BACTERIAL LEAF BLIGHT OF RICE PLANT. IV.
Effect of Bacteriophage on the Infectivity of *Xanthomonas oryzae*[1]

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Abstract

Some factors associated with the infection of *X. oryzae* was clarified and the effect of the phage on the infection of *X. oryzae* was studied. In the greenhouse condition when two-month old rice plants (Taichung Native 1) were inoculated with *X. oryzae*, only 1×10^1 cells per ml of *X. oryzae* was required to obtain 43% infection. When six-day old seedlings were used, 1×10^6 cells per ml of *X. oryzae* was required to obtain 30% infectivity. Obviously, six-day old seedlings were more resistant than two-month old plants. When the phage was applied 1 day before *X. oryzae* inoculation, all inoculated leaves were free from infection. However, when phage was applied with *X. oryzae* or 24 hours after *X. oryzae* infection, the effect was not so great. Apparently the application of phage has significant protective effect on rice plant from the infection of *X. oryzae*, but less therapeutic effect.

Introduction

The concentration of bacterial cells in the water of paddy field or on the site of infection is believed to be very important to the infection of rice plants by bacterial leaf blight caused by *Xanthomonas oryzae*. For successful infection by this organism a certain concentration of bacterial cells is required; however, the information concerning this point is still obscure. If this information can be clarified and if there is any approach which can keep the bacterial concentration in the paddy water or the site of infection below this critical concentration, the rice plants may be protected from the attack by this disease.

Previous studies showed that phages of *X. oryzae* were widely distributed in the water of paddy fields (Kuo *et al.*, 1968b) and at least six morphologically distinctive phages were isolated and their properties studied (Kuo *et al.*, 1967,

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Kuo *et al.*, 1968a, Kuo *et al.*, 1969). These phages were very easy to be cultivated and could produce 6×10^{12} pfu per ml by conventional cultivation method. The phages could also be kept in lysates or on dry filter papers at room temperature for at least six months without losing their activities. These properties are very helpful when they are used for the biological control of this disease.

Many attemps have been made to use phages for disease control. Some of them have succeeded but others have failed. They have been extensively reviewed by Okabe and Goto (1963). Recently, Stonier *et al.*, (1967) have demonstrated that bacteriophage PF_2 of *Agrobacterium tumefaciens* had its inhibitory effect on tumor induction, and Civerolo and Keil (1969) confirmed that leaf infection of bacterial spot of peach was significantly reduced when the phage was applied as a spray and allowed to dry before inoculation with *Xanthomonas pruni*.

In this investigation, an attempt was made to use phages for disease control. For the preliminary information, some factors associated with the infection of *X. oryzae* were clarified, and the effect of the phage on the infection of *X. oryzae* was studied.

Materials and Methods

Xanthomonas oryzae strain 604 was originally isolated from infected rice plants collected from Taichung in 1970. The culture was maintained in PS agar slants (Kuo *et al.*, 1967) and stored in a refrigerator at 4°C. The phage used was Xp12, a virulent phage active against *X. oryzae*. It was originally isolated from diseased specimens and preserved at the Institute of Botany, Academia Sinica.

Cultures of *X. oryzae* grown in PS medium for 16 hr at 28°C were diluted with sterilized water and used to inoculate rice plants. High titer stocks of the phage were prepared by inoculating an early log phase (4×10^8 cells/ml) culture of bacteria in PS medium at 28°C, and this culture was aerated continuously until lysis was complete. Such lysates contained approximately 3×10^{12} pfu phage per ml. Bacterial debris was removed by centrifugation and passed through Millipore membrane filters (mean pore size 0.45 μ). Lysates were stored at 4°C.

The standard two-layer agar technique (Adams, 1959) was used to determine phage titers. A mixture containing 0.5 ml of *X. oryzae* indicator and 0.1 ml phage sample was added into 3 ml of melted semisolid PS agar (0.6% agar) medium. The mixture was poured over a layer of the same medium, except that the concentration of agar was 1.6%. Plaques were counted after incubating the plates at 28°C overnight.

Rice plants used were variety Taichung Native 1 (a highly susceptible variety). Since the susceptibility of seedlings was known to differ from that of aged plants, one-week old seedlings and two-month old plants were selected for comparison. For the inoculation of young seedlings, six-day old seedlings grown in petri dishes were removed and injured by cutting off the tips of leaves. The injured seedlings were immediately immersed in bacterial suspension for one hour, then planted in pots under greenhouse condition. The infectivity was examined 10 days after inoculation. Since the infected young seedlings wilted and died rapidly, the amount of infection was expressed in terms of percentage as calculated by dividing the number of infected plants by the total number of plants originally inoculated. For inoculation of two-month old plants, uniform size leaves were selected and inoculated by cutting off the leaf tips and dipped into bacterial suspension for one hour. After ten days the severity of disease was examined. Based on the development of symptom, the severity of disease was divided into 5 grades; they were: no symptom =0, infected area became yellow and restricted to a small area =1, infected area became yellow and symptom developed slowly =2, infected area became water soaked, and the symptom developed fast =3, whole leaf shrinked and wilted rapidly =4. The percentage of disease infection was determined by the following equation.

$$\text{Percentage of infectivity} = \frac{(a\times 0)+(b\times 1)+(c\times 2)+(d\times 3)+(e\times 4)}{n\times 4}\times 100$$

a, *b*, *c*, *d*, *e*, express the number of inoculated leaves at grades 0, 1, 2, 3, 4, respectively, and *n* expresses the total number of inoculated leaves.

