APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4364–4370
0099-2240/94/$04.00+0

Vol. 60, No. 12

Parallel Formation and Synergism of Hydrolytic Enzymes and Peptaibol Antibiotics, Molecular Mechanisms Involved in the Antagonistic Action of *Trichoderma harzianum* against Phytopathogenic Fungi

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Received 31 May 1994/Accepted 11 October 1994

Chitinase, β-1,3-glucanase, and protease activities were formed when *Trichoderma harzianum* mycelia, grown on glucose as the sole carbon source, were transferred to fresh medium containing cell walls of *Botrytis cinerea*. Chitobiohydrolase, endochitinase, and β-1,3-glucanase activities were immunologically detected in culture supernatants by Western blotting (immunoblotting), and the first two were quantified by enzyme-linked immunosorbent assay. Under the same conditions, exogenously added [U-^{14}C]valine was incorporated in acetone-soluble compounds with an apparent M_r of <2,000. These compounds comigrated with the peptaibols trichorzianines A_1 and B_1 in thin-layer chromatography and released [U-^{14}C]valine after incubation in 6 N HCl. Incorporation of radioactive valine into this material was stimulated by the exogenous supply of α-aminoisobutyric acid, a rare amino acid which is a major constituent of peptaibols. The obtained culture supernatants inhibited spore germination as well as hyphal elongation of *B. cinerea*. Culture supernatants from mycelia placed in fresh medium without cell walls of *B. cinerea* did not show hydrolase activities, incorporation of [U-^{14}C]valine into peptaibol-like compounds, and inhibition of fungal growth. Purified trichorzianines A_1 and B_1 as well as purified chitobiohydrolase, endochitinase, or β-1,3-glucanase inhibited spore germination and hyphal elongation, but at concentrations higher than those observed in the culture supernatants. However, when the enzymes and the peptaibols were tested together, an antifungal synergistic interaction was observed and the 50% effective dose values obtained were in the range of those determined in the culture supernatants. Therefore, the parallel formation and synergism of hydrolytic enzymes and antibiotics may have an important role in the antagonistic action of *T. harzianum* against fungal phytopathogens.

Trichoderma spp. have received major attention as agents for the biological control of phytopathogenic fungi and are excellent candidates for successful exploitation (5, 36). However, the molecular basis of biocontrol is not clearly understood. Proposed mechanisms of antagonism resulting in biocontrol are antibiosis (6, 8, 9, 14, 35), mycoparasitism (1, 4, 5, 40), and competition (41). Evidence for a fungicidal action of selected antibiotics or hydrolytic enzymes of *Trichoderma* spp. under in vitro conditions is available (13, 25, 28, 29). In most cases the concentrations required are far beyond those occurring in vivo, although combinations of enzymes with different modes of action substantially improved the inhibitory effect (14). Interestingly, the in vitro 50% effective dose for chitinases was considerably reduced by the simultaneous addition of selected fungicides (30). Similarly, a synergism between *Trichoderma* enzymes and antibiotics could occur in vivo.

In the present study, we investigated this possibility, using the peptaibols (trichorzianines) (Fig. 1) of *Trichoderma harzianum* as model antibiotics. Trichorzianines and a number of closely related peptaibols are produced by several *Trichoderma* spp. (3, 12, 15). These antibiotics form voltage-gated ion channels in black lipid membranes (34) and modify the membrane permeability of liposomes in the absence of applied voltage (13, 26). They induce leakage of compounds from *Rhizoctonia solani* and lysis of *Phytophthora cactorum* (13, 25). However, there is no evidence that peptaibols are produced by *T. harzianum* during its antagonistic action or under conditions inducing formation of cell wall lytic enzymes.

In this paper we show that (i) the synthesis of both hydrolytic enzymes and peptaibols is triggered by cell walls of *Botrytis cinerea* and (ii) peptaibols act synergistically with chitinases and a β-1,3-glucanase in the inhibition of fungal spore germination and hyphal elongation.

MATERIALS AND METHODS

Fungal strains and culture conditions. *T. harzianum* Rifai (ATCC 36042), which is a potent mycoparasite, was used

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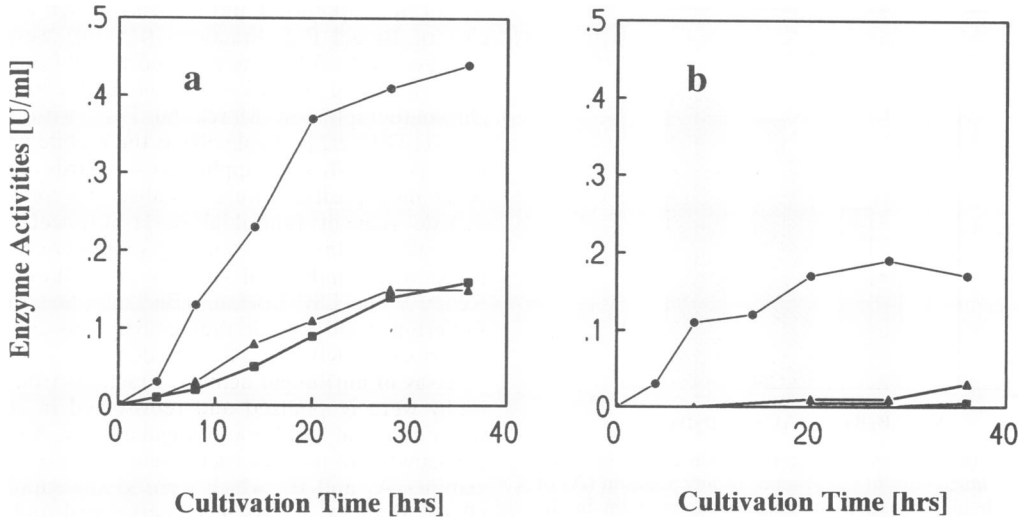
TA_1 : Ac Aib Ala Ala Aib Aib Gln Aib Aib Aib Ser Leu Aib Pro Val Aib Ile Gln **Gln** Trpol

TB_1 : Ac Aib Ala Ala Aib Aib Gln Aib Aib Aib Ser Leu Aib Pro Val Aib Ile Gln **Glu** Trpol

FIG. 1. Amino acid structure of peptaibol antibiotics trichorzianines A_1 (TA_1) and B_1 (TB_1). Ac, *N*-acetyl; Trpol, —CH_2OH; Boldface indicates those amino acids which differ in TA_1 and TB_1.



