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Synergistic Interaction Between Cell Wall Degrading Enzymes and Membrane Affecting Compounds

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A number of cell wall degrading enzymes (CWDEs) and cell membrane affecting compounds (MACs) that alter cell membrane structure or permeability have been assayed in vitro against phytopathogenic fungi and bacteria. Osmotin, gramicidin, valinomycin, phospholipase B, trichorzianine A1, trichorzianine B1, gliotoxin, flusilazole, and miconazole were tested in combination with three endochitinases, four exochitinases, and one glucan 1,3-β-glucosidase from fungi, bacteria, or plants. Every combination of MAC + CWDE showed a high level of inhibition against *Botrytis cinerea* and *Fusarium oxysporum* and the interaction between the two kinds of compounds was of a synergistic nature. Different levels of synergism were obtained among the compound combinations depending upon the antifungal activity of the enzyme. When the enzyme treatment was applied subsequent to the MAC, the level of synergism was lower, indicating that degradation of the cell wall is needed to establish the synergistic interaction. The synergism with MACs was also present when the fungal cell wall was altered in a non-enzymatic manner by including L-sorbose in the growth media. The sensitivity of bacterial strains to the two trichorzianines depended upon the nature of their cell wall and could be synergistically enhanced by partial digestion of the wall. Some of the combinations showed a high level of synergism, suggesting that the interaction between MACs and CWDEs could be involved in biocontrol processes and plant self-defense mechanisms.

Additional keywords: Gliocladium, Trichoderma.

The antifungal activity of some compounds is due to their ability to affect the function or the structure of the plasmalemma and other membranes of the fungal cell. These compounds, often indicated as antibiotics, may include small molecules and peptides, proteins, enzymes, and chemical pesticides. Cell wall degrading enzymes (CWDEs) produced by plants, bacteria, and fungi are also powerful antifungal agents in vitro, especially if mixtures of different enzymes are applied (Broadway et al. 1995; Mauch et al. 1988; Lorito et al. 1994a; Lorito et al. 1993a). Some membrane affecting compounds (MACs) and CWDEs are able to interact synergistically in the inhibition of pathogenic fungi and it has been suggested that this synergism is involved in both plant defense and microbial biocontrol mechanisms (Lorito et al. 1994c; Schirmböck et al. 1994). In previous studies, we showed that certain enzymes from *Trichoderma* spp. and *Gliocladium* spp. strongly enhance the fungicidal effect of commonly used inhibitors of sterol synthesis, which alter membrane integrity and structure (Lorito et al. 1994c). Gliotoxin, which selectively attacks thiol groups located on cell membranes (Jones and Hancock 1988), was found to be synergistic in the inhibition of fungi with the addition of endochitinase from *G. virens* in vitro (Di Pietro et al. 1993; Lorito et al. 1994c). Moreover, the antifungal activity of peptaibol antibiotics, trichorzianine A1 and trichorzianine B1, was strongly enhanced by the addition of CWDEs purified from biocontrol fungi (Schirmböck et al. 1994).

The purpose of the present work was to determine if many different kinds of MACs and CWDEs are able to interact synergistically against different fungi and bacteria and to investigate the mechanism of this interaction. We examined synergism between CWDEs from fungi, bacteria, and plants in combination with gramicidin, valinomycin, osmotin, phospholipase, peptaibol antibiotics, or azole fungicides. Gramicidin, valinomycin, and phospholipase B are known to induce depolarization or damage the lipid structure of the cell membrane, and they can be considered typical MACs. Osmotin, a pathogenesis related (PR)–protein that is produced by plants under different stress conditions, is apparently involved in plant defense against pathogens (Liu et al. 1994). There is evidence suggesting that osmotin alters the plasma membrane and the osmotic equilibrium of the cell (Woloshuk et al. 1991; Liu et al. 1994). Peptaibol antibiotics, such as trichorzianines, depolarize the cell membrane by forming voltage-gated ion channels and modify membrane permeability (El-Hajji et al. 1989). They are active against fungi (Schirmböck et al. 1994) and Gram-positive bacteria, while Gram-negative cells are normally less sensitive since the antibiotics have difficulty in penetrating the outer membrane (OM) (El-Hajji et al. 1989). We tested combinations of trichorzianines and compounds that alter bacterial cell walls or OM to determine if there is a relationship between penetration of inhibitory compounds and the occurrence of synergism. Flusilazole and miconazole are azole fungicides widely used against fungal diseases of plants, animals, or humans. They affect membrane integrity

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by inhibiting a demethylation step in the synthesis of sterols in fungi (Köller 1992). L-sorbose added to the growth medium alters the cell wall integrity of *Trichoderma* by affecting β-1,3 glucan synthase activity and thereby reducing the wall content of β-1,3 glucans (Kubicek 1983). L-sorbose was included in this study in combination with some MACs to determine if there was a synergistic effect caused by a nonenzymatic alteration of the cell wall. We demonstrated that a synergistic interaction between CWDEs and MACs inhibits the growth of bacterial cells according to the complexity of the cell wall and reduces the germination of fungi when L-sorbose is used to alter the cell wall instead of enzymes. We propose a hypothetical model to explain the synergism observed and consider the interaction between CWDEs and MACs for the engineering of disease-resistant crops and new biocontrol agents.