Results

Age of plants and infectivity of X. oryzae

Susceptibility of six-day old seedlings to bacterial leaf blight was different from that of two-month old plants. When two-month old plants were inoculated with 1×10^8 bacterial cells per ml, the symptom appeared 4 days after inoculation, but symptom developed slowly. However, when young seedlings were infected, the symptom usually appeared 10 days after inoculation, but once infected, the whole seedling wilted and died within a few days.

Inoculum concentrations and infectivity of X. oryzae

Since the symptom developments of six-day old seedlings and two-month old plants were different, rice plants of both ages were examined for their responses to different inoculum concentrations. The inoculum concentrations tested were 0, 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9 bacterial cells per ml. The plants were inoculated by injuring the leaf tips

and dipped in different concentration of bacterial suspensions for one hour, they were then planted in pots under greenhouse condition for observation. As indicated in Table 1, the response of young seedling and aged plants to different concentrations of bacterial suspension was quite different. When young seedlings were inoculated, much higher concentration was required for successful infection. If inoculum concentration was below 1×10^5 cells per ml, seedlings were almost all free from infection. When two-month old plants were inoculated, 43% of inoculated leaves became infected even the inoculum concentration was as low as 1×10^1 cells per ml. However, when plants were inoculated with low concentration of inoculum, the symptom appeared rather late and developed slowly.

For young seedlings at the concentration of 1×10^6 cells per ml, the infectivity was around 30% and progressively increased with increasing inoculum concentration. As the concentration reached 1×10^8 cells per ml, all seedlings were completely killed. For aged plants, when they were inoculated with inoculum at concentration of 1×10^1 cells per ml, the symptom appear slowly and the severity of symptom was gradually increased when the inoculum concentration increased. At the concentration of 1×10^2 cells per ml, the majority of symptom was yellow and restricted to the leaf tip of inoculated site. When the inoculum concentration was above 1×10^4 cells per ml, the symptom appeared earlier and spread fast, and always caused wilting of the whole leaf.

Effect of bacteriophage on the infection of ***X. oryzae***

The two-month old plants grown under greenhouse condition were injured by cutting off leaf tips and dipped in bacterial suspension at 1×10^8 cells per ml. The phage was applied at following 5 different times: (1) 1 day prior to *X. oryzae* inoculation; (2) 3 days prior to *X. oryzae* inoculation; (3) 7 days prior to *X. oryzae* inoculation; (4) Same time as *X. oryzae* inoculation; (5) 1 day after *X. oryzae* inoculation. Application of phage was done by injuring the leaf tips and then dipping into phage suspension (1×10^9 pfu/ml) for 24 hours. As indicated in Table 2, a marked protective effect occurred when phage was applied before inoculation with *X. oryzae*. When the phage was applied 1 day before *X. oryzae* inoculation, all inoculated leaves were free from infection. The disease reduction was 100%. When phage was applied 3 days before *X. oryzae* inoculation, the infectivity was 3% compared to 97% of the control plants, which corresponded to 96% disease reduction. When phage was applied 7 days before inoculation, the infectivity was 13% as compared with the control, the disease reduction was 86%. Apparently, the phage was stable for 7 days in site of infection in the absence of host bacteria, and still effective in protecting seedlings from *X. oryzae* infection. When rice plants

Table 1. *Effect of inoculum concentration on the infection of* ***Xanthomonas oryzae*** *on different age of rice plants*

Inoculum concentration (No. of cells/ml)	Six-day old seedlings			Two-month old plants						
	No. of plants inoculated	No. of plants infected	% infectivity	No. of leaves inoculated	No. of infected leaves in different grades					% infectivity
					0	1	2	3	4	
0	98	0	0	100	100	0	0	0	0	0
1×10^{1}	102	0	0	100	27	17	26	35	5	43
1×10^{2}	110	0	0	96	22	11	37	22	4	43
1×10^{3}	101	0	0	92	0	2	21	41	28	75
1×10^{4}	100	4	4	100	0	0	2	6	92	97
1×10^{5}	98	6	6	100	0	0	0	0	100	100
1×10^{6}	100	30	30	98	0	0	0	0	98	100
1×10^{7}	100	54	54	102	0	0	0	0	102	100
1×10^{8}	92	92	100	116	0	0	0	0	116	100
1×10^{9}	94	94	100	98	0	0	0	0	98	100

Table 2. *Effect of Xp12 phage on the infection of* ***Xanthomonas oryzae*** *on different age of rice plants*

