Note

Combined Use of the Biocontrol Bacterium *Pseudomonas fluorescens* Strain LRB3W1 with Reduced Fungicide Application for the Control of Tomato Fusarium Wilt

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Received 1 October 2005/Accepted 24 December 2005

*Pseudomonas fluorescens* strain LRB3W1 inhibited the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* and suppressed the Fusarium wilt of tomato. The chemical fungicide, benomyl, did not suppress the disease incidence at low concentrations. However, the disease incidence was decreased by the combined application of benomyl at low concentrations with strain LRB3W1. Combined application of benomyl with the bacterium was more effective than treatment with the bacterium alone. The survival of strain LRB3W1 was not influenced by the presence of benomyl. This combined use of the biocontrol bacterium, strain LRB3W1, and a fungicide, benomyl, should be an attractive approach for suppressing tomato wilt.

Key words: Biocontrol/ *Fusarium oxysporum*/ *Pseudomonas*/ Synergistic action/Tomato wilt.

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici*, is a vascular disease of tomato plants (*Lycopersicon esculentum* Mill.) that causes losses in both yield and quality of tomatoes (Jones and Woltz, 1982; Kuniyasu, 1990). Although the use of Fusarium-resistant cultivars can provide some degree of disease control, alternative disease control methods are required for many susceptible commercial cultivars. In addition, ecological damage and pesticide resistance resulting from repeated use of chemical fungicides for control of plant diseases have prompted a search for alternative control methods. With regard to the potential of biocontrol agents for the suppression of tomato Fusarium wilt, some fungal or bacterial agents have been reported (De Cal et al., 1995; Larkin and Fravel, 1998; Toyoda et al., 1993; Yamaguchi et al., 1992). However, the effect of biocontrol agents alone is not always satisfactory, and many researchers have stated that the synergistic phenomenon involved in the control of diseases using a combined application of fungicides and biocontrol agents is an attractive alternative (Duffy, 2000; Elmer and McGovern, 2004; Kondoh et al., 2000).

*Pseudomonas fluorescens* strain LRB3W1 (LRB3W1 hereafter) was isolated from the lettuce rhizosphere. This bacterium produces the antibiotics 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN), and inhibits the growth of several phytopathogenic fungi and bacteria (Tazawa et al., 2000; Sugisawa et al., 2004; Tsuchiya et al., 1997). In the present study, we examined the antagonistic activity of the strain LRB3W1 against the Fusarium wilt pathogen, *F. oxysporum* f. sp. *lycopersici*, and subsequently demonstrated its suppressive effect on Fusarium wilt of tomato. We also demonstrated that the combination of the bacterium with the chemical fungicide, benomyl, at low concentrations enhanced the suppressive effect of the bacterium against the same disease.

Strain LRB3W1 was isolated from the lettuce
rhizosphere and stored at the National Institute for Agro-Environmental Sciences, Ibaraki, Japan. Strain LRB3W1-rif/nal/str is a rifampicin-, nalidixic acid-, and streptomycin-resistant derivative of strain LRB3W1. King B medium agar (KBA; Eiken Chemical Co., Ltd., Tokyo, Japan) was used for bacterial culture.

The tomato wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici* race 2 880621a-1, was incubated on potato dextrose (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 PD liquid medium at 25°C for 10 d on a reciprocal shaker (140 strokes/min). The culture was sieved through four layers of sterile gauze to remove the mycelia 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 ca. 1 × 10⁶ cells/ml for use as an inoculum.

Bacterial suspension [ca. 10⁶ colony forming units (CFU)/ml] or sterile water (control), was inoculated on diluted nutrient broth plus yeast extract medium (dNBYG) agar plates (Duffy and Defago, 1999). Plates were then incubated for 72 h at 27°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 opposite the bacterial colonies, 40 mm away. Fungal growth was measured after incubation in the dark for 7 d at 27°C. For comparison, fungal growth was also measured on dNBYG agar plates amended with the fungicide, benomyl (benlate®; Sumika Takeda Agrochemical Company Ltd., Tokyo, Japan) at 1, 10, and 100 μg/ml. Three replicate plates were tested for each treatment, and each experiment was replicated three times.

