Relationship Between Phyllosphere Population Sizes of *Xanthomonas translucens* pv. *translucens* and Bacterial Leaf Streak Severity on Wheat Seedlings

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ABSTRACT

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The relationship between leaf-associated population sizes of *Xanthomonas translucens* pv. *translucens* on asymptomatic leaves and subsequent bacterial leaf streak (BLS) severity was investigated. In three experiments, *X. translucens* pv. *translucens* was spray-inoculated onto 10-day-old wheat seedlings over a range of inoculum densities (10^4 , 10^5 , 10^6 , 10^7 , and 10^8 CFU/ml). Lesions developed most rapidly on plants inoculated with higher densities

of *X. translucens* pv. *translucens*. Leaf-associated pathogen population sizes recovered 48 h after inoculation were highly predictive of BLS severity 7 days after inoculation ($R^2 = 0.970$, $P < 0.0001$). The relationship between pathogen population size on leaves and subsequent BLS severity was best described by the logistic model. Leaf-associated *X. translucens* pv. *translucens* population size and BLS severity from a particular pathogen inoculum density often varied among experiments; however, the disease severity level caused by a particular leaf-associated *X. translucens* pv. *translucens* population size was not significantly different among experiments. Biological and disease control implications of the *X. translucens* pv. *translucens* population size-BLS severity relationship are discussed.

Xanthomonas translucens pv. *translucens* (18,33) causes bacterial leaf streak (BLS) and black chaff of wheat when it infects the glumes and lemma. BLS is an important bacterial disease of wheat in many wheat-growing regions of the world and can cause up to a 30 to 40% loss in yield (9,29). Symptoms of BLS include water-soaked streaks on leaves. Lesions can exude copious amounts of bacteria that are visible under humid conditions as a yellow slimy mass. Current control strategies for BLS include treating seeds with heat to ensure a clean seed source (9,10) and developing resistant cultivars (26,27). Leaf-associated populations of *X. translucens* pv. *translucens* are thought to be important in dispersing the pathogen in the absence of disease within and among plants (8), because *X. translucens* pv. *translucens* has not been reported to spread systemically within infected plants (25). Duveiller (8) observed that populations of 10^6 CFU of *X. translucens* pv. *undulosa* (a closely related pathovar that also causes bacterial leaf streak of cereals, including wheat) per leaf were detectable up to 30 days prior to initial BLS symptoms. Populations of 10^8 CFU of *X. translucens* pv. *undulosa* per leaf accompanied the development of visible lesions. However, a large component of this population may have been derived from incipient lesions (8). A specific quantitative relationship between leaf-associated population sizes of *X. translucens* pv. *translucens* on asymptomatic leaves and subsequent BLS severity has not been determined.

Leaf-associated populations of phytopathogenic bacteria can serve as direct sources of inoculum for infection under a favorable environment (15,20,21). The existence of leaf-associated phytopathogenic xanthomonads has been well documented on both hosts (12, 24,30) and nonhosts (16,32). However, conflicting evidence exists as to the role these populations play in pathogenesis. On citrus, leaf-associated populations of *X. campestris* pv. *citrumelo* were not shown to play a critical role in the infection process in field studies (11). Research on field-grown tomatoes indicated that a positive correlation existed between population sizes of *X. campestris* pv. *tomato* recovered from leaves and bacterial leaf spot several days later (17), although a specific quantitative relationship was not described. Furthermore, leaf-associated populations of *X. campestris* pv. *pruni* on symptomless peach leaves were reported to be important in the sudden, widespread appearance of bacterial spot of stone fruits (30).

