***Pseudomonas* Lipodepsipeptides and Fungal Cell Wall–Degrading Enzymes Act Synergistically in Biological Control**

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Pseudomonas syringae pv. *syringae* strain B359 secreted two main lipodepsipeptides (LDPs), syringomycin E (SRE) and syringopeptin 25A (SP_{25A}), together with at least four types of cell wall–degrading enzymes (CWDEs). In antifungal bioassays, the purified toxins SRE and SP_{25A} interacted synergistically with chitinolytic and glucanolytic enzymes purified from the same bacterial strain or from the biocontrol fungus *Trichoderma atroviride* strain P1. The synergism between LDPs and CWDEs occurred against all seven different fungal species tested and *P. syringae* itself, with a level dependent on the enzyme used to permeabilize the microbial cell wall. The antifungal activity of SP_{25A} was much more increased by the CWDE action than was that of the smaller SRE, suggesting a stronger antifungal role for SP_{25A}. In vivo biocontrol assays were performed by using *P. syringae* alone or in combination with *T. atroviride*, including a *Trichoderma* endochitinase knock-out mutant in place of the wild type and a chitinase-specific enzyme inhibitor. These experiments clearly indicate that the synergistic interaction LDPs–CWDEs is involved in the antagonistic mechanism of *P. syringae*, and they support the concept that a more effective disease control is given by the combined action of the two agents.

Additional keywords: plasma membrane.

Recent research on necrosis-inducing phytopathogenic pseudomonads has led to the isolation and structural characterization of two different groups of cyclic lipodepsipeptides (LDPs) that are toxic for plants and microorganisms. One group is formed by nonapeptide lactones acylated by a long-chain, 3-hydroxy fatty acid and includes the syringomycins (SRs) (Ballio et al. 1988; Fukuchi et al. 1990a; Scaloni et al. 1994; Segre et al. 1989), the syringotoxins (STs) (Ballio et al. 1990, 1994a; Flamand et al. 1996; Fukuchi et al. 1990b), the syringostatins (Fukuchi et al. 1990c, 1992), and the pseudomycins (Ballio et al. 1994b; Coiro et al. 1998; Harrison et al. 1991). The other group contains lipopeptides formed by a long and highly hydrophobic peptide chain, C-terminated by a polar lactonized penta- or octa-peptide moiety; this group includes the tolaasins (Nutkins et al. 1991), the syringopeptins (SPs) (Ballio

et al. 1991, 1995; Isogai et al. 1995; Scaloni et al. 1997), the fuscopeptins (FPs) (Ballio et al. 1996; Baré et al. 1999), and the corpeptins (Emanuele et al. 1998).

The phytotoxicity and the antimicrobial activity of all these metabolites is due to their specific interference with the plasma membrane, as suggested for SR by Takemoto (1992), and also demonstrated by their effect on some membrane functions in higher plants (Che et al. 1992; Di Giorgio et al. 1994). Recent biophysical studies have demonstrated that LDPs form trans-membrane ion channels and consequently increase membrane permeability (Agner et al. 2000a, 2000b; Blasko et al. 1988; Brodey et al. 1991; Dalla Serra et al. 1999a, 1999b; Fegin et al. 1996, 1997; Hutchison and Gross 1997; Hutchison et al. 1995; Kaulin et al. 1998; Schagina et al. 1998).

The simultaneous synthesis of representatives of the two LDPs groups has been observed in some species of the genus *Pseudomonas*. This is particularly well documented for isolates of *P. syringae* pv. *syringae*, in which SRs or STs co-occur with SPs (Ballio et al. 1991), and for *P. fuscovaginae*, which produces both STs and FPs (Flamand et al. 1996).

A first comparative evaluation of some biological activities of SRs and SPs has demonstrated that syringomycin E (SRE) and syringomycin G (SRG) are more effective than syringopeptin 25A (SP_{25A}) in inhibiting the growth of *Rhodotorula pilimanae*, while SP_{25A} is much more phytotoxic than are the two nonapeptides (Iacobellis et al. 1992). These results have led to the generalization that the nonapeptides are mainly responsible for the antimicrobial activity and the syringopeptins for the phytotoxicity of partially purified extracts of *P. syringae* pv. *syringae* (Iacobellis et al. 1992). From a more extended comparison of the effects of the two groups of toxins on several physiological processes in higher plants (Di Giorgio et al. 1994, 1996a, 1996b) and some microorganisms (Lavermicocca et al. 1997), it seems that SPs are intrinsically more active than SRs (Camoni et al. 1995). However, SPs will be hindered in the access to the plasma membrane by the cell wall, the outer membrane of gram-negative bacteria, or both, because of their larger size and higher hydrophobicity than SRs.

