Biological Control of Cyclamen Soilborne Diseases by *Serratia marcescens* Strain B2

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ABSTRACT

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Cyclamen plants were treated with a highly chitinolytic bacterium, *Serratia marcescens* strain B2, and then challenge inoculated with *Rhizoctonia solani* sclerotia or *Fusarium oxysporum* f. sp. *cyclaminis* conidia. The bacterium suppressed these fungal diseases of cyclamen plants, especially the damping off caused by *R. solani*, in a greenhouse. Strain B2 survived at approximately 10^6 to 10^7 CFU/g in soil for 4 months after the initial application under greenhouse conditions. Chitinolytic enzymes and antifungal low-molecular-weight compounds were present in filtrates of *S. marcescens* B2, which suppressed germination of *R. solani* sclerotia in vitro.

Additional keywords: antifungal low-molecular-weight compounds, chitinolytic enzymes, *Fusarium oxysporum* f. sp. *cyclaminis*, *Rhizoctonia solani*

Damping off, caused by *Rhizoctonia solani*, and Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cyclaminis*, are the most common soilborne diseases of cyclamen plants in greenhouses. Most cyclamen cultivars are highly susceptible to these fungal pathogens. Typically, these soilborne diseases are controlled by repeated applications of fungicides; however, repeated applications of broad-spectrum or persistent fungicides may result in soil contamination, fungicide resistance, or harmful effects to nontarget organisms. Thus, biological control is an attractive alternative strategy for the control of cyclamen diseases.

We recently reported that a highly chitinolytic bacterium, *Serratia marcescens* B2, isolated from tomato phylloplane, suppressed the growth of *Botrytis* spp. in vitro (1) and controlled broad bean chocolate spot, caused by *B. fabae*, in a growth chamber (4,21) and cyclamen gray mold, caused by *B. cinerea*, in a greenhouse (5). *S. marcescens* B2 suppressed growth and infection of the fungicide-resistant isolates as effectively as the fungicide-sensitive isolates, both in vitro and in vivo (1,4,5,21). Also, the isolate persisted on leaf discs placed at the base of cyclamen plants and on the phylloplane under humid conditions (5). *S. marcescens* is a ubiquitous organism in water, soil, and food (3); thus, the bacterium may survive well in a greenhouse environment.

Our study was initiated to control cyclamen soilborne pathogens *R. solani* and *F. oxysporum* f. sp. *cyclaminis* with *S. marcescens* B2 under greenhouse conditions. Although some soilborne diseases develop in cyclamen plants throughout the cultivation period, no effective controls for them have been developed. Efficacious control methods are urgently needed for commercial growers. Our objectives were to establish an efficient strategy for biocontrol of soilborne diseases in cyclamen plants, and evaluate integrated control of *R. solani* and *F. oxysporum* f. sp. *cyclaminis* with *S. marcescens* B2.

MATERIALS AND METHODS

Inoculum production. *S. marcescens* strain B2, isolated from the phylloplane of tomato plants growing in a field near Kyusyu University, Japan, was stored as freeze-dried samples at Ibaraki University. The bacterium was routinely cultured at 28°C on chitin-amended Luria broth (LB) agar medium (LBCA; 10 g of tryptone, 5 g of NaCl, 5 g of yeast extract, 1.8 g of colloidal chitin, and 15 g of Bacto-agar [Difco Laboratories, Detroit] in 1 liter of distilled water; 1). For applications, the bacteria, which were incubated in liquid LB medium at 28°C on a reciprocal shaker (125 strokes/min) for 48 h, were collected by centrifugation at 10,000 rpm for 10 min, and diluted with sterile water to approximately 1×10^9 CFU/ml. *R. solani* AG-4 was obtained from the Institute for Fermentation (IFO, Osaka, Japan). To produce sclerotia, the fungus was grown on potato sucrose agar (PSA) medium at 25°C in the dark for 2 to 3 weeks. The sclerotia (approximately 3 mm in diameter) were incubated in potato sucrose (PS) liquid medium at 25°C for 24 h on a reciprocal shaker (125 strokes/min) before use as inoculum.

F. oxysporum f. sp. *cyclaminis* IHF-1 was isolated from Fusarium wilt-diseased cyclamen plants in Ibaraki Horticultural Research Institute greenhouses (Minori-machi, Ibaraki, Japan). To produce the inoculum, the fungus was incubated on PSA at 25°C in the dark for 4 days. Mycelial discs (5 mm in diameter), cut with a cork borer from the colony, were cultured in PS liquid medium at 25°C for 5 days on a reciprocal shaker (140 strokes/min). The culture was centrifuged at 5,000 rpm for 5 min and the pellet resuspended in sterile distilled water, then passed through four layers of sterile tissue paper to remove mycelial fragments. The conidial suspension was adjusted to a concentration of approximately 1×10^6 conidia/ml for use as inoculum.

Plant materials. Cyclamen (*Cyclamen persicum* Miller) cv. Pias was used in this study. Seed was sown on artificial soil (Metro-Mix 360, Scotts-Sierra Horticultural Products Co., Marysville, OH) and kept in the dark for 4 weeks at 25°C with high moisture. Seedlings grown for 2 to 4 weeks under greenhouse conditions were used in experiments with *R. solani*. Plants grown for approximately 5 months in pots (15 cm in diameter and 10 cm tall) under greenhouse conditions were used in experiments with *F. oxysporum* f. sp. *cyclaminis*.