RESULTS

All combinations of fungal CWDEs and MACs inhibited spore germination of *Botrytis cinerea* Pers.:Fr. and *Fusarium oxysporum* Schlechtend.:Fr. more strongly than the compounds used alone (Tables 1 and 2). Results for the inhibition of germ tube elongation were essentially consistent with those for the inhibition of spore germination of both fungi (data not shown). The calculated values for effective dose producing 50% inhibition (ED_{50}) were always lower for the

Table 1. Fifty percent effective dose (ED_{50}) for inhibition of *Botrytis cinerea* spore germination and relative level of synergism (RS) of mixtures containing varying concentrations of membrane affecting compounds (MACs) and given concentrations of cell wall degrading enzymes (CWDEs)

Compound mixtures[a]	ED_{50} (µg/ml)[b]	RS[c]	Compound mixtures[a]	ED_{50} (µg/ml)[b]	RS[c]
Osmotin +	10.0 (8 – 12)	–	Endoc G	13.1 (10.5 – 15)	20.0 (23.8 – 19)
Endoc T (11)	0.7 (0.6 – 0.8)	30.0 (34.6 – 27.4)	Chitob T	27.0 (24.5 – 29)	15.3 (17.5 – 14)
Endoc G (3.5)	0.5 (0.2 – 0.6)	37.3 (40 – 34)	Chitob G	29.0 (26 – 32)	14.0 (16.6 – 13)
Chitob T (25)	1.0 (0.9 – 1.2)	22.0 (23.8 – 21)	NAGas T	22.2 (20 – 24)	18.3 (19 – 17)
Chitob G (29)	3.0 (2.5 – 3.4)	15.7 (17.5 – 13)	Glucos T	22.0 (19 – 25)	18.5 (19 – 17.5)
NAGas T (17)	0.6 (0.4 – 0.7)	34.6 (36 – 30)	Endoc P	29 (26.5 – 31)	14.0 (16.5 – 12)
Glucos T (20)	0.3 (0.2 – 0.5)	40.0 (40 – 37.3)	Chitob S	20.1 (18 – 22)	19.1 (18 – 18.5)
Endoc P (32)	0.3 (0.1 – 0.5)	40.0 (40 – 37.3)			
Chitob S (4)	5.0 (4.2 – 5.5)	11.0 (13 – 10.4)	Trich B1 +	95.2 (92 – 99)	–
			Endoc T	25 (23 – 27)	23.8 (25.6 – 22)
Gramicidin +	>300	–	Endoc G	30 (28 – 31.5)	19.5 (21 – 18)
Endoc T	25 (20 – 29)	34.6 (36.4 – 30.1)	Chitob T	35 (34 – 36)	16.8 (15 – 17)
Endoc G	28 (25 – 30)	31.0 (34.6 – 29.2)	Chitob G	36 (33 – 38.5)	16.5 (18 – 15.5)
Chitob T	47 (46 – 49)	22.9 (24.7 – 22.1)	NAGas T	28 (24 – 31)	21.9 (26 – 18)
Chitob G	60 (57 – 62)	17.5 (21.2 – 16.6)	Glucos T	33 (32 – 34)	17.3 (18 – 17)
NAGas T	30 (29 – 31)	29.2 (30.1 – 28.3)	Endoc P	52 (50 – 55)	11.2 (13 – 10)
Glucos T	27 (24 – 29)	32.8 (35.5 – 30.1)	Chitob S	30 (27 – 32)	19.5 (22 – 18)
Endoc P	47 (44 – 49)	22.9 (24.7 – 22.1)			
Chitob S	30 (27 – 33)	29.2 (32.8 – 28.3)	Gliotoxin +	1.2 (1.1 – 1.3)	–
			Endoc T	0.1 (0.01 – 0.2)	39.1 (40 – 39)
Valinomycin +	7.3 (7.0 – 7.6)	–	Endoc G	0.01(0.01 – 0.04)	40.0 (40 – 40)
Endoc T	1.0 (0.9 – 1.1)	40.0 (40 – 39.1)	Chitob T	0.7 (0.6 – 0.8)	38.5 (39 – 38)
Endoc G	1.5 (1.3 – 1.7)	36.4 (39.1 – 35.5)	Chitob G	0.9 (0.8 – 1)	32.8 (38 – 22)
Chitob T	5.1 (4.8 – 5.3)	26.5 (28.3 – 22)	NAGas T	0.18 (0.1 – 0.2)	39.1 (40 – 39)
Chitob G	5.5 (5.4 – 5.7)	23.8 (24.7 – 18.4)	Glucos T	0.8 (0.8 – 0.9)	37.3 (37.3 – 32)
NAGas T	1.8 (1.7 – 1.9)	32.8 (35.5 – 31.9)			
Glucos T	1.2 (1.0 – 1.4)	39.1 (40 – 37.3)	Flusilazole +	68 (61 – 85)[d]	–
Endoc P	2.5 (2.2 – 2.6)	29.2 (31 – 28.3)	Endoc T	0.6 (0.4 – 0.8)	40.0 (40 – 39)
Chitob S	5.6 (5.5 – 5.7)	19.7 (23.8 – 18.4)	Endoc G	0.7 (0.7 – 0.8)	40.0 (40 – 39)
			Chitob T	1.9 (1.6 – 2.1)	32.8 (34 – 30)
Phospholip +	>300	–	Chitob G	5 (4 – 6.5)	23.8 (26 – 20)
Endoc T	25 (21 – 28)	40.0 (40 – 40)	NAGas T	0.9 (0.8 – 1)	38.8 (39 – 37)
Endoc G	40 (36 – 44)	38.2 (40 – 31.9)	Glucos T	0.8 (0.7 – 1)	39.0 (40 – 37)
Chitob T	55 (51 – 58)	31.0 (31.9 – 29.2)			
Chitob G	43 (42 – 46)	32.8 (34.6 – 31.9)	Miconazole +	3.0 (2 – 4)	–
NAGas T	32 (29 – 34)	40.0 (40 – 40)	Endoc T	0.07 (0.06 – 0.08)	37.3 (39 – 36)
Glucos T	31 (30 – 32)	40.0 (40 – 40)	Endoc G	0.01 (0.01 – 0.03)	39.5 (40 – 39)
Endoc P	120 (107 – 139)	22.9 (23.8 – 18.4)	Chitob T	0.06 (0.04 – 0.08)	38.2 (39 – 36)
Chitob S	63 (60 – 66)	26.1 (26.5 – 25.6)	Chitob G	0.06 (0.05 – 0.07)	38.2 (39 – 36)
			NAGas T	0.1 (0.06 – 0.2)	36.4 (39 – 31)
Trich A1 +	90.5 (85 – 95)	–	Glucos T	0.2 (0.1 – 0.3)	31.0 (32 – 30)
Endoc T	13.0 (11 – 14.8)	20.2 (23 – 20)			

