**Growth Dynamics of *Xanthomonas campestris* pv. *oryzae* in Leaves of Rice Differential Cultivars**

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**ABSTRACT**

Bacterial multiplication and spread were monitored in race-specific interactions of *Xanthomonas campestris* pv. *oryzae* and rice (*Oryza sativa*). Bacterial numbers in compatible and incompatible interactions increased equally until levels reached 10^10–10^11 colony-forming units/leaf. Thereafter, the bacterial growth rates in incompatible interactions slowed in comparison with those in compatible interactions. Maximum bacterial numbers per leaf were dependent on host cultivar. In both compatible and incompatible interactions, bacteria spread from the inoculation point; however, bacteria in compatible interactions spread more rapidly.

Pathogenic specialization of *Xanthomonas campestris* pv. *oryzae*, the causal agent of bacterial blight, has been demonstrated on rice cultivars that have specific genes for resistance to the compatible and incompatible races of *Xanthomonas campestris* pv. *oryzae*. Differences in lesion development in different rice cultivars also are important factors in determining the host's response to bacterial multiplication. Symptoms were not observed in advance of bacteria. Lesion lengths, in general, were positively correlated with bacterial numbers. When leaves were inoculated with bacterial mixtures (1:1 of race 1 or 6 [compatible] to race 2 [incompatible]), the growth rate of race 2 isolates was severely restricted compared with that of the control (race 2 alone). The same effects were observed in a host in which all races were compatible. The apparent interaction between bacterial isolates confounded analysis of the effect of mixed inoculations on race-specific resistance induction.

**MATERIALS AND METHODS**

**Bacterial isolates.** Isolates PXO61 (race 1), PXO86 (race 2), and PXO99 (race 6) of Philippine *X. c. oryzae* were obtained from...
T. W. Mew at the International Rice Research Institute (IRRI, Los Baños, Philippines). Bacterial cultures were grown on peptone sucrose agar (25) for immediate use. For long-term storage, bacteria were suspended in 15% glycerol (10) and frozen at -80°C or suspended in sterile skim milk (Difco Laboratories, Detroit, MI) and lyophilized. Virulence of _Xanthomonas oryzae_ to rice is lost upon repeated transfer of cultures (22). Therefore, cultures for experiments were grown fresh (one to two transfers) from glycerol or lyophilized stocks.

To select streptomycin (Sm)-resistant PXO61 or PXO99 strains (PXO61 and PXO99), 1-ml aliquots of 17-hr-old bacterial cultures (about 10^10 cfu/ml) were inoculated into 15 ml of peptone sucrose broth containing 50 µg of streptomycin per milliliter. After incubation overnight as described above, streptomycin-resistant colonies were selected on peptone sucrose agar containing 100 µg of streptomycin per milliliter. A rifampicin (Rif)-resistant PXO86 strain (PXO86^Rif) was obtained by a gradient plate technique (24).

Growth of antibiotic-resistant strains was compared to that of wild-type parents in culture (peptone sucrose broth and nutrient broth) and in plants (as described below).

**Rice cultivars.** Seed of rice (_Oryza sativa L._) cultivars IR8 (Xa-11, gene for resistance to _X. oryzae_), IR1545-339 (Xa-4), Cas 209 (Xa-10) (27), and IR1545-339 (xa-5) (16) were supplied by T. Mew (IRRI). The interactions of these cultivars with isolates of races 1, 2, and 6 of _X. oryzae_ are shown in Table 1. Two rice seeds were planted per 8.9-cm-sq pot in Bacto potting soil (Michigan Peat Co., Houston, TX) supplemented with complete fertilizer (Peters 20-20-20, W. R. Grace, Cambridge, MA). Plants were grown in a greenhouse (28-32°C days and 22-26°C nights). After 2 wk, pots of seedlings were individually transplanted into flats containing 5 cm of sterile soil and fertilizer.

**Preparation of inocula.** Suspensions for inoculations were prepared by growing bacteria in nutrient broth (Difco Laboratories) overnight on a rotary shaker (200 rpm) at 28°C. Cells were collected by centrifugation at 17,000 g for 10 min at 22°C. The pellet was washed twice and resuspended in distilled water. The inoculum was adjusted to desired concentrations using a Klett-Summerson spectrophotometer with a red #66 filter (Klett Manufacturing Co., New York, NY).