In vitro antifungal activity assay. Four paper discs (7 mm in diameter) inoculated with a 20-µl suspension (approximately 10^9 CFU/ml) of *S. marcescens* strain B2 were placed on LB agar (LBA), LBCA, PSA, and chitin-amended potato sucrose agar (PSCA) media on Petri dishes (90 mm in diameter). After incubation at 28°C for 24 h in the dark, a mycelial disc (5 mm in

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diameter) of either 4-day-old *R. solani* AG-4 or *F. oxysporum* f. sp. *cyclaminis* IHF-1, both collected from colonies on PSA, was placed singly on the center of each Petri dish containing the bacterium. Three replicate plates were used in each assay. Fungal colony diameters were measured after incubation at 25°C in the dark for 7 days, and mycelial growth was observed daily under a light microscope.

Biocontrol test. To test the ability of *S. marcescens* B2 to control damping off caused by *R. solani*, cyclamen seedlings were treated with the bacteria in one of the following two ways: either a 20-ml bacterial suspension (approximately 1×10^9 CFU/ml) was added to the artificial medium in 120-ml pots containing 2-week-old seedlings produced as described above, or cyclamen seeds were incubated in the bacterial suspension at 20°C for 24 h on a reciprocal shaker (125 strokes/min) before being germinated as described and grown for 6 to 8 weeks. Two comparison treatments were benomyl, applied in a 100-ppm suspension at 20 ml/pot, or sterile water. Four sclerotia of *R. solani* were buried approximately 1 cm deep in each pot. All treatment combinations were replicated six times, and placed in a growth chamber at 20°C, arranged in randomized complete blocks. After 1 week, disease incidence was calculated as the percentage of seedlings damping off.

To test the ability of *S. marcescens* B2 to control wilt caused by *F. oxysporum* f. sp. *cyclaminis*, the bacterium was applied as a suspension of approximately 1×10^9 CFU/ml added to the artificial medium in pots (15 cm in diameter) containing cyclamen plants grown as described above. Four comparison treatments were: benomyl, applied in a 100-ppm suspension at 160 ml/pot; 0.2% colloidal chitin solution, 160 ml/pot; a mixture of 80 ml of bacterial suspension (approximately 2×10^9 CFU/ml) and 80 ml of 0.4% colloidal chitin suspension; and sterile water. *F. oxysporum* f. sp. *cyclaminis* was applied to each pot in 60 ml of a conidial suspension (approximately 1×10^6 conidia/ml). Each treatment combination was replicated three times. The experiment was placed in a greenhouse in a completely randomized arrangement and maintained at 20 to 25°C for 3 to 4 weeks until typical wilt symptoms appeared. Disease incidence was evaluated according to a damage index, as follows: 0 = no yellowing on the leaves, 1 = yellowing of approximately one third of leaves, 2 = yellowing of approximately one half of leaves, and 3 = yellowing of the whole leaf. Disease incidence (percent) was calculated by the following formula: Disease incidence (%) = $[(0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3)/3 \times (n_0 + n_1 + n_2 + n_3)] \times 100$ (n_{0-3} = number of index-0 to -3 leaves).

Population dynamics of *S. marcescens* B2 in cyclamen rhizosphere. *S. marcescens* B2 was applied to pots containing 6-week-old or 10-month-old cyclamen plants in 20 ml of bacterial suspension (approximately 1×10^9 CFU/ml) per 100 g of artificial medium. Plants were kept in the greenhouse at 15 to 20°C. Each week after the application, a 1-g sample was taken from each pot at 1 and 5 cm depth, approximately 2 cm from the cyclamen tuber, and transferred to a 25-ml bottle. These samples were washed with 10 ml of sterile water, and serial dilutions were cultured on LBCA plates for 24 h. Colonies with reddish pigment and chitinolytic halo were counted to estimate the *S. marcescens* B2 population. Samples were also collected from pots containing a cyclamen seedling grown from seed incubated in the bacterial suspension for 24 h at 20°C ("seed bacterization"), and from pots with no cyclamen plants ("non-rhizosphere").

Chitinolytic and antifungal assays of the culture filtrate. Chitinolytic activity in the culture filtrate of *S. marcescens* B2 was assayed using the following procedure. The bacterium was incubated in liquid LB medium at 28°C on a reciprocal shaker (125 strokes/min) for 72 h. The culture was centrifuged at 10,000 rpm for 5 min, and the supernatant was separated into low- and high-molecular-weight fractions with a dialysis tube (12,000-14,000 molecular-weight cutoff, 25-mm diameter, regenerated cellulose, Sanko Co., Tokyo, Japan). Fractions were assayed after treatment as follows: heating for 48 h at 28 or 37°C, or for 2 h at 60°C; autoclaving for 25 min at 121°C; or treating with 1% proteinase K (EC 3.4.21.14, WAKO Co., Osaka, Japan) for 48 h at 37°C. The chitinolytic activity was assayed with *p*-nitrophenyl-β-D-N-acetylglucosaminide (pNP-NAG1), *p*-nitrophenyl-β-D-N,N′-diacetylchitobiose (pNP-NAG2), and *p*-nitrophenyl-β-D-N,N′,N″-triacetylchitotriose (pNP-NAG3) as substrates. Reaction mixtures containing 100 µl of each fraction diluted 10 times and 10 µg of substrate in 100 µl of NcIl vaine buffer, pH 6.0, were incubated at 37°C for 60 min. The reaction was stopped with 100 µl of 1 M Na_2CO_3 and absorbance of released *p*-nitrophenyl (pNP) was measured at 405 nm with a spectrophotometer.

