

Survival, Seed Transmission, and Epiphytic Development of *Xanthomonas campestris* pv. *glycines* in the North-Central United States

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ABSTRACT

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Xanthomonas campestris pv. *glycines* located in plant debris placed on the soil surface or buried at a depth of 15 cm survived through the winter in Iowa. Epiphytic populations of the pathogen developed on both resistant and susceptible seedlings that grew from seeds with either external or internal populations of *X. c.* pv. *glycines*. The pathogen dispersed from a diseased source plant in the field equally in all directions and at roughly the same rate in plots of either resistant or susceptible soybeans. The size of the epiphytic population did not differ significantly between resistant and susceptible cultivars during the first 3 wk after inoculation. However, 35 days after inoculation, external populations of *X. c.* pv. *glycines* were 20- to 50-fold greater on the susceptible plants. Eventually, disease developed on susceptible but not on resistant plants.

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326 Plant Disease/Vol. 73 No. 4

Bacterial pustule of soybeans (*Glycine max* (L.) Merr.) is caused by *Xanthomonas campestris* pv. *glycines* (Nakano) Dye. Symptoms are restricted to leaves and consist of small yellow to brown lesions with raised pustules in the center. When the disease becomes severe, lesions may merge to form large necrotic patches. The pathogen survives in crop residues (4,5,8) as well as on seed (4,5) and enters the plant through wounds and stomates (10).

Much of the information about this disease comes from research conducted in the southern United States or the tropics (4,5,8) and may not be directly applicable to the situation in the north-central states. Here we describe our assessment of the survival in plant debris, dispersal in resistant and susceptible cultivars, and potential for seed transmission of *X. c.* pv. *glycines* under field conditions in central Iowa. In addition, we hoped to determine the extent to which epiphytic populations of the pathogen developed on resistant and susceptible soybean cultivars.

MATERIALS AND METHODS

Inoculum. Xcg-8, the strain of *X. c.* pv. *glycines* used in these tests, was obtained from a typical bacterial pustule lesion on a soybean plant (cv. BSR 301) grown near Ames, Iowa, in 1980. The strain was characterized as a pathovar of *X. campestris* according to the criteria of Dye (3). A naturally occurring mutant of Xcg-8 resistant to 50 µg/ml of the

antibiotic rifampin was also used. This strain, Xcg-Ra, appeared to have pathogenic capabilities (i.e., growth rate in host tissue and aggressiveness in infectivity titrations) identical to those of Xcg-8 (6).

For inoculum production, stock cultures that had been suspended in sterile 5% skim milk and frozen in liquid nitrogen were rapidly thawed, then spread on yeast extract-calcium carbonate agar (20 g agar, 10 g yeast extract, and 2.5 g calcium carbonate per liter of distilled water) (YCA). After incubation for 24–48 hr at 20 C, the plates were flooded with sterile distilled water, the colonies were suspended, and the suspensions were adjusted to 10^8 cfu/ml on the basis of spectrophotometric measurements. These suspensions were then diluted as necessary.

Detection of *X. c. pv. glycines*. Populations of strain Xcg-8 were detected by use of a selective medium originally developed by Mulrean and Schroth (9) for *X. c. pv. juglandis*. When spread on this medium, cells of *X. c. pv. glycines* grew into small (1 mm diameter) blue colonies surrounded by a zone of starch hydrolysis. The YCA medium was amended with 50 μ g/ml of rifampin (Sigma Chemical Co., St. Louis, MO) (YCRA) for use in detection of strain Xcg-Ra. Cycloheximide was added to both the Mulrean and Schroth medium and YCRA at 100 μ g/ml to inhibit fungal growth.

To detect epiphytic populations of *X. c. pv. glycines*, individual leaflets were placed into flasks containing 50 ml of a solution of sterile 0.06 M phosphate buffer (pH 7.0) and 0.1% Triton X-100 (Fisher Scientific Co., Fair Lawn, NJ) (Triton buffer). The flasks were then shaken at room temperature (20–22 C) for 1 hr on a reciprocal shaker (60 cycles per minute). Next, the buffer was serially diluted in sterile distilled water and plated on the appropriate medium. The plates were incubated at 20–24 C, and bacterial colonies were counted 4–5 days later. Only symptomless plant tissues were used for determination of epiphytic populations.