[a] Endoc T, Endoc G, and Endoc P = endochitinase from *Trichoderma harzianum, Gliocladium virens,* and *Nicotiana tabacum,* respectively; Chitob T, Chitob G, and Chitob S = chitin 1,4-β-chitobiosidase from *T. harzianum, G. virens,* and *Streptomyces albidoflavus,* respectively; NAGas T = *N*-acetyl-β-glucosaminidase from *T. harzianum*; Glucos T = glucan 1,3-β-glucosidase from *T. harzianum*; Trich A1 = trichorzianine A1; Trich B1 = trichorzianine B1; Phospholip = phospholipase B. Values in parentheses indicate concentrations (µg/ml) giving 10% inhibition for each enzyme alone that was used in combination with increasing amounts of MAC.

[b] Values in parentheses are the ED_{50} for the lower and upper 95% fiducial limits for 95% probability.

[c] Values in parentheses are the RS values for the lower and upper 95% fiducial limits for 95% probability. The RS was not calculated for the inhibition of germ tube elongation.

[d] ED_{50} values for flusilazole are expressed in ng/ml.

combined treatments than for the single compound treatments (Tables 1 and 2). For instance, the ED_{50} values on *B. cinerea* spore germination for osmotin were reduced from 10 to 0.3 μg/ml by adding 32 μg/ml of tobacco endochitinase (endoc P), the effective dose that gave about 10% inhibition when used alone ($ED_{\leq 10}$). Similarly the ED_{50} values of gramicidin, valinomycin, phospholipase B, trichorzianine A1 and B1, gliotoxin, flusilazole, and miconazole were significantly lowered by the addition of small doses of any of the CWDEs from fungi, bacteria, or plants tested in our work. In the case of phospholipase B and gramicidin a substantial level of inhibition was observed only with the addition of a CWDE, since these two compounds alone had relatively little effect on *B. cinerea* and *F. oxysporum* (Tables 1 and 2). When Limpel's formula was applied to the bioassay data, every combination of CWDE + MAC showed an observed effect (Eo) higher than the expected effect (Ee) for an additive response, thus indicating the presence of synergism for the inhibition of both test fungi (Tables 1 and 2). The values calculated for the relative level of synergism (RS) were variable among the different compound combinations (Tables 1 and 2). The endochitinases from *Trichoderma* and *Gliocladium* (endoc T and endoc G, respectively), as well as the glucan 1,3-β-glucosidase (glucos T) and the *N*-acetyl-β-glucosaminidase (NAGase T), both from *Trichoderma*, were the most synergistic among the CWDEs. Gliotoxin, flusilazole, miconazole, and osmotin were the most synergistic among the MACs. Lower levels of synergism were observed for the chitobiosidase from *G. virens* (chitob G), endoc P among the CWDEs, and trichorzianine A1 and B1 among the MACs (Tables 1 and 2).

The highest inhibition was observed when both the CWDE and the MAC were applied together at the start of the biosas-

Table 2. Fifty percent effective dose (ED_{50}) for inhibition of *Fusarium oxysporum* spore germination and relative level of synergism (RS) of mixtures containing varying concentrations of membrane affecting compounds (MACs) and given concentrations of cell wall degrading enzymes (CWDEs)

