

Pseudomonas fluorescens

PfG32の浸根処理によるトマト青枯病の発病抑制

抗菌物質およびシデロフォアの役割

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Suppression of Bacterial Wilt Disease of Tomato by Root-dipping with *Pseudomonas fluorescens* PfG32 —The Role of Antibiotic Substances and Siderophore Production—

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Abstract

Pseudomonas fluorescens strain PfG32 isolated from the rhizosphere of the onion actively suppressed the occurrence of bacterial wilt disease of tomato (BWT) in vermiculite amended natural soil and produced antibiotic substance(s) and siderophore. To investigate the role of the substances in the biocontrol, several mutants defective in antibiotic substances or siderophore production were isolated from spontaneous Rif^r mutant, PfG32R, by Tn5 insertion. Based on pot experiment, the suppression of BWT by Ant⁻Sid⁺ (antibiotics non-producing but siderophore producing) mutants was lower compared to its parental strain and to Ant⁺Sid⁻ (siderophore non producing but antibiotics producing) mutants. However, Ant⁻Sid⁺ mutants still express a significant effect. The suppression of BWT by PfG32R was correlated to the suppression of the population of the pathogen observed on the surface of root and to the delay of appearance of detectable population of the pathogen in root tissues. We consider that the ability of PfG32 to produce antibiotics and siderophore is the important factor on determining its biological control and other factor may act as secondary factor.

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Key words : *Pseudomonas fluorescens*, antibiotics, siderophore, bacterization, biocontrol.

INTRODUCTION

Certain soil-inhabiting pseudomonads colonize on the surface of roots and inhibit the infection by some soil-borne plant pathogens. Most of the antagonist strains produce antibiotic and other compound (siderophore, volatile substance) thought to account in part for their antimicrobial properties^{5,7,18,20}. *Pseudomonas fluorescens* is well-known as biocontrol agent of soil-borne fungal diseases. The mechanisms involved in the biocontrol by this antagonist were suppression of pathogen population, interference of infection and induction of plant disease resistance^{17,21}. The relative importance of these mechanisms may differ considerably among the strains. For example, *P. fluorescens* strain 3551 and strain HV37a controlled damping-off disease of cotton caused by *Pythium* spp. at greenhouse experiment ; siderophore production was mainly involved in the disease suppression by strain 3551²², whereas in strain HV37a the antibiotic oomycin A was more importantly involved⁹.

In contrast to the role of antibiotics which was clearly demonstrated in several biocontrol mechanism, the role of siderophore in natural condition is still vague^{5,11}. Keel *et al.*¹⁵ suggested that iron sufficiency is necessary for the metabolism of *P. fluorescens* strain CHA0 to suppress black root rot of tobacco. In this case, siderophore indirectly takes a part in biocontrol mechanism at low iron extractable condition. However, evidence for the involvement of the siderophore in suppression of black root rot by strain CHA0 was not observed in a natural, non-sterile soil pot⁵.

Bacterial wilt disease of tomato (BWT) caused by *P. solanacearum* is one of the important diseases distributed world wide especially in the temperate area¹². Several strategies have been developed to control this disease including biological control by introducing biocontrol agent^{1,4,8,10,25,27}. It was reported that some strains of *P. fluorescens* actively suppress the disease occurrence of BWT when they were introduced to plant rhizosphere by root dipping. From our preliminary study we found that some of these strains produce both antibi-

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otics and siderophore at different level. To elucidate the role of antibiotics and siderophore in the biocontrol mechanism of BWT by *P. fluorescens* strain PfG32R in nonsterile soil pots, some mutants which defect in each the active substance was isolated from the antagonist. The results showed that antibiotics and siderophore production may play main role on the biocontrol and other factor may act as a secondary factor.

