Histology of Onion Leaves Infected with Pseudomonas cepacia

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ABSTRACT

Pseudomonas cepacia moved into the leaf sheaths through the intercellular spaces when stab-inoculated into the base of onion leaf blades. Although large bacterial masses developed, separate small groups of bacteria frequently were formed in the intercellular spaces of both blade and sheath. The small group type of distribution was observed when the lesions were periodically sprinkled with water. Except for the xylem vessels and epidermal cells, host cells were compressed and there were large bacterial masses in the intercellular spaces in close association with these collapsed cells. In the blade, the large parenchyma cells were the first to collapse; the smaller, more compactly packed parenchymatous cells near the periphery of the blade collapsed later. Tissues composed of these cells eventually were macerated. In the sheath, bacteria were widespread among the loosely organized parenchyma cells adjacent to the adaxial epidermis. Bacterial masses and scattered bacteria in close association with host cell walls were commonly observed in this area. Bacteria in the intercellular spaces of the closely packed parenchyma cells beneath the abaxial epidermis usually were restricted to dense, compact masses.

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Pseudomonas cepacia Burkh. is a soft rot pathogen of onion (*Allium cepa* L.). Burkholder (1) noted that only bulbs appeared susceptible and young growing plants had no symptoms. He suggested that the bacteria probably entered the bulb through neck wounds created when the tops were removed at harvest. However, bulbs naturally infected with *P. cepacia* have been observed in the field before harvest in recent years in New York. The appearance of these naturally infected bulbs suggests that ingress also occurs in the upper parts of the plant, either directly or through wounds in the leaves or neck resulting from cultural procedures or environmental conditions. Repeated inspection of plants growing in the field indicated that infection frequently occurs before harvest. In experiments preliminary to the present study, the addition of water to the site of inoculation after initial pathogenesis greatly increased the rate of spread of bacteria in the onion leaf. The leaf axils form natural basins which collect and hold water. Inoculation of the leaf axils followed by the addition of water resulted in lesions which rapidly expanded into the leaf sheaths which constitute the neck and bulb of the onion.

The purpose of this study was to determine how the bacteria move from the leaf blade into the leaf sheath.

MATERIALS AND METHODS.—One-month-old onion bulbs sprouted under greenhouse conditions, and 5-month-old, greenhouse-grown Downing Yellow Globe onions were inoculated with an 18-hr-old culture of *P. cepacia* (isolate 64-22). The bacteria were maintained in glass-distilled water and were transferred twice on nutrient agar before use as inoculum. We made inoculations by stabbing the leaf axils with a needle bearing the bacteria. Noninoculated leaf axils and leaf axils stabbed with a sterilized needle were used as two series of controls. Portions of the leaves were collected immediately after inoculation, and after 1 and 3 days. The plants were sprinkled with water before the collection of the first set of samples, and immediately after the collection of the second set of samples. After each of the two sprinklings, the plants were incubated in plastic bags at 27-30 C until sampling. Other plants were likewise incubated in plastic bags, but were not sprinkled with water.

The samples were fixed in Navashin's fixative (6) and dehydrated in dioxan (9), or were fixed and dehydrated by using the acrolein to *n*-butanol series described by Feder & O'Brien (3). The samples in *n*-butanol were brought to room temperature and placed under vacuum until most of the air in them was removed. The samples then were transferred to dioxan (diethylene dioxide) by means of the following series: (i) two-thirds *n*-butanol: one-third dioxan; (ii) one-third *n*-butanol: two-thirds dioxan; (iii) two changes of dioxan; (iv) dioxan with 5% xylene. The samples were exposed to each solution for 4 hr, then were embedded in Tissuemat (melting point = 55 C) by the procedure for dioxan-dehydrated material described by Sass (9). The blocks were frozen, and 10-μ sections cut on a rotary microtome. The serial sections were affixed to slides with Haupt's adhesive (6). Staining was done with Harris' hematoxylin and orange G (6), using a schedule devised by Nelson & Dickey (7). Conant's quadruple stain (6) was used for some sections of healthy tissue; and toluidine blue (3), for some from the inoculated plants. The Harris' hematoxylin-orange G staining procedure proved to be the most satisfactory.

