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Co-utilization of *Bacillus subtilis* and Flutolanil in controlling damping-off of tomato caused by *Rhizoctonia solani*

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Abstract

Damping-off of tomato caused by *Rhizoctonia solani* was controlled in a pot test using the biological agent, *Bacillus subtilis* RB14-C, and the chemical pesticide, Flutolanil. The co-utilization of *B. subtilis* RB14-C, and Flutolanil decreased the amount of Flutolanil used from 375 μg/pot when Flutolanil was used alone to 94 μg/pot, while exerting the same effect of reducing disease occurrence.

Introduction

Excessive use of chemical pesticides has caused a number of environmental problems. Because of this, the use of biological control agents has been promoted. Many applications of fungus and bacteria for plant disease control have been attempted (Weller 1988, Cook 1990, Gutterson 1990, Shoda 2000). However, it is difficult to assess the 100% suppressibility of plant disease in soil by application of only microorganisms, mainly due to the complexity of the plant and soil system. Therefore, the co-utilization of chemical pesticides and microorganisms may be one of the practical methods for use in the field. By such co-utilization, a reduction in the amount of chemical pesticides to be used is anticipated (Hwang 1994, Hwang *et al.* 1996, Mandeel 1996).

Bacillus subtilis RB14-C was isolated as a microbial agent to suppress the growth of plant pathogens both *in vitro* and in a plant test and the characteristics of this bacterium were described in detail in previous papers (Hiraoka *et al.* 1992a,b, Phae *et al.* 1992, Huang *et al.* 1993, Asaka & Shoda 1996). In this study, this bacterium is co-utilized with the chemical pesticide, Flutolanil, which is a commercially available chemical agent to suppress the damping-off caused by *Rhizoctonia solani*, and the usefulness of this method is investigated.

Materials and methods

Strain and medium used

Bacillus subtilis RB14-C is a spontaneous, streptomycin-resistant mutant of the parent strain RB14 and is a co-producer of antifungal antibiotics iturin A and surfactin, which are associated with the suppression of plant diseases (Hiraoka *et al.* 1992a). The growth rate and the antifungal activity of *B. subtilis* RB14-C were confirmed to be the same as those of the parent strain *B. subtilis* RB14. L medium consisting of 10 g Polypepton (Nippon Pharmaceutical Co., Ltd., Tokyo) 5 g yeast extract and 5 g NaCl (per liter) was used for the growth of the bacterium. Number 3 (No. 3) medium used for antifungal antibiotic production contains (per liter) 10 g Polypepton, 10 g glucose, 1 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$ adjusted to pH 6.8.

As a fungal plant pathogen, *Rhizoctonia solani* K-1, which was isolated at Kanagawa Horticultural Experiment Station, Kanagawa, Japan, was used (Asaka & Shoda 2000) as a severe damping-off pathogen in many plants. The fungus was grown on

potato/dextrose (PD) medium containing (per liter) potato infusion 200 g and glucose 20 g (pH 5.6).

Chemical pesticide used

A 25% solution of Flutolanil (v/v), (α,α,α-trifluoro-3′-isopropoxy-*o*-toluanilide) (Nihon Noyaku Co., Ltd., Tokyo) was used.

Growth of B. subtilis *RB14-C on plate containing Flutolanil*

After *B. subtilis* RB14-C was cultivated in L medium at 37 °C for 16 h with shaking, 50 μl of culture broth suitably diluted was spread on L-agar (L medium plus 1.5% agar) plate containing 100 mg Flutolanil l^{-1}. The number of colonies that appeared on the plates was counted after 24 h incubation at 37 °C and compared with that of the control plate containing no Flutolanil.

Inhibitory effect of mixture of iturin A and surfactin from supernatant of B. subtilis *RB14-C and Flutolanil on growth of* R. solani in vitro

B. subtilis RB-14C was grown in No. 3 medium at 30 °C for five days. Then, the culture broth was acidified with 12 M HCl at pH 2.0 and centrifuged at 12 000 $\times$ *g* for 25 min. The precipitate was extracted with 10 ml methanol and centrifuged again. The supernatant was filtered through a 0.2 μm pore size polytetrafluoroethylene (PTFE) membrane (JP020, Advantec Ltd., Tokyo) and the filtrate was injected into a HPLC column (octadecylsilanolate)-2, 4.6 mm diam $\times$ 250 mm; GL Sciences, Tokyo, Japan) to measure the concentrations of iturin A and surfactin (Asaka & Shoda 1996). Different concentrations of the filtrate and 0.2 mg Flutolanil l^{-1}were poured into a plastic plate containing PDA (potato/dextrose + 1.5% agar) medium. As a control, PDA containing only 0.2 mg Flutolanil l^{-1} was prepared. Then, 5 mm plugs were taken from a PDA culture of *R. solani* K-1 and placed on the center of the plate prepared above. After incubation at 28 °C for seven days, the relative areas of fungal growth on the plate containing the mixture of iturin A and surfactin and Flutolanil to that on the plate containing only Flutolanil were measured.

