Multiplication of *Pseudomonas syringae* pv. *glycinea* on Soybean Primary Leaves Exposed to Aerosolized Inoculum

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ABSTRACT

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Aerosol generated from a suspension containing 2×10^8 colony-forming units (CFU) of *Pseudomonas syringae* pv. *glycinea* per milliliter was admitted into a stirred-settling chamber containing potted cultivar Acme soybean plants with unifoliolate leaves fully unfolded and one-half to two-thirds expanded. Epiphytic populations averaging 10^3 and 10^4 CFU · cm^{-2} developed in 9 and 14 days, respectively, on leaves that were wetted gently with atomized water before inoculation and kept at 20 C without further wetting. Infection did not take place on these leaves. Bacterial blight symptoms developed consistently on leaves that were either wounded and wetted or water-soaked and wetted immediately before inoculation. Interposition of up to 48 hr between water-soaking and inoculation did not prevent infection if leaves were wetted again immediately before exposure to aerosol. Bacteria did not become established either upon or within untreated leaves.

Additional key words: bacterial blight, *Glycine max.*

Bacterial blight disease of soybean (*Glycine max* L.), which is induced by *Pseudomonas syringae* pv. *glycinea* (Coerper) Young et al (6) (*P. syringae* van Hall, according to Buchanan and Gibbons [1]) (hereafter referred to as pv. *glycinea*), usually first becomes obvious in the field as water-soaked lesions on unifoliolate leaves of scattered seedling plants. With the advent of relatively cool, damp weather, the blight spreads and foci consisting of individual infected plants are no longer discernible in a general epidemic involving the entire canopy of leaves. At this stage, the presence of pv. *glycinea* in aerosols captured at the edge of infected plots during rainstorms and during sprinkler irrigation can be demonstrated (18). Further, infection of injured soybean leaves exposed to aerosols of pv. *glycinea* occurs in both laboratory and greenhouse experiments (17).

On soybean primary leaves gently wetted with a water suspension of the bacterium, pv. *glycinea* has been shown to be inherently capable of epiphytic multiplication (13). Therefore, it also would be significant to know whether the pathogen can establish epiphytic populations on leaves exposed to aerosolized inoculum. Experiments that address this question are described in this paper.

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MATERIALS AND METHODS

Plant material. Potted Acme soybean plants were grown from pv. *glycinea*-free seed (13) in pasteurized soil in a greenhouse at 18–26 C and 50–65% relative humidity under natural light supplemented with 12,000 lux from HLRG-400 W lamps (N. V. Philips, Eindhoven, The Netherlands) for 16 hr during the day. Plants were thinned to two or three in each pot and exposed to aerosolized inoculum of pv. *glycinea* when unifoliolate leaves were fully unfolded and one-half to two-thirds expanded. The leaves were subjected to different treatments before inoculation, and the resulting conditions were as follows: dry (untreated, no free water visible on either upper or lower leaf surfaces); wet (sterile tap water atomized over and around the plants with a Ceccato paint spray

gun fitted with a No. 1.5 nozzle [Ceccato and C., S.p.A., 36100 Alte Ceccato, VI, Italy] operated at 0.8 atm gauge pressure from a distance of 1 m until water droplets could be seen on both surfaces); water-soaked (sterile tap water atomized with the same gun at 1 atm from a distance of approximately 10 cm against the abaxial surface until the first signs of water congestion appeared); wounded (leaves inserted between two layers of coarse sandpaper and squeezed gently between thumb and forefinger). With the exceptions noted below under Results, experiments were quadruplicated and repeated at four different times of the year between July 1978 and March 1980.

Inoculation procedure. Soybean plants were inoculated by exposure to an aerosolized suspension of pv. *glycinea*. The 48-hr growth of a strain of pv. *glycinea* race 2 was washed from plates of medium 523 (12) and suspended in sterile distilled water at a concentration of 2×10^8 colony-forming units (CFU) per milliliter. To produce aerosol, 1 L of the suspension was introduced into a 3-L flask and sprayed against the inner surface of the flask wall by an atomizer (DeVilbiss Co., Somerset, PA 15501) delivering 0.38 ml · sec^{-1} of liquid under 1 atm gauge pressure. Inoculum escaping through the top of the flask was conveyed inside 4-mm (ID) glass tubing into the air portion of a trap consisting of 500 ml of water in a 2-L flask, and then into the bottom center of a 65 × 65 × 60 (height)-centimeter metal stirred-settling aerosol chamber constructed as described by Venette (17) in accordance with recommendations by Dimmick (5). The chamber was connected to an Andersen Viable Particle Sampler (Andersen 2000 Inc., Atlanta, GA 30320) through a three-way, quick-acting valve that allowed samples of air to be withdrawn from the chamber for short (5–15 sec) periods without significantly disturbing the flow of air through the chamber.