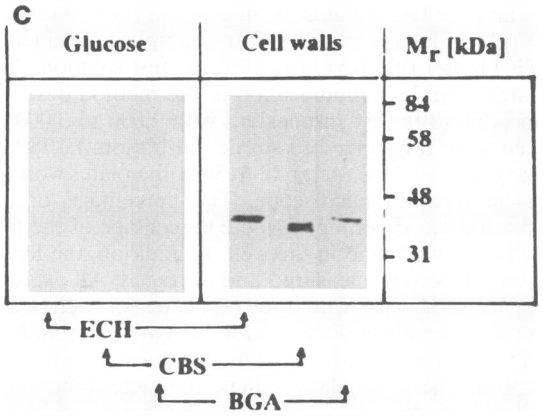


FIG. 2. Formation of cell wall hydrolytic enzymes by *T. harzianum* on transfer to replacement medium with (a) or without (b) *B. cinerea* cell walls. Circles, protease activity; triangles, β-1,3-glucanase activity; squares, chitinase activity. Values shown are from a single experiment only, while similar differences between the results shown in panels a and b were obtained in three separate experiments. (c) SDS-PAGE and immunostaining of extracellular hydrolases. CBS, chitobiosidase; ECH, endochitinase; BGA, β-1,3-glucanase. Culture filtrates from mycelia incubated for 37 h were used for these experiments.

throughout the present study. The organism was maintained on malt agar slants and subcultured bimonthly.

For enzyme and peptaibol production, *T. harzianum* was grown for 20 h on a rotary shaker (250 rpm, 28°C) in 1-liter flasks containing Mandels-Andreotti medium (32), with glycerol (0.5% [vol/vol]) as the carbon source. The fungal biomass was removed by filtration through a sintered (G1 porosity) funnel without suction (to avoid hyphal breakage) but with continuous aeration (by stirring with a sterile spatula). The biomass was transferred to fresh Mandels-Andreotti medium (32), lacking carbon and nitrogen sources, and supplemented with 0.5 g of *B. cinerea* cell walls per liter, 2 g of $NaNO_3$ per liter, and 0.14 g of glutamine per liter, to give a mycelial density of 1 ± 0.3 g (wet weight)/liter. Incubation was continued for 36 h, and 10-ml samples were taken at appropriate times. The samples were centrifuged (15 min, 4°C, 12,000 × *g*) and stored at −20°C until use for analysis.

To monitor peptaibol formation, the contents from a 1-liter flask of replacement culture (time zero) were distributed in 10-ml aliquots into 100-ml Erlenmeyer flasks and further incubated on a shaking water bath (100 rpm, 28°C). After 30 min, they were pulsed with a total of 10 μCi of [U-^{14}C]valine (specific radioactivity, 9.25 GBq/mmol; Amersham, United Kingdom), and incubation was continued for 24 h. Duplicate flasks were harvested at 0, 20, 28, and 36 h (calculated from the time of the pulse labelling), and the supernatant was analyzed for peptaibol formation.

For enzyme production, *T. harzianum* was grown for 4 days on a rotary shaker in Richard's modified medium, which contained 10 g of KNO_3, 5 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 2 mg of $FeCl_3$, 1% (wt/vol) crab shell chitin (Sigma), 1% (wt/vol) polyvinylpyrrolidone (Polyclar AT; GAF Corp., Wayne, N.J.), 150 ml of V8 juice, and 1 liter of H_2O at pH 6.0 (19). Thereafter, the biomass was removed by filtration and used for enzyme purification.

To isolate *B. cinerea* cell walls, the fungus was grown for 72 h in 1-liter flasks containing 200 ml of medium (20 g of glucose per liter, 10 g of ammonium sulfate per liter, 10 g of KH_2PO_4 per liter, 1 g of NaCl per liter, 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter [pH 6.5]). The mycelial mass was harvested by filtration, dried between filter paper, and stored at −20°C until use for cell wall preparations.

For bioassays, *B. cinerea* 26, isolated from grapes (provided by R. Pearson, Cornell University), and *Fusarium oxysporum* f. sp. *phaseoli* isolated from beans (Naples, Italy) were used. These fungi were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.). Spores were suspended in 5 mM Tris-HCl buffer (pH 6.5) and filtered through sterile filter paper (if necessary to remove mycelial fragments), and their concentration was adjusted to 10^5 to 10^6 propagules per ml.

***B. cinerea* cell wall preparation.** To prepare cell walls of *B. cinerea*, the mycelia were homogenized in 10 volumes of 0.1% (wt/vol) sodium dodecyl sulfate (SDS) in a Potter Elvehjem Pistill homogenizer. Purification of cell walls was carried out as reported previously (32a). The purified cell walls were ground to a fine powder under liquid nitrogen and stored at −20°C until use.

