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Short communication

The interaction of endochitinase, a synthetic peptide and resveratrol in controlling fungi *in vitro*

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Abstract

The efficacy of two-way combinations of *Trichoderma* endochitinase (CHIT42), synthetic peptide ESF12 and resveratrol upon the control of growth of *Botrytis cinerea* and *Penicillium digitatum* were evaluated *in vitro*. CHIT42 had effective concentration for 50% growth inhibition (EC_{50}) values of 1.5 and 0.5 μM against *B. cinerea* and *P. digitatum*, respectively. CHIT42 also caused swelling, hyperbranching and bursting of *P. digitatum* at concentrations as low as 0.003 μM. The EC_{50} values of ESF12 were 30.9 and 29.2 μM against *B. cinerea* and *P. digitatum*, respectively. Resveratrol had EC_{50} values of 178.6 and 343.2 μM against *B. cinerea* and *P. digitatum*, respectively. Combinations of CHIT42 + ESF12, CHIT42 + resveratrol and ESF12 + resveratrol showed additive activity (interaction ratios between 0.5 and 1.5) against *B. cinerea* and *P. digitatum*.

Antifungal compounds may be combined in mixtures for three main reasons: (i) to exploit the synergistic or additive interaction among the compounds, when dosage rates of one or two or more of the components can be reduced without loss of effective control of the target pathogen (Lobeer, 1996), (ii) to reduce the potential for the development of resistance in the target pathogen to either of the components (Samoucha and Cohen, 1988) and (iii) to widen the spectrum of antifungal activity to control several diseases occurring simultaneously (Gisi, 1996). However, antifungal compounds may interact synergistically, additively or antagonistically, depending upon their mode of action, mode of uptake and translocation and probably their direct interaction. Synergistic and additive interactions are desirable while antagonistic interactions are not.

The production of transgenic plants with several antifungal genes is likely to result in a reduction of the probability of a pathogen overcoming the antifungal gene products and also may result in the simultaneous enhancement of resistance against different fungal pathogens. However, before transferring cloned antifungal genes to plants, it is useful to determine (i) whether the end products of the antifungal genes control the target fungal pathogens, and (ii) the nature of the interaction between the end products.

Trichoderma harzianum produces a variety of chitinolytic enzymes (Chet, 1987), including endochitinase, which inhibits spore germination and hyphal elongation of several fungal pathogens (Lorito et al., 1993a,b). It can also cause morphological changes such as swelling, branching, necrosis and vacuolization of hyphae of the treated fungus (Lorito et al., 1993b). *Trichoderma* endochitinase (CHIT42) interacts synergistically with other chitinolytic and glucanolytic enzymes (Lorito et al., 1993a,b; 1994; 1996) as well as membrane-affecting compounds (MACs) such as flusilazole and valinomycin (Lorito et al., 1996).

ESF12 is a synthetic antimicrobial peptide (Powell et al., 1995) that mimics the amphipathic α-helical structure of the magainins and cecropins (Broekaert et al., 1997; Soravia et al., 1988). ESF12 inhibited the germination of conidia of *Cryphonectria parasitica*, *Fusarium oxysporum* f.sp. *lycopersici*, and *Septoria musiva* at micromolar concentrations and also had antibacterial activity (Powell et al., 1995).

Resveratrol is a stilbene phytoalexin produced by grapevines (Langcake and Pryce, 1976), pines, certain legumes and forest trees (Hart, 1981). It has been associated with the prevention of wood decay (Hart, 1981) and with disease resistance (Aguamah et al., 1981; Dercks and Creasy, 1989). Direct evidence for the involvement of resveratrol in the plant defense system comes from the enhancement of disease resistance in transgenic plants constitutively expressing resveratrol. The stilbene synthase gene from grapevine has been transferred into tobacco (Hain et al., 1993), tomato (Thomzik et al., 1997) and potato (Stahl et al., 1994) resulting in elevated resveratrol levels, that, in the case of tomato and tobacco, resulted in enhanced disease resistance.