Survival of *X. c. pv. glycines* in leaf debris. Diseased soybean leaves (cv. Clark) were collected in early October from plants that had been inoculated with Xcg-8 or Xcg-Ra in July 1982. The leaves were air-dried and broken into small pieces (1–5 mm), and 1-g samples of this leaf tissue, containing either Xcg-8 or Xcg-Ra, were placed in each of 84 nylon mesh bags. On 30 October 1982, the bags were taken to the field; one-half were placed on the soil surface and one-half were buried at a depth of 15 cm. Placement was in a split-plot design with pathogen strain as the subplot factor. At 1-mo intervals throughout the winter and spring, six bags (three of Xcg-8 and three of Xcg-Ra) were collected randomly

from each of the placements. Each bag was washed with tap water to remove excess soil, and its contents were removed, placed in 50 ml of sterile Triton buffer, and reduced to a suspension in a Waring Blender. The suspensions were then serially diluted and plated on the appropriate medium. One hundred randomly selected colonies from tissues sampled in May were tested for pathogenicity. Expanding leaflets on greenhouse-grown seedlings of BSR 301 were lightly dusted with 600-mesh Carborundum, then gently rubbed with an inoculum suspension containing approximately 10^7 cfu/ml in sterile distilled water. This and all other field experiments were conducted at Curtiss Experimental Farm, Ames, Iowa. Plots were located in fields on which corn had been grown the previous year.

Transmission of *X. c. pv. glycines* from seed onto plants. In 1981, seeds of a susceptible cultivar (BSR 301) were surface-sterilized in 70% ethanol for

3 min, then immersed for 1 min in an aqueous suspension of Xcg-Ra at 10^4 or 10^7 cfu/ml. A second sample of seeds was contaminated internally by vacuum infiltration. Seeds submerged in bacterial suspensions were placed under a partial vacuum for 1 min, then removed and treated in 70% ethanol for 1 min. Control seeds were treated with 70% ethanol for 3 min, then immersed in sterile distilled water for 1 min. All seeds were air-dried at room temperature and immediately planted in the field on 11 May 1981. The treatments were arranged in a randomized complete block design with four blocks. Seeds were planted in hills (10 seeds per hill) located 1.52 m apart. Each hill was surrounded by hills of uninoculated resistant soybeans (cv. BSR 302).

Three symptomless seedlings from each hill were sampled when primary leaves had just become fully expanded. Plants were divided into four parts—hypocotyl, cotyledon, primary leaf, and apical bud—each of which was processed