Enzyme assay and production. Endochitinase, the 40-kDa chitin-1,4-β-chitobiosidase (chitobiosidase), and glucan-β-1,3-glucosidase (β-1,3-glucanase) were purified to electrophoretic homogeneity as previously described (19, 29). Enzyme solutions were kept at 4°C and were utilized for bioassays within 2

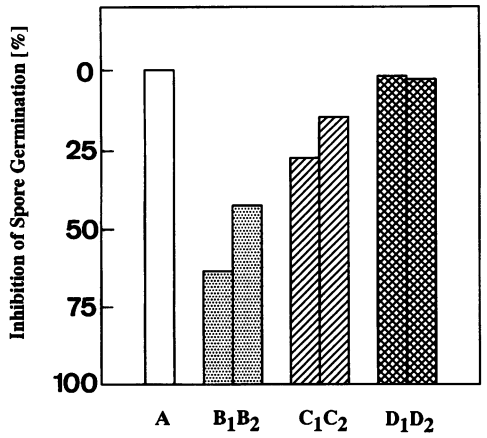


FIG. 3. Inhibition of *B. cinerea* spore germination caused by crude culture filtrate of *T. harzianum* in the absence of any addition (A) or harvested after 37 h of incubation in replacement medium in the presence (B) or absence (D) of *B. cinerea* cell walls or after ultrafiltration through an Amicon YM 10 membrane (cutoff, 10 kDa) (C). Subscripts 1 and 2 refer to 25 and 50% (vol/vol), respectively, of the crude culture filtrate in the spore germination assays.

weeks. Otherwise they were concentrated until dry in a Speed-Vac apparatus (Savant Instruments, Fermingdale, N.Y.) and stored at −20°C until use.

Endochitinase activity was measured by determining the release of *N*-acetyl-β-D-glucosamine under the conditions described by Elad et al. (11), by the Elson-Morgan method (37). β-1,3-Glucanase was determined by measuring the release of reducing groups from a 0.1% (wt/vol) solution of laminarin in 50 mM phosphate buffer (pH 6.7) (Sigma) (33). Protease activity was measured by the azocasein hydrolysis method (17). The endochitinase and β-1,3-glucanase activities are expressed in units (micromoles of product formed per minute) under the conditions used. For protease, 1 U is defined as the increase in A_{365} per minute. Protein contents were determined according to the method of Bradford (2).

Electrophoretic and immunological methods. Samples from the culture filtrate of *T. harzianum* were mixed with a double volume of ethanol and left at −20°C for 3 h. The precipitated protein was harvested by centrifugation in an Eppendorf centrifuge (10 min) and resuspended in SDS-sample buffer (24) to give a final protein concentration of about 0.1 mg/ml. For electrophoresis, the samples were heated to 100°C for 5 min, and occurring precipitates were removed by centrifugation. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (24), and the separated proteins were blotted onto nitrocellulose (4). Enzyme bands were visualized by immunostaining (18), with polyclonal antibodies raised in rabbits against endochitinase, chitobiosidase, and glucan-β-1,3-glucosidase (19, 29).

Quantification of chitobiosidase and endochitinase by ELISA. Enzyme-linked immunosorbent assays (ELISA) were carried out as described previously (22a), with polyclonal antibodies raised in rabbits in a final dilution of 1:1,000 and anti-rabbit immunoglobulin G coupled to alkaline phosphatase as a second antibody. Purified enzymes (19, 29) were used to calibrate the assay.

Determination of peptaibol formation. Samples (10 ml) from the culture filtrate were lyophilized and extracted three times each with 1 ml of acetone. To analyze the peptaibols by gel chromatography, the acetone extract was concentrated to 0.5 ml by evaporation and passed through a column (10 by 1 cm) of Biogel P-2. Fractions (0.5 ml) were collected and analyzed in the following way. Individual fractions were lyophilized, taken up in 20 μl of acetone, and subjected to thin-layer chromatography on Merck 60 F_{254}, with *n*-butanol–acetic acid–H_2O (6/2/2 [vol/vol/vol]) as the mobile phase. Trichorzianines A_1 and B_1 were applied as standards on separate tracks. After their positions were visualized by spraying with anisaldehyde reagent (anisaldehyde-H_2SO_4-acetic acid [1/0.5/20] [vol/vol/vol]), the corresponding spots were scraped out of the tracks of the individual samples. After they were mixed with Ready Value LSC Cocktail (Beckman Scientific Instruments, Fullerton, Calif.), their radioactivity was counted in an LSC (Pharmacia-LKB, Uppsala, Sweden).

Assay of antifungal activity. Fractions from gel chromatography were lyophilized and redissolved in 50 μl of distilled water and analyzed for antifungal activity. Analyses of antifungal activities of purified trichorzianines were done with trichorzianines A_1 and B_1, which were kindly supplied by B. Bodo (Paris, France). Assays were performed under sterile conditions. Equal volumes of spore suspension, 3× potato dextrose broth, and the test solution or suspension (in 5 mM Tris-HCl buffer [pH 6.5]) were mixed. Control samples contained 5 mM Tris-HCl buffer (pH 6.5) instead of the test solution. The test was carried out by a modification of the method described by Lorito et al. (30): test suspensions with 1,000 to 3,000 spores per well were transferred to sterile flat-bottom ELISA plates and incubated at 25°C. After 22 to 30 h, the plates were placed under an inverted microscope. The percentage of conidia germinating was determined as the percentage of the first 100 spores randomly found in the well. In addition, the lengths of 20 germ tubes were measured and averaged. All experiments were performed twice, with three replicates for each treatment. The inhibition values obtained for the two experiments were combined and averaged, and standard deviations were calculated from these six data values. To determine the 50% effective dose, the dose-response curves were subjected to regression analysis by using a binomial regression of the third order, with R^2 ranging between 0.95 and 0.99. According to Richer (39), the following formula was used to determine an antifungal synergistic effect between lytic enzymes and peptaibols: if synergism exists, $E_o(xA + yB) > E_o(x + y)A$ and $E_o(xA + yB) > E_o(x + y)B$, where E_o is the percentage of inhibition, A and B are enzymes and peptaibols, respectively, and x and y are the concentrations of each component in the mixture. The E_o values were calculated by regression analysis of the dose-response curves.

