Histopathology of Pelargonium Species Infected with Xanthomonas pelargonii

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ABSTRACT

The histology of *Pelargonium* spp. susceptible, moderately resistant, and resistant to *Xanthomonas pelargonii* was studied. The mode of spread of the pathogen was similar in all species, initially involving movement of the pathogen throughout the plant in the xylem vessel elements and subsequent movement laterally into adjoining parenchyma cells. The relative numbers of the pathogen and the numbers of fascicles initially invaded were low in resistant species and high in susceptible species. In susceptible species, bacterial pockets formed around affected protoxylem vessel elements, enlarging to encompass all xylem cells in the fascicle, and finally portions of the cambium, phloem, cortex, and epidermis. *Pelargonium* spp. responded to infection by proliferation of a ring of cells around affected portions of fascicles, with cells immediately inside this ring having a suberinlike material formed on their walls. This appears to be a secondary defense reaction, restricting the lateral spread of the pathogen. Tanninlike materials were found in the tissues of all *Pelargonium* spp. tested, but appear to be of different types in the susceptible and resistant species. It is suggested that tanninlike substances may be, in part, responsible for disease resistance through their actions as bacteriostatic agents and enzyme inhibitors.

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Additional key words: Pelargonium × *hortorum, P. radens, P. fulgidum, P. graveolens, P. acerifolium.*

Bacterial blight of *Pelargonium* spp. incited by *Xanthomonas pelargonii* (Brown) Starr & Burkh. is recognized as one of the most important diseases of florist's geranium, *Pelargonium* × *hortorum* Bailey, in the United States (18). This vascular wilt pathogen is disseminated primarily during vegetative propagation. As symptom expression is suppressed under the cool conditions prevailing at propagation (24), many symptomless infected plants are produced and sold. In warm weather, symptom expression occurs and many of these apparently healthy plants suddenly wilt and die. Several *Pelargonium* spp. are known to possess significant resistance to *X. pelargonii* (13), but none of the cultivars of the highly desirable *P.* ×

hortorum are resistant. All attempts to incorporate disease resistance into *P.* X *hortorum* have failed. This investigation was designed to determine whether anatomical differences exist in resistant and susceptible *Pelargonium* spp., and to study the differences between the reaction of resistant and susceptible *Pelargonium* spp. to infection by *X. pelargonii*.

MATERIALS AND METHODS.—Isolates of *X. pelargonii* were obtained from diseased *P.* X *hortorum* plants from several areas of Pennsylvania. The isolates were tested for pathogenicity on *P.* X *hortorum* 'Improved Ricard', using the method of Nichols (21). The most virulent isolate was selected for use in this study. Cultures of *X. pelargonii* were maintained on potato-dextrose agar (PDA), pH 7.2. We prepared inoculum from cultures grown on PDA slants for 48 hr at 27 C by washing the bacterial cells from a slant with 30 ml of sterile distilled water. This resulted in a nonstandardized bacterial suspension approximating 30% transmittance at 600 nm. We inoculated plants by injecting 0.02 ml of the bacterial suspension into the plant stem with a microliter hypodermic syringe (Hamilton, Model 705N syringe). The area to be inoculated was swabbed with 95% ethyl alcohol prior to injection. Two-month-old plants were inoculated at a point approximately six internodes below the youngest macroscopically visible leaf. Inoculation was made just below the node and into the vascular tissues. Sterile distilled water was injected into stems of check plants.

Pelargonium X *hortorum* 'Improved Ricard' (susceptible), *P. radens* H. E. Moore 'Dr. Livingston' (moderately resistant), *P. fulgidum* (L.) L'Her. ex Ait. 'Brilliant' (moderately resistant), *P. graveolens* L'Her. ex Ait. 'Rober's Lemon Rose' (resistant), and *P. acerifolium* L'Her. (resistant) were used in this study (13). In addition the healthy tissues of *P.* X *domesticum* Bailey 'Madame Layal' (resistant), *P. graveolens* 'Gray Lady' (moderately resistant), and three cultivars of *P. peltatum* (L.) L'Her. ex Ait. (susceptible) were studied. All plants were derived from culture-indexed stock. Munnecke's culture-index technique was used (19). Cuttings were rooted under intermittent mist in BR-8 blocks (American Can Co.) and potted in a steam-treated 1:1:1 peat, perlite, soil mix in 5-inch clay pots. We maintained greenhouse temperatures at about 21 C (day and night) in order to get rapid disease development, but temperatures were higher in the summer, ca. 21 to 30 C.