The antibiotic properties of the SPs have been little investigated. SP_{25A} has been reported to be much less active than SRE and SRG on *R. pilimanae* (Iacobellis et al. 1992) and to have negligible activity on *Geotrichum candidum* (Lavermicocca et al. 1997). Fuscopeptin A was nearly as active as syringopeptin 22A (SP_{22A}) on *Botrytis cinerea*, moderately active on *R. pili-*

manae, and inactive on *G. candidum* (Ballio et al. 1996). Gram-negative bacteria insensitive to SRE and ST are also insensitive to SP_{22A} and SP_{25A}. Instead, both SPs inhibit the growth of the three gram-positive bacteria, which are only partially sensitive to SRE and ST (Lavermicocca et al. 1997). Tolaasin, the peptin produced by *P. tolaasii*, has a similar selectivity in antibacterial tests (Rainey et al. 1991, 1992). It becomes highly toxic to gram-negative bacteria when tested in a mixture with subminimal inhibitory quantities of polymyxin B, an antibiotic permeabilizing the outer membrane of gram-negative bacteria (Zhang et al. 2000); thus, this cell structure might represent the barrier responsible for the resistance to tolaasin. Accordingly, Rainey and colleagues (1992) found that tolaasin disrupts the protoplasts of both gram-positive and gram-negative bacteria.

In this respect, we speculate how the addition of cell wall-degrading enzymes (CWDEs) may enhance the efficacy of *Pseudomonas* LDPs by facilitating their access to the cell membranes. In this work, the inhibitory activity on both fungal growth and germination of *Pseudomonas* LDPs in combination with CWDEs was assayed against various species of plant-pathogenic and nonpathogenic fungi differing in cell wall composition. Enzymes used included an endochitinase (CHIT42) and a glucan β -1,3-glucosidase (GLUC78) purified from the biocontrol fungus *Trichoderma atroviride* (*harzianum*) strain P1, and CWDEs produced by the same *P. syringae* pv. *syringae*

strain from which the LDPs were purified. Mixtures of LDPs-CWDEs were also tested both on the CWDEs-producing *T. atroviride* strain, which has a low sensitivity to its own enzymes, and on the LDP-producing *P. syringae* pv. *syringae* strain, in the attempt to alter the sensitivity of this microorganism to its own toxins. The results presented indicate that enzymatic degradation of the cell wall permits both type of toxins, especially those with higher molecular weight (SPs), to reach their target and alter cell membrane functions much more effectively than in the absence of the enzymes. In addition, data are presented indicating that the synergism between CWDEs and LDPs may support the antagonistic mechanism of a *Pseudomonas* species or its positive interaction with a *Trichoderma* species during biological control.

RESULTS

LDPs and CWDEs produced by *P. syringae* pv. *syringae* strain B359.

High-pressure liquid chromatography-mass spectrometry (HPLC-MS) analysis of an acetone extract of the bacterial culture supernatant (Fig. 1A) confirmed the presence of two major LDPs: SRE and SP_{25A} with a molecular weight of 1,225 (Fig. 1B) and 2,400 (Fig. 1C), respectively (Ballio et al. 1991). HPLC analysis indicates that strain B359 produces a complex set of LDPs including different SRs and SPs. Medium replace-

Fig. 1. High-pressure liquid chromatography-mass spectrometry electrospray analysis of a culture filtrate of *Pseudomonas syringae* pv. *syringae* strain B359. **A**, Chromatographic profile of crude culture filtrate. **B**, Mass spectrum of a peak eluted at 20.7 min. **C**, Mass spectrum of a peak eluted at 36.0 min.

ment (discussed below) raised the level of SRs and SPs in the culture filtrate up to 35 and 60 $\mu\text{g ml}^{-1}$, respectively; thus almost doubling the highest value obtained without replacement. A similar effect was obtained with the addition of 1% glucose to the minimal medium (MM).

Under the cultural conditions used for the production of LDPs, strain B359 also accumulated different chitinolytic and glucanolytic enzymes. The enzyme activities detected in the culture filtrate included β -*N*-acetylhexosaminidase (EC 3.2.1.52) (or *N*-acetylglucosaminidase), chitin 1,4-chitobiosidase (hereafter designated chitobiosidase) (Harman et al. 1993), endochitinase (EC 3.2.1.14), and glucan β -1,3-glucosidase (EC 3.2.1.58). The production of CWDEs was already detectable after 1 day of growth and generally increased up to the seventh day of cultivation. The addition of 1% glucose did not significantly alter enzyme production. Medium replacement in glucose minimal medium (MMG) or chitin minimal medium (MMC) provided the highest levels of CWDEs in the culture liquid, particularly glucanase and endochitinase, respectively.

Chitin increased the level of glucanase and produced a culture filtrate with 30% more protein and additional bands in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). The production of CWDEs was very low when the bacterium was cultivated in the rich medium of Luria and Bertani (LB), where LDPs were not synthesized.

HPLC separation of the culture filtrate provided a fraction with *N*-acetylglucosaminidase and glucanase activities and showing two protein bands of 42 kDa (glucanase) and 45 kDa (β -*N*-acetylhexosaminidase) on SDS-PAGE (Fig. 2).

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Pseudomonas syringae* pv. *syringae* culture filtrate obtained on chitin minimal medium and after high-pressure liquid chromatography (HPLC) purification. Lane 1, Molecular mass markers (numbers indicate kDa); lane 2, HPLC-purified β -*N*-acetylhexosaminidase and glucan β -1,3-glucosidase; and lane 3, crude culture filtrate.

