Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination

Matteo Lorito,† Clemens Peterbauer,‡ Christopher K. Hayes and Gary E. Harman

Author for correspondence: Gary E. Harman. Tel: +1 315 787 2246. Fax: +1 315 787 2320. e-mail (internet): Gary_Harman@Cornell.Edu

Different classes of cell wall degrading enzymes produced by the biocontrol fungi Trichoderma harzianum and Gliocladium virens inhibited spore germination of Botrytis cinerea in a bioassay in vitro. The addition of any chitinolytic or glucanolytic enzyme to the reaction mixture synergistically enhanced the antifungal properties of five different fungitoxic compounds against B. cinerea. The chemicals tested were gliotoxin, flusilazole, miconazole, captan and benomyl. Dose response curves were determined for each combination of toxin and enzyme, and in all cases the ED₅₀ values of the mixtures were substantially lower than ED₅₀ values of the two compounds used alone. For instance, the addition of endochitinase from T. harzianum at a concentration of 10 µg ml⁻¹ reduced the ED₅₀ values of toxins up to 86-fold. The level of synergism appeared to be higher when enzymes were combined with toxins having primary sites of action associated with membrane structure, compared with pesticides having multiple or cytoplasmic sites of action. Among enzymes tested, the highest levels of synergism with synthetic fungicides were detected for the endochitinase from T. harzianum strain P1, which, when used alone, was the most effective chitinolytic enzyme against phytopathogenic fungi of those tested. The use of hydrolytic enzymes to synergistically enhance the antifungal ability of fungitoxic compounds may reduce the impact of some chemical pesticides on plants and animals.

Keywords: Botrytis cinerea, biocontrol fungi, synergistic interaction, antifungals, cell wall degrading enzymes

INTRODUCTION

The use of synthetic chemicals as fungicides is a primary method of control of disease-causing fungi in animals, including humans, and crop plants. However, the exposure of human populations and natural habitats to increasing amounts of pesticides is becoming unacceptable, and new strategies are required in the attempt to eliminate or reduce the doses of chemicals needed (Cook & Granados, 1991). The majority of pathogenic fungi contain chitin and 1,3-β-glucans in their cell walls (Bartnicki-Garcia, 1968), and dissolution or perturbation of these structural polymers has adverse effects upon the growth and differentiation of fungi (Poulose, 1992). Cell wall degrading enzymes (Broglie et al., 1991;ordentlich et al., 1988), especially chitinolytic enzymes produced by biocontrol micro-organisms such as species of Trichoderma and Gliocladium (Di Pietro et al., 1993; Lorito et al., 1993a), are able to effectively control plant pathogenic fungi. These enzymes act synergistically in combination (Lorito et al., 1993a). In addition they should not be toxic to vertebrates or plants, since these organisms do not contain the target polymer. Combinations of cell wall degrading enzymes and pesticides may also act synergistically in the control of pathogenic fungi (Collins & Pappagianis, 1974; Köller, 1992; Roberts et al., 1988; Watanabe et al.,...
1988). Enzymic lysis of the cell wall may increase the uptake of chemicals into the target cell (Poulouse, 1992).

The purpose of this study was to determine the occurrence of synergistic antifungal interactions between a number of fungal hydrolases and different classes of fungitoxic compounds, and to determine if the level of synergism may be related to the mechanism of action of the toxins used.