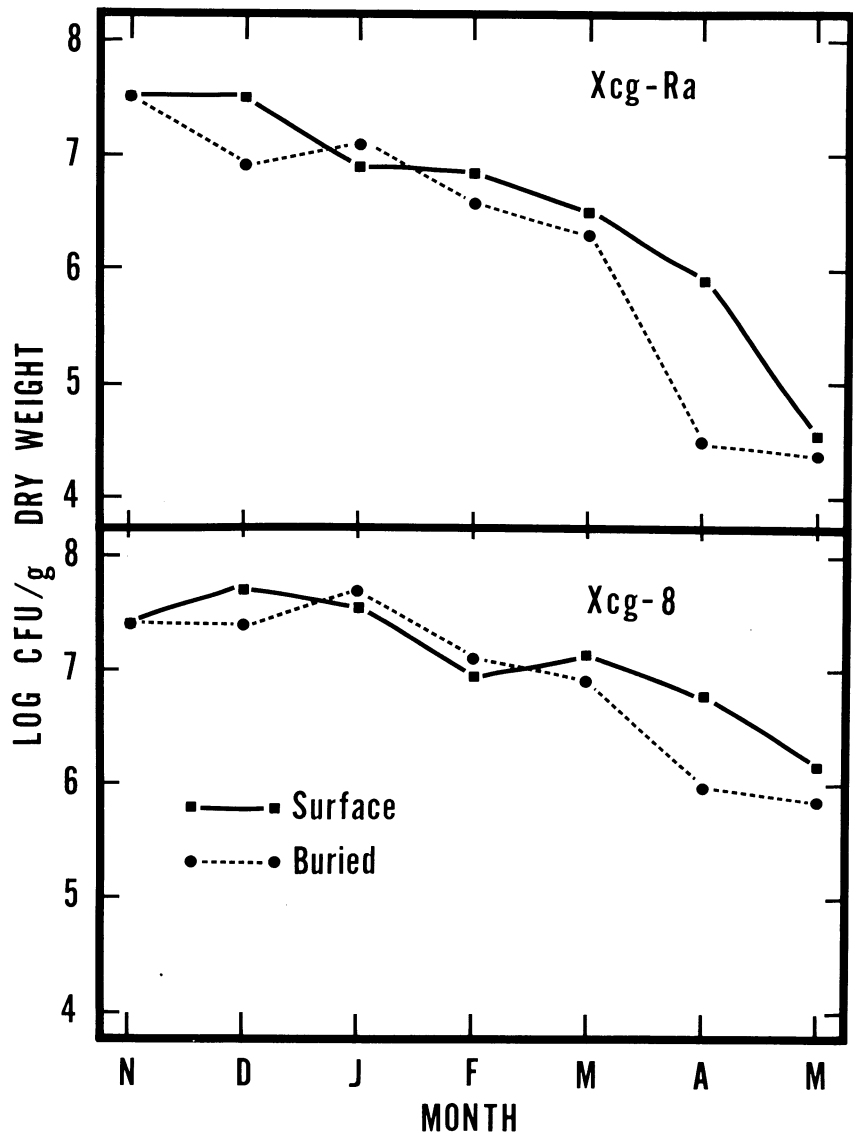


Fig. 1. Survival of *Xanthomonas campestris* pv. *glycines* in surface or buried (15 cm) soybean leaf debris in Iowa, from November 1982 to May 1983, based on initial dry weight of debris. Xcg-Ra is a rifampin-resistant strain derived from strain Xcg-8.

separately to determine epiphytic pathogen populations. After the plants had developed six trifoliolate leaves (9 July 1981), the most recently expanded symptomless leaflets were also sampled and examined for the presence of epiphytic populations of Xcg-Ra. In 1982, the experiment was repeated using susceptible (BSR 301) and resistant (cv. Williams) soybeans inoculated with 10^4 cfu/ml, but only the primary leaves and sixth trifoliolate leaves were sampled. In addition, late in the season (10 August), susceptible plants were examined to determine if disease was caused by the seedborne inoculum. Several diseased leaflets were collected from each hill and

surface-sterilized with 70% ethanol. Fifty individual pustules per hill were pierced with a sterile needle, which was then touched to plates of YCA and YCRA. The proportion of pustules containing strain Xcg-Ra was estimated by dividing the number of colonies that developed on YCRA by the number observed on YCA. Plants in this experiment were physically isolated from other plots in which the rifampin-resistant strain was used.

Seed infestation. Seeds from heavily diseased plants, which had been inoculated with Xcg-Ra, were harvested by hand or combine. In the hand harvest, seeds were aseptically removed from each pod. Four 50-seed samples were collected

from both the combine-harvested seed and the hand-harvested seed. Twenty-five seeds from each sample were placed in 125-ml flasks containing 25 ml of Triton buffer and shaken on a rotary shaker until the seed coats loosened (1–2 hr). The buffer was serially diluted and the dilutions were plated on YCRA. Another 25 seeds from each sample were washed individually in 2 ml of Triton buffer, and the wash liquid was serially diluted and plated on YCRA.

Contamination of seeds during threshing was examined by harvesting four samples of healthy plants in a clean single-plant thresher, then four samples of plants heavily infected with Xcg-Ra, and finally four samples of healthy plants. Twenty-five seeds were collected separately from each sample and assayed by the single-seed method just outlined.