The antifungal activity of the fractions was assayed using the following procedure. Sclerotia of *R. solani* AG-4 were incubated in each fraction at 25°C for 72 h on a reciprocal shaker (60 strokes/min). After sterilization of the sclerotia with 70% ethanol, they were incubated on water agar (WA, 15 g of agar in 1 liter of distilled water) in a Petri dish at 25°C in the dark. Germination of the sclerotia was examined after 48 h of incubation.

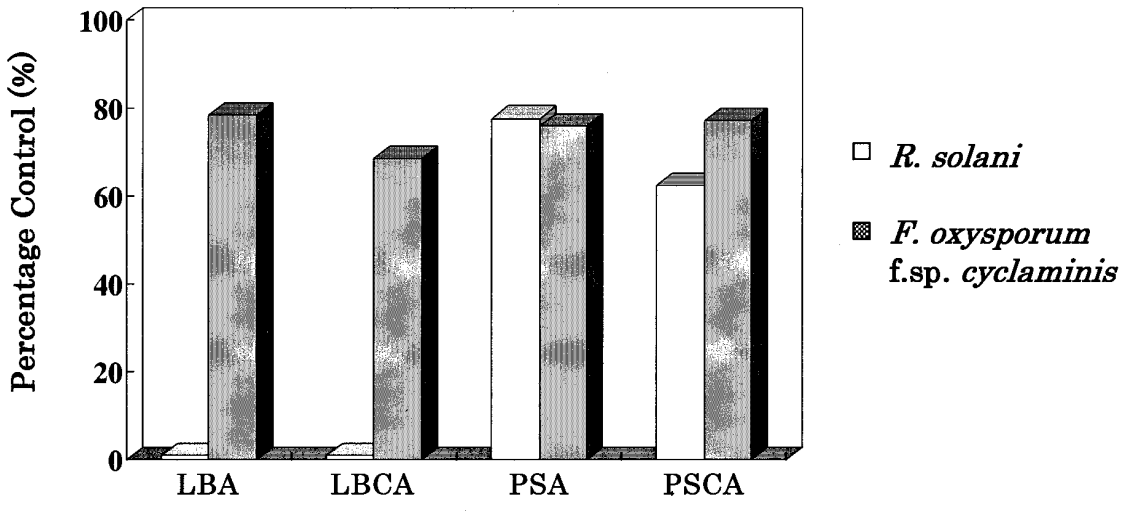

Fig. 1. Radial growth inhibition of *Rhizoctonia solani* AG-4 and *Fusarium oxysporum* f. sp. *cyclaminis* IHF-1 co-cultured with *Serratia marcescens* strain B2 at 25°C for 7 days in the dark. Fungal growth inhibition was calculated as follows: percent control = (average fungal colony diameter in culture with the bacterium/average colony diameter in culture without the bacterium) × 100. LBA = 10 g of tryptone, 5 g of NaCl, 5 g of yeast, and 15 g of agar; LBCA = LBA amended with 1.8 g of colloidal chitin; PSA = potato sucrose agar; and PSCA = PSA amended with 1.8 g of colloidal chitin/liter.

