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Synergistic Interactions Between Chitinase ChiCW and Fungicides Against Plant Fungal Pathogens

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Antifungal activity of ChiCW and synergistic interactions between ChiCW with fungicides were investigated. Conidial germinations of phytopathogenic fungi, *Alternaria brassicicola*, *Botrytis elliptica*, and *Colletotrichum gloeosporioides*, were inhibited by ChiCW but *A. longipes* was not. In addition, ChiCW showed synergistic effect with fungicides Switch (cyprodinil+fludioxonil) and tebuconazole to inhibit fungal conidial germinations. The level of synergism of ChiCW with tebuconazole was higher than that with Switch. The results indicate that ChiCW may exhibit a higher level of synergism with fungicides that have a primary effect upon membranes.

Keywords: ChiCW, synergistic interaction, fungicide, phytopathogenic fungi, tebuconazole

ChiCW of *Bacillus cereus* 28-9, as a modular endochitinase, consists of a signal peptide, a catalytic domain, a fibronectin type III-like domain, and a chitin-binding domain [4, 5]. In the previous study, ChiCW showed antifungal activity effectively against conidial germination of *Botrytis elliptica*, the causal agent of lily leaf blight. Based on this result, ChiCW was proposed to play an important role in the antagonism of *B. cereus* 28-9 against *B. elliptica* [5].

Synergistic interactions between fungal cell-wall degrading enzymes from *Trichoderma harzianum* and *Gliocladium virens* and antifungal compounds have been demonstrated [9, 10]. Cell-wall degrading enzymes from *Trichoderma virens* and a Gram-negative bacterium, *Serratia marcescens* strain B2, exhibited synergistic antifungal activities with bacterial antibiotics [14, 16]. To date, many chitinases have been identified from bacteria and showed antifungal activities [3, 5]. In our previous study, the antifungal activity of a biocontrol strain, *Bacillus subtilis* F29-3, was enhanced by expressing the *chiA* gene of *Bacillus circulans* WL-12 in strain F29-3 [2]. However, synergistic interactions between chitinases from Gram-positive bacteria and antifungal compounds against plant pathogenic fungi were not reported previously, to our memory. Therefore, the aim of this study was to investigate the antifungal activity of ChiCW and synergistic interactions between ChiCW with fungicides against several phytopathogenic fungi.

Four fungal species were used as test fungi in this study: *Alternaria brassicicola* (causing black spot disease on *Brassica* species), *A. longipes* (causing brown spot of tobacco), *B. elliptica* B061, and *Colletotrichum gloeosporioides* (causing anthracnose disease). *A. brassicicola*, *A. longipes*, and *C. gloeosporioides* were cultured on potato dextrose agar (PDA, Difco) at 25°C. *B. elliptica* was cultured on V-8 juice agar [20% V8 juice (Campbell Soup Co.), 0.3% $CaCO_3$, 1.8% agar] at 25°C.

For testing fungicide sensitivities of the test fungi, six formulated fungicides, benomyl (Benlate, 50% wettable powder; DuPont), carbendazim (Bavistin, 50% wettable powder; BASF), dichlorofluanid (Euparen, 50% wettable powder; Bayer), cyprodinil+fludioxonil (Switch, 62.5% water-dispersible granules; Syngenta), iprodione (Rovral, 50% wettable powder; Bayer), and tebuconazole (Folicur, 25.9% emulsifiable concentrate; Bayer), were selected. Stock solutions of the commercial formulations in sterile water were added to molten PDA, and plates were infested with mycelial disks of fungal strains obtained from actively growing cultures. Subsequently, plates were incubated at 25°C to examine fungicide sensitivities of the test fungi.

ChiCW was purified from the periplasmic protein of *E. coli* DH5α (pNTU55) according to the method of Huang *et al.* [5]. Protein concentration was determined by Bradford's method [1] using bovine serum albumin as the standard.