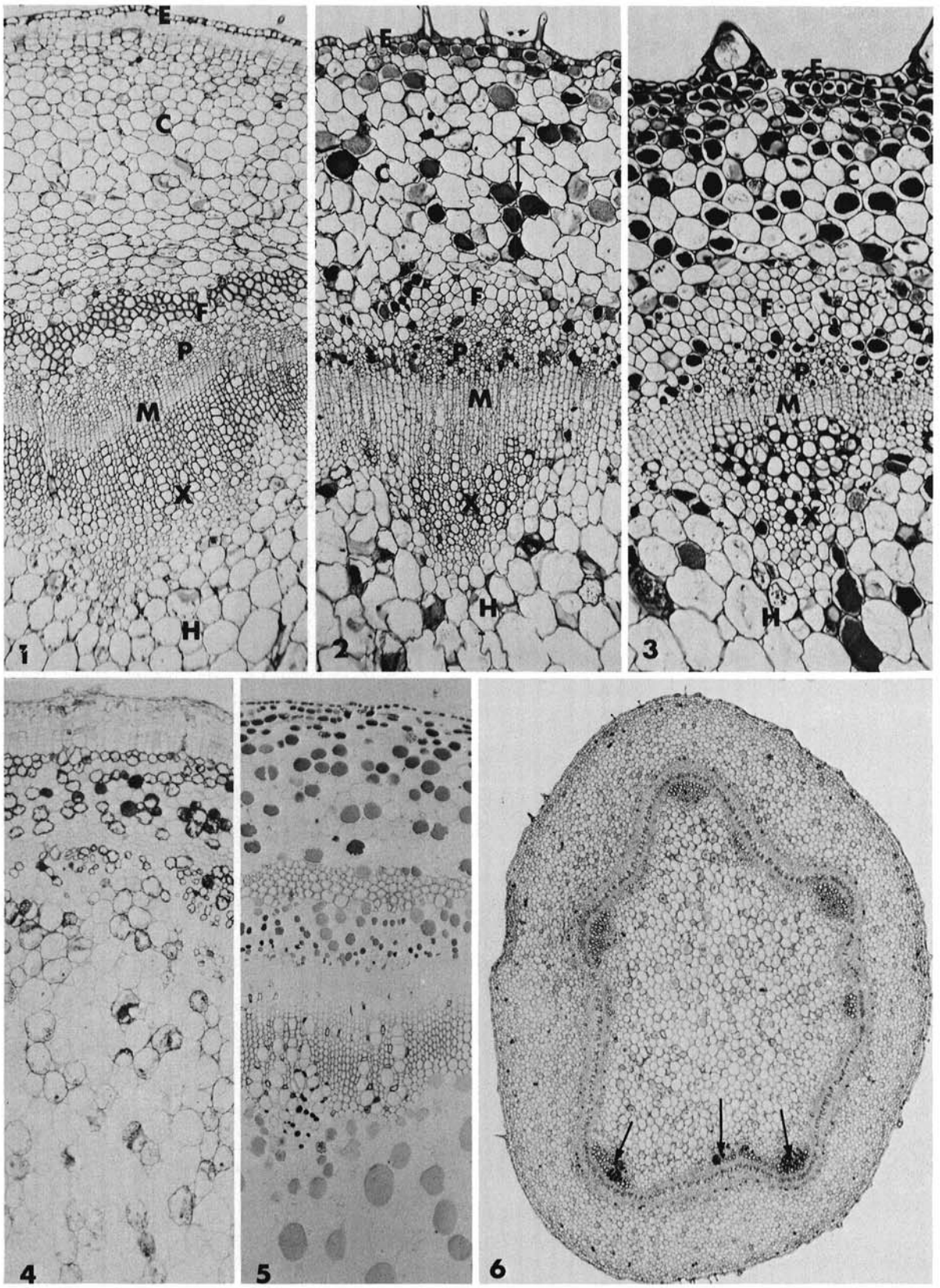
Inoculated plants were sampled before and during symptom appearance. Branches to be sampled were removed aseptically, and the leaves cut off. We surface-sterilized branches by placing them in a 15% solution of Clorox (5.25% sodium hypochlorite) plus a trace of detergent for 5 min. No water rinse was used. Branches were cut aseptically into ca. 20 5-mm pieces. Alternate pieces were placed in vials of fixative, and the remaining pieces were put in individual tubes of nutrient broth plus 1.5% dextrose (10) and incubated at 27 C. At 3 and 10 days, a loop of the broth solution from each tube was streaked onto PDA plates which were incubated at 27 C. Microbial growth on PDA was recorded. Colonies characteristic of *X. pelargonii* were creamy-yellowish, excessively slimy, and were composed of gram-negative, rod-shaped cells (9). Pieces adjacent to those yielding the pathogen were prepared for sectioning. Check plants were treated in the same manner.

Plant materials were killed and fixed in Rawlins' alcohol-formalin-acetic acid solution No. 1 (22), dehydrated using a tertiary butyl alcohol schedule (12), and embedded in Paraplast (Curtin Scientific Co.). Stem pieces were softened 48 hr or more in an aqueous solution of 90 ml of 1% sodium lauryl sulfate (Dreft) and 10 ml of glycerol (1), and sectioned on a rotary microtome. Microscope slides were cleaned in an acid cleaning solution (12) prior to use. Sections were affixed on slides with Haupt's adhesive (12).

Sections were stained with a modified Johansen's quadruple stain (12) or with Harris' hematoxylin and orange G (13). Johansen's quadruple stain was most useful in studying the healthy and diseased tissues of the stem. Harris' hematoxylin and orange G aided in determining the location of bacteria (20). Histochemical tests were performed for the detection of starch, cellulose, suberin, cutin, lignin, pectic substances, and tannins (11, 12, 23). The polarized light microscope was used as an aid in observing starch, cellulose, and crystals. Maceration of healthy tissues was done using Jeffrey's method (12), and the cells were stained with safranin 0. Stomata, substomatal cavities, epidermis, and trichomes were observed from epidermal peels of healthy plants mounted in a solution of cotton blue in lactophenol. A Leitz Ortholux research microscope was used for observations with polarized and unpolarized light. Photographs were taken with a Leitz Aristophot camera with a 4 X 5 inch Graflex back using selected Kodak Wratten gelatin filters.

RESULTS.—*Anatomy of the healthy stem tissues.*—The stems of all *Pelargonium* spp. observed were anatomically similar, and had similar distribution of tissues within the stem and cells within a tissue (Fig. 1-3). Observations were made from portions of the stems approximately 6 inches from the apex, and in an internode. At this point in the stem, the cortex and epidermis formed a mantle around the vascular tissues and pith. The originally

Fig. 1-6. 1, 2, 3) Portions of transverse stem sections of *Pelargonium* spp. showing healthy stem tissues, epidermis (E), cortex (C), phloem fibers (F), phloem (P), cambium (M), xylem (X), pith (H), and tanninlike materials (T). **1)** *P.* X *hortorum* (S) (X 40). **2)** *P. radens* (MR) (X 40). **3)** *P. acerifolium* (R) (X 70). **4, 5)** Transverse sections of healthy stem tissue of *Pelargonium* spp. fixed in an aqueous solution of ferrous sulfate and formalin to test for the presence of tanninlike materials. **4)** *P.* X *hortorum* (S) showing material which stained blue-black; and **5)** *P. acerifolium* (R) showing material which stained brown in the stem tissues. (X 40) **6)** Transverse stem section of *P.* X *hortorum* infected with *Xanthomonas pelargonii* showing several fascicles (arrows) displaying infected xylem vessel elements (X 18). S = susceptible; MR = moderately resistant; R = resistant.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discrete fascicles were joined by secondary tissues derived from the developing interfascicular cambium to form a continuous cylinder of vascular tissues within the stem. The pith cylinder was surrounded by the xylem. The epidermis was a single layer of compact cells with somewhat thickened walls, and glandular or simple trichomes. It was covered by a thin, but continuous, cuticle. The stomata of all species corresponded to the anomocytic type (4), and were overarched by the cuticle. The cortex was a cylinder extending from the epidermis to the phloem fibers, and was composed of parenchyma with intercellular spaces. Druses were observed in the cortex of all species, usually in vertical files immediately adjacent to the phloem fibers. The outermost layer of phloem was a continuous or discontinuous ring of thick-walled fibers, whereas the inner layers of the phloem were all thin-walled cells. All secondary phloem cells were thin-walled. The xylem was composed of vessel elements and parenchyma cells, and, in the secondary xylem, nucleate fibers. In the secondary xylem, the vessel elements and nucleate fibers predominated, whereas in the primary xylem, the parenchyma cells appeared most numerous. Secondary and metaxylem vessel elements were scalariform or reticulate, and had slanted ends with a single large perforation. Protoxylem vessel elements had helically thickened secondary walls, which when viewed in longitudinal section appeared as a coiled spring. However, in preparing transverse sections, often only a portion of a single coil was included in the section, and this should be kept in mind when interpreting certain illustrations. The pith was composed of large, thin-walled cells with large intercellular spaces.

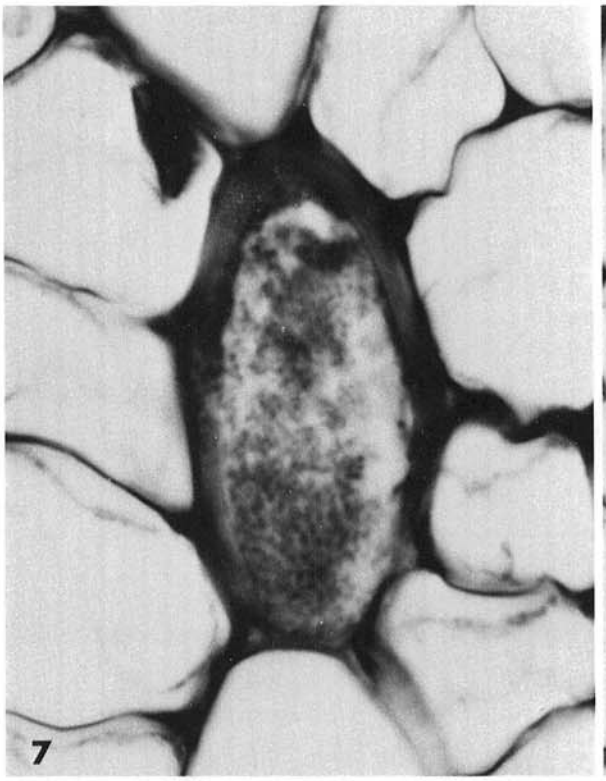
In moderately resistant and resistant *Pelargonium* spp., amorphous materials were present in stem tissues stained with Johansen's quadruple stain (Fig. 2, 3). In stems of all *Pelargonium* spp. fixed in an aqueous solution of ferrous sulfate and formalin (12), materials giving a positive reaction occurred in all tissues except the cambium in both inoculated and noninoculated plants (Fig. 4, 5). In the susceptible *P.* × *hortorum* (Fig. 4) and *P. peltatum*, blue-black deposits occurred in the cells or around the inside of the cell walls. In the moderately resistant and resistant *P. fulgidum, P. graveolens, P. acerifolium* (Fig. 5), and *P. domesticum*, brown deposits occurred except in the xylem parenchyma where the color was blue-black to brown.

Histopathology of the susceptible response in P. × *hortorum*.—The pathogen was detected initially in the stem as a mass of cells occluding the lumen of one or more xylem vessel elements in several fascicles (Fig. 6, 7). The bacterial masses occurred in both primary and secondary xylem vessel elements, although further spread of the pathogen occurred only from the protoxylem vessel elements. Bacterial masses filled the pit chambers and/or the lumen of xylem vessel elements. The bacteria occurred in long strands which passed through several adjacent vessel elements via the large perforations in the ends. Affected vessels did not show any noticeable morphological changes due to the presence of the pathogen.

Later, the pathogen was observed in one or more xylem parenchyma cells adjacent to the affected vessels (Fig. 8). The pathogen seemed to occur at random in any or all adjacent cells (Fig. 9, 10) rather than in a specific cell or cells. Affected xylem parenchyma cells had portions of their walls missing; generally, those walls were nearest the masses of bacteria (Fig. 9). The pathogen partially (Fig. 9) or completely filled the lumen of affected parenchyma cells (Fig. 8). The pathogen was clearly intracellular and intercellular at this point. The originally affected xylem vessel element and nearby vessel elements were soon surrounded by bacterial masses formed in space previously occupied by xylem parenchyma cells (Fig. 11). There was no accumulation of cell wall material at the periphery of the bacterial pocket, nor was there any apparent lysis of the secondary walls of the vessel elements. Microscopic observation using polarized light showed that birefrigence of walls of the xylem vessel elements was not affected by the presence of the bacterial masses, and that no birefringent materials had accumulated at the periphery of the pocket (Fig. 12).

The pocket increased in size as the pathogen was observed in additional adjacent xylem cells (Fig. 13). The pocket soon encompassed all the xylem tissues of a fascicle. The pathogen then was observed in the cambium, and appeared to spread rapidly into the phloem, cortex, and epidermis. Invaded cells collapsed, and were partially or completely decomposed. Eventually, even the cellulosic secondary walls of the phloem fibers disintegrated. As opposed to localized pocket formation in the xylem, extensive tissue collapse occurred in the pith, cortex, and epidermis after invasion of a portion of the tissue by *X. pelargonii* (Fig. 14). At this stage, the invaded area extended from the edge of the pith to the epidermis in a portion of the stem. Further invasion of the remaining healthy tissues brought about the death of all of the stem tissues, and eventually the plant. The progression of disease development in stems of *P.* × *hortorum* from the occlusion of a single xylem vessel element in a fascicle (Fig. 6) to the occlusion of numerous xylem vessel elements and initiation of pocket formation (Fig. 10) to the development of externally visible lesions (Fig. 14) and collapse of

Fig. 7-10. Transverse stem sections of *Pelargonium* × *hortorum* infected with *Xanthomonas pelargonii.* **7)** Closeup of an occluded protoxylem vessel element showing the granular mass of bacteria (× 1,870). **8)** Closeup of a xylem vessel element filled with bacteria and adjacent parenchyma cells, two of which are filled with bacteria (× 1,660). **9)** Portion of a transverse stem section showing parenchyma cells containing bacteria adjacent to infected xylem vessel elements. Bacteria appear scattered (S) throughout the affected parenchyma cells as well as massed (N) in areas. Note the apparent dissolution (arrows) of parenchyma cell walls (× 780). **10)** Continued development of *X. pelargonii* within the stem and initiation of pocket formation (arrow) (× 14).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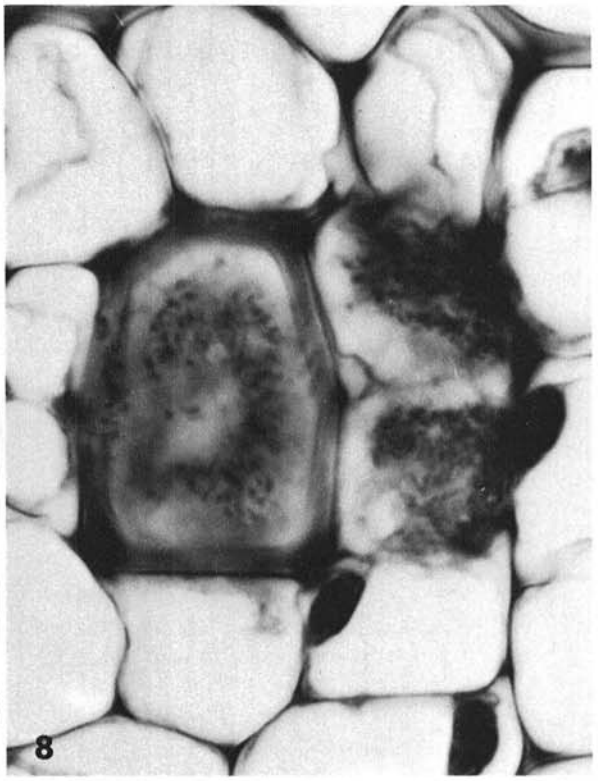
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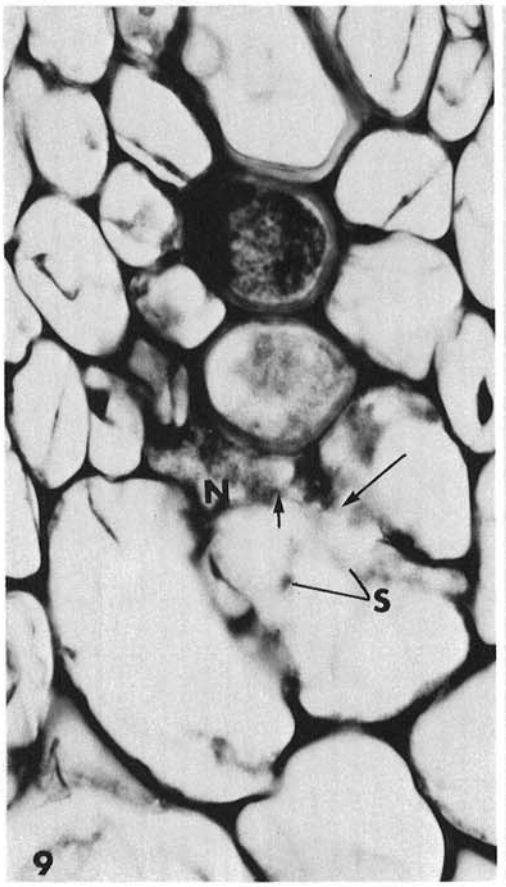
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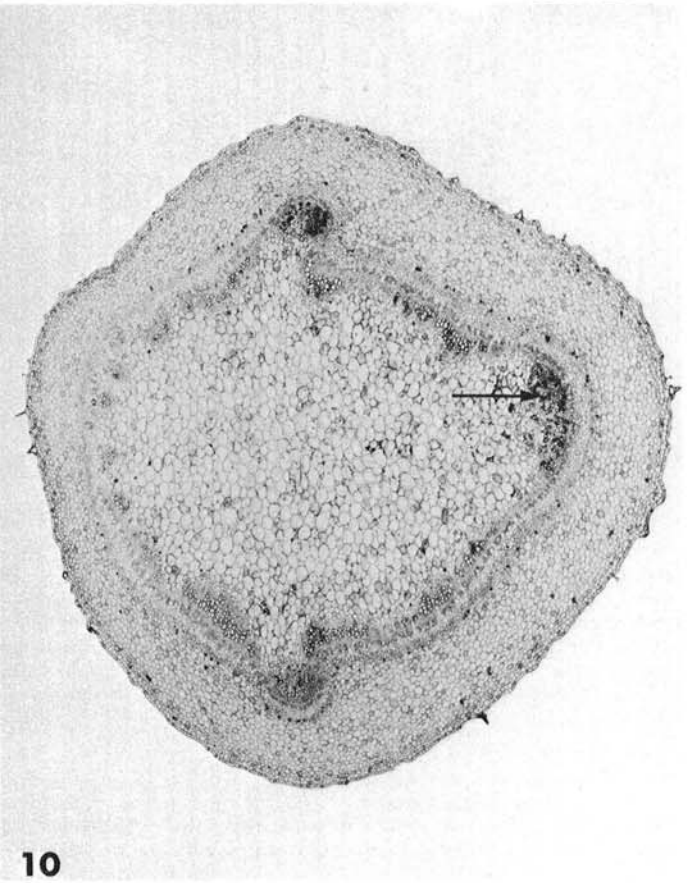
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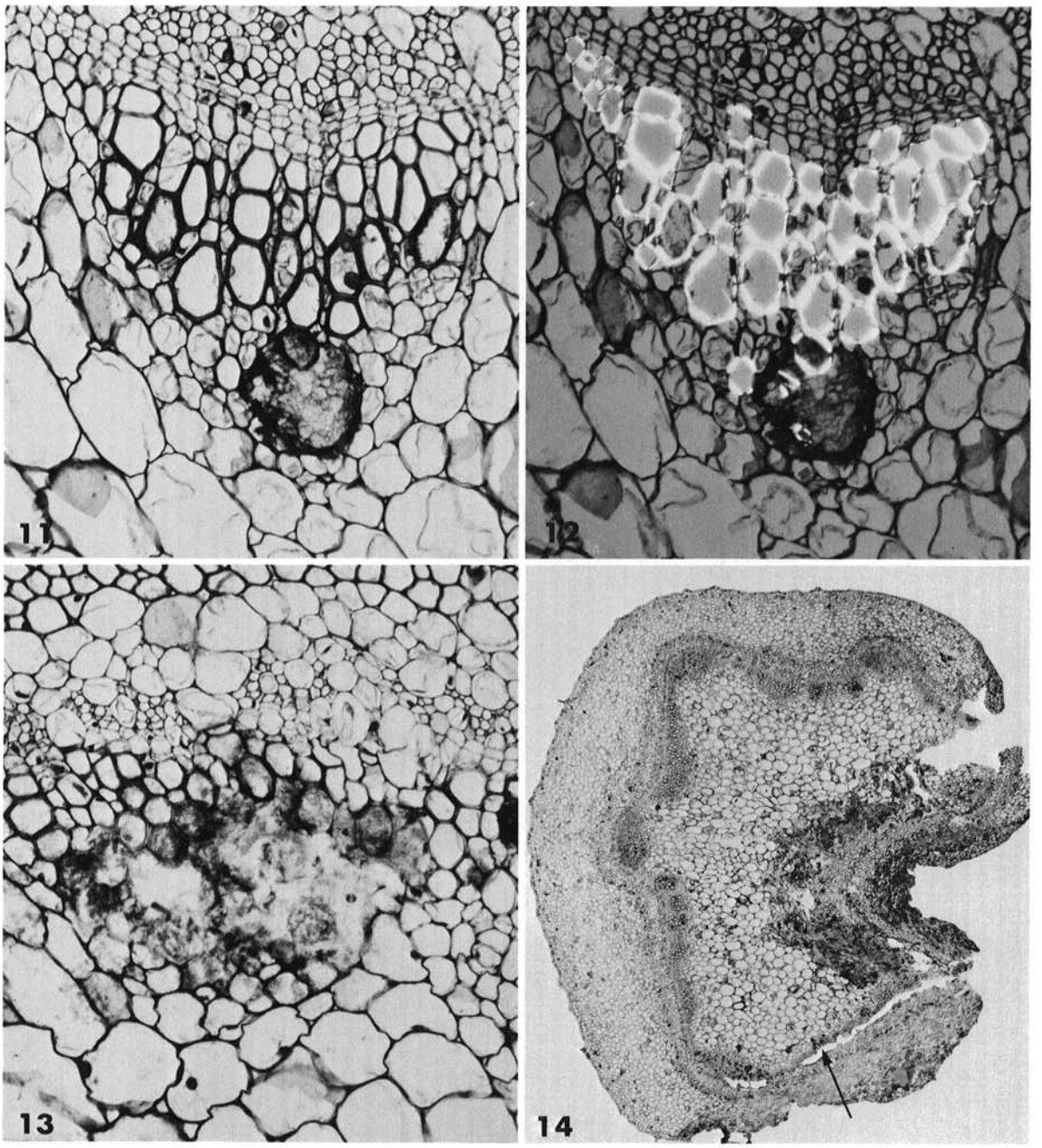
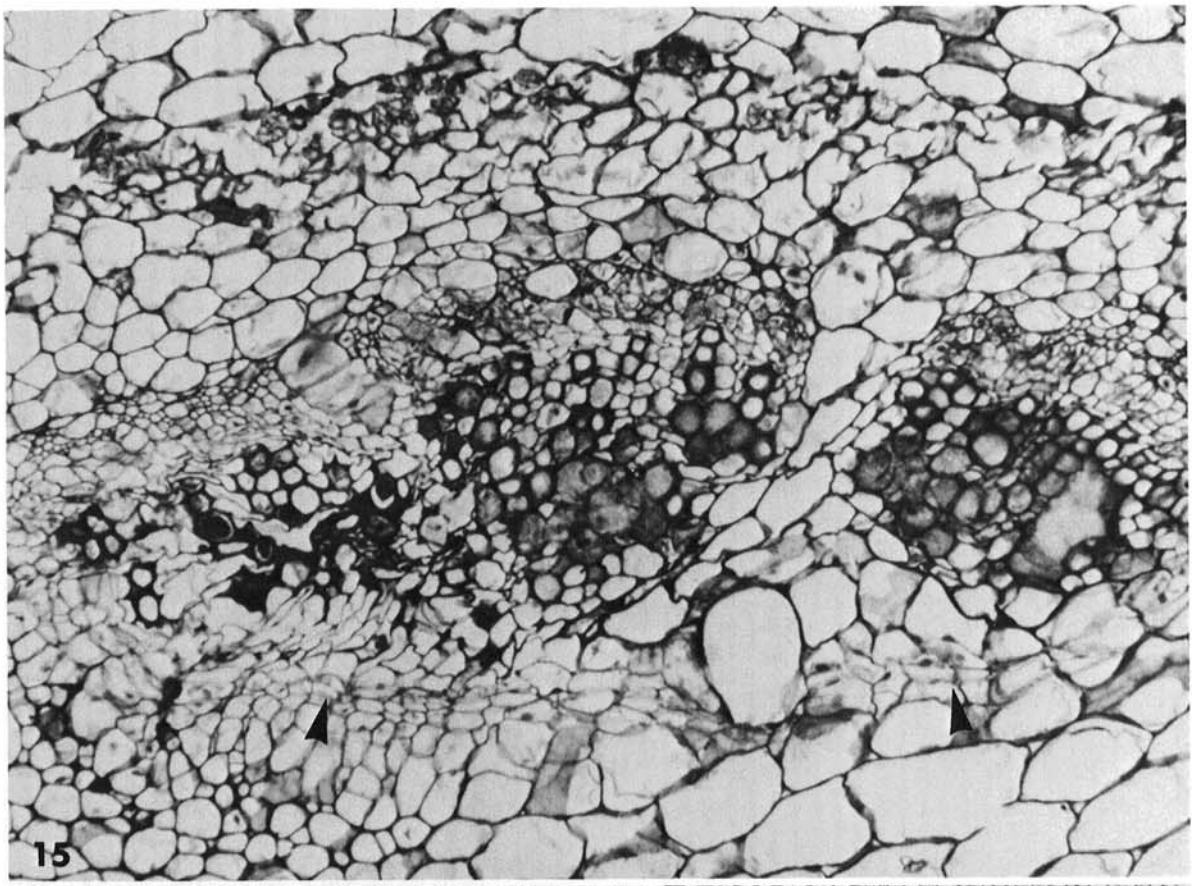


Fig. 11-14. Transverse stem sections of *Pelargonium* × *hortorum* infected with *Xanthomonas pelargonii*. **11, 12)** Portion of a transverse stem section showing early development of the bacterial pocket in the protoxylem and surrounding tissue viewed under transmitted light (11) and partially polarized light (12). Note the birefrigence of the cell walls of xylem vessel elements in the bacterial pocket. (× 170). **13)** Portion of a transverse stem section showing disintegration of the xylem tissues as the bacterial pocket enlarges (× 170). **14)** Transverse stem section showing collapse of invaded tissues in a section of the stem. Note that cells with secondary walls persist, whereas cells with only primary walls do not; note the rapid dissolution of the cambium (arrow) and collapse of phloem, cortex, and epidermis in the affected area (× 11).

Fig. 15-17. Portions of transverse stem sections of *Pelargonium* spp. infected with *Xanthomonas pelargonii* showing affected fascicles surrounded by several layers of cells (arrowheads). **15)** *P.* × *hortorum* (S) (× 135). **16)** *P. fulgidum* (MR) (× 225). **17)** *P. acerifolium* (R) (× 130). S = susceptible; MR = moderately resistant; R = resistant.



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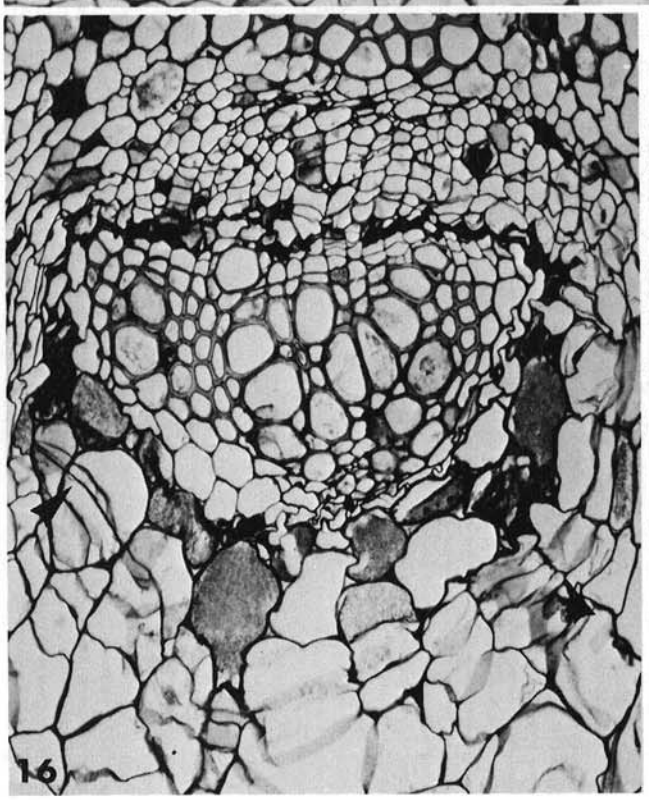

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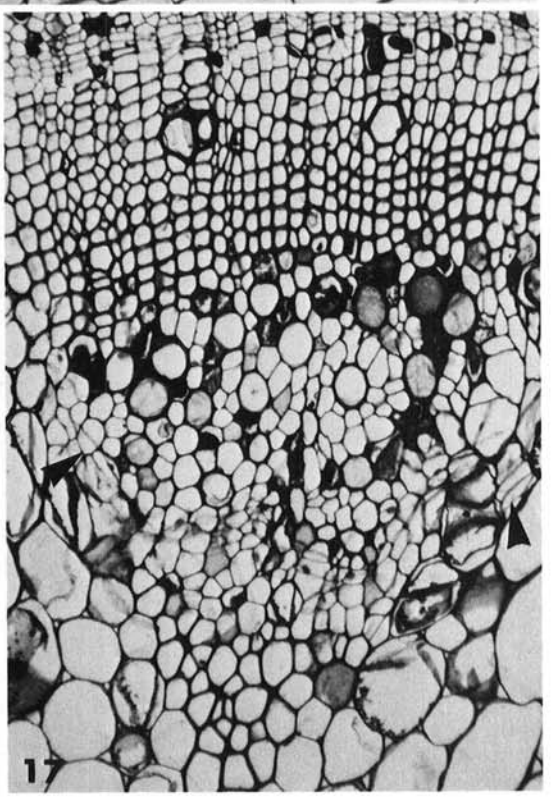

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most or all stem tissues was characteristic of this disease.

Affected portions of fascicles were often surrounded by a proliferated ring of several cell layers (Fig. 15-17). This ring of cells was usually continuous with the fascicular cambium of a bundle. Just inside this ring were several cell layers which were not filled with bacterial cells. Histochemical tests with $ZnCl_2$-KI (23) showed a normal cellulose reaction for all cells except those just inside the proliferated ring. These cells gave a positive reaction for suberin when stained with Sudan IV, but a negative reaction to all other histochemical tests.

Tyloses (Fig. 18-20) were present in the xylem vessel elements of inoculated plants. Tyloses were also noted in the vessels of noninoculated plants. Frequently, vessel elements not containing bacterial cells displayed a deposit on their walls (Fig. 21). This deposit generally appeared as a roughened layer somewhat constricting the lumen. It gave a positive reaction for lignin and a negative reaction to all other tests.

Histochemical tests for cellulose (Zn-Cl-I) showed the absence of cellulose walls in the bacterial pockets, and the absence of cellulose materials accumulated at the periphery of the pocket. Tests for pectic compounds with ruthenium red indicated the presence of pectic compounds between the walls of partially degraded cells and the absence of such materials from badly decomposed areas, such as the bacterial pockets. In collapsed and partially decomposed tissues, a generalized faint positive reaction was observed. There was no apparent difference in starch content due to the presence of the pathogen as determined by tests with iodine-potassium iodide.

The pathogen was observed above and below the point of inoculation, and was present in the apical 5 mm of the shoot as well as at the crown. The pathogen could always be isolated from inoculated plants.

Histopathology of the resistant response in P. acerifolium, P. fulgidum, P. graveolens, P. radens.—All of the moderately resistant and resistant species responded similarly to infection as did *P. X hortorum*, differing only in degree of development of the pathogen. The pathogen was observed above and below the point of inoculation, and was present in the apical 5 mm of the shoot as well as at the crown of the plant. The pathogen could be readily isolated from all inoculated plants throughout the duration of this study. The resistant response was characterized by the limited establishment and proliferation of the pathogen in the xylem tissues, and by reduced lateral spread of the pathogen.

DISCUSSION.—The gross anatomical aspects of the epidermis, cortex, phloem, cambium, xylem, and pith of healthy plants were very similar in all the species observed. There were no differences, particularly in the xylem, apparently great enough to account for the differences in resistance. The distribution of paratracheal axial parenchyma cells was similar. The mode of spread of the pathogen was similar in all species, initially involving movement throughout the plant in the xylem vessel elements and subsequent spread laterally out of affected xylem vessel elements to adjoining cells. The invasion of adjacent xylem parenchyma cells, rapidity of such spread, and the extent of further development of the pathogen varied with the species.

Xanthomonas pelargonii was consistently isolated from stems of both susceptible and resistant species throughout the 2-month duration of this study, indicating that *X. pelargonii* was surviving and reproducing even in resistant plants. Thus, we may conclude that disease resistance does not simply involve eradication of the pathogen in resistant plants.

Initiation of bacterial pockets occurred only from affected protoxylem vessel elements, although both primary and secondary xylem vessel elements were invaded initially. No breakdown of the helically thickened secondary walls of protoxylem vessels was observed, and no other obvious alterations were noted. Protoxylem vessel elements are the first xylem tissues to be differentiated in the stem. Their differentiation and maturation takes place before internodal elongation in the stem is completed. These nonliving vessel elements do not keep pace with the elongation by active growth, and thus are stretched passively (5). This often results in complete destruction of the protoxylem vessel elements, but our observations on the protoxylem of *Pelargonium* spp. indicate that such vessel elements are stretched and crushed, but not completely obliterated. It is from these functional vessel elements that bacterial pockets are initiated. The bacterial cells occluding the lumen of such vessel elements are separated from the lumens of adjacent parenchyma cells only by a thin, three-layered structure consisting of primary wall-middle lamella-primary wall. The pathogen may escape through discontinuities in the stretched, crushed primary wall of affected protoxylem vessel elements and/or through degraded portions of the primary wall. The primary pit fields would seem particularly vulnerable to tearing and/or dissolution.

No host cell wall materials, other than lignin, could be discerned in bacterial pockets by microscopic observation and histochemical techniques, indicating their dissolution during pathogenesis. The failure of cell wall materials to accumulate at the periphery of bacterial pockets, and the disorganization and disorientation of xylem vessel elements in bacterial pockets, tends to substantiate the hypothesis that dissolution of host cell walls occurs during pathogenesis. Goto & Okabe (8) report that xanthomonads produced cellulolytic enzymes in vitro, as evidenced by the liquefaction of a carboxymethylcellulose gel medium. Dye (3) showed that *X. pelargonii* produces pectolytic enzymes in vitro, especially calcium pectate-liquefying enzymes, polygalacturonase, and protopectinase. In addition, most species of *Xanthomonas* are reported to be strongly lipolytic. Thus, while further work is required to conclusively demonstrate the relationship between enzymes and pathogenicity, the available evidence supports the hypothesis that dissolution of host cell walls is an essential part of pathogenesis of the susceptible response.

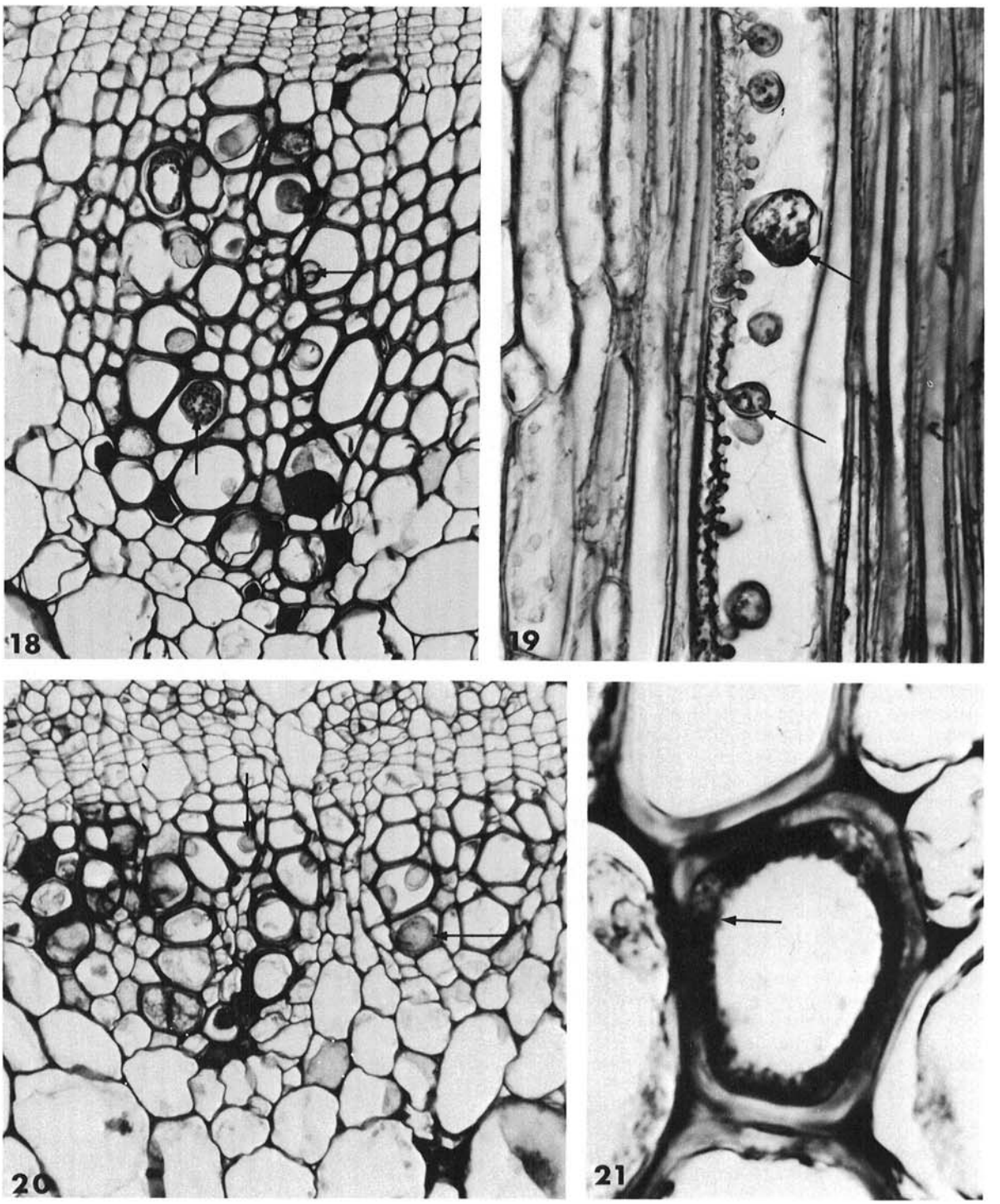

Fig. 18-21. Portions of stem sections of *Pelargonium* spp. infected with *Xanthomonas pelargonii.* **18)** Transverse stem section of *P. radens* (MR) showing tyloses (arrows) formed in the lumens of the xylem vessel elements (× 220). **19)** Longitudinal stem section of *P. graveolens* (R) showing tyloses (arrows) formed in the lumen of a xylem vessel element (× 340). **20)** Transverse stem section of *P.* × *hortorum* (S) showing tyloses (arrows) formed in the lumens of the xylem vessel elements (× 265). **21)** Xylem vessel element of *P.* × *hortorum* (S) showing deposition of ligninlike materials (arrow) on the inside of the cell wall (× 1,230). S = susceptible; MR = moderately resistant; R = resistant.

The origin and role of the ligninlike deposits on vessel walls of affected fascicles was not determined. It appears that these deposits correspond to the "hyperlignification" of vessel walls of *P. zonale* infected by *X. pelargonii* as described by Lemattre (15, 16). The role of tyloses in this disease was not clear, as tyloses were found in equal numbers in resistant and susceptible species.

The formation of a ring of cells around affected portions of a fascicle and the deposition of suberinlike materials on walls of cells within this ring appear to be responses of the host to the pathogen, as no such phenomena occurred in noninoculated plants. Lemattre (15, 16) reported the same phenomena in *P. zonale* infected with *X. pelargonii*. Bugbee & Anderson (2) reported meristematic activity of leaf mesophyll cells of *P.* × *hortorum* following infection by *X. pelargonii*. This resulted in the elevation of the leaf blisters above the surface of the leaf, when the blistered areas were found to be composed of corksuberized cells and "crushed" mesophyll cells. They proposed this as a defense mechanism preventing spread of the pathogen. These responses in the stems and leaves of *Pelargonium* spp. appear similar. They both have features common to the wound-healing response of plants (4) in which fatty substances, including suberin, are deposited on walls of affected cells followed by meristematic activity of adjacent cells. There seemed to be a positive correlation between the amount of host tissue affected by the pathogen and the degree of development of this host response; the response was well developed in the susceptible and moderately resistant species, and only weakly developed in the highly resistant species. We interpret this as a secondary defense reaction restricting the lateral spread of the pathogen. It would appear particularly effective in the moderately resistant species, where the primary defense reactions slow the development of the pathogen and allow complete development of this response in the early stages of pathogenesis.

Tanninlike materials were found in stem tissues of all inoculated and noninoculated *Pelargonium* spp. tested. The tanninlike materials occurred as blue-black deposits in susceptible species and brown deposits in resistant species. The significance of the color difference is not known, but may indicate that a different type of tanninlike material occurs in the resistant species and may be, in part, responsible for resistance to *X. pelargonii*.

A great deal of literature deals with the role of tannins (phenolics) in plant disease resistance. The best known example for the protective role of performed phenolics is the onion-*Colletotrichum circinans* complex (25), where resistance of onion cultivars is correlated with red or yellow pigmentation of the bulb scales. Resistance was reportedly due to the ability of the phenolics associated with these pigments to diffuse into the infection drop, inhibit germination of *C. circinans*, and prevent penetration. There does not appear to be a similar example involving preformed phenolics and disease resistance to bacterial pathogens. However, phenolics have been reported as bacteriostatic agents and enzyme inhibitors (7), generally inhibiting pectolytic and cellulolytic enzymes (6, 14, 17). In the *Pelargonium* spp. infected with *X. pelargonii*, tannin-induced bacteriostasis could account for the inability of the pathogen to become established and proliferate widely in resistant plants. Tannin-induced cellulolytic and pectolytic enzyme inhibition could account for the lack of lateral spread and bacterial pocket formation. Based on the evidence presented in this paper, we suggest that resistance of *Pelargonium* spp. to *X. pelargonii* is due to preformed substances present in resistant species prior to infection, and to their effect on the pathogen and/or the effect on the host-parasite physiology. This study points out the need for a more detailed investigation on the nature of the substances involved and their effects on the pathogen. The usefulness of histopathology in elucidating the nature of plant disease relationships is clearly demonstrated.

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