RESULTS

Formation of hydrolytic enzymes by *T. harzianum* incubation with *B. cinerea* cell walls. In order to demonstrate the biochemical changes occurring on contact of *T. harzianum* with the surface of a plant pathogen, we have used a mycelium replacement system. Briefly, this consists of pregrowing *T. harzianum* in a medium allowing rapid growth but not leading to expression of antagonistic activity and then transferring the mycelia to fresh media containing cell walls of the phytopathogenic host. This technique reduces incubation times and avoids interferences caused by different growth rates. A similar approach has been successfully used to study enzyme productions in *Trichoderma reesei* (42) or penicillin formation by *P. chrysogenum* (20). When pregrown mycelia were transferred to new medium containing *B. cinerea* cell walls as the only carbon source, they started to secrete chitinases, β-1,3-glucanases, and proteases with no apparent delay (Fig. 2a). Enzyme activities

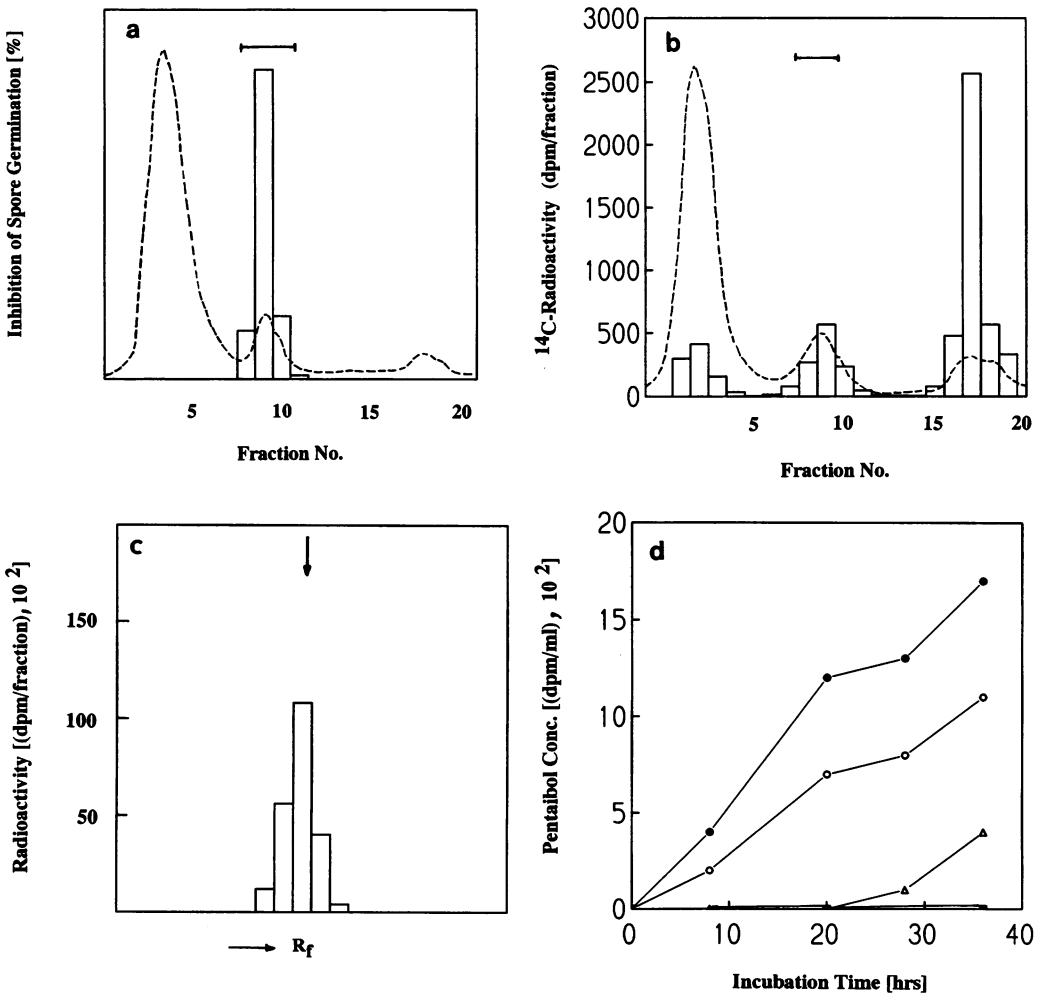


FIG. 4. (a and b) Gel chromatography on Biogel P-2 of acetone extract from extracellular culture supernatant of *T. harzianum* incubated for 37 h in replacement medium in the presence of *B. cinerea* cell walls. The elution profile for A_{280} is indicated by the dashed line. (a) Activity of individual fractions inhibiting *B. cinerea* spore germination; (b) radioactivity from [U-^{14}C]valine in individual fractions. The elution profile of trichorzianines A_1 and B_1, as determined in a separate experiment, is indicated by a horizontal bar. (c) Separation of the second peak from gel permeation chromatography (see panel b) by thin-layer chromatography. The arrow indicates the position of trichorzianines A_1 and B_1, as determined in a separate experiment. (d) Incorporation of radioactivity from [U-^{14}C]valine into putative peptaibols during incubation of *T. harzianum* in replacement medium in the presence (closed symbols) or absence (open symbols) of α-aminoisobutyrate (10 mM). ● and ○, replacement to cell wall-containing medium; △, replacement to glucose-containing medium; ■, with cycloheximide (50 μg/ml). Values shown are from a single experiment, but similar differences between the four curves were obtained in four separate experiments.