**METHODS**

**Strains and media.** *Botrytis cinerea* Pers. ex Fr. strain 26, provided by R. Pearson, Cornell University, was used as model test fungus. This strain was isolated from grapes, and was used previously to assay synergistic antifungal activity of chitinolytic enzymes from *Trichoderma harzianum* (Lorito et al., 1993a). Conidia from *B. cinerea* were produced by growing the fungus at 20–25 °C on potato dextrose agar (PDA) (Difco) under a 16 h/8 h light/dark cycle. They were suspended in water, filtered through sterile Kimwipes (Kimberly-Clark) to remove mycelial fragments, and adjusted to 10^6 propagules ml^{-1}. *T. harzianum* strain P1 (ATCC 74058), which was selected for its resistance from a strain originally isolated from wood chips (Tronsmo, 1991), and *Gliocladium virens* strain 41 (ATCC 20906), isolated from *Aphanomyces* suppressive soil (Smith et al., 1990), were used for the production of the chitinolytic and glucanolytic enzymes. Both strains are biocontrol agents of root diseases (Smith et al., 1993) for the production of glucosidase from *B. cinerea* (Tronsmo, 1991), whereas *G. virens* strain 41 is effective against *Phytophthora* diseases (Smith et al., 1990) as well as other plant pathogenic fungi, including *B. cinerea* (G. E. Harman & W. F. Wilcox, unpublished results).

**Enzymes, enzyme assays and enzyme purification.** Enzyme assays and enzyme nomenclature reported by Tronsmo & Harman (1993) and by Harman et al. (1993) were used in this study. Four enzymes were tested. Two of these were a 41 kDa endochitinase and a 40 kDa chitin 1,4-β-chitobirosidase (hereafter designated chitobirosidase), both from *T. harzianum* strain P1. These enzymes are strong inhibitors of a number of chitin-containing fungi, especially when used in combination (Lorito et al., 1993a, Lorito et al., 1994), and were able to synergistically increase the effectiveness of a bacterial biocontrol agent (Lorito et al., 1993b). The third enzyme, a 78 kDa glucan 1,3-β-glucosidase (hereafter designated glucosidase) from *T. harzianum* strain P1. The fourth enzyme was an endochitinase from *G. virens* strain 41 that inhibited spore germination and germ tube elongation of *B. cinerea*, although at a lower level than the endochitinase from *T. harzianum* (Di Pietro et al., 1993).

All four enzymes were purified by the basic method described by Harman et al. (1993) and modified by Di Pietro et al. (1993) for the endochitinase from *G. virens*. Briefly the two fungal strains were grown for 5–6 d at 25 °C in modified Richard's medium (Harman et al., 1993) for the production of chitobirosidase and endochitinase from *T. harzianum* or in SMCS medium (Di Pietro et al., 1993) for the production of glucosidase from *T. harzianum* and endochitinase from *G. virens*. The culture filtrates obtained by centrifugation and filtration were dialysed, concentrated and fractionated by gel-filtration chromatography on Sephacryl S-300 (Pharmacia LKB Biotechnology). Peak fractions containing the enzyme activity of interest were dialysed, concentrated and further purified by chromatofocusing, followed, if necessary, by isoelectric focusing in a Rotofor cell (Bio-Rad). For the glucosidase, several peaks with glucan 1,3-β-glucosidase activity were detected after the chromatofocusing step, and fractions selected from the major activity peak were pooled, dialysed, concentrated and applied to the Rotofor cell to obtain an electrophoretically pure glucosidase, as determined by using SDS and native electrophoresis on a Phast gel electrophoresis unit (Pharmacia) (Lorito et al., 1994). Protein concentration in the enzyme preparations was determined using the Micro BCA protein assay (Pierce) with trypsin inhibitor from soybean (Sigma) as the standard protein. Enzyme solutions were kept at 4 °C and utilized for the bioassays within two weeks, or concentrated to dryness in a SpeedVac apparatus (Savant Instruments) and stored at -20 °C until used.

**Chemical fungitoxic compounds.** Different classes of fungitoxic compounds were tested: (1) gliotoxin [2,3,5a,6-tetrahydro-6-hydroxy-3-(hydroxymethyl)-2-methyl-10H-3,10a-epipithiopyrazino[1,2-a]-indole-1,4-dione] (Sigma), a naturally occurring epipolythiodiketopiperazine produced by fungi such as *G. virens* (including the strain used in this study for enzyme production) (Di Pietro et al., 1993) that selectively acts on thiol groups located on membranes (Jones & Hancock, 1988); (2) the triazole flusilazole [bis(4-fluorophenyl)methyl-(1H-1,2,4-triazol-1-yl)-methyl]islan (E. I. duPont de Nemours), which specifically inhibits a demethylation step in the synthesis of sterols in fungi and consequently affects membrane structure and integrity, and which is widely used in agriculture (Koller, 1992); (3) the imidazole miconazole (1-[2,4-dichloro-β-[2,4-dichlorobenzyl]oxy]-phenethyl)imidazole) (Sigma) used in medical and veterinary therapies of fungal diseases, and which has a similar mode of action and effect to that of flusilazole (Koller, 1992); (4) benomyl (E. I. duPont de Nemours) used to control plant disease, and which inhibits mitosis by binding to β-tubulin (Ishii, 1992); (5) captan [N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide] (Stauffer), a plant protectant that binds to thiol groups and has a non-specific mode of action (Lukens, 1969).

