Histopathology of a Symptomless Chrysanthemum Cultivar Infected by *Erwinia chrysanthemi* or *E. carotovora* subsp. *carotovora*

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ABSTRACT

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A histological study was made on rooted cuttings of *Chrysanthemum morifolium* 'White Marble' infected with either *Erwinia carotovora* subsp. *carotovora* (*E. c.* subsp. *carotovora*) or *E. chrysanthemi* to determine why infected plants of that cultivar remain symptomless under normal growing conditions. Both *Erwinia* species were distributed rapidly in the host vascular system and caused breakdown of the xylem parenchyma cells and formation of hypertrophied, hyperplastic and chromophilic xylem parenchyma cells around the infected vascular bundle. Vascular plugging also was present in the later stages of host reaction. Histochemical tests revealed suberin in the host cells surrounding infected vascular bundle and a decrease in pectic material in infected xylem parenchyma cells.

Host reaction to isolates of *E. c.* subsp. *carotovora* was more intense than that caused by *E. chrysanthemi*. *E. c.* subsp. *carotovora*, but not *E. chrysanthemi*, was restricted to the initially infected vascular bundle. Suberin was present earlier and in greater amounts in plants infected with *E. c.* subsp. *carotovora*. The host reaction of hypertrophied, hyperplastic, and chromophilic cells around the infected vascular bundle and the formation of suberin may explain, in part, the lack of symptoms in cultivar White Marble infected with *E. c.* subsp. *carotovora* and *E. chrysanthemi*.

Erwinia chrysanthemi, the causal organism of bacterial blight of *Chrysanthemum morifolium* Ramat. (2) and *E. c.* subsp. *carotovora*, incitant of bacterial soft rot commonly have been isolated from chrysanthemums showing symptoms of these diseases (3). The soft rot symptoms induced in chrysanthemum by *E. chrysanthemi* and *E. c.* subsp. *carotovora* under conditions of high humidity and temperature are similar. Under dryer environmental conditions, however, chrysanthemum cultivar White Marble shows some tolerance to these two pathogens (9). Smith (12) reported that cuttings taken from symptomless infected stock plants exhibited basal rot and hollow stem symptoms after being placed on the propagation bench. Such cuttings are worthless to chrysanthemum propagators. When *E. c.* subsp. *carotovora* was introduced into the vascular tissue of White Marble chrysanthemum, tissue breakdown was not evident unless the bacteria had access to pith tissue (13).

This study was initiated to investigate the absence of bacterial blight symptoms in plants of chrysanthemum cultivar White Marble infected with either *E. c.* subsp. *carotovora* or *E. chrysanthemi*.

MATERIALS AND METHODS

Rooted cuttings of *Chrysanthemum morifolium* 'White Marble' (Yoder Brothers, Barberton, OH 44203) were potted in 10.2-cm (4-inch) diameter clay pots containing a steam-treated peat:perlite:soil (1:1:1, v/v) mixture and placed on a greenhouse bench under continuous incandescent light to prevent flower bud initiation.

The plants were inoculated with either *E. chrysanthemi* (strain EC-16 from chrysanthemum) or *E. c.* subsp. *carotovora* (strain 311 from chrysanthemum) 23 days after potting. The inoculum was grown for 48 hr on Difco nutrient agar slants. Thirteen plants were inoculated with *E. chrysanthemi*, 39 plants were inoculated with *E.*

c. subsp. *carotovora* and four plants were uninoculated. The inoculation technique, described previously (9), consisted of stabbing the midrib of the petiole of the third youngest leaf with a fine sewing needle smeared with bacterial cells. A sterile needle was inserted into the petiole of the uninoculated controls. Following inoculation, the plants were placed under continuous light and mist in a heated mist chamber. The temperature in the chamber ranged from 25 to 34 C and the relative humidity was maintained at 100% as determined by wet bulb/dry bulb copper-constantan thermocouples and recorded on a Honeywell multipoint strip chart recorder.

Plants were sampled at 2, 4, 6, 8, 10, 12, 24, 31, 48, 55, 72, and 96 hr after inoculation. Each sample consisted of three plants inoculated with *E. c.* subsp. *carotovora* strain 311 and one plant inoculated with *E. chrysanthemi* strain EC-16. Once every 24 hr an uninoculated control plant was sampled. The leaves and roots were removed from the plants to be sampled and the main stems were outlined on paper. The stems were surface sterilized in a 10% solution of Clorox (5.25% sodium hypochlorite) for 5 min, and the stem was sectioned into 5-mm pieces with sterile razor blades (9). Alternate pieces were fixed in Rawlins formalin-acetic acid-alcohol (FAA) solution No. 1 (10) and the remaining pieces were placed aseptically into individual tubes containing Difco nutrient broth. The broth tubes were incubated at 21 C for 10 days and examined for bacterial growth.