For use with plants, 18–24 pots were placed in the chamber under suspended paper cups that were then lowered onto the soil surface without touching the plants. Water was atomized inside the chamber until visible condensation appeared on the walls, at which time the chamber door was closed and the fan was turned on. Five minutes later, the cups were lifted and the aerosol was admitted into the chamber. Cups were left in place over control plants. The aerosol supply was discontinued after 60 min, the fan was left on for an additional 120 min, then the plants were removed and returned to the greenhouse. The procedure described above will be referred to as inoculation throughout the rest of this paper. Care was taken to avoid injury to leaves before, during, and after passage of plants through the chamber. No symptoms of infection or epiphytic populations of pv. *glycinea* were detected either on control plants that were included in each experiment or on plants that were subjected to one of the preinoculation treatments described above and exposed to aerosol generated from sterile water.

Temperature in the chamber was maintained at 24–26 C and ~95–96% relative humidity as long as plants were enclosed. Measurements with the Andersen sampler indicated that the average and range of recovery of pv. *glycinea* from the air inside the chamber were 520 (range, 481–565) CFU·L^{-1} at the time when aerosol supply was discontinued, and 2 (range, 0–3) and 0 CFU·L^{-1} 1 and 2 hr later, respectively. Aerosols were estimated to have mass median diameters of 1.2–2.6 μm, with particles having a negatively skewed distribution (5).

Assay of leaves for pv. *glycinea*. Plants were assayed immediately after inoculation and periodically up to 22 days afterward. Each sample consisted of six or nine leaves suspended in 30 or 40 ml, respectively, of sterile phosphate buffer (0.05 M, pH 6.5) and washed for 1 hr in 250-ml flasks on a reciprocal shaker at room temperature. Washed leaves were rinsed in sterile buffer, both upper and lower leaf surfaces were pressed against the agar surface of plates of SVCA (13) containing 50 μg ml^{-1} of cycloheximide, then each leaf was suspended in 30 ml of sterile buffer and ground at 16,000 rpm in an Omni Mixer (Du Pont-Sorvall Inc., Newtown, CT 06470) for 1 min. Separate counts of pv. *glycinea* were taken after 4–5 days of incubation at 26 C on plates in which leaf impressions had been made and on plates of the same medium inoculated with leaf wash water and leaf slurry. In some experiments, duplicate samples were prepared and separate counts of pv. *glycinea* were taken on plates inoculated with leaf wash water and with the slurry of unwashed leaves.

RESULTS

Calibration experiments. Several combinations of factors, including treatment of leaves as described above, length of exposure to aerosol, postaerosol incubation of plates at graded temperatures under supplemental light, and daily wetting of leaves after inoculation, were tested in order to provide a suitable working procedure for the experiments described below. Relevant findings from this work are summarized in the following paragraphs:

Atomization of water over and around plants daily after inoculation increased the number of saprophytic bacteria on the phylloplane, but had little, if any, effect on populations of pv. *glycinea*. Those that developed on leaves wetted by the same method and allowed to dry before inoculation did not differ appreciably from untreated leaves. Consequently, leaves were allowed to dry after inoculation.

Pathovar *glycinea* did not survive on the phylloplane when plants were incubated under supplemental light and the day temperature reached 28–30 C. Therefore, plants were incubated in a greenhouse section maintained at 16–18 C (night) and 20–22 C (day) without supplemental light after inoculation.

In a total of seven experiments, bacterial blight infection occurred consistently on leaves that were either water-soaked or wounded and wetted immediately before inoculation. Symptoms appeared more quickly and were more severe on plants kept at 25 instead of 20–22 C after inoculation. In some experiments, water-soaked plants were allowed to recover for different periods of time on the greenhouse bench before inoculation. Interposition of such a period of recovery of up to 48 hr between water-soaking and inoculation did not prevent infection if leaves were wetted as indicated above immediately before inoculation. Examination of leaves at different times after water-soaking showed that macroscopic signs of water congestion disappeared within 4 hr, but observation under a dissecting microscope revealed small, scattered areas of water-congested tissues up to 24–30 hr after water-soaking.