The specific role of leaf-associated populations in influencing disease symptoms remains largely unknown for most phytopathogenic xanthomonads. Civerolo (4) found that the number of lesions produced by *X. campestris* pv. *pruni* was directly proportional to inoculum concentration in growth chamber studies. However, inoculation was performed by directly injecting inoculum into the leaves and was not directly analogous to infection processes in the field, where the pathogen must go from the leaf surface into the leaf itself. Previous work on phytopathogenic pseudomonads has shown that disease is positively correlated with phyllosphere population sizes prior to symptom development. An apparent threshold of *Pseudomonas syringae* pv. *syringae* of 10^4 CFU/g of leaf tissue was required before symptoms of bacterial brown spot of bean occurred under field conditions (21). Incidence of halo blight of oats was also shown to be strongly positively correlated with the phyllosphere population size of *P. syringae* pv. *coronafaciens* prior to disease onset (15). It is not known whether a similar relationship exists for most phytopathogenic xanthomonads.

The specific objective of this study was to determine the quantitative relationship between leaf-associated population sizes of *X.*

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translucens pv. *translucens* and subsequent disease severity in growth chamber experiments.

METHODS AND MATERIALS

Rifampin-resistant mutant. A spontaneous rifampin-resistant mutant of *X. translucens* pv. *translucens* (strain 4) isolated from a BLS lesion from wheat in Minnesota (R. Jones, University of Minnesota) was generated by plating 100-μl aliquots of cell suspension (~10⁹ CFU/ml) on Difco nutrient agar (Difco Laboratories, Detroit) amended with 2.5 g of dextrose per liter and 100 μg of rifampin (NADR; Sigma Chemical, Co., St. Louis) per ml. The mutant, *X. translucens* pv. *translucens* strain 4Rif-2, was stable after several serial transfers and caused disease as well as or better than the parent strain. Strain 4Rif-2 was stored at -80°C as a suspension in a 20% (vol/vol) sterile glycerol solution.

Host plants and growing conditions. The highly susceptible spring wheat cultivar Angus was used in all experiments. Wheat seedlings were grown in vermiculite in square plastic pots (6.7 cm wide by 6.7 cm tall), four seedlings per pot. The plants were placed in a controlled environment chamber maintained at 22°C, 88% relative humidity (RH), day and 18°C, 95% RH, night, with a 16-h day

and 224 mmol m⁻² s⁻¹ photon flux light density. Preliminary evaluation of environmental conditions indicated that the environmental conditions employed in the growth chamber were sufficient for the colonization of the phyllosphere by strain 4Rif-2 and subsequent BLS symptom development (data not shown). Plants were watered as needed to keep the vermiculite moist and fertilized every 7 days with 20-20-20 N-P-K.

Preparation of bacterial inoculum. Inoculum was prepared from cultures of *X. translucens* pv. *translucens* 4Rif-2 grown on nutrient agar amended with 2.5 g of dextrose without rifampin (NAD) per liter for 48 h. Cells were harvested with a sterile inoculation loop or sterile cotton swab and suspended in sterile phosphate buffer (0.1 M, pH 7.00). Inoculum density was adjusted turbidimetrically (λ = 600 nm) to a concentration of 10⁸ CFU/ml with sterile phosphate buffer and verified by dilution plating onto NAD. Dilutions of inoculum were then made with sterile phosphate buffer to yield final *X. translucens* pv. *translucens* 4Rif-2 inoculum concentrations of ~10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ CFU/ml.

Relationships among inoculum density, strain 4Rif-2 populations, and BLS severity. Seedlings (10 days old) were spray-inoculated with sterile phosphate buffer and placed in the growth chamber in a completely randomized design. Because these experiments were developed to find a suitable pathogen inoculum density to study the potential for the biological control of *X. translucens* pv. *translucens* (31), this preinoculation step was included to simulate the application of a putative biocontrol agent to the leaf surface. After 48 h, seven pots of wheat seedlings were each spray inoculated until runoff with each density of *X. translucens* pv. *translucens* 4Rif-2 (10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ CFU/ml). Pots were then placed randomly in the growth chamber. After another 48 h, 10 first seedling leaves were collected from each inoculum density treatment for population quantification (described below). Seedlings were examined daily for the presence of BLS symptoms for the duration of the experiment. At 7 days after strain 4Rif-2 inoculation, BLS severity was estimated visually on all of the remaining first seedling leaves using pictorial disease assessment keys for percent leaf area occupied by BLS lesions (7). The experiment was repeated three times.