Antifungal synergism between *Pseudomonas* LDPs and *Trichoderma* CWDEs.

Both SRE and SP_{25A} showed antifungal activity against all the fungi tested at final concentrations ranging from about 0.15 to 1.5 $\mu\text{g ml}^{-1}$ (0.12 to 1.2 μM for SRE and 0.06 to 0.62 μM for SP_{25A}). The inhibitory effect of SRE on both spore germination and hyphal elongation was detectable at concentrations lower than those of SP_{25A} (50% effective dose [ED₅₀] values ranged from about 0.6 to 0.9 $\mu\text{g ml}^{-1}$ for SRE and about 0.8 to >1.5 $\mu\text{g ml}^{-1}$ for SP_{25A}), which often did not give a complete inhibition even at concentrations as high as 1.5 $\mu\text{g ml}^{-1}$ (Table 1).

Spore germination and hyphal elongation of the two vascular pathogens *Fusarium oxysporum* and *Verticillium dahliae* were inhibited by both toxins, although the effect of SRE appeared more dose dependent (Fig. 3A and B; data for *V. dahliae* not shown). The addition of endochitinase or glucanase purified from a *Trichoderma* species synergistically enhanced the antifungal activity of the *Pseudomonas* LDPs (Fig. 3A and B; Table 1). The resulting enhancement of antifungal activity was significantly higher with SP_{25A} compared with SRE, as indicated by the difference in relative synergism (RS) values shown in Table 1. For instance, the addition of endochitinase or glucanase to 0.1 μg of toxin per ml augmented six to eight times the inhibitory effect of SP_{25A} on spore germination and only twice that of SRE. A similar effect was obtained when the two polyphagous pathogens *B. cinerea* and *Penicillium expansum* were used as test fungi (Fig. 3C and D; data for *Penicillium expansum* not shown). In this case, the mixture of *Trichoderma* endochitinase and SP_{25A} was particularly synergistic at low concentrations (Fig. 3D; Table 1). Like for the other fungi, the sporangia germination of the oomycete *Phytophthora infestans* was inhibited by the treatment with the *Pseudomonas* LDPs, but the synergistic interaction occurred only with the glucanase (Fig. 3E and F; Table 1), in agreement with the fact that chitin is considered to be substantially absent in the cell wall of this fungus. Interestingly, the addition of endochitinase abolished the antifungal activity of the toxin against *Phytophthora infestans*. The synergistic interaction of SRE or SP_{25A} with *Trichoderma* CWDEs also affected the nonpathogenic yeast *R. pilimanae*. This microorganism was much more sensitive to SRE than to SP_{25A}, but the addition of a low amount of *Trichoderma* glucanase made the inhibitory effect of SP_{25A} significantly higher than that of SRE on the same dose basis (Table 1; data not shown).

The chitinase-specific inhibitor allosamidin (at 5 $\mu\text{g ml}^{-1}$) completely abrogated the endochitinase–LDPs synergistic interaction with all target fungi. This effect was not observed with the glucanase–LDPs combination (data not shown).

As previously reported (Lorito and Woo 1998; Lorito et al. 1993), *T. atroviride* strain P1 was not sensitive to its own 42-kDa endochitinase and 78-kDa glucanase, but its conidial germination and hyphal elongation were inhibited by the two *Pseudomonas* LDPs (particularly by SRE) applied alone (Fig. 4A and B). The addition of the two enzymes did not improve the inhibitory activity of the two toxins, even if the two enzymes were applied simultaneously in the mixture with the *Pseudomonas* LDPs (Fig. 4A and B; data not shown). Instead, a strong synergistic interaction was found when the toxins, and in particular SP_{25A}, were combined with a mixture (Novozyme; Novo Nordisk, Bagsvaerd, Denmark) of various CWDEs commonly used to obtain fungal protoplasts (Fig. 4B).

The growth of *P. syringae* pv. *syringae* strain B359 was not affected by the two toxins in the in vitro bioassay. The presence in the reaction mixture of a sublethal dose of lysozyme (Sigma, St. Louis, MO, U.S.A.), together with LDPs, resulted in a strong reduction of the bacterial growth (Fig. 4C and D). Similar to the results obtained with the fungal tar-

gets, the synergistic improvement of the inhibitory activity was higher for SP_{25A}, which was more effective already at low doses, than for SRE.

Antifungal synergism between *Pseudomonas* LDPs and CWDEs.

Pseudomonas culture filtrates that showed chitinolytic and glucanolytic activity were also inhibitory to *B. cinerea* spore germination and germ tube elongation in vitro. This activity was detectable at a concentration of about 60 µg ml⁻¹, with an ED₅₀ value ranging from 300 to about 600 µg ml⁻¹, depending on the medium used for the culture. A similar level of antifungal activity was reached by testing a HPLC fraction that contained both a β-*N*-acetylhexosaminidase and a glucanase (Fig. 2).

