Original

Co-inoculation of an Antibiotic-Producing Bacterium and a Lytic Enzyme-Producing Bacterium for the Biocontrol of Tomato Wilt Caused by *Fusarium oxysporum* f. sp. *lycopersici*

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The antifungal compound 2,4-diacetylphloroglucinol-producing bacterium, *Pseudomonas fluorescens* strain LRB3W1, inhibits the growth of *Fusarium oxysporum* f. sp. *lycopersici*, and controls Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici*. On the other hand, *Serratia marcescens* strain B2, which produces cell wall-degrading enzyme chitinases, did not inhibit fungal growth and the suppressive effect of strain B2 against tomato Fusarium wilt was less than that of strain LRB3W1. Combined inoculation of strain LRB3W1 with strain B2 was more effective than treatment with strain LRB3W1 alone. When 2,4-diacetylphloroglucinol and the chitinolytic enzymes were applied in combination, a synergistic inhibitory effect against the pathogen was observed. It was possible that bacteria which produce cell wall-degrading enzymes enhanced the biocontrol effect of the antibiotic-producing bacterium against tomato Fusarium wilt.

Key words : Biocontrol/Cell wall degrading enzymes/2,4-diacetylphloroglucinol/Pseudomonad/
Synergistic action.

INTRODUCTION

Microorganisms produce antimicrobial compounds or proteins that ensure their survival in a variety of environments. The traits of producing antimicrobial factors have been utilized for the biological control of plant pathogens in agriculture (Haas and Défago, 2005). A biocontrol bacterium, *Pseudomonas fluorescens* strain LRB3W1, produces antifungal fac-

tors such as antibiotics, 2,4-diacetylphloroglucinol (2,4-DAPG) and hydrogen cyanide (Tazawa et al., 2000; Tsuchiya et al., 1997). Strain LRB3W1 controlled several plant diseases including tomato Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Someya et al., 2006). On the other hand, a chitinolytic bacterium, *Serratia marcescens* strain B2, also inhibited various phytopathogenic fungi, and controlled several plant fungal diseases (Someya et al., 2000). Therefore, both strain LRB3W1 and strain B2 are expected to be useful biocontrol agents (BCAs). However, the effect of a BCA alone is not

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always satisfactory, and many researchers have stated that improving the biocontrol efficacy of BCAs by combining them with fungicides or other BCAs is an attractive alternative. In the present study, we examined the effect of the combined application of the bacteria, *P. fluorescens* strain LRB3W1 and *S. marcescens* strain B2, for the control of tomato Fusarium wilt. We also demonstrated that the lytic enzyme-producing bacterium strain B2 enhanced the biocontrol efficacy of the antibiotic-producing bacterium strain LRB3W1 against *F. oxysporum* f. sp. *lycopersici* via the synergistic activity of lytic enzymes and the antibiotic 2,4-DAPG.

MATERIALS AND METHODS

Microbial strains and culture conditions

Pseudomonas fluorescens strain LRB3W1 (LRB3W1) was isolated from the lettuce rhizosphere and was stored at the National Institute for Agro-Environmental Sciences, Ibaraki, Japan. King B medium agar (KBA; Eiken Chemical Co., Ltd., Tokyo, Japan) was used for strain LRB3W1 culture. *Serratia marcescens* strain B2 was isolated from tomato plants and was stored at Ibaraki University, Ibaraki, Japan. For strain B2 culture, LB (Sigma-Aldrich Japan, Tokyo, Japan) medium was used.

The tomato wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici* race 2 880621a-1, was incubated on potato dextrose medium (PD; Becton, Dickinson and Company, Sparks, MD) agar at 25°C in the dark. To produce the inoculum, the fungus was incubated on PD agar at 25°C in the dark for 5 d. Mycelial discs (5 mm in diameter), cut with a cork borer from the colony, were cultured in liquid PD medium at 25°C for 10 d on a reciprocal shaker (140 strokes/min). The culture was filtered through four layers of sterile gauze to remove mycelial fragments, and was centrifuged at 3,000 rpm for 20 min. The pellet was resuspended in sterile distilled water. The suspension of bud-cells was adjusted to a concentration of about 1×10^6 cells/ml for use as inoculum (Someya et al., 2006).

In vitro antifungal activity assay

Bacterial suspension [1×10^9 colony forming units (CFU)/ml] or sterile distilled water (control), were inoculated on diluted nutrient broth plus yeast extract medium (dNBYG) agar plates (Duffy and Défago, 1999). Plates were then incubated for 72 h at 28°C in the dark. A mycelial disc (5 mm in diameter) of *F. oxysporum* f. sp. *lycopersici*, cut from colonies grown on PD agar, was placed on the plate opposite to the bacterial colonies 40 mm away. The growth of fungi was measured after incubation in the dark for 10 d at

25°C. Three replicate plates were tested for each treatment, and each experiment was replicated three times.