increased to about 30 to 35 h of incubation and then decreased slowly (data not shown). With the exception of protease, no other enzyme activities were found when mycelia were incubated in the same medium containing glucose instead of cell walls (Fig. 2b). Since these enzyme activities are the result of a number of isoenzymes, we used antibodies against endochitinase, chitobiosidase, and β-1,3-glucanase to demonstrate the presence of specific, well-characterized (19, 29) components. All three enzymes were present in the supernatants from the cell wall-containing cultures but absent from the culture medium containing glucose (Fig. 2c). By using the same antibodies in an ELISA, the concentrations of chitobiosidase and endochitinase were determined as 5.9 and 2.7 mg/liter in filtrates from cell wall-containing cultures after 37 h. In order to demonstrate a de novo protein synthesis, experiments were also carried out with cycloheximide, an inhibitor of translation (43). In its presence, no enzyme activities were found, and no enzymes were detected with the aid of immunological techniques (data not shown).

When the extracellular supernatant from a cell wall-induced culture (harvested after 36 h of incubation) was added to spores of *B. cinerea*, it inhibited their germination (Fig. 3). However, the fungistatic activity was partially maintained when extracellular enzymes were removed by ultrafiltration (Amicon YM 10; cutoff size, 10 kDa; see Fig. 5), suggesting that other antifungal compounds were present in the culture filtrate. No inhibitory activity was present in culture filtrates obtained in the presence of glucose or in filtrates obtained after incubating the cell wall-containing medium without *T. harzianum* for 37 h.

Peptaibol formation in *T. harzianum* on incubation with *B. cinerea* cell walls. The results described above suggest that one or more antibiotics were formed by *T. harzianum* during incubation with *B. cinerea* cell walls. The culture supernatant was lyophilized and subjected to gel chromatography, and the

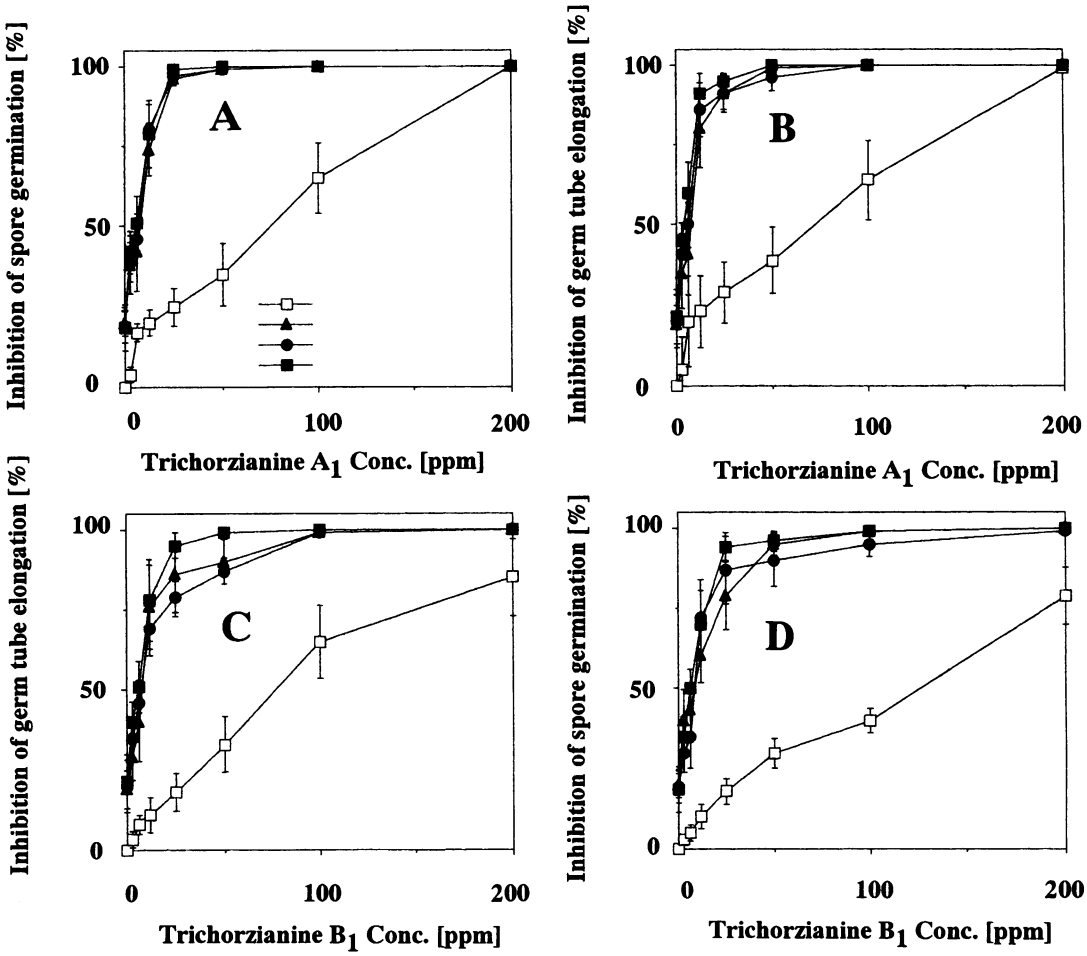


FIG. 5. Effect of addition of endochitinase (▲, 25 μg/ml), chitobiosidase (●, 50 μg/ml), and β-1,3-glucanase (■, 25 μg/ml) on inhibition of *B. cinerea* spore germination (A and C) and hyphal elongation (B and D) by trichorzianine A_1 (A and B) and trichorzianine B_1 (C and D). □, control without added enzyme. Vertical bars indicate standard deviations (n = 6).