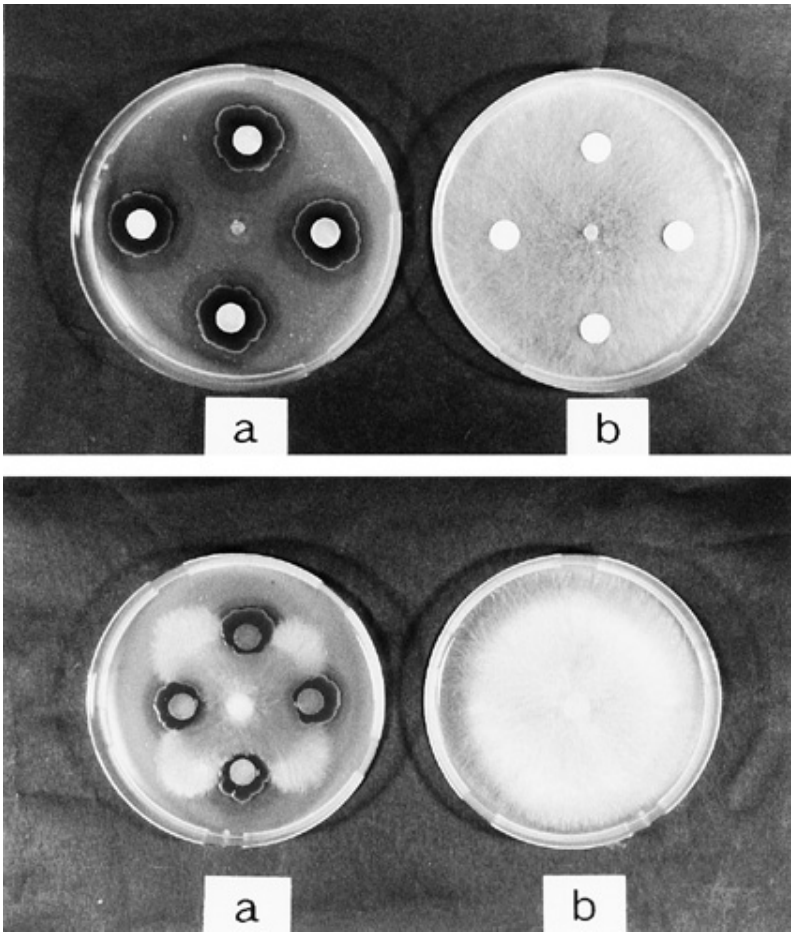

Fig. 2. Radial growth of *Rhizoctonia solani* AG-4 (upper) and *Fusarium oxysporum* f. sp. *cyclaminis* IHF-1 (lower) co-cultured **(A)** with *Serratia marcescens* B2 or **(B)** without the bacterium. The bacterium produced reddish pigment and formed clear zones on LBCA (10 g of tryptone, 5 g of NaCl, 5 g of yeast, and 15 g of agar, amended with 1.8 g of colloidal chitin).

RESULTS

Growth inhibition of *R. solani* and *F. oxysporum* f. sp. *cyclaminis* in vitro. Growth of *R. solani* was almost completely inhibited, and growth of *F. oxysporum* f. sp. *cyclaminis* was reduced to approximately 70% compared with that in the absence of the bacterial colonies (Figs. 1 and 2). However, the growth inhibition of these fungi by isolate B2 was markedly reduced on PSCA, where neither hydrolyzation of colloidal chitin nor production of reddish pigment by isolate B2 were detected.

Light microscopy showed abnormal forms of *R. solani* mycelia (e.g., swelling, curling, or bursting), which suggested degradation of the hyphal cell wall or hyphal cell death, on the clear zones around isolate B2 colonies in LBCA (Fig. 3). In *F. oxysporum* f. sp. *cyclaminis* mycelia, however, these phenomena were rarely observed.

Suppressive effect of *S. marcescens* B2 on damping off. In a growth chamber, no damping off was observed on cyclamen seedlings in soil treated with either the bacterial suspension or benomyl for up to 5 days after inoculation; whereas, a 33% incidence of damping off was observed in the control with the pathogen alone (Fig. 4). On the seedlings grown from seed treated with B2, damping-off incidence was 19%, but still lower than in the control. *S. marcescens* B2 did not cause any significant damage to cyclamen seedlings during the test periods.

Suppressive effect of *S. marcescens* B2 on Fusarium wilt. Under greenhouse conditions, Fusarium wilt incidence was reduced to 55% on cyclamen plants grown in