Growth of B. subtilis *RB14-C and production of iturin A and surfactin in a medium containing Flutolanil*

A 300 μl of cell culture of *B. subtilis* RB14-C prepared in L medium was poured into 20 ml of No. 3 medium containing 10 mg Flutolanil l^{-1} in shaking flasks and the optical density at 660 nm which was converted to dry cell weight was monitored at 37 °C by shaking at 120 spm for five days. At each sampling time, one flask was taken and the entire 20 ml of the culture broth was treated in the same manner to measure the concentrations of iturin A and surfactin by HPLC.

Plant test in pot

The characteristics of the soil used in this study were the same as those described previously (Asaka & Shoda 1996). The soil was autoclaved for 60 min at 121 °C four times at 12-h intervals. Sterilized soil (150 g) was put into a plastic pot with a diameter of about 90 mm, and moisture was kept at 60% of the maximum water holding capacity by the daily addition of sterilized water.

R. solani K-1 was incubated statically in the dark at 30 °C for one week in PD medium and the mycelial mats that formed on the surface of the medium were homogenized (4000 rpm, 2 min) in sterile water and inoculated into the soil at a ratio of one piece of the mat to one pot five days before planting the germinated tomato seeds.

The plant test followed the procedure previously reported (Asaka & Shoda 1996). Seeds of tomato (Ponderosa) were disinfected with 70% (v/v) ethanol and then with 0.5% sodium hypochlorite. After rinsing with sterile water, the seeds were germinated on a 2% (w/v) agar plate at 30 °C for two days. Each pot was sown with nine germinated seeds after the samples of *B. subtilis* RB14-C culture and Flutolanil were introduced into soil and placed in a growth chamber at 30 °C with 80% relative humidity under 16 h of light (about 12 000 lux). After two weeks, the percentage of diseased seedlings per pot was determined. Furthermore, the shoots were clipped off at the soil surface level and their lengths and dry weights were measured.

Preparation of B. subtilis *RB14-C and Flutolanil for plant test*

The following four samples of the culture broth of *B. subtilis* RB14-C grown in No. 3 medium for five days and Flutolanil were introduced into the soil two days after *R. solani* inoculation.
(1) Flutolanil solution to give 94 μg/pot.
(2) Flutolanil solution to give 375 μg/pot.
(3) Thirty ml of culture broth of *B. subtilis* RB14-C.
(4) A mixture of 94 μg Flutolanil/pot and 30 ml of culture broth.

Table 1. Effects of treatments of *B. subtilis* RB14-C and/or Flutolanil on the suppression of damping-off of tomato seedlings caused by *R. solani* 14 days after planting.

R. solani (3.0 g/pot)	Flutolanil (μg/pot)	RB-14-C (ml)	Shoot length (mm)	Dry weight of shoots (mg/pot)	Percentage of diseased seedlings (%)
+	–	–	9.2^{a}	41^{a}	90^{a}
+	94	–	30^{b}	109^{ab}	64^{b}
+	375	–	67^{c}	192^{b}	19^{cd}
+	0	30	48^{bc}	227^{bc}	51^{b}
+	94	30	71^{c}	286^{c}	21^{c}
–	–	–	107^{d}	307^{c}	0^{d}
LSD $p = 0.05$			20	87	21

For each treatment, each datum is an average of results from four experiments. Means in any column with different letters are significantly different ($p = 0.05$) according to Fisher's protected least significant difference (LSD) analysis.

Table 2. Populations of *B. subtilis* RB14-C in soil treated with RB14-C and/or Flutolanil, 0, 3 and 14 days after planting.

Treatment	Viable cell number ($10^8 \times$ cfu g^{-1} dry soil)		
	0 day	3 days	14 days
RB14-C (30 ml)	5.6	3.7	2.4
RB14-C (30 ml) + Flutolanil (94 μg/pot)	4.5	4.1	2.3

Each value is an average of results from two pots in one experiment.

Counting of viable cell number in soil

Soil, 3 g was sampled at 0, 3 and 14 days after planting and suspended in 8 ml of 0.85% NaCl solution (pH 7.0) in a 50-ml Erlenmeyer flask, and then shaken for 15 min at 140 spm at room temperature. The suspension was serially diluted in 0.85% NaCl solution and plated onto L agar plates containing 100 μg ml^{-1} streptomycin. After incubation at 37 °C for 12 h, the number of viable cells was counted and expressed as cfu (colony forming units).

Quantitative analysis of iturin A and surfactin recovered from soil

Soil, 3 g was suspended in 21 ml of acetonitrile/3.8 mM trifluoroacetic acid (4:1 v/v) in a 50-ml Erlenmeyer flask and then shaken for 1 h at 140 spm at room temperature. The soil in the suspension was then removed by filtration with filter paper (Toyo Roshi Co., Ltd., Tokyo) and the filtrate was evaporated. The precipitate was extracted with 2 ml methanol for 2 h and subjected to HPLC analysis described above.