Compound mixtures[a]	ED_{50} (μg/ml)[b]	RS[c]	Compound mixtures[a]	ED_{50} (μg/ml)[b]	RS[c]
Osmotin +	11.0 (8 – 12)	–	Endoc G	3 (2.5 – 3.5)	30.5 (33 – 28.5)
Endoc T (10)	0.2 (0.1 – 0.3)	38.0 (40 – 36.4)	Chitob T	12 (10.5 – 13.5)	15.8 (18 – 13)
Endoc G (5)	0.2 (0.1 – 0.4)	38.5 (40 – 34.5)	Chitob G	15 (11 – 19)	10.2 (16 – 7)
Chitob T (30)	0.9 (0.7 – 1.1)	24.0 (26 – 21)	NAGas T	14 (12 – 16)	13.2 (15.8 – 9.5)
Chitob G (33)	1.0 (0.9 – 1.1)	23.1 (24 – 21)	Glucos T	8 (7 – 9)	27.5 (30 – 23)
NAGas T (12)	0.6 (0.4 – 0.7)	28.8 (34.5 – 26)	Endoc P	12 (10.5 – 14)	15.2 (18 – 13.2)
Glucos T (14)	0.1 (0.1 – 0.2)	40.0 (40 – 38.5)	Chitob S	11 (10 – 13)	16.0 (18 – 13.5)
Endoc P (38)	0.1 (0.1 – 0.1)	40.0 (40 – 40)			
Chitob S (8)	2.3 (2.2 – 2.5)	8.0 (11.2 – 6.5)	Trich B1 +	95.5 (92 – 99)	–
			Endoc T	15 (10 – 19)	16.0 (23 – 13)
Gramicidin +	>300	–	Endoc G	10 (8 – 11.5)	23.0 (25.5 – 21)
Endoc T	30 (26 – 33)	31.0 (32 – 22)	Chitob T	22 (20 – 24)	10.0 (12 – 8.5)
Endoc G	18 (16 – 22)	40.0 (40 – 38)	Chitob G	24 (21 – 27)	8.5 (11 – 7.5)
Chitob T	97 (95 – 100)	12.0 (13 – 11)	NAGas T	20 (18 – 22)	12.0 (14 – 10)
Chitob G	102 (97 – 112)	10.0 (11 – 5.5)	Glucos T	11 (10 – 13)	22.0 (23 – 21.5)
NAGas T	32 (29 – 35)	22.8 (25 – 20)	Endoc P	20.5 (17 – 24)	11.4 (15 – 8.5)
Glucos T	26 (23 – 29)	32.0 (34 – 25)	Chitob S	20 (16 – 24)	12.4 (16 – 8.5)
Endoc P	110 (104 – 121)	6.0 (8 – 5)			
Chitob S	27 (23 – 30)	30.4 (34 – 24)	Gliotoxin +	1.1 (1.0 – 1.2)	–
			Endoc T	0.05 (0.01 – 0.09)	40.0 (40 – 39)
Valinomycin +	8.5 (8.0 – 9.0)	–	Endoc G	0.01 (0.01 – 0.02)	40.0 (40 – 40)
Endoc T	1.5 (1.4 – 1.7)	31.9 (34 – 28.5)	Chitob T	0.8 (0.7 – 0.9)	36.5 (38 – 30)
Endoc G	1.0 (0.3 – 1.2)	40.0 (40 – 38.5)	Chitob G	0.9 (0.8 – 1)	30.0 (36.5 – 26)
Chitob T	2.5 (2.1 – 2.7)	25.0 (27 – 23.5)	NAGas T	0.2 (0.1 – 0.3)	38.5 (38.5 – 38.5)
Chitob G	3.0 (2.9 – 3.1)	20.4 (21.5 – 19)	Glucos T	0.1 (0.08 – 0.3)	38.5 (39.5 – 38.5)
NAGas T	3.5 (3.3 – 3.7)	18.2 (18.5 – 17)			
Glucos T	1.1 (1.0 – 1.3)	40.0 (40 – 38)	Flusilazole +	59.0 (51 – 65)[d]	–
Endoc P	4.0 (3.9 – 4.1)	7.6 (9 – 6)	Endoc T	0.3 (0.1 – 0.5)	40.0 (40 – 39)
Chitob S	1.6 (1.5 – 1.7)	30.0 (31.9 – 28.5)	Endoc G	0.1 (0.1 – 0.2)	40.0 (40 – 40)
			Chitob T	1.0 (0.8 – 1.2)	34.0 (37 – 30)
Phospholip +	>300	–	Chitob G	4.0 (3. – 4.5)	28.5 (29 – 27)
Endoc T	29 (26 – 31)	40.0 (40 – 38.5)	NAGas T	0.9 (0.8 – 1)	35.5 (37 – 34)
Endoc G	35 (31 – 38)	37.5 (38.5 – 34)	Glucos T	0.7 (0.6 – 0.8)	38.5 (39 – 37)
Chitob T	49 (45 – 55)	30.5 (31 – 27)			
Chitob G	65 (57 – 70)	20.4 (22 – 19)	Miconazole +	5.0 (4.5 – 5.5)	–
NAGas T	40 (35 – 44)	32.8 (37.5 – 31.2)	Endoc T	0.1 (0.07 – 0.2)	38.0 (39.5 – 36.5)
Glucos T	49 (40 – 55)	30.5 (32.8 – 27)	Endoc G	0.06 (0.05 – 0.07)	40.0 (40 – 40)
Endoc P	70 (67 – 79)	19.0 (19.5 – 18.5)	Chitob T	0.6 (0.4 – 0.8)	33.0 (34 – 27.5)
Chitob S	44 (38 – 50)	31.2 (34 – 29)	Chitob G	0.7 (0.6 – 0.8)	28.5 (33 – 27.5)
			NAGas T	0.4 (0.3 – 0.5)	34.0 (35.5 – 33)
Trich A1 +	87 (83 – 91)	–	Glucos T	0.3 (0.1 – 0.5)	35.5 (38 – 33)
Endoc T	10 (9 – 11.8)	17.5 (23 – 15.5)			

[a] Abbreviation of compounds as per Table 1. Values in parentheses indicate concentrations (μg/ml) giving 10% inhibition for each enzyme alone that was used in combination with increasing amounts of MAC.

[b] Values in parentheses are the ED_{50} for the lower and upper 95% fiducial limits for 95% probability.

[c] Values in parentheses are the RS calculated for the lower and upper 95% fiducial limits for 95% probability. The RS was not calculated for the inhibition of germ tube elongation.

[d] ED_{50} values for flusilazole are expressed in ng/ml.

say (Fig. 1). If the MAC was applied at the beginning of the assay and the CDWE was added 8 h later, the percent inhibition and the level of synergism were substantially reduced (Fig. 1A). However, if the MAC was applied 8 h later than the enzyme, the response curve was similar to that obtained when the two compounds were both applied at the beginning of the assay (Fig. 1A). In addition, our data indicated that the presence of the enzyme is needed for at least 4 to 8 h to obtain the highest level of synergism, suggesting that the synergistic interaction occurred after there was considerable damage of the cell wall (Fig. 1B). This result was consistent for both the inhibition of spore germination or germ tube elongation caused by 15 different combinations of MAC + CWDE, including gliotoxin + endo G, gliotoxin + glucos T, osmotin + endoc P, osmotin + endoc T, trichorzianine A1 + endoc T, trichorzianine B1 + NAGase T, flusilazole + endoc G, and miconazole + endoc T (Fig. 1 and data not shown).

