Histopathology of Chrysanthemum Vascular Tissue Infected with *Erwinia carotovora* subsp. *carotovora*

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We thank Yoder Brothers, Inc., Barberton, OH 44203, for supplying the chrysanthemums, H. Kunoh for assisting in the scanning electron microscopy, and H. Lyon for preparing the photomicrographs.
Accepted for publication 25 July 1980.

ABSTRACT

Smith, C. M., and Dickey, R. S. 1981. Histopathology of chrysanthemum vascular tissue infected with *Erwinia carotovora* subsp. *carotovora*. Phytopathology 71:148-151.

Chrysanthemum morifolium 'White Marble' plants were inoculated with *Erwinia carotovora* subsp. *carotovora* via the petiole of the first fully expanded leaf, whereas unrooted cuttings were inoculated via the petiole or at the base. All were placed under mist at 25 ± 5 C. Plants were also inoculated at the apex and grown in a greenhouse without mist at 23–30 C. Sections of vascular tissue of stems examined by light microscopy also were observed by scanning electron microscopy. *Erwinia carotovora* subsp. *carotovora* colonized but was restricted to, and caused limited or no degradation of, vascular tissue. Vascular infection did not lead to pith maceration although a host reaction of hypertrophic, hyperplastic parenchyma cells surrounding infected xylem was not evident. Individual bacteria were attached to vessel walls while masses of cells were present in some lumina. Unidentified material with a spongy appearance in scanning electron microscopy occurred within and between some infected vessels in the vicinity of pectic substances identified histochemically under light microscopy. The mechanism of tolerance of White Marble to *E. carotovora* subsp. *carotovora* is not clear. Whereas the hypertrophic, hyperplastic host response does not appear to be required in all cases, bacterial attachment to vessel walls and aggregation into masses surrounded by pectic material may be involved in confining the pathogen to vascular tissue.

Additional key words: bacterial soft rot disease, pathological anatomy.

The symptoms of bacterial soft rot disease of chrysanthemums incited by *Erwinia carotovora* subsp. *carotovora* in cuttings and plants consist of initial pith maceration and vascular discoloration with subsequent epidermal and cortical necrosis of the stem. High moisture content of rooting media and frequent misting are required for symptom development (10).

In addition, *E. carotovora* subsp. *carotovora* can inhabit vascular tissue without symptom development. The pathogen survived for more than 2 mo in the vascular system and either remained localized or migrated throughout the plant (10). The absence of symptoms during vascular infection in chrysanthemums of cultivar White Marble appears to be due to the restriction of the pathogen to resistant vascular tissue and its exclusion from susceptible pith (7). Pennypacker et al (7) suggested that a host reaction involving the development of hypertrophic, hyperplastic parenchyma cells and formation of suberin around infected vascular tissue may be responsible for the tolerance of White Marble chrysanthemums to *E. carotovora* subsp. *carotovora*.

This study was undertaken to investigate vascular infection and survival of *E. carotovora* subsp. *carotovora* in chrysanthemum. In particular, the role of the hypertrophic, hyperplastic response of

0031-949X/81/02014804/$03.00/0

White Marble chrysanthemums in restricting the pathogen to vascular tissue was studied.

MATERIALS AND METHODS

Growth and inoculation of chrysanthemums. Unrooted cuttings of *Chrysanthemum morifolium* Ramat. 'White Marble' were supplied by Yoder Brothers, Inc., Barberton, OH 44203 and