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Biological Control of the Bacterial Wilt of Tomato with Antibiotic-Producing Strains of *Pseudomonas glumae*

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Pretreatment of the roots of tomato seedlings with the suspension of antibiotic productive strains of *Pseudomonas glumae* suppressed the severity of wilt incited by challenge inoculation with *P. solanacearum*. The disease suppression was positively correlated with length of dipping time and concentration of the bacterial suspension used for pretreatment. Dipping of the roots of tomato seedlings into the bacterial suspension of ca. 10^{10} cfu/ml for 24 h showed highest suppressiveness. The pretreatment of tomato roots with nonantibiotic-producing strain or heat-killed cells of *P. glumae* also showed protection, suggesting that some mechanisms other than antibiotic productivity were involved in the suppression of the disease. When the roots of tomato seedlings were treated with lipopolysaccharide (LPS) or extracellular polysaccharide (EPS) obtained from *P. glumae* before inoculation with *P. solanacearum*, the disease suppression was remarkable. Especially, disease occurrence was suppressed significantly by application of EPS solution. This result indicated that EPS could be involved in the mechanisms of disease suppression.

INTRODUCTION

Bacterial wilt caused by *Pseudomonas solanacearum* E. F. Smith has been reported in virtually all solanaceous plant producing regions of the world and considered to be the most serious disease (Kelman, 1953; Buddenhagen and Kelman, 1964). At present, various measures such as cultivation of resistant varieties (Matsuda, 1977), crop rotation every other year, and soil disinfection by chemical application have been recommended to control this disease. However resistant cultivars are not immune and there has been some damage during the harvest period. Soil fumigants also have unfavorable effects on environmental factors. As a result none of these procedures is sufficiently effective. Some other effective methods may be developed in the future.

In nature, there are many species of antagonistic micro-organisms living in close contact with other micro-organisms, plants and so on. These antagonistic micro-organisms by antibiosis, competition and exploitation, control the population of other micro-organisms including plant pathogenic ones. The use of this phenomenon i.e. biological control, may possibly become an ideal disease control method (Cook and Baker, 1983).

Since successful application of the bacteriocin productive strain 84 of *Agrobacterium radiobacter* against crown gall of stone fruits (Kerr, 1972 and 1974), the studies have emphasized biological methods to control bacterial diseases of plants, and a number of attempts to control bacterial diseases with antibiotic-producing strains have been made.

Chen et al. (1981 and 1984) reported the efficacy of using an avirulent bacteriocin

-producing strain of *P. solanacearum* on the control of bacterial wilt of tobacco. Tanaka et al. (1990) also showed that tobacco plants were protected from bacterial wilt with an avirulent strain M4S of *P. solanacearum* and its bacteriophages. Moreover, Hara and Ono (1991) reported the possibility of biological control on bacterial wilt of tobacco by dipping the root system of seedlings in suspension of a weakly-virulent bacteriocin-producing strain of *P. solanacearum* prior to transplanting. They concluded that further study was necessary to clarify the mechanism of protection from bacterial wilt and to develop superior strains which would provide effective control.

In general, *Pseudomonas* spp. are well-documented as effective biological control agents of plant diseases caused by soil borne fungi and bacteria (Weller, 1988).

The authors (1986) previously demonstrated that strains of *P. glumae* Kurita et Tabei, the causal bacterium of grain rot of rice, produced antibacterial substances showing a wide activity spectrum against plant pathogenic bacteria including *P. solanacearum*. These findings led us to this current study on the application of *P. glumae* for the biological control of bacterial disease of plants. This investigation focused on the efficacy of *P. glumae* strains on the protection of tomato seedlings from the infection by *P. solanacearum* under controlled environmental conditions, and its mechanisms.

MATERIALS AND METHODS

Pseudomonas glumae

Six strains of *P. glumae* shown in Table 1 were used in this experiment. These strains were cultured on slants of PSA medium (potato (300 g) decoction 1 liter, 2 g Na, $\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.5 g $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 5 g peptone, 15 g sucrose, 15 g agar, pH 7.0) at 30°C for 2 to 3 days, suspended in sterile distilled water at a concentration of ca. 10^7 cfu/ml in test tubes, and preserved at 22-25°C during the experiment. For long term preservation, the bacteria were lyophilized with PS broth and kept at 4°C.

