Pseudomonas lachrymans Adsorption, Survival, and Infectivity Following Precision Inoculation of Leaves

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ABSTRACT

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Precise quantities of *Pseudomonas lachrymans* suspensions were deposited on leaf surfaces and the effects of drying and other factors were measured. Assay of survival was by dilution plating of wash water and by direct counts of infections on the inoculated leaves. Both techniques had a minimum detection level of about 1,000 cells per leaf. Over a range of 10^4 to 10^{11} cells/4 cm^2 a constant proportion (93%) of the inoculum was adsorbed, indicating nonspecific adsorption sites. During a 16-hour post-inoculation drying period, survival was highest on pubescent suscept (cucumber) leaves, less on pubescent nonhost (potato) leaves, and least on glaborous nonhost (pear) leaves; both leaf surface morphology and innate plant resistance influenced the death rate. The pathogen's resistance to dry conditions governed survival on all three crops after the initial drying period; death rates were similar on all three crops. On cucumber, survival after drying for up to 16 hours was less at 10 C than at 25 C. With increasing periods of artificial dew on leaves before inoculation, short-term survival of the pathogen decreased.

Additional key words: epidemiology, cucumber, angular leaf spot, leaf surface.

Bacteria produced within or upon infected plant parts are mobilized in water and provide inoculum which spreads to surfaces of noninfected plant parts (3). When infection does not occur immediately, this inoculum, to be effective, must survive until penetration occurs. Survival of plant-pathogenic bacteria has been the subject of several recent reviews (1, 4, 8, 12, 21). Much contemporary work has been concerned with leaf surface (phylloplane) phenomena.

Survival studies with *Pseudomonas lachrymans* (Smith and Bryan) Carsner (2, 22), and other pathogens, were first conducted on nonliving materials like glass slides. Survival times were short, but in vivo viability is probably maintained longer. On leaves, transpiration provides moisture which retards bacterial desiccation; stomata provide micro-infection sites where pathogens can survive in a protected niche; various nutrients are present which provide substrates for bacterial metabolism; and survival is affected by diverse microorganisms which normally occur on leaf surfaces and interact with the pathogen. The relative importance of each of these influences is unknown and undoubtedly varies with different pathogen-suscept-environment combinations.

Previous experiments on survival of pathogenic bacteria in the phyllosphere have been done with relatively imprecise numbers of cells per cm^2 of leaf surface (14, 17). Either natural inoculum was measured (3, 7) or a cell suspension was sprayed on the leaves (16). When the inoculum was applied by spraying, the concentration of cells in suspension could be controlled precisely, but the inoculum actually impacting the leaf surface was not known. Much valuable information has been obtained with these methods, but certain phenomena can be detected only by using precision inoculation devices. Adsorption of *P. lachrymans* to cucumber leaf surfaces and short-term survival of these adsorbed cells were chosen for investigation.

MATERIALS AND METHODS

Suscept and pathogen.—Bacterial survival was studied on *Cucumis sativus* L. 'Elem' seedlings grown in a greenhouse until one to two true leaves had developed. Twenty-four hours before inoculation plants were transferred to growth chambers for acclimatization, removed for inoculation, and immediately returned to the same chambers. The major environmental parameters were 24 C, 50-60% relative humidity, and 16/8-hour light/dark photoperiod.

Pseudomonas lachrymans inoculum was produced on nutrient-glycerine agar slopes incubated for 24-48 hours at 28 C. When the bacteria were washed from the agar with deionized water and agitated on a Vortex mixer, many cells were found in aggregates of two to 10. The clumping was effectively eliminated by 30-second immersion of a 16 × 150-mm test tube containing the bacterial suspension in an ultrasonic cleaner bath at 80

kHz (Model G100, Ultrasonics Instruments International, Farmingdale, N.Y.); viability was not reduced even after 10 minutes of treatment at this frequency. The bacterial suspensions were usually prepared more concentrated than required, diluted with deionized water to 0.2 absorbance at 400 nm in a Bausch and Lomb Spectronic 20 spectrophotometer, and further diluted by 10^{-2} to the inoculum concentration used in most experiments. By dilution-plate procedures, 0.2 absorbance was found to be 7.5×10^8 colony-forming units (CFU)/ml. For inoculum suspensions with more than 7.5×10^8 CFU/ml, portions of the concentrated suspensions were diluted to 0.2 absorbance and the original concentration determined by calculation from the dilution factor.