TABLE 1. Counts (colony-forming units per leaf) of *Pseudomonas syringae* pv. *glycinea* for soybean primary leaves subjected to different treatments before inoculation by exposure to bacterial aerosol

Pretreatment[a]	Days after inoculation				
	0	2	5	9	15
A: dry (untreated)	$1.2\times10 + 0$[b]	0 + 0	0 + 0	0 + 0	0 + 0
B: water-soaking	$2.0\times10^2 + 1.8\times10^3$	$2.8\times10^3 + 3.0\times10^5$	$3.7\times10^5 + 1.4\times10^7$	$2.6\times10^5 + 2.8\times10^6$	$7.1\times10^5 + 1.0\times10^7$
C: water-soaking, 4-hr recovery, and wetting	$3.3\times10^2 + 2.8\times10^3$	$2.3\times10^2 + 3.6\times10^3$	$2.9\times10^4 + 3.3\times10^4$	$3.5\times10^4 + 2.5\times10^5$	Nd[c]
D: water-soaking, 15-hr recovery, and wetting	$1.3\times10^2 + 2.8\times10^3$	$2.7\times10^2 + 5.8\times10^3$	$1.2\times10^4 + 9.7\times10^4$	$7.0\times10^4 + 1.0\times10^6$	Nd

[a] Operations in treatments C and D were performed sequentially without interruption.
[b] The frequency of isolation from the wash water of leaves and that from the slurry of washed leaves are given before and after the + sign, respectively.
[c] Nd = not determined.

Effect of preinoculation treatment of leaves on development of populations of pv. *glycinea*. Table 1 shows the results of a set of experiments in which the following leaf treatments were compared: (i) dry; (ii) water-soaking immediately before inoculation; (iii) water-soaking followed by a 4-hr period of recovery and wetting again immediately before inoculation; and (iv) as indicated under iii, except the period of recovery was extended to 15 hr.

Except for a limited number of colonies obtained by washing freshly inoculated leaves, pv. *glycinea* was never isolated from leaves subjected to the dry treatment. All other treatments resulted in higher counts of pv. *glycinea*. These counts were similar immediately after inoculation, but their rate of increase with time was greater for leaves inoculated immediately after water-soaking than for those allowed to recover after water-soaking before inoculation. A greater proportion of the total populations of pv. *glycinea* was invariably isolated by grinding than by washing leaves. In addition, pv. *glycinea* was detected only occasionally by agar impression of freshly exposed leaves, but many (often uncountable) colonies of the pathogen were obtained when leaves were examined by the same technique $\geqslant$2 days after inoculation. This suggests that at least a part of bacterial population of each leaf was developing in the mesophyll. The suggestion was confirmed by appearance of bacterial blight symptoms, beginning 9 and 5 days after exposure of leaves that were and were not allowed to recover after water-soaking, respectively.

In another set of experiments, treatments were as follows: dry; wet; water-soaking immediately before inoculation; water-soaking followed by 18-, 24-, 48-, or 72-hr periods of recovery before inoculation; or water-soaking followed by 24-, 48-, or 72-hr periods of recovery and by wetting again immediately before inoculation. In the experiments, leaves were assayed only by grinding without previous washing.

The frequency of isolation of pv. *glycinea* upon withdrawal of plants from the chamber immediately after inoculation (Table 2) was lowest from leaves subjected either to the dry treatment or to recovery for 48–72 hr after water-soaking without further wetting, and highest from leaves that either were not allowed to recover after water-soaking or were wetted again after recovering for any length of time. Only leaves that were visibly wet when inoculated yielded pv. *glycinea* 12 days after exposure. At this time, bacterial counts for leaves inoculated immediately after water-soaking were higher by a factor of $\sim 10^3$ than those for other leaves. Severe bacterial blight symptoms developed within 5–6 days on all leaves inoculated immediately after water-soaking. Occasionally, symptoms also developed after 9 or more days on leaves allowed to recover for 24–48 hr after water-soaking and wetted again before inoculation. However, no such leaves were among those assayed for pv. *glycinea* (Table 2).