The bacterial cells were initially grown on PSA slants at 30°C for 48 h, suspended in 10 ml of sterile distilled water, added to 200 ml of PS broth in a Sakaguchi flask,

Table 1. Bacterial strains used in this experiment.

<i>Pseudomonas</i> Species	Strain	Isolated from	Supplied from or isolated at
<i>P. glumae</i>	YN7825	<i>Oryza sativa</i>	NIAES ^{a)}
	750	„	„
	752	„	„
	805	„	„
	P1-22-4	„	„
	Ku8117	„	KU ^{b)}
<i>P. solanacearum</i>	c319	<i>Nicotiana tabacum</i>	KTES ^{c)}

a) National Institute of Agro-Environmental Sciences.

b) Kyushu University.

c) Kagoshima Tobacco Experimental Station.

and shaken at 30°C for 48 h. The bacterium was harvested by centrifugation at 6,000 \times g for 20 min, and resuspended in sterile distilled water at a concentration of ca. 10^{10} cfu/ml. The suspension was diluted 10 and 100 times just before use. Viable cells were counted by plating on the PSA medium. Bacterial suspensions thus prepared were used for protecting tomato seedlings from infection with *P. solanacearum*.

Pseudomonas solanacearum

P. solanacearum strain C319 was maintained in sterile distilled water at 25–27°C. Stock suspension was spread on TTC medium (Kelman, 1954) (10g peptone, 1 g casein hydrolysate (Difco), 5 g glucose, 17 g agar, 0.05 mg triphenyl tetrazolium chloride, distilled water 1 liter), and the plates were incubated at 30°C for 48 h in order to select virulent colonies. Typical virulent colonies were isolated and grown in PS broth on a rotary shaker at 30°C for 48 h. Cells were harvested by centrifugation at 6,500 \times g for 20 min and resuspended in sterile distilled water to produce concentrations of ca. 10^8 , 10^9 and 10^{10} cfu/ml to be used as inoculum. Viable cells were counted by the plating method on TTC medium. When the inoculum concentration was lower than 10^8 cfu/ml, the disease symptom did not appear clearly (data not shown).

Induction of non-antibiotic-producing mutants of *P. glumae* by NTG treatment.

A wild strain of *P. glumae*, 750, was cultured on the YPDA medium containing streptomycin sulfate (500 μ g/ml) to obtain streptomycin resistant (SR) mutants. The streptomycin resistant parental strain, 750-SR, thus obtained was grown in YPD broth under shaking conditions at 30°C for 24 h. The bacterial cells were washed twice in sterile phosphate buffer (0.067 M, pH 7.0) by centrifugation. The final pellet was resuspended to be the concentration of ca. 10^7 cfu/ml, which was treated with *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (NTG) at the concentration of 50 μ g/ml in the same buffer at 30°C for 20 min. After treatment, cells were washed twice in the same buffer to remove NTG. A part of the bacterial suspension was transferred to YPD broth and incubated overnight to multiply surviving cells. The culture was then diluted, spread on YPDA plates containing streptomycin sulfate (500 μ g/ml) and incubated for 3 to 4 days at 30°C. Single colony was isolated and each isolate was measured for antibiotic activity by the plating chloroform method (Chen et al., 1981; Wakimoto et al., 1986) using various media containing *P. solanacearum* as an indicator. Thus, a streptomycin resistant and non--antibiotic-productive strain, 750-SR-NP, was obtained.

Heat-killed cells of *P. glumae*

The bacterial cells of *P. glumae* strain 750 grown on the slant of PSA medium at 30°C for 48 h were suspended in sterile distilled water (conc. ca. 10^{10} cell/ml). The suspension was incubated at 100°C for 10 min in a water bath and used to test its suppressiveness to the tomato wilt.

