

Suppressing Effects of *Erwinia herbicola* on Infection by *Xanthomonas oryzae* and on Symptom Development in Rice

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ABSTRACT

Symptoms were reduced or prevented and the incubation period for symptom appearance was prolonged when rice plants were inoculated with *Xanthomonas oryzae* mixed with *Erwinia herbicola* by needle-prick or root-dip methods. Symptom development was retarded most when low concns of *X. oryzae* (10^6 /ml) were combined with high concns of *E. herbicola* (10^8 or more/ml). However, no delay in symptom development was observed when rice leaves were inoculated

with mixed inocula of *X. oryzae* and heat-killed *E. herbicola* or mixed inocula of streptomycin-resistant *X. oryzae* and viable *E. herbicola* suspended in streptomycin solution.

E. herbicola lowered the pH of a liquid medium to inhibitory levels but otherwise did not produce substances antagonistic to *X. oryzae* in liquid or solid media.

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When rice (*Oryza sativa* L.) leaves with bacterial blight symptoms are freshly collected and kept in plastic bags for a few days, the pathogen, *Xanthomonas oryzae*, becomes very difficult to isolate. Furthermore, it is not easy to isolate the pathogen from old bacterial ooze droplets found on diseased leaves, because this ooze is mixed with yellow saprophytic bacteria. Fast-growing, fermentative, yellow bacteria have been isolated from diseased rice leaves and identified as *Erwinia herbicola* (8). Whether *E. herbicola* has any effect on *X. oryzae* or on infection and disease development was investigated by growth and inoculation experiments with the two organisms. Preliminary results of this study appeared in an abstract (9).

MATERIALS AND METHODS.—*Preparation of inocula.*—*E. herbicola* isolates 14 and 16 (Philippines), and *X. oryzae* isolates P (Ceylon) and VN (Vietnam) were grown on Wakimoto's medium (20) for 48 h and suspended in sterile distilled water. VNS, a streptomycin-resistant mutant originating from VN (10), was grown on the same medium supplemented with 1% streptomycin sulfate (w/v) and 100 ppm nystatin (w/v) for 48 h, and its cells were washed twice with distilled water and centrifuged at 3,000 rpm (Sorvall RC2-B) for 5 min. Bacterial suspensions were adjusted turbidimetrically to approximately 5×10^8 cells/ml using a spectrophotometer (Bausch and Lomb's Spectronic 20) at 625 μ m. The suspensions were then diluted or mixed to required concns. *E. herbicola* in suspension was heat-killed in a water bath at 80 C for 10 min. In one of the experiments, bacterial suspensions were prepared in 1% streptomycin solution (w/v) in which the growth of *E. herbicola*, but not that of the VNS isolate of *X. oryzae*, was inhibited.

Inoculation.—Leaves of greenhouse-grown plants of rice cultivars IR8 and Aichi-asahi were inoculated by a single needle-prick method at maximum tillering stage. IR8 plants at seedling stage were inoculated by a root-dip method; root tips were cut before seedlings were immersed in a bacterial suspension for 30 min. Inoculated plants were kept in a greenhouse where temp ranged from

27 to 40 C. Observations for appearance of symptoms were made daily, starting 5 days after inoculation. Comparisons were made of incubation period and of percentages of plants showing lesion development, but not of lesion size.

RESULTS.—*Effect of E. herbicola on symptom development of bacterial blight of rice.*—Development of symptoms was prevented and the incubation period was prolonged when *X. oryzae* was mixed with *E. herbicola* and inoculated on rice plants by either single needle-prick or root-dip inoculation methods (Table 1). Pure inoculum of *X. oryzae* gave 100% infection on Aichi-asahi leaves by needle-prick inoculation, and 92% infection on IR8 seedlings by root-dip inoculation. However, when it was mixed equally with the saprophyte and inoculated, much lower frequencies were obtained, ranging from 6.7% to 36%. Leaves inoculated with *X. oryzae* alone showed symptoms within 6 to 8 days after inoculation, while the incubation period was prolonged nearly 3-fold following inoculation with mixed inocula. The majority of seedlings inoculated with pure inoculum of *X. oryzae* developed 'kresek' (complete wilt). Only a few seedlings inoculated with a mixed inoculum developed 'kresek.' Thus, *E. herbicola* reduced not only incidence but also severity of the disease.

