

Soybean Primary Leaves as a Site for Epiphytic Multiplication of *Pseudomonas glycinea*

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ABSTRACT

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A suspension containing 10^3 or 10^4 colony-forming units of either a motile or a nonmotile strain of *Pseudomonas glycinea* per milliliter was sprayed on the abaxial surface of soybean primary leaves. The distribution of inoculum between the phylloplane and the mesophyll ("epiphyllous" and "endophyllous" inoculum, respectively) was determined immediately after inoculation. Colony counts up to 28 days

after inoculation showed that bacterial populations derived from the total dose of inoculum were significantly greater than those developing from the endophyllous portion of inoculum only. Symptomless leaves and leaves bearing up to four bacterial blight lesions supported significantly higher populations arising from epiphyllous inoculum than did leaves with five such lesions.

Additional key words: bacterial blight, motility, ultraviolet irradiation.

Bacterial blight, which is incited by *Pseudomonas glycinea* Coerper, affects soybean [*Glycine max* (L.) Merr.] worldwide and is especially prevalent in the upper United States. The bacterium is commonly seed-borne (12), but under some conditions may overwinter in association with diseased plant material in the field (3, 9, 13). During the growing season, disease symptoms may be present on stems, petioles, and pods, but are particularly conspicuous on leaves where they consist of small angular lesions, usually water-soaked at the center and surrounded by a yellow-green halo (14).

In Minnesota, spots usually develop at first on primary leaves of isolated seedlings and, with the onset of relatively cool, damp weather, become prevalent suddenly on the trifoliolate leaves of most plants. This epidemiological pattern suggests that the bacteria may multiply on the surface of primary leaves before epidemic outbreaks. The present investigation was undertaken to determine whether soybean primary leaves are inherently capable of supporting epiphytic multiplication of *P. glycinea*. A special effort was made to trace the origin of epiphytic populations of *P. glycinea* to initial cells on the phylloplane and to exclude interference from latent infections.

MATERIALS AND METHODS

Media.—In addition to conventional media indicated below, the following medium (SVCA) was used: nutrient broth 8.0 g; sucrose 50.0 g; agar 16.0 g; crystal violet 0.4 ml of a 1.0% aqueous solution; distilled water 990 ml; cycloheximide 10.0 ml of a 1.0% aqueous solution, added

aseptically after sterilization of other ingredients at 121 C for 15 min. On this medium, *P. glycinea* forms domed mucoid colonies differing only in having a darker coloration than those formed on 5% sucrose nutrient agar (21).

Bacteria.—These were a newly isolated race 2 (1) strain of *P. glycinea*, designated R2, and a nonmotile mutant of R2, designated M7, produced with 250 $\mu\text{g/ml}$ N-methyl-N'-nitro-N-nitrosoguanidine as a mutagen (22). Cultures of both isolates were maintained on nutrient agar + 2.0% glycerol (NAG) slopes at 4 C. Cultures of M7 were purified periodically to prevent accumulation of motile revertants (22). The log median effective dose ($\log \text{ED}_{50}$) and the slope (b) of the log dose - probit response line at the ED_{50} point (4) did not differ significantly ($P = 0.05$) with the two strains. The two parameters were estimated at $\log \text{ED}_{50} = 2.2297 \pm 0.300$ and $b = 1.726 \pm 0.089$ for R2, and $\log \text{ED}_{50} = 2.2715 \pm 0.302$ and $b = 1.745 \pm 0.082$ for M7.

Plant material.—Acme soybean plants were raised individually in pots of pasteurized garden soil under natural light supplemented with 20,000 lux provided by fluorescent lamps in a greenhouse at 21-26 C and 45-60% relative humidity. Seed tested by plating on SVCA was never found to harbor *P. glycinea*. Neither visible nor latent infections of bacterial blight were ever detected in plants grown from this seed.