Tomato plants (*L. esculentum* Mill. cv. Momotaro) were used in this study. Seeds were sown in 300 cm³ of artificial soil [Kureha-soil (Kureha Chemical Industry Co., Ltd., Tokyo, Japan); vermiculite = 3:1, v/v] in plastic pots (10.5 cm in diameter and 9 cm tall), and were grown in a glasshouse at 27°C. Two-wk-old seedlings were used for bioassays.

Fifteen ml of bacterial suspension (ca. 1 × 10⁹ CFU/ml) or distilled water (control) was added into the soil of the tomato rhizosphere 1 d before the challenge inoculation with the pathogen. To compare disease suppression with that of the bacterial treatment alone, 15 ml of benomyl solution at 1, 10, and 100 μg/ml were applied into the soil with or without bacterial treatment (1 × 10⁹ CFU/ml). One d after application of the treatment, 6 ml of a bud-cell suspension of *F. oxysporum* f. sp. *lycopersici* (ca. 10⁶ cells/ml) was inoculated into each pot. Three plants were tested for each treatment, and each experiment was replicated three times.

Disease severity was evaluated according to the proportion of diseased (yellowing, wilting, or collapsed) leaves 4 wk after pathogen inoculation. Disease severity = number of diseased leaves / 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. The mean of disease incidence for each experiment was statistically analyzed using Tukey's method (Tukey, 1984).

Fifteen ml of strain LRB3W1-rif/nal/str cell suspension (ca. 1 × 10⁹ CFU/ml) and 15 ml of benomyl solution at 1, 10, and 100 μg/ml were simultaneously applied into the pots containing 2-wk-old tomato plants to compare the bacterial populations to those in the case of the treatment with bacteria alone. Plants were kept in a glasshouse at 27°C. Following the bacterial application, a 1-g soil sample was taken each wk from the tomato rhizosphere. These soil samples were mixed with 9 ml of sterile 15mM phosphate buffer (pH 7.0), and serial dilutions were cultured for 3 d in the dark on KBA plates containing 20 μg/ml nalidixic acid, 20 μg/ml rifampicin and 50 μg/ml streptomycin. Colonies were counted to estimate populations of LRB3W1-rif/nal/str.

The growth of *F. oxysporum* f. sp. *lycopersici* was inhibited in the presence of the bacterial colonies (Figs. 1, 2). A fungal growth-inhibiting zone of approximately 15 to 20 mm was formed around the bacterial colonies (Fig. 1, right). While the growth of the pathogen was inhibited by the fungicide, benomyl, at

![Control](image1.png)  ![with LRB3W1](image2.png)

FIG. 1. Antifungal activity of *Pseudomonas fluorescens* strain LRB3W1 (LRB3W1) on the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* (Fol). The plates were inoculated for 10 d at 27°C. The fungal growth was examined on the dNBYG medium agar plate. Bar 1 cm.
FIG. 2. Fungal growth of *F. oxysporum* f. sp. *lycopersici* co-cultured with *P. fluorescens* strain LRB3W1 (LRB3W1) and its growth without the bacterium (Control). The plates were incubated for 10 d at 27°C. For comparison, the fungal growth of the pathogen was examined on the dNBYG medium plates containing the fungicide, benomyl, at 1, 10, and 100 μg/ml. Error bars indicate standard deviations.

10 μg/ml, the pathogen was tolerant to benomyl at 1 μg/ml concentration (Fig. 2).