Lipopolysaccharide (LPS) extract

For phenol extraction, 5 g of dry bacterial cells was suspended in 100 ml of distilled water at 65°C and emulsified with 100 ml of warm (65°C) 88% phenol. The

emulsion was stirred frequently for 30 min and then cooled in an ice bath. The cooled solution was centrifuged for 30 min at 10,000 **X g**. During centrifugation, the emulsion separated into three phases: an aqueous phase on the top, a precipitate at the interface and the phenol phase at the bottom of the tube. The aqueous phase was collected; the precipitate and phenol phases were extracted again with 100 ml of distilled water at 65°C. After centrifugation, the aqueous phase was collected and combined with the first extract. After dialysis against distilled water for 48 h, the volume of extract was reduced to one fourth the original volume by evaporation under vacuum at 50°C. Saturated sodium acetate had to be added (1 ml/100 ml) to the same preparations to precipitate lipopolysaccharide. The precipitate was collected by centrifugation, dissolved in 25 ml of distilled water, and precipitated again by the addition of six volumes of cold 95% ethanol. The ethanol precipitate was collected by centrifugation, washed with ether, and dried under vacuum. The preparation of LPS was dissolved in distilled water at 10, 5 and 2.5 mg/ml, and used for testing its suppressiveness to tomato wilt.

Extracellular polysaccharide (EPS) extract

Colonies of *P. glumae* were streaked heavily onto plates of PSA medium. The plates were incubated at 30°C for 3 days; the surface growth was scraped off into 40 ml of sterile distilled water and the mixture was stirred until uniform. The suspension was centrifuged at 20,000 **X g** for 1 h, and the precipitate, containing mainly bacterial cells, was discarded. The supernatant fluid was recentrifuged for 2 h, the precipitate was discarded, and three volume of cold 95% ethanol was added slowly with stirring to the supernatant fraction. The precipitate which formed was recovered by centrifugation at 3,000 **x g** for 30 min, washed twice with 95% and once with absolute ethanol, and finally dried under vacuum. Samples requiring further purification were dissolved in a 0.1 M tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.4 at 5 mg/ml. A 1.0-mg amount of Pronase per ml of buffer was added, and incubation was carried out at 37°C for 48 h. The digest was then dialyzed for 17 h against two changes of 40 volumes of distilled water. The extracellular polysaccharide (EPS) was precipitated with three volumes of 95% ethanol as above, and dried. The preparation of EPS was dissolved in distilled water at 10, 5 and 2.5 mg/ml, and used to test its suppressiveness to tomato wilt.

Tomato plants

To evaluate the suppressiveness of *P. glumae* cells to bacterial wilt of tomato, the seeds of the Japanese domestic tomato cultivar 'Toko' were used. The seeds were disinfected by being dipped in a solution of 3% sodium hypochlorite (Antiformin) at room temperature for 15 min, rinsed with tap water, sown on the autoclaved soil in pots 10 cm in diameter, and incubated in a plant-growth chamber at 30°C. Two weeks later, plants 10 cm in height were used.

Pot assay for bacterial wilt suppression

After pulling out the tomato seedlings, the roots were rinsed with tap water and dipped in a suspension of *P. glumae* (conc. ca. 10^8 , 10^9 and 10^{10} cfu/ml) for periods from 10 min to 24 h (pretreatment). The roots thus treated were then dipped in *P. solanacearum* suspension (conc. ca. 10^8 , 10^9 and 10^{10} cfu/ml) for 10 min (post-inoculation). Each

plant thus treated was transplanted in a plastic pot (6X 6 X 4.5 cm) containing heat sterilized artificial soil (Kumiai). The plants were grown in a plant-growth chamber at 30°C. The soil in the pots was kept constantly under wet conditions. The disease severity was recorded at 5-day intervals using the following index: 0; no wilt, 1; 1-50% of leaves wilted, 2; more than 50% of leaves wilted, 3; entire plant wilted or dead.

Monitoring the population of *P. glumae* and *P. solanacearum* in/on the roots of tomato seedlings

Tomato roots were dipped in a suspension of *P. glumae* (conc. 7.0×10^9 cfu/ml) or distilled water as a control for 24 h, followed by *P. solanacearum* (conc. 1.0×10^{10} cfu/ml) for 10 min before transplanting. The treated tomato plants were removed from pots with roots 5, 10 and 15 days after transplanting. Each root sample was shaken in 500 ml of sterile distilled water, weighed and ground in 10 ml of sterile distilled water with a sterile glass rod to permit the release of bacteria from the tissue. Population of *P. glumae* and *P. solanacearum* in/on root was determined using a dilution-plate-counting on the S-PG medium (Tsushima et al., 1986) and TTC medium (Kelman, 1954), respectively.

