Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol

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Abstract

The interactions between biocontrol fungi and bacteria may play a key role in the natural process of biocontrol, although the molecular mechanisms involved are still largely unknown. Synergism can occur when different agents are applied together, and cell wall degrading enzymes (CWDEs) produced by fungi can increase the efficacy of bacteria. *Pseudomonas* spp. produce membrane-disrupting lipodepsipeptides (LDPs) syringotoxins (SP) and syringomycins (SR). SR are considered responsible for the antimicrobial activity, and SP for the phytotoxicity. CWDEs of *Trichoderma* spp. synergistically increased the toxicity of SP_{25-A} or SR_E purified from *P. syringae* against fungal pathogens. For instance, the fungal enzymes made *Botrytis cinerea* and other phytopathogenic fungi, normally resistant to SP_{25-A} alone, more susceptible to this antibiotic. *Pseudomonas* produced CWDEs in culture conditions that allow the synthesis of the LDPs. Purified bacterial enzymes and metabolites were also synergistic against fungal pathogens, although this mixture was less powerful than the combination with the *Trichoderma* CWDEs. The positive interaction between LDPs and CWDEs may be part of the biocontrol mechanism in some *Pseudomonas* strains, and co-induction of different antifungal compounds in both biocontrol bacteria and fungi may occur.

Abbreviations: LDP – lipodepsipeptide; CWDE – cell wall degrading enzyme; SP – syringotoxin; SR – syringomycin

Introduction

Many *Pseudomonas* strains produce cyclic lipodepsipeptides (LDPs) belonging to two different groups: the nonapeptide toxins such as the syringomycins (SRs), and the syringopeptines (SPs) family which includes molecules with an aminoacidic moiety of 25 (SP_{25A}) or 22 (SP_{22A}) residues (Ballio et al. 1990; Scaloni et al. 1994). *Pseudomonas* LDPs have antibiotic properties due to their ability to interfere with plasma membrane and act in the nanomolar range by forming transmembrane ion channels (Di Giorgio et al. 1994). They prove to be quite effective in the growth inhibition of a wide array of fungi (Lavermicocca et al. 1997; Bull et al. 1998). However, the sensitivity of the target organisms to the various LDPs has been found to be very different. The activity against fungal growth of the 'small' nonapeptides was higher