MATERIALS AND METHODS

Bacterial strains. *P. fluorescens* PfG32, which was isolated from the rhizosphere of onion, provided from Bacterial Culture Collection of Faculty of Agriculture, Tokyo University of Agriculture and Technology was maintained on Yeast-Peptone (yeast extract, 5 g/l, peptone 10 g/l, pH 7.0; YP) medium for routine preculture. Sucrose-L-asparagine (SA)²⁶⁾, succinic acid minimal medium (SMC)²⁴⁾ and King's B medium (KB)¹⁹⁾ were used routinely for the assay of the antagonistic activity and siderophore production. Spontaneous rifampicin resistant mutant (PfG32R) was isolated from the PfG32 strain by culturing an overnight bacterial suspension on KB-gradient rifampicin (0–200 ppm) plates. After several transfers on the same plates the single colony was purified and preserved in skim milk solution (skim milk 100 g/l, Na-glutamate 15 g/l) at -80°C . *P. solanacearum* strain Ps27 and T-11 were maintained on the cassamino-peptone-glucose (CPG)¹⁷⁾ medium for routine experiment, except for the inoculation experiment where the pathogen was cultured in potato semi-synthetic (PS)³⁰⁾ broth. Spontaneous nalidixic acid mutant of *P. solanacearum* Ps27 was isolated by culturing an overnight bacterial suspension on CPG-gradient nalidixic acid (0–75 ppm) plates. After several transfers on the same plates the single colony was purified and stock in skim milk solution (skim milk 100 g/l, Na-glutamate 15 g/l) at -20°C . *E. coli* was maintained at 37°C on Luria-Bertani (LB)²³⁾ medium. For long-term preservation, bacteria were maintained in skim milk solution and preserved at -20°C or in 20% glycerol preserved at -80°C .

Mutagenesis and characteristic of the mutants. *P. fluorescens* PfG32R, a spontaneous rifampicin resistant mutant was used as parental strain. PfG32R was mutated with Tn5 by using suicide plasmid pJB4JI: Tn5³⁾. Recipient cells and donor were grown separately overnight at 27°C in YP broth containing appropriate antibiotics with aeration. A 50 μl of bacterial culture was transferred into 1.5-ml eppendorf tube and gently mixed. Bacteria were collected by centrifugation for 15 second at $8000 \times g$ and suspended in 10 μl fresh LB broth. After incubation for 3 hours, suspension was diluted 100 times and inoculated onto KB plates containing rifampicin and kanamycin at concentration of 100 ppm. Transconjugants were picked up from the plates after 2 or 3 days incubation.

Antibiotics non-producing mutants were identified by

replicating transconjugants onto succinic minimal medium supplemented by 100 μM Fe-ammonium sulphate. Each plate contained 9 transconjugants arranged. After 2 days, cells were killed by chloroform fumigation and challenged by spraying the pathogen suspension. Antibiotics non-producing mutants were selected 2 days later by scoring for failure to inhibit the growth of pathogen at 26°C . Selected mutants were examined for their siderophore production as described below.

To determine the site of Tn5 insertion, total DNA from each mutant was isolated by using CTAB/NaCl solution²⁾. The total DNA was digested to completion with *Eco*RI, electrophoresed in 0.7% agarose, and transferred to nylon membrane. The membrane was hybridized with ^{32}P -labeled DNA of pRZ102¹⁴⁾ at 68°C as described by Maniatis *et al.*²³⁾ Autoradiography was carried out at room temperature on X-ray film (Fuji Medical, Fuji Photofilm Co. Inc.).

Siderophore and antibiotic assays. The presence of siderophore in the culture filtrate of the antagonist was detected spectrophotometrically as described by Scher and Baker²⁶⁾. Each mutant was grown at 28°C on SA broth with aeration. Culture filtrates were adjusted to pH 5.5 by 1 N HCl. Spectrum of Fe-siderophore