To determine if *X. c. pv. glycines* was able to infect the seeds within pods in the field and to provide infected seeds for a survival experiment, pods of susceptible soybeans (BSR 301) were artificially inoculated on 10 August 1981. The pods were inoculated by spraying with a suspension of Xcg-Ra (10^7 cfu/ml), by scratching the surface of the pods with a needle previously dipped into the suspension, or by injecting 0.1 ml of the suspension into the pod through the suture. Controls were treated with sterile distilled water in the same way. Each inoculation treatment and control treatment was performed on 100 pods. Seeds were hand-harvested in mid-October, and eight 10-seed samples were tested for the presence of Xcg-Ra as described. In order to determine if the pathogen was borne on the seed surface or internally, an additional four 10-seed samples were collected from treatments containing contaminated seeds. These samples were surface-sterilized in 70% ethanol for 5 min, then assayed for the presence of the pathogen. The treatments containing contaminated seeds were stored at room temperature, and another four 10-seed samples were assayed in May 1982. Finally, four 10-seed samples from each of these treatments were planted in the field in hill plots. Ten randomly selected leaflets from each treatment were sampled for epiphytic populations of Xcg-Ra 5 wk after planting.

Development and dispersal of epiphytic populations. Plots consisted of either resistant (Williams) or susceptible (cv. Oakland or BSR 301) soybeans planted in hills (10 seeds per hill) 0.76 m apart, with an inoculated hill of susceptible plants in the center of each plot to serve as the source of inoculum. Each plot was square and contained 121 hills. Plots were separated from each other by four rows of resistant (BSR 302) soybeans and were arranged in a randomized complete block design with two replications of each cultivar. Plots were planted on

Table 1. Establishment of a rifampin-resistant strain of *Xanthomonas campestris* pv. *glycines* (Xcg-Ra) on field-grown seedlings of susceptible soybean cultivar BSR 301 developing from artificially inoculated seeds

Plant part	Log cfu/plant part ^a			
	External inoculation ^b with:		Internal inoculation ^c with:	
	1×10^4 cfu/ml	1×10^7 cfu/ml	1×10^4 cfu/ml	1×10^7 cfu/ml
Hypocotyl	3.45	4.06	4.13	3.90
Cotyledon	2.76	3.32	2.72	3.02
Primary leaf	2.30	1.82	2.77	3.21
Apical bud	0.31	0.94	0.46	0.64

^a Epiphytic populations of Xcg-Ra were determined by leaf-washing, dilution-plating method using yeast extract-calcium carbonate agar amended with 50 μ g/ml of rifampin and 100 μ g/ml of cycloheximide. Plants were sampled when primary leaves had just become fully expanded. Values are means obtained from populations on three symptomless seedlings. Strain Xcg-Ra was not detected on any control plants.

^b Seeds were surface-sterilized in 70% ethanol for 3 min, immersed in aqueous suspension of Xcg-Ra for 1 min, air-dried at room temperature, and planted immediately. Controls were treated the same except for immersion in sterile distilled water instead of bacterial suspension.

^c Seeds were surface-sterilized in 70% ethanol for 3 min, immersed in aqueous suspension of Xcg-Ra and placed under partial vacuum for 1 min, removed, placed in 70% ethanol for 1 min, air-dried, and planted immediately.