TABLE 2. Counts (colony-forming units per leaf) of *Pseudomonas syringae* pv. *glycinea* for the slurry of soybean primary leaves subjected to different treatments before inoculation by exposure to bacterial aerosol

Pretreatment[a]	Days after inoculation	
	0	12
A: dry (untreated)	1.2×10	0
B: wetting	1.6×10^2	4.5×10^4
C: water-soaking	1.7×10^{10}	7.2×10^7
D: water-soaking and 18-hr recovery	1.3×10^2	0
E: water-soaking and 24-hr recovery	1.5×10^2	0
F: water-soaking and 48-hr recovery	4.4×10	0
G: water-soaking and 72-hr recovery	1.5×10	0
H: water-soaking, 24-hr recovery, and wetting	1.6×10^3	5.4×10^4
I: water-soaking, 48-hr recovery, and wetting	1.4×10^3	5.2×10^4
J: water-soaking, 72-hr recovery, and wetting	9.8×10^2	8.1×10^3

[a]Operations in treatments D–J were performed sequentially without interruption.

Because more bacteria invariably were isolated immediately after inoculation of wetted leaves than comparable dry leaves, attempts were made to determine whether differences were due to a matter of physical deposition of aerosol particles on the phylloplane and/or their retention there, or, perhaps, to a biological event or sampling phenomenon. In two experiments, leaves were first subjected either to the dry or to the wet treatment, then exposed to an aerosolized 3.0% aqueous solution of sodium fluorescein as described before for bacterial suspensions, and finally washed to remove the dye completely upon removal of the plants from the aerosol chamber. Examination of the wash water at 475 nm in a spectrophotometer showed that the quantity of dye on leaves exposed to aerosol before and after wetting was significantly different and amounted to 22.9 and 35.7 μg of sodium florescein per leaf, respectively. This corresponds to deposition of approximately 0.73 and 1.19 μl of aerosolized solution per leaf, respectively. Comparison of these figures with those from experiments with bacterial aerosol indicates that differences in the numbers of bacteria isolated immediately after inoculation of dry and wet leaves were not due to a purely physical phenomenon.

Attempts also were made to evaluate the relative effect of water-soaking, with or without recovery and further wetting before inoculation, on the frequency of isolation of pv. *glycinea* from freshly inoculated leaves. A 0.5% aqueous solution of Congo red was atomized, as described above, over and around leaves previously subjected to one of the following treatments: dry; water-soaked; or water-soaking followed by 5-, 24-, 48-, or 72-hr periods of recovery. Three hours later, the leaves were rinsed quickly in water and observed macroscopically and by leaf sectioning. Large portions of leaves exposed to the dye immediately after water-soaking were stained (Fig. 1). Comparatively smaller areas of leaves allowed to recover 5, 24, or 48 hr after water-soaking also were stained. No staining was usually observed either macroscopically or microscopically inside leaves that were not water-soaked or were allowed to recover for 72 hr after water-soaking before exposure to the dye. Leaf sections showed that the stain diffused through intercellular spaces and accumulated against cell walls in the mesophyll of stained leaves. This indicates that wetting leaves up to at least 48 hr after water-soaking results in the formation of a film of water extending from the leaf surface into the mesophyll, and that the magnitude of this effect decreases with increasing time after water-soaking. The results further suggest that spread of bacteria from the phylloplane into the mesophyll also may be enhanced by the presence of a film of water. However, direct evidence of motility

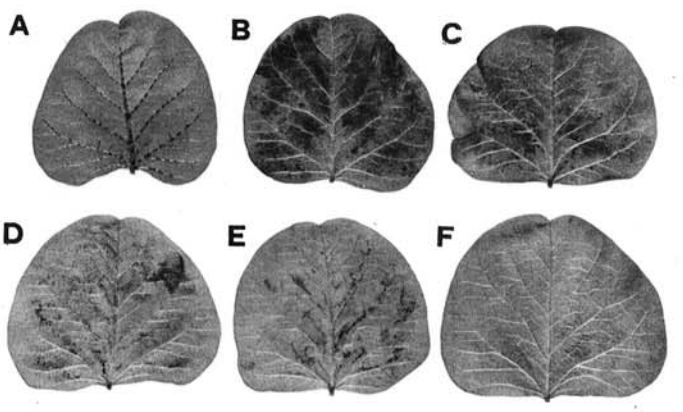

Fig. 1. Retention of 5% aqueous Congo red solution atomized onto soybean primary leaves previously subjected to one of the following treatments: **A,** dry (untreated); **B,** water-soaking; and **C–F,** water-soaking followed by 5-, 24-, 48-, and 72-hr periods of recovery, respectively. All leaves, except A, were quickly rinsed in water before the photograph was taken. The dye was retained on the phylloplane of leaf A and in the mesophyll of leaves B–E.

of bacterial inoculum on the phylloplane was not obtained.