Bacterial population enumeration. Leaves were harvested aseptically using a flame-sterilized scalpel and forceps and placed individually in 18 ml of sterile phosphate buffer. Leaves were sonicated for 10 min and the leaf wash decanted immediately into sterile 50-ml test tubes to prevent re-adhesion of bacteria to the leaf surface. Appropriate dilutions of the leaf wash were then made using a spiral plater (Spiral Biotech, Bethesda, MD) and suspensions were plated onto NADR plates. Plates were incubated for 72 h at 28°C and *X. translucens* pv. *translucens* 4Rif-2 colonies counted.

Statistical analyses. In each experiment, mean log-transformed (13) *X. translucens* pv. *translucens* 4Rif-2 population sizes and mean BLS severity were estimated for each treatment. One-way analysis of variance (ANOVA) (6) was performed using the GLM

Fig. 1. Effect of *Xanthomonas translucens* pv. *translucens* strain 4Rif-2 inoculum density (CFU/ml) on the time until first visible symptoms of bacterial leaf streak (BLS). Incidence = percentage of inoculated leaves with water-soaked or necrotic lesions of BLS. A total of 18 leaves per inoculum density were evaluated for the presence of BLS for each sampling time from 3 days after inoculation onward. A total of 28 leaves per inoculum density were evaluated for the presence of BLS 1 and 2 days after inoculation. Data are shown for a representative experiment.

TABLE 1. Leaf-associated *Xanthomonas translucens* pv. *translucens* strain 4Rif-2 population sizes recovered 48 h after inoculation with five different inoculum densities among three different experiments^y

Experiment	Strain 4Rif-2 inoculum density (CFU/ml)				
	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
1	2.456 A d ^z	2.700 B d	3.897 B c	5.434 A b	5.863 B a
2	2.566 A d	3.602 A c	3.952 B c	5.282 A b	6.453 A a
3	2.206 A e	3.402 A d	4.859 A c	5.648 A b	6.458 A a

^y Average log_{CFU} per leaf of *X. translucens* pv. *translucens* 4Rif-2 recovered from symptomless wheat leaves 48 h after inoculation. Population sizes of *X. translucens* pv. *translucens* 4Rif-2 recovered from 10 individual leaves were averaged for each inoculum density.

^z Population sizes in the column for each inoculum density that are followed by the same capital letter are not significantly different (*P* < 0.05), as determined by Fisher's least significant difference (LSD) test. Population sizes in the same row followed by the same lowercase letter are not significantly different (*P* < 0.05), as determined by Fisher's LSD test.

TABLE 2. Bacterial leaf streak (BLS) severity 7 days after inoculation for five different *Xanthomonas translucens* pv. *translucens* strain 4Rif-2 inoculum densities among three different experiments^y

Experiment	Strain 4Rif-2 inoculum density (CFU/ml)				
	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
1	1.28 C d ^z	5.44 C d	16.06 B c	59.17 B b	78.12 B a
2	2.47 B e	9.17 B d	20.78 B c	62.50 AB b	83.89 B a
3	3.50 A e	13.11 A d	37.56 A c	70.65 A b	91.24 A a

^y Average BLS severity 7 days after inoculation with *X. translucens* pv. *translucens* 4Rif-2. BLS severities from 18 individual leaves were averaged for each inoculum density.