The purpose of the present study was to determine the nature of the two-way interactions among endochitinase, ESF12 and resveratrol in their ability to reduce growth of *Botrytis cinerea* and *Penicillium digitatum in vitro*. This information should lead to the identification of suitable combinations of genes that could enhance disease resistance in transgenic plants. It was hypothesized that the interactions of two-way mixtures of (i) endochitinase + ESF12 (ii) endochitinase + resveratrol and (iii) ESF12 + resveratrol would be synergistic.

B. cinerea was isolated from grapevine and provided by J. Burr (Cornell University) and *P. digitatum* was provided by Dr. G. Harman. They were grown on V8 agar and potato dextrose agar (PDA), respectively, at 25 °C under continuous white fluorescent light. Conidial suspensions were prepared from 7-day-old cultures and adjusted to a concentration of 10^5 spores ml^{-1}.

The endochitinase CHIT42 was obtained from a transgenic strain of *T. harzianum* strain P1 (ATCC 74058) that contained the endochitinase gene (*ech42*) driven by a cellobiohydrolase (*cbh1*) promoter (Emilio et al., 1996). After induction by cellulose, strain P1 primarily produced endochitinase in the culture filtrate. There were small mixtures of other proteins, but based on native polyacrylamide gel electrophoresis, the enzyme was about 80% pure and its activity was associated with a 40-kD band (Deng et al., 1997). The concentrated culture filtrate was used in assays and hereafter designated CHIT42. The unconcentrated culture filtrate contained approximately 1.5 $\mu g\, ml^{-1}$ endochitinase (Deng et al., 1997).

ESF12, a synthetic amphipathic antimicrobial peptide consisting of 18 amino acids (Powell et al., 1995) was synthesized at the Cornell Biotechnology Analytical/Synthesis Facility, Cornell University, to a purity level of more than 95%. The lyophilized ESF12 powder was dissolved in sterile distilled water to a concentration of 500 μM and stored at -20°C. Resveratrol was purchased from Sigma.

An automated colorimetric method, which uses the absorbance (optical density) of microcultures in a microtiter plate as a function of growth (Daeschel, 1992; Wilson et al., 1997), was adapted. Preliminary tests using CHIT42 and ESF12 with *B. cinerea* and *P. digitatum* confirmed that growth and absorbance were directly correlated. Antifungal assays were performed in 96-well sterile flat bottom microplates. Each well contained appropriate amounts of antifungal compound(s), 35 μl of 2$\times$ potato dextrose broth (PDB) (Difco) and approximately 4000 spores of either *B. cinerea* or *P. digitatum* in a total mixture volume of 70 μl. The plates were covered with sterile lids and placed in polystyrene boxes lined with moistened filter paper to maintain high humidity. The boxes were placed in polystyrene containers and incubated at 25 $\pm$ 2 °C in the dark. The absorbance of each well was determined as optical density (OD) at 492 nm at the beginning of the experiment and after 36 h of incubation. ESF12, resveratrol and endochitinase were tested at five concentrations alone and in two-way combinations against *B. cinerea* and *P. digitatum*. For each compound, preliminary experiments were performed to determine the approximate effective concentration for 50% growth inhibition (EC_{50}) levels. All experiments were performed twice with three replications of each concentration combination, except for experiments with ESF12 + CHIT42 and resveratrol + CHIT42 *versus B. cinerea* which were performed once.

Since resveratrol is sensitive to light, which causes conversion from the *trans* to the *cis* form, all experiments involving resveratrol were performed under dim light. In order to reduce the potential effect of the ethanol from the resveratrol stock solution, the final concentration of ethanol in the assay mixture was adjusted to approximately 0.08% at which point

ethanol had no effect on fungal growth. Control wells contained 0.08% ethanol.