RESULTS

Protection efficacy shown by *P. glumae*

Pretreatment with *P. glumae* showed a significantly lower incidence and severity of wilt, compared with the untreated control. However, the suppressiveness of *P. glumae* to bacterial wilt varied depending upon the duration of the dipping for pretreatment of tomato seedlings. The suppression was slight when seedlings were dipped in a bacterial suspension of *P. glumae* for 10 min, while it increased in parallel with the duration of dipping. When the seedlings were dipped for 12 or 24 hr, the control was marked (Fig. 1). The suppressive effect also varied depending upon bacterial concentrations in both pretreatment and post-inoculation. The effect was the highest at ca. 10^{10} cfu/ml of *P. glumae*, while it decreased at 10^8 and 10^9 cfu/ml. When the inoculum concentration was ca. 10^8 cfu/ml, the higher suppressive effect was obtained than for 10^{10} cfu/ml (Fig. 2). Although *P. glumae* strains Ku8117, 805, 752, PI-22-4 and YN7825 showed a different degree of antibiosis in vitro, they did not show any significant variation in disease suppressiveness (Fig. 3). The non-antibiotic-producing strain (750-SR-NP) also suppressed disease to the same level as that shown by the antibiotic-producing wild strain (750) (Fig. 4). Furthermore, the heat-killed cells suspension of *P. glumae* 750 also manifested suppressiveness similar to that of living bacteria (Fig. 5).

Effect of LPS and EPS from *P. glumae* on the protection of tomato from bacterial wilt

As shown in Table 2, tomato plants treated with LPS or EPS showed a more significant decrease in the severity of the disease than the water-treated control 5 days after inoculation. However the effectiveness of LPS decreased with the lapse of time. There was no significant reduction in the severity of disease in tomato plants treated with LPS compared with the water-treated control at 30 days after challenge-inoculation. In contrast, the disease severity of tomato seedlings treated with EPS was

significantly reduced during the 30-days observation period.

Population of *P. glumae* and *P. solanacearum* in/on root

Colonization of *P. glumae* and *P. solanacearum* in/on root is shown in Fig. 6. The population of *P. glumae* was ca. 10^8 cfu/g of fresh weight of root after the treatment with *P. glumae* (conc. ca. 10^{10} cfu/ml) for 24 h followed by transplanting in steam-sterilized soil. The population of *P. solanacearum* in treated roots and untreated roots was ca. 10^8 and 10^9 cfu/g in fresh roots, respectively, immediately after the tomato