The decrease of disease incidence was most pronounced when lower concns of the pathogen (10^7 cells/ml or less) were combined with higher concns of the saprophyte (10^8 cells/ml or more) (Table 2). *E. herbicola* at 10^6 cells/ml or less, did not reduce symptom development regardless of the concns of *X. oryzae*. Pure inoculum of *X. oryzae* at 10^7 cells/ml resulted in 100% infection. However, at 10^6 cells/ml, infection decreased to 44%; at 10^5 cells/ml there was no infection, either with pure or mixed inocula.

When *E. herbicola* was killed by heat treatment or when its growth was inhibited by streptomycin, infection from mixed inocula was not reduced (Table 3).

Population changes of X. oryzae (VNS) and E. herbicola 14 in vivo.—Isolates VNS and 14 at 10^8 cells/ml were used to inoculate surface-sterilized IR8

TABLE 1. Effect of *Erwinia herbicola* on suppression of symptom development in rice plants following inoculation with *Xanthomonas oryzae*

Inocula ^a	Leaves or plants infected (%)		Average incubation period (days)	
	Needle prick ^b	Cut root ^c	Needle prick	Cut root
<i>X. oryzae</i>	100.0	92.0	6.7	11.5
<i>X. oryzae</i> + <i>E. herbicola</i> (Isolate No. 14)	6.7	22.0	17.3	18.7
<i>X. oryzae</i> + <i>E. herbicola</i> (Isolate No. 16)	36.2	32.0	18.1	20.0

^aThe concn of each bacterial species was 10^8 /ml.

^bBased on 40 to 58 inoculated leaves of rice cultivar Aichi-asahi.

^cBased on 50 inoculated seedlings of rice cultivar IR8.

leaves. The population changes of VNS and 14 within the leaf tissue were estimated following grinding of surface-sterilized leaf samples and serial dilution plating. Leaf samples consisted of 8 mm of leaf length on each side of the needle-prick site.

A lag phase of 5-6 h occurred before *E. herbicola* started to grow in inoculated rice leaves. Its population then rapidly reached 5×10^5 /16 mm of leaf length, after which no further net multiplication occurred during the 22-day test period (Fig. 1). The population changes of *E. herbicola* were almost identical, whether it was inoculated alone or with *X. oryzae*. When inoculated alone, *X. oryzae* had about a 12-h lag phase in the rice plant, after which it increased gradually to a maximum population of 10^8 /16 mm leaf length within 10 days. By that time, symptoms were visible around inoculation sites, and bacteria had developed beyond the sample area, reaching a very high total population size. With interference of *E. herbicola*, the initial inoculated population of *X. oryzae* remained unchanged or declined slightly. However, in a few samples from leaves inoculated with mixed inocula, the population of *X. oryzae* showed an increase. These leaves probably would have developed symptoms later, had they not been sacrificed.

Effect of E. herbicola on the growth of X. oryzae in vitro.—*X. oryzae* and *E. herbicola* were streaked in separate parallel lines 1 cm apart on plates of Wakimoto's medium. Additionally, equal concns of these bacteria were mixed, serially diluted, and plated on the same medium to observe growth interaction. Growth of *X. oryzae* on the streaked plates was as vigorous as when it was grown alone. And at low densities, *X. oryzae* colonies immediately adjacent to *E. herbicola* colonies were not inhibited. However, when colony density of the saprophyte was high, *X. oryzae* colonies did not appear.

In another experiment, *E. herbicola* was grown in nutrient broth or Wakimoto's liquid medium and after 3 days a filter-sterilized culture filtrate was prepared and adjusted to pH 7. Equal volumes of this culture filtrate and double-strength Wakimoto's medium were mixed and poured into plates. Approximately 100 cells of *X. oryzae* in 0.1 ml of suspension were spread evenly on the agar surface. The number and size of colonies of *X. oryzae* were almost identical on plates supplemented with culture filtrate of *E. herbicola* as on control plates. *E. herbicola* had shifted the pH of the original liquid medium from 7.0 to 4.3-4.6 after growing in it for 3 days. If this low pH of the culture filtrate was not adjusted

TABLE 2. Percentage of leaves infected following inoculation with different concn combinations of *Erwinia herbicola* and *Xanthomonas oryzae*

No. cells/ml of inoculum		Infected leaves ^a (%)
<i>X. oryzae</i>	<i>E. herbicola</i>	
10^8	10^9	10
10^8	10^8	14
10^8	10^7	66
10^8	10^6	100
10^8	10^5	100
10^8	0	100
10^7	10^9	4
10^7	10^8	8
10^7	10^7	40
10^7	10^6	96
10^7	10^5	100
10^7	0	100
10^6	10^9	0
10^6	10^8	2
10^6	10^7	22
10^6	10^6	40
10^6	10^5	48
10^6	0	44

^aBased on 50 inoculated leaves of rice cultivar Aichi-asahi, following single needle-prick inoculation.

upward before it was added to the medium, growth of *X. oryzae* was prevented, or limited to a few small colonies.