To assay bacterial growth in rice leaves, plants were inoculated with bacterial suspensions containing 5 x 10^5 cfu/ml. For mixed inocula, suspensions of PXO61 and PXO86 or PXO99 and PXO86 containing 1 x 10^6 cfu/ml were combined in equal amounts (final concentration: 5 x 10^6 cfu/ml of each isolate). A second inoculum was obtained by combining the 1:1 bacterial mixture with an equal portion of water to yield a mixture containing a total population (combined isolates) of 5 x 10^6 cfu/ml of bacteria.

**Inoculation and sampling of rice plants.** A double sewing machine needle (Schmetz, 2.0/80, Herzogenrath, West Germany) dipped into inoculum (prepared as described above) was used to stab fully expanded rice leaves (30-40 days past sowing, four- to five-leaf stage) once between the margin and midrib, perpendicular to the veins, 10 cm from the leaf tip. The double-needle inoculation technique is adapted from that of Muko and Yoshida (13). Only the second-youngest leaf per plant was inoculated. After inoculation, plants were placed in flats of water and incubated in a growth chamber (32°C days and 22°C nights, 12-hr photoperiod, 70% RH). Lesion lengths were measured on each sampling day.

**RESULTS**

**Antibiotic resistant strains.** Streptomycin- and rifampicin-resistant strains of _X. oryzae_ were selected to expedite detection and allow differentiation of isolates in mixed inoculations. Antibiotic-resistant strains had growth rates similar to those of the wild types in cultivars Cas 209 (Fig. 1), IR1545-339, and IR8 (data not shown) and produced similar lesion lengths on rice leaves (data not shown). In addition, growth rates were identical in culture (data not shown).

**Sampling procedures.** Grinding leaves in phosphate buffer instead of water did not increase the number of bacteria recovered (data not shown). Recovery was 2.5 ± 0.4, standard error) x 10^8 cfu/leaf from leaves after double-needle inoculations with 5 x 10^5 cfu/ml bacterial suspensions. After Hagborg infiltration of Cas 209, bacteria were recovered at 1.6 ± 0.1 x 10^6 cfu/leaf when a 1 x 10^6 cfu/ml suspension was used and 2.7 ± 0.6 x 10^6 when a 1 x 10^5 cfu/ml suspension was used.

**Bacterial multiplication in rice leaves.** Although starting
Inoculum concentrations varied between experiments, the overall shapes of the bacterial growth curves in all interactions tested were very reproducible. The growth curves shown are from representative experiments.

In compatible and incompatible interactions with Cas 209 (Fig. 2A) and IR1545-339 (Fig. 2B), bacteria multiplied steadily and at similar rates in rice leaves for 4-6 days after inoculation, reaching $10^{5}-10^{6}$ cfu/leaf. Thereafter, bacterial growth in incompatible interactions with these hosts slowed in comparison with bacterial growth in compatible interactions. Bacterial numbers in compatible interactions with Cas 209 (Fig. 2A) and IR1545-339 (Fig. 2B) reached $10^{11}-10^{13}$ cfu/leaf within 8-10 days after inoculation. Inoculation of Cas 209 with PXO99Sm (race 6, compatible) and PXO86Rf (race 2, incompatible) or with PXO99Sm (compatible) and IRN793Rf (race 2, incompatible) resulted in the same multiplication pattern (data not shown) as those shown in Figure 2A. In IR20, the differences in multiplication were less definitive in that bacterial numbers did not exceed $10^7$ cfu/leaf in compatible interactions, which was only about 10-fold higher than in incompatible interactions (Fig. 2C).