compared to that of SPs, while the activity of SPs was higher when they were injected inside the plant. These observations suggest that SRs have mainly antifungal activity while SP are more phytotoxic. It can be hypothesized that the low activity of SP on fungi found in vitro is due to the barrier effect of the fungal wall, and that the efficacy of these toxins in vivo is mediated by the action of enzymes capable of increasing cell permeability and, therefore, susceptibility to SP. The inhibitory activity, both on fungal growth and germination, of *Pseudomonas* LDPs in combination with cell wall degrading enzymes (CWDEs) was tested against several species of plant pathogenic and non-pathogenic fungi differing in cell wall composition. Our results indicate that enzymatic degradation of the cell wall permits both toxins, especially the one with higher molecular weight (SPs) to reach its target, and alter cell membrane functions, much more effectively
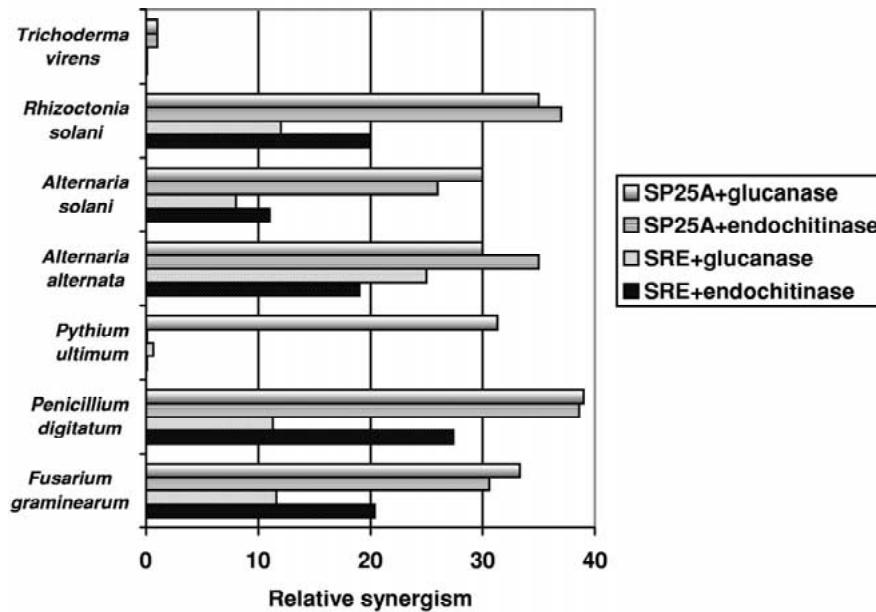
Figure 1. Relative level of synergistic inhibition (RS) of various phytopatogenic fungi calculated for in vitro bioassays of mixtures containing different concentrations (μg ml^{-1}) of *Pseudomonas syringae* LDPs (SR$_E$ and SP$_{25A}$) and given concentrations of cell wall degrading enzymes from *Trichoderma virens*. RS values for spore germination (mycelia growth for *Rhizoctonia solani*) are indicated. Similar results were obtained for the inhibition of hyphal elongation. Endochitinase and glucanase =42 kDa endochitinase and 78 kDa glucose 1,3-β-glucosidase from *T. virens* strain G41, respectively. Cell wall degrading enzymes (CWDEs) were applied at a concentration giving about 10% inhibition when used alone; RS values were calculated as described by Lorito et al. (1996a) and range from 0 (no synergism) to 40 (maximum value).

than in the absence of the enzymes. We also suggest that the synergism between CWDEs and LDPs may support the antagonistic mechanism of *Pseudomonas* or its positive interaction with *Trichoderma* during biocontrol.

Material and methods

Strain B359 of *P. syringae* pv. *syringae*, from the collection of the Department of Plant Pathology, University of California, was used as source of LDPs. All the fungal strains were from the collection of the Institute of Plant Pathology of the University of Naples, Italy. For production of LDPs the bacteria were grown at 28 °C in static conditions on a medium containing 55 mM mannitol and 20 mM histidine (Surico et al. 1988). LDPs were identified by HPLC coupled with an API-100 single quadrupole electrospray mass spectrometry (Sciex Instruments) equipped with an atmospheric pressure ionization source. A probe voltage of 4.8 kV and a declustering potential of 70 V were used. Quantification of toxins was performed by ELISA as described by Fogliano et al. (1999). The fungicidal 42 kDa endochitinase and 78