Strain LRB3W1 produces the antibiotics DAPG, HCN and fluorescent siderophores (Tazawa et al., 2000; Tsuchiya et al. 1997). These antifungal factors producing rhizobacteria are well known as effective biocontrol agents against various soilborne phytopathogens including fusaria (He et al., 2004; Raaijmakers et al., 2002). It is thought that antibiotics play a role in the antagonism of strain LRB3W1 against *F. oxysporum* (Sugisawa et al., 2004). Biocontrol activity by antagonistic microbes is often influenced by environmental factors including fungicide applications. However, the *in vitro* growth of the strain LRB3W1 was not influenced by the presence of benomyl at 100 μg/ml (data not shown).

Under glasshouse conditions, disease symptoms appeared 2 wk after pathogen inoculation, and the disease incidence of plants in control increased 4 wk after pathogen inoculation. Three-fourth of the leaves in the control showed yellowing, wilting, or fell off 4 wk after inoculation of the pathogen (Fig. 3). In contrast, the bacterial treatment suppressed the disease incidence by ca. 40% (Fig. 4). The pathogen, *F. oxysporum* f. sp. *lycopersici*, was detected from all diseased petioles collected from the control plants. In the bacterium-treated plants, the pathogen was detected from the lower leaves, but not from the upper leaves at the time of sampling (data not shown). Treatment with fungicide, benomyl, at 100 μg/ml reduced the disease incidence by ca. 30% (Fig. 4). These results show that strain LRB3W1 has potential as an effective biocontrol agent for the control of Fusarium wilt of tomato.

FIG. 3. Suppression of Fusarium wilt of tomato in potting soil by *P. fluorescens* strain LRB3W1 (+LRB3W1) 4 wk after pathogen inoculation. Distilled water (Control) or 15 ml of the bacterial suspension (ca. 1 x 10^9 CFU/ml) was applied to the soil 1 d before pathogen inoculation.

FIG. 4. Comparison of effectiveness of the chemical fungicide (Benomyl) at different concentrations, 1, 10, and 100 μg/ml, and biological control by *P. fluorescens* strain LRB3W1 (LRB3W1), used alone or in combination, against Fusarium wilt of tomato. The columns with different letters are significantly different (*P* = 0.05).

It is considered that the antibiotics, DAPG, HCN, and fluorescent siderophores produced by strain LRB3W1 played important roles in suppression of disease development as in the cases of the antifungal activity by the bacterium *in vitro* (Tsuchiya et al., 1997). In addition to the direct mechanisms, other mechanisms by indirect action have a possibility to reduce the disease incidence. It has been reported that many bacterial and fungal biocontrol agents induced systemic resistance in tomato plants against...
fungal diseases such as Fusarium wilt (Amemiya et al., 1986; Duijff et al., 1998; Komada et al., 1994; Yamaguchi et al., 1992). The strain LRB3W1 also induced systemic resistance to the host plants against phytopathogens, and DAPG produced by the strain was proven as one of the systemic resistance inducers (lavicoli et al. 2003; Someya et al., 2005b). We hypothesize that various factors such as antibiotics as well as induced systemic resistance caused by strain LRB3W1 play synergistic roles in suppression of disease development.

The disease incidence in plants treated with benomyl at 1 μg/ml was not significantly different from that in the control plants (P = 0.05), but there was a difference in the disease incidence between plants treated with benomyl at 10 and 100 μg/ml (Fig. 4). The bacterial treatment also significantly reduced disease incidence. In the presence of the bacterium, the disease incidence in plants treated with a low dosage of benomyl (1 and 10 μg/ml) was significantly lower than that in plants treated with fungicide alone (Fig. 4). The bacterial treatment in combination with 10 μg/ml of benomyl was as effective as the benomyl treatment at 100 μg/ml. These results point to the possibility of combining biological and chemical treatment to reduce the fungicide application required for control of Fusarium wilt of tomato.