Combination of either culture filtrates or purified enzymes synergistically enhanced the antifungal activity of both LDPs, and in particular that of SP_{25A}, as reported above for *Trichoderma* CWDEs. The filtrates, obtained from cultures in MM, MMG, and MMC, tested at a concentration giving 10 to 15% inhibition, were assayed in combination with the two LDPs at a concentration of 0.15 µg ml⁻¹. The inhibition values obtained for *B. cinerea* spore germination in combination with SP_{25A} were 35% with the MM filtrate, 77% with the MMG filtrate, and 100% with the MMC filtrate; the values in combination with SRE were 20% for MM, 49% for MMG, and 65% for MMC. The amount of enzyme needed to obtain complete inhibition of *B. cinerea* in combination with the LDPs was much higher for bacterial than fungal CWDEs. Allosamidin in the assay mixture caused an almost complete loss of the synergistic interaction between the bacterial filtrate or the chitinase-containing HPLC fraction and either one of the LDPs (data not shown), indicating that chitinase activity increases the fungal cell susceptibility to the toxins.

Biocontrol synergism in vivo between *P. syringae* and *T. atroviride*, and role of chitinase.

In vivo bioassays on apples artificially infected with *B. cinerea* spores were performed in conditions that gave a modest level of disease control when either the *P. syringae* pv. *syringae* strain B359 or the *T. atroviride* strain P1 were used alone (Fig. 5). A similar effect was obtained with the *T. atroviride* endochitinase-minus mutant D11. In these conditions, simultaneous application of *P. syringae* and *T. atroviride* resulted in a strongly synergistic improvement of the biological control (Fig. 5). When the endochitinase-minus mutant was used instead of the wild type, much lower pathogen control was obtained, compared with strain P1 treatment. A similar low level of synergism was found when the two biocontrol agents, *P. syringae* and *T. atroviride* (wild type), were applied together with the chitinase inhibitor allosamidin. This indicates an involvement of chitinase activity in the positive biocontrol interaction of a *Pseudomonas* species and a *Trichoderma* species against a *Botrytis* species. The biocontrol effect of the bacterium alone was also diminished by the presence of the chitinase inhibitor, suggesting that, in our test conditions, CWDEs play a role in the antagonistic mechanism of *P. syringae*.

DISCUSSION

P. syringae pv. *syringae* strain B359 is a well-known producer of LDPs in static culture (Monti et al. 2001; Surico and DeVay 1982). Production was improved by transferring the biomass grown in LB medium to a fresh MM. This procedure might be useful in studies requiring large amounts of crude or purified LDPs, like those concerning their potential use in human medicine (De Lucca et al. 1999; Zhang et al. 2001) or the

development of new biocontrol products (Bull et al. 1998). Interestingly, the replacement culture technique also positively influenced the synthesis of CWDEs, in particular glucanase and endochitinase. A further stimulation of enzyme production, together with a general increase of protein content in the culture filtrates, was obtained when chitin was the main carbon source. The higher concentration of CWDEs is of practical value, since it makes their purification easier.

Previous work on *Trichoderma* metabolites has shown that CWDEs and membrane-acting peptides (i.e., trichorzianines and other peptaibols), concurrently produced during biological control, interact synergistically as antifungal agents (Lorito et al. 1996a). 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. It has been recently reported that LDPs bind to chitin, β-1,3-glucans, or both, and are thus adsorbed by the fungal cell (De Lucca et al. 1999); this interaction is impaired by adding appropriate CWDEs and, thus, the unbound LDPs can affect the adjacent plasma membrane.

When assayed on various fungi as inhibitory agents without the enzyme, the “smaller” SR was generally more effective than the “larger” SP, being the ED₅₀ for SRE was always lower than that for SP_{25A} (Fig. 3; Table 1). CWDEs used at a concentration causing a limited inhibition of the target fungi dramatically increased the efficacy of both LDPs, with SP_{25A} being four to ten times more active than SRE on a molar basis. The higher activity levels of SP_{25A} compared with SRE is in agreement with the fact that SP_{25A} is much more active than SRE when tested on unilamellar liposomes (Camoni et al. 1995) and that SP_{25A} has a higher affinity than SRE for a preformed lipid layer (Dalla Serra et al. 1999a).

The effectiveness of the various combinations of LDPs and CWDEs was correlated with the cell wall composition of each target fungus. In the *Fusarium* and *Botrytis* species, the addition of glucanase or chitinase determined a marked increase of LDPs activity, and in particular of SP_{25A}. As expected, the synergistic effect on the *Phytophthora* species was observed only with glucanase, while chitinase seemed to inhibit the action of LDPs.

The experiments with the *Trichoderma* and *Pseudomonas* species as targets further confirmed that the integrity of the cell wall strongly reduces the efficacy of LDP toxins. No synergism was found on the *Trichoderma* species, because the fungus cell wall is protected from the action of its own enzymes, unless an appropriate CWDE mixture is applied. Similarly, the addition of lysozyme at a sublethal dose was required to make *P. syringae* pv. *syringae* strain B359 sensitive to its own toxins. The particularly high increase of the SP_{25A} effect on strain B359 induced by lysozyme suggests a very low permeability of the bacterial outer membrane to LDPs.