Preparation of inocula.—The bacteria were subcultured twice on NAG slants at 25 C for 24 hr, collected from the second slant in sterile distilled water (SDW), adjusted turbidimetrically to a concentration of 10^8 colony-forming units (CFU)/ml, diluted to the required concentration, and used immediately. When needed, bacterial cells were washed by centrifugation and resuspension twice in SDW before adjusting the

concentration of the stock suspension to 10^8 CFU/ml. The titer of all suspensions was checked by plate counts and found not to differ from the expected concentration by more than 10%.

Inoculation techniques.—Depending on the purpose of the experiments, the inoculum was administered to both primary leaves of 10- to 12-day-old plants either by spraying or by infiltration. In both cases, a Paasche Model H airbrush (Paasche Airbrush Co., Chicago, IL 60614) fitted with a No. 3 aircap and liquid adjuster was used. Spraying was carried out by delivering the bacterial suspension onto the abaxial surface from a distance of approximately 20 cm under a pressure of 0.6 atm, until just before run-off. Infiltration was achieved by enclosing the plants in plastic bags, uncovering them after 3 hr, and applying inoculum under a pressure of 1.5 atm to the abaxial surface from a distance of approximately 6 cm. Infiltrated leaves became uniformly water-soaked, but resumed their normal appearance within 3–4 hr. The quantity of inoculum deposited by spraying on the leaf surface and introduced by infiltration into the mesophyll was approximately 8.0 and 4.0 μ liters/cm², respectively. Inoculated and control plants treated with SDW were kept under observation for 4 wk under the same greenhouse conditions described above.

Ultraviolet (UV) irradiation.—In some of the experiments described below, in which it was desirable to kill the cells of *P. glycinea* deposited on the leaf surface after inoculation, each leaf surface was exposed to 0.17 W/cm² incident UV energy at 253.7 nm from a Strato-Ray SP91-L30 unit (Strato-Ray Co., Minneapolis, MN 55426) in the dark as soon as the inoculum dried. After irradiation, the plants were kept in the dark at 25 C for 1 hr to minimize UV damage repair by the bacteria. Before this procedure was used routinely, its effects on the bacteria and on the plant were calibrated by tests, as summarized under Results.

Isolation procedures.—Inoculated and control leaves were screened for *P. glycinea* by one or more of three different techniques, indicated as “printing”, “washing”, and “grinding”, respectively. Printing was performed by taking first an enlarged (23) and then a direct (16) print of the same leaf on SVCA, and by recording the presence or absence of *P. glycinea* after 72 hr at 25 C. Washing was carried out by shaking 10 leaves in 100 ml of SDW in 250-ml flasks at room temperature in the dark for 2 hr, plating 1 ml of the wash water and appropriate dilutions of it with 14 ml of molten (48 C) SVCA, and counting *P. glycinea* colonies after 72 hr at 25 C. Grinding consisted in comminuting single leaves in 2.0 ml of sterile 0.05 M potassium phosphate buffer (pH 6.5) and assaying the homogenate for *P. glycinea* as above. Since the weight-to-surface-area ratio of leaves changed with age, bacterial counts were expressed as CFU/unit leaf area, the latter being defined as the total (adaxial + abaxial) surface of a square portion of leaf of 1 cm on a side.

Identity of *P. glycinea* in the colonies was verified by purifying random colonies, characterizing them according to the LOPAT scheme (18, 21), and testing them for virulence by spraying suspensions of 10^5 CFU/ml on young Acme trifoliolate leaves in the greenhouse. Controls (2) indicated that no appreciable errors due to bacterial multiplication in the wash water were made when the washing technique was used.