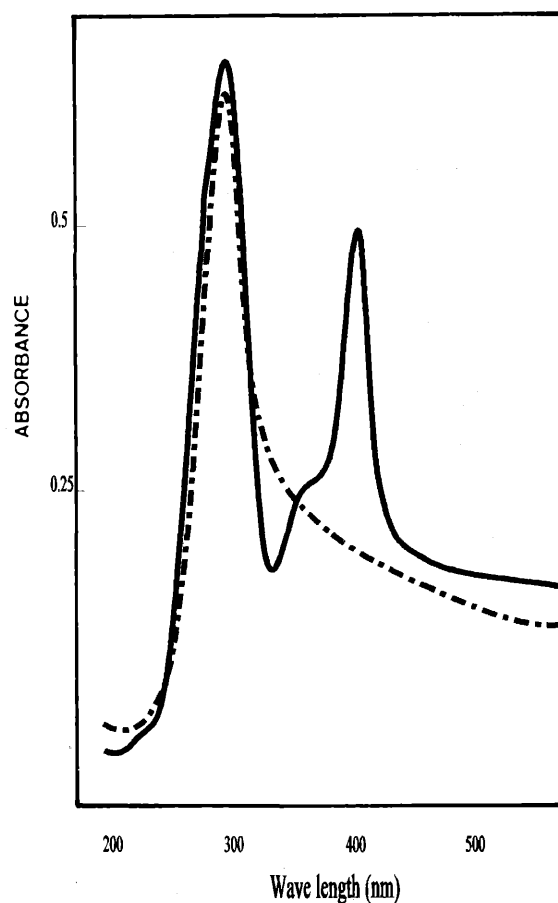


Fig. 1. Absorption spectra for the culture filtrate of *Pseudomonas fluorescens* PfG32R in the presence (dashed line) and absence (solid line) of 200 μM Fe-ammonium sulphate.

complex was read (from 250 up to 450 nm). Culture filtrates obtained from iron supplemented broth was served as control. No peak at 400 nm was observed if iron had previously been added to culture medium (Fig. 1). The quantity of siderophore production was calculated from peak height at $\lambda = 400$ nm.

To examine the production of antibiotics, each mutant was grown on SMC broth supplemented by 100 μ M Fe-ammonium sulphate for 2 days. Bacterial cells were separated from the culture by centrifugation at $8000 \times g$ at 4°C. After the supernatant was lyophilized, the active substance was extracted by 70% MeOH from the residue. The diluted solution was spotted onto SMC plates seeded by the pathogen suspension. Production of antibiotics was expressed as active units/OD₆₀₀. One active unit was defined as a highest titer of 70% MeOH extract required for complete inhibition of pathogen growth.

Examination of bacterial wilt disease suppression. Suppression of bacterial wilt disease was evaluated in greenhouse experiment. Seeds of tomato cv. Fukuju No. 100 were sown in a 15-cm diameter pot containing a mixture of natural soil and vermiculite (5 : 1) amended with fertilizer (N : P : K = 2 : 1 : 1) at rate 10 g per pot. Pots were maintained in a greenhouse at 18–37°C under natural light. All tested plants were arranged in 4 plants per pot. Soil moisture was maintained by watering several times daily. Three weeks after sowing, healthy and uniform plants were selected for the experiment.

Antagonist and its mutants were grown overnight with aeration at 28°C in YP broth supplemented with appropriate antibiotics. Cells were harvested by centrifugation at $4000 \times g$ for 20 min and the pellet was suspended in 20 mM phosphate buffer (pH 7.0). The bacterial suspension was roughly adjusted optically to concentration at *ca.* 10^9 cfu/ml (OD₆₀₀ = 1.0). Actual colony forming unit was determined by dilution plating method with YP. One month old plants were removed from the pots, and they were shook gently to remove loosely adhering soil. Roots, then, were washed under running water for 30 min. The washed roots were dipped into antagonist suspension for 30 min. One hundred ml of the antagonist suspension was expended to treat 25 plants. Treated plants were transplanted and kept in greenhouse. Irrigation was done daily to maintain soil moisture.

Five ml of *P. solanacearum* precultured for overnight at 28°C in PS broth was inoculated into 250 ml of the same medium and grown for overnight with aeration. Cells were harvested by centrifugation at $4000 \times g$ for 20 min and suspended in sterile distilled water. The suspension was adjusted to concentration at approximately 10^9 cfu/ml. Challenge inoculation was done on 4 days after the plants were transplanted by soil drenching, 100 ml suspension per pot. Each treatment contained 20 plants and was arranged in 3 block replication. The experiment was repeated 3 times separately.

Change of the pathogen population in root system.

Spontaneous nalidixic acid resistant mutant of Ps27 was used to monitor the growth of the pathogen in the rhizosphere in the presence or absence of the antagonist. The introduction of antagonist and the inoculation of pathogen were done as described above. Four of the individually cultivated tomato plants with one treatment were removed from the pots at 0, 4, 7, 14 and 21 days after challenge inoculation. After soil loosely adhering root was removed by slightly shaking, root samples were divided into two parts and treated as follows: (1) One part of sample was separated into two root systems *i.e.* treated root and new root. Each root system was placed in 100 ml of distilled water per plant for 5 min, and shook to remove all the soil adhering roots. Each sample was stirred intensively in 100 ml sterile distilled water (SDW), each suspension was served for counting bacterial population on root surface. (2) The other parts of root samples were washed as above without intensive stirring. The washed roots were surface-sterilized with 7% EtOH for 10 sec and 1% sodium hypochlorite for 5 min, then washed twice with SDW. Plant roots were ground in 2 ml of SDW. Populations were assayed on TTC¹⁷⁾ medium supplemented with nalidixic acid (50 ppm).