Quantitative inoculation.—Precise and accurate quantities of inoculum were applied to leaves with Schein's inoculator (19) as modified by one of us (J.R.). The inoculator consists of a pressure-regulated source of CO_2 passed through a timer-controlled solenoid valve and then a DeVilbiss sprayer (Model No. 15). In our experiments the bacterial suspension was sprayed at low velocity to minimize forcing the pathogen into stomata. With a carbon dioxide pressure of 1 bar, and the timer operated for 1 second per inoculation, 0.015 ml of liquid was deposited on the 4-cm^2 circular target employed.

Wet and dry incubation.—Moist periods longer than 1 minute were maintained by misting water on the plants and enclosing them in polyethylene bags. For periods longer than 24 hours the plants were re-misted after 1 day. For dry incubation periods, plants were maintained in the growth chamber or, for short intervals (< 10 minutes), in the laboratory.

Measurement of bacterial survival.—Number of lesions formed and dilution-plate procedures were the basis of estimates of bacterial survival. Lesions were counted on the leaves subjected to the various incubation treatments and the data therefore were a measure of survival and infectivity. After incubation, the inoculated leaves were injured by rubbing with the finger to facilitate establishment of infections. Leaf surfaces were misted if required so all leaves were wet when injured. The rubbing caused no damage visible to the unaided eye; however, lesions per leaf were approximately tenfold those formed on leaves inoculated and kept moist for 24 hours, but not injured. Lesions, 1- to 2-mm diameter water-soaked areas with a small white crust, were counted 4-6 days after injury. Some of the lesions formed outside the 4 cm^2-inoculation site; these resulted from infection by surviving *P. lachrymans* cells spread to other portions of the leaf when it was rubbed.

Survival was also estimated by direct isolation and plating (3). Excised leaf laminae were washed in jars with one to six leaves and 20 ml of sterile deionized water per leaf. The capped jars were oscillated at 190 strokes per minute for 10 minutes. Serial dilutions (1:99) of the wash water were prepared and 0.1 ml samples spread on predried modified sucrose agar (SA) in plates. The plates were incubated at 28 C and colonies counted 4-6 days after plating. Modified sucrose agar was a semi-selective medium containing 8 gm nutrient agar, 50 gm sucrose, 2.1 gm $MnSO_4$, and 4 mg crystal violet per liter. *Pseudomonas lachrymans* formed typical colonies on this medium (3, 7). Six times during the experimentation, presumed *P. lachrymans* colonies were tested for pathogenicity; all produced typical angular leaf spot lesions on cucumbers.

RESULTS

Adsorption of Pseudomonas lachrymans on leaf surface.—Preliminary adsorption studies and the experiments detailed below indicated a 100-fold loss in CFU between the number thought to have been applied to the leaf and the number removed in washing. Two phenomena could account for this difference: (i) bacteria were killed during the spraying procedure, and (ii) bacteria were adsorbed to the leaf and not removed by washing. Inoculum was sprayed on several target materials (polystyrene plastic, glass plate, waxed paper, Millipore membrane filter), washed as described for