Distribution of inoculum.—The distribution of inoculum between the phylloplane and the mesophyll (“epiphyllous” and “endophyllous” inoculum, respectively) was determined after spraying a suspension containing 10^3 , 10^4 , or 10^5 CFU of either R2 or M7/ml on the leaves of 25 plants. As soon as the inoculum dried on the leaf surface, the leaves were examined for *P. glycinea* by one of the following techniques: (i) grinding (this was intended to determine the total dose of inoculum); (ii) irradiation followed by grinding (this was intended to determine the proportion of endophyllous inoculum); (iii) washing first, and then grinding after rinsing in three changes of SDW. [The first step was intended to measure the combined epiphyllous inoculum thus recoverable and the endophyllous inoculum that leaked out of the mesophyll. The second step was intended to estimate, by comparison with data from (ii), epiphyllous inoculum not recoverable by washing]; (iv) irradiation followed by the same steps as under (iii). [Comparison of these data with those from (ii) and (iii) was intended to give an estimate of epiphyllous inoculum contributing to colony counts obtained by the first step under (iii)].

The experiments were carried out four times. The results did not differ significantly and were combined.

Epiphytic multiplication of *Pseudomonas glycinea* on lightly infected leaves.—This was monitored following spray inoculation of plants with suspensions containing 10^3 or 10^4 CFU of either R2 or M7/ml. For each of the four strain-concentration combinations, three groups of 50 plants each were used. Group No. 1 received no further treatment after inoculation, group No. 2 was inoculated and irradiated, and group No. 3 was processed in a manner similar to group No. 2, except that inoculation was delayed until 2 hr after the end of the dark treatment.

As soon as the epiphyllous inoculum dried, or (within group No. 2) at the end of the dark treatment, the leaves from five plants for each treatment were ground to determine the total dose of inoculum (groups No. 1 and No. 3) and the endophyllous proportion of it (group No. 2) in each leaf. Further groups of 10 leaves for each treatment were examined after 1, 2, 4, 6, 8, 11, 14, 21, and 28 days with the same techniques.

Within each set of counts taken on any group of 10 leaves, interference from abnormally high values associated with latent infections was excluded by disregarding all such counts, the omission of which significantly diminished the coefficient of variation (CV) of the set (25).

Experiments with each of the four strain-concentration combinations were conducted once in summer and once in winter. The results did not differ significantly and were combined. Since the results obtained with group No. 3 never differed significantly from those with group No. 1, the former were omitted.

Epiphytic multiplication of *Pseudomonas glycinea* on heavily infected leaves.—Strain R2 was infiltrated into the leaves of three groups of 900, 200, and 600 plants at a concentration of 3.3×10^3 , 5.5×10^3 , and 15.6×10^3 CFU/ml, corresponding to a dosage of 1ED₇₀, 1ED₈₅, and 1ED₉₉, respectively. Two hr after the leaves had resumed their normal appearance, they were sprayed with a suspension containing 10^4 CFU of M7/ml. The distribution of epiphyllous and endophyllous inoculum of M7 in these leaves did not differ significantly from that

in leaves not subjected to infiltration previously.

On the 14th day after inoculation, populations of R2 and M7 were measured in six random groups of 10 leaves showing none, one, two, three, four, and five bacterial blight lesions each within each group. Each leaf was examined separately by removing a 4-mm diameter disk of tissue around each lesion, grinding the remaining tissue, and screening the resulting colonies for motility (22).

The rate of appearance of the motile phenotype within populations developing from M7 inocula in vivo in 14 days was estimated at $\leq 10^{-5}$ /generation by pilot assays done under the conditions described previously. Assays also indicated that populations of M7 arising from inoculum administered to leaves that had been washed (5) after inoculation with R2 were not significantly different in size from those recorded with unwashed leaves. All experiments were conducted twice in the spring. The results did not differ significantly and were combined.