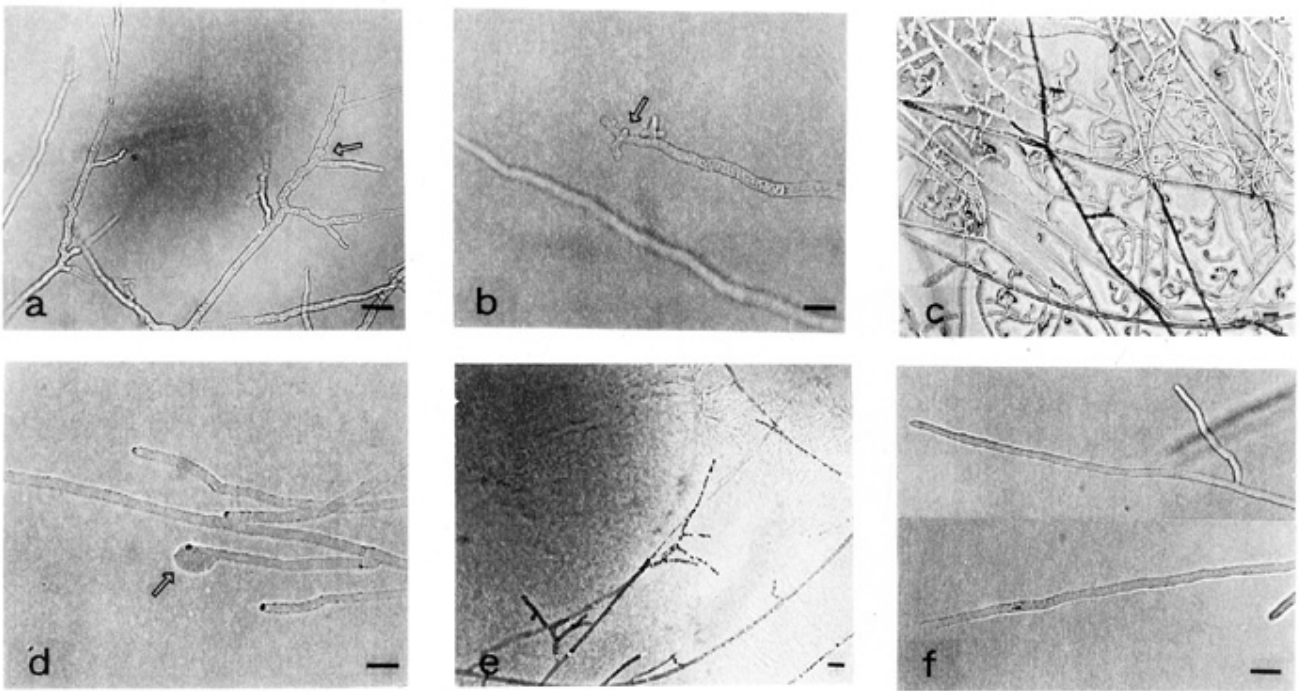

Fig. 3. Light microscopy of *Rhizoctonia solani* mycelia co-cultured with *Serratia marcescens* B2. The mycelia showed abnormal forms suggesting degradation of the hyphal cell wall: partial swelling (arrows) **(A)** in the hyphae and **(B)** at the tip; **(C)** hyphal curling; **(D)** burst (arrow) at the hyphal tip; or **(E)** hyphal collapse. **(F)** These abnormal forms were not found in mycelia cultured without the bacterium. Scales: 40 µm.

soil treated with B2 for up to 21 days after inoculation; whereas, 90% incidence was observed in the control with the pathogen alone (Fig. 5). In the presence of chitin, the efficacy of B2 increased 20% over that of treatment with the bacterium alone (Fig. 5). *S. marcescens* B2 did not cause any significant damage to cyclamen plants during the test periods.

Survival of *S. marcescens* B2 in the cyclamen rhizosphere. In pots with the plants cultivated for 6 weeks and 10 months, the populations of B2 remained near 10^5 and 10^7 CFU/g, respectively, for more than 12 weeks under greenhouse conditions (Fig. 6). However, B2 was markedly reduced in the unplanted soil, and the bacterium was not detected in the soil at 7 weeks after application. In the soil sown with the seed, the population level remained at 10^3 to 10^4 CFU/g for 14 weeks (Fig. 6). *S. marcescens* B2 and other chitinolytic organisms were not detected in the nontreated control soil.

Chitinolytic and antifungal activities in the *S. marcescens* culture filtrate. After 72 h in liquid shake culture, the high-molecular-weight fraction of B2 culture filtrate released pNP from pNP-NAG1, pNP-NAG2, and pNP-NAG3 (Fig. 7). The chitinolytic activity was highest with pNP-NAG1. Interestingly, activity with pNP-NAG3 was higher than with pNP-NAG2. After heating at more than 60°C or treating with proteinase K, the high-molecular-weight fraction showed very low chitinolytic activity. Chitinolytic activity was not detected in the low-molecular-weight fraction of B2 culture filtrate (Fig. 7).

When sclerotia of *R. solani* AG-4 were treated with the low- and high-molecular-weight fractions of B2 culture filtrate, more than 70% of the sclerotia failed to germinate on WA plates (Table 1). After sclerotia were heated at up to 60°C or treated with proteinase K, the high-molecular-weight fraction showed very low antifungal activity, whereas that of the low-molecular-weight fraction remained high (Table 1).

DISCUSSION

Damping off, caused by *R. solani*, is one of the soilborne diseases in cyclamen plants that develops within 10 weeks after sowing. The fungus does not sporulate; thus, the sclerotia are the survival and dissemination units. Sclerotia in soil are very hard to control with fungicides. Also, this fungus can infect an extremely wide range of crop plants. Because of these characteristics, the complete control of this disease with fungicides or the use of resistant cultivars was difficult.

In our experiment, *S. marcescens* B2 survived in the artificial media used for growing cyclamen seedlings under greenhouse conditions. Treatment with the bacterium suppressed damping off in cyclamen seedlings. Also, fungal growth and sclerotial germination were inhibited in the presence of the bacterium in vitro. These results suggested that *S. marcescens* B2 could be used as an effective biocontrol agent against cyclamen damping off under greenhouse conditions.

Fusarium wilt, caused by *F. oxysporum* f. sp. *cyclaminis*, is also an important soilborne disease of cyclamen plants in Japan. This disease has been controlled with methyl bromide, chloropicrin, or benomyl. These chemicals, however, are undesirable due to sudden reduction of the fungicidal activity, environmental pollution, and cost.