Treatments	Six-day old seedlings				Two-month old plants							
	No. of plant inoculated	No. of plant infected	% infectivity	% disease reduction	No. of leaf inoculated	No. of infected leaves in different grade					% infectivity	% disease reduction
						0	1	2	3	4		
Applied phage 1 day prior to inoculation	100	0	0	100	30	30	0	0	0	0	0	100
Applied phage 3 days prior to inoculation					30	28	1	0	1	0	3.3	96.6
Applied phage 7 days prior to inoculation					31	26	0	1	2	2	12.9	86.7
X. oryzae and Xp12 mixture	100	78	78	10	32	2	0	3	6	21	84.4	13.1
Applied phage 1 day after inoculation	100	74	74	14	30	6	1	0	5	18	73.3	24.5
X. oryzae	100	86	86	0	34	1	0	1	2	31	97.1	0
Phage Xp12	100	0	0	—	36	36	0	0	0	0	0	—

were inoculated with a mixture of *X. oryzae* and the phage, or with *X. oryzae* 1 day after the phage application, the percentage of infectivity was 84 and 73%. As compared with the control, the disease reduction was 13 and 24%. Obviously the effect was much less than that of applying phage prior to *X. oryzae* inoculation.

In order to confirm the above results, six-day old seedlings were tested for the same purpose. The seedlings were injured by cutting off leaf tips and dipped in bacterial suspension at 1×10^8 cells per ml. The phage was applied at following 3 different times: (1) 1 day prior to inoculation; (2) same as *X. oryzae* inoculation; (3) 1 day after inoculation. Application of phage was also done by cutting off the leaf tips and dipping in phage suspension (1×10^8 pfu/ml) for 24 hours. The results are showed in Table 2, there was no infection occurred when phage was applied before *X. oryzae* infection. However, when phage was applied with *X. oryzae* or 24 hours after *X. oryzae* infection, the degree of infection is similar to *X. oryzae* infected control. Again, the evidence showed that the application of the phage has significant protective effect on rice plant from the infection of *X. oryzae*, but only little therapeutic effect.

Discussion

From the results presented, it was found that the susceptibility of aged rice plants and young seedlings to leaf blight disease was different, especially the response to different inoculum concentrations. When young seedlings were inoculated with *X. oryzae*, an inoculum concentration with 1×10^6 cells per ml was required to obtain 30% infection, when the inoculum concentration was below this concentration, all seedlings were free from the disease. Whereas, if the two-month old plants were inoculated, only 1×10 cells per ml was required to obtain 43% infection. Apparently, six-day old seedlings was more resistant than two-month old plants. The mechanism of the resistant is till unknown; however, it is an interesting problem to be further investigated.

In the summer of 1969, samples of paddy water were randomly collected from the healthy rice field. The existance of phage in these samples were examined by two-layer agar technique. It was found that the phage activity was very easy to be detected among these samples. If the specificity of the phage to *X. oryzae* is very high, the existance of phage should represent the existance of *X. oryzae*. Consequently, it means that *X. oryzae* should be widely distributed in the rice field. If this assumption is true, the infection of *X. oryzae* should be decided by the inoculum concentration. Bacterial concentration as high as 10^6 is rarely found in paddy water, except the water drops just exuded from the hydropore of diseased leaves. However, we have frequently

encountered with paddy waters which have a concentration of 1×10^1 cells per ml. Therefore, the chance for seedlings to be attacked by *X. oryzae* is rather low. On the contrary, the chance for two-month old plant to be attacked by *X. oryzae* was much higher. Since very susceptible rice variety Taichung Native 1 was used in this experiment, the critical concentration for disease infection was very low. If resistant variety is used, the critical concentration of disease infection should be higher.

The application of phage on rice leaves can reduce the amount of *X. oryzae* infection. But there was no significant effect on the amount of infection when rice leaves were inoculated with *X. oryzae* prior to phage application. Apparently the phage can not come in contact with the bacteria. Thus disease therapy by means of the bacteriophage is of no value. It is possible that the bacteria rapidly occupied sites within the intercellular spaces, which made them relatively inaccessible to the phage. However, when the bacteriophage was applied, first they can widely distributed in intercellular spaces at the presumed sites of *X. oryzae* infection. In this case, the phage was able to infect more bacteria, in addition, phage infected bacterial cells could release more phage particles within the intercellular spaces, and infected more bacteria, thereby the amount of infection reduced.

水稻白葉枯病之研究 IV

噬菌體對水稻白葉枯病病原細菌 *Xanthomonas oryzae* 感染力之影響

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在溫室栽培狀況下，當二個月的水稻成株（臺中在來一號）葉片接種水稻白葉枯病病原細菌 *Xanthomonas oryzae*，其濃度在 1×10^1 cells/ml 時可達到 43% 感染率；如以一星期的水稻幼苗接種時，細菌濃度需在 1×10^6 cells/ml 才能達到 30% 感染率，因此可以明顯地看出一星期的幼苗較二個月的成株具有抗病性。應用噬菌體來作生物防治，即將噬菌體在接種細菌之前一天處理稻葉，則所有經過接種的葉片均無感病，然而，當噬菌體和細菌混合同時接種或細菌接種一天後再以噬菌體處理之，則其效果沒有前者大，由此結果明白地顯示應用噬菌體對於水稻白葉枯病的防治上具有預防之效果，但治療的效果則較少。

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