RESULTS

Isolation and characterization of antibiotic and siderophore non-producing mutants

Frequency of conjugation was 2.3×10^{-7} per recipient. The transconjugants were first screened on succinic acid-minimal medium (SMC) supplemented with iron for the ability to produce antibiotics. Colonies which did not show a clear zone on the lawn of the pathogen were selected as antibiotics non-producing mutants. No antibiotics activity was found in the culture filtrate of all of the mutants, but a trace antibiotics activity was found in cell lysate of PfG32-52. Seven mutants defective in antibiotics production were selected and further characterized for siderophore production. Siderophore production was quantitatively varied among these mutants (Table 1).

Siderophore-non producing mutants were obtained by selecting non fluorescent colonies on solidified sucrose-L-asparagine medium (SA amended with 1.5% agar). Two selected mutants did not show Fe-siderophore peak when their SA culture filtrates were examined spectrophotometrically (Fig. 1). These mutants maintained their capability of producing antibiotics comparable to the parental strain (Table 1). Single Tn5 insertion was indicated only in PfG32-2 by Southern analysis of total DNA using pRZ102¹⁴⁾ as a probe, but not in other mutants (Fig. 2). Thus, though mutation in PfG32-2 was suggested to be due to Tn5 insertion, mutation in other mutants was due to some other mechanism, possibly spontaneous one. These mutants were stable in their phenotypic characters such as kanamycin resistance,

Table 1. Characteristics of *Pseudomonas fluorescens* PfG32R and its mutants

Strain	Production of		Disease Index ^{a)}		Population of ^{b)}	
	Siderophore ^{c)}	Antibiotic au/OD ₆₀₀ ^{d)}	Days after challenge inoculation		Pathogen (10 ⁴ cfu/g)	Antagonist
			7	21		
PfG32R	+(100.00)	56 a ^{e)}	0.00 e	33.33 d	1100 a	8.24 a
PfG32-2	+(85.26)	0 b	2.78 cd	68.75 b	9270 c	6.35 ab
PfG32-11	+(56.16)	0 b	4.17 bc	62.42 b	9270 c	5.49 ab
PfG32-52	+(80.78)	0 b	4.86 bc	66.58 b	ND ^{f)}	5.26 ab
PfG32-67	+(58.20)	0 b	4.17 bc	72.92 b	ND	3.22 bc
PfG32-6.44	+(88.92)	ND	1.39 cd	62.25 b	9670 c	4.59 abc
PfG32-6.57	+(56.45)	ND	6.95 ab	66.58 b	ND	3.14 bc
PfG32-6.59	+(82.47)	ND	6.25 ab	72.92 b	ND	2.71 c
PfG32-001	-(0.00)	48 a	0.00 d	41.67 c	1230 a	3.15 ab
PfG32-444	-(0.00)	56 a	0.00 d	43.75 c	2230 b	3.31 bc
None	ND	ND	14.58 a	97.92 a	9670 c	ND

a) Disease Index (DI) was calculated as: $DI = \frac{\sum(n \times SI)}{4 \times N} \times 100$, where SI (severity index), 0=healthy plant~4=dead plant; n, number of plants which showed the same SI value; N, total number of observed plants. Each treatment consists of 20 plants and replicated 3 times.

b) Bacterial population on the unwashed root surface was counted on 21 days after the challenge inoculation.

c) Siderophore production (+ and -) was judged by the presence of fluorescence on SA medium observed on uv illuminator. The amount of siderophore produced (SP) was calculated by the following equation and shown in parentheses: $SP = \frac{\text{Peak height } (\lambda_{400}) \text{ of mutant/cell density (OD}_{600})}{\text{Peak height } (\lambda_{400}) \text{ of parental strain/cell density (OD}_{600})} \times 100\%$

d) One active unit was defined as the greatest dilution point of crude preparation required for growth inhibition of pathogen. Specific activity was calculated as units per OD₆₀₀. The values of antibiotic production are the average of four separate experiments.

e) Means followed by the same letter are not significantly different ($p=0.001$) according to Duncan's multiple range test.

f) ND: not done.