Assuming (i) independent actions of these antifungal compounds and (ii) additive interactions, the expected efficacy of a mixture may be determined by the Abbott formula: $I_{exp} = X + Y - (XY/100)$, where I_{exp} is the expected percent inhibition for an additive interaction, and X and Y are percent fungal growth inhibited by the components in a mixture when used alone (Abbott, 1925). The value XY represents the overlapping inhibition effected by the components together. Empirical response rates for each treatment were determined by the (untreated − treated/untreated) formula. The empirical response values were analyzed by the probit procedure of SAS software (SAS Institute, Cary, NC). For mixtures of the three compounds, the empirical response rates for each component were analyzed when used alone and at the four tested concentrations of the second component and *vice versa*. The resultant probit equations were used to determine the predicted percentage inhibition (I_{obs}) for each treatment. The predicted I_{obs} values were used for the determination of interaction ratios (IRs) which were computed as $IR = I_{obs}/I_{exp}$. The data were also analyzed by the Wadley approach (Gisi et al., 1985): $EC_{70exp} = [a + b]/[(a/EC_{70A}) + (b/EC_{70B})]$, where EC_{70exp} is the expected effective dose resulting in 70% growth inhibition of the mixture. EC_{70A} and EC_{70B} are the observed effective doses of component A and B, respectively , resulting in 70% growth inhibition. A, B are the components and, a and b are the ratios of these components in the mixture. The level of interaction is calculated as: $IR = EC_{70exp}/EC_{70obs}$, with EC_{70obs} representing the observed effective dose.

Five increasing concentrations of each compound were used individually for the determination of the efficacy of each compound against *B. cinerea* and *P. digitatum*. CHIT42 inhibited the growth of both test fungi with average EC_{50} values of 1.5 and 0.5 μM against *B. cinerea* and *P. digitatum*, respectively (Table 1). The EC_{50} values of ESF12 and resveratrol against both fungi were 20–686-fold greater than that of CHIT42 (Table 1). On a molar basis, CHIT42 was three times more effective against *P. digitatum* than against *B. cinerea*. On the contrary, resveratrol was twice as effective against *B. cinerea* as against *P. digitatum*. ESF12 was equally effective against both fungi.

The nature of the interactions between ESF12, resveratrol and endochitinase in two-way mixtures were investigated *in vitro*. Table 2 shows the effective concentrations of each component required for 70% growth inhibition (EC_{70}) of *B. cinerea* and *P. digitatum* for each compound alone and in combination. All interaction ratios were within the range of 0.5–1.5, suggesting additive interactions. Since none of the IRs were more than 1.5, the combined activities of the two-way mixtures of CHIT42, ESF12 and resveratrol did not appear to be synergistic.

Kosman and Cohen (1996) suggested that the nature and level of interaction between compounds in a mixture must be confirmed by applying several mathematical methods. Therefore, the data were also analyzed using the Abbott formula (Abbott, 1925). All interaction ratios were again within the range of 0.5–1.5, confirming the additive nature of interaction in the mixtures (data not shown). When the predicted percentage growth inhibition values for each mixture were plotted against the increasing concentrations of one component in a mixture, the intercepts increased progressively with increasing concentrations of the second component, demonstrating that each compound inhibited fungal growth separately (data not shown). These growth inhibition graphs do not appear to deviate dramatically from parallelism, also indicative of solely additive interactions.

Antimicrobial compounds are known to induce abnormal morphological changes in the hyphae of test fungi (Cavallarin et al., 1998). In order to determine the effect of the antifungal compounds on fungal growth characteristics, each fungus was examined under the microscope after treatment with each compound individually and in two-way combinations. Morphological abnormalities of *P. digitatum* were observed when grown with CHIT42 and ESF12, either alone or in combination. CHIT42 caused hyperbranching around the germinated spores of *P. digitatum* (Figure 1). At higher concentrations of CHIT42, the spores of *P. digitatum* turned into swollen spherical protoplasts, supportive of the cell wall degrading activity of the CHIT42. Also, noteworthy were the smaller sizes of

Table 1. EC_{50} values of CHIT42, ESF12 and resveratrol against *B. cinerea* and *P. digitatum* as determined by OD at 492 nm

Enzyme/compound	*B. cinerea* (μM)	*P. digitatum* (μM)
CHIT42	1.5 (46.1)[1]	0.5 (14.6)
ESF12	30.9 (57.7)	29.2 (54.5)
Resveratrol	178.6 (40.8)	343.2 (78.3)

[1]Values in parentheses are in μg ml^{-1}.