RESULTS.—*Histology of noninoculated plants.*—The onion leaf consists of a sheath and a rounded

hollow blade. The thickened bases of the sheaths comprise the bulk of the bulb. The sheaths encircle the next inner leaf which emerges through an orifice at the base of the blade of the encircling leaf. Thus, the blade is situated to one side of the sheath. The crotch formed by the blade and the next younger leaf will be referred to as the leaf axil in this study. Two parts of the onion leaf were studied, the base of the blade and the portion of the sheath between the blade and the bulb. Only observations pertinent to the pathological study are discussed. The anatomy of the healthy onion plant has been studied by Hoffman (5) and others (2, 4), and the following is primarily a confirmation of certain aspects of these reports. There was little difference between the anatomy of leaves on plants grown from bulbs and those on plants grown from seed.

Base of the blade.—The base of the blade is highly meristematic (5), but the outer wall of the epidermal cells in this region are very thick (approximately 4 μ thick at the center of the abaxial wall and thicker at the corners). The other three walls of the epidermal cells are less than 1.1 μ thick. Scott et al. (11) described these walls as composed mainly of cellulose and pectic substances. The epidermal cells are basically rectangular in shape, with the side walls slightly bowed out and the end walls squared off. These cells are arranged in well-defined columns parallel to the length of the leaf, and in irregular rows laterally. The stomata are located at the ends of the epidermal cells. The guard cells are sunken. The base of the adaxial side of the leaf blade is bounded by a ligule which forms a semicircular collar around the next inner leaf. Leaves of resprouted bulbs occasionally have thick-walled fibers arranged in a semicircle around the adaxial side of the blade near the base of the ligule. These fibers apparently have not been reported previously and were not observed in plants grown from seed.

The more mature sections of the blade have columnar cells located just beneath the epidermis, but at the base of the blade these cells have not yet differentiated. The precursors of these cells are short, cylindrical cells with their long axes oriented parallel to the length of the leaf. The lacunar cavity of the hollow onion leaf ends at the base of the blade in the mature leaf. The parenchyma cells of the central portion of the blade have very thin walls, and tear and collapse easily during formation of the cavity (Fig. 1).

The vascular bundles are generally uniformly scattered near the periphery of the blade. Most of these bundles run parallel to the longitudinal axis of the leaf, but there are several branches and cross-connections between the longitudinal bundles. The larger bundles have bundle sheaths consisting of long cylindrical parenchyma cells. These bundle cells are oriented with their long axes parallel to the vascular bundle.