The data presented here explain the apparent discrepancy concerning the different antimicrobial activities of the two classes of LDPs (Bender et al. 1999; Iacobellis et al. 1992; Lavermicocca et al. 1997), which contrast with the similar pore-forming ability of the two types of toxins on protoplasts, erythrocytes, and artificial bilayers (Hutchinson and Gross 1997; Hutchinson et al. 1995). We propose that the biological activity of each toxin on intact cells is conditioned by the permeability of the cell wall/outer membrane, which influences the access of LDPs to the cell.

The possible use of some *Pseudomonas* strains or their toxins as biocontrol agents on postharvest citrus and pome fruit also sustains the research interest about the peptides produced by these bacteria. It has been recently reported (Bull et al. 1998; Whipps 1997) that SRE may be the metabolite mainly responsible for the biocontrol activity of two *P. syringae* strains

contained as active ingredients in commercially available biopesticide formulations (EcoScience Corporation, Orlando, FL, U.S.A.). Despite the evidence presented so far, the role played by LDPs in the antifungal activity of these bacteria is not fully understood. These toxins are proposed to be major virulence determinants in *Pseudomonas* pathogenic strains (Bender et al. 1999; Scholz-Schroeder et al. 2001; Takemoto 1992), but their production during plant tissue colonization is still not proven. Attempts to recover the *Pseudomonas* toxins from attacked plants were unsuccessful, although infected tissues gave a positive reaction, with antibodies raised against SPs and SRs (Fogliano et al. 1999; Gallo et al. 2000).

The molecular mechanism that may support the antifungal capabilities of *P. syringae* has been attributed mainly to the production of toxins such as LDPs (Bender et al. 1999). Although this bacterium and other pseudomonads are known to be CWDE producers, the role of such enzymes in biological control as well as in pathogenicity is poorly understood (Whipps 1997). The results presented here suggest that CWDEs are used by *P. syringae* to synergize the fungicidal action of its toxins during interaction with other microbes. In fact, (i) LDP and CWDE production can be induced at the same time; (ii) the main LDPs produced by strain B359 synergistically interact with the enzymes against a variety of soilborne and leaf fungi, with different efficacy depending on the level of cell wall degradation; (iii) the use of CWDEs more potent than those from the *Pseudomonas* species further increases the synergism and the inhibitory effect of the mixture; (iv) specific inhibition of chitinase activity in CWDE–LDP mixtures knocks out the synergism and significantly reduces the antifungal effect; and (v) chitinase inhibition reduces the biocontrol effect of *P. syringae* in vivo.

Despite the many years of research spent to understand the antagonistic mechanisms of the *Pseudomonas* and *Trichoderma* species, two of the most commonly found microorganisms in the agricultural environment, there is a profound lack

of knowledge on how this bacterium–fungus interaction affects plant health and disease development. A positive, possibly synergistic, interaction between the *Trichoderma* species and biocontrol bacteria, including some *Pseudomonas* species, has been previously reported for combined applications in the control of plants pathogens (Whipps 1997). Furthermore, *Trichoderma*- or *Pseudomonas*-based biopesticides may be labeled as compatible with each other and even proposed for a joint use. Our findings suggest that the interaction between the *Pseudomonas* and *Trichoderma* species, established in appropriate application conditions, involves a synergism between the antimicrobial compounds produced by the two microorganisms, possibly in addition to other biocontrol mechanisms (i.e., exclusion of the pathogen from the infection site or competition for the substrate). The synergism between *Pseudomonas* LDPs and *Trichoderma* CWDEs that we found in vitro against a variety of fungi seems to also occur in vivo and supports a combined and more effective biocontrol action of the two agents. In addition, in vivo experiments with a chitinase inhibitor and a gene knock-out mutant clearly indicate the involvement of specific CWDEs (chitinase) activity in the biocontrol interaction between the two microbes. Therefore, it is tempting to speculate that the membrane-affecting toxins and CWDEs, simultaneously released by the *Pseudomonas* and *Trichoderma* species, form inhibitory mixtures with much more pronounced activity on microbes other than the two producing strains.

MATERIALS AND METHODS

Organisms and culture conditions.

Strain B359 of *P. syringae* pv. *syringae* (from the collection of the Department of Plant Pathology, University of California, Davis, U.S.A.) was used as a source of LDPs and for postharvest biocontrol experiments (Surico and DeVay 1982). For induction of LDPs and CWDEs, the bacteria were grown in three different media at 28°C in static conditions: namely in a MM,

Table 1. Fifty percent effective dose (ED₅₀) for inhibition of spore germination or bacteria colony growth and the relative level of synergism (RS) of mixtures containing varying concentrations of *Pseudomonas* lipodepsipeptides (LDPs) and given concentrations of cell wall-degrading enzymes (CWDEs)^a