Biocontrol test

Tomato (*Lycopersicon esculentum* Miller) cultivar Momotaro was used in this study. Seeds were sown on 300 cm³ artificial soil [Kureha-Fertilized granulated soil (Kureha Chemical Industry Co., Ltd., Tokyo, Japan) and vermiculite at a 3:1 ratio, v/v] in plastic pots (7.5 cm in diameter and 6 cm tall), and grown in a glasshouse at 25°C. Two-wk-old seedlings were used for bioassays.

Fifteen milliliters of bacterial suspension (about 1×10^5 , 1×10^7 , and 1×10^9 CFU/ml) or distilled water (control) were added to the pot containing a tomato plant 1 d before challenge inoculation with the pathogen. Each bacterium (strains LRB3W1 and B2) was inoculated alone or in combination into the soil. For comparison, 15 ml of benomyl (benlate®; Sumika Takeda Agrochemical Company Ltd., Tokyo, Japan) solution at 1, 10 or 100 µg/ml was applied into the soil to compare its disease suppression with that of bacterial treatment. One d after bacterial application, *F. oxysporum* f. sp. *lycopersici* was applied to each pot at 3 ml of a bud-cell suspension (1×10^6 cells/ml). Pots were kept for 4 wk under glasshouse conditions. Three plants were tested for each treatment, and each experiment was replicated five times.

Disease severity was evaluated according to the proportion of diseased (yellowing, wilting, or collapsed) leaves 4 wk after pathogen inoculation. Disease severity was defined as the number of diseased leaves divided by the number of total leaves in each plant. Disease severity of the control treatment (distilled water) was calculated and set to 100% to represent the disease incidence for the control (Someya et al., 2006). The mean of disease incidence for each experiment was statistically analyzed using Tukey's method (Tukey, 1984).

Effect of lytic enzymes on the antifungal activity of 2,4-diacetylphloroglucinol against pathogen

Strain B2 was incubated in liquid LB medium with 0.2% colloidal chitin for up to 72 h at 25°C on a reciprocal shaker (125 strokes/min). The bacterial cells were centrifuged, and the supernatant was filtered through a 0.20 µm filter, and the extracellular proteins were then precipitated with ammonium sulfate (70% saturation). The precipitate was collected and dissolved in 15 mM phosphate buffer (pH 7.0), dialyzed and lyophilized. The extracellular protein was dissolved in 15mM phosphate buffer, and the endochitinolytic activity of the solution was adjusted

to about 20 nkatal. Endochitinolytic activity was measured using a modification of Schales' procedure (Someya et al., 2001).

The suspension of bud-cells of pathogen was adjusted to a concentration of 2×10^6 cells/ml. Equal volumes of a bud-cell suspension and a 2,4-diacetylphloroglucinol (Toronto Research Chemicals Inc., ON, Canada) solution at 0.2, 1, 2, 10, 20, 100, 200 and 500 μ g/ml in the presence or absence of extracellular proteins containing chitinolytic enzymes from strain B2 were mixed. Mixtures were incubated for 12 h at 25°C in darkness. After incubation, mixtures were centrifuged at 3,000 rpm for 20 min, and the supernatant was discarded. The bud-cells were washed with sterile 15mM phosphate buffer, and serial dilutions were cultured on *Fusarium*-selective medium for 5 d at 25°C in darkness (Nishimura, 2001). Colonies were counted to estimate the survival rate of the pathogen. The rate of the bud-cell germination (about 1000 bud-cells) was examined, and each experiment was replicated three times. The number of bud-cells germinated in the control treatment was calculated and set to 100% to represent the bud-cell germination for the control. The mean number of bud-cells germinated for each experiment was statistically analyzed using Tukey's method (Tukey, 1984).