**Assay procedures.** The various chemicals were dissolved or suspended in appropriate solvents. Flusilazole was dissolved in acetone, benomyl in 70% (v/v) ethanol, miconazole in 50% (v/v) ethanol, gliotoxin in 100% (v/v) ethanol and captan in deionized sterile water. In all cases pure compounds were employed, except for captan and benomyl, where commercially available formulations were tested. For captan two formulations were tested: a dry powder (Stauffer) and a liquid flowable formulation (Gustafson) containing 50% (w/w) and 37% (w/v) active ingredient, respectively. The formulation of benomyl tested was Benlate (E. I. duPont de Nemours), a dry powder containing 50% (w/w) active ingredient. Concentrated stock solutions or suspensions were made and progressively diluted in sterile deionized water to provide appropriate concentrations for the assay and to reduce the amount of solvent (ethanol or acetone) to non-toxic levels. Finally, diluted solutions of toxins as well as enzyme solutions (the enzymes were also dissolved in sterile deionized water) were sterilized by filtration before use in bioassays.

Assay mixtures contained 20 μl of a conidial suspension (10^6 to 10^8 conidia ml⁻¹) of the test fungus *B. cinerea*, 20 μl of 3 x potato dextrose broth, (PDB) (Difco), 18 μl of an enzyme solution (made at appropriate concentration) and 2 μl of a solution or suspension of a chemical toxin (made at appropriate concentration). In the controls, sterile water was used instead of the solution containing either the enzyme or the toxin or both. In addition, the various solvent solutions were tested at the final concentrations employed to determine if they had an effect in the bioassay. Mixtures prepared as indicated above were placed in
Table 1. ED₉₀ values for inhibition of spore germination of B. cinerea for different toxins used either alone or with different amounts of fungal cell wall degrading enzymes