Cells of *X. oryzae* (isolate VNS) and *E. herbicola* (isolate 14) from 48-h slant cultures were incubated separately or together on a reciprocal shaker at room temp of about 28 C. Periodically, the population changes of both species were estimated by dilution plating. The plating medium was supplemented with 1% streptomycin to estimate the population of VNS. The lag period and generation time of both organisms were calculated based on growth curves obtained from this experiment. When both organisms were inoculated separately, the lag phase for isolate 14 was about 75 min and for isolate VNS, about 6 h; the exponential period for 14 was only about 12 h, while for VNS it lasted for about 24 h (Fig. 2). The average generation time for 14 was only 40 min, while that for VNS was about 2 h. When both organisms were inoculated together in the same medium, the growth curve for 14 was almost identical to that when it was grown alone, but for VNS, it was entirely different. VNS grew very slowly after passing the lag phase; retarded

TABLE 3. Percentage of rice (cultivar IR8) leaves infected following inoculation with *Xanthomonas oryzae* (VNS) in combination with streptomycin-treated, heat-killed, or living cells of *Erwinia herbicola* (Isolates 14 or 16)

Inocula ^a	Number of leaves diseased/inoculated	Infected leaves (%)
VNS in Distilled Water (DW)	66/66	100
VNS in 1% Streptomycin (Sm)	63/63	100
VNS + 14 in DW	5/65	8
VNS + 14 in Sm	64/64	100
VNS + 16 in DW	8/65	12
VNS + 16 in Sm	61/64	95
VNS + 14 (heat killed) in DW	69/70	99
VNS + 16 (heat killed) in DW	64/65	98

^aFinal concn of *X. oryzae* (VNS) was 5×10^8 cells/ml, and of *E. herbicola* (Isolates 14 or 16) was 10^9 cells/ml; inoculated by a single needle prick.

growth was evident within 9 h and the population declined gradually shortly thereafter.

DISCUSSION.—*Erwinia herbicola* is a very common microorganism in nature. It has been isolated from various plants, seeds, paddy rice, animals, and human

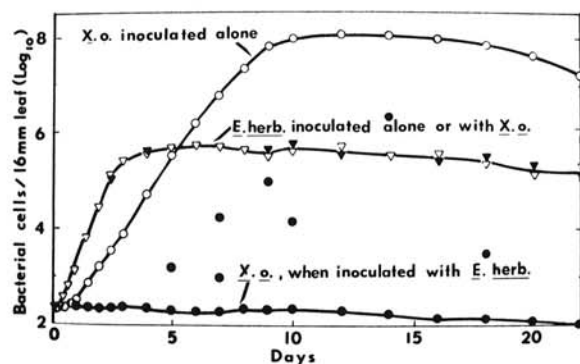


Fig. 1. Population changes of *Xanthomonas oryzae* (VNS) and *Erwinia herbicola* (Isolate 14) in rice (cultivar IR8) leaves following needle-prick inoculation. Scattered solid circles indicate the population of *X. oryzae* in individual leaves inoculated with mixed inoculum, which might have developed symptoms later.

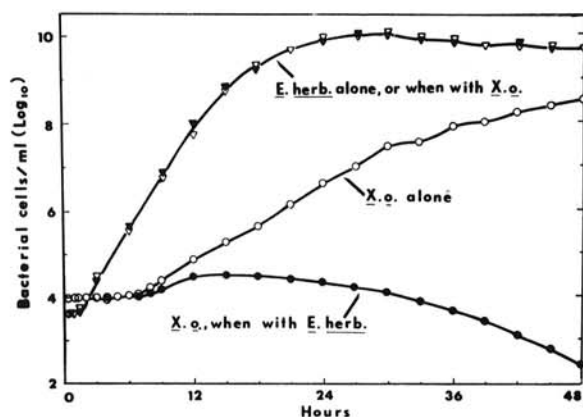


Fig. 2. Population changes of *Xanthomonas oryzae* (VNS) and *Erwinia herbicola* (Isolate 14) in Wakimoto's liquid medium at 28 C.