Antifungal activities of chitinase and fungicides were assayed by the method of Huang *et al.* [5] with a slight modification. Assay mixtures contained 10 μl of a conidial suspension (1×10^5 conidia/ml) and an equal volume of an enzyme or a fungicide solution. Cyprodinil+fludioxonil

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and tebuconazole were used in bioassays for *B. elliptica* and the other test fungi, respectively. In the controls, sterile distilled water was used instead of the tested solution. On the other hand, the procedure to analyze synergistic interactions between ChiCW and fungicides was conducted as described by Lorito *et al.* [9, 10] with some modifications. Assay mixtures contained 10 µl of a conidial suspension, 9 µl of an enzyme solution (made at appropriate concentration), and 1 µl of a fungicide solution (made at appropriate concentration). In the controls, sterile distilled water was used instead of the solution containing either the enzyme or the fungicide or both. Conidial germination was examined under a light microscope and the percentage of inhibition was calculated after incubation of prepared assay mixtures at 25°C for 12 h. Each assay was triplicated.

The presence of synergism between enzyme and fungicides was determined by using Limpel's formula [13]: $E_e=(X+Y-XY)/100$, where E_e is the expected effect from additive responses of two inhibitory compounds, and X and Y are the percentages of inhibition relative to each compound used alone. If the combination of the two agents produces any value of inhibition greater than E_e, then synergism exits.

Fungicide sensitivities of our fungal strain had to be investigated firstly in this study. Among 6 different fungicides, only tebuconazole could inhibit all test fungi (data not shown). Tebuconazole was used for further assays of all test fungi but not for *B. elliptica*. Switch (cyprodinil+ fludioxonil) was selected to perform further assays of *B. elliptica* B061 because this fungicide was recommended to control *B. elliptica* by the manufacturer, although *B. elliptica* B061 was also sensitive to tebuconazole.

Fifty % effective concentrations (EC_{50}) of fungicides and ChiCW used alone to inhibit conidial germination were investigated (Table 1). Switch effectively inhibited conidial germination of *B. elliptica*, and tebuconazole can inhibited conidial germinations of *A. brassicicola*, *A. longipes*, and *C. gloeoporioides*. ChiCW was effective against *A. brassicicola*, *B. elliptica*, and *C. gloeosporioides* but not able to inhibit *A. longipes* (Fig. 1).

Every fungicide/ChiCW combination tested inhibited conidial germination of the test fungi and showed a substantial level of synergism (Table 2). When ChiCW (at uninhibitory concentration) was applied with Switch,

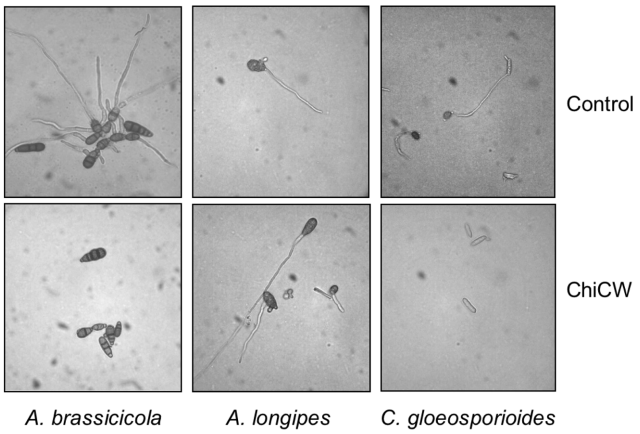

Fig. 1. Effect of ChiCW on conidial germinations of *A. brassicicola*, *A. longipes*, and *C. gloeosporioides*.
Conidial suspensions were treated with purified ChiCW with sterile distilled water as the control.

the EC_{50} of Switch decreased about four times ($E_e=12.5$). The fungicide Switch contains two kinds of fungicides, cyprodinil and fludioxonil, with different modes of action. Cyprodinil (4-cyclopropyl-6-methyl-*N*-phenylpyrimidine) is an anilinopyrimidine fungicide and has been proposed to inhibit amino acid methionine biosynthesis [11]. Fludioxonil [4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1*H*-pyrrole-3-carbonitrile], a synthetic analog of the bacterial metabolite of pyrrolnitrin, is a phenylpyrrole fungicide and demonstrates its fungicidal effect through hyperaction of the Mak protein kinase in osmotic signal transduction [6, 12]. Using a very small amount of Switch (0.47 ppm) was effective against 50% conidial germination of *B. elliptica* (Table 1). Furthermore, when ChiCW was applied with Switch, the EC_{50} of Switch decreased four times, indicating that ChiCW can synergistically interact with Switch to inhibit conidial germination of *B. elliptica*.