^z Disease severity values in the column for each inoculum density that are followed by the same capital letter are not significantly different (*P* < 0.05), as determined by Fisher's least significant difference (LSD) test. Disease severity values in the same row that are followed by the same lowercase letter are not significantly different (*P* < 0.05) as determined by Fisher's LSD test.

procedure in SAS (release 6.04; SAS Institute, Inc., Cary, NC) to determine the effect of inoculum density on leaf-associated population size of strain 4Rif-2. One-way ANOVA was also used to determine the effect of *X. translucens* pv. *translucens* 4Rif-2 inoculum density on BLS severity. The consistency among experiments in resulting pathogen population size and BLS severity at each inoculum density of strain 4Rif-2 was evaluated using the one-way ANOVA. Regression analyses between mean log-transformed strain 4Rif-2 population size and mean BLS severity were performed using the R-code application in the XSlip-Stat statistical programming language (5). Comparisons of regression lines for each experiment were performed using the generalized *F* test (34).

RESULTS

Pathogen inoculum density determined the time required for visible BLS symptoms to develop on wheat leaves (Fig. 1). Symptoms of BLS first became visible as small, water-soaked spots that later coalesced and became necrotic as the lesion aged. Increasing inoculum concentration decreased the amount of time until water-soaked lesions first became visible. High inoculum densities (10^7 and 10^8 CFU/ml) of the pathogen resulted in nearly 100% of the inoculated leaves showing symptoms by 4 days after inoculation. Plants inoculated with lower inoculum densities did not start developing BLS symptoms until 4 (10^6 CFU/ml) and 5 (10^5 and 10^4 CFU/ml) days after inoculation. At 6 days after inoculation, 33% of the leaves from seedlings inoculated with 10^4 CFU of the pathogen per ml remained void of visible symptoms.

There were significant differences in leaf-associated population sizes of the pathogen and disease severity for different inoculum densities in all three experiments (Tables 1 and 2). In general, as inoculum density increased, pathogen population sizes and BLS severity also increased, although the increases in pathogen population size or disease severity among inoculum densities were not always significant for the lower inoculum densities (Tables 1 and 2, experiments 1 and 2). Pathogen population sizes and disease severity often varied among experiments for a particular pathogen inoculum density. Significant differences ($P < 0.05$) in pathogen population sizes recovered from leaves were detected at inoculum densities of 10^5 , 10^6 , and 10^8 CFU/ml among experiments (Table 1).

Fig. 2. Relationship between leaf-associated population sizes of *Xanthomonas translucens* pv. *translucens* strain 4Rif-2 recovered from symptomless wheat leaves 48 h after inoculation and bacterial leaf streak (BLS) severity assessed 7 days after inoculation. Strain 4Rif-2 population size (logCFU per leaf) averaged over 10 individual leaves per inoculum density; BLS severity expressed as mean BLS severity for 18 individual leaves per inoculum density.

BLS severity differed significantly ($P < 0.05$) among the three experiments at each pathogen inoculum density studied (Table 2).

The relationship between mean pathogen population size recovered from the phyllosphere and mean BLS severity 5 days later at each pathogen inoculum density was best described by the logistic model (Figs. 2 and 3). The logistic model suggests that the potential of a leaf-associated pathogen population to cause disease changes as a function of population density. Analysis of residuals generated from linear regression performed on these data indicated that a simple linear model fit poorly. The shape of the relationship suggested that the logistic or Gompertz models would more accurately describe the relationship (3). Appropriate transformations of BLS severity were made and a regression line generated for each model. Analysis of residuals generated from both models indicated that the logistic model was best at describing the relationship. Thus, the logit-transformation (3) of BLS severity was used in subsequent analyses to describe the relationship between leaf-associated population sizes of *X. translucens* pv. *translucens* 4Rif-2 and BLS severity (Fig. 3).