RESULTS

Calibration of ultraviolet (UV) irradiation.—Tests made to calibrate the effects of the UV treatment on the

bacteria and on the plant indicated that:

(i) the rate at which R2 and M7 survived irradiation did not differ significantly;

(ii) with one exception (this being the isolation of one colony of R2 from a leaf sprayed with the highest concentration of inoculum), *P. glycinea* was never recovered by the printing technique from any of more than 1,500 leaves that, at widely different times, were irradiated after spray inoculation with suspensions containing between 10^3 and 10^5 CFU of either R2 or M7/ml;

(iii) *P. glycinea* was never detected by spraying several groups of 40 plants with suspensions containing 10^4 CFU of either R2 or M7/ml, irradiating them and washing the leaves of 10 plants in each group 0, 24, 48, or 72 hr after the end of the dark treatment. When comparable groups of leaves were irradiated and examined as indicated above after infiltration with similar bacterial suspensions, the recovery of R2 and M7 2 hr after the end of the dark treatment ranged from 2.9 to 5.9 and from 3.6 to 7.4 CFU/unit leaf area, respectively;

(iv) a substantial proportion of leaves irradiated 30 min or longer after drying of inocula containing between 10^3

TABLE 1. Mean and range of recovery of *Pseudomonas glycinea* from soybean primary leaves inoculated by spraying with suspensions containing 10^3 , 10^4 , and 10^5 colony-forming units (CFU) of either a motile (R2) or a nonmotile strain of the bacterium per milliliter

Isolate	Concentration of inoculum (bacteria/ml)	Treatment ^a	Recovery (CFU/unit leaf area) from:			
			Wash water		Ground tissue	
			Mean ^b	Range ^c	Mean ^b	Range ^c
R2	10^3	(i)			5.9	4.4-7.1
		(ii)			0.029	0.020-0.038
		(iii)	5.9	4.9-7.3	0.033	0.024-0.046
		(iv)	0	...	0.028	0.023-0.033
	10^4	(i)			52	43-62
		(ii)			0.28	0.20-0.36
		(iii)	63	58-73	0.26	0.23-0.31
		(iv)	0	...	0.27	0.13-0.34
	10^5	(i)			680	510-860
		(ii)			3.8	2.3-5.1
		(iii)	610	490-710	2.7	1.0-4.4
		(iv)	0.21	0-0.84	2.6	1.3-4.1
M7	10^3	(i)			5.8	4.0-6.7
		(ii)			0	...
		(iii)	5.9	4.9-7.5	0	...
		(iv)	0	...	0	...
	10^4	(i)			58	46-73
		(ii)			0.021	0-0.084
		(iii)	54	48-64	0.034	0-0.070
		(iv)	0	...	0.012	0-0.048
	10^5	(i)			630	530-750
		(ii)			0.28	0.17-0.39
		(iii)	570	490-640	0.23	0.12-0.35
		(iv)	0	...	0.21	0.18-0.28

^aThe leaves were subjected to one of the following treatments as soon as the inoculum dried on the phylloplane: (i) grinding; (ii) ultraviolet irradiation followed by grinding; (iii) washing followed by grinding; (iv) irradiation followed by washing and grinding.

^bArithmetic mean of values recorded in two experiments.

^cRange of arithmetic means of values recorded in each of two experiments.

and 10^5 CFU/ml, yielded *P. glycinea* when examined as indicated under (iii);

(v) neither visible tissue damage nor changes in the number of epidermal and mesophyll cells permeable to Evans blue (7) were detected in any of more than 300 leaves examined up to 30 days after irradiation;

(vi) measurements of leaf resistance with a Model LI-60 diffusion resistance meter (Lambda Instruments Co., Inc., Lincoln, NE 68504) did not differ significantly on plants subjected to irradiation 2 hr earlier and on control plants;

(vii) reducing the amount of incident energy below standard resulted in recovery of *P. glycinea* from leaves inoculated and examined as indicated under (iii), whereas increasing it above standard caused visible bronzing and chlorotic vein banding of primary and oldest trifoliate leaves;

(viii) the growth pattern (5) of R2 and M7 *in vivo* over a period of 2 wk after inoculation was not affected by irradiating the leaves immediately before infiltration of suspensions containing 10^2 , 10^3 , and 10^4 CFU/ml of

either isolate; infectivity parameters of both isolates for irradiated leaves also were not significantly different from those for nontreated leaves.