Results and discussion

Growth of B. subtilis *RB14-C in solid and liquid media containing Flutolanil*

The number of viable cells of *B. subtilis* RB14-C on L-agar plate containing 100 mg Flutolanil l^{-1} after 24 h incubation was almost the same as that on L-agar plate without Flutolanil (data not shown). When the growth of *B. subtilis* RB14-C in liquid L medium containing 10 mg Flutolanil $l^{-1}$was compared with that in the same medium without Flutolanil no difference in growth pattern was observed (data not shown). From the results of experiments, *B. subtilis* RB14-C was found to be resistant to Flutolanil.

Productivity of iturin A and surfactin in medium containing Flutolanil

The final concentrations of iturin A after incubation for six days were about 148 mg l^{-1} in liquid medium containing 10 mg Flutolanil l^{-1}, and 160 mg l^{-1} in the Flutolanil-free medium. Surfactin concentration was 80 mg l^{-1} in the Flutolanil-containing medium

Table 3. Concentrations of iturin A and surfactin recovered from soils 0, 3 and 14 days after planting when *B. subtilis* RB14-C and/or Flutolanil were treated.

Treatment		Concentration of indicated antibiotic (μg g^{-1} dry soil)		
		0 day	3 days	14 days
RB14-C (30 ml)	Iturin A	13.3	3.9	0.4
	Surfactin	75.5	60.2	19.1
RB14-C(30 ml) + Flutolanil (94 μg/pot)	Iturin A	15.6	3.0	0.3
	Surfactin	67.5	47.6	13.1

Each value is an average of results from two pots in one experiment.

and 430 mg l^{-1}in the Flutolanil-free medium. This suggests that the synthesis of surfactin was adversely affected by 10 mg Flutolanil l^{-1}, but that of iturin A was almost unaffected by Flutolanil. Since the antifungal effect of iturin A was about 100 times stronger than that of surfactin, decreased in surfactin production may have a minor effect on the ability of *B. subtilis* RB14-C to suppress plant pathogen growth.

Comparison of growth of R. solani *on plates containing a mixture of iturin A and surfactin of* B. subtilis *RB14-C and Flutolanil*

As the filtrate of the culture broth contained 900 mg iturin A l^{-1}and 2500 mg surfactin l^{-1}, different concentrations of the filtrate were prepared by diluting it. When the areas of growth of *R. solani* on plates were compared after incubation for seven days, the growth of *R. solani* was found to decrease in proportion to the increase in iturin A concentration in plates. Flutolanil, 0.2 mg l^{-1} reduced the growth of *R. solani* by 60% compared with no Flutolanil in PDA plate. When different concentrations of iturin A were mixed with 0.2 mg Flutolanil l^{-1}, significant growth inhibition was observed. In the iturin A concentration range of 5–20 mg, growth of *R. solani* was reduced by 95%, indicating the synergistic effects of iturin A and Flutolanil.

Plant test

The results of the plant test are shown in Table 1. The data are average values of four experiments. The percentage of diseased seedlings of the pot containing *R. solani* reached 90%. When Flutolanil was introduced into the pot, the percentage of diseased seedlings was reduced to 19.4–64%, depending on the introduced concentrations. The culture broth of RB14-C suppressed the percentage of diseased seedlings to 51%. Co-utilization of the culture broth of RB14-C and Flutolanil (94 μg/pot) reduced the percentage of diseased seedlings to 20%, which is almost equivalent to that when 375 μg Flutolanil/pot was introduced to soil. This indicates that the co-utilization of *B. subtilis* RB14-C and Flutolanil reduces the amount of Flutolanil used to one-fourth of that when only Flutolanil is used, while exerting the same effect of reducing disease occurrence

The viable cell number of *B. subtilis* RB14-C in soil during the 14-day plant test is shown in Table 2. Although a slight decrease in viable cell number was observed, the same order of 10^8 cfu was detected, indicating that *B. subtilis* RB14-C was not influenced by the existence of Flutolanil in soil. Table 3 shows the concentrations of iturin A and surfactin extracted from soil. When the culture broth of *B. subtilis* RB14-C and Flutolanil were mixed into soil, about 16 μg iturin A introduced into soil was decreased to 3 μg after three days of incubation and almost no iturin A was detected after 14 days. Similarly, 68 μg surfactin on day 0 of incubation was reduced to 13 μg after 14 days. These degradation patterns were similar to that in soil which contained only the culture broth of *B. subtilis* RB14-C (Asaka & Shoda 1996).

As the biodegradability of iturin A and surfactin is significantly higher than that of Flutolanil, the co-utilization of *B. subtilis* RB14-C will reduce the burden of chemicals to the environment. According to the data of practically used amount of Flutolanil in the field of agriculture, we estimated that the concentration of Flutolanil in one pot is about 13 000 μg which is far larger than that in our experiment. Therefore, in practical test in the field of agriculture which is under way, based on these experimental results, a drastic decrease of the use of Flutolanil will be expected.

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