<table>
<thead>
<tr>
<th>Enzyme and source</th>
<th>Conc. (µg m⁻¹)</th>
<th>Enzyme</th>
<th>Gliotoxin ED₉₀ (µg m⁻¹)</th>
<th>Fluconazole ED₉₀ (µg m⁻¹)</th>
<th>Micrococcin ED₉₀ (µg m⁻¹)</th>
<th>Captoxin ED₉₀ (µg m⁻¹)</th>
<th>Benomyl ED₉₀ (µg m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>1250 (1180-1360)</td>
<td>68 (61-85)</td>
<td>3000 (2890-3160)</td>
<td>980 (850-995)</td>
<td>4500 (4390-4900)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endochitinase from T. harzianum P1</td>
<td>41</td>
<td>190 (163-220)</td>
<td>12 (10-15)</td>
<td>0.65 (043-1)</td>
<td>9 (9-9)</td>
<td>70 (64-89)</td>
<td>9 (9-10)</td>
</tr>
<tr>
<td>25</td>
<td>140 (132-170)</td>
<td>29 (29-29)</td>
<td>0.31 (022-045)</td>
<td>29 (29-29)</td>
<td>46 (38-51)</td>
<td>29 (28-29)</td>
<td>60 (45-80)</td>
</tr>
<tr>
<td>Chitinase from T. harzianum P1</td>
<td>152</td>
<td>1020 (920-1100)</td>
<td>30 (26-30)</td>
<td>19 (16-21)</td>
<td>21 (19-21)</td>
<td>62 (49-80)</td>
<td>16 (14-17)</td>
</tr>
<tr>
<td>90</td>
<td>220 (199-255)</td>
<td>24 (24-24)</td>
<td>0.3 (021-05)</td>
<td>24 (22-25)</td>
<td>39 (23-49)</td>
<td>26 (25-28)</td>
<td>74 (65-86)</td>
</tr>
<tr>
<td>25</td>
<td>980 (885-1010)</td>
<td>22 (19-23)</td>
<td>31 (19-42)</td>
<td>17 (15-19)</td>
<td>230 (198-260)</td>
<td>16 (15-17)</td>
<td>180 (169-197)</td>
</tr>
<tr>
<td>75</td>
<td>49 (39-69)</td>
<td>41 (39-42)</td>
<td>0.1 (008-020)</td>
<td>39 (32-38)</td>
<td>1.5 (1.2-2.5)</td>
<td>45 (44-45)</td>
<td>19 (12-29)</td>
</tr>
<tr>
<td>Endochitinase from G. fujikuroi</td>
<td>195</td>
<td>710 (605-796)</td>
<td>2 (1-4)</td>
<td>2.9 (1.6-4.1)</td>
<td>13 (10-16)</td>
<td>82 (69-98)</td>
<td>4 (6-9)</td>
</tr>
<tr>
<td>100</td>
<td>240 (200-283)</td>
<td>35 (35-36)</td>
<td>0.2 (011-032)</td>
<td>36 (36-37)</td>
<td>19 (7-32)</td>
<td>36 (34-37)</td>
<td>27 (19-41)</td>
</tr>
</tbody>
</table>

*Values obtained by probit analysis.
† ED₉₀ values for each toxin combined with the indicated amount of cell wall degrading enzyme. The initial value is the quantity of toxin required for 50% inhibition for each quantity of enzyme (with lower and upper 95% fiducial limits).
‡ Eₙ is the expected value for an additive response according to Limpel’s formula, expressed as percentage inhibition (Richer, 1969). Values are provided for the expected value for additive effects of each quantity of each toxin (with similar value for the fiducial limits). The values used in Limpel’s formula were those obtained from regression of percentage inhibition vs increasing toxin or enzyme concentration. In every case except with glucosidase at 75 µg m⁻¹, values for Eₙ are less than 50% inhibition, and so synergy was evident.

The results are the means of at least two experiments with three replicates for each experiment. The standard deviations were calculated from at least two experiments with 300 observations per experiment. The values of spore germination obtained for the control ranged from 70 to 95%. For each experiment the means of the control values were calculated, then taken as 0% inhibition; all other values were divided by these values and multiplied by 100 to obtain percentage inhibition. According to Richer (1987), the following (Limpel’s) formula was used to determine the presence of antifungal synergistic interactions between chitinases and fungicides: Eₙ = (X + Y – XY)/100, where Eₙ is the expected effect from additive responses of two inhibitory compounds, and X and Y are the percentages of inhibition relative to each compound used alone. If the combination of the two agents produces any value of inhibition greater than Eₙ, synergy exists, and the difference is proportional to the level of synergy observed. For each toxin, for each enzyme or for each toxin/enzyme combination, dosage response curves were obtained by probit analysis of the data. The lower and the upper 95% fiducial limits for 95% probability were obtained by probit analysis. ED₉₀ values for each enzyme, each toxin and each combination of enzyme and toxin were also calculated by probit analysis.

RESULTS

Every pesticide/enzyme combination tested inhibited spore germination of B. cinerea and showed a substantial level of synergism (Table 1, Figs 1 and 2). In Table 1 we present the quantities of toxin required to obtain 50% inhibition of B. cinerea spore germination, together with the 95% fiducial limits for these values. For enzyme/fungicide mixtures we also present the inhibition levels (Eₙ) expected for these quantities of enzymes and chemicals if only additive effects occurred. In most cases, Eₙ was substantially lower than the value of inhibition actually observed for the mixtures, indicating that synergy did occur (Table 1). Further, Eₙ values were outside the values calculated for the fiducial limits, indicating that synergy was significant.