When L-sorbose alone was included in the assay medium, a modest level of inhibition was observed under our bioassay conditions (Fig. 2). The application of L-sorbose together with CWDEs did not improve the activity of the enzymes, while the combinations L-sorbose + MAC were clearly synergistic, as indicated by both spore germination and germ tube elongation (Fig. 2). The MACs tested with 1 or 3% L-sorbose were flusilazole, gliotoxin, osmotin, gramicidin, valinomycin, and trichorzianine A1, and all these bioassays gave similar results (Fig. 2 and data not shown). For instance, the values for the Ee and the Eo on *B. cinerea* spore germination were, respectively, 9 and 50% for L-sorbose + gliotoxin and 17 and 50% for L-sorbose + trichorzianine A1.

A synergistic effect of MACs and CWDEs was also obtained with trichorzianine A1 and B1 against Gram-positive and Gram-negative bacteria (Fig. 3 and data not shown). The two trichorzianines at low concentrations were active against Gram-positive but not against Gram-negative bacteria. However, the growth of Gram-negative strains was synergistically inhibited by combining trichorzianine A1 and B1 with $ED_{\leq 10}$ concentrations of: 0.5 mM EDTA; 0.001% Triton X-100; 50 µg phospholipase B per ml; EDTA + 2 µg lysozyme per ml; Triton X-100 + lysozyme; or phospholipase B + lysozyme (Fig. 3 and data not shown). For example, the Ee and the Eo values for the inhibition of *Erwinia carotovora* subsp. *carotovora* growth were, respectively, 5.5 and 50% for trichorzianine A1 + EDTA, 7 and 50% for trichorzianine A1 + EDTA + lysozyme, 13 and 50% for trichorzianine A1 + Triton X-100, and 12 and 50% for trichorzianine A1 + Triton X-100 + lysozyme. The combination trichorzianine A1 or B1 + lysozyme was not synergistic on Gram-negative strains, but it was synergistic on Gram-positive strains (data not shown).

DISCUSSION

Many of the natural mechanisms of antibiosis involved in plant defense, fungal attack, fungal biocontrol, and bacterial antagonism may rely on a concurrent degradation of the cell wall and cell membranes. It is a common finding that organisms utilizing CWDEs for either defense or attack also produce MACs, and vice versa. In this study we show that a number of CWDEs and MACs from plants, fungi, or bacteria can produce synergistic mixtures of antifungal or antibacterial agents.

Among the combinations of MACs + CWDEs tested, different levels of synergism were observed. This may be partially due to differences in the specific activity of the enzyme because CWDEs with higher biological activity were usually more synergistic, or required significantly lower doses of application to produce high RS values (Table 1). However, the level of synergism may also depend on the source of the compounds, since endoc P from tobacco was much more synergistic with osmotin from tobacco than with other MACs. It is conceivable that MACs and CWDEs produced by the same organism and involved in the same mechanism are adapted to act in concert and to generate a high biological effect.

CWDEs are also synergistic with other biologically active compounds that are not known as MACs (Collins and Pappagianis 1974; Köller 1992; Watanabe et al. 1988). However, when we tested fungicides that do not specifically affect membranes (i.e., benomyl and captan) there was an absence of synergism, or substantially lower synergism than for combinations of MACs + CWDEs (Lorito et al. 1994c, and data not shown).

CWDEs produced by *Trichoderma* spp. and *Gliocladium* spp. are synergistic with trichorzianines, gliotoxin, micona-

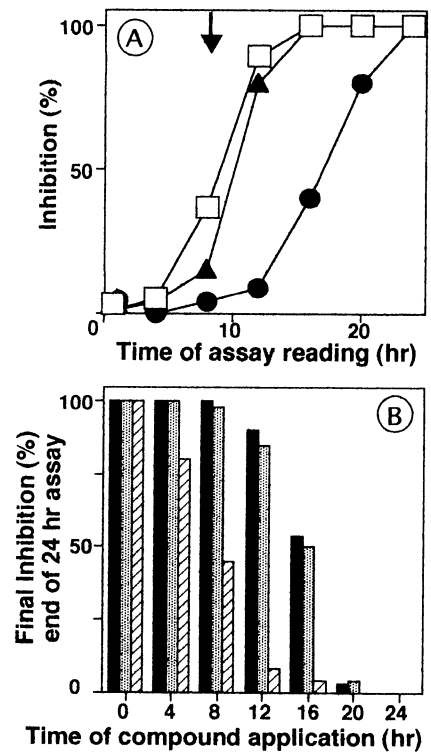

Fig. 1. Effect of a delayed application in the bioassay of endoc P (32 µg/ml) and osmotin (0.5 µg/ml) on the inhibition of *Botrytis cinerea* spore germination. Indicated rates of application gave 100% inhibition for the two compounds applied together and little inhibition for compounds applied singly in a standard assay. **A,** percent inhibition when the assay was interrupted and read at different times (standard assay length = 24 h). Compound applications: endoc P time = 0 and osmotin time = 0 (□); endoc P time = 0 and osmotin time = +8 h (▲); and osmotin time = 0 and endoc P time = +8 h (●). Arrow indicates time of delayed application (+ 8 h). **B,** Effect of varying time (4, 8, 12, 16, 20, 24 h) of compound application on final inhibition at end of standard 24-h assay. Compound applications: endoc P time = 0 and osmotin time = 0 (■); endoc P time = 0 and osmotin time = varying (▨); and osmotin time = 0 and endoc P time = varying (▨). Similar curves were obtained for other MAC + CWDE combinations.

zole, or flusilazole and are presumably involved in biocontrol mechanisms (Di Pietro et al. 1993; Lorito et al. 1994c; Schirmböck et al. 1994). This study indicates that CWDEs produced by plants or bacteria also behave similarly when combined with a variety of MACs. These results support the hypothesis, also suggested by other studies (Schirmböck et al. 1994; Lewis and Papavizas 1987), that the two kinds of compounds cooperate during the antagonistic process and that the alteration of the cell membrane is an important factor in this mechanism. In a biocontrol perspective, this synergistic interaction could also be considered (i) for the genetic engineering of new biocontrol agents able to produce synergistic mixtures of MACs and transgenic CWDEs, and (ii) for the formulation of biologically based pesticides containing CWDEs and a low amount of chemicals.