kDa glucan 1,3-β-glucosidase were purified from the culture filtrate of *T. virens* strain G41 following a protocol already developed for *T. harzianum* (Lorito et al. 1994). The tests conducted with mixtures of CWDEs and bacterial LDPs contained an increasing dose of toxins and a concentration of enzyme producing 10% inhibition when applied alone. This bioassays were based on the inhibition of spore germination and/or mycelia growth, and were performed as previously described (Lorito et al. 1994, 1996a). The relative level of synergism (RS) for each CWDE + LDP combination was calculated as described by Lorito et al. (1996a) by applying the formula RS = 50–(10+Y–Y/10), where Y is the effect (percent inhibition) of a LDP used alone at a concentration giving 50% inhibition in combination with a CWDE applied at a concentration giving 10% when used alone. For less synergism the RS value approaches zero, while 40 is the highest possible value. The chitinase-specific inhibitor allosamidin (kindly provided by Dr. Sakuda, University of Tokyo) was used in the in vitro antifungal assay by adding a 5–10 μl aliquot of the buffer-dissolved drug (in 0.1 M phosphate buffer pH 6.5) at a final concentration in the assay of 5 μg ml^{-1}. This concentration was previously determined to be
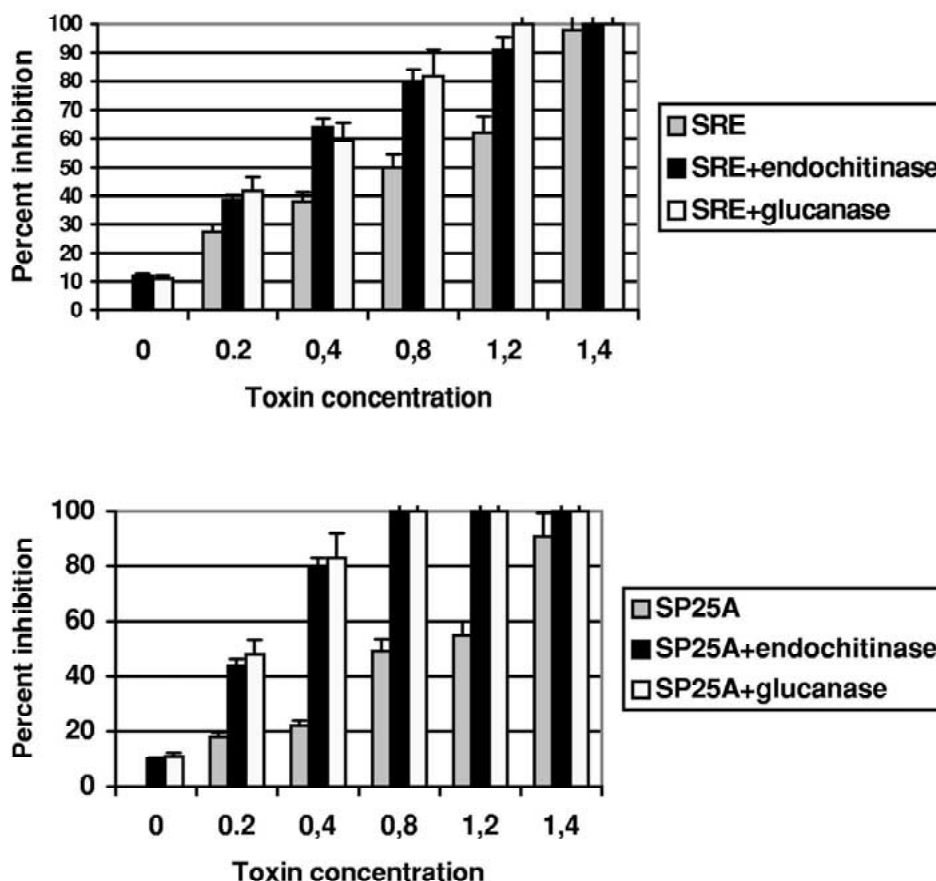
Figure 2. Synergistic effect of *Pseudomonas syringae* LDPs (SR$_E$ and SP$_{25A}$), applied alone and in combination with an endochitinase or a glucose 1,3-β-glucosidase from *Trichoderma virens*, on spore germination of *Penicillium digitatum*. Cell wall degrading enzymes (CWDEs) were applied at a concentration giving about 10% inhibition when used alone. Toxin concentration values indicate μg ml^{-1}. Data are presented in the figure as means ± standard deviation. Similar results were obtained for inhibition of hyphal elongation.

sufficient for at least a 90% inhibition of chitinolytic activity in the culture filtrates of both *T. virens* and *P. syringae*.

