Control of Tobacco Bacterial Wilt by an Avirulent Strain of *Pseudomonas solanacearum* M4S and Its Bacteriophage

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Key words: biological control, *Pseudomonas solanacearum*, tobacco, bacterial wilt.

Treatments with heat-killed cells of *Pseudomonas solanacearum*¹⁾, a living avirulent strain of *P. solanacearum* M4S²⁾ or avirulent bacteriocin-producing strains of *P. solanacearum*^{3,4)} have been used in attempts to control bacterial wilt of tobacco, caused by *P. solanacearum*. Similarly, avirulent, non-bacteriocin-producing strains of *P. solanacearum* have been tested for controlling brown rot of potato, caused by the same pathogen⁵⁾. Although tobacco plants were protected with treatment of M4S²⁾, its efficacy was not sufficiently high for commercial use.

Although bacteriophages in *P. solanacearum* have been previously described⁶⁾, they have not been used as a means to control bacterial wilt of tobacco. The possibility of disease control using bacteriophage alone or in combination with other control measures was examined in this study.

Seeds of Japanese domestic tobacco cultivar 'Shiroenshu 1' were sowed in a greenhouse seedling bed. Six weeks after sowing, seedlings were transplanted to clay pots 12 cm in diameter and maintained at 28 C in the greenhouse.

Virulent strains of *P. solanacearum* used in this study were U-10 and U-166, both were isolated from diseased tobacco in Oyama City in Tochigi Prefecture and Ohmiya Town in Ibaraki Prefecture in Japan, respectively. An avirulent strain of *P. solanacearum* M4S²⁾, described previously, was also used. The bacteria were grown on Kelman's TZC medium⁷⁾ at 28 C and maintained at room temperature in sterilized distilled water.

The phage was isolated from the stems of diseased tobacco as follows: ten grams of stems were cut into small pieces (each side approximately one cm) and shaken in 100 ml of sterilized water in an Erlenmeyer flask. Ten ml of filter sterilized extracts were added to 100 ml of CPG broth⁷⁾ inoculated with the host bacterium. After shake-culturing on a rotary shaker (150 rpm) at 28 C for 2 days, the culture was centrifuged at 10,000 rpm for 20 min. Phages were detected and isolated by plaque formation on a lawn of *P. solanacearum* U-10. The phage designated P4282 was purified by isolating single plaques three times. It was stored at 5 C in CPG broth from shake-cultures of *P. solanacearum* M4S, removing host cells via 0.22 μ m membrane filtration.

A culture of M4S was grown in CPG broth in a jar-fermenter. Cells were harvested by centrifugation and stored in a freezer at –20 C until use. The cells were thawed by the addi-

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tion of sterilized distilled water, stirred on a magnetic stirrer at room temperature and adjusted to 10^8 cfu/ml at use. Virulent bacterial strains were grown on TZC agar medium at 28 C for 2 days and the cells were transferred to sterilized distilled water. The concentration of virulent cells was adjusted to 10^7 cfu/ml. *P. solanacearum* M4S mixed with the phage was grown in CPG broth on a rotary shaker (120 rpm) at 28 C for 2 days. Bacterial cells were removed by centrifugation followed by membrane filtration. The broth was diluted 100 times with sterilized distilled water (10^7 plaque forming units/ml). In some experiments, phage particles were partially purified by precipitation with polyethylene glycol (PEG 6000) following the method of Maniates *et al.*⁸⁾

Culture filtrate was used as a sample for electron microscopic observation. The sample was negatively stained with 2% uranylacetate on carbon-coated collodion film. The specimen was examined in a JEM-100B (JEOL) electron microscope at 80 kV.

The phage P4282 in culture filtrate had a polygonal head (*ca.* 69 nm) and a short tail (*ca.* 20 nm) (Fig. 1), the same shape as Psso 154 and Psso NCL reported by Hendrick and Sequeira⁹⁾. P4282 lysed both U-10 and M4S but not U-166.

For pre-inoculation with avirulent strain M4S, roots of tobacco plants were placed in the bacterial suspension for one hour before transplanting to pots. Immediately after transplanting, 100 ml of diluted culture filtrate containing phage (CF) was poured into the pot.

Four days after transplanting, plants were inoculated with virulent *P. solanacearum*. Ten ml of bacterial suspension was applied to roots injured prior to inoculation by insertion of a knife.

Disease incidences were recorded 2 weeks after inoculation with virulent *P. solanacearum* using the following index: 0=no symptoms, 1=up to 25% of the foliage wilted, 2=25–50% of the foliage wilted, 3=50–75% of the foliage wilted, 4=75–100% of the foliage wilted, and 5=dead.

Tobacco plants were protected from wilting by pre-treatment with M4S and subsequent treatment with CF (Table 1). Pre-inoculation with M4S alone showed significantly lower incidence and severity of wilt, compared to the untreated control. Treatment with both M4S and CF was more efficacious than either treatment alone in decreasing wilting. There were no significant differences in efficacy among the CF treatments applied at different times after inoculation with M4S.

Tobacco plants were significantly protected by treatment with a combination of M4S and

Fig. 1. Electron micrograph of bacteriophage of *P. solanacearum*, P4282. Bar, 100 nm.