In addition, ChiCW also exhibited synergistic interaction with tebuconazole to inhibit *A. brassicicola*, *A. longipes*, and *C. gloeosporioides*. Tebuconazole [1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazole-1-yl-methyl)pentane-3-ol] is a demethylation inhibitor fungicide and inhibits C14-demethylase in sterol biosynthesis [7, 8]. When ChiCW (at uninhibitory concentration) was applied with tebuconazole,

Table 1. Fifty percent effective concentrations (EC_{50}) of two fungicides[a] and ChiCW against four fungal species.

Fungus	EC_{50} (µg/ml)		
	ChiCW	Switch	Tebuconazole
Alternaria brassicicola	3.25	–[b]	1785
Alternaria longipes	No effect	–	3570
Botrytis elliptica	3.25	0.47	–
Colletotrichum gloeosporioides	3.25	–	28.6

[a]Fungicides Switch and tebuconazole were used in bioassays for *B. elliptica* and other fungi, respectively.
[b]–: not tested.

Table 2. Fifty percent effective concentrations (EC_{50}) of individual fungicides[a] used either alone or with ChiCW against four fungal species.

Fungus	EC_{50} of fungicide (μg/ml)	
	Without ChiCW	With ChiCW (1.5 μg/ml[b])
Alternaria brassicicola	1,785	11.2
Alternaria longipes	3,570	22.4
Botrytis elliptica	0.47	0.12
Colletotrichum gloeosporioides	28.6	1.4

[a]Fungicides Switch and tebuconazole were used in bioassays for *B. elliptica* and other fungi, respectively.
[b]At this concentration, ChiCW does not inhibit spore germination of test fungi.

the EC_{50} of tebuconazole for inhibition of *C. gloeosporioides* and *A. brassicicola* decreased 20 and 159 times (both of $E_e=0$), respectively (Table 2). The result reveals that ChiCW can significantly interact with tebuconazole to synergistically inhibit conidial germination, especially against *A. brassicicola*. Furthermore, the EC_{50} of tebuconazole for inhibition of *A. longipes* dramatically decreased 159 times when using the ChiCW/tebuconazole combination ($E_e=0$). However, ChiCW used alone did not inhibit conidial germination of *A. longipes*.

The level of synergism may be affected by the mode of action of the compounds. In the study of Lorito *et al*. [10], the highest levels of synergism occurred in fungal cell wall degarding enzymes with two sterol demethylation inhibitors and with gliotoxin. Sterols are required for the structure and function of membranes [15]. Combining the effect of these toxins with partial digestion of cell walls may be particularly damaging for the targeted cells and may reduce the lethal doses of the toxins [10]. In this study, the level of synergism of ChiCW with tebuconazole was higher than that with Switch (Table 2), indicating that ChiCW exhibited higher synergism with the fungicides that have primary effect upon membranes.

On the other hand, the synergistic antifungal activity of the cell-wall degrading enzyme and the antifungal compound was correlated with the cell wall composition of the target fungus [16]. In this study, three different species of fungi were used and their cell wall compositions were not exactly known. ChiCW showed inhibitory effect (when used alone) and synergistic effect (when used with fungicides) against conidial germination of *A. brassicicola*, *B. elliptica*, and *C. gloeosporioides* (Tables 1 and 2). The results suggest that ChiCW is able to hydrolyze the cell walls of these fungal conidia and enhance conidia to uptake fungicides.