individual fractions were assayed for their ability to inhibit *B. cinerea* spore germination. This inhibiting activity corresponded to an M_r of about 2,000 ± 500 (Fig. 4a), suggesting that peptide antibiotics or peptaibols (15) may be the components responsible for this fungicidal effect. The absence of antifungal activity in the high-molecular-weight (i.e., enzyme) fraction is not in contradiction to the findings shown in Fig. 3, since chitinases and β-1,3-glucanases were inactivated by the acetone treatment. To determine if the 2-kDa material had a peptaibol structure, we added [U-^{14}C]valine, which is an amino acid present in the *T. harzianum* peptaibol trichorzianine, to the medium (12). After incubation for 8 h, the obtained supernatant was lyophilized and subjected to gel chromatography. The radioactive label appeared in three peaks with different M_rs (Fig. 4b), which conceivably represent extracellular protein, peptaibols, and free valine. This putative peptaibol peak eluted from Biogel P-2 at the same position as trichorzianines A_1 and B_1 (Fig. 4b) and migrates to the position of trichorzianines A_1 and B_1 in thin-layer chromatography (Fig. 4c). Upon hydrolysis in 6 N HCl, the [^{14}C]valine label appeared in the valine peak on Biogel P-2 only (data not shown). All these data clearly indicate that the 2-kDa chromatography peak contains peptaibols. Furthermore, the incorporation of radioactivity into this peak was stimulated by the addition of α-L-aminoisobutyric acid (AIB) to the medium (Fig. 4d). As with the induction of hydrolases, no formation was observed in the presence of glucose, and cycloheximide also prevented the formation (Fig. 4d).

With trichorzianine A_1 as a standard, the A_{280} peak from gel filtration allowed the calculation that the original culture filtrate had contained 25 (±10) mg of peptaibols per liter.

Synergistic effect of hydrolases and peptaibols on fungal growth. The parallel formation of hydrolytic enzyme and antibiotics in *T. harzianum* in the presence of cell walls of a potential host suggests that these two classes of compounds may cooperate in mycoparasitism. Therefore, the effect of different concentrations of trichorzianines A_1 and B_1 on spore germination and hyphal elongation of *B. cinerea* and *F. oxysporum* was investigated in the presence of purified endochitinase, chitobiosidase, and β-1,3-glucanase from *T. harzianum*. As a prerequisite, the 50% effective doses for trichorzianines A_1 and B_1 with respect to spore germination and germ tube elongation of *B. cinerea* and *F. oxysporum* were determined. The values obtained were in the range of 70 to 100 μg/ml, with spore germination being less sensitive than germ tube elongation (Fig. 5). The same range of peptaibol concentration has previously been reported to inhibit growth of other fungi (13, 25). When one of the three enzymes was added, the concen-

tration of trichorzianines needed for complete inhibition was strongly reduced. About 200 μg of trichorzianine A_1 per ml was needed to completely inhibit conidial germination in the absence of enzymes, whereas the presence of 25 μg of endochitinase per ml reduced this concentration to 30 μg/ml. The same concentration of endochitinase resulted in only 35% inhibition (28). When the data were evaluated by the method of Richer (39), evidence of a synergistic action was obtained.

Essentially consistent data were obtained when *F. oxysporum* was used as a test organism.

In order to investigate, whether the concentrations of enzymes and peptaibols formed under the present experimental conditions would be sufficient to explain the observed inhibitory effect, a further experiment was carried out in which endochitinase, chitobiosidase, and trichorzianine A_1 were added at final concentrations of 6, 3, and 25 mg/ml, respectively, to the spore germination assay. Under these conditions, spore germination was inhibited by 41% (±17%). Although this value is somewhat lower than the inhibition obtained by the crude culture filtrate (57%) (see Fig. 3), we conclude that synergism of hydrolytic enzymes and peptaibols contribute significantly to the antagonism of *T. harzianum* against phytopathogens.

DISCUSSION

There have been several attempts to explain the antagonistic action of *T. harzianum* against phytopathogenic fungi either by the secretion of cell wall hydrolytic enzymes or by the production of antibiotics. Our results indicate that in fact both types of compounds are involved in this process and that antibiotics (at least some) and hydrolytic enzymes cooperate synergistically in antagonism. Di Pietro et al. (10) reported on a similar type of synergism between an endochitinase and a diketopiperazine antibiotic (gliotoxin) from the biocontrol fungus *Gliocladium* (*Trichoderma*) *virens*, closely related to *T. harzianum*. The investigators interpreted the synergism as a result of a weakening of the cell wall of *B. cinerea*, which improved the rate of diffusion of the antibiotic towards the cell surface. It is intriguing that both gliotoxin and the peptaibols affect the cytoplasmic membrane (13, 22), hence pointing to the cell membrane as an important target, as also supported by other studies (27). Similarly, many plant pathogenic fungi attack plant cells via a modification of their cell membrane (7).

Although the antibiotic compounds, whose formation was triggered by *B. cinerea* cell walls, have not been fully identified in the course of this study, we assume that they belong to the peptaibol family for the following reasons: (i) they exhibit similar M_rs, (ii) they migrate to the same position in thin-layer chromatography, (iii) exogenously added [U-^{14}C]valine becomes incorporated into them and is released under conditions of hydrolysis of peptide bonds, and (iv) their formation is stimulated by the exogenous addition of AIB. AIB is a nonprotein amino acid, a major component of all peptaibols, and its exogenous addition has been shown to increase the formation of these antibiotics (3, 38). However, we have not proven that these compounds are identical to trichorzianine A_1 or B_1, but it is possible that the formation of different peptaibols is strain specific.