The nature of the chemical fungitoxicant affected the level
of synergism. For most of the combinations of pesticide/enzyme, the level of synergism was higher when a cell wall degrading enzyme was associated with gliotoxin, flusilazole or miconazole as opposed to captan or benomyl (Table 1). The ED50 values of the combinations between enzymes and gliotoxin, flusilazole or miconazole were lower compared with mixtures containing enzymes and captan or benomyl (Table 1, Fig. 1). For instance, the addition of endochitinase (10 µg ml⁻¹) from *T. harzianum* strain P1 reduced the ED50 values 6.5-, 104- and 42.8-fold for gliotoxin, flusilazole and miconazole, respectively, whereas the reduction for either captan or benomyl was 3.1-fold (Table 1, Figs 1 and 2). When cell wall degrading enzymes were combined with the same pesticide, the level of synergism expressed was higher for endochitinase from *T. harzianum*. This enzyme, when used alone, was more effective than any other enzyme (Lorito et al., 1993a) (Table 1). Similarly, the addition of 1 ng flusilazole ml⁻¹ reduced the ED50 value 6.8-fold for endochitinase from *T. harzianum*, and 4.6-, 1.3- and 3.9-fold for chitobiosidase and glucosidase from *T. harzianum* and endochitinase from *G. virens*, respectively.

There were no differences between the two formulations of captan used in this study, and the data shown were obtained with the dry formulation.

**DISCUSSION**

This paper used spore germination as the criterion of fungitoxicity of pesticides and enzymes. However, both chemical fungicides and enzymes have effects upon other stages of the fungal life cycle. In our earlier work (Lorito et al., 1993a), we showed that effects on hyphal elongation were very closely correlated with inhibition of spore germination. In addition, both enzymes and chemicals induce a number of morphological abnormalities within hyphae, including, in the case of enzymes, bursting of vegetative cells (Lorito et al., 1993a).

The ability of cell wall degrading enzymes to interact synergistically in degradation of substrates has been reported by several authors (De La Cruz et al., 1992; Lorito et al., 1993a; Mauch et al., 1988; Nevalainen et al., 1991). In addition, plant chitinases were synergistic with
inhibitors of chitin synthesis in fungi, such as polyoxin B and nikkomycin (Roberts et al., 1988; Poulose, 1992). The results obtained in this study showed that different cell wall degrading enzymes substantially enhanced the activity of a variety of chemical and natural fungitoxic compounds. The chemicals chosen had different modes of action (Köller, 1992; Jones & Hancock, 1988; Miller, 1969; Ishii, 1992); thus the ability of the enzymes to synergistically interact with the pesticides tested was not associated with a single class of compounds. The mechanism(s) for the synergism observed in this study is unknown. One possibility is that digestion of the cell wall may enhance the uptake of the chemicals. However, differences in the level of synergism were detected between toxins having direct effects on membranes versus other antifungal compounds with non-specific modes of action or with specific sites of action on cytoplasmic components. We found that the highest levels of synergism occurred with two sterol demethylation inhibitors and with gliotoxin. Gliotoxin has a direct effect upon membranes (Jones & Hancock, 1988), while demethylation inhibitors inhibit sterol synthesis (Köller, 1992). Sterols are required for structure and function of membranes (Vanden Bossche, 1989), and so both classes of highly synergistic toxicants have significant and primary effects upon membranes. These findings suggest that combining the effect of these toxins with partial digestion of cell walls may be particularly damaging for the targeted cells and, therefore, may reduce the lethal doses of the toxins. In addition, fungi, including Botrytis allii, treated with sterol demethylation inhibitors showed an irregular deposition of chitin in the fungal cell wall possibly as result of a functional imbalance in the chitin synthase system (Köller, 1992). Therefore, the ability of cell wall degrading enzymes to increase the intracellular dose of the toxins may also lead to a further disorganization of the cell wall and consequently to more effective lytic action of the enzyme. This synergistic feedback loop may be started at relatively low doses of the two agents and may also be responsible for the high level of synergistic antifungal interaction observed with sterol demethylation inhibitors.