Regression lines generated for each experiment for the relationship between the logit-transformation of BLS severity and pathogen population size recovered from leaves 48 h after inoculation were compared using the *F* test. The *F* test indicated that a single common regression line generated from all three experiments described the relationship as well as regression lines with different slopes for each experiment ($F = 0.754$, $P > 0.05$), different intercepts ($F = 1.375$, $P > 0.05$), and different slopes and intercepts ($F = 0.918$, $P > 0.05$) for each experiment. Biologically, this implies that disease severity resulting from a given population of leaf-associated *X. translucens* pv. *translucens* 4Rif-2 was consistent among experiments.

DISCUSSION

In preliminary experiments, we tested the effects of pre- and postinoculation leaf wetness on leaf colonization and infection by *X. translucens* pv. *translucens*. We found that, although continuous

Fig. 3. Relationship between leaf-associated population size of *Xanthomonas translucens* pv. *translucens* strain 4Rif-2 recovered 48 h after inoculation from symptomless wheat leaves and subsequent bacterial leaf streak (BLS) severity transformed to fit the logistic model for three different experiments. Strain 4Rif-2 population size (logCFU per leaf) averaged over 10 individual leaves per inoculum density; BLS severity expressed as the logit-transformed average BLS severity for 18 individual leaves per inoculum density. Linear regression lines were not significantly different among three different experiments ($y = 1.386x - 6.976$; $R^2 = 0.970$, $P < 0.0001$).

leaf wetness 48 h pre- and postinoculation enhanced both colonization and infection, leaf wetness was not essential to either process (data not shown). We consistently obtained high levels of disease and *X. translucens* pv. *translucens* colonization on plants maintained at high RH in the absence of visible leaf wetness beyond the immediate postinoculation period (6 h). Several previous studies involving phytopathogenic bacteria have indicated a need to cover plants with plastic bags to maintain free moisture on leaf surfaces (32), provide a dew period after inoculation (1), or otherwise make the environment more conducive to bacterial growth (19,22,23). Results from experiments conducted on plants maintained in the unsaturated environment used in this study may be more relevant to the field, since free moisture may not exist for several days at a time in the field. The lack of observable free moisture several hours after pathogen inoculation on leaves of seedlings and subsequent development of BLS supports the observation of Duveiller (8) that long periods of free moisture on leaf surfaces are not required for BLS development.

The time interval between inoculation and development of BLS symptoms depended on inoculum concentration of the pathogen. A single phytopathogenic bacterium has a very low, but non-zero, probability of causing disease, indicating that a large population of bacteria on the leaf surface may be required for successful infection (14,21). If a large leaf-associated population of *X. translucens* pv. *translucens* 4Rif-2 is required to successfully infect a wheat leaf, the population size of the pathogen immigrating onto or growing on the leaf surface would influence the time required for strain 4Rif-2 to reach a sufficient population density necessary to infect the leaf. Further research should be conducted to determine specific factors responsible for the relationship between leaf-associated *X. translucens* pv. *translucens* population size and the time required for BLS development. Understanding the specific mechanism or mechanisms responsible for this relationship may direct efforts to control BLS. For example, if a threshold population size of leaf-associated *X. translucens* pv. *translucens* is required to induce infection, efforts to control BLS should focus on keeping *X. translucens* pv. *translucens* population sizes below this threshold or to identify wheat cultivars that maintain a higher threshold for infection to occur. In either case, the effect would be to delay leaf penetration and keep the leaf-associated populations of *X. translucens* pv. *translucens* on the surface of the leaves and susceptible to inhibition by the physical and biological environment as long as possible.

Population sizes and disease severity attained on leaves following inoculation with a particular pathogen inoculum density often differed among experiments (Tables 1 and 2). Variability in establishment of pathogen populations and BLS severity among experiments may make spray inoculation techniques difficult to use in assessing cultivar susceptibility (27). However, the use of a spray inoculation technique may be more relevant than infiltration approaches to predicting cultivar susceptibility in the field, because *X. translucens* pv. *translucens* is often deposited on the wheat leaf surface by wind, rain splash, or sprinkler irrigation (1,29,35), and bacterial cells must penetrate the leaf surface to successfully infect. The use of infiltration inoculation techniques artificially removes this key step in the infection process. Although BLS severity and pathogen population size on the leaf surface resulting from inoculation with a particular pathogen density often varied, BLS severity resulting from a given leaf-associated pathogen population size did not differ significantly among experiments.