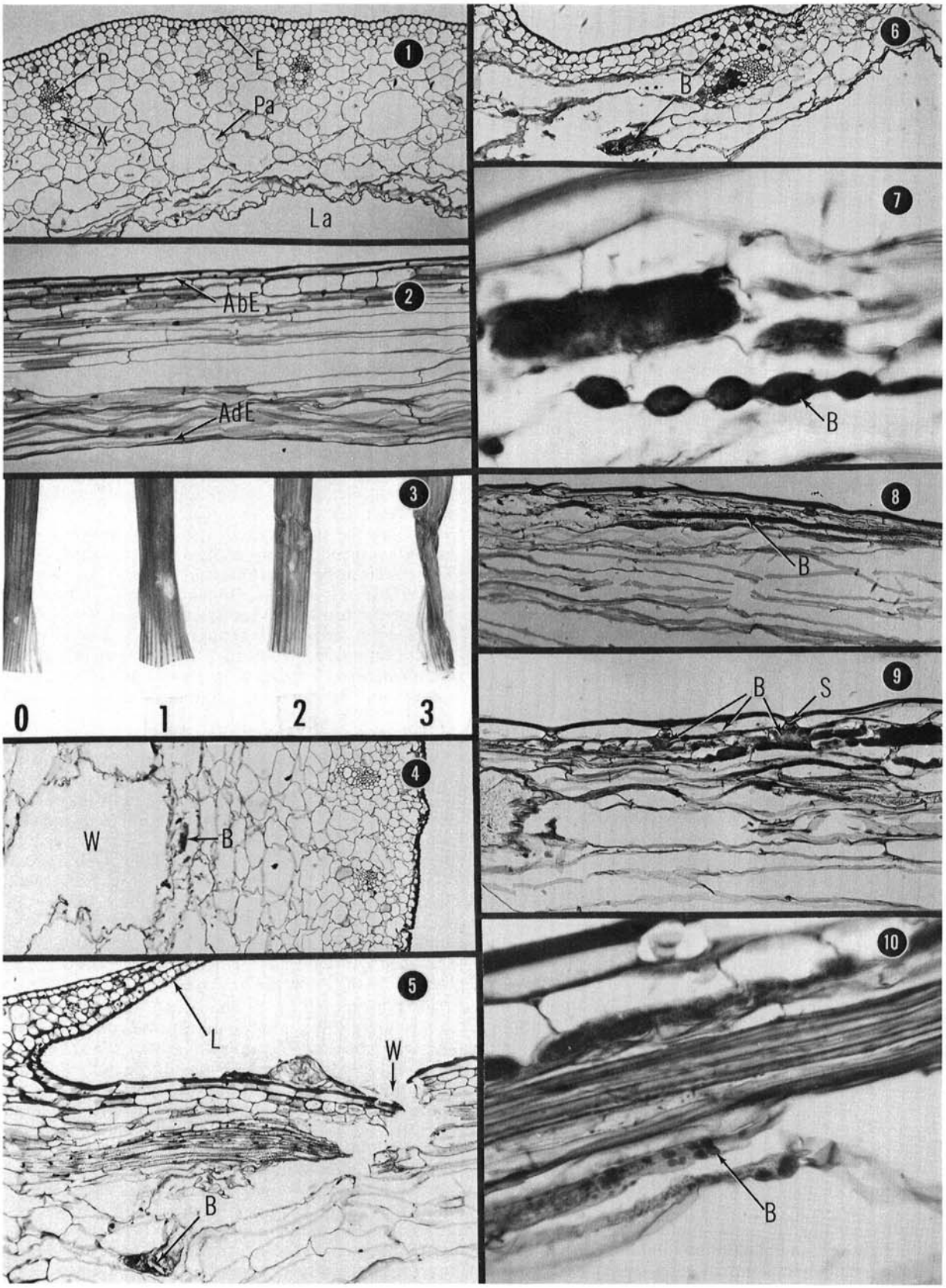
The laticifers are located two or three cell layers beneath the epidermis and longitudinally oriented in the leaf. They are long, cylindrical, articulated, non-anastomosing cells which frequently have prominently pitted transverse septa (8). The latex within these cells is alcohol-soluble, and was dissolved from the tissue during dehydration and staining, so identification of the laticifers was based upon the other characters listed above.

Sheath.—The sheath remains meristematic longer than does the blade (4). The epidermal cells of the abaxial side of the sheath are thick-walled like those of the blade, whereas those of the adaxial side of the sheath are thin-walled and delicate (Fig. 2). The epidermis of the adaxial side of the sheath has beneath it several layers of loose, collapsed parenchyma (Fig. 2), and consequently this inner epidermis is easily sloughed off with handling of the samples. The majority of sheath samples had lost their inner epidermis by the time the samples were embedded in paraffin, although these samples were handled as a unit with several inner sheaths. Stomata are present on the abaxial epidermis, though less common than on the blade. There are no stomata on the adaxial epidermis. The vascular bundles are arranged around the periphery of the abaxial epidermis.

Histology of inoculated plants.—Disease development was very rapid in inoculated leaves sprinkled with water. One day after inoculation, most of these leaves had large, spreading lesions, although a few exhibited small, sharply defined, lens-shaped lesions. These were assigned ratings of "2" and "1", respectively (Fig. 3). By the 3rd day, lesions girdled the base of the blades and spread into the sheath. These leaves were assigned a rating of "3". Leaves with disease ratings from "0" to "2" were sampled at the leaf blade axil. Samples from leaves with a "3" rating were from the margin of the disease area in the leaf sheath.