Methyl bromide had played an important role in soilborne plant diseases control systems. However, there is a pressing need for effective alternatives to methyl bromide application (Martin, 2003). There are few effective chemical fungicides against soilborne phytopathogens, but benomyl is one of the few really effective fungicides against soilborne phytopathogens such as fusaria (Channon and Thomson, 1973; Mihuta-Grimm et al., 1990). Benomyl functions as an antimitotic agent by binding to fungal β-tublin and preventing microtubule polymerization (Davidse, 1986). However, benomyl at low concentrations does not suppress the disease sufficiently for agricultural use (Fig. 4). Therefore, biocontrol is likely to supplement the application of chemical fungicides at a low dosage for integrated disease control. However, the fungicidal chemical, benomyl, has a possibility to inhibit the fungal antagonists as well as phytopathogens in agricultural use (Kay and Stewart, 1994). Therefore, many researchers have tried to improve the efficacy of fungal antagonists where benomyl is employed (Ogawa et al., 2000). In the present study, the bacterial agent, strain LRB3W1, was not adversely influenced by combination with benomyl at high concentrations. On the contrary, a synergistic effect was observed with treatment of the bacterium in combination with benomyl. We have previously reported that a chitinolytic enzyme-producing bacterium, Serratia marcescens, controlled some fungal plant diseases (Someya et al., 2000; 2005a), and demonstrated that a low dosage of antifungal compounds including antibiotic and chemical fungicides enhanced the antifungal activity of the chitinase-producing bacterium (Someya et al., 2001; 2005c). In the cases of combined application of lytic enzymes and antifungal compounds, cell wall degradation by lytic enzymes has a possibility to enhance the uptake of antifungal compounds into fungal cells (Someya et al., 2001; 2005c; Woo et al., 2002). However, although strain LRB3W1 does not produce lytic enzymes such as chitinases, synergistic biocontrol activity was observed. Other strains of antibiotic-producing bacteria also showed effectiveness in combination with chemical fungicides, but the synergistic mechanisms have not yet been identified (Duffy, 2000; Elmer and McGovern, 2004; Kondoh et al., 2000). We are currently focusing on the role of interactions between the chemical and bacterial factors on the suppressive mechanisms.

In the tomato rhizosphere, the population of LRB3W1-rif/nal/str remained at ca. 10^7 CFU/g soil 4 wk after bacterial inoculation under glasshouse conditions (Fig. 5). Not only did the bacterium not cause any significant damage to tomato plants, but strain LRB3W1 enhanced the growth of several crop plants (data not shown). The bacterial population was not influenced by treatment with benomyl at high concentration (Fig. 5). In the case of co-utilization of a biocontrol bacterium Bacillus subtilis and a fungicide, flutolanil, the bacterium also was not influenced by the existence of flutolanil in soil (Kondoh et al.,...
2000). While the integrated use of many fungal biocontrol agents with chemical fungicides such as benomyl is restricted, bacterial agents such as strain LRB3W1 can be used in combination with reduced fungicide application. The results suggest the strain LRB3W1 would be an effective and persistent biocontrol agent for Fusarium wilt of tomato in the presence or absence of the fungicide, benomyl.

Biocontrol of soilborne phytopathogens by introducing microorganisms has been studied for a long period. However, biocontrol alone is not sufficiently effective in agricultural systems while a low dosage of chemical fungicides also fails to achieve an adequate level of disease control. Therefore, many researchers have stated that the synergistic integrated use of fungicides and biocontrol agents for the control of phytopathogens may be more efficient and longer lasting than that achieved with biological agents or a low dosage of fungicides alone. The present study indicated that synergistic effects between the antagonistic bacterium _P. fluorescens_ strain LRB3W1 and a low dosage of the fungicide, benomyl, played a role in the integrated control of the Fusarium wilt of tomato. Our results demonstrated the possibility that the combined use of a biocontrol agent and chemical fungicide is an attractive approach to reduce the chemical dosages for disease management.

**REFERENCES**


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SUPPRESSION OF TOMATO _FUSARIUM_ WILT 79