RESULTS AND DISCUSSION

Growth inhibition of *F. oxysporum* f. sp. *lycopersici* by antagonistic bacteria

The growth of *F. oxysporum* f. sp. *lycopersici* was inhibited by *P. fluorescens* strain LRB3W1 on both dNBYG and LB medium agar (Fig. 1). Although *S. marcescens* strain B2 slightly inhibited the growth of *F. oxysporum* f. sp. *lycopersici* on LB medium agar, the bacterium did not inhibit the growth of the patho-

FIG. 1. Fungal growth of *F. oxysporum* f. sp. *lycopersici* co-cultured with *P. fluorescens* strain LRB3W1 (LRB3W1), *S. marcescens* strain B2 (B2) and its growth without bacteria (Control) on the dNBYG and LB medium agar, respectively. The plates were incubated for 10 d at 25°C. Error bars indicate standard deviations.

gen on dNBYG medium agar. Strain LRB3W1 produced antibiotics, 2,4-diacetylphloroglucinol (2,4-DAPG) and hydrogen cyanide, and strain B2 also produced an antibiotic, prodigiosin (Someya et al. 2001; Tazawa et al. 2000; Tsuchiya et al. 1997). Furthermore, 2,4-DAPG produced by fluorescent pseudomonads including strain LRB3W1 is well known as an effective inhibitor against various soilborne phytopathogens including fusaria (Keel et al., 1990). Okamoto et al. (1998) reported that an antibiotic, prodigiosin, produced by *S. marcescens* had antagonistic activity against several phytopathogenic fungi. We also previously reported that the prodigiosin from strain B2 also had antifungal activity against phytopathogenic fungi (Someya et al., 2001). However, Okamoto et al. (1998) indicated that the antifungal activity of prodigiosin against *Fusarium* species was lower than that of other phytopathogenic fungi such as *Phytophthora* spp., *Pythium* spp. and *Rhizoctonia solani*. In the present study, the prodigiosin produced by strain B2 did not inhibit the growth of *F. oxysporum* f. sp. *lycopersici*. Strain B2 also produced lytic enzymes, chitinases, but the antifungal activity of chitinases from strain B2 was not sufficient to inhibit the fungal growth of *F. oxysporum* (Someya et al., 2000).

Comparisons of the use of single and multiple bacterial strains for the biocontrol of tomato *Fusarium* wilt

Disease symptoms appeared about 2 to 3 wk after pathogen inoculation, and the disease incidence of control plants increased 4 wk after pathogen inoculation. The disease incidence of plants treated with strain LRB3W1 was decreased (Fig. 2). The suppressive effect by strain LRB3W1 depended on the density of the inoculated bacteria. It has been reported that the 2,4-DAPG production by *P. fluorescens* was dependently regulated by the bacterial population density (Raaijmakers et al., 1999). In the tomato rhizosphere, strain LRB3W1 survived at about 10^7 CFU/g soil for 4 wk after bacterial inoculation (Someya et al., 2006). The results showed that strain LRB3W1 may be effective as a biocontrol agent for the control of tomato *Fusarium* wilt, but strain LRB3W1 alone is not sufficiently effective in agricultural systems. The suppressive effect of strain LRB3W1 inoculated at 10^7 CFU/ml was lower than that of the benomyl application at 100 μ g/ml (Fig. 2). On the other hand, the disease incidence of the plants treated with strain B2 was also decreased, but the suppressive effect of strain B2 was lower than that of strain LRB3W1. The suppressive effect of strain B2 at 10^9 CFU/ml was lower than that of strain

FIG. 2. Suppression of *Fusarium* wilt of tomato in potting soil by *P. fluorescens* strain LRB3W1 (LRB3W1), *S. marcescens* strain B2 (B2) and mixture of strain LRB3W1 and B2 (LRB3W1+B2) 4 wk after pathogen inoculation. Distilled water (Control), 15 ml of each bacterial suspension (about 1×10^9 CFU/ml) or bacterial mixture (about 1×10^9 CFU/ml each) was applied to the soil 1 d before pathogen inoculation.

LRB3W1 at 10^5 CFU/ml (Fig. 2). However, the disease incidence significantly decreased compared with that of each bacterial treatment when each bacterium was co-inoculated into the tomato rhizosphere at a concentration about 1×10^7 CFU/ml and 1×10^9 CFU/ml (Fig. 3). The suppressive effect of the

FIG. 3. Biological control by *P. fluorescens* strain LRB3W1 (LRB3W1) and *S. marcescens* strain B2 (B2), used alone or in combination, against *Fusarium* wilt of tomato. Each bacterium was inoculated into the pots at different concentrations (15 ml of bacterial suspension at about 10^5 , 10^7 and 10^9 CFU/ml). For comparison, 15 ml of benomyl solution at 1, 10 or 100 μ g/ml was applied into the soil to compare its disease suppression with that of the bacterial treatment. The columns with different letters are significantly different ($P = 0.05$) by Tukey's method.

bacterial mixture at 10^9 CFU/ml was the same as that of benomyl treatment at 100 μ g/ml.