Results and discussion

Pseudomonas syringae strain B359 was found to produce two main LDPs: a SR$_E$ with a molecular mass of 1254 uma and a SP$_{25A}$ with a molecular mass of 2400 uma. High levels of LDP production in the media were obtained by using a cultivation method based on a medium replacement. Addition of 1% glucose to the minimal medium also increased toxins production. SR$_E$ and SP$_{25A}$ showed antifungal activity against all the fungi tested at final concentrations in the assay varying from about 0.15 – 1.5 μg ml^{-1} (0.12 – 1.2 μM for SR$_E$ and 0.06 – 0.62 μM for SP$_{25A}$). Combination of LDPs and CWDEs dramatically increased the antifungal activity particularly for SP$_{25A}$ (Figures 1 and 2). The relative synergism values (RS) for phytopathogenic fungi were all positive except for *Pythium ultimum* treated with endochitinase. In fact, the synergistic inhibitory effect was correlated with the fungal cell wall composition of the target microorganisms. LDPs were also effective on *Trichoderma virens*, but no synergism with the CWDEs produced from the same strain was found, unless an enzyme mixture normally used to digest *Trichoderma* cell walls (Novozyme from Novo Nordisk) was applied in combination with the bacterial toxins (data not shown). On *Pseudomonas*, which was not sensitive to its own LDPs applied alone, the addition of lysozyme at sublethal doses made the LDPs inhibitory for the bacterium (data not shown). The addition of the chitinase inhibitor allosamidin in the *in vitro* bioassay

always blocked the effect of the fungal enzymes and completely annulled the CWDEs-LDPs synergistic interactions with all the target fungi tested when the endochitinase, but not the glucanase, was applied (data not shown).

Previous work on *Trichoderma* metabolites has shown that CWDEs and membrane-acting peptides (i.e. trichorzianines and other peptaibols) are produced concurrently during biocontrol and interact synergistically as antifungal agents (Lorito et al. 1996b). Since *Pseudomonas* LDPs also act by altering membrane functions, we considered that the addition of CWDEs may enhance the efficacy of the toxins by facilitating their access to the cell membranes. In fact, LDPs can be partially adsorbed to the fungal cell wall by binding chitin and β-1,3-glucans, from where they could be released by the action of appropriate CWDEs and reach the nearby plasmalemma.

CWDEs used at a concentration giving a limited inhibition of the target fungi caused a dramatic increase of the efficacy of both LDPs, with SP_{25A} being 4 to 10 times more active than SR_E on a molar basis. This may be due to the fact that SP_{25A} is more efficient in altering membrane permeability because of its stronger lipophilic character respect to SR_E, and is in agreement with the fact that SP_{25A} is much more active than SR_E when tested on unilamellar liposomes.

The potential application of some *Pseudomonas* strains and their toxins, as post-harvest biocontrol agents on citrus and pome fruit (Bull et al. 1998), supports a growing research interest about the peculiar peptides produced by these bacteria. It has been recently reported (Bull et al. 1998) that SR_E may be the metabolite mainly responsible for the biocontrol activity of *P. syringae* strains that are the active ingredients of two biopesticide furmulations commercially available (EcoScience Corporation, Orlando FL). Despite the evidence presented so far, the role played by LDPs in the antifungal activity of this bacterium is not fully understood.

After many years of study on the antagonistic mechanism of *Pseudomonas* and *Trichoderma*, two of the most commonly found microorganisms in the agricultural environment, there is still a profound lack of knowledge on how this bacterium–fungus interaction affects plant health and disease development. A positive, possibly synergistic, interaction between these agents may frequently occur in nature and be responsible for a better biocontrol of plant pathogens. Furthermore, *Trichoderma*- or *Pseudomonas*–based biopesticides may be labeled as compatible with each other, and eventually proposed for a joint use. Such interaction between *Pseudomonas* and *Trichoderma* may involve, in addition to other mechanisms, a synergism between the antimicrobial compounds produced by the two agents. Further work, performed also with knock-out mutants, will eventually demonstrates if the synergism between *Pseudomonas* LDPs and *Trichoderma* CWDEs found in vitro also occurs in vivo, supporting a combined and highly effective biocontrol action.

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