Samples taken at inoculation and assigned disease rating "0".—Bacteria were deposited during inoculation in wounded cells and intercellular spaces in the immediate area of the wound caused by the inoculating needle (Fig. 4, 5). In general, the bacteria appeared to be sparse, with large bacterial masses occurring infrequently. Occasionally, a xylem vessel was broken during inoculation and the bacteria were distributed in the vessel both above and below the break in the vessel. Distribution of bacteria in the injured xylem vessels at the time of inoculation extended considerably further from the wound than bacteria in the intercellular spaces.

Disease rating "1".—The most apparent histological difference between freshly inoculated leaves and leaves with a "1" rating was that the latter exhibited extensive development of bacteria in the intercellular spaces and wounded cells (Fig. 6). The bacteria were observed farther away from the wound, and the intercellular spaces often appeared to be expanded by masses of bacteria (Fig. 7). Occasionally, laticifers were found filled with bacteria (Fig. 8). This apparently was due to the chance occurrence of wounding. Bacterial masses occasionally were found in the substomatal cavities (Fig. 9). These were interconnected by thin strands of bacteria in the intercellular spaces beneath the epidermal cells separating the stomata. Occasionally, other strands of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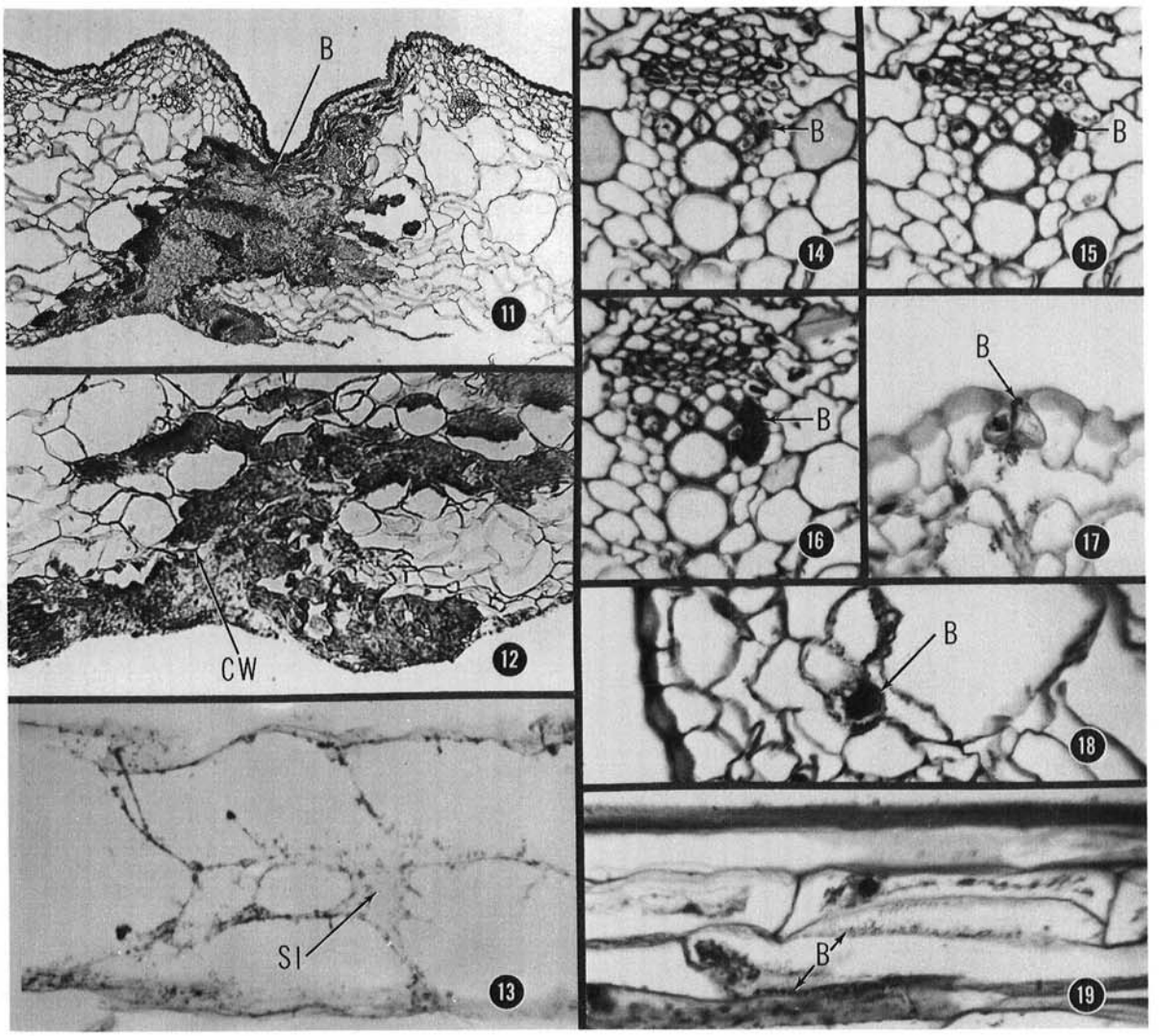