Several authors have suggested that combinations of introduced BCAs showed both positive and negative interactions in plant disease control. For example, it was reported that the mixtures of bacterial strains antagonistic to phytopathogens enhanced their suppressive activity (de Bouer et al., 2003; Fukui et al., 1999; Szczech and Shoda, 2004; Toyoda et al., 1993); however, it was also reported that there was a negative interaction between BCAs in the biocontrol of pathogens (Fukui et al., 1999; Roberts et al., 2005). It was also suggested that some saprophytic *Pseudomonas* spp. enhanced the disease severity of a phytopathogen (Fernando et al., 1994). These results suggested that selecting the correct BCAs in a mixture is important for the control of phytopathogens. Regarding the combination between strain LRB3W1 and strain B2, *in vitro* data showed that each bacterium did not inhibit the growth or metabolite production of the other (data not shown).

Synergistic antifungal activity of the 2,4-diacetylphloroglucinol and chitinolytic enzymes against the bud-cell viability of the tomato wilt pathogen, *F. oxysporum* f. sp. *lycopersici*

The tested isolate, *F. oxysporum* f. sp. *lycopersici* race 2 880621a-1, was susceptible to 2,4-DAPG (Schouten et al., 2004). 2,4-DAPG significantly decreased the viability of bud-cells at a concentration of more than 50 μ g/ml (Fig. 4). On the other hand, application of extracellular proteins containing chitinolytic enzymes from strain B2 did not inhibit the bud-cell germination. It has been reported that

FIG. 4. Inhibition of *F. oxysporum* f. sp. *lycopersici* bud-cell germination by 2,4-diacetylphloroglucinol (2,4-DAPG) in the presence or absence of extracellular proteins from *S. marcescens* strain B2. The germination of about 1,000 bud-cells was examined after 5 d of incubation at 25°C. The values for bud-cell germination are means from three replicates of each experiment. The column with different letters are significantly different ($P = 0.05$) by Tukey's method.

chitinolytic enzymes are effective inhibitors against phytopathogenic fungi containing chitin (Herrera-Estrella and Chet, 1999). We also previously reported that chitinolytic enzymes produced by strain B2 caused lyses of phytopathogenic fungal hypha, but the lytic phenomena were rarely observed in *F. oxysporum* (Someya et al., 2000). In the present study, the antifungal activity of extracellular proteins from strain B2 alone was not definitely observed (Fig. 4). However, the germination of bud-cells treated with 2,4-DAPG at 5 and 10 μ g/ml was significantly decreased in the presence of extracellular proteins containing chitinolytic enzymes from strain B2. We previously reported that the synergistic antifungal activity of chitinolytic enzymes plus antibiotic or chemical pesticides against phytopathogenic fungi (Someya et al. 2001; 2005). Such synergistic antagonism was also observed for chitinolytic enzymes in combination with various other antifungal compounds (Lorito et al., 1994). We hypothesized that the synergistic effect between 2,4-DAPG and chitinolytic enzymes against *F. oxysporum* f. sp. *lycopersici* occurred in the tomato rhizosphere. On the other hand, synergisms between antifungal compounds such as antibiotics or fungicides against microorganisms also occurred (Jawetz et al., 1952; Scardavi, 1966). Strain LRB3W1 produced antifungal compounds, 2,4-DAPG and hydrogen cyanide, and strain B2 also produced an antibiotic, prodigiosin (Someya et al., 2001; Tazawa et al., 2000; Tsuchiya et al., 1997). The combination of these antifungal compounds and chitinolytic enzymes also may play a role in the suppression of the phytopathogen, *F. oxysporum*, in soil.

Conclusion

Recently, the combined application of protective microorganisms in agriculture has received much attention. It has recently been reported that some plant-colonizing bacteria enhanced the nodulation by rhizobia (Sindhu and Dadarwal, 2001), mycorrhization by ectomycorrhizal fungi (Founoune et al., 2002) and nitrogen fixing by symbiotic bacteria (Derylo and Skorupska, 1993). Regarding the bacteria-mycorrhiza interactions, these bacteria are called mycorrhiza helper bacteria (MHB). On the other hand, it has been reported that some helper bacteria increased rather than decreased pathogenicity (Dewey et al., 1999). Our results show that the lytic enzyme-producing bacterium combined with other BCAs improved total disease suppression. In cases like this, we suppose that lytic enzyme-producing bacterium acts as the biocontrol helper bacterium (BHB) in the biocontrol of phytopathogens

with an antibiotic-producing bacterium. Interactions between microorganisms in the rhizosphere include both synergistic and antagonistic relationships. Selecting the optimum mixture of protective microorganisms is the next step in the biological control of plant diseases.

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