After incubation, the position of stem pieces in the nutrient broth tubes showing evidence of bacterial growth was recorded on the stem diagram and a sample from each was streaked onto nutrient agar in petri plates and incubated at 21 C. Bacterial colonies morphologically resembling *Erwinia* were transferred to nutrient agar slants. To determine the spread of the pathogen in the host stem, all cultures were subjected to the potato tuber rot test (9). Cultures inducing a rot in potato tuber slices were tested for their ability to cause bacterial blight symptoms in chrysanthemum. All cultures suspected of being *E. chrysanthemi* were tested for pathogenicity on the susceptible chrysanthemum cultivar Giant No. 4 Indianapolis White (9). Cultures suspected of being *E. c.* subsp. *carotovora* were tested on unrooted cuttings of the

chrysanthemum cultivar White Marble, under conditions suitable for symptom expression.

The pathogenicity test for *E. c.* subsp. *carotovora* was carried out by soaking two freshly cut, unrooted White Marble cuttings in a suspension of the suspected isolate for 20 min and then sticking the cuttings in a flat of steam-treated peat:perlite (1:1, v/v) mixture. The bacterial suspension consisted of bacterial cells from two 48-hr-old nutrient agar cultures of the suspected isolate shaken with 60 ml of sterile water. Cuttings inoculated with isolates suspected of being *E. c.* subsp. *carotovora* were widely spaced in the flat to minimize bacterial spread among cuttings. Cuttings inoculated with known isolates of *E. c. carotovora* and *E. chrysanthemi* and noninoculated cuttings were also incorporated into this test. After 7 days under mist, the cuttings were removed and examined for basal soft rot. Presence of basal rot was considered positive evidence that the inoculated bacterial isolate was *E. c.* subsp. *carotovora*. Information from both the potato tuber rot test and the chrysanthemum pathogenicity test was recorded on the appropriate stem diagram. These procedures allowed us to trace the movement of the pathogen in the chrysanthemum stem.

The 5-mm pieces in FAA were dehydrated in a tertiary butyl alcohol series, infiltrated, and embedded in Paraplast (Scientific Products, Obetz, OH 43207) (7). The tissue was softened for 24 hr in a solution of 90 ml of 1% sodium lauryl sulfate (Dreft, Proctor and Gamble, Cincinnati, OH 45202) and 10 ml of glycerol (1) prior to sectioning at 10 μm on a Leitz rotary microtome. Transverse and longitudinal serial sections were mounted on chemically clean slides with Haupt's adhesive and stained with Harris' Hematoxylin and Orange G (7). A few slides were stained with Johansen's Quadruple stain (7).

Histochemical tests were conducted on selected tissue for pectin (iron absorption method), lignin (Maüle reaction), cellulose (zinc-chlor-iodide reaction and polarized light), suberin (Sudan IV), and gums (orcinol and phloroglucinol) (6,11).

RESULTS

Isolations and pathogenicity tests. Bacteria were isolated from both inoculated and uninoculated chrysanthemum stems. Although we isolated bacteria with colony morphology resembling that of *Erwinia* sp. from the inoculated chrysanthemum, the large number of unidentified bacteria associated with the chrysanthemums made pathogenicity tests necessary to verify the identity of the *Erwinia* isolates.

Fifty percent of the Erwinialike bacterial isolates from plants inoculated with *E. chrysanthemi* rotted potato tuber tissue. However, only 32% of those bacteria caused bacterial blight symptoms in plants of chrysanthemum Giant No. 4 Indianapolis White. *E. chrysanthemi* was recovered 40 mm basally from the inoculation point 2 hr after inoculation, and at varying distances after that time. Isolation of the pathogen was inconsistent after 31 hr following inoculation.

Seventy-five percent of the bacteria isolated from plants inoculated with *E. c.* subsp. *carotovora*, had colony morophology resembling *Erwinia* and caused rot in potato tubers. All of the bacteria that induced tuber rot also caused a basal soft rot on unrooted cuttings of White Marble, and were thus identified as *E. c.* subsp. *carotovora*. *E. c.* subsp. *carotovora* was recovered 45–50 mm basally from the inoculation point 2 hr following inoculation and throughout the study was isolated at various distances into the stem from the point of entry.

Histology of uninoculated plants. The anatomy of the stem of *C. morifolium* 'Giant No. 4 Indianapolis White' was described (8). The anatomy of stems of cultivar White Marble closely resembled that of Giant No. 4 Indianapolis White, differing only in the pith tissue. Pith of cultivar White Marble had large intercellular spaces between the parenchyma cells (Fig. 1) whereas Giant No. 4 Indianapolis White had small intercellular spaces in the pith tissue (Fig. 2). Random amphivasal vascular bundles occurred infrequently in the pith of White Marble plants and were absent in Giant No. 4 Indianapolis White plants.

Histology of plants inoculated with *E. c.* subsp. *carotovora*. The

bacteria entered the main stem of the chrysanthemum via the xylem vessel elements of the leaf trace and colonized the vessel elements of the vascular bundle from which the trace emerged. Although *E. c.* subsp. *carotovora* was recovered at a few cm basally from the inoculation point 2 hr after inoculation, there was no histological evidence of its presence in stems until 24 hr after inoculation. Bacteria were observed in several xylem vessel elements and many of the xylem parenchyma cells had disintegrated 24 hr after inoculation (Fig. 3). The cells surrounding the xylem cavity were chromophilic and slightly hyperplastic. Although the vascular cavity formed by the breakdown of the xylem parenchyma cells was smaller further down the stem from the inoculation point, bacteria were still present in several vessel elements (Fig. 4). Hyperplasia of the parenchyma cells around the cavity and their chromophilic reaction were reduced as the distance from the point of entry increased.