In our biological control test, fungal growth was barely inhibited in the presence of the bacterium in vitro; whereas, bacterial treatment of the soil suppressed Fusarium wilt to 50% in cyclamen plants. B2 showed no suppression against some isolates of *Fusarium*, *Verticillium*, or *Gibberella* spp. in in vitro tests (21), although *Pseudomonas* isolates expressing *S. marcescens* chitinase were reported to be

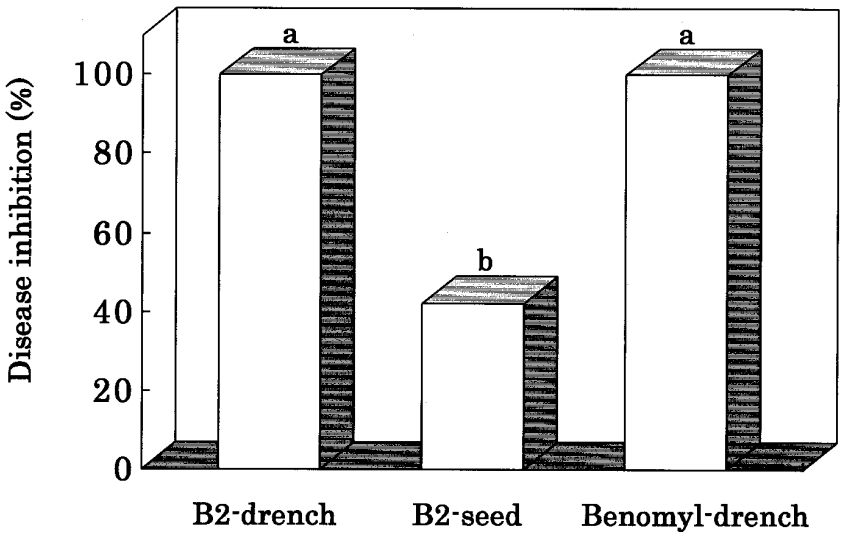

Fig. 4. Effect of *Serratia marcescens* B2 on *Rhizoctonia solani* AG-4-induced damping off of cyclamen seedlings. The treatments were as follows: 20-ml bacterial suspension applied to the medium in which seedlings were growing (B2-drench); 100 ppm benomyl applied in a 20-ml suspension (benomyl-drench); or seedlings growing from seed incubated in the bacterial suspension at 20°C for 24 h (B2-seed). Four *R. solani* sclerotia were buried 1 cm deep in each pot. The disease severity was calculated as the ratio of numbers of seedlings damping off to total number of seedlings 1 week after inoculation. Different letters indicate statistical significance at the 5% level by Tukey's method.

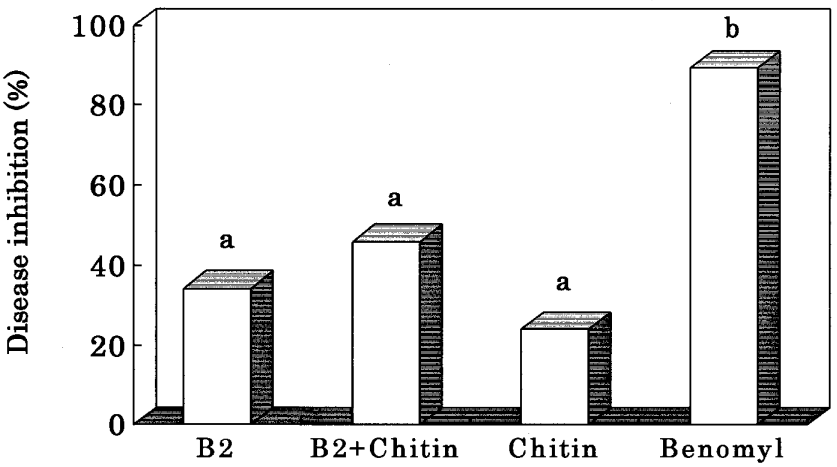

Fig. 5. Effects of *Serratia marcescens* B2 on Fusarium wilt of cyclamen plants with *Fusarium oxysporum* f. sp. *cyclaminis* IHF-1. The treatments were as follows: 160-ml bacterial suspension applied to the medium in which approximately 10-month-old plants were growing (B2-drench); 100 ppm benomyl applied in a 160-ml suspension (benomyl-drench); 0.2% colloidal chitin solution in a 160-ml suspension (chitin-drench); or mixtures of bacterial suspension and colloidal chitin solution (B2 + chitin-drench). A 60-ml conidial suspension was applied to the medium in each pot. The disease severity was calculated with the following formula: disease incidence (%) = $[(0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3)/3 \times (n_0 + n_1 + n_2 + n_3)] \times 100$ (n_{0-3} = number of index-0 to -3 leaves) at 3 weeks after inoculation. Different letters indicate statistical significance at 5% level by Tukey's method.

suitable for biological control of *F. oxysporum* (17). These results suggested that an indirect effect of B2 was responsible for the inhibition of cyclamen Fusarium wilt rather than a direct antifungal action. Such an idea is supported by reports (8,9,19,20) that systemic resistance against plant diseases was induced in plants by application of plant growth-promoting rhizobacteria, such as *P. fluorescens*.