Table 2. Fungicidal activities (EC_{70} values) of CHIT42, ESF12 and resveratrol alone and in mixtures, and the level of interactions of the mixtures tested against *B. cinerea* and *P. digitatum*

Enzyme/compound	*B. cinerea* (μM)			*P. digitatum* (μM)		
	Observed	Expected	IR[1]	Observed	Expected	IR[1]
CHIT42	1.7	—	—	0.6	—	—
ESF12	43.3	—	—	42.0	—	—
Resveratrol	230.3	—	—	453.5	—	—
CHIT42 + ESF12 (1 : 100)[2]	33.7	24.2	0.7	46.9	25.3	0.6
CHIT42 + resveratrol (1 : 400)	189.1	109.2	0.6	377.9	233.5	0.6
ESF12 + resveratrol (1 : 4)	206.3	123.6	0.6	469.9	217.1	0.5

[1]Interaction ratios calculated according to Wadley (Gisi, 1996; Gisi et al., 1985).
[2]Values in parentheses are the ratios of the individual component in the mixture.

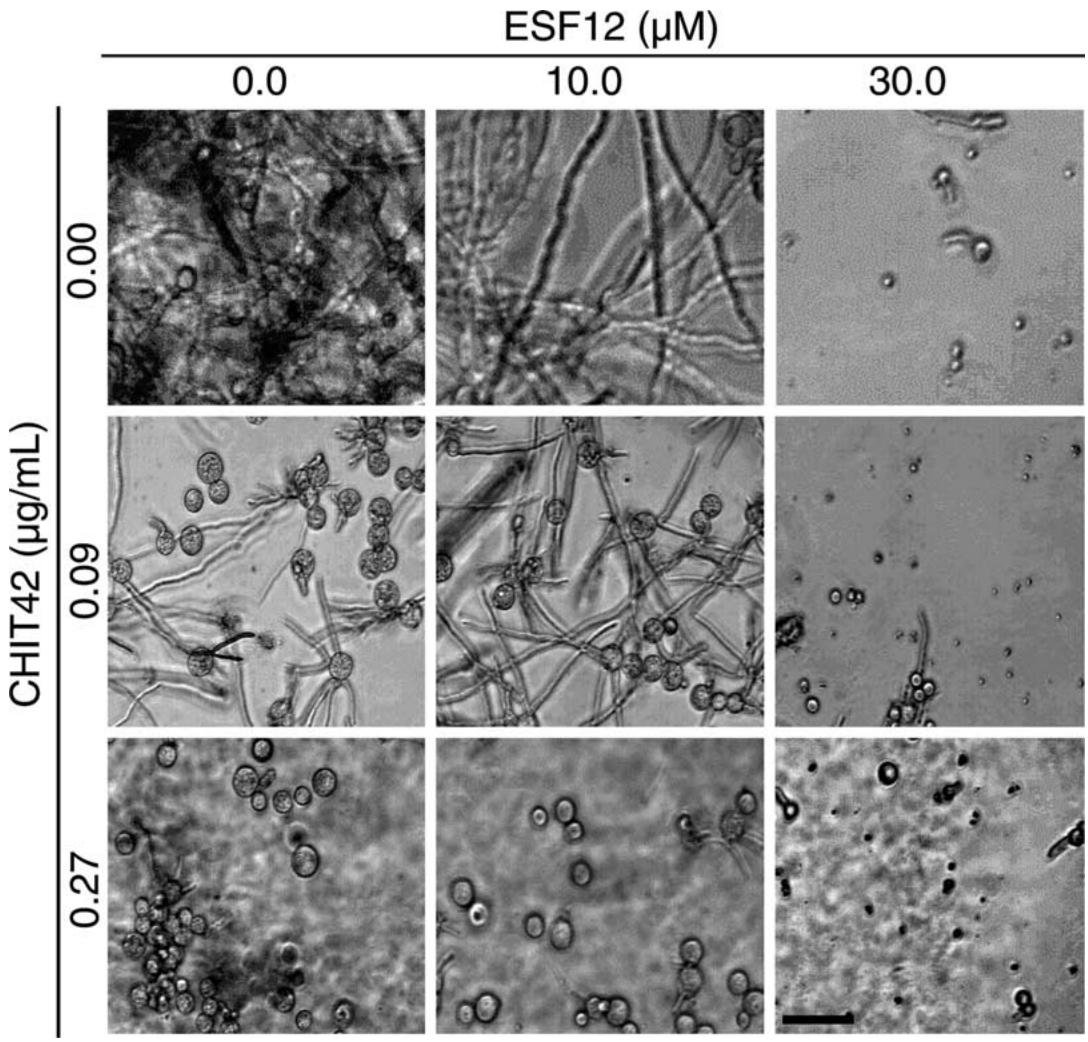

Figure 1. Effects of ESF12 and CHIT42 alone and in combinations at the indicated concentrations on the growth and morphology of *P. digitatum*. Pictures were taken after 18 h of incubation of *P. digitatum* spores with ESF12 and/or CHIT42. Bar represents 10 μm.

the protoplasts produced by CHIT42 at higher levels of ESF12 (Figure 1). A visual comparison of growth inhibition due to CHIT42 and ESF12 alone and in combination does not indicate any dramatic difference, indicating that CHIT42 and ESF12 do not act synergistically (Figure 1). No morphological changes were detected in the remaining fungus/antifungal compound combinations.

Antifungal compounds in a mixture may act synergistically, additively, or antagonistically (Levy et al., 1986). Endochitinase degrades fungal cell walls (Lorito et al., 1993b), while ESF12 supposedly interferes with cell membrane permeability (Powell et al., 1995). Lorito et al. (1996) reported a synergistic interactions between CHIT42 and several MACs in control of *B. cinerea* and *Fusarium oxysporum*. However, in all experiments involving CHIT42 and ESF12 (a MAC) reported here, no synergistic interactions were observed. Since the mechanism by which ESF12 and other related antimicrobial peptides permeabilize the membrane is not completely understood (Nissen-Meyer and Nes, 1997), it could be suggested that ESF12 acts in a way that does not affect the activity of CHIT42 or *vice versa*. It can also be suggested that some *Penicillium* and *Botrytis* strains are less susceptible to the potential synergy between chitinase and ESF12.