Microorganism tested	SRE alone	Enzyme added ^b	SRE + CWDEs ^c		SP _{25A} alone	Enzyme added	SP _{25A} + CWDEs	
	ED ₅₀		ED ₅₀	RS	ED ₅₀		ED ₅₀	RS
<i>Fusarium oxysporum</i>	0.56	Endochitinase	0.24	19.3	0.8	Endochitinase	0.05	35.1
		Glucanase	0.19	25.6		Glucanase	0.06	33.7
<i>Verticillium dahliae</i>	0.61	Endochitinase	0.22	22.5	0.95	Endochitinase	0.10	36
		Glucanase	0.25	18.5		Glucanase	0.11	35.5
<i>Botrytis cinerea</i>	0.92	Endochitinase	0.29	20.2	>1.5	Endochitinase	0.12	38
		Glucanase	0.43	16.7		Glucanase	0.32	31.5
<i>Penicillium expansum</i>	0.48	Endochitinase	0.14	21.5	1.15	Endochitinase	0.03	38.5
		Glucanase	0.18	16.5		Glucanase	0.06	37
<i>Phytophthora infestans</i>	0.61	Endochitinase	>1.5	<0	0.76	Endochitinase	>1.5	<0
		Glucanase	0.26	13.9		Glucanase	0.06	33.7
<i>Rhodotorula pilimanae</i>	0.29	Endochitinase	0.22	4.5	1.45	Endochitinase	1.29	7
		Glucanase	0.16	17.5		Glucanase	0.10	36
<i>Trichoderma atroviride</i> (harzianum)	0.38	Endochitinase	0.36	<0	>1.5	Endochitinase	>1.50	0.5
		Glucanase	0.30	<0		Glucanase	1.42	1.5
		Novozyme	0.12	15.8		Novozyme	0.05	35.6
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	>1.5	Lysozyme	0.31	30.5	>1.5	Lysozyme	0.10	32

^a The varying concentrations are given as micrograms per milliliter. The *Pseudomonas* LDPs are syringomycin E (SRE) and syringopeptin 25A (SP_{25A}) and the given concentrations of CWDEs are from various sources (discussed in text). Similar results were obtained for the inhibition of hyphal elongation in the filamentous fungi tested.

^b Endochitinase and glucanase = 42-kDa endochitinase and 78-kDa glucan β -1,3-glucosidase, respectively, from *Trichoderma atroviride* (harzianum) strain P1; and novozyme and lysozyme = commercial preparations of degrading enzymes from Novo Nordisk (Bagsvaerd, Denmark) and Sigma (St. Louis, MO, U.S.A.), respectively.

^c CWDEs were applied at a concentration of 1 to 50 μ g ml⁻¹ giving about 10% inhibition when used alone (except for endochitinase and glucanase used on *T. atroviride* and endochitinase on *Phytophthora infestans*, applied at 300 μ g ml⁻¹); RS values were calculated as described by Lorito and colleagues (1996a) and range from 0 (no synergism) to 40 (maximum value).

prepared as described by Surico and colleagues (1988) containing 55 mM mannitol and 20 mM histidine, or MM supplemented with 1% glucose (MMG) or with 2% crab shell colloidal chitin (MMC). The production of toxins and CWDEs was also tested on standard LB medium. Replacement cultures were made with bacteria grown in LB medium for 4 days, recovered by centrifugation, and transferred to MMG or MMC. The production of LDPs and CWDEs was monitored daily for up to 9 days.

Strain P1 of *T. atroviride* (*harzianum*) (ATCC 74858, morphologically classified as *T. harzianum* and as *T. atroviride* according to recent genome sequence analysis) was grown in the previously described synthetic liquid medium used for the preparation of endochitinase and glucanase (Harman et al. 1993; Lorito et al. 1994). Mutant D11, obtained from strain P1 by homologous gene disruption of the endochitinase-encoding *chit42* gene, produces the same enzymes of the wild type but not of the endochitinase (Woo et al. 1999).

Fig. 3. Effect of *Pseudomonas syringae* pv. *syringae* lipodepsipeptides (LDPs) (syringomycin E [SRE] and syringopeptin 25A [SP_{25A}]), applied alone and in combination with an endochitinase or a glucan β -1,3-glucosidase from *Trichoderma atroviride*, on spore germination of **A and B**, *Fusarium oxysporum*; **C and D**, *Botrytis cinerea*; and **E and F**, *Phytophthora infestans*. Cell wall-degrading enzymes were applied at a concentration giving about 10% inhibition when used alone. Data are presented as means $\pm$ standard deviation. Similar results were obtained for inhibition of hyphal elongation.

