's action was in inhibition of cell-free protein synthesis through its action on large ribosomal RNA[9]. There is good reason to believe that the *in vivo* action of tricholin vs. *R. solani* might be identical to that of determined *in vitro*. The analysis of the polysomal profile in tricholin treated cells has further proven that the tricholin is, indeed, an effective inhibitor of the translational machinery *in vivo*.

Preliminary receptor analysis and proteolytic digestion data also revealed that tricholin entered *R. solani* cells whereas in an experiment with a human hepatoma cell line, tricholin showed no effect on growth or on binding to hepatoma cells (data not shown). This implyed that the antagonistic action of tricholin to *R. solani* is specific and that the action could be mediated by a specific receptor interaction. Currently, we are investigating this mechanism that involves in possible receptor interaction. We are also studying the antifungal spectrum of tricholin vs. other pathogenic fungi. This information could lead to an understanding of the *in vivo* action of tricholin.

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