High antifungal activities were detected in both fractions of *S. marcescens* B2 cul-

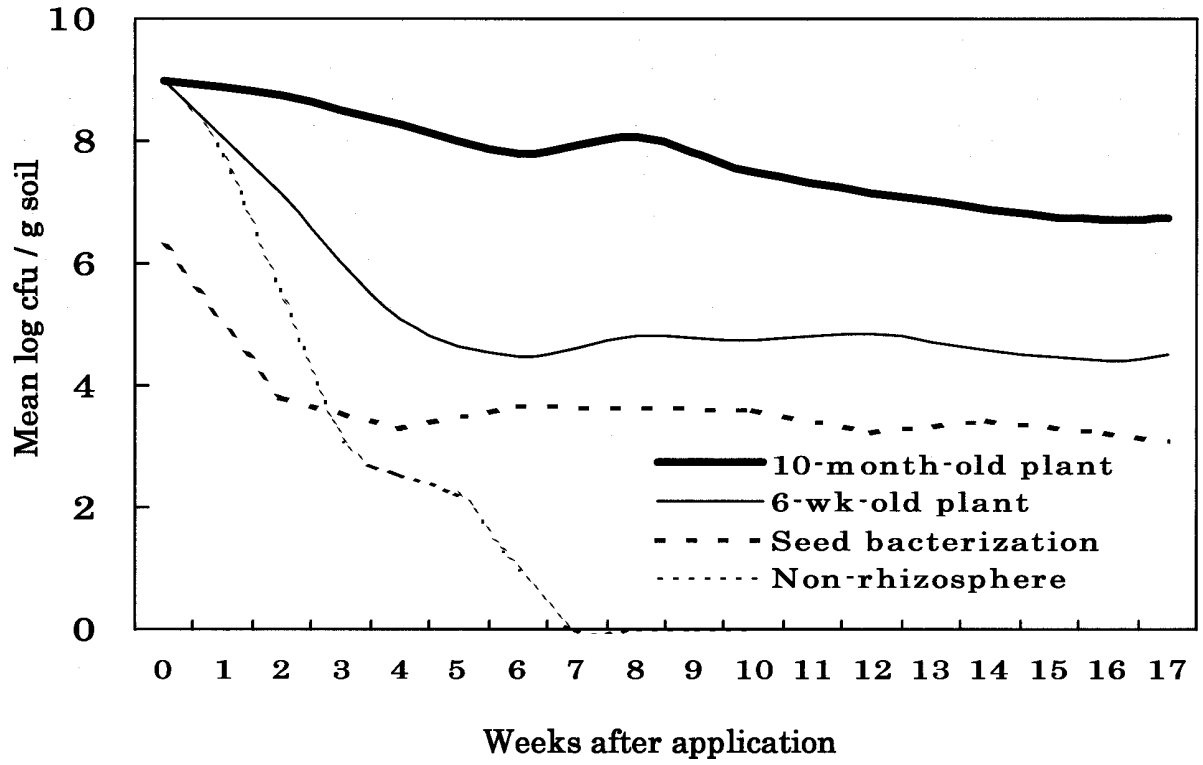

Fig. 6. Population dynamics of *Serratia marcescens* B2 in pot containing 6-week- or 10-month-old cyclamen plants, a cyclamen seedling from seed incubated in a suspension of *S. marcescens* B2 at 20°C for 24 h (seed bacterization), or no cyclamen plant (non-rhizosphere).