The following microorganisms, obtained from the culture collection of Department of Arboriculture, Botany and Plant Pathology of the University of Naples, were used as targets for the in vitro bioassay: (i) *F. oxysporum* isolated from bean, *V. dahliae* isolated from carnation, *B. cinerea* isolated from grape, *Penicillium expansum* isolated from orange, and *T. atroviride* (*harzianum*) strain P1: conidia of all these fungi were harvested from potato dextrose agar (PDA) cultures grown at 25°C, resuspended in water, and filtered to remove hyphal fragments; (ii) *Phytophthora infestans* isolated from potato: sporangia were obtained by pregrowing the strain on potato dextrose broth, transferring the culture in a salt-based medium at the required temperature, and harvesting after 4 days by centrifugation, and they were then used for germination tests; (iii) *R. pilimanae*: the cells were grown on PDA at 25°C, harvested after 1 week, and resuspended in sterile distilled water; (iv) *P. syringae* pv. *syringae* strain B359: bacterial cells were grown as described above, harvested after 48 h of incubation, and resuspended in sterile distilled water. A strain of *B. cinerea* originally isolated from apple was used for postharvest experiments.

Identification of LDPs and their quantification in culture filtrates.

LDPs were identified by HPLC carried out on acetone extracts of culture supernatants by using a Perkin-Elmer apparatus equipped with two series 200 micro LC pumps and UV detection coupled with an API-100 single quadrupole mass spectrometer (Sciex, Ottawa, Ontario, Canada) and an atmospheric pressure ionization source. After filtration through an Acrodisc 0.22- μ m disk (Millipore, Bedford, MA, U.S.A.), each sample (20 μ l) was fractionated by reverse-phase-HPLC according to Ballio and colleagues (1991) on an Aquapore RP300 column (2.1-mm inside diameter; Brownlee [Perkin-Elmer], Boston, MA, U.S.A.) at a flow rate of 0.2 ml min⁻¹. The eluate was monitored at 220 nm, and 30 μ l was split into the ion source; a probe voltage of 4.8 kV and a declustering potential of 70 V were used for LDP ionization. The mass-to-charge ratio scale of the instrument was calibrated with the ions of polypropylene glycol ammonium adducts. Acquisition was made in the positive ion mode with a dwell time of 0.5 ms and a step size of 0.5 atomic mass unit (amu). Each scan was acquired from 500 to 2,500 amu. Data were processed through the Bio Multi View software (Sciex).

Fig. 4. Effect of *Pseudomonas syringae* pv. *syringae* lipodepsipeptides (LDPs) (syringomycin E [SRE] and syringopeptin 25A [SP_{25A}]) **A and B**, on spore germination of *Trichoderma atroviride* strain P1 and **C and D**, on growth of *P. syringae* pv. *syringae* strain B359. Bacterial toxins were used either alone or in combination with an endochitinase; a glucan β -1,3-glucosidase obtained from the same *Trichoderma* strain; **A and B**, the commercial cell wall-degrading enzymes preparation of Novozyme; or **C and D**, a lysozyme. The enzymes were applied at a concentration giving about 10% inhibition when used alone. Data are presented as means $\pm$ standard deviation. Similar results were obtained for inhibition of hyphal elongation for *T. atroviride*.

Preparative isolation of the toxins was performed as previously described (Ballio et al. 1991). Toxin purity was checked by HPLC with diode-array detection. The amount of the two types of LDPs in the culture filtrates was determined by competitive enzyme-linked immunosorbent assay (ELISA), using polyclonal antibodies specific either for SRs or SPs (Fogliano et al. 1999; Gallo et al. 2000).

Production of *Trichoderma* CWDEs.

The 42-kDa endochitinase and the 78-kDa glucanase were purified from the culture filtrate of *T. atroviride* (*harzianum*) strain P1 as described earlier (Harman et al. 1993; Lorito et al. 1994) and dissolved in sterile distilled water. The purity of enzyme solutions was assessed by SDS-PAGE, followed by silver staining. Assays and nomenclature of chitinolytic and glucanolytic enzymes were as reported by Harman and colleagues (1993). The endochitinase activity was determined by measuring the percent reduction in turbidity of a suspension of colloidal chitin, and the glucosidase activity was determined by assaying the reducing sugars released from laminarin (Sigma) (Harman et al. 1993; Lorito et al. 1994). Enzyme solutions utilized for the bioassays were kept for no more than 2 weeks at 4°C or concentrated to dryness in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY, U.S.A.) and stored at -20°C.

Production and purification of *Pseudomonas* CWDE.

P. syringae pv. *syringae* strain B359 culture filtrate obtained in the conditions described above for the production of LDPs were dialyzed and lyophilized, and the solid residue was resuspended in 20 mM sodium phosphate buffer, pH 7.4, at a protein concentration of 5 mg ml⁻¹. The crude mixture was fractionated by HPLC on a DEAE-5PW anion exchange column (Bio-Rad Laboratories, Richmond, CA, U.S.A.), run with 10 mM sodium phosphate buffer, pH 7.4, at a flow rate of 1 ml min⁻¹.

After 10 min, further elution was achieved by a 45-min linear gradient of up to 0.5 M sodium chloride. The eluate was monitored at 220 nm and each peak was manually collected, dialyzed, and lyophilized. Fractions were examined by SDS-PAGE and assayed for CWDE activities as described above for *Trichoderma* enzymes.

Biological assays.