The question as to which antibiotics are involved in antagonism has not been conclusively answered until now. The present results show that the concentrations of trichorzianines found can account for a major portion of the effect. However, it is well possible that other antibiotics may also be formed under these conditions and thereby further reduce the concentration needed by individual components. The fact that an isolate of *T. harzianum* can produce various different antibiotics (3, 9, 38) could explain why strains defective in the production of only one of them, e.g., 6-*n*-pentyl pyrone, are not impaired in biocontrol (16). It would be intriguing to know whether there is also synergism between different secondary metabolites produced by *Trichoderma* spp. This will explain why the effective dose of inhibitory compounds is particularly low in vivo (5, 15). This topic should be considered for future studies as well as for strain improvement and strain application.

The fact that the presence of cell walls of *B. cinerea* triggers the production of both cell wall hydrolytic enzymes and peptaibols suggests that the cascade of antagonistic events may be regulated by a common mechanism. We do not know whether this is caused by the release of cell wall oligosaccharides or carbon catabolite derepression, by a physical elicitation, or by a combination of all these factors. The first hypothesis is supported by the evidence that in *T. reesei* the degradation of cellulose and xylan is triggered by degradation products (23, 31). However, the initial coiling of *T. harzianum* around its victim is triggered solely by recognition of a lectin on the cell surface of the pathogen (21). The experimental approach used in this study may be an additional tool to investigate this process.

ACKNOWLEDGMENTS

This study was supported by the Austrian Federal Ministery of Agriculture and Forestry (C.P.K.), by the Istituendo Centro CNR di Studio delle Tecniche di Lotta Biologica, 80055 Portici (Napoli), Italy (M.L.), and in part by BARD grants US-1723-89 and CRG 931173 from the NATO Collaborative Research Grants Programme, which are gratefully acknowledged, to G.E.H.

We are also very grateful to B. Bodo (Paris) for the gift of trichorzianines A_1 and B_1.

REFERENCES

1. **Ayers, W. A., and P. B. Adams.** 1981. Mycoparasitism and its application to biological control of plant diseases, p. 91–103. *In* G. C. Papavizas (ed.), Biological control in crop production. Allanheld, Osmun, Totowa, N.J.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Brewer, D., F. G. Mason, and A. Taylor.** 1987. The production of alamethicins by *Trichoderma* sp. Can. J. Microbiol. **33:**619–625.
4. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
5. **Chet, I.** 1987. Trichoderma—application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi, p. 147–160. *In* I. Chet (ed.), Innovative approaches to plant disease control. John Wiley & Sons, New York.
6. **Claydon, N., M. Allan, J. R. Hanson, and A. G. Avent.** 1987. Antifungal alkyl pyrones of *Trichoderma harzianum*. Trans. Br. Mycol. Soc. **88:**503–513.
7. **Daly, J. M., and B. J. Deverall.** 1983. Toxins in plant pathogenesis. Academic Press, New York.
8. **Dennis, C., and J. Webster.** 1971. Antagonistic properties of species groups of *Trichoderma*. I. Production of non-volatile antibiotics. Trans. Br. Mycol. Soc. **57:**25–39.
9. **Dennis, C., and J. Webster.** 1971. Antagonistic properties of species groups of *Trichoderma*. II. Production of volatile antibiotics. Trans. Br. Mycol. Soc. **57:**41–48.
10. **Di Pietro, A., M. Lorito, C. K. Hayes, R. M. Broadway, and G. E. Harman.** 1993. Endochitinase from *Gliocladium virens*: isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. Phytopathology **83:**308–313.
11. **Elad, Y., I. Chet, and Y. Henis.** 1982. Degradation of plant

pathogenic fungi by *Trichoderma harzianum*. Can. J. Microbiol. **28:** 719–725.

12. **El Hajji, M., S. Rebuffat, D. Lecommandeur, and B. Bodo.** 1987. Isolation and determination trichorzianines A antifungal peptides from *Trichoderma harzianum*. Int. J. Peptide Protein Res. **29:**207–215.
13. **El Hajji, M., S. Rebuffat, T. Le Doan, G. Klein, M. Satre, and B. Bodo.** 1989. Interaction of trichorzianines A and B with model membranes and with amoeba *Dictyostelium*. Biochim. Biophys. Acta **978:**97–104.
14. **Ghisalberti, E. L., M. J. Narbey, M. M. Dewan, and K. Siva sinthamparam.** 1990. Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. Plant Soil **121:**287–291.
15. **Ghisalberti, E. L., and K. Sivasinthamparam.** 1991. Antifungal antibiotics produced by *Trichoderma* spp. Soil Biol. Biochem. **23:** 1011–1020.
16. **Graeme-Cook, K. A., and J. L. Faull.** 1991. Effect of ultraviolet-induced mutants of *Trichoderma harzianum* with altered production on selected pathogens *in vivo*. Can. J. Microbiol. **37:**659–664.
17. **Haab, D., K. Hagspiel, K. Szakmary, and C. P. Kubicek.** 1990. Formation of the extracellular proteases from *Trichoderma reesei* QM 9414 involved in cellulase degradation. J. Biotechnol. **16:**187–198.
18. **Hagspiel, K., D. Haab, and C. P. Kubicek.** 1989. Protease activity and proteolytic modification of cellulases from a *Trichoderma reesei* QM 9414 selectant. Appl. Microbiol. Biotechnol. **32:**61–67.
19. **Harman, G. E., C. K. Hayes, M. Lorito, R. M. Broadway, A. Di Pietro, C. Peterbauer, and A. Tronsmo.** 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology **83:**313–318.
20. **Hönlinger, C., and C. P. Kubicek.** 1989. Regulation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and isopenicillin N biosynthesis in *Penicillium chrysogenum* by the α-aminoadipate pool size. FEMS Microbiol. Lett. **65:**71–76.
21. **Inbar, J., and I. Chet.** 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. J. Bacteriol. **174:**1055–1059.
22. **Jones, R. W., and J. G. Hancock.** 1988. Mechanism of gliotoxin action and factors mediating gliotoxin sensitivity. J. Gen. Microbiol. **134:**2067–2075.