Calibration of inoculum distribution.—Grinding, and irradiation followed by grinding provided a correct estimate of the total dose of inoculum and of epiphyllous inoculum, respectively (Table 1).

Assays indicated that estimates of endophyllous inoculum thus obtained could be combined with the results of infectivity titration of R2 and M7 to determine the percentage of leaves expected to show bacterial blight symptoms by the 20th day after spray inoculation. Expected values were approximately 0% after spraying with 10^3 CFU of either isolate/ml or with 10^4 CFU of M7/ml, and 5% after spraying with 10^4 CFU of R2/ml.

Epiphytic multiplication of *Pseudomonas glycinea* on lightly infected leaves.—Significantly lower counts were always obtained with irradiated than with nonirradiated leaves (Fig. 1).

Colony counts remained approximately constant for the duration of experiments with irradiated leaves, but

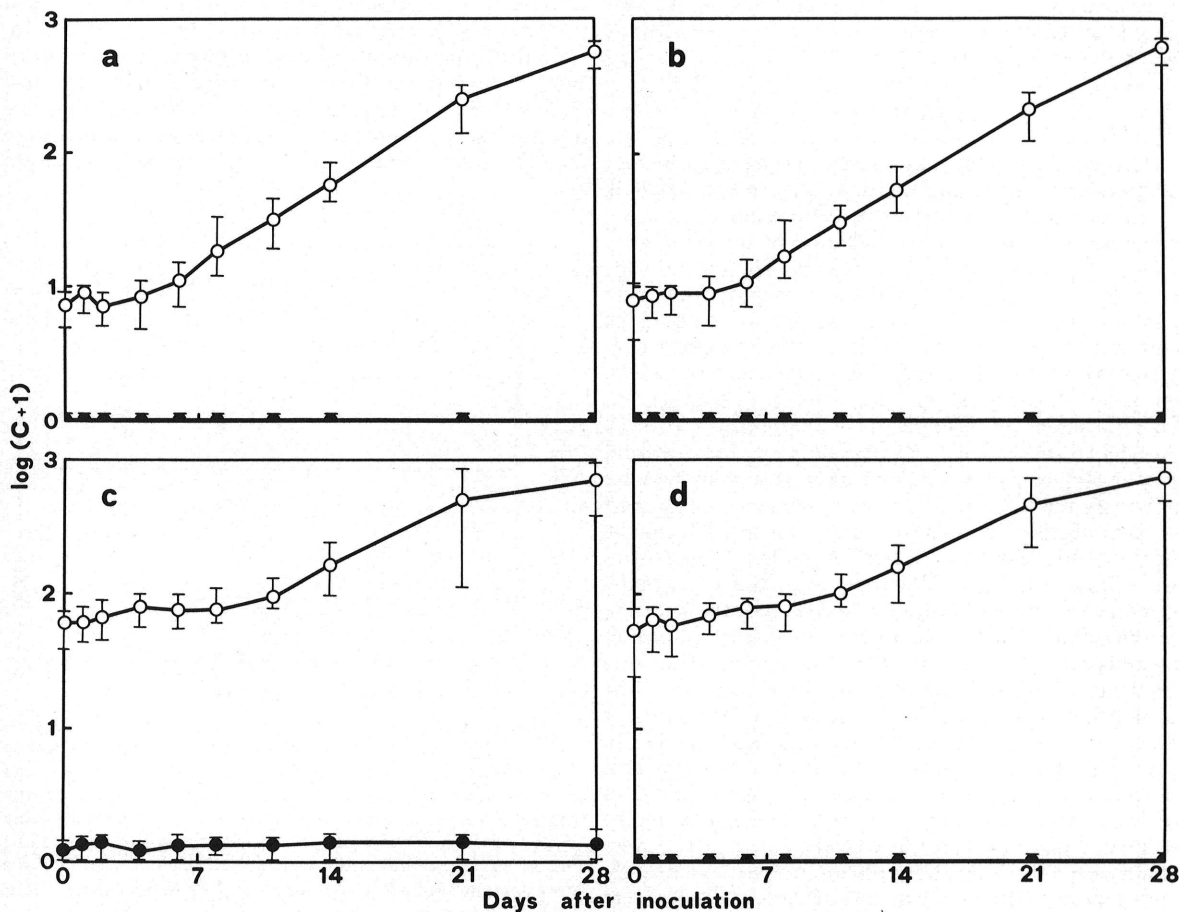


Fig. 1—(a to d). Arithmetic mean and range of recovery [C = colony-forming units (CFU)/unit leaf area] of *Pseudomonas glycinea* from the ground tissue of soybean primary leaves. The leaves were left nontreated (open circles) or subjected to ultraviolet irradiation (closed circles) immediately after spray inoculation. The inocula contained 10^3 (a and b) or 10^4 (c and d) CFU of either a motile (a and c) or a nonmotile (b and d) strain of the bacterium/ml. The lines connecting open and closed circles in each panel show variations in total and endophyllous populations, respectively.

increased significantly with time after inoculation when the leaves were not irradiated (Fig. 1). The ratio between colony counts on nonirradiated leaves on two consecutive samplings tended to increase during the first 3 wk after inoculation and decreased during the fourth. The overall ratio between counts on the 28th day and those taken on the day of inoculation was approximately 10 for leaves inoculated with 10^4 CFU/ml and 100 for those inoculated with 10^3 CFU/ml.

Colony counts on the 1st, 7th, 14th, and 28th day after inoculation indicated that inoculation with washed bacteria reduced the occurrence of abnormally high counts associated with latent infections and lowered the recovery of R2 from leaves irradiated after inoculation with 10^3 CFU/ml.