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CF, prior to challenge with virulent strain U-10, susceptible to the phage. In the case of virulent strain U-166, tolerant to the phage, the effects of M4S and CF were greatly diminished (Table 2).

There was no difference in severity or incidence of wilt, when tobacco plants were pre-treated with M4S and subsequently treated with CF or partially purified phages (Table 3).

Cross sections of stems (*ca.* 1 cm in thickness) were crushed in sterilized distilled water. Presence of M4S was determined by culture on *P. solanacearum* selective medium¹⁰⁾. The presence of phages were examined by plaque formation on double layered plates using M4S as an indicator. Among 20 plants pre-treated with both M4S and CF, the phage was recovered from 9 of the 18 plants from which M4S was recovered (data not shown).

Table 1. Protection of tobacco plants from bacterial wilt by avirulent *Pseudomonas solanacearum* M4S and culture filtrate containing bacteriophage (CF)

Treatment ^{a)}			% Wilted plant	Disease index ^{b)}
M4S (Primary)	CF (Secondary)	Interval		
+	+	1 hr	17.6 A ^{c)}	0.58 AB
+	+	3 days	14.1 A	0.43 A
+	+	5 days	17.6 A	0.53 AB
+	+	7 days	14.5 A	0.52 AB
+	—	—	39.5 B	1.26 B
Untreated control			95.8 C	4.06 C

a) M4S: *P. solanacearum* M4S, CF: Culture filtrate containing bacteriophage.

+: Treated, —: Untreated.

All plants were inoculated with *P. solanacearum* U-10 and experiments were repeated five times.

b) Disease index based on a scale from 0 (no visible symptoms) to 5 (dead).

c) Values followed by the same letter do not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

Table 2. Efficacy of M4S and phage treatment against the wilt diseases caused by phage-susceptible and phage-tolerant pathogenic *P. solanacearum* strains

Treatment	Inoculum (Virulent strain)	% Wilted plant	Disease index
M4S and CF	U-10	7.5*** ^{a)}	0.18***
	(Phage susceptible)		
Untreated control	U-10	87.5	4.10
M4S and CF	U-166	75.0 NS	3.33*
	(Phage tolerant)		
Untreated control	U-166	95.0	4.63

a) ***,*: Statistically different by paired *t*-test, $P < 0.001$ and 0.05 respectively, NS: Not significantly different by paired *t*-test. Experiments were repeated four times.

Table 3. Comparison of the efficacy of CF and partially purified phage

Treatment	% Wilted plant	Disease index
M4S and CF	11.7 ^{a)}	0.22
M4S and Phage	5.0	0.06

a) There is no significant difference between the treatments by paired *t*-test. Experiments were repeated six times.

The results of this study indicate some enhancement of control of bacterial wilt of tobacco by *P. solanacearum* phage. Lack of involvement of bacteriocins or other kinds of antibacterial substances in culture filtrate was suggested for the following reasons (data not shown): (1) there was no *in vitro* antibacterial activity against U-10 or U-166 in culture filtrates of M4S devoid of the phage, (2) no protection of tobacco plants was afforded by the treatment with the phage-free M4S culture filtrate and (3) CF did not inhibit growth of U-166, tolerant to the phage, indicating antibacterial substances were absent in the CF preparation. Possible involvement of phage in protection of tobacco was suggested by: (1) degree of pathogen lysis was correlated to the degree of the protection (data not shown), (2) the protection was afforded by the treatment with the partially purified phage and (3) CF was not effective to U-166, phage tolerant strain.

Control of bacterial wilt of tobacco and brown rot of potato with avirulent strains of *P. solanacearum* has been reported on a greenhouse scale²⁻⁵⁾ and also in a limited field trial^{3,4)}. However these control measures have not been reported for commercial production of these plants. Often single biological control measures are less efficacious than those afforded by conventional chemical control, and the use of avirulent strains of *P. solanacearum* is probably not an exception. Effective phage strains with wider host ranges, found by survey from nature or genetic manipulation, may offer an effective means for enhancing biological control of bacterial wilt.

We are grateful to Dr. Y. Takanami, Life Science Research Laboratory of Japan Tobacco Inc., for the electron micrograph of the bacteriophage. We are also grateful to Dr. R.V. Miller, Plant Pathologist of Mycogen Corporation, Ruston, Louisiana, USA, for reading and correcting the manuscript.

和 文 摘 要

田中 博・根岸秀明・前田初枝：非病原性タバコ立枯病菌 *Pseudomonas solanacearum* M4S とバクテリオファージによるタバコ立枯病の生物防除

非病原性立枯病菌 M4S 菌の懸濁液にタバコ苗を浸根、鉢植えし、ファージを含む培養濾液 (CF) を添加、さらに病原性立枯病菌を接種した。M4S 菌のみを前接種した場合、無処理にくらべて発病が抑制されたが、M4S 菌の前接種に加え CF を前処理した場合、さらに発病が抑制された。しかし、ファージが病原性菌に感染しない場合には発病抑制効果はみられなかった。CF に替えて部分精製したファージを用いた場合、CF 処理の場合との間に発病抑制効果に差は認められなかった。以上の結果から、ファージが発病抑制に関与する可能性が示唆された。

(Received March 22, 1989)