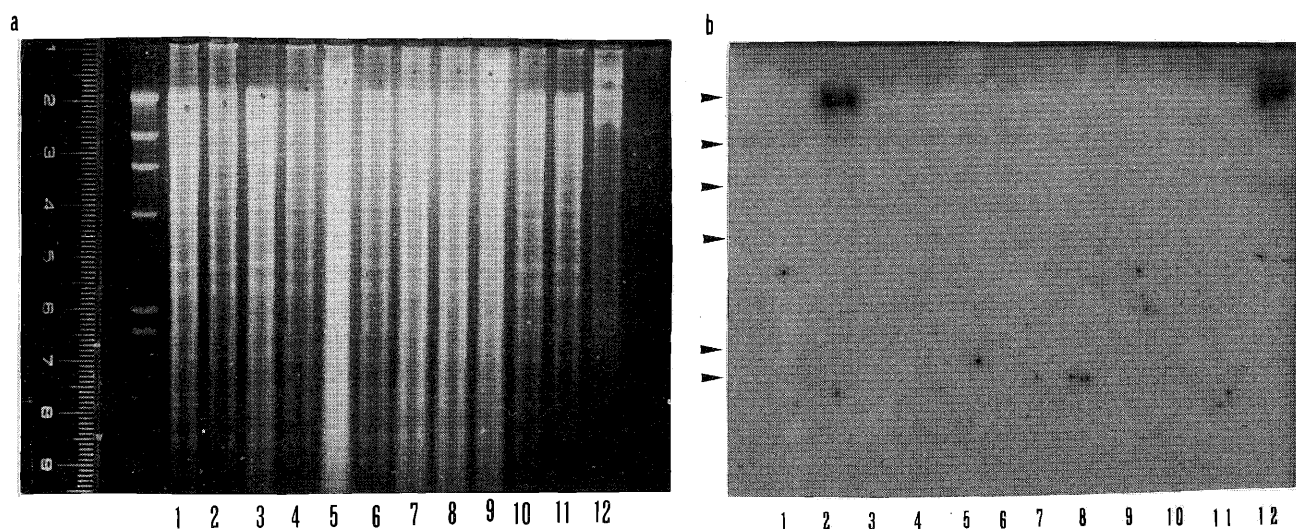


Fig. 2. Southern blot analysis of Tn5 insertion present in the mutants of PfG32R. Total DNA samples digested with *Eco*RI were electrophoresed for 12 hr at 20 V in 0.7% agarose (a), transferred onto nylon membrane, and hybridized with ³²P-labeled pRZ102 (b). Lane: 1, PfG32R (parental strain); 2, PfG32-2; 3, PfG32-11; 4, PfG32-52; 5, PfG32-67; 6, PfG32-6.44; 7, PfG32-6.57; 8, PfG32-6.59; 9, PfG32-444; 10, PfG32-001; 11, PfG32R (parental strain); 12, undigested DNA of pJB4J1. Arrows indicated the position of λ -HindIII fragments.

growth rate and colonization activity in rhizosphere.

Suppression of bacterial wilt disease of tomato by PfG32R and its mutant

PfG32R and its mutants were compared in the pot

experiment for their capability of suppression of BWT. Plants grown after dipping their roots in the suspension of PfG32R showed three times lower severity of BWT than the control that was treated only with buffer.

Table 2. The effect of *Pseudomonas fluorescens* PfG32R and its mutants on the colonization of tomato root system by *Pseudomonas solanacearum*