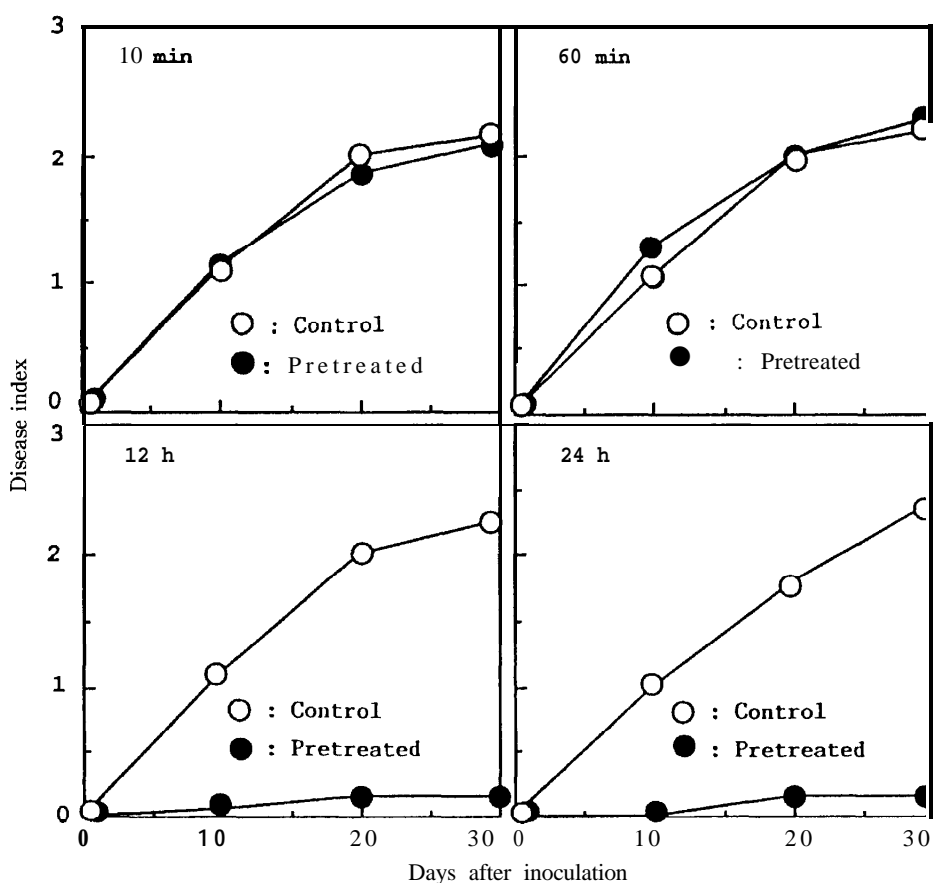


Fig. 1. Protection of tomato seedlings from bacterial wilt by pretreatment with *Pseudomonas glumae* strain 750. Tomato seedlings were pretreated with a suspension of *P. glumae* (conc. ca. 10^{10} cfu/ml) and challenge-inoculated with *P. solanacearum* strain C319 (con. ca. 10^8 cfu/ml) by dipping their roots just after pretreatment. Sterile distilled water was used instead of a suspension of *P. glumae* in the case of the check. Disease index based on a scale from 0, no visible symptom to 3, entire plant wilted or dead.

seedlings were dipped in the suspension of *P. solanacearum* (conc. ca. 10^{10} cfu/ml) for 10 min. Though the population of *P. glumae* gradually decreased in/on roots, the population of *P. solanacearum* remained constant (10^{7-8} cfu/g) for 15 days after transplanting. The population of *P. solanacearum* in/on roots of untreated plants remained at 10 times higher level than that of the plants treated with *P. glumae* for 15 days.

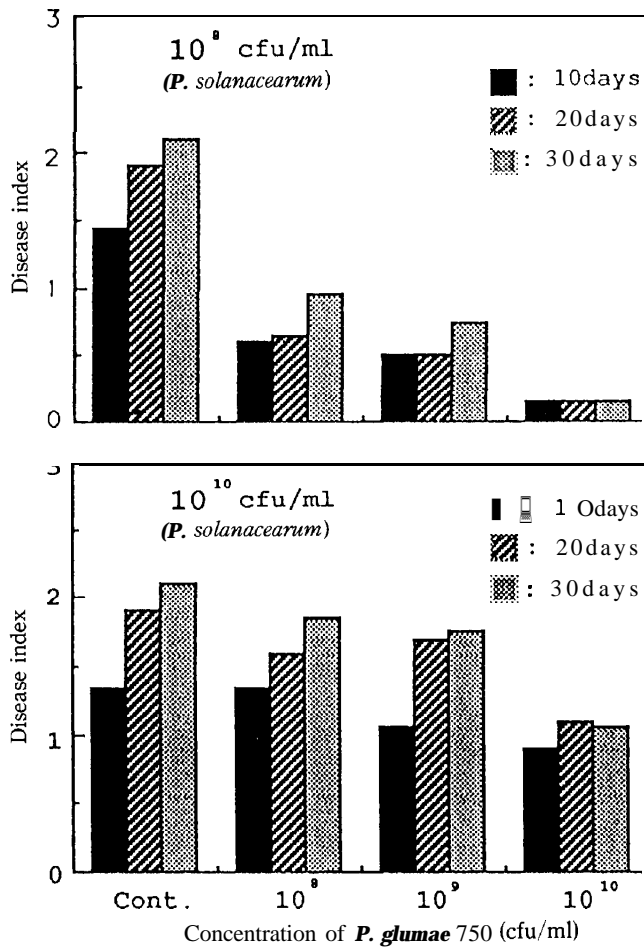


Fig. 2. Suppression of the bacterial seedling rot at different bacterial concentrations of *Pseudomonas glumae* and *P. solanacearum* used for pretreatment and inoculation, respectively. Tomato seedlings were pretreated with a suspension of *P. glumae* for 24 h and inoculated with *P. solanacearum* strain C319 for 10 min by dipping their roots just after pretreatment. Disease index based on a scale from 0, no visible symptom to 3, entire plant wilted or dead.