Evidence of multiplication of pv. *glycinea* on the phylloplane of plants exposed to aerosolized inoculum. In these experiments, leaves subjected only to the wetting treatment were inoculated, and the subsequent development of populations of pv. *glycinea* was monitored by grinding duplicate samples of leaves with and without previous washing. When populations of less than 50 CFU of pv. *glycinea* were detected upon removal of plants from the chamber, counts were taken again 1 and 2 days later on the entire quantity of slurry obtained from the leaf samples.

Similar counts of pv. *glycinea* were invariably obtained either by washing or by grinding previously unwashed leaves. Furthermore, few (<2), if any, colonies of pv. *glycinea* were isolated by agar impression of washed leaves. For reasons given elsewhere (13), the combined evidence from these observations was taken to indicate that pv. *glycinea* was multiplying only on the phylloplane. Bacterial blight symptoms were never observed on inoculated plants.

The development of epiphytic populations of pv. *glycinea* arising from aerosolized inoculum is shown in Fig. 2. The number of bacteria declined at first, but returned to approximately the initial values by the third to fourth day after inoculation and continued to increase for the duration of the experiments. The specific growth rate of the bacteria was greatest between the second and the ninth day after inoculation and decreased later. The relative range of variation of colony counts among replicated experiments decreased from its maximum immediately after inoculation to a minimum 2 days later and then increased. This trend was confirmed when occasional counts were taken on the slurry of single leaves by using techniques described elsewhere (13). In some experiments (not included in Fig. 2) in which <50 CFU of pv. *glycinea* per leaf were counted immediately after inoculation, bacterial populations became extinguished within 1 day.

DISCUSSION

Airborne particles containing viable bacteria have been indicated as a possible form of effective inoculum for infection of diverse agricultural plants; eg, pome fruit trees by *Erwinia amylovora* (16), potato by *E. carotovora* (8,14,15), and soybean by *P. syringae,* pv. *glycinea* (17). The results of the present work suggest that the physical condition of the leaf tissue is important in determining whether the immediate effect of exposure of soybean primary leaves to aerosolized inoculum of pv. *glycinea* includes the formation of bacterial blight lesions, or is restricted to the establishment of epiphytic bacterial populations. This appears to be the first record of initiation of ephiphytic colonization of leaves by a bacterial plant pathogen from an aerosol source.

The effect of preinoculation treatment of leaves on delivery of inoculum and subsequent development of bacterial populations was striking, but not readily explainable. While leaves exposed only to the wetting treatment retained the same quantity of viable inoculum as leaves allowed to recover for 24 hr without further wetting after water-soaking, bacterial populations subsequently declined and failed to become established on the latter as well as on untreated leaves.

Methods used in this work were not designed primarily to discriminate between the relative importance of individual factors in determining the number of bacteria present on the phylloplane upon withdrawal of plants from the aerosol chamber. However, experiments with sodium fluorescein indicated that survival of bacteria after deposition was not the only limiting factor. Because the water-soaking procedure used in this work required force to drive water into the mesophyll, mechanical damage to delicate leaf structures (9,10) may have modified leaf characteristics that affect the frequency of impact of aerosol particles onto the phylloplane and their retention there. These characteristics include the electric field around leaves and heat and mass transfer by the movement of air across dry and wet leaf structures (4,7). That substantial differences exist between dry untreated leaves and apparently dry leaves that were water-soaked up to 48 hr earlier also was indicated by the results of experiments with Congo red. Mechanical damage is an important factor in increasing the wettability of plant surfaces (11). Therefore, the interval during which the mesophyll could be penetrated by the staining solution atomized onto the phylloplane might be a measure of the time necessary for leaves to recover from mechanical damage associated with water-soaking and the reestablishment of a high contact angle between water droplets and the phylloplane. Spread of bacteria through films of water thus formed may explain, in part at least, the observed occurrence of infection in leaves that were allowed to recover for up to 48 hr after water-soaking and were wetted again immediately before inoculation. Water congestion by forceful spray of water against leaves has long been associated with increased rate of invasion of leaf tissues by bacteria (2,3). However, the residual effect of the treatment recorded in the present work does not appear to have been studied in detail before.