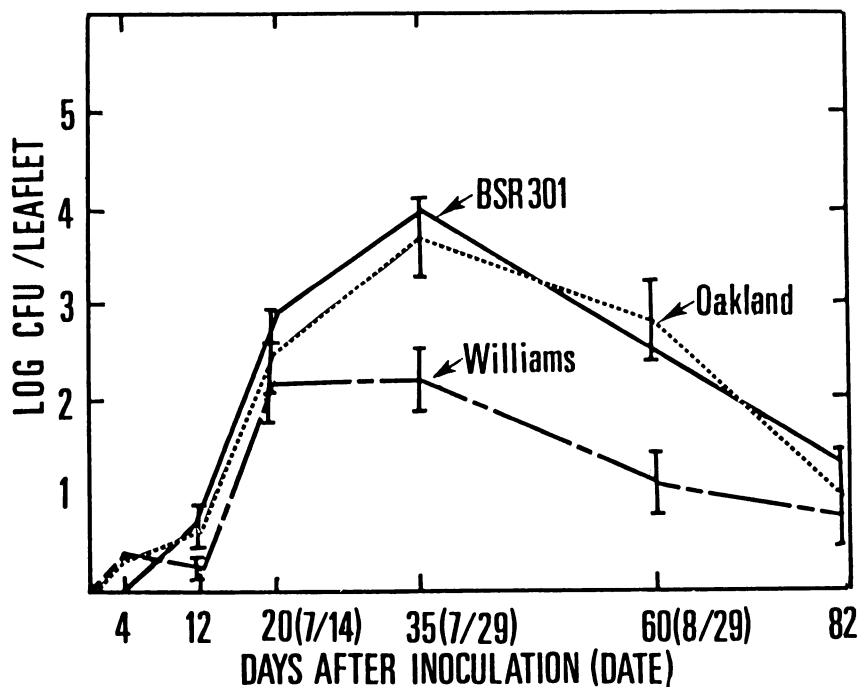


Fig. 2. Colonization of leaflets of resistant (Williams) and susceptible (BSR 301 and Oakland) cultivars of soybean by epiphytic populations of a rifampin-resistant strain of *Xanthomonas campestris* pv. *glycines*. All sampled leaflets were symptomless. Data points represent means of four leaflets from each of three hills in four directions from infection foci. Bars = standard error.

10 May 1982, and plants in the central hill in each plot were sprayed with a suspension of Xcg-Ra (10^7 cfu/ml) on 25 June. A portable settling tower was used to minimize spray drift to adjacent hills.

At 4, 12, 20, 35, 60, and 80 days after inoculation, four symptomless leaflets from each hill at various distances (1.07, 2.14, and 3.21 m) and directions (NW, NE, SE, and SW) from the inoculated hill were sampled to detect epiphytic populations of Xcg-Ra. These hills were located along the diagonal axes within the square plots. Leaflets were placed in separate bags, transported to the laboratory in a cooler, and processed individually on the same day by means of the leaf-washing method. Precautions were taken to avoid physically spreading the pathogen within these plots. Equipment was not taken into the plots, and every attempt was made to avoid touching the plants during sampling. A hygrothermograph and rain gauge were placed at the edge of the plot to record temperature, relative humidity, and rainfall.

RESULTS

Survival in leaf debris. Populations of *X. c. pv. glycines* strains Xcg-Ra and Xcg-8 survived equally well in surface and buried debris (Fig. 1). Pathogen populations gradually declined through the winter and into spring. In general, fewer bacteria were recovered from the buried samples, but only at the April sampling was there a significant difference ($P=0.05$) between populations in surface and buried tissue. One hundred single colonies from tissue samples collected in May were used to inoculate susceptible soybean plants (BSR 301); all caused typical pustule lesions.

Transmission of *X. c. pv. glycines* from seed onto plants. Epiphytic populations of strain Xcg-Ra developed on susceptible soybean seedlings from either external or internal seedborne inoculum (Table 1). Inoculum placement and inoculum concentration (10^4 vs. 10^7 cfu/ml) did not significantly (ANOVA, $P=0.05$) affect the magnitude of the epiphytic populations recovered 2 wk after planting. The pathogen was not present on the uninoculated control seedlings at this time. When the plants were sampled at the six-leaf stage, epiphytic pathogen populations were detected on trifoliolate leaves in the upper canopy in all inoculated treatments as well as on some of the control plants. In the subsequent test, epiphytic populations of strain Xcg-Ra became established on the primary leaves of resistant as well as susceptible plants from both internal and external seedborne inoculum. Once again, the pathogen was not found on control seedlings. Average populations ranged from 30 to 400 cfu per primary leaf. Later in the season, 90% of the pustules that developed on susceptible plants contained

Xcg-Ra. Because plants in this experiment were physically isolated from other plots in which the rifampin-resistant strain was used, it is evident that seedborne inoculum can result in infections occurring several weeks after planting.