clinical material (4, 7, 14, 16). Due to its ubiquitous nature, and its association with diseases of both plants and animals, it would be worthwhile to investigate more thoroughly the ecology of *E. herbicola*. Association of yellow saprophytic bacteria with *X. oryzae* in diseased rice leaves has been known for a long time (12). This yellow bacterium was named *Bacillus oryzae* Hori & Bokura by Ishiyama; however, no further experiments were made. *B. oryzae* could be the same as *E. herbicola*. Unfortunately, the original descriptions of *B. oryzae* were not sufficient to compare it with *E. herbicola*.

Modification of infection by plant pathogenic bacteria when associated with saprophytic bacteria has been found in some diseases (1, 2, 3, 5, 6, 11, 19). *E. herbicola* in the present study interfered with the growth of *X. oryzae* in vitro and in vivo. Because *X. oryzae* can grow side by side with *E. herbicola*, and also grows normally in a medium containing a culture filtrate from *E. herbicola*, it seems that this saprophytic bacterium does not produce substances antagonistic to *X. oryzae* in vitro. In mixed culture, *E. herbicola* can probably rapidly deplete the culture medium and reduce the pH of the medium to levels unfavorable for growth of *X. oryzae*.

In vivo, after an initial lag phase of 5-6 h, *E. herbicola* multiplied rapidly to a moderate population level (5×10^5 /16 mm leaf) with or without the presence of *X. oryzae*. However, for some unknown reason it was unable to increase beyond that moderate level. Multiplication of *E. herbicola* may occur only in the area around the inoculation site. *X. oryzae*, when not limited by interference with *E. herbicola*, appears to be able to grow continuously inside the rice leaves and to migrate (in xylem vessels) from the inoculation site to adjacent areas. With the interference of *E. herbicola*, *X. oryzae* was unable to grow in most inoculated leaves. In the few leaves where it did grow, it was delayed for some time and the incubation period was prolonged.

One explanation for the inhibition of symptom development with a mixed inoculum is that *E. herbicola* may shift the pH to unfavorable levels, and may make nutrients limiting for *X. oryzae*. Another hypothesis, that of phytoalexins (1, 13, 15, 17), may explain the inhibition. Such a hypothesis remains to be explored with regard to *X. oryzae*, rice tissue and *E. herbicola* interaction. For bacterial blight of rice to develop, the pathogen must become established within the xylem vessels. In leaves it is uncertain if the inhibitory interaction is exerted on the pathogen while it is in the parenchyma before it invades

the vessels, or after it is in the vessels. Since *X. oryzae* is the bacterium uniquely able to invade, move, and multiply within rice xylem vessels, it may be that *E. herbicola* evokes a host response normal to nonxylem bacteria when they enter vessels. Use of a new leaf-clipping inoculation technique (18) should enable more precise interpretation of this interaction.

The total numbers of cells developed was higher from mixed inocula than with *X. oryzae* alone. Crosse (3) obtained a slight reduction in incidence and severity of bacterial canker of stone fruits when killed *E. herbicola* cells were present in inocula. He explained that killed cells might block the xylem elements, thus preventing extensive migration of the pathogen. However, no reduction of symptom development of bacterial blight of rice was found when heat-killed or streptomycin-treated *E. herbicola* cells were present in the inocula. Thus, a hypothesis of spatial interference cannot be postulated to explain the results obtained here.

The role, if any, of *E. herbicola* in influencing the amount of field infection of rice by *X. oryzae* remains unknown. Inoculum for spread of the disease in the field is probably largely dependent on the quantity of pathogenic bacteria which are exuded from diseased leaves. If the number of cells of *X. oryzae* arriving at the infection court of the hydathodes is small, and microcolonies of *E. herbicola* are also present, infection by *X. oryzae* might be inhibited. It is at this site, with normal ingress occurring, where studies of interaction should be pursued to obtain results extendable to natural disease occurrence. Also, in the ecological cycle, *X. oryzae* in ooze droplets on the leaves is often mixed with *E. herbicola*, resulting in an intimately associated mixture of bacteria to compete together when the droplets become liquefied.

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