Because leaves water-soaked or wetted with water immediately before inoculation were still wet when plants were withdrawn from the aerosol chamber, bacterial inoculum may have moved during this period to a location different from that where it first came into contact with the phylloplane. The consistent decrease in the range of variation of pv. *glycinea* counts from immediately after inoculation to 1–2 days later suggests that a limited number of suitable sites is available in which the bacteria can survive on the phylloplane. If this is true, transition of bacteria from a random distribution to one coincident, at least in part, with that of sites compatible with bacterial survival and subsequent multiplication, may help to explain the establishment of aerosolized inoculum of pv. *glycinea* on wet, but not on dry leaves.

The relative efficiency of wetting uninjured leaves with inoculum compared to aerosolized inoculum of pv. *glycinea* in generating epiphytic populations and causing infection on soybean primary leaves is difficult to determine because pertinent data from this and a previous study (13) were obtained under different conditions of incubation of the plants after inoculation. Available evidence indicates that survival of approximately 50 CFU of pv. *glycinea* on the phylloplane at the end of exposure of soybean primary leaves to

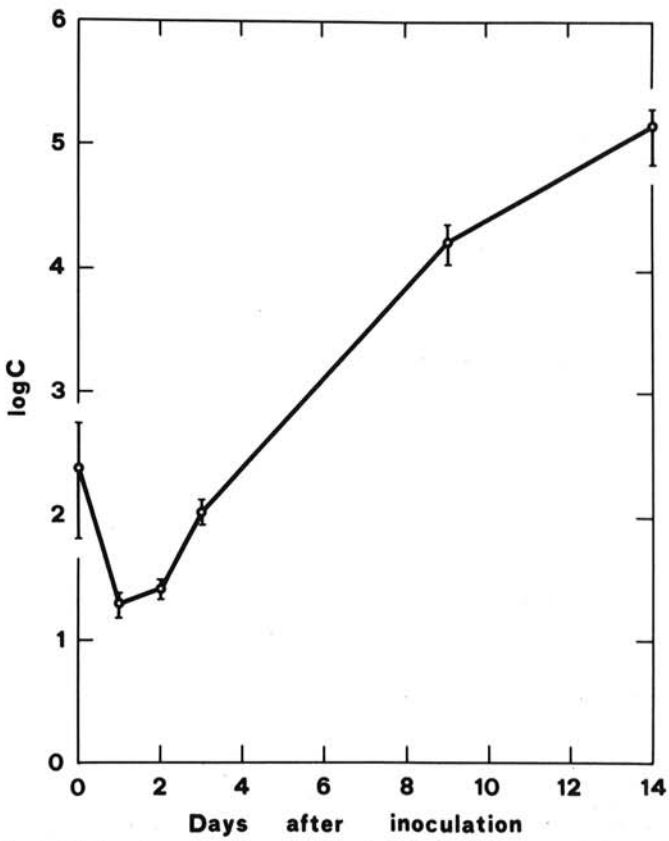

Fig. 2. Arithmetic mean and range of variation of epiphytic populations of *Pseudomonas syringae* pv. *glycinea* (C = colony-forming units per leaf) on soybean primary leaves inoculated by exposure to a bacterial aerosol. Leaves were wetted gently with atomized water immediately before inoculation.

an aerosol source is sufficient to generate dense epiphytic populations averaging 10^3 and 10^4 CFU · cm^{-2} after 9 and 14 days of incubation at 20 C, respectively.

Colonization of the phylloplane of soybean primary leaves of pv. *glycinea* at relatively low temperatures may have significant epidemiological implications in view of the possible establishment of extensive epiphytic populations of the pathogen in the field early during the cooler part of the soybean growing season. It also is tempting to speculate that other events similar to those we observed may provide an additional clue to the epidemic nature of bacterial blight of soybean, but their occurrence in nature has not been investigated. This applies especially to the finding that daily wetting of leaves previously exposed to aerosol failed to result in an increased epiphytic population—which might imply minimal effects of dew on leaf surface populations in the field. However, the demonstration that pv. *glycinea* in aerosols can initiate an epiphytic phase of growth suggests that becoming aerosolized may add significantly to the overall epidemic potential of the pathogen on its natural host.

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