Seed infestation. When plants heavily infected with strain Xcg-Ra were harvested with a combine (12% grain moisture), only 2–3% of the seeds harbored the bacterium, and populations were quite low (5–20 cfu per seed). When similar pods were hand-harvested and the seeds removed aseptically, the pathogen was not detected.

Strain Xcg-Ra was not detected on seeds from healthy plants processed through a clean single-plant thresher. However, two of four samples from heavily diseased plants (5% of leaf area symptomatic) contained Xcg-Ra, as did one of four samples from healthy plants threshed after the diseased plants.

When pods were inoculated, contaminated seeds were detected only in the wound-inoculation treatments. Seeds removed from pods inoculated by the scratch method contained 420 ± 183 cfu per seed (mean \pm standard error), whereas those from pods inoculated by injection contained 450 ± 313 cfu per seed. In both instances, the bacteria were not detected if the seeds were surface-sterilized. After the seed samples were stored for 7 mo, 166 ± 115 cfu per seed were still detectable. When these seeds were planted in the field, epiphytic populations of the pathogen were found on the seedlings after emergence.

Development and dispersal of *X. c. pv. glycines* populations. Epiphytic pathogen populations were dispersed at the same rate (0.1 m/day) in plots of resistant and susceptible soybean plants. There was also no significant difference in rate of dispersal with respect to direction in any of the plots. During the first 20 days after inoculation, the epiphytic populations of Xcg-Ra were comparable on the resistant (Williams) and susceptible (Oakland, BSR 301) plants (Fig. 2). At 35 days after inoculation, epiphytic populations of Xcg-Ra were 20- to 50-fold greater on susceptible plants than on resistant plants (Fig. 2). Populations of Xcg-Ra appeared to remain on the leaves until physiological maturity. On susceptible plants, the percentage of pustules containing Xcg-Ra decreased from an average of 64% on the hills nearest the infection foci to 32 and 4% of the pustules on the hills 2.1 and 3.2 m away, respectively. All resistant plants remained symptomless throughout the season, whereas 2–3% of the total leaf area of the susceptible cultivars was symptomatic. Temperature and rainfall data were recorded for the duration of this experiment (Fig. 3).

DISCUSSION

X. c. pv. glycines strains Xcg-8 and Xcg-Ra survived in exposed and buried plant debris through the winter in central Iowa. Lehman (8) and Graham (5) also found that *X. c. pv. glycines* could overwinter in diseased debris in studies carried out in North Carolina. In the tropics, however, plant debris is not