The PR-protein osmotin produced from tobacco under salt stress, wounding, and virus or fungal attack was synergistic with eight different CWDEs obtained from tobacco, biocontrol fungi, or bacteria. The high levels of synergism observed and the fact that the expression of both tobacco chitinase and osmotin can be induced by the same stimulus strongly suggest the involvement of this MAC/CWDE interaction in the plant defense mechanism against fungi. Similarly, a synergistic interaction has been reported between nikkomycin Z, which alters cell wall structure by inhibiting chitin synthase, and zeamatin, which is similar to osmotin and acts by causing membrane permeabilization (Vigers et al. 1991). A synergism between MACs involved in plant defense and CWDEs may be of interest for the production of transgenic plants with improved resistance to pathogens. For instance, we are producing transgenic crop plants able to overexpress the tobacco osmotin gene and to constitutively express the gene encoding for the antifungal endoc T used in this study (Hayes et al. 1994; Lorito et al. 1994b). If the synergism observed in vitro exists also in vivo, it is likely that these plants will show a high resistance to fungal pathogens. Moreover, transgenic plants overexpressing antifungal CWDEs may be protected from fungi by applying low doses of commercially available MACs (i.e., azole fungicides) synergistic with the transgenic CWDEs.

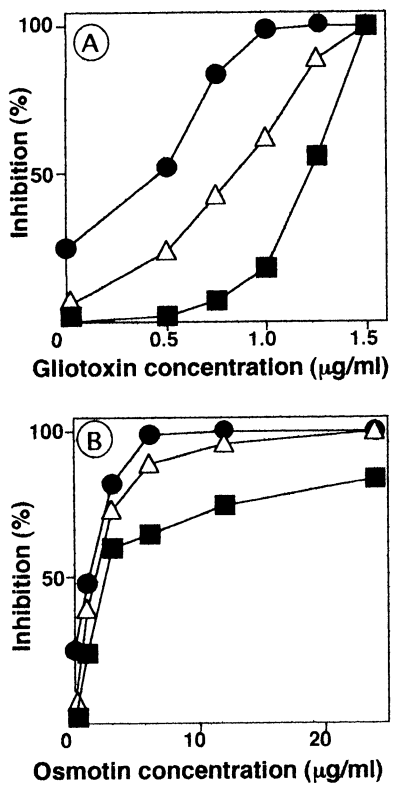

Fig. 2. Effect on *Botrytis cinerea* germ tube elongation with the combined application of membrane affectin compound (MAC) (gliotoxin or osmotin) and L-sorbose. **A,** Gliotoxin: alone (■); + 1% L-sorbose (△); + 3% L-sorbose (●). **B,** Osmotin: alone (■); + 1% L-sorbose (△); + 3% L-sorbose (●). Similar results were observed for other MAC + L-sorbose combinations.

In this study, we propose a hypothetical model to explain the interaction between CWDEs and MACs based on the idea that synergism arises because the action of each compound is enhanced by the effect of the other. The degradation of the cell wall by the CWDE should facilitate the penetration of the MAC. In turn, the MAC activity on some membrane-associated functions, such as chitin synthesis, should reduce the synthesis and the repairing of the cell wall, thus facilitating the action of the CWDE. Several lines of evidence presented in this study support this model. If the CWDE is applied late during the assay, the synergism is lower or absent. This indicates that degradation of the cell wall is required to enhance the activity of the MAC, to establish the synergistic effect, and to obtain the highest level of inhibition. Conversely, delay in the application of the MAC does not modify the response curve, probably because its action is fast and the synergism occurs as soon as the cell wall is degraded enough to allow the penetration of increasing amounts of MAC. The fact that the combination of L-sorbose + MAC was synergistic in the inhibition of *B. cinerea* and *F. oxysporum* fits the model since it indicates that synergism is not necessarily associated with the presence of a CWDE and that a nonenzymatic alteration of the wall can also enhance the penetration and the action of a MAC. The following results are also in agreement with the model: (i) only Gram-positive bacteria, which have a cell wall that is less permeable to antibiotics than that of Gram-negative bacteria, are sensitive to low doses of trichorzianines; (ii) compounds able to partially degrade the cell wall of Gram-negative bacteria made these cells much more sensitive to the peptaibols and were synergistic with these antibiotics; (iii) the inhibitory activity of trichorzianines on Gram-positive strains was synergistically enhanced by a partial digestion of the bacterial cell wall with sublethal doses of lysozyme; and (iv) lysozyme was not synergistic with peptaibols on Gram-negative cells, probably because it was unable to permeabilize the cell wall enough to allow the penetration of the MAC; this indicates that a substantial alteration of each layer of the bacteria cell wall is required to facilitate the penetration of the trichorzianines. Other experiments should be performed in order to confirm the model proposed in this study. For instance, it could be determined if the treatment with enzymes improves the penetration rate of some MACs or if the treatment with MACs that alter membrane-associated synthesis of cell wall components (e.g., chitin synthesis) makes the cell wall more sensitive to CWDEs.

MATERIALS AND METHODS

Organisms and culture conditions.

Strain 12 of *B. cinerea* and strain Fop 1 of *F. oxysporum*

isolated from grape and from bean, respectively, were used as target fungi in the in vitro bioassays. Conidia of both fungi were harvested from potato-dextrose agar cultures at 25°C, resuspended in water, and filtered through sterile Kimwipes to remove hyphal fragments, if necessary. *Erwinia carotovora* subsp. *carotovora* strain 009 and *Pseudomonas syringae* pv. *syringae*, both Gram-negative, *Bacillus cereus* strain ATCC 11778 and *Clavibacter michiganensis* pv. *michiganensis* strain 007, both Gram-positive, were grown in Luria broth (LB) at 27°C on a rotary shaker (New Brunswick, Edison, NJ) at 200 rpm to mid log phase (OD_{495} = 0.4 – 0.6) and 5 µl were used as inoculum for the bioassay.