Epiphytic multiplication of *Pseudomonas glycinea* on heavily infected leaves.—Colony counts of M7 on leaves showing five bacterial blight lesions were significantly lower than those on other leaves (Fig. 2). No significant differences were detected between symptomless leaves and leaves showing up to four lesions each.

The concentration of inoculum of R2 had no significant effect on the magnitude of populations of M7 measured within each disease severity class. Colony counts of R2 (not reported here) were never significantly greater than those of M7.

DISCUSSION

The results suggest that soybean primary leaves may support epiphytic multiplication of *P. glycinea*. The bulk of information leading to this conclusion came from experiments in which UV irradiation of soybean leaves soon after spray inoculation reduced significantly the number of *P. glycinea* colonies recovered from these leaves during the next 4 wk. As far as it could be determined, the protocol for irradiation suppressed *P. glycinea* on the phylloplane without damaging the leaf or inducing formation of appreciable quantities of bacteriostatic or bactericidal compounds (10, 11). Accidental survival of *P. glycinea* on irradiated leaves did not invalidate the technique used in this study because survivors and their progeny, if any, were recorded as part of endophyllous populations and subtracted from the total number of bacteria/leaf when epiphytic populations were estimated. Bacteriological examination of leaves by grinding rather than washing also enhanced the precision of the estimates by eliminating errors due to leakage of bacteria out of the mesophyll and to retention of bacteria on the phylloplane. In addition, advance estimates of the proportion of diseased leaves allowed high counts of bacteria in these leaves to be identified readily. In principle, it could not be excluded that endophyllous inoculum multiplied to some extent in the mesophyll of all leaves. This possibility, however, was ruled out by the combined findings that (i) bacterial recovery from leaves irradiated after inoculation did not increase significantly with time, and (ii) colony counts of the same order were obtained with nonirradiated leaves and with leaves irradiated before inoculation.