Root system ^{a)} /strain	Log cfu/g fresh root Days after the challenge inoculation					
	0	4	7	14	21	28
Treated root surface						
PfG32R (Wild type)	8.47 ^{b)} b	6.26 f	8.13 c	7.77 de	7.90 cd	7.60 de
PfG32-2 (Ant ⁻ Sid ⁺)	8.53 b	6.60 f	8.50 b	8.50 b	8.63 b	— ^{c)}
PfG32-444 (Ant ⁺ Sid ⁻)	8.53 b	6.56 f	8.63 b	8.63 b	7.50 e	7.90 cd
None	8.47 b	6.40 f	8.70 ab	8.90 ab	9.03 a	—
New root surface						
PfG32R (Wild type)	8.20 de	6.27 h	8.10 e	8.27 de	8.23 de	8.35 de
PfG32-2 (Ant ⁻ Sid ⁺)	8.30 de	6.27 h	8.33 de	8.70 bc	8.77 bc	—
PfG32-444 (Ant ⁺ Sid ⁻)	8.13 de	6.17 h	7.80 f	8.37 de	8.53 cd	8.73 bc
None	8.23 de	6.57 g	9.20 a	9.03 ab	8.97 ab	—
In root tissue						
PfG32R (Wild type)	0.00 i	0.00 i	0.00 i	4.03 f	4.77 d	5.70 c
PfG32-2 (Ant ⁻ Sid ⁺)	0.00 i	1.20 h	3.73 f	4.80 d	5.67 c	—
PfG32-444 (Ant ⁺ Sid ⁻)	0.00 i	0.00 i	1.37 h	4.40 e	4.97 d	6.20 b
None	0.00 i	2.53 g	5.37 c	6.20 b	6.93 a	—

a) Washed root was prepared as described in Materials and Methods.

b) The values of log population are the average of three separately replications. Means followed by the same letter are not significantly different ($p=0.01$) according to Student-Newman-Keul's test.

c) — : Not done.

Ant⁺Sid⁻ (siderophore non-producing but antibiotics producing) mutants were significantly effective to suppress BWT occurrence than the Sid⁺Ant⁻ (antibiotics non-producing but siderophore producing) mutants. However, the latter retained its capability of disease suppression significantly compared to the control. These results suggest that antibiotics may be the main factor but siderophore may also be a factor involved in the biocontrol of BWT by strain PfG32.

Population of pathogen in the rhizosphere was reduced drastically when the plants were protected by antibiotic producing bacteria, either wild type or siderophore non-producing mutants. Population of pathogen in the rhizosphere of the plants that were treated with antibiotics non-producing mutant reached at the same level of the control ($9.3-9.7 \times 10^7$ cfu/g unwashed root). While, population of pathogen on the rhizosphere of the plants that were treated with antibiotics producing bacteria was 10 times lower than the control that were treated with buffer (Table 1). The populations of PfG32R and of its mutants in the rhizosphere were not significantly different. These results indicated that the capability of the mutant to suppress the disease occurrence did not depend merely on the capability of the bacteria to colonize on the rhizosphere.

Change of pathogen population in each root system

As the population of pathogen was suppressed by the PfG32R as described above, the change of population of pathogen in root system was investigated further by counting viable cells which colonized on the surface of the roots and in the root tissues. PfG32-2 and PfG32-444

were chosen as the representative strains of antibiotics non-producing mutant and of siderophore non-producing one, respectively. As shown in Table 2, after 21 days from the challenge, the population of the pathogen on the surface of the roots of the plants treated by either PfG32R or PfG32-444 was 2-order lower than the population of the pathogen on the surface of the roots of the plants treated with only buffer. PfG32-2 suppressed the pathogen population at 1-order lower than the control. In the case of newly developed roots, only PfG32-444 suppressed the population of pathogen at the same rate as the parental strain did (Table 2). These results indicate that the effect of antibiotics on the suppression of the population of the pathogen on the surface of both of the treated root and the newly developed roots may be stronger than the effect of siderophore.

The pathogen was detectable in the root tissues of the plants treated by PfG32R after 14 days from the challenge, while it was detectable after 7 days in the case of PfG32-444. In contrast to these results, the delay in the appearance of the pathogen in root tissues was not observed when plants were treated by PfG32-2. After 21 days from the challenge, PfG32R and PfG32-444 suppressed the growth of pathogen in root tissues at 2-order lower than the control and 1-order lower than in the plants treated by PfG32-2, respectively. These results indicated the antibiotic production by PfG32R may be responsible for the suppression of the pathogen population on root surface at least partly.

DISCUSSION

Our results indicate that *P. fluorescens* PfG32 strain actively suppresses the occurrence of BWT planted in vermiculite amended non-sterile soil. While the antagonist produced both siderophore and antibiotics, the results suggested that the antibiotics might take a main role on the biocontrol. The evidence of the importance of antibiotic on the suppression of BWT was also demonstrated in the case of *Bacillus subtilis* and of *P. glumae*^{8,25)}. Phae *et al.* demonstrated that the antibiotic iturin produced by *B. subtilis* NB22 was a single factor in the biocontrol mechanism²⁵⁾. In the case of PfG32, the antibiotic is considered not as a single factor on the suppression of the disease occurrence but siderophore may also be responsible.