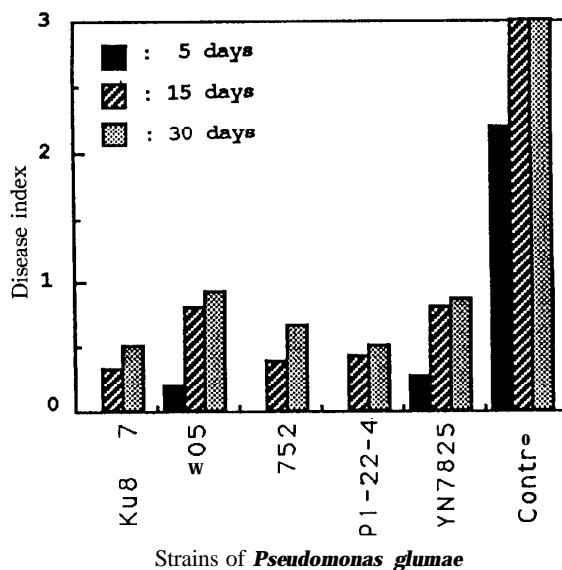


Fig. 3. Comparison of *Pseudomonas glumae* strains on the protection of tomato seedlings from the bacterial wilt infection. The roots of tomato seedlings were dipped in suspension of *P. glumae* ca. 10^{10} cfu/ml for 24 h and then dipped in *P. solanacearum* suspension (conc. ca. 10^8 cfu/ml) for 10 min. Disease index based on a scale from 0, no visible symptom to 3, entire plant wilted or dead.

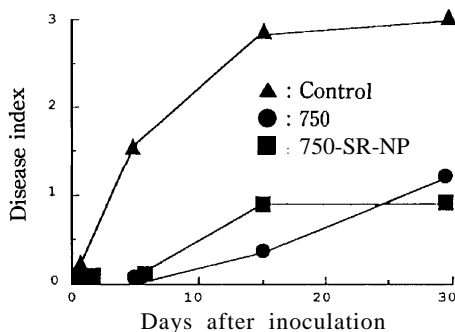


Fig. 4. Effect of non-antibiotic-producing mutant (750-SR-NP) of *Pseudomonas glumae* on the protection of tomato seedlings from bacterial wilt. The roots of tomato seedlings were dipped in suspension of *P. glumae* at ca. 10^{10} cfu/ml for 24 h and then dipped in *P. solanacearum* suspension (conc. ca. 10^8 cfu/ml) for 10 min. Disease index based on a scale from 0, no visible symptom to 3, entire plant wilted or dead.

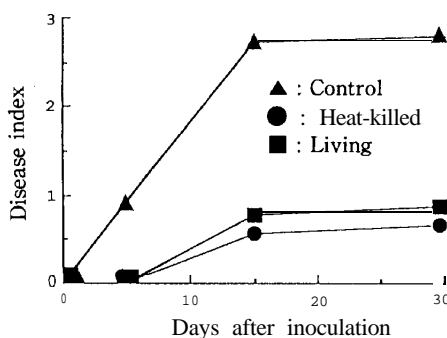


Fig. 5. Effect of the heatkilled cells of *Pseudomonas glumae* 750 on the protection of tomato seedlings from bacterial wilt. The roots of tomato seedlings were dipped in suspension of living bacteria or heatkilled bacteria of *P. glumae* for 24 h and then dipped in *P. solanacearum* suspension (con. ca. 10^8 cfu/ml) for 10 min. Disease index based on a scale from 0, no visible symptom to 3, entire plant wilted or dead.