Compared with *A. brassicicola*, conidial germination of *A. longipes* was not inhibited by ChiCW (Table 1, Fig. 1). Although both fungi belong to the genus *Alternaria*, ChiCW exhibited different effects on the conidial germinations of *Alternaria* spp. The result, indicate that the digestion of conidial cell walls of *A. longipes* by ChiCW may be partial and not enough to directly damage *A. longipes* cells, revealing that the cell wall compositions and/or structures of *A. brassicicola* and *A. longipes* may be different. However, synergism of ChiCW with tebuconazole against *A. brassicicola* and *A. longipes* was demonstrated (Table 2), suggesting that the partial digestion of conidial cell walls of *A. longipes* by ChiCW is enough for tebuconazole to target membranes and to inhibit sterol biosynthesis.

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References

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
2. Chen, C. Y., Y. H. Wang, and C. J. Huang. 2004. Enhancement of the antifungal activity of *Bacillus subtilis* F29-3 by the chitinase encoded by *Bacillus circulans chiA* gene. *Can. J. Microbiol.* **50:** 451–454.
3. Dahiya, N., R. Tewari, and G. S. Hoondal. 2006. Biotechnological aspects of chitinolytic enzymes: A review. *Appl. Microbiol. Biotechnol.* **71:** 773–782.
4. Huang, C. J. and C. Y. Chen. 2005. High-level expression and characterization of two chitinases, ChiCH and ChiCW, of *Bacillus cereus* 28-9 in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **327:** 8–17.
5. Huang, C. J., T. K. Wang, S. C. Chung, and C. Y. Chen. 2005. Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. *J. Biochem. Mol. Biol.* **38:** 82–88.
6. Kojima, K., Y. Takano, A. Yoshimi, C. Tanaka, T. Kikuchi, and T. Okuno. 2004. Fungicide activity through activation of a fungal signalling pathway. *Mol. Microbiol.* **53:** 1785–1796.
7. Kwok, I. M. Y. and R. T. Loeffler. 1993. The biochemical mode of action of some newer azole fungicides. *Pestic. Sci.* **39:** 1–11.
8. Lamb, D. C., D. E. Kelly, N. J. Manning, D. W. Hollomon, and S. L. Kelly. 1998. Expression, purification, reconstitution and inhibition of *Ustilago maydis* sterol 14α-demethylase (CYP51; $P450_{14DM}$). *FEMS Microbiol. Lett.* **169:** 369–373.
9. Lorito, M., C. K. Hayes, A. Zoina, F. Scala, G. Del Sorbo, S. L. Woo, and G. E. Harman. 1994. Potential of genes and gene products from *Trichoderma* sp. and *Gliocladium* sp. for the development of biological pesticides. *Mol. Biotechnol.* **2:** 209–217.
10. Lorito, M., C. Peterbauer, C. K. Hayes, and G. E. Harman. 1994. Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. *Microbiology* **140:** 623–629.

11. Masner, P., P. Muster, and J. Schmid. 1994. Possible methionine biosynthesis inhibition by pyrimidinamine fungicides. *Pestic. Sci*. **42:** 163–166.
12. Okada, A., S. Banno, A. Ichiishi, M. Kimura, I. Yamaguchi, and M. Fujimura. 2005. Pyrrolnitrin interferes with osmotic signal transduction in *Neurospora crassa*. *J. Pestic. Sci.* **30:** 378–383.
13. Richer, D. L. 1987. Synergism - a patent view. *Pestic. Sci*. **19:** 309–315.
14. Someya, N., M. Nakajima, K. Hirayae, T. Hibi, and K. Akutsu. 2001. Synergistic antifungal activity of chitinolytic enzymes and prodigiosin produced by biocontrol bacterium, *Serratia marcescens* strain B2, against gray mold pathogen, *Botrytis cinerea*. *J. Gen. Plant Pathol*. **67:** 312–317.
15. Vanden Bossche, H. 1990. Importance and role of sterols in fungal membranes. *In*: *Biochemistry of Cell Walls and Membranes in Fungi*. Springer-Verlag, Berlin.
16. Woo, S., V. Fogliano, F. Scala, and M. Lorito. 2002. Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. *Antonie Van Leeuwenhoek* **81:** 353–356.