In the case of resveratrol, the results reported here suggest that it acts independently of CHIT42 and ESF12 and that the nature of the interaction is additive. The EC_{50} value for resveratrol with *P. digitatum* was approximately twice as high as with *B. cinerea*. Adrian et al. (1997) reported an EC_{50} of approximately 400 μM for spore germination of *B. cinerea*, which is twice the concentration found in the present study. The difference may be due to different systems of fungal growth measurement and other biological parameters. Seventeen *Vitis* spp. produced from 5.7 to 567 μg resveratrol per gram of leaf fresh weight (Dercks and Creasy, 1989) which approximately corresponds to per milliliter of aqueous solution. Since the resveratrol synthase gene could be expressed to a higher level under the constitutive 35S promoter, it appears that resveratrol could be produced at least up to 567 $\mu g\,ml^{-1}$ in transgenic grapevines. This level of expression is well above the EC_{50} of 40.8 $\mu g\,ml^{-1}$ for *B. cinerea* (Table 1), supporting the hypothesis that constitutive expression of resveratrol in grapevines would lead to enhanced resistance against fungal pathogens. Other evidence in support of this hypothesis is provided by the constitutive expression of resveratrol in tobacco (Hain et al., 1993), rice (Stark-Lorenzen et al., 1997) and tomato (Thomzik et al., 1997) which resulted in enhanced resistance to *B. cinerea, Pyricularia oryzae* and *Phytophthora infestans*, respectively. For perennial crops such as grapevines, it is important that disease resistance remains effective for a long period of time because of the amount of resources put into the production of improved version of old cultivars. To achieve durable resistance, one approach would be to pyramid defense-related genes into one single, elite cultivar. Since the products tested here apparently have different modes of action, the transfer of genes responsible for the production of these compounds might result not only in the durability of resistance but might also provide simultaneous protection against several fungal pathogens.

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