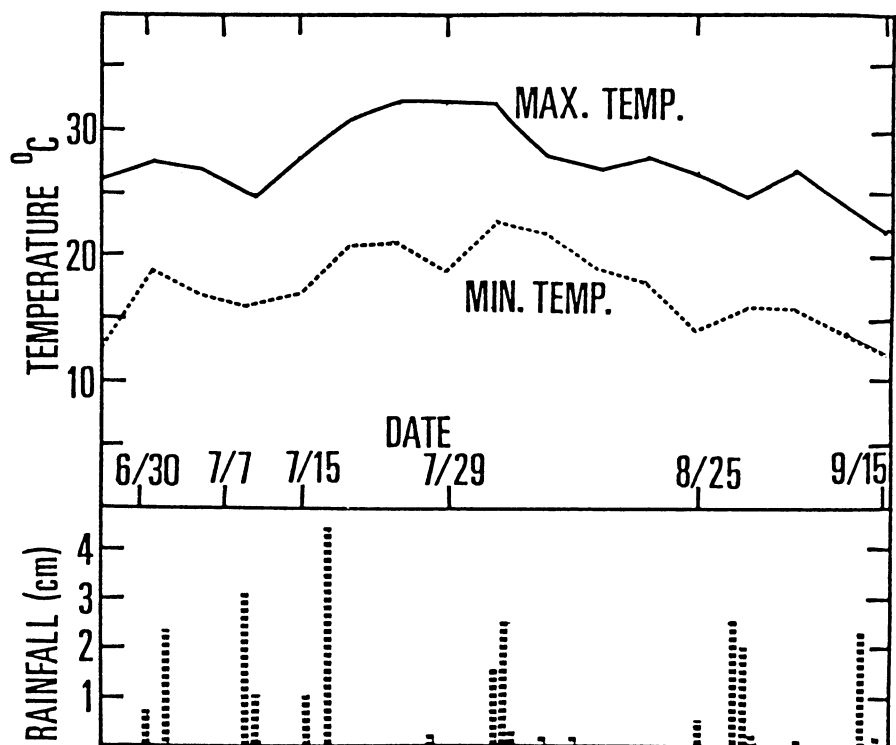


Fig. 3. Temperature and rainfall over duration of experiment to determine colonization of resistant and susceptible soybeans by epiphytic populations of *Xanthomonas campestris pv. glycines*.

considered an important survival site because of the rapid decay of infected leaves and competition from soil microbes (4). In the midwestern United States, microbial activity in the soil is low during the winter, and decay of plant debris is minimal. As a result, populations of *X. c. pv. glycines* decreased little during that period. During the spring, when microbial activity was greater, pathogen populations decreased at a greater rate. Large populations were still present in the debris, however, and these bacteria were highly virulent.

Epiphytic populations of *X. c. pv. glycines* became established on the foliage of soybean plants grown from both internally and externally inoculated seeds. These epiphytic populations spread upward in the canopy with time and eventually caused disease under favorable environmental conditions.

In our study, seeds did not seem to become naturally infected in the pod. Seeds did become externally contaminated during threshing of infected plants, however. Even though pathogen populations were quite small on these contaminated seeds, epiphytic populations did become established on seedlings when these seeds were planted in the field. Further studies will be needed to critically assess the relative importance of seed and debris as sources of inoculum of the bacterial pustule pathogen.

We were somewhat surprised to find that prevailing wind direction had no effect on the rate of dispersal of epiphytic populations of *X. c. pv. glycines*. Although wind direction was not directly monitored, the storms we were able to observe during this experiment had gusty winds that blew in several directions. Every attempt was made to avoid moving the bacteria from plant to plant during sampling procedures. A large proportion

of the pustules that developed on susceptible plants in this experiment did not contain strain Xcg-Ra. These plots were bordered on three sides by other soybean research plots containing a wide variety of genotypes. Bacterial pustule disease was common in other parts of the field, and these diseased plants were a likely source of natural inoculum for the plants in our plots.

In the field, populations of *X. c. pv. glycines* were 20- to 50-fold higher on the susceptible soybean cultivars Oakland and BSR 301 than those on the resistant cultivar Williams. Only symptomless leaflets were sampled in this study, and we feel that the bacteria recovered were primarily epiphytes. However, one cannot rule out the possibility that some of the bacteria were actually being exuded from presymptomatic infections. A similar relationship between host resistance and size of epiphytic populations has been noted with several plant-pathogenic pseudomonads and xanthomonads (1,7). Under natural conditions in the field, bacterial pustule lesions rarely appear on resistant plants. When artificially inoculated, however, *X. c. pv. glycines* grows equally well in leaves of resistant and susceptible plants, although a sixfold larger inoculum dose is needed to initiate infection in resistant plants (6). On the basis of these observations, we believe that the mechanism of bacterial pustule resistance involves the combined effects of smaller epiphytic pathogen populations (reduced inoculum potential) and greater inoculum concentrations necessary for infection (reduced infection efficiency). Crosse (1,2) has suggested that similar factors may be associated with the resistance of some cherry cultivars to *Pseudomonas mors-prunorum*.

Epiphytic populations may also play

an important role in the survival of *X. c. pv. glycines* during certain portions of the disease cycle. It has been well documented that this pathogen causes disease only under very warm conditions (8). In Iowa, the disease does not develop before the hottest days of midsummer. We have demonstrated that epiphytic bacteria can spread significantly throughout the growing season and that the pathogen can survive as an epiphyte from the time of seedling emergence until mid-July, when hot conditions allow disease to develop.

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