**Mixed inoculations.** In mixtures of PXO61Sm (compatible) and PXO86Rf (incompatible) in Cas 209, the PXO61Sm population grew similarly to when it was inoculated alone (Fig. 2A). However, the PXO86Rf growth rate was significantly reduced in the mixture compared with its growth in single inoculations, and the final population did not exceed $10^5$ cfu/leaf. Similar patterns were observed in mixed inoculations of IR1545-339 with PXO86Rf (incompatible) and PXO99Sm (compatible) (Fig. 2B). In cultivar IR20, both bacterial populations resulting from mixed inoculations (PXO86Rf, compatible, and PXO61Sm, incompatible) were about 10% lower than those of PXO86Rf alone (compatible) and were similar to the levels reached by PXO61Sm alone (incompatible) (Fig. 2C). In IR8, in which both race 1 and 2 isolates result in compatible interactions, populations followed a pattern similar to that observed in interactions of race 1 and 2 with Cas 209 and IR1545-339. That is, the race 1 isolate (PXO61Sm) in mixed inoculation...
inoculations increased at a rate equal to that when the isolate was inoculated alone (Fig. 3), but populations of the race 2 isolate (PXO86Rif, compatible) did not increase above \(10^6\) cfu/leaf. When inoculated alone, PXO86Rif reached populations of \(10^5-10^6\) cfu/leaf by 8 days after inoculation. The number of total bacteria in the 1:1 inoculum mixtures (\(5 \times 10^4\) cfu/ml of each isolate or a total of \(5 \times 10^5\) cfu/ml of the combined isolates) did not affect the shape of the growth curves or the rates of growth of any isolate (data not shown).

Symptom development. Lesions on all hosts began to appear 6–8 days after inoculation. Average lesion lengths at day 12 differed among cultivars (Fig. 4). Until extensive tissue necrosis occurred, lesion lengths corresponded with bacterial numbers of the compatible race in the interactions; that is, the higher the bacterial numbers were, the longer the lesions were. Lesion lengths on Cas 209 and IR1545-339 resulting from mixed inoculations were not substantially different from those resulting from inoculations only with the compatible race (Fig. 4). On IR20, mixed inoculations resulted in shorter lesion lengths than in the compatible control. Individual inoculations of IR8, a compatible host, with races 1 and 2 produced lesions of similar lengths. Mixed inoculations of IR8 also resulted in lesion lengths similar to those resulting from individual inoculations.

Spread of bacteria. Bacteria in both compatible (PXO61 sm) and incompatible (PXO86 Rif) interactions with Cas 209 multiplied and spread outward from the inoculation point (Table 2). In the incompatible interactions, however, bacteria multiplied less rapidly and moved to adjacent sections at a much slower rate. Symptoms were never observed in advance of the bacteria; rather, bacterial numbers in a given section approached \(10^8\) cfu/2-cm section before symptom expression. The same trends were observed in all experiments.

**Fig. 3.** Multiplication of isolates of *Xanthomonas campestris* pv. *oryzae* isolates PXO61sm (○ = PXO61sm compatible) and PXO86 Rif (□ = PXO86 Rif compatible) in leaves of rice cultivar IR8. Rice leaves were inoculated individually with each isolate (dashed lines) or with a mixture of the two bacteria at a 1:1 ratio (solid lines). Data are log of the means and standard errors of four replications from each treatment of a representative experiment.

**Fig. 4.** Lesion lengths (cm) on four rice cultivars 12 days after inoculation with individual isolates of incompatible or compatible races of *Xanthomonas campestris* pv. *oryzae* or 1:1 mixtures. Cas 209, IR20, and IR8 were inoculated with PXO86 Rif (race 2) and PXO61sm (race 1). IR1545-339 was inoculated with PXO86 Rif (race 2) and PXO99sm (race 6). Data are the means and standard errors of four replications from each treatment of a representative experiment.

**TABLE 2.** Spread of isolates PXO61 sm (race 1, compatible) and PXO86 Rif (race 2, incompatible) of *Xanthomonas campestris* pv. *oryzae* in adjacent leaf sections (A–F) of rice cultivar Cas 209. Progression from the inoculation point was monitored in the upper 12 cm of the leaf at 2-day intervals by cutting 2-cm sections above and below the point of inoculation (section E).