The following microorganisms were used to obtain different CWDEs: (i) *Trichoderma harzianum* strain P1 (ATCC 74058), isolated from wood chips, is an iprodione-resistant strain with biocontrol activity against several pathogenic fungi in vivo (Tronsmo 1991); the strain was grown on a synthetic medium (SMSC) for enzyme production, as described previously (Harman et al. 1993; Lorito et al. 1994a); (ii) *Gliocladium virens* strain 41 (ATCC 20906), isolated from *Aphanomyces* suppressive soil (Smith et al. 1990), is effective against diseases caused by *Phytophthora* and other fungi; for enzyme production this strain was grown on SMSC as described elsewhere (Di Pietro et al. 1993, Lorito et al. 1994c).

Enzymes, enzyme purification, and enzyme assay.

Chitinolytic enzymes from microorganisms were named and assayed according to Harman et al. (1993). *Trichoderma harzianum* strain P1 was used to obtain endochitinase (EC 3.2.1.14; designated as endoc T) (Bielka et al. 1984), *N*-acetyl-β-glucosaminidase (EC 3.2.1.30; designated as NAGase T), chitin 1,4-β-chitobiosidase (designated as chitob T) and glucan 1,3-β-glucosidase (EC 3.2.1.58; designated as glucos T). *Gliocladium virens* strain 41 was used to obtain endochitinase (EC 3.2.1.14; designated as endoc G) and chitin 1,4-β-chitobiosidase (designated as chitob G). Enzyme purification was conducted by procedures similar to those described by Harman et al. (1993) and Lorito et al. (1994a), consisting of a chromatography on Sephacryl S-300 HR (Pharmacia LKB Biotechnology, Uppsala, Sweden) and a chromatofocusing on PBE 94 (Pharmacia), followed by isoelectric focusing on a Rotofor apparatus (BioRad Laboratories, Richmond, CA). The endoc G of this study had a biological activity higher than previously published (Di Pietro et al. 1993; Lorito et al. 1994c) due to optimization of the purification protocol. A chitin 1,4-β-chitobiosidase from *Streptomyces albidoflavus* strain NRRL E-16746 (designated as chitob S) was purified by anion exchange chromatography (Broadway et al. 1995) and kindly provided by R. M. Broadway. Endochitinase from *Nicotiana tabacum* (designated as endoc P) was purified by high-performance liquid chromatography and kindly provided by R. Bressan. Lysozyme (Sigma Chemical Co., St. Louis, MO) was also tested against the four bacterial strains used in this study, either alone or in combination with peptaibols, EDTA (Sigma), phospholipase B (Sigma), or Triton X-100 (Serva Fein Biochemica, Heidelberg, Germany). Chitinolytic enzyme activity was determined by the release of *p*-nitrophenol from different *p*-nitrophenyl substrates (Sigma) and by measuring turbidity reduction of a colloidal chitin suspension (Harman et al. 1993; Lorito et al. 1994a). Endo P was determined by using the method of Legrand et al. (1987). Gluco T activity was determined by measuring the amount of reducing sugar released from laminarin (Sigma), following the method of Ashwell (1957).

Membrane affecting and nonenzymatic compounds.

All the MACs used, except osmotin and peptaibols, were commercially available formulations. Osmotin was purified from induced tobacco plants and kindly provided by R. Bressan. Peptaibol antibiotics trichorzianine A1 and trichorzianine B1 were produced by different species of *Trichoderma*, including *T. harzianum,* and they were kindly provided by B. Bodo. To enhance the action of the trichorzianines on Gram-negative bacteria, the following compound combinations were used to alter the cell wall or permeabilize the OM (Wooley et al. 1984): EDTA; Triton X-100; phospholipase B; EDTA + lysozyme; Triton X-100 + lysozyme; or phospholipase B + lysozyme. Other compounds used were: gramicidin, valinomycin, gliotoxin, L-sorbose, phospholipase B, miconazole (all from Sigma), and flusilazole (E. I. DuPont de Nemours, Brussels). The chemicals were dissolved or suspended as follows: osmotin, phospholipase B, trichorzianine A1, trichorzianine B1, and L-sorbose were dissolved in sterile deionized water; gramicidin and gliotoxin were dissolved in 100% ethanol (vol/vol); miconazole in 50% ethanol (vol/vol); flusilazole and valinomycin in acetone. When the solvent was other than water, concentrate stock solutions or suspensions were made and progressively diluted in sterile deionized water to provide appropriate concentrations for the assays and to reduce the amount of solvent to nontoxic levels. All the solutions were filter sterilized before use in bioassays.