Use of the nonmotile strain proved helpful not only to obtain different ratios of epiphyllous to endophyllous inoculum, but also to determine the relationship between disease severity and epiphytic multiplication of bacteria

originating from outside the infected tissue. Since the two strains used in this study survived UV irradiation at the same rate, it seems reasonable that the higher recovery of the motile strain from freshly inoculated, irradiated leaves was caused by penetration of more motile than nonmotile cells into the mesophyll by the time of irradiation. This conclusion is supported by the fact that seven of the leaves inoculated with the motile, but none of those inoculated with the nonmotile strain, showed either overt or latent infection at the time of sampling. The restriction of nonmotile populations on the phylloplane of leaves heavily infected by the motile strain is less readily explained. Comparative colony counts of both strains suggest that chemical changes associated with development of bacterial blight lesions influence epiphytic multiplication of *P. glycinea* adversely.

The inverse relation existing between concentration of inoculum and rate of increase of epiphytic populations in other systems (6) was confirmed in this study. Raising the concentration of inoculum from 10^3 to 10^4 CFU of either isolate/ml increased endophyllous populations of *P. glycinea*, but did not affect the final size of epiphytic populations on nonirradiated leaves. This shows that *P. glycinea* is highly efficient in colonizing the phylloplane of primary soybean leaves from small doses of inoculum. The declining rate of increase of epiphytic populations with time suggests that the chemical (8) and physical status of the phylloplane had a less restricting influence on developing populations of *P. glycinea* as leaves were aging. However, transition of CFU from isolated cells to

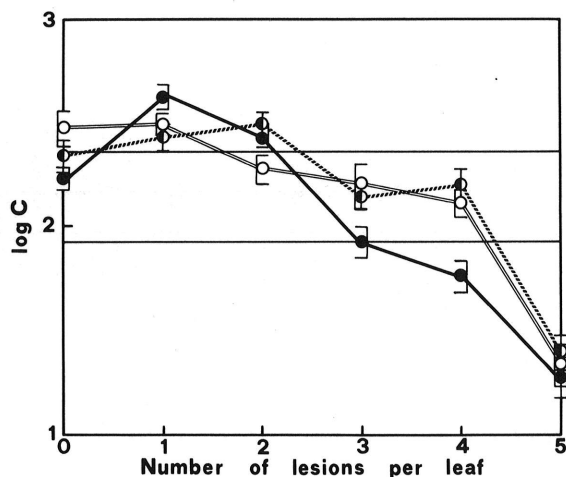


Fig. 2. The relation, on the 14th day after inoculation, between the number of bacterial blight lesions induced in soybean primary leaves by a motile strain of *Pseudomonas glycinea*, and the arithmetic mean and range of recovery [C = colony-forming units (CFU) unit leaf area] of a nonmotile strain of the same bacterium. The motile strain was injected into the mesophyll at a dose corresponding to $1ED_{70}$ (open circles and twin lines), $1ED_{85}$ (half-closed circles and discontinuous line), and $1ED_{99}$ (closed circles and continuous line). The nonmotile strain was sprayed onto the same leaves at a concentration of 10^4 CFU/ml approximately 5 hr after inoculation of the motile strain. The two horizontal lines delimit the range of recovery of the nonmotile strain on the 14th day after spraying onto leaves not subjected to previous inoculation.

cell aggregates (15, 17) also may have contributed to the observed effect.

As a result of the above and possibly other circumstances, the rate at which epiphytic populations of *P. glycinea* increased on primary leaves was lower than that reported earlier for trifoliolate leaves (19, 20, 24). It remains to be determined to what extent the relative epidemiological importance of colonization of primary and trifoliolate leaves by *P. glycinea* under field conditions depends on the rate of proliferation of epiphyllous bacteria. However, the inherent ability of primary leaves to support epiphytic multiplication of *P. glycinea* suggests that this may be a relevant factor in the increase of the inoculum of the bacterial blight pathogen from overwintered propagules.

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