The logistic model best described the relationship between pathogen population size on symptomless leaves and subsequent BLS severity (Fig. 2). The ability of small population sizes of the pathogen to cause BLS symptoms suggests that, under conditions conducive to bacterial growth, even very small population sizes of *X. translucens* pv. *translucens* have the potential to cause disease. However, the relationship indicates that, during the time frame of the experiment (7 days), the efficiency of a small population of the pathogen to cause BLS symptoms is less than that of larger popu-

lation sizes. The dramatic increase in BLS severity between leaves having an average *X. translucens* pv. *translucens* population size of 4.0 log_{CFU} per leaf to those having a mean population size of 5.0 log_{CFU} per leaf (Fig. 2) prior to disease development may reflect a shift in the probability that an individual *X. translucens* pv. *translucens* cell will elicit disease, perhaps as a function of density-dependent autoinduction of a pathogenicity factor or factors (2,28) or density-dependent induction of factor or factors influencing *X. translucens* pv. *translucens* ingress. Alternatively, the dramatic increase in BLS severity with increasing pathogen population size over this range may be due to density-dependent colonization or survival on the phyllosphere by *X. translucens* pv. *translucens* as has been reported for *P. syringae* on bean (36). A third possibility is that host resistance mechanisms may be more easily overcome by pathogen population sizes on leaves greater than 4.0 log_{CFU} per leaf.

From the perspective of disease control, the logistic relationship suggests that BLS severity can be minimized if leaf-associated pathogen population sizes are kept small, despite the presence of disease-conducive conditions. The relationship between leaf-associated pathogen population sizes and subsequent BLS severity (logit-transformed) provides a quantitative method to evaluate the factors that may influence the ability of a pathogen population to cause disease including plant resistance, pathogen aggressiveness, and the presence of leaf-associated antagonist populations. For example, past work on soybean (12) and tomato (24) has suggested that two important mechanisms of resistance to bacterial diseases in these crops are the ability of resistant cultivars to reduce leaf-associated pathogen population sizes (decrease inoculum density) and to require greater pathogen population sizes on resistant than on susceptible cultivars to cause comparable disease severities (reduce infection efficiency). Control strategies that reduce the infection efficiency of leaf-associated populations of *X. translucens* pv. *translucens* would significantly alter the logistic relationship between pathogen population size on the leaf surface and subsequent disease severity.

Although these experiments were conducted in a growth chamber, the results have important implications for understanding disease development in the field. When variation due to environmental factors is controlled, there is a strong correlation between numbers of cells of *X. translucens* pv. *translucens* that can be recovered from wheat leaves 48 h after a single inoculation event and the severity of disease that develops 5 days later. This shows that, other things being equal, treatments that restrict the ability of *X. translucens* pv. *translucens* to survive and multiply on leaf surfaces will both reduce subsequent disease severity and delay the development of symptoms. Furthermore, the relationship between early population sizes of *X. translucens* pv. *translucens* on wheat leaves and later disease severity is logistic rather than linear. This means that it is not necessary to completely eliminate the pathogen from surfaces of wheat leaves to greatly limit disease development. Of course, environmental conditions in the field will influence both the population size of *X. translucens* pv. *translucens* and the susceptibility of leaves to infection. In our experiments, we used a highly susceptible cultivar of wheat. Most currently grown cultivars will not become infected as readily as 'Angus'. On the other hand, environmental conditions were employed that were not overly conducive to infection. In our experiments, the leaves were visibly wet for only a few hours after inoculation, which indicates that extended periods of high RH should be sufficient for infection to occur in the field even in the absence of visible free moisture on leaves.

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