22a. **Kolbe, J., and C. P. Kubicek.** 1990. Qualification and quantification of the main components of the *Trichoderma reesei* cellulase complex by an enzyme-linked immunosorbent assay (ELISA). Appl. Microbiol. Biotechnol. **34:**26–30.

23. **Kubicek, C. P.** 1993. From cellulose to cellulase inducers: facts and fiction, p. 181–188. *In* P. Suominen and T. Reinikainen (ed.), *Trichoderma reesei* cellulases and other hydrolases: enzyme structures, biochemistry, genetics and applications. Foundation of Biotechnical and Industrial Fermentation Research, Helsinki, Finland.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:** 680–685.
25. **Lederer, W., H.-K. Lorenz, and E. Seemüller.** 1992. Studies on antagonistic effects of *Trichoderma* isolates against *Phytophthora cactorum*. J. Phytopathol. **136:**154–164.
26. **Le Doan, T., M. El-Hajji, S. Rebuffat, M. R. Rajeswari, and B. Bodo.** 1986. Fluorescein studies of the interaction of trichorzianine A III_c with model membranes. Biochim. Biophys. Acta **858:**1–5.
27. **Lewis, J. A., and G. C. Papavizas.** 1987. Permeability changes in hyphae of *Rhizoctonia solani* induced by germling preparations of *Trichoderma* and *Gliocladium*. Phytopathology **77:**699–703.
28. **Lorito, M., G. E. Harman, C. K. Hayes, R. M. Broadway, A. Tronsmo, S. L. Woo, and A. Di Pietro.** 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathology **83:**302–307.
29. **Lorito, M., C. K. Hayes, A. Di Pietro, S. L. Woo, and G. E. Harman.** 1994. Purification, characterization, and synergistic activity of a glucan-β-1,3-glucosidase and a *N*-acetyl-β-glucosaminidase from *Trichoderma harzianum*. Phytopathology **84:**398–405.
30. **Lorito, M., C. K. Peterbauer, C. K. Hayes, and G. E. Harman.** 1994. Synergistic interaction between fungal cell-wall degrading enzymes and different antifungal compounds on spore germination. Microbiology **140:**623–629.
31. **Mach, R. L., A. Butterweck, M. Schindler, R. Messner, P. Herzog, and C. P. Kubicek.** 1993. Molecular regulation of formation of xylanase (XYN) I and II by *Trichoderma reesei*, p. 211–216. *In* P. Suominen and T. Reinikainen (ed.), *Trichoderma reesei* cellulases and other hydrolases: enzyme structures, biochemistry, genetics and applications. Foundation of Biotechnical and Industrial Fermentation Research, Helsinki, Finland.
32. **Mandels, M., and R. E. Andreotti.** 1978. The cellulose to cellulase fermentation. Proc. Biochem. **13:**6–13.

32a. **Messner, R., and C. P. Kubicek.** 1990. Synthesis of cell wall glucan, chitin, and protein by regenerating protoplasts and mycelia of *Trichoderma reesei*. Can. J. Microbiol. **36:**211–217.

33. **Miller, G. L.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31:**426–428.
34. **Molle, G., H. Duclohier, and G. Spach.** 1987. Voltage dependent and multistate ionic channels induced by trichorzianines, antifungal peptides related to alamethicin. FEBS Lett. **224:**208–212.
35. **Okuda, T., A. Fujiwara, and M. Fujiwara.** 1982. Correlation between species of *Trichoderma* and production patterns of isonitril antibiotics. Agric. Biol. Chem. **46:**1811–1822.
36. **Papavizas, G. C., and R. D. Lumsden.** 1980. Biological control of soilborne fungal propagules. Annu. Rev. Phytopathol. **18:**389–413.
37. **Reissig, J. L., J. L. Strominger, and L. F. Leloir.** 1955. A modified colorimetric method for the determination of *N*-acetylamino sugars. J. Biol. Chem. **217:**959–966.
38. **Reusser, F.** 1967. Biosynthesis of antibiotic U-22,324, a cyclic polypeptide. J. Biol. Chem. **242:**243–247.
39. **Richer, D. L.** 1987. Synergism: a patent view. Pestic. Sci. **19:**309–315.
40. **Singh, J., and J. L. Faull.** 1990. Hyperparasitism and biological control, p. 167–179. In K. G. Mukerji and K. L. Garg (ed.), Biocontrol of plant pathogens. CRC Press, Boca Raton, Fla.
41. **Sivan, A., and I. Chet.** 1989. The possible role of competition between *Trichoderma harzianum* and *Fusarium oxysporum* on rhizosphere colonization. Phytopathology **79:**198–203.
42. **Sternberg, D., and G. R. Mandels.** 1979. Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose. J. Bacteriol. **139:**761–769.
43. **Woodruff, H. B., and I. M. Miller.** 1963. Antibiotics, p. 23–51. *In* R. M. Hochster and J. H. Quastel (ed.), Metabolic inhibitors: a comprehensive treatise. Academic Press, New York.