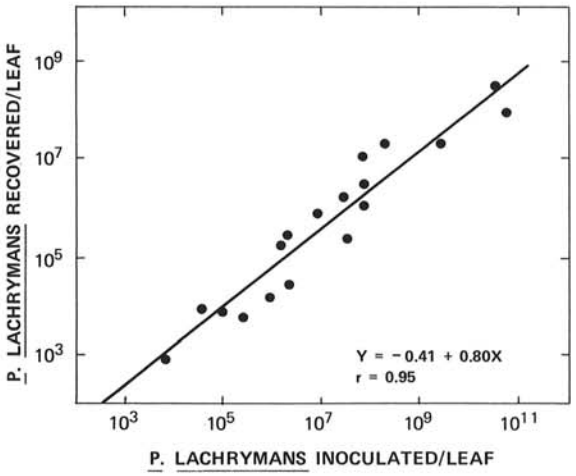

Fig. 1. Recovery of *Pseudomonas lachrymans* from surface of cucumber leaves inoculated and immediately washed in water for 10 minutes.

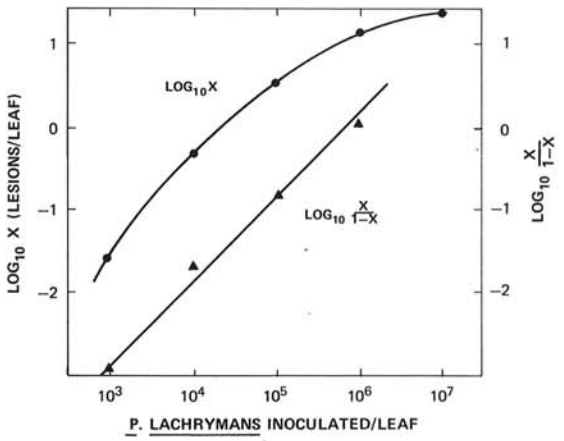

Fig. 2. Infectivity of *Pseudomonas lachrymans* on cucumber after inoculation, washing, and injury by rubbing. For $\log_{10}$ X, X = mean number of lesions per leaf. For $\log_{10}$ X/(1-X) (multiple infection transformation), X = (mean no. lesions)/no. lesions at maximum inoculum dose.

leaves, diluted, and plated. About 10 percent loss in CFU was found. It was gravimetrically determined that the amount of spray deposited on the smooth target materials was also about 10 percent less than that which adhered to the hirsute cucumber leaf surface.

The loss of CFU by nonremoval of bacteria adsorbed on cucumber leaf surfaces was determined by preparing tenfold dilutions of bacteria and inoculating them on 10 replicate first-true leaves. The experiment was repeated four times with different inoculum concentrations each time. If there were specific *P. lachrymans*-adsorption sites on cucumber leaves, the proportion of cells removed during washing would increase rapidly once the sites were saturated. Nonspecific adsorption would be indicated by constant proportions being removed. Leaf washing was begun within 1 minute of inoculation and, after appropriate dilution, the wash water was plated. Concurrently, samples of the inoculum suspension were diluted and plated to estimate the number of bacteria applied to leaves; from 10^3 to 10^{11} CFU per 4 cm^2 leaf-target-area were applied.

Most of the inoculated bacteria were adsorbed strongly enough not to be removed by the washing procedure. However, the proportion remaining on the surface was almost constant ($0.93 \pm .06$) over the wide range of inoculum concentrations (Fig. 1). The regression line of the ratio of number of bacteria recovered per number of bacteria applied had a slope < 1.0; if specific adsorption sites were involved a straight line with slope > 1.0 or a curvilinear equation would fit the data. The fit to a straight line was excellent (Fig. 1). The slope (< 1.0) indicated that at high inoculum doses either the dilution plate procedure tended to underestimate the number of bacteria recovered in the wash water or the estimate of bacterial populations applied was too high. With the large dilution factors required to get separate countable colonies in the dilution plates or measureable absorbance readings in the spectrophotometer, both effects were possible at very high inoculum doses. At lower doses, the data still fit a straight line and the slope was closer to unity. Upon eliminating from the regression the three highest inoculum levels actually used in the experiments, the correlation coefficient (r) became 0.93 and the regression coefficient (slope) became 0.99 ± 0.11.