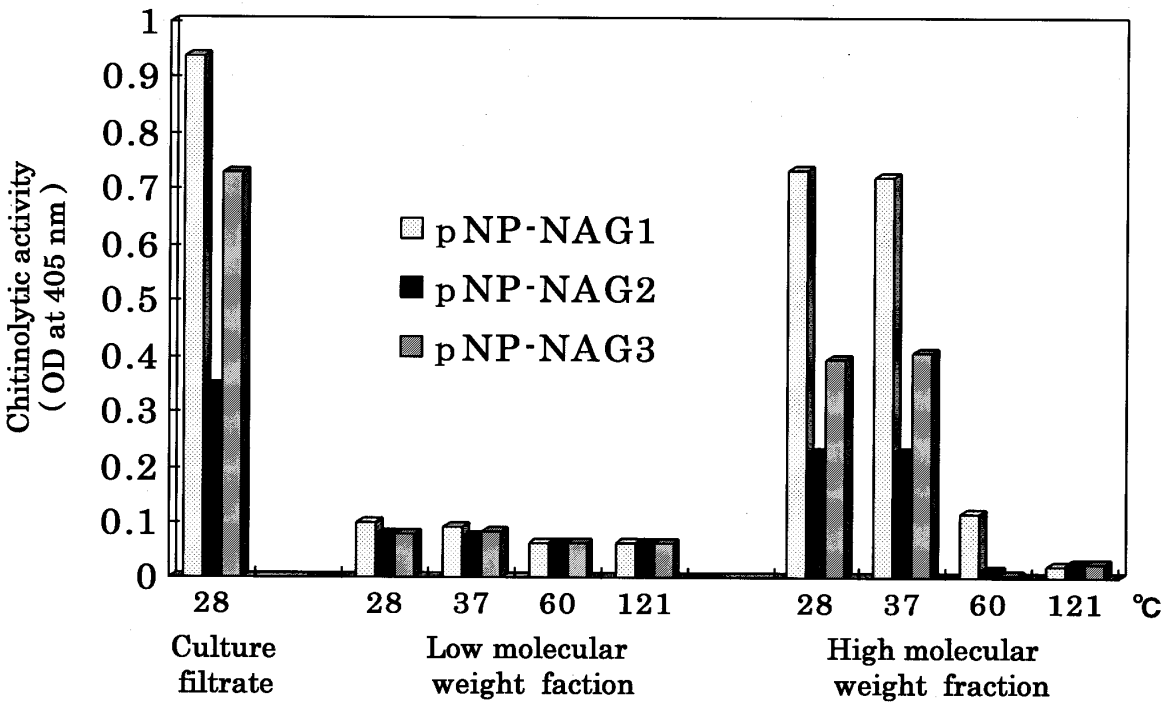

Fig. 7. Chitinolytic activity in low- and high-molecular-weight fractions of *Serratia marcescens* B2 culture filtrate. The activity was measured by optical density (405 nm) of *p*-nitrophenyl (pNP) released from pNP-NAG1, pNP-NAG2, and pNP-NAG3 after 1 h of incubation at 37°C. The fractions were assayed after treatment as follows: heating for 48 h at 28°C or autoclaving for 25 min at 121°C.

ture filtrate; the low-molecular-weight molecule was heat stable and non-proteinaceous, and the high-molecular-weight molecule was heat unstable and proteinaceous. The low-molecular-weight molecule may be the same substance with the reddish pigment, prodigiosin, produced by *S. marcescens* (12,13). This pigment was secreted only at the time when the antifungal activity was detected, and the pigment was fungitoxic to *Phytophthora capsci* and *Pythium aphanidermatum*. Both Oomycetes contain little chitin in the cell walls (1,21). Structural analysis of the low-molecular-weight molecule is now in progress.

In our study, activity with pNP-NAG3 was higher than with pNP-NAG2. These results indicate the presence of both exochitinase and endochitinase activities, because exochitinase (N-acetyl-β-glucosaminidase) can release N-acetyl-D-glucosamine from the non-reducing end of the chitin chain quite slowly and endochitinases can cut chain oligomers longer than trimer quite rapidly. It is suggested that *S. marcescens* B2 produced two types of chitinolytic enzymes, endo- and exotypes. These results are compatible with previous reports on the antifungal action of *S. marcescens* and its chitinase toward other fungi (6,14,16,17). In our study, however, although *F. oxysporum* f. sp. *cyclaminis* was hardly inhibited with *S. marcescens* B2 in vitro, application of the bacterium as a drench suppressed Fusarium wilt to approximately 50% in the cyclamen plants. These results suggested that the control of cyclamen Fusarium wilt by the bacteria was by indirect action (e.g., induction of systemic acquired resistance in the plants) rather than direct antifungal action.

Oligosaccharide-elicitors, which are produced by the degradation of the fungal cell wall with chitinase or β-1,3-glucanase, have been reported to play an important role in signal transduction essential for various plant defense mechanisms (e.g., production of phytoalexins, lignification, and induction of hypersensitive reactions; 2,7,10,18). Our results showed that *S. marcescens* B2 secretes both exo- and endochitinase. Also, antifungal substances were detected from the leaves of cyclamen plants treated with isolate B2, but not from untreated cyclamen leaves, by thin-layer chromatography bioassay (*data not shown*). Based on these facts, we propose that bacterial application to the cyclamen growing medium has an inductive effect on defense reactions but no direct effect on the fungi. This effect is thought to be related to the production of elicitor-like oligosaccharides from the fungal cell walls by hydrolytic action of chitinolytic enzymes of B2. On the other hand, various factors produced by bacterium, *S. marcescens* B2, and *Pseudomonas* spp. (11,15), have been reported as elicitors inducing systemic resistance to plant diseases. The structural analysis of elicitors produced by *S. marcescens* B2 is now in progress.

S. marcescens B2 has already been proven useful for biocontrol of gray mold, an airborne disease in cyclamen plants (5). The bacterium produces chitinolytic enzymes, which cause degradation of the fungal cell walls and induction of plant defense reaction, in addition to the antifungal low-molecular-weight molecule. These results suggest that this bacterium may be an effective and persistent biocontrol agent for both airborne and soilborne diseases in cyclamen plants.

Table 1. Antifungal activities against *Rhizoctonia solani* sclerotia by *Serratia marcescens* strain B2 culture filtrate[a]

Fraction, temperature (°C)[b]	Treatment[c]	Inhibition (%)[d]
Low		
28	–	95.3
37	–	93.9
37	+	68.3
60	–	94.6
121	–	83.8
High		
28	–	73.3
37	–	72.1
37	+	14.0
60	–	11.6
121	–	9.8

[a] Sclerotia of *R. solani* AG-4 were incubated in each fraction treated (as indicated above) at 24°C for 72 h on a reciprocal shaker (60 strokes/min). After sterilization of the sclerotia with 70% ethanol, they were incubated on water agar at 24°C in the dark for 48 h.

[b] Culture filtrate of *S. marcescens* strain B2 was separated into low- and high-molecular-weight fractions with a dialysis tube (12,000 to 14,000 molecular weight cutoff, 25-mm-diameter regenerated cellulose, Sanko Co.). Each fraction was heated for 48 h at 28 or 37°C, for 2 h at 60°C, or for 25 min at 121°C.

[c] Each fraction was treated with 1% proteinase K for 48 h at 37°C.

[d] Inhibition of sclerotial germination = ratio of germinated sclerotia to total incubated sclerotia.

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