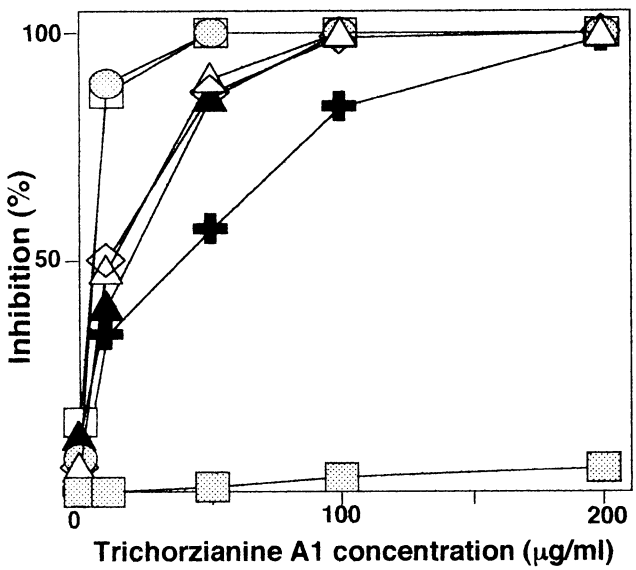

Fig. 3. Effect of trichorzianine A1 alone and in combination with compounds that alter the bacterial cell wall on the growth of Gram-negative bacteria (*Erwinia carotovora* subsp. *carotovora*). EDTA (1 mM), Triton X-100 (0.001%), phospholipase B (50 µg ml^{-1}) and lysozyme (2 µg/ml), all applied at a concentration giving <10% inhibition when used alone, were combined with increasing amounts of trichorzianine A1. Trichorzianine A1: alone (□); + EDTA (△); + EDTA + lysozyme (○); + Triton X-100 (✚); + Triton X-100 + lysozyme (□); + phospholipase B (▲); phospholipase B + lysozyme (◇). Similar curves were obtained for another Gram-negative bacterial strain (*Pseudomonas syringae* pv. *syringae*) and for the trichorzianine B1.

Biological assays.

The bioassays with fungal strains were conducted as described by Lorito et al. (1993b) and Schirmböck et al. (1994), with some modifications. The assay mixtures (45 to 60 µl), containing only 2,000 to 3,000 conidia of the test fungi in 5 mM Tris-HCl pH 6.0 or 5 mM potassium phosphate buffer pH 6.7, were incubated in a flat-bottomed enzyme-linked immunosorbent assay (ELISA) plate at 25°C. The plates were observed at different times by using an inverted microscope to determine the effect on spore germination and on germ tube elongation (Lorito et al. 1993b). If necessary, the assay mixture could be lowered from 45 to 30 µl and, in this case, the ELISA plate was incubated in a humid chamber. This method permitted an efficient assay of a large number of different antifungal compound combinations at one time. Standard assays were also performed by applying the CWDE at the beginning and adding the MAC 4, 8, 12, 16, or 20 h after the beginning of the assay, and vice versa. The assays with bacterial strains were performed by measuring the reduction in absorbance of the bacteria culture grown in a sterile ELISA plate at 27°C on a rotary shaker (New Brunswick) at 200 rpm. The assay mixtures (100 µl) contained about 1,000 bacteria cells suspended in LB medium and 66 mM potassium phosphate buffer, pH 6.2. The bacterial growth was first monitored by measuring, over a time frame of 20 h, the absorbance at 495 nm and the number of cells. Then the bioassays were conducted by determining the OD_{495} after 6 to 8 h, which corresponds, in the untreated control, to a mid to late log phase depending on the strain tested. All the bioassays included controls containing sterile water instead of the compounds to be tested. In addition, the other solvents were tested at the final concentration employed to determine if they had an effect in the bioassay. All treatments for a single MAC and the controls were performed simultaneously in a single experiment. Each experiment was repeated on two separate days and contained three treatment replicates each time. The values from treatment replicates and the data from the two experiments were combined for the statistical analyses.

Analysis of the data.

The control was considered as 0% inhibition and all other values were divided by the control values and multiplied by 100 to obtain percent inhibition for fungal spore germination and germ tube elongation. Similarly, the percent inhibition of bacterial growth was calculated by using the OD_{495} values of the control as 0% inhibition, since there was a linear relationship between the OD_{495} values obtained in the bioassays and the number of bacterial cells in the growing cultures. For each treatment, dosage response curves were calculated by probit analysis of the data collected from two separate experiments with three treatment replicates. The lower and upper 95% fiducial limits for 95% probability and the ED_{50} values for each compound alone and each combination were also obtained by probit analysis. This method of presenting data was chosen because it summarizes entire probit analyses from a number of determinations in a single figure. The relative level of synergism (RS) was determined for each CWDE + MAC combination by applying Limpel's formula: $Ee = X + Y - (XY/100)$, where Ee is the expected effect for an additive, nonsynergistic effect, and X and Y are the percentages of inhibition relative to each compound used alone (Richer 1987). If the combination of the two compounds produces any value of inhibition or observed effect (Eo) greater than Ee, then synergism exists and the greater the difference between Eo and Ee, the greater the level of synergism (Richer 1987; Lorito et al. 1994c). To obtain the X, Y, Ee, and Eo values, we first determined the dose response curves for each MAC and each CWDE singly, and then we combined each enzyme (at a concentration giving 10% inhibition when used alone) with increasing amounts of each MAC. The threshold of 10% was arbitrarily chosen in consideration of the sensitivity of the assay and, consequently, the X value was =10 for all the enzymes. Then, we calculated the ED_{50} values of the MAC (and the upper and lower 95% fiducial limits for 95% probability) for each combination and therefore the observed effect was 50 (Eo = 50). From these ED_{50} values and the dose response curves of single MACs, we obtained Y values (also for the upper and lower 95% fiducial limits for 95% probability) as the effect of each MAC (at a concentration giving 50% inhibition when used alone) in combination with an enzyme. Subsequently, the Ee values were calculated using Limpel's formula and subtracted from the Eo values (= 50) to determine the level of synergism. A modified version of Limpel's formula which includes Eo = 50, X = 10 and the Δ(Eo–Ee) was used for all the calculations described above: RS (relative level of synergism) = $50\ (10 + Y - Y/10)$, where Y is the effect (percent inhibition) of a MAC used alone at a concentration giving 50% inhibition in combination with a CWDE applied at a concentration giving 10% when used alone. If less synergism exists, the RS value approaches zero, while 40 is the highest possible value. The level of synergism calculated is not absolute since each enzyme was used at only one concentration and the comparison between Eo and Ee values was arbitrarily done for Eo = 50. The relative level of synergism was calculated for the inhibition of spore germination including the lower and the upper 95% fiducial limits but was not calculated for the inhibition of germ tube elongation.

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