To determine the tenacity with which the bacteria were adsorbed a second wash was performed with some leaves. Only $2 \pm 1.7\%$ of the original inoculum was removed. It was also possible that the original inoculum had been dislodged from the site of inoculation and, during the washing procedure, was readsorbed on other parts of the laminae. After inoculation and washing twice, leaves were momentarily pressed onto SA. More *P. lachrymans* colonies than could be counted developed from the target area and only 5 to 10/cm^2 from the noninoculated leaf surface. Some redistribution had occurred, but most bacteria were fixed at the inoculation site.

Infectivity of adsorbed bacteria.—Graded doses (10^1 to 10^7/4 cm^2) of the pathogen were applied to leaves in situ. The usual washing procedure was replaced by a thorough wash with a water spray. Inoculated leaves were injured by rubbing and the plants were incubated in a moist chamber. Twenty-five leaves were used for each inoculum concentration and the experiment was repeated twice. Adsorbed bacteria were infective and lesions developed at all doses of 10^3 or more *P. lachrymans*/4 cm^2 target area. This minimum detection level was greater than the theoretical detection limit of the dilution plate procedure we employed where, for one colony per plate, 215 bacterial cells per leaf would have to be present. However, because of redistribution and adsorption of washed bacteria on leaves in the wash water, and perhaps other effects, the dilution plate technique rarely detected inoculum applied that was $< 10^3$ per leaf.

The number of lesions that developed at succeedingly higher doses of inoculum (Fig. 2) was not linear on a log-log scale. At the higher inoculum doses, lesions were the result of infection by more than one *P. lachrymans* cell. A multiple infection transformation (9, 23) was performed in which it was assumed that the number of lesions counted on leaves with the maximum inoculum dose was the highest number which could be formed on the leaves (Fig. 2 and caption). Regressing the data for transformed lesion counts on inoculum concentration yielded a straight line ($r = .89$) with a slope of 1. Using the regression equation the number of bacteria required to cause one lesion per leaf was calculated by the following steps:

(i) maximum mean number of lesions = 25.2 per leaf

(ii) multiple infection transformation for 1 lesion per leaf

$$\log_{10} \frac{X}{1-X} = \frac{1/25.2}{1-1/25.2} = \log_{10} 0.0413 = 1.384$$

(iii) solving regression equation,

$$\log_{10} \frac{X}{1-X} = -5.9 + \log_{10} N,$$

where N = number of *P. lachrymans* cells inoculated per leaf. $N = 3.28 \times 10^4$ cells

(iv) using previously established proportion of inoculated cells remaining on leaf after washing (0.93), $3.28 \times 10^4 \times 0.93 = 3.05 \times 10^4$ bacteria caused one lesion per leaf when plants were injured by rubbing.

Survival of Pseudomonas lachrymans on dry leaf surfaces.—The quantitative assessment of survival on cucumber leaves was accompanied by measurements on leaves of the nonhomologous potato (*Solanum tuberosum* L.) and pear (*Pyrus communis* L.). Potato was selected because, like cucumber, its leaves are pubescent; pear leaves have a glaborous waxy surface. Beginning 2 hours after inoculation, when the original spray suspension had dried completely, 16 leaves were removed from each species, washed, and the wash water plated on SA. Samples were taken again after 17 hours, 3 days, and 6 days. *Pseudomonas lachrymans* persisted on all three crops for 6 days, but the mortality rates were different (Fig. 3). On cucumber the population declined gradually until after 6 days one-tenth of the original (2 hours) number of bacteria was present. Small water-soaked lesions developed then and sampling was terminated. The largest difference between the three species was the death rate during the first 24 hours after inoculation. On the smooth exposed surface of pear leaves mortality was greatest, on potato intermediate, and cucumber the least. The greater survival on cucumber was not due to bacterial growth in lesions. During the succeeding 5 days the death rates were similar on the surface of all crops although

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