Fig. 11-19. 11-13) Sections of onion leaf blade tissue with 1-day-old bacterial infections at the "2" rating stage. **11)** Transverse section near site of inoculation showing extensive development and consolidation of the bacteria. **12)** Transverse section of bacterial mass shown in Fig. 11 showing host cell wall within the bacterial mass. **13)** Longitudinal sections showing bacteria adhering to slime. **14-16)** Transverse sections of bacterial mass in onion leaf blade with 1-day-old bacterial infection at the "2" rating stage. Sections taken at 20-μ intervals moving from the base of the blade towards the sheath. **14)** Bacteria in intercellular space between xylem parenchyma cells. **15)** Bacterial mass larger; two adjacent parenchyma cells collapsed. **16)** Bacterial mass has completely collapsed three cells and partially collapsed two others, but xylem vessels remain intact. **17-19)** Sections of onion leaf sheath tissue with 3-day-old bacterial infections at the "3" rating stage. **17)** Transverse section showing bacteria in stomatal cavity exuding out between guard cells. **18)** Transverse section showing small bacterial mass in intercellular space. **19)** Longitudinal section showing various sized aggregations of bacteria in close association with host cell walls. B = bacteria; CW = host cell wall; Sl = slime.

Fig. 1-10. 1) Transverse section of healthy onion leaf blade. **2)** Longitudinal section of healthy onion leaf sheath section showing the collapse of parenchyma beneath adaxial epidermis. **3)** Disease ratings of onion leaves inoculated with *Pseudomonas cepacia*: 0 = check; 1 = lens-shaped lesion; 2 = spreading lesion; 3 = leaf girdled. **4-5)** Sections of inoculated onion leaf blade tissue at time of inoculation. **4)** Transverse section. **5)** Longitudinal section. **6-10)** Sections of onion leaf blade tissue with 1-day-old bacterial infections at the "1" rating stage. **6)** Transverse section showing bacteria in wound, xylem, and intercellular spaces. **7)** Longitudinal section showing expansion of intercellular spaces by bacteria. **8)** Longitudinal section showing bacteria in laticifer. **9)** Longitudinal section showing bacteria beneath epidermis extending from stoma to stoma. **10)** Longitudinal sections showing small, separate bacterial masses on parenchyma cell walls. AbE = abaxial epidermis; AdE = adaxial epidermis; B = bacteria; E = epidermis; L = ligule; La = lacunar cavity; O = phloem; Pa = parenchyma; S = stoma; W = wound; X = xylem.

bacteria interconnected the bacterial masses in the substomatal cavities to bacterial masses deeper within the leaf. The large parenchyma cells of the interior of the leaf blade were collapsed by the bacteria. The bundle sheath cells and the smaller, more compactly packed parenchyma cells near the epidermis were less severely collapsed. The xylem vessels and epidermal cells did not appear to be distorted in this manner.

