Colonization and Movement of Pseudomonas syringae on Healthy Bean Seedlings

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ABSTRACT

Pseudomonas syringae was recovered from terminal buds and from newly unfurled leaves and stipules of healthy bean seedlings 9-22 days after buds were inoculated and seedlings were placed in a humid atmosphere. The pathogen also was recovered from these leaves and stipules after plants were in a less humid atmosphere for 7-16 additional days. In other tests, *P. syringae* spread over the wet leaf lamina within 2 days after the lamina was inoculated at one point near the petiole junction. Motility of the pathogen and physical forces could account for movement on individual leaves. Under suitable conditions in nature, we suggest (i) that there is epiphytic colonization of healthy buds by *P. syringae*; (ii) that from this source the pathogen may be distributed on newly forming plant parts as the plant grows; and (iii) that motility of the pathogen and physical forces result in additional spread on the plant when it is wet. Phytopathology 60:677-680.

Diseases incited by *Pseudomonas syringae* cause serious damage on a wide range of herbaceous and woody plants in the temperate regions of the world. Brown spot of bean, incited by this pathogen, occasionally is very destructive, especially in humid situations. The sporadic nature of the disease suggests that inoculum may arise from several sources. Some inoculum probably originates in pre-existing lesions, and is moved to new locations by splashing rain, irrigation water, or by dew. It is also possible that inoculum is provided by the epiphytic growth of the organism on healthy parts of a plant or on healthy plants.

We present results of studies on (i) the colonization of buds of healthy bean seedlings by *P. syringae*; and (ii) the movement of the pathogen on wet leaves. Part of the information has appeared in abbreviated form (7, 10).

MATERIALS AND METHODS.—*Plants.*—Greenhouse-grown bean (*Phaseolus vulgaris* L. 'Red Kidney') seedlings from one lot of seed were used. Plants were grown at 22-26 C in an autoclaved sand:peat mixture in 8-cm-diam clay pots (1 plant/pot). Seedlings were inoculated 10-14 days after planting for bud tests, and 14-21 days for leaf tests.

As a matter of convenience, a terminal bud is here defined as the bud plus the smallest leaf, provided the leaf is <1 cm long.

Inoculum.—P. syringae, isolate 622 (Is-622), was obtained from a diseased lima bean leaf in Salinas Valley, California, in 1968. Inoculum was prepared from 1-day-old cells grown on YDCP agar containing (g/liter): yeast extract 2.5; peptone, 2.5; NaCl, 0.5; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; glucose, 20; $CaCO_3$, 10; and agar, 20.

Bud tests.—These tests were made in wire mesh chambers, 52 cm wide, 94 cm long, and 84 cm high, which were enclosed with transparent polyethylene film. A small amount of fresh air was introduced 2 min every 10 min. Light (350 ft-c at plant height) was provided for 16 hr a day by fluorescent tubes above the chambers. Temperature inside chambers was 25-27 C.

The bud exposed when the unifoliolate leaves unfurled was inoculated by inserting into the bud a 4-mm fiberglass cloth disc (warp and woof, 22 threads/cm) containing a suspension of about 10^4 viable units of Is-622. Pots containing plants were elevated 3 cm from the chamber floor. Usually 16-20 seedlings were examined in one chamber: 10-12 were inoculated with the pathogen, and the remainder were control seedlings. Pieces of wet cheesecloth were placed on the sides of the chamber to produce a relative humidity of about 100%. The cloths were removed after 1 day and 1.3-1.8 kg of reagent grade NaCl were scattered on the chamber floor to maintain a relative humidity of 85-95%, as determined by a membrane-type RH meter. Although some water condensed on the sides of the chamber near the floor, plant surfaces were dry.

Leaf tests.—Attached unifoliolate leaves were affixed with narrow strips of cellophane tape to the bottoms of 14-cm plastic petri dishes that had been cut to admit petioles. Pieces of heavy fiberglass cloth were affixed to the insides of dish covers.

At the start of an experiment, the cloth was saturated with water, the opening admitting the petiole was loosely sealed with a piece of aluminum foil, and an 8-mm fiberglass inoculum disc containing a suspension of 10^4-10^5 viable units of Is-622 was placed on the dry, upper surface of the leaf in the midrib depression 1 cm below the petiole-lamina junction. Within a few hr after closing dishes, water was visible on leaf and dish surfaces. Dishes were not disturbed during the course of an experiment. Light was provided for 16 hr

each day by fluorescent tubes 30 cm above dishes. Temperature in the laboratory room where tests were made was 24-26 C.

Detection of P. syringae in vitro.—With practice Is-622 was easily recognized by previously described techniques when grown on an agar medium containing tetrazolium and cycloheximide, "TTCC" (9).

Detection of Is-622 in buds was effected by crushing the buds with a spatula in about 2 ml of sterile water and streaking the suspension on TTCC. Detection on stipules was accomplished by stripping a stipule from a bud or leaf with sterile forceps and immediately streaking the inside of the flared tip-half of the stipule for 20-30 cm on TTCC.

In leaf tests, a replica print method was used to note the distribution of Is-622 on a leaf. A sterile velvet-covered plate 8 cm in diam was pressed momentarily onto the wet leaf and then momentarily against the surface of TTCC in a petri dish. This method was satisfactory, provided the elevation of the water drops on the leaf was no greater than the length of the velvet pile. In other tests in which distribution information was not sought, we used leaf prints. These were made by pressing dry leaves momentarily against the surface of the agar.

Serial dilution counts of Is-622 on TTCC were employed to estimate numbers of the pathogen (8). Cells of Is-622, like other *P. syringae* isolates did not become separated when they divided in vitro, and chains of up to 100 cells often were found in water suspensions made from cultures of several ages. Since the length of chains could not be predicted, dilution counts were subject to error. However, the method was useful for assaying "viable units" in inoculum suspensions, in which most chains were < 10 cells in length, and in leaf tests for obtaining rough estimates of Is-622 populations.

Pathogenicity tests were made on Fordhook lima bean seedlings. Seedlings in the crookneck stage were inoculated by puncturing the stem near the top of the crook with a needle bearing bacterial cells. Pathogenic isolates caused a collapse of the stem and death of the seedling in 3-5 days.

RESULTS.—*Bud colonization.*—Seven experiments were made to determine the extent of bud colonization. Crushed terminal buds were examined 9-12 days after buds had been inoculated. Is-622 was recovered from 9 of 10, 4 of 11, and 11 of 12 terminal buds in three experiments, respectively. The low proportion in the second test is attributed to the fact that the buds were so small (< 3 mm long) that the inoculum disc was laid on rather than inserted into the bud.

In four experiments, seedlings were examined 15-22 days after bud inoculation. Stipules from terminal buds were streaked on TTCC to detect the pathogen. Most sampled stipules were 40-60 cm above the point of inoculation, and 3-4 trifoliolate leaves had unfurled. Is-622 was found on stipules from terminal buds of 37 of 44 seedlings examined.

No colonies resembling Is-622 were derived from control plants. However, there were numbers of other kinds of bacteria colonizing buds and stipules of all except a few control and inoculated plants. These organisms are presumed to be the "normal flora"; additional research will be required to determine their identity.

The chaining habit of *P. syringae* proved of value in providing further evidence of the colonization of the stipule surface by Is-622. When these organs were bleached, stained, and examined with the microscope (2), chains of bacterial cells were seen on the inside surface of stipules from terminal buds and from upper trifoliolate leaves of plants 15-22 days after inoculation. On some stipules, chains were side-by-side in swirling masses, as may be seen at the edges of Is-622 colonies on agar. More often, chains were in colonies of 10^2-10^3 cells; these groups usually lay in pools of lightly staining material presumed to be an extracellular matrix produced by the bacterium. Groups of chaining cells were not seen on stipules from control plants, although usually short, nonchaining rods were found on these stipules as well as on those from inoculated plants. Presumably, these rods were members of the normal flora.

Evidence that the chaining cells on stipules were indeed Is-622 was provided in one test by observations of the only two inoculated seedlings on which the normal flora was absent. One of the two stipules of the 5th trifoliolate leaf of each of these plants was streaked on TTCC; a pure culture of Is-622 resulted. The other stipules were bleached, stained, and examined with the microscope. We saw only chaining cells, both in masses and in smaller colonies.

Leaf colonization.—Is-622 also was found on leaves that had unfurled after buds were inoculated. In most bud colonization tests, leaf prints of one leaflet from two of the topmost expanded leaves of a plant were made. When Is-622 was detected in the terminal bud, it usually was present also on one or more leaves below. The pathogen also was present on stipules of these leaves.

Survival of Is-622 under moderate humidity.—It was of interest to determine the persistence of the pathogen on bud-colonized leaves and stipules under conditions less humid than those of the chamber. At the conclusion of most of the experiments reported above, plants were repotted in larger pots and transferred from the chamber to the greenhouse, where the relative humidity usually was 50-60%. Because of their use in previous assays, terminal buds had either been removed or killed by the removal of stipules for streaking. Is-622 was found on leaves of one or more inoculated plants in six experiments, after plants had been in the greenhouse 7-16 days. Also, leaf stipules from 20 plants in three of these tests were streaked. The pathogen was detected on 17 plants. The normal flora also was present on leaves and stipules.

In all of the experiments that are reported in this and the preceding sections, there were no symptoms

of the brown spot disease on either control or inoculated plants.

In vivo verification of Is-622.—Data that are presented on epiphytic colonization by Is-622 are based on the recognition of this isolate on TTCC agar. The reliability of this method was assessed by testing presumed colonies of Is-622 for pathogenicity. Out of 178 representative colonies derived from a number of experiments, bacteria from 175 colonies were pathogenic.

Movement of P. syringae on leaves.—In leaf tests, replica prints of 24 wet leaves were made 1 or 2 days after the inoculum discs had been placed on leaves. Composite distribution pictures of Is-622 after these times are given in Fig. 1. Generalized figures are presented because the distribution of the pathogen was not the same on all leaves; at times, some members of the normal bacterial flora prevented the detection of Is-622 by inhibiting or overgrowing the pathogen. The distribution and numbers of the normal flora varied from leaf to leaf.

P. syringae (Fig. 1) after 1 day was mainly in depressions over veins and on some of the horizontal areas adjacent to the inoculum disc and veins. The tip of the leaf usually was reached by this time. The pathogen was distributed over most of the lamina after 2 days (Fig. 1).

Gravitational forces could have influenced the movement of the pathogen, as was noted by direct observation of condensing water on the leaf. Gravity and capillarity probably were responsible for much of the distribution of the pathogen noted after 1 day. We suggest, also, that motility of Is-622 was important, particularly on the more horizontal parts of the leaf. Thus, in experiments in which dye solutions replaced the pathogen in the discs, dyes were confined largely to depressions over veins, in contrast to a wider distribution of the pathogen indicated by replica prints. Is-622 was vigorously motile in vitro. This was demonstrated in 10 tests by seeding about 10^7 viable units of Is-622 in 25 mm^2 in the center of a petri dish containing a soft agar medium consisting of (g/liter): beef extract, 0.3; peptone, 0.5; sucrose, 1.0; and Bacto agar, 1.5. In 24-30 hr at 26-28 C, the organism had reached the edge of the dish. Examination of portions of the medium taken at various distances from the dish center revealed motile chains of bacteria. Motile chains closely resembling these chains also were observed in water droplets taken from the leaf surface at various distances from the inoculum disc; no chains were seen on control leaves.

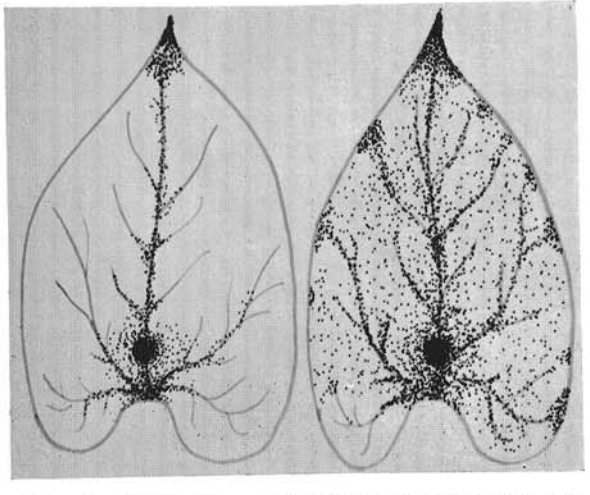

Fig. 1. Distribution of *Pseudomonas syringae* on wet, unifoliolate bean leaves 1 day (left) and 2 days (right) after an inoculum disc had been placed in the midrib depression 1 cm from the petiole-lamina junction. The pathogen distribution was revealed by replica printing on an agar medium.

Attempts were made to determine if movement of Is-622 on the leaf was accompanied by increase in numbers, as measured by dilution counts. After the 2-day test period, an inoculum disc was removed from the wet leaf and swirled in a Vortex mixer for about 1 min in 10-ml buffer, which was then assayed. Ten 10-ml portions of buffer then were forcefully directed at the leaf surface from a pipette, and the portions were combined and assayed (3 tests), or a leaf was shaken about 3 min in 100 ml of buffer for assay (1 test). Less than 12% of the recovered Is-622 remained on discs of the 17 leaves examined ($< 5\%$ remained on 14). Essentially no increases in viable units over those applied were noted on four leaves, $<$ 100-fold increases were noted on 10 leaves, and $<$ 1,000-fold increases were noted on three leaves. Even though these data are considered rough estimates because of the chaining habit of Is-622, they indicate that great increases in populations did not take place. It is possible, however, that as Is-622 moved over the leaf, increases were balanced by deaths, and the washing procedures did not remove all Is-622.

DISCUSSION.—The experiments reported in this paper suggest that *P. syringae* can grow on the healthy bean plant and thus possess a "resident phase" (5, 6) in its life cycle. Expanded leaves did not appear to be important sites of colonization. Rather, this organism under some conditions can grow in the bud, especially on the inside surface of stipules. How long a bud may remain colonized is not known, but this probably depends on environmental conditions. The pathogen probably also multiplies on the surface of small leaves within the bud and is distributed on leaves as the plant grows. After leaves expand, *P. syringae* is capable of remaining viable on leaves and stipules for a period of time, as shown in our tests. Moreover, the pathogen may be distributed still farther on stationary wet leaves by motility and by gravitational and capillary forces.

A resident phase in the life cycle of *P. syringae* offers an explanation for many sudden attacks by this pathogen on agriculturally important plants. On such hosts as citrus, almond, and pear, extensive outbreaks of disease in California have followed a night of frost or a hail storm, indicating that abundant inoculum was present before the predisposing conditions occurred. In England, freezing injury predisposed pear blossoms to a blight incited by *P. syringae,* which was present natu-

rally on healthy blossoms (13). This pathogen and the related *P. mors-prunorum* have been isolated from leaves and other parts of healthy fruit trees, and the probability of an epiphytic habit of growth has been suggested (1, 4, 11). Attention also has been directed to the bud; *P. syringae* was associated with dormant peach and pear buds (3, 12), and *P. mors-prunorum* ". . . appeared suddenly at bud break . . ." in rain water draining from plum and cherry trees (1). A resident phase may also explain the periodic isolation of *P. syringae* in California (by M. N. Schroth and D. C. Hildebrand) from miscellaneous, apparently-healthy plant materials. These considerations indicate that *P. syringae* may be a resident on many hosts in addition to bean and that the bud, in particular, would bear close scrutiny in future work.

Our data probably represent underestimations of the distribution of Is-622. This is because there was a variety of other types of bacteria also on the seedling. Many of these organisms from the plant did not seem to influence the in vitro growth of Is-622, because on occasion Is-622 was detected easily among 6-8 types of slow-growing or noninhibitory forms. Some of these other organisms, however, inhibited or overgrew Is-622 when colonies of the two were in proximity on assay dishes. This raises questions as to the interactions among members of the normal flora and the pathogen in nature. Perhaps a biological control of the brown spot disease can be achieved by selective management of one or more antagonistic residents. Altering the epiphytic microflora will not be accomplished without difficulties, as has been noted (6). We conclude, however, that the study of the plant surface offers many opportunities for increasing our basic understanding of interactions among bacteria and between bacteria and the plant, and that this new information may lead to the biological control of disease.

LITERATURE CITED

1. Crosse, J. E. 1966. Epidemiological relations of the pseudomonad pathogens of deciduous fruit trees. Annu. Rev. Phytopathol. 4:291-310.
2. Daft, G. C., & C. Leben. 1966. A method for bleaching leaves for microscope investigation of microflora on the leaf surface. Plant Dis. Reptr. 50:493.
3. Dowler, W. M., & D. H. Peterson. 1967. Transmission of *Pseudomonas syringae* in peach trees by bud propagation. Plant Dis. Reptr. 51:666-668.
4. English, H., & J. R. Davis. 1960. The source of inoculum for bacterial canker and blast of stone fruit trees. Phytopathology 50:634 (Abstr.).
5. Leben, C. 1963. Multiplication of *Xanthomonas vesicatoria* on tomato seedlings. Phytopathology 53: 778-781.
6. Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. 3:209-230.
7. Leben, C. 1969. Movement of *Pseudomonas syringae* on bean leaves. Phytopathology 59:1037 (Abstr.).
8. Leben, C., G. C. Daft, & A. F. Schmitthenner. 1968. Bacteria blight of soybean: population levels of *Pseudomonas glycinea* in relation to symptom development. Phytopathology 58:1143-1146.
9. Leben, C., V. Rusch, & A. F. Schmitthenner. 1968. The colonization of soybean buds by *Pseudomonas glycinea* and other bacteria. Phytopathology 58:1677-1681.
10. Leben, C., & M. N. Schroth. 1969. Movement of *Pseudomonas syringae* on bean seedlings. Phytopathology 59:1037 (Abstr.).
11. Panagopoulos, C. G. 1966. Studies on the source of inoculum for blast and black pit of citrus. First Congress Mediterranean Phytopath. Union Proc. Bari-Naples: 340-345.
12. Panagopoulos, C. G., & J. E. Crosse. 1964. Blossom blight and related symptoms caused by *Pseudomonas syringae* van Hall on pear trees. Rept. East Malling Research Sta., 1963:119-122.
13. Panagopoulos, C. G., & J. E. Crosse. 1964. Frost injury as a predisposing factor in blossom blight of pear caused by *Pseudomonas syringae* van Hall. Nature 202:1352.