Evidence of the role of siderophore in the biocontrol was usually supposed by presence of the effect of the disease suppression under the limitation of iron. However, the mechanism of siderophore on the suppression of disease is still vague. Keel *et al.*¹⁵⁾ suggested that siderophore was involved in the suppression of disease occurrence by indirect effect where the siderophore served the bacteria to supply iron which was required for induction of antibiotics production.

Howell and Stipanovic¹³⁾ also reported that *P. fluorescens* Pf5 required complete available nutrient for production of pyoluteorin in soil. All of mutants defective in antibiotics production varied in the quantity of siderophore production. If siderophore took place in the biocontrol mechanism, the effect on disease suppression may become lower as the quantity of siderophore becomes lower. However, we did not see a correlation between the capacity of disease suppression and the quantity of siderophore among siderophore producing mutants. This is probably due to the fact that vermiculite was reported to be able to release the trace iron extractable by bacteria into soil^{5,15)}.

Since comparable numbers of mutants and parental strain were isolated from the rhizosphere of tomato, the reduction of the suppressiveness of the mutants was not due to their inability to establish and maintain effective population in the rhizosphere. Studies with other biocontrol agents also have demonstrated that loss of antibiotic or siderophore production does not affect the ability of the bacteria to colonize root^{22,28)}.

Although the suppression of the growth of the pathogen by the PfG32R was observed on the surface of the treated roots, the antagonist suppressed the population of the pathogen more actively on the surface of the newly developed roots³¹⁾. Previous studies showed that most of the antagonistic bacteria moved along the roots and have counted higher population at root tips^{6,31)}. On the root tips probably the root exudate was rich, and it induced the bacteria to move to that place. The root exudate was known as a source of nutrient for the activity of bacteria on root surface.

Although the growth of the pathogen on both the surface of treated roots and of newly roots were suppressed by the PfG32R, the population of the pathogen is still high. The minimum threshold of the colonization for wilt induction in susceptible tobacco by *P. solanacearum* was approximately 5×10^5 cfu/g root in greenhouse experiment⁴⁾.

Our data indicated that the pathogen survived at the level of 2-order higher than the minimum threshold. So, the PfG32R probably suppressed the pathogen at the site of infection rather than the total root surface. It was also showed that PfG32R and PfG32-444 delayed the colonization of the pathogen and the growth of pathogen in root tissues. We suggest that the suppression of the growth of the pathogen in the root tissues is the important factor on the suppression of disease appearance. Keel *et al.* (1992)¹⁶⁾ suggested that 2,4-diacetylphloroglucinol produced by *P. fluorescens* strain CHA0 that inhibited the growth of pathogen and also affected the physiology of seed germination may induce the plant resistance and cause the suppression of disease occurrence. The evidence of induced disease resistance on plant was also come from the observation of HCN produced by the CHA0 strain²⁹⁾. Therefore, adequate observation on the effect of released active substance by PfG32R on the physiology of plant is needed for clarification of the biocontrol mechanism of PfG32R.

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和 文 摘 要

Karden MUYA・渡辺 実・後藤正夫・瀧川雄一・露無慎二：
Pseudomonas fluorescens PfG32の浸根処理によるトマト青枯
病の発病抑制—抗菌物質およびシデロフォアの役割

Pseudomonas fluorescens PfG32は、バーミキュライトを含む非殺菌処理土壌で育成されたトマトにおいて青枯病の発病を抑制した。PfG32は抗菌物質とシデロフォアを産生することがわかったので、上記発病抑制におけるこれらの物質の役割を明らかにするため、Tn5挿入によるそれぞれの非産生変異株を分離した。ポット試験の結果、親株を浸根処理したトマトの青枯病発病度は33.3であり、対照と比較して約1/3と低かった。シデロフォア非産生変異株で処理した場合、発病度が41.7~43.7であったのに対し、抗菌物質非産生変異株に浸根したトマトの青枯病発病度は62.3~72.9となり、抑制効果が低下したが、完全にはなくならなかった。従って、PfG32による青枯病発病抑制の主要な要因は抗菌物質の生産であると推定されるが、他の要因も存在するものと考えられる。