Disease rating "2".—Lesions with ratings of "2" exhibited extensive development of bacteria in the vicinity of the wound (Fig. 11). The bacterial masses in this area merged, filling the intercellular spaces and wounded cells. The host parenchyma cells were collapsed by the bacterial masses. Host cell walls could be seen within the bacterial mass (Fig. 12). These cell walls usually were walls of entire cells which had been compressed by the bacteria. Cell walls which appeared to be fragments of cells were seen infrequently. Parenchyma cells were washed out of some of the samples during preparation for sectioning, indicating that the tissue had become macerated. Most of the bacteria also were washed out of these samples. Those that remained were attached to slime (Fig. 13). This was the first time in pathogenesis that slime was observed. Smaller bacterial masses were found collapsing the surrounding host cells farther down the leaf towards the sheath and away from the main bacterial mass (Fig. 14, 15, 16). The bacteria in the wounded xylem vessels did not invade tissue beyond that at the time of inoculation.

Disease rating "3".—Collapse of the parenchyma beneath the adaxial epidermis of the sheath caused difficulty in the obtaining of complete sections of the sheath. No longitudinal sections were obtained of the sheath with the adaxial epidermis attached. Nonetheless, transverse sections of intact sheath samples revealed few differences between infection patterns there and in the base of the blade. Bacteria were observed in substomatal cavities, intercellular spaces, and in close association with cell walls (Fig. 17, 18, 19). The bacteria near the abaxial epidermis were usually grouped in dense, compact masses or strands (Fig. 18), whereas the bacteria located among the collapsed parenchyma adjacent to the adaxial epidermis were less densely packed and more dispersed. Bacteria were not observed in the xylem vessels of 16 leaf sheaths examined. This follows observations of samples with "2" ratings that bacterial movement in the wounded xylem vessels is limited.

DISCUSSION.—Absence of any marked difference between the infection patterns of the bacteria in the "1" stage and the "2" stage suggests that the lens-shaped lesion of the "1" stage was an artifact of inoculation. That the lesion initially was lens-shaped is probably a function of the intercellular spaces and the shape and arrangement of the host cells. The parenchymatous cells beneath the epidermis are cylindrical, with their long axes oriented longitudinally, forming columns. Each cell in the column is arranged so that the end walls of these cells are irregularly staggered. Death of a group of these cells will naturally result in more necrosis on the longitudinal axis than the lateral axis, even if an equal number of cells were affected in both directions. However, more cells are killed on the longitudinal axis because most of the intercellular spaces also are oriented longitudinally. The bacteria probably flowed into these spaces with the cell sap expressed by the inoculating needle, and thus spread farther longitudinally than laterally. The later movement of the bacteria from the lens-shaped lesion was uneven, and resulted in the irregularly shaped lesion of the "2" rating. Lactophenol on a needle stabbed into the leaf produced a lens-shaped lesion if the leaf was washed off 1 or 2 min after wounding. Longer application of the lactophenol resulted in larger irregularly shaped lesions.

The primary avenues of movement of the bacteria were the intercellular spaces. Intracellular movement was negligible, and was observed only in the xylem vessels and perhaps the laticifers. The lesions increased in size most rapidly when water was periodically added to the diseased area. This rapid increase in size was related to the movement of the bacteria in the intercellular spaces. It seems from the distribution of the bacteria that the intercellular spaces contained water or expressed cell sap, and that the bacteria flowed or swam through these spaces. Scattered bacteria and small bacterial masses were observed entirely separated from the main masses of bacteria and in close association with host cell walls. These bacteria could not have separated from the main mass of bacteria except in a liquid environment. This may partially explain the requirement of free moisture for rapid lesion expansion at temperatures ranging from 21 to 32 C. Sasser et al. (10) proposed that the high osmotic potential of intercellular fluid inhibited reproduction of *Xanthomonas vesicatoria* in pepper leaves, and that water-congestion reduced this osmotic potential to levels suitable for reproduction of bacteria. Such a system may also apply to *P. cepacia*, but at present it appears that the main effect of moisture is to enable more rapid spread within the onion tissues.