<table>
<thead>
<tr>
<th>Day</th>
<th>Isolate</th>
<th>Race</th>
<th>A (cm)</th>
<th>B (cm)</th>
<th>C (cm)</th>
<th>D (cm)</th>
<th>E (cm)</th>
<th>F (cm)</th>
<th>Total lesion length (cm)</th>
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<tr>
<td>0</td>
<td>PXO61 sm</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.2(0.1)</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>PXO86 Rif</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.2(0.1)</td>
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<tr>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.7(0.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>PXO86 Rif</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>4.4(0.1)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>4</td>
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<td>0</td>
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<td>5.7(0.3)</td>
<td>5.7(0.3)</td>
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<td>0</td>
<td>0</td>
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<td>6.1(0.1)</td>
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<td>7.4(0.5)</td>
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*Data (four replications per treatment) were log transformed before calculating the means ± standard errors.*
Effect of inoculum density on expression of resistance. The initial water soaking from Hagborg infiltration of Cas 209 leaves disappeared within 4 hr. At both high and low inoculum levels, PXO61 (compatible) multiplied steadily (after a lag of 1–2 days) through days 8–10 (Fig. 5). After this time, multiplication reached a plateau. Numbers of PXO61 (incompatible) at high inoculum did not exceed the initial $10^2$–$10^6$ cfu/leaf. At low inoculum, the bacteria multiplied to $10^2$–$10^6$ cfu/leaf by day 6 and did not increase beyond that level. After Hagborg inoculation with a high inoculum density, water soaking was observed in the compatible interaction at 48–72 hr. In incompatible interactions, either no symptoms appeared by day 4 or the leaf tissue that was infiltrated became dry and gray in color. The same trends were observed in all experiments.

**DISCUSSION**

Our investigations indicate that incompatibility in the interaction between isolates of *X. oryzae* and cultivars Cas 209, IR1545-339, and IR20 was reflected in lower bacterial numbers and reduced lesion lengths, although these correlations were much less pronounced in cultivar IR20. In IR20, compatible interactions resulted in lower bacterial populations (Fig. 2C) and reduced lesion development (5, Fig. 4) when compared with compatible interactions in cultivars Cas 209 and IR1545-339, suggesting that some level of resistance to the compatible race exists. It is unclear whether the reduction of final PXO61 populations in compatible interactions is a feature of the Xa-4 gene for resistance carried by IR20. Resistance to *X. oryzae* conferred by Xa-4 is not complete; that is, clear differences between compatible and incompatible interactions are not always apparent. Horino et al. (5) reported that gene Xa-4 is influenced by temperature. At high temperatures (33 C days, 25 C nights), lesions in incompatible interactions with PXO61 were up to five times longer than lesions at low temperatures (29 C days, 21 C nights). At these high temperatures, lesion lengths did not increase in compatible interactions with PXO86.

The effect of temperature on bacterial multiplication was not measured. Our conditions (32 C days, 22 C nights) approached those that would favor longer lesions in incompatible interactions and, thus, may have minimized the expected differences in compatible and incompatible interactions.

To assess whether resistance expression was dependent on time or a threshold number of bacteria, varied inoculum levels were used and bacterial multiplication was monitored. As shown in Figure 2A (initial inoculum about $10^7$ cfu/leaf) and in the inoculum-density experiment (Fig. 5, initial inoculum about $10^5$ and $10^7$ cfu/Cas 209 leaf), bacteria in incompatible interactions multiplied to $10^2$–$10^6$ cfu/leaf and did not increase further, regardless of the time necessary to reach that number. This suggested that bacterial concentration was critical in the activation of rice defense mechanisms. Although reduction of the growth rate was observed when bacterial numbers had reached $10^2$–$10^6$ cfu/leaf, activation of host defenses probably took place at a lower population level.

Parry and Callow (18) measured bacterial movement basipetally in rice leaves by plating sections and scoring for presence or absence of bacterial growth from the ends of those sections. Bacteria were found in the same number of leaf sections in both compatible (PXO71) and incompatible (PXO86) interactions with Cas 209. They concluded that, because in both interactions bacteria were present throughout the leaf, the difference between compatible and incompatible interactions was in the expression of symptoms by the host; that is, longer lesions did not necessarily result from more bacterial growth and spread. However, they did not enumerate bacteria in the leaf sections. In our experiments, isolates of both compatible and incompatible races of *X. oryzae* colonized leaf tissues. However, in the incompatible interaction, bacteria did not invade the leaf as aggressively as did those in the compatible interaction (Table 2). Also, in the incompatible interaction, populations of bacteria if present in a section were always one or two log units lower than those in the compatible interaction. Previous reports indicated that differences in bacterial numbers between compatible and incompatible interactions were two log units or less (12,14,18,20,26). However, in those studies, leaf sections of 10 cm or less were sampled. Our experiments suggest that whole-leaf samples give a more reliable estimate of the true differences in compatible and incompatible interactions for the following reasons: 1) bacteria in incompatible interactions did not grow and move in leaves to a limited extent; 2) bacterial numbers in both interactions were similar at the inoculation site (Table 2, E); and 3) compatible bacteria accumulated to fairly high concentrations in the lower portions of the leaf before symptom expression (17; our data, not shown). No symptoms were seen in sections with less than $10^5$ cfu/leaf, indicating a minimum bacterial number necessary for symptom expression.