Table 2. Protection of tomato seedlings from bacterial wilt by lipopolysaccharide (LPS) and extracellular polysaccharide (EPS) from *Pseudomonas glumae*.

Concentration of LPS and EPS (ppm) ^{a)}	Disease index ^{b)}		
	5	15	30 days
LPS			
2,500	0.13BC ^{c)}	1.60AB	1.60AB
5,000	0.60B	1.40AB	1.40AB
10,000	0.13BC	1.00BC	1.40AB
EPS			
2,500	0.00C	1.00BC	1.00BC
5,000	0.13BC	0.20CD	0.40C
10,000	0.00C	0.00D	0.00C
Water control	1.20A	2.20A	2.20A

a) Tomato seedlings were pretreated with a suspension of LPS or EPS at each concentration and inoculated with *P. solanacearum* strain C319 (ca. 10^{10} cfu/ml, 10 min) by dipping their roots just after pretreatment.

b) Disease index based on a scale from 0, no visible symptom to 3, entire plant wilted or dead.

c) Within columns, means with same letter are not significantly different ($P=0.05$) according to the Duncan's multiple range test.

DISCUSSION

Non-pathogenic and bacteriocin productive strains of *P. solanacearum* have been reported to be applicable for biological control of bacterial wilt of solanaceous plants caused by *P. solanacearum* (Chen et al., 1984 and 1981; Trigalet, 1990). The mechanism of the suppression of the disease above mentioned was considered to be the inhibition

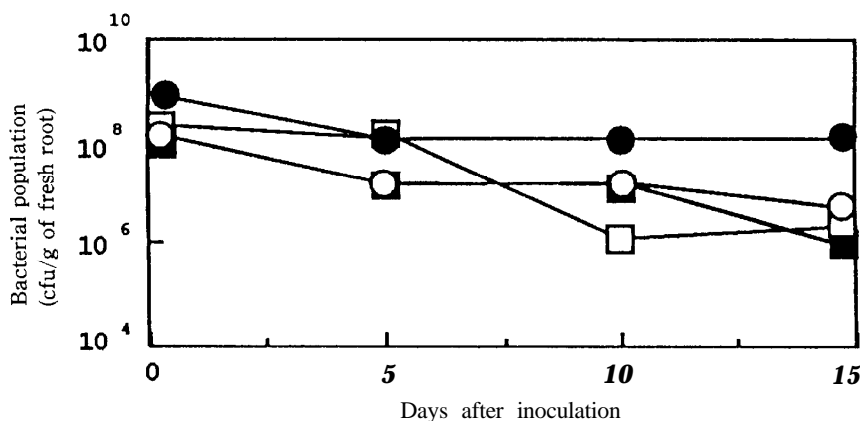


Fig. 6. Growth competition between *Pseudomonas glumae* and *P. solanacearum* on/in roots of tomato seedlings. Tomato roots were dipped in the suspension of *P. glumae* (7×10^9 cfu/ml) for 24 h and followed by *P. solanacearum* (1×10^{10} cfu/ml) for 10 min.
 ● : *P. solanacearum* in mixed culture ■ : *P. glumae* in mixed culture
 ○ : *P. solanacearum* in single culture □ : *P. glumae* in single culture

of the multiplication of pathogenic bacteria by bacteriocin producing non-pathogenic bacteria. Of the interest, *P. glumae* produced antibiotics which inhibit growth of various plant pathogenic bacteria including *P. solanacearum* (Wakimoto *et al.*, 1986). All strains of *P. glumae* showed antibiotic activity against *P. solanacearum* on TTC medium *in vitro*. This finding led us to the attempt to use *P. glumae* as a potential agent for biological control of bacterial wilt of tomato. Dipping the roots of tomato seedlings in suspension of *P. glumae* just before inoculation with *P. solanacearum* showed marked disease suppression. The efficacy of suppression varied with bacterial concentration used for pretreatment (*P. glumae*) and post-inoculation (*P. solanacearum*), and the duration of the dipping for pretreatment. The highest suppression was observed when tomato seedlings were treated with *P. glumae* by dipping at the concentration of ca. 10^{10} cfu/ml for 12-24 h. This result suggested that the mechanism of disease suppression could relate with the adhesion of *P. glumae* to the roots and the induction of resistance, because several hours were needed to express the efficacy for the disease control. The data agreed, in part, with the work of Averre and Kelman (1964) and indicated that the disease severity is related to the ratio of *P. solanacearum* to *P. glumae* cells in the inoculum solution. In this study with *P. glumae*, the suppression or reduction of disease severity clearly related with the low concentration of *P. solanacearum* to *P. glumae* cells. Trigalet *et al.* (1990) also showed that Tn-5 induced avirulent mutants of *P. solanacearum* competed with virulent strains *in planta*, and tomato plants were protected from the bacterial wilt. In this case, the degree of competition increased as the concentration of avirulent strains increased. In general, effectiveness of the control by bacterization is obtained when the concentration of the antagonist is higher than that of the pathogen.