The relative sensitivity of Pythium ultimum and Rhizoctonia solani to gliotoxin was affected by the presence or the integrity of diffusion barriers such as the fungal cell wall (Jones & Hancock, 1988). Di Pietro et al. (1993) discovered the ability of this natural fungicide to interact synergistically with an endochitinase produced from a strain of G. virens during the inhibition of B. cinerea which may be involved in the biocontrol ability of these fungi. Our findings confirmed these results and also showed that several cell wall lytic enzymes from different biocontrol fungi, including strain P1 of T. harzianum, which does not produce gliotoxin, were able to increase the antifungal effect of five different toxins. This suggests that synergism between toxins and enzymes may be a quite common event, since the co-production of cell wall lytic enzymes and toxic metabolites probably occurs commonly among biocontrol agents.

A number of authors have suggested the possibility of using cell wall degrading enzymes as adjuvants of fungicidal or fungitoxic compounds (Poulose, 1992). The results of this study expand the range of useful combinations of fungicides and enzymes and indicate that the level of synergism may be affected by the mode of action of the compounds. These findings may also be useful in designing effective combinations to be tested, in which synergism is likely to occur at high levels. In some cases, either the enzyme or the producing organism may be used in association with antimycotic drugs. For instance,
combining captan with T. harzianum, which is resistant to this fungicide, resulted in greater control of Verticillium dahliae on potato than either agent used singly (Ordentlich et al., 1990). The reasons for this interaction in vivo between captan and Trichoderma have not been investigated, although our results suggest that cell wall degrading enzymes could play a role in this synergism. While this study examined effects of enzymes and antifungal compounds only on B. cinerea, chitinolytic enzymes from T. harzianum have been shown to act synergistically against a wide range of disease-causing fungi in the Ascomycetes, Deuteromycetes and Basidiomycetes (Lorito et al., 1993a). Therefore, the synergistic activities of enzymes and chemical fungicides reported here are also likely to apply to a wide range of chitinous fungi.

These results, therefore, suggest that combinations of fungal cell wall degrading enzymes and synthetic pesticides may be useful in a range of agricultural applications, and perhaps even in veterinary or human medicine. Davies & Pope (1978) demonstrated that a mixture of chitinolytic enzymes and chemical antifungal agents had useful antimycotic activity in immunosuppressed laboratory animals. Enzymes and chemicals might be topically applied or introduced in other ways. For example, the possibility of producing transgenic plants resistant to plant pathogenic fungi via production of fungal cell wall degrading enzymes has been attempted, but only a moderate level of resistance has been achieved (Broglie et al., 1991). This difficulty may be overcome by introducing several genes coding for synergistic antifungal enzymes; our data indicate that ED₉₀ levels may be reduced 10-fold by such combinations (Lorito et al., 1993a). Alternatively, or in addition, pesticides synergistic with the expressed enzymes may be applied. The data in this work indicate that the presence of even low levels of enzymes may increase the sensitivity of pathogenic fungi by 1–2 orders of magnitude. Thus, very low levels of demethylatation inhibitors may be sufficient to eliminate plant disease that does occur on transgenic plants. Assuming that levels of synergy in vivo equivalent to the synergy in vitro reported here can be obtained, a number of strategies can be envisioned for use of synergistic chemical and enzymatic agents with a substantial reduction in the rate of chemical protectant applied.

ACKNOWLEDGEMENTS

The authors thank W. Koller, Cornell University, Geneva, NY, for helpful information, suggestions and discussion, R. M. Broadway, Cornell University, for the critical revision of the manuscript, R. Pearson, Cornell University, for providing the strains of Botrytis cinerea used in this study and G. Nash and P. Nielsen, Cornell University, for their technical assistance. This research was supported in part by BARD Grant US-172389. The first author was supported by a grant from the National Council of Research (CNR), Italy.

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Chitinolytic enzyme and fungicide synergism


Received 18 August 1993; accepted 28 September 1993.