In mixed inoculation experiments of Cas 209 or IR1545-339, when the mixture contained equal numbers of bacteria from both races, bacterial multiplication of isolates representing the compatible interaction was not restricted compared with bacterial growth in leaves inoculated with only the compatible isolate (Fig. 2A and B). However, multiplication of bacteria representing incompatible races was inhibited, and bacterial numbers dropped over time. We observed the same phenomenon in IR8 (Fig. 3), which carries no known genes for resistance to these isolates, suggesting that inhibition of growth of isolates of incompatible races in Cas 209, IR1545-339, and IR8 after mixed inoculations probably was due to some interaction between the isolates. No inhibition of one isolate by another was observed in intra mixed cultures (nutrient broth, peptone sucrose broth), but the race 2 isolate (PXO86) multiplied at a slightly slower rate than did the race 1 isolate (PXO61, data not shown). Other isolates (IRN793, race 2, and PXO99, race 6) in Cas 209 produced growth curves similar to those of PXO86 and PXO61 in single and mixed inoculations. It is possible that, in the host plant, isolates such as PXO61 or PXO99 can outcompete other isolates for available nutrients. Restricted growth of the isolate from the incompatible race might prevent that isolate from reaching the threshold level ($10^4$–$10^6$ cfu/leaf) necessary for resistance induction. Thus, susceptibility would be the observed phenotype. Lesion-length data obtained from some cultivars
(Cas 209 and IR1545-339) were consistent with this hypothesis. When these cultivars were inoculated with 1:1 mixtures of compatible and incompatible races, the resulting lesion lengths were not significantly different from those of leaves inoculated with only bacteria from the compatible race (Fig. 4). Other researchers using different host cultivar-bacterial race interactions reported similar results (18, 21).

In IR20, initial growth of the isolate PXO86 R (compatible) in mixed inoculations with PXO61 sm (incompatible) was not reduced to the extent that it was in mixed inoculations of cultivars Cas 209 and IR8. However, the final PXO86 R population in IR20 was about one log unit lower in mixed inoculations than in individual control inoculations. Multiplication patterns of isolate PXO61 sm (incompatible) in IR20 were similar in mixed and single inoculations. The reduction of PXO86 R populations in IR20 could be explained in two ways: Expression of specific resistance to PXO61 sm reduced the populations of that isolate to the extent that it was in mixed inoculations of cultivars Cas 209 and IR8. Alternatively, the resistance induced by PXO61 sm also affected growth of PXO86 R, that is, that resistance was physiologically predominant over susceptibility. Our data do not favor either scenario because measurement of resistance in IR20 by differences in bacterial growth and final populations was not definitive under our conditions (Fig. 2C). Lesion lengths resulting from mixed inoculations on IR20 were reduced in leaves in comparison with the compatible control, but in our conditions, lesion lengths between compatible and incompatible interactions were not significantly different (Fig. 4).

With the exception of IR20, the population and lesion length data collectively suggest that incompatibility is not physiologically predominant to compatibility in the interaction between rice and X. c. oryzae. The inhibitory or competitive interaction between bacterial isolates in mixed inoculations, however, confounds this conclusion. In an attempt to avoid or mask the effects of isolate-isolate interactions, several approaches are under way. Preliminary evidence (authors, unpublished) indicates that, if higher ratios (incompatible to compatible) of bacteria are inoculated into rice leaves of cultivars Cas 209 and IR1545-339 or if incompatible races are inoculated before inoculation with compatible races, then lesion lengths and multiplication of bacteria from both races are reduced. In addition, we are developing isogenic mutants of X. c. oryzae differing only in the gene responsible for incompatibility to a particular host. These will be used in concert with newly released host isolines, which differ only slightly from both races are reduced. In addition, we are developing isogenic mutants of X. c. oryzae differing only in the gene responsible for incompatibility to a particular host. These will be varieties of rice. Ann. Phytopathol. Soc. Jpn. 40:93-97.

19. Petpisit, V., Khush, G. S., and Kauffman, H. E. 1977. Inheritance of resistance in IR20 by differences in bacterial growth and final populations as effective under our conditions (Fig. 2C).


**LITERATURE CITED**