The early (24–48 hr) evidence of invasion by *E. c.* subsp. *carotovora* consisted primarily of xylem vessel element colonization, some bacterial plugging of vessel elements, a reduction in wall thickness in xylem parenchyma cells surrounding infected vessel elements, and occasional breakdown of those cells (Fig. 5). There was a slight chromophilic reaction in the cells surrounding the infected vascular bundle, but this reaction was less distinct as the distance from the inoculation point increased.

Forty-eight hours after inoculation with *E. c.* subsp. *carotovora*, hypertrophied, hyperplastic parenchyma cells were observed around all or part of the infected vascular bundle. A strong chromophilic reaction also was present in the hypertrophied, hyperplastic cells nearest the infected xylem vessel elements (Fig. 6). Inside this ring of altered cells the xylem parenchyma cells and xylem vessel elements were broken down. The walls of the vessel elements of this infected vascular bundle had collapsed over the distance of a few micrometers whereas above and below the area they were essentially intact (Fig. 7–10). Such restricted areas of collapse might occur several times in one vessel element or in several vessel elements at different locations. Vessel element collapse of this nature released bacteria into the vascular cavity formed by the disintegration of xylem parenchyma cells.

Below the area of intensive host/pathogen interaction, the bacteria were confined to vessel elements and the surrounding xylem parenchyma cells were thin walled (Fig. 11). In this area a slight hyperplastic response was still present in the cells surrounding the vascular bundle, but the extensive host reaction described above was lacking. Bacteria escaped from the vessel elements at intervals down the stem and caused disintegration of xylem parenchyma cells resulting in cavities in the vascular tissue.

As the time after inoculation increased, plants inoculated with *E. c.* subsp. *carotovora* showed an increase in vessel element plugging, the continued presence of bacteria in the vessel elements and less breakdown of xylem parenchyma cells in the infected vascular bundle. Hypertrophied, hyperplastic cells continued to surround the infected vascular bundle.

Host reaction to the presence of *E. c.* subsp. *carotovora* was more pronounced at or near the juncture of the leaf trace and main stem (Fig. 12). Host response to invasion in this area consisted of hypertrophied, hyperplastic cells surrounding the infected vascular bundle. The cells nearest the infected vascular tissue also were chromophilic. The host reaction in areas farther from the inoculation point was limited to the presence of chromophilic cells surrounding the infected vascular bundle (Fig. 13). As the time from inoculation increased, the anatomical reaction of the host to infection was less intense.

Ninety-six hours after inoculation, the only evidence of infection was the presence of plugged vessel elements (Fig. 14) and thin-walled xylem parenchyma cells. The anatomical response of the host was restricted to some hypertrophy and hyperplasia in the cells surrounding the infected vascular bundle at the point of juncture with the inoculated leaf trace. Below this area the cells described above showed only hypertrophy.