The bacterial strains used in this experiment were those producing antibiotics effective against *P. solanacearum* *in vitro* experiment. However, it is not known

whether the mechanisms of the protective effect were caused by antibiotics produced by *P. glumae* which colonized around tomato roots, or by some other mechanisms such as the protection of tomato roots from the invasion of *P. solanacearum* or by systemic resistance induction by pretreatment with *P. glumae*. Main (1968) pretreated susceptible tobacco cuttings with avirulent mutants of *P. solanacearum*, and observed protection when they were subsequently challenged by virulent bacteria. Kempe and Sequeira (1983) demonstrated that induced resistance through pretreatment with pseudomonads may be involved in the mechanism of suppression of bacterial wilt of potato.

To investigate the mechanisms of disease suppression, a non-antibiotic-producing mutant of *P. glumae* (750-SR-NP) induced by NTG treatment and heat-killed bacterial cells were used. Both non-antibiotic-producing living bacteria and heat-killed bacteria suppressed disease severity like antibiotic producing viable wild strain, as in the case of heat-killed cells of *P. solanacearum* reported by Tanaka (1983). Therefore, the protective effect was not considered to be caused simply by antibiotics produced by *P. glumae* but that some other complicated mechanisms were involved.

The multiplication of *P. glumae* and *P. solanacearum* under mixed culture on/in roots of tomatoes was compared with that under a single culture. Both bacteria multiplied showing almost the same pattern. This is additional evidence to support the conclusion that the failure of pathogenesis to progress in the plants could not be attributed to the inhibition or elimination of *P. solanacearum* population in the host by *P. glumae*. Further, no fundamental alteration in the inherent virulence of *P. solanacearum* occurred, since inoculations with pure cultures obtained in reisolations were equal in virulence to the original culture of *P. solanacearum*.

The possibility of disease control using lipopolysaccharide (LPS) or extracellular polysaccharide (EPS) was examined in this study. When the roots of tomato seedlings were treated with LPS or EPS obtained from *P. glumae* before inoculation with *P. solanacearum*, the disease suppression was evident. In particular, occurrence of disease was suppressed significantly by application of EPS solution. This result indicates that EPS plays an important role in the suppression of bacterial wilt of tomato seedlings through pretreatment with *P. glumae*. It was demonstrated that lipopolysaccharides on bacterial cell wall were responsible for induced-resistance in tobacco leaves (Graham et al., 1977). However, Chen et al., (1984) reported that LPS did not play a major role in protecting tobacco plants treated with avirulent strain from bacterial wilt. Further study should be conducted to clarify the mechanism of protection in detail.

Although tomato plants have been protected by treatment of *P. glumae*, cost efficiency has not been sufficient for commercial use. The present strains of *P. glumae* need to be improved before they can be used effectively for control of bacterial wilt. It may be possible to develop superior strains through selection or genetic methods which would develop high adaptivity, rapid movement and multiplication, and good biocontrol in the rhizoplane and rhizosphere of plants.

Control of bacterial wilt of tobacco, tomato and brown rot of potato with avirulent strains of *P. solanacearum* has been reported on greenhouse and also in a limited field trial. However these control measures have not been reported for commercial production of these plants. Often single biological control measures are less beneficial than those afforded by conventional chemical control, and the use of non-pathogenic strains of *P. glumae* is probably in the same category. An effective means for enhancing

biological control of bacterial wilt must be developed.

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