In vitro bioassays were performed as described by Lorito and colleagues (1996b). The flat-bottomed ELISA plates containing the assay mixtures were observed at different times with an inverted microscope to follow spore germination and germ tube elongation. The assays with *P. syringae* pv. *syringae* strain B359 were performed by measuring the reduction in absorbance of the culture grown in a sterile ELISA plate at 27°C on a rotary shaker at 200 rpm. The assay mixtures (100 µl) contained about 1,000 bacterial cells suspended in LB medium dissolved in 66 mM potassium phosphate buffer, pH 6.2. A calibration curve correlating the number of cells with the absorbance at 495 nm were included in all assays. Bioassays were conducted by reading the absorbance at 6 and 12 h, times that correspond, in the untreated control, to a mid to late log phase. The initial absorbance was measured for each assay mixture, and the plate was incubated in a humid chamber. Bacterial growth was measured by subtracting the reading at zero time from that at 6 or 12 h. Bacterial cell density was also evaluated microscopically by transferring aliquots of each sample to a Hausser counting chamber (Hausser Scientific Company, Hoarsham, PA, U.S.A.).

In addition to the 42-kDa endochitinase and the 78-kDa glucanase from the *Trichoderma* species, two commercial preparations of CWDEs commonly used to digest fungal (Novozyme) or bacterial (lysozyme) cell walls were tested. The assays conducted with mixtures of CWDEs and bacterial LDPs contained an increasing dose of toxins and a given

Fig. 5. Postharvest biological control of *Botrytis cinerea* on apple fruit by *Pseudomonas syringae* pv. *syringae* (strain B359) in combination with *Trichoderma atroviride* strain P1. The effect of chitinase inhibition was assessed with the biocontrol assay containing strain D11, an endochitinase-minus mutant of wild-type strain P1. Controls included treatments with water in the absence of biocontrol microbes or allosamidin (allos). Data are presented as means $\pm$ standard deviation.

amount of enzyme preparation at a concentration producing 10% inhibition when applied alone. This concentration was determined by first performing dose-response experiments with the enzymes alone, as already reported for the *Trichoderma* endochitinase or glucanase with various fungal pathogens (Lorito et al. 1993). All bioassays included controls containing sterile water instead of the compounds to be tested. Treatments and controls were performed simultaneously in a single experiment. Each experiment was repeated on two separate days in triplicate. The values from the replicates and the data from the two experiments were combined for statistical analyses.

Postharvest assays were performed on apple var. Annurca against *B. cinerea*. Fruits with no visible wounds were selected, stored at 15°C, and washed in water before the experiment. A surface-sterilized steel rod was used to make three 2-mm-deep × 1-mm-wide punctures on each fruit. *B. cinerea* spores, obtained on PDA, were suspended in a small volume of sterile water at a concentration of 1×10^6 spores per ml. A 20-μl drop of this suspension was placed in the wounds and allowed to dry before application of the biocontrol agents. *P. syringae* cells obtained from an overnight culture (27°C on MMC) were centrifuged and resuspended in sterile water at 1 to 3×10^9 CFU ml⁻¹, and a 20-μl drop of this suspension was applied to the wounds and allowed to dry. Spore suspensions of *T. atroviride* strain P1 and the its endochitinase-minus mutant D11 (Woo et al. 1999) were also obtained on PDA, suspended in water at 1×10^6 spores per ml, and applied, alone or together with the bacteria, as described for the pathogen *B. cinerea*. Inoculated apples were properly spaced in plastic trays and incubated for 5 days at 21°C and 80% relative humidity.

The chitinase-specific inhibitor allosamidin (a kind gift of S. Sakuda, University of Tokyo) was used both in in vitro and postharvest biocontrol tests. The drug, dissolved in 0.1 M phosphate buffer, pH 6.5, was used in the assay at a final concentration of 5 μg ml⁻¹. This concentration was previously determined to be sufficient for an at-least-90% inhibition of chitinolytic activity in the culture filtrates of both *T. atroviride* and *P. syringae*.

Analysis of data.

The values relative to spore germination and germ tube elongation obtained for the control were considered as no inhibition; all other values were divided by these and multiplied by 100 to obtain percent inhibitions. The percent inhibition of bacterial growth was calculated in a similar way by taking the absorbance value of the control as no inhibition, since there was a linear relationship between the values of the absorbance at 495 nm obtained in the bioassays and the number of bacterial cells in the growing cultures. For each treatment, dosage-response curves and ED₅₀ values were calculated by probit analysis of the data. The relative level of synergism (RS) for each CWDE + LDP combination was calculated as described by Lorito and colleagues (1996a), applying the formula $RS = 50 - (10 + Y - Y/10)$, in which 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% inhibition when used alone. For less synergism, the RS value approaches zero, while 40 is the highest possible value. It should be noted that the level of synergism calculated is not absolute, since each enzyme preparation was used at only one concentration, and the comparison between Eo (observed effect) and Ee (expected effect) values was arbitrarily done for Eo = 50 (Richer 1987).

For postharvest assays on apple, each treatment was performed in triplicate on 25 fruits, and the percentage of infected

wounds was determined for each replicate and averaged. Each experiment was repeated twice, and the results of the two experiments were combined for calculating final mean and standard deviations.

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