Host response to invasion by *E. c.* subsp. *carotovora* consisted primarily of the formation of hypertrophied, hyperplastic, and (occasionally) chromophyilic cells around the infected vascular
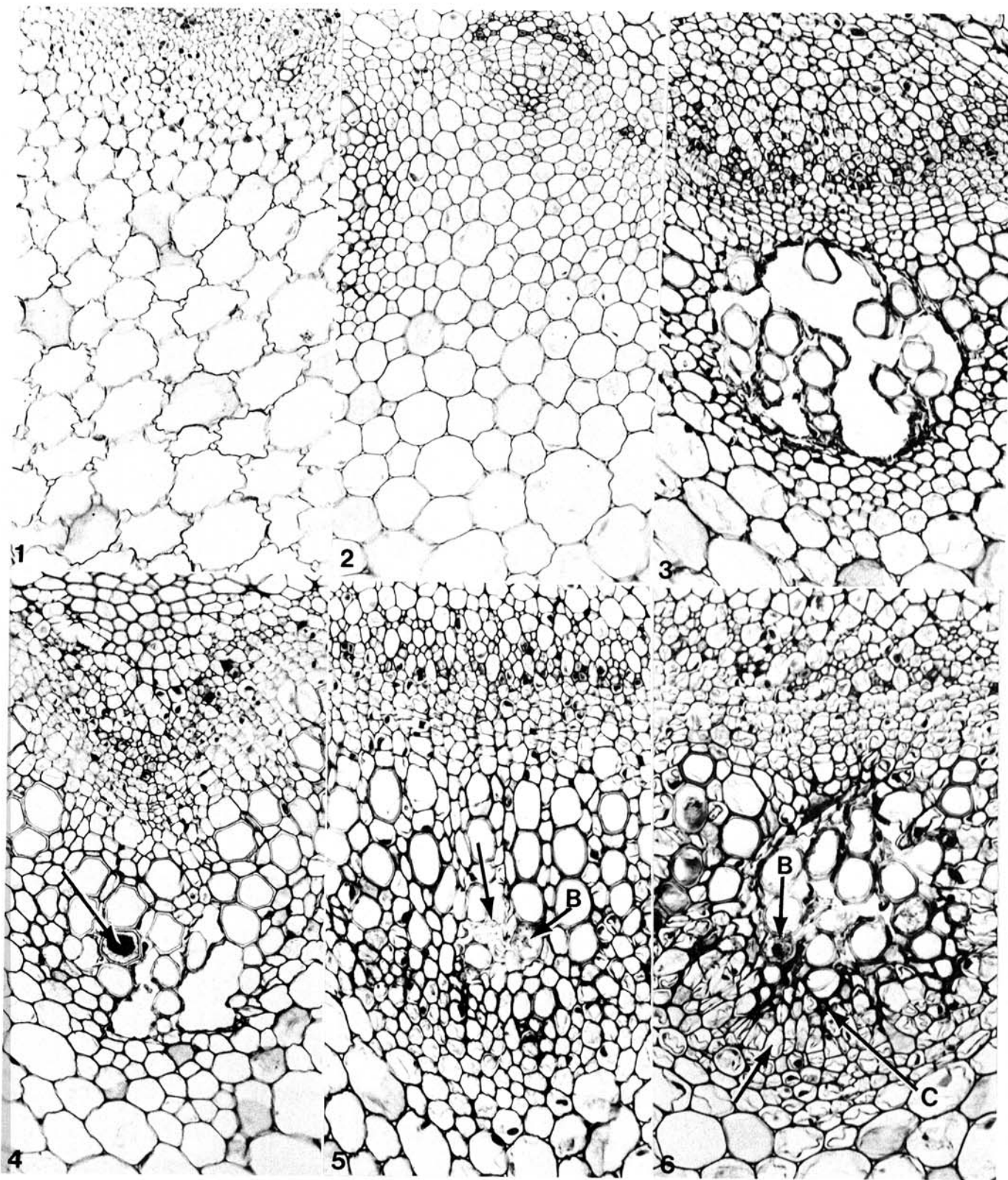
Figs. 1–6. 1, Portion of a cross section of a stem of an uninoculated plant of *Chrysanthemum morifolium* 'White Marble' showing the loosely packed cells in the pith with many large intercellular spaces (×175). **2,** Portion of a cross section of a stem of an uninoculated plant of *C. morifolium* 'Giant No. 4 Indianapolis White' showing the tightly packed cells in the pith with only a few small intercellular spaces (×175). Figs. 3–6 are portions of cross sections through stems of *C. morifolium* 'White Marble' inoculated with *E. c.* subsp. *carotovora.* **3,** Extensive xylem parenchyma cell breakdown caused by *E. c.* subsp. *carotovora* 24 hr after inoculation near the point of inoculation (×238). **4,** The amount of xylem parenchyma cell breakdown decreases as the distance from the inoculation point increases. Note the bacterial mass in one of the xylem vessel elements (arrow) (×219). **5,** *Erwinia c.* subsp. *carotovora* (B) has colonized one vessel element 31 hr after inoculation. Note the reduction in cell wall thickness in the xylem parenchyma cells near the infected vessel element (arrow) (×250). **6,** Hypertrophied, hyperplastic cells surround the infected vascular bundle (arrow) and the cells interior to the hypertrophied, hyperplastic cells are chromophyllic (C) 48 hr after inoculation. Breakdown of xylem parenchyma cells and bacteria filled xylem vessel elements (B) can be seen in the infected vascular bundle (×225).

bundle. These altered cells became noticeable 24 hr after the host was inoculated with the pathogen. Host response was most intense 48 hr after infection and was present thereafter for the duration of the study.

The activity of *E. c.* subsp. *carotovora* in the host was evidenced by the presence of bacterial cells in some vessel elements and the breakdown of vessel elements and xylem parenchyma cells. Pathogen destruction of host tissue varied in intensity throughout
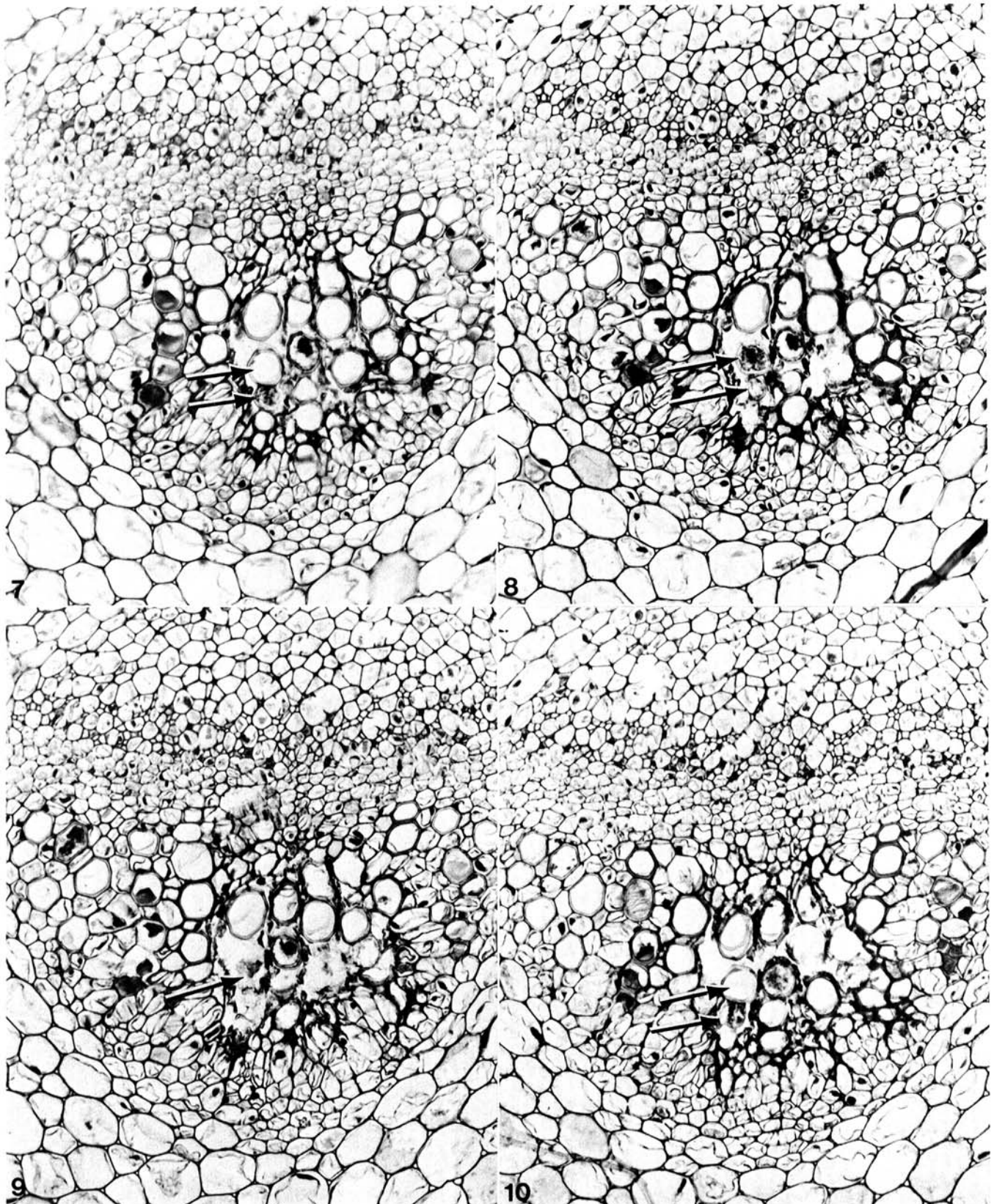

Figs. 7-10. Selected cross sections from an 80 μm portion of *Chrysanthemum morifolium* 'White Marble' 48 hr after inoculation with *E. c.* subsp. *carotovora.* **7,** The walls of the two xylem vessel elements (arrows) are intact in the initial section (×238). **8,** The walls of two xylem vessel elements (arrows) are breaking down 20 μm from the section in Fig. 7 (×238). **9,** The walls of two xylem vessel elements have disappeared (arrow) 30 μm from the section in Fig. 7 (×238). **10,** The walls of two xylem vessel elements are evident again (arrows) 80 μm away from the section in Fig. 7.

the length of each stem and was most pronounced 48 hr after inoculation. Breakdown of host tissue diminished after 48 hr and at 96 hr after inoculation was totally absent. At no time was *E. c.* subsp. *carotovora* found in pith tissue.

Histochemical tests on tissue from plants infected with *E. c.* subsp. *carotovora* indicated decreased pectic material in the xylem
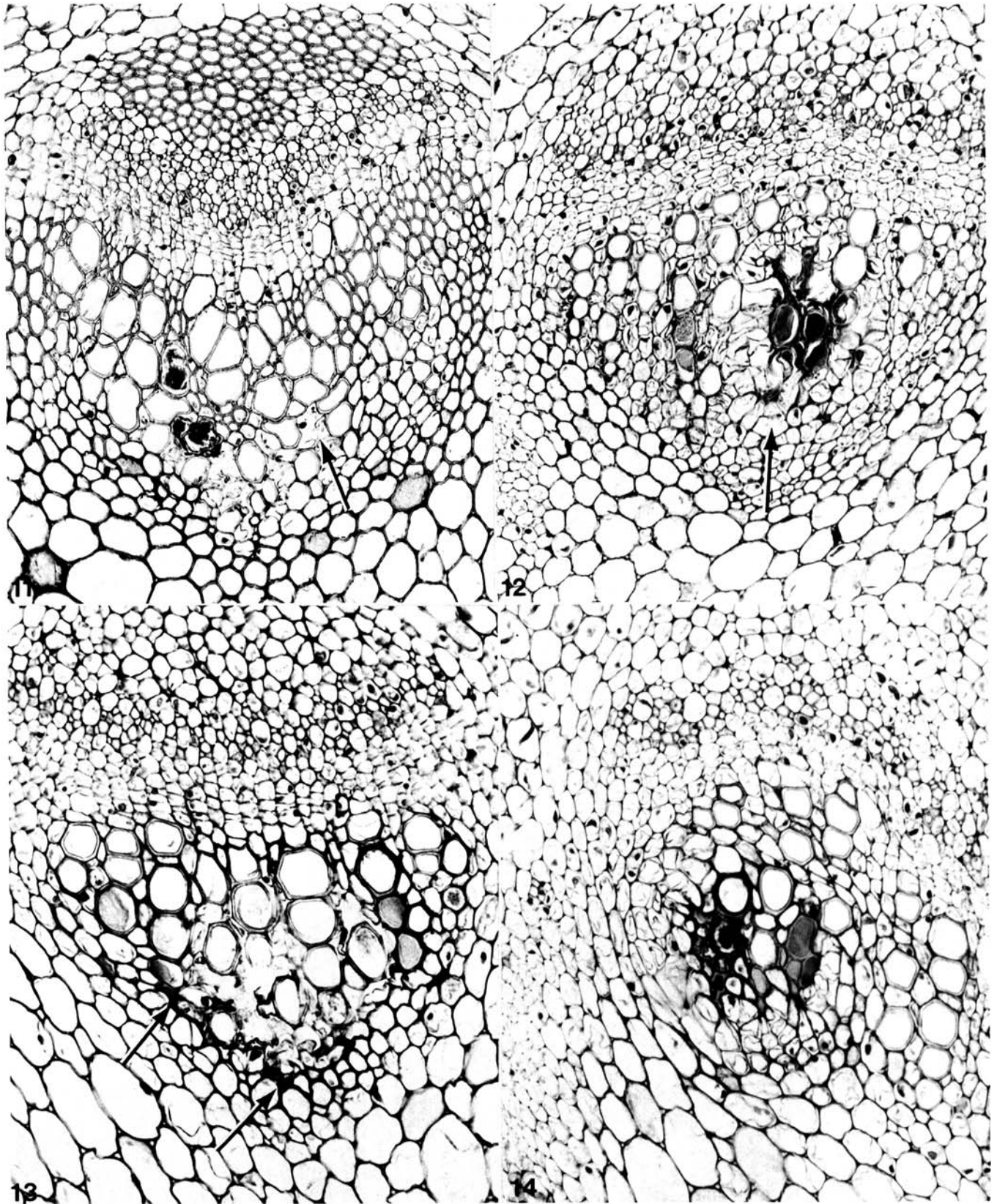
Figs. 11–14. Portions of cross sections through stems of *Chrysanthemum morifolium* 'White Marble' infected with *E. c.* subsp. *carotovora.* **11,** Near the base of the plant 48 hr after inoculation, the bacteria are evident in several vessel elements and the xylem parenchyma cells have thin walls (arrow) (×180). **12,** Occurrence of plugging in a few xylem vessel elements and extensive hypertrophy (arrow) and some hyperplasia in the cells around the infected vascular bundle 55 hr after inoculation (×230). **13,** Several millimeters down the stem from the section in Fig. 12, only chromophillic cells (arrows) surround the disintegrating vascular bundle (×350). **14,** Ninety-six hr after inoculation, the only evidence of infection is plugging in the xylem vessel elements and some hypertrophy in the cells surrounding this area (×294).

parenchyma cells of some of the infected vascular bundle. Suberin was produced and accumulated around the infected bundles surrounded by hyperplastic, hypertrophied, and chromophilic cells. It was present frequently in the vascular plugs and in the xylem vessel element walls as well as in the tissue surrounding the infected area. Suberin was initially detected 24 hr after inoculation with *E. c.* subsp. *carotovora,* and was consistently present through the remainder of the study.
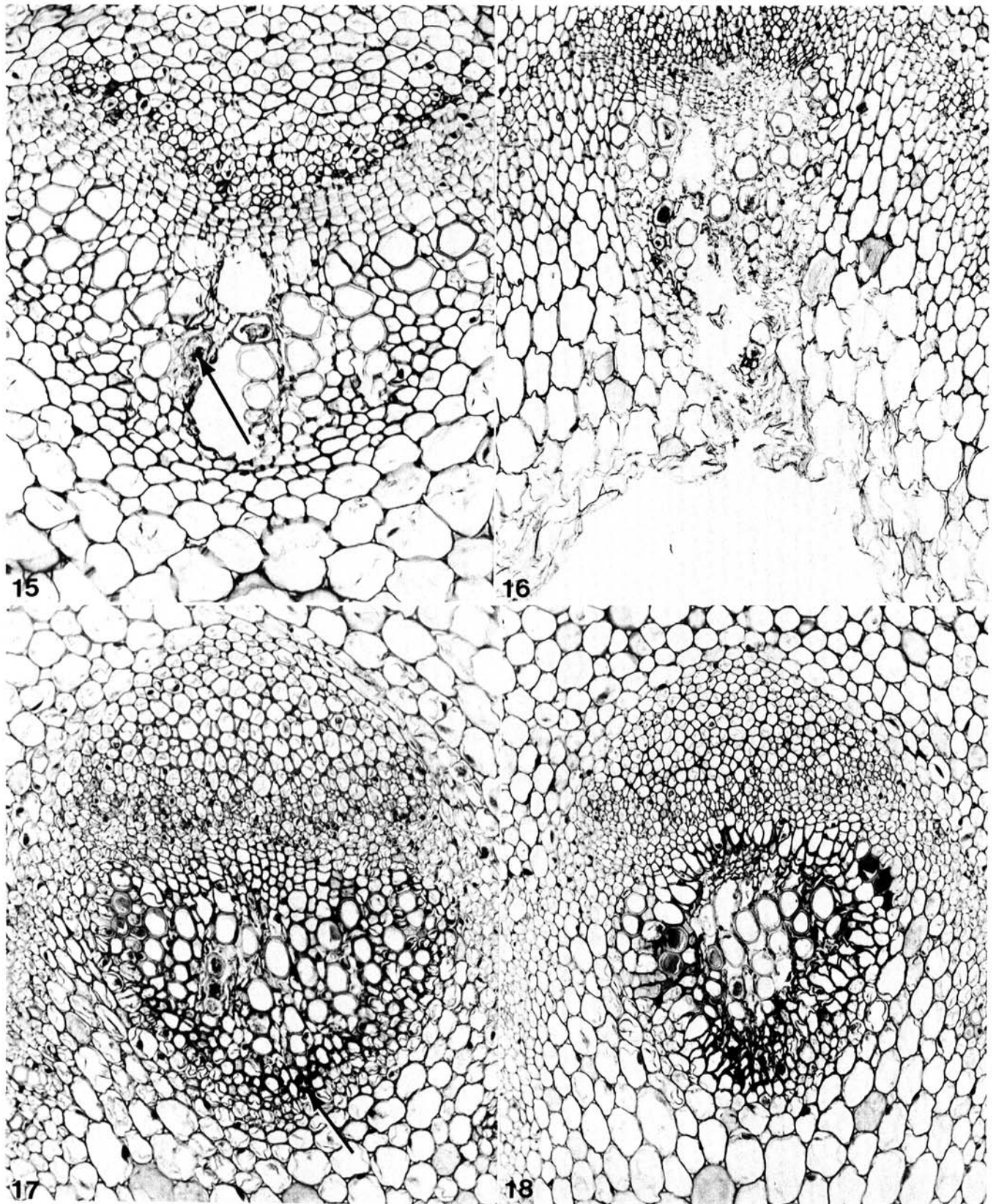
Figs. 15-18. Portions of cross sections through stems of *Chrysanthemum morifolium* 'White Marble' infected with *E. chrysanthemi* 28-96 hr after inoculation. **15,** Breakdown of some xylem parenchyma 28 hr after inoculation. Note the bacteria in one of the xylem vessel elements (arrow) (×238). **16,** Destruction of a vascular bundle and breakdown of pith tissue 48 hr after inoculation (×150). **17,** Hyperplastic and chromophillic (arrow) cells surrounding the infected vascular bundle 55 hr after inoculation (×180). **18,** Hypertrophied and chromophillic cells surrounding the vascular bundle 96 hr after inoculation (×175).

The zinc-chlor-iodine test and polarized-light microscopy indicated that the crystalline structure of the cellulose in some vessel elements was altered. Phloroglucinol and orcinol confirmed the presence of gums in the vascular plugs and the Maüle reaction indicated no differences between the lignin content of older, healthy tissue and of the corresponding infected tissue.

Histology of plants inoculated with *E. chrysanthemi*. The reaction of plants inoculated with *E. chrysanthemi*, was generally similar to that caused by *E. c.* subsp. *carotovora*. The initial evidence of infection consisted of breakdown of xylem parenchyma cells, the presence of bacteria in the lumens of xylem vessel elements (Fig. 15), and the presence of chromophilic xylem parenchyma cells. No hyperplastic cells were present during the early stages of infection. In one instance, this initial reaction was followed by complete breakdown of several vascular bundles and pith tissue (Fig. 16).

Later, the host reaction consisted of hyperplastic, chromophilic cells surrounding the infected vascular bundle in the area where the infected leaf trace joined the stem (Fig. 17). Although disintegration of xylem parenchyma cells occurred and bacteria were present throughout the length of the stem, host response was restricted to the area described above.

Bacteria were still abundant in both the xylem vessel elements of the infected vascular bundle and the cavities formed by breakdown of the xylem parenchyma cells 96 hr after inoculation. Hypertrophied, chromophilic cells occurred around the infected vascular bundle in the area of bacterial entry into the stem (Fig. 18); below this area hyperplastic, chromophilic cells surrounded the vascular bundle. Further down the stem only bacterial masses in xylem vessel elements and thin-walled xylem parenchyma cells were observed. Bacteria were present in the initially infected vascular bundle throughout the length of the stem basal to the inoculation point and invaded surrounding vascular bundles causing additional breakdown of xylem parenchyma cells.
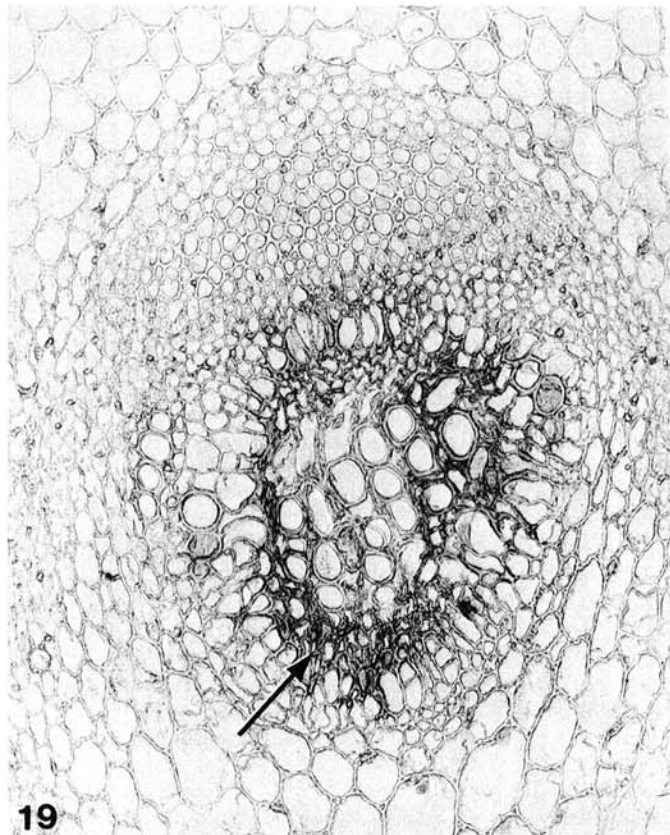

Fig. 19. Portion of a cross section of a stem of *Chrysanthemum morifolium* 'White Marble' 96 hr after inoculation with *E. chrysanthemi* stained with Sudan IV, showing the presence of large quantities of suberin around the disintegrating vascular bundle near the point of inoculation (×175).

The histochemical test for pectic material was less intense and birefringence of cellulose was reduced in the xylem parenchyma cells of infected vascular bundles as compared to these reactions in uninfected vascular tissue. Formation of vascular plugs of pectic material and gum was observed in infected vascular bundles. Suberin was more frequently noted in the hyperplastic cells of plants infected with *E. chrysanthemi* than in similar cells of plants infected with *E. c.* subsp. *carotovora* (Fig. 19). Suberin was first noted in plants infected with *E. chrysanthemi* 28 hr after inoculation but was not present consistently until 55 hr after infection.

DISCUSSION

Chrysanthemum cultivar White Marble failed to exhibit any symptoms when inoculated via the vascular system with isolates of either *E. c.* subsp. *carotovora* or *E. chrysanthemi* and grown under environmental conditions favorable for symptom expression. Although symptoms were absent, isolations indicated that both pathogens survived in the host plant. Smith and Dickey (13) reported that the pith of White Marble is extremely susceptible to maceration by either species of *Erwinia*, and Garibaldi and Bateman (5) reported the production of pectic enzymes by *E. chrysanthemi*. Thus, although *E. c.* subsp. *carotovora* and *E. chrysanthemi* can macerate pith tissue, in this experiment the pith was intact in most cases, indicating that these two pathogens did not come in contact with pith cells.

The histological studies reported herein provide a possible explanation for the tolerance of the chrysanthemum cultivar White Marble to *Erwinia* species. Under the environmental conditions of this study, White Marble plants appeared to contain *E. c.* subsp. *carotovora* in infected vascular bundles. These bundles were frequently surrounded by hypertrophied, hyperplastic and chromophilic cells. Breakdown of xylem parenchyma cells was often present within the infected bundle but similar cell destruction was never found external to the ring of altered cells.

Plants of the chrysanthemum cultivar White Marble were unable to contain *E. chrysanthemi* in the initially infected vascular bundle to the same degree as *E. c.* subsp. *carotovora*. On one occasion *E. chrysanthemi* broke out of the initially infected vascular tissue and penetrated pith and additional vascular tissue. The result in that case was identical to that described for *E. chrysanthemi* in the cultivar Giant No. 4 Indianapolis White (8).

The host reaction of hypertrophied, hyperplastic, and chromophilic cells was always most intense near the point where the pathogen entered the main stem. Few cells of *E. c.* subsp. *carotovora* were detected in stained stem cross sections below this area, although the observation of the breakdown of xylem parenchyma cells indicated that some pathogen cells were probably present. It is possible that *E. c.* subsp. *carotovora* was not present in sufficient numbers to breach the hypertrophied, hyperplastic cells formed by the host plant even though the host reaction was reduced in intensity further from the inoculation point.

Esau (4) reported that cork cells frequently form around diseased tissue within the host. Such cells are characterized by hypertrophy followed by hyperplasia and contain suberin as a coating on the cell wall. Suberin was identified histochemically in many of the cells of the host reaction layer in infected White Marble plants. Wainwright and Nelson (14) also reported the formation of similar hyperplastic cells around infected vascular bundles of *Pelargonium* species infected with *Xanthomonas pelargonii*. Wellhausen (15) reported marked differences in the histological reaction of the vascular bundle to invasion by bacteria in inbred lines of maize differing in resistance to *E. stewarti*. In moderately susceptible lines infection of the protoxylem stimulated cell division and subsequent lignification in adjacent parenchyma resulting in the formation of heavily lignified cells around the point of infection. In very susceptible lines, invasion was so rapid that destruction of vascular bundles occurred before the host could react, while in resistant lines the morphology of the bundle was unchanged.

In chrysanthemum White Marble plants infected with a strain of *E. c.* subsp. *carotovora*, the hypertrophied, hyperplastic, suberized

cells surrounding the infected vascular bundle may constitute a wound-healing reaction which may play an important role in containing the pathogen.

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