Bacteria were observed rather frequently in the substomatal cavities. All evidence indicates that the bacteria invaded the substomatal cavities from within the leaf through the intercellular spaces. The bacteria in these substomatal cavities were connected by thin strands of bacteria to other masses of bacteria, and were usually traced to larger bacterial masses nearer the site of inoculation. Other artificial inoculations made to test ingress through the stomata failed to result in symptoms. No symptoms were produced when the bacteria were sprayed on leaf surfaces at different times of the day under various temperature and humidity regimes. Water-soaking the leaves before spraying with bacteria produced a few lesions, but usually the epidermis was found to be broken.

The bacteria sometime were observed to extend beyond the guard cells from the substomatal cavity (Fig. 17). This was observed in a leaf sheath sample taken from within the false stem or neck of the onion bulb. This raises the possibility that bacteria in one leaf sheath may invade the adjacent outer sheath by escaping through the stomatal pore and entering the

next outer sheath through breaks in the epidermis. Previously it was thought that breaks in both sheaths were required for spread from sheath to sheath. Breaks in the epidermis of the leaf sheaths probably occur when the tops of the plants topple due to high winds or to maturity of the bulb. Thus, an onion bulb with several infected bulb scales may have been the result of a single initial infection rather than of multiple infections. This is probably often the case, since isolations from such bulbs seldom produced more than one species of soft rot bacteria, although isolations were made from each decayed scale.

The onion leaves used in this study contained both very hard and very succulent tissues, as well as numerous air spaces which made fixation and sectioning difficult. The acrolein fixation and dehydration schedule of Feder & O'Brien (3) proved to be far superior to the procedure using Navashin's fixative (6) and dioxan dehydration (8). The latter combination caused much plasmolysis and shrinkage in the tissues; the former caused little plasmolysis and shrinkage. Plasmolysis might have been eliminated from the former procedure if smaller samples had been cut or if the samples had been vacuum-infiltrated in an ice bath at the first stage of dehydration. Some of the larger samples were probably incompletely dehydrated because they floated on the dehydrating solutions.

The thick outer wall of the epidermis of onion leaves is quite hard and rigid, as evidenced by the difficulties encountered during sectioning. The Tissuemat did not lend sufficient support to the samples unless the blocks were frozen before sectioning; in unfrozen blocks, the epidermis would tear off in large chunks when sectioned. This thick outer wall must present a barrier to wounding, and, since the bacteria require wounds for inoculation, hinder infection under most field conditions.

LITERATURE CITED

1. BURKHOLDER, W. H. 1950. Sourskin, a bacterial rot of onion bulbs. Phytopathology 40:115-117.
2. ESAU, K. 1953. Plant anatomy. John Wiley & Sons, Inc., New York. 735 p.
3. FEDER, N., & R. P. O'BRIEN. 1968. Plant microtechnique: Some principles and new methods. Amer. J. Bot. 55:123-142.
4. HAYWARD, H. 1938. The structure of economic plants. The Macmillan Co., New York. 674 p.
5. HOFFMAN, C. A. 1933. Developmental morphology of Allium cepa. Bot. Gaz. 95:279-299.
6. JOHANSEN, D. A. 1940. Plant microtechnique. McGraw-Hill, New York. 523 p.
7. NELSON, P. E., & R. S. DICKEY. 1966. Pseudomonas caryophylli in carnation. II. Histological studies of infected plants. Phytopathology 56:154-163.
8. RENDLE, A. B. 1889. On the vesicular vessels of the onion. Ann. Bot. 3:169-178.
9. SASS, J. E. 1958. Botanical microtechnique [3rd ed.]. Iowa State College Press, Ames, Iowa. 228 p.
10. SASSER, J. M., R. W. MILLER, & D. J. FIELDHOUSE. 1970. Osmotic potential, a controlling factor in the development of bacterial spot disease. Phytopathology 60:1311-1312.
11. SCOTT, F. M., K. C. HAMNER, E. BAKER, & E. BOWLER. 1958. Electron microscope studies of the epidermis of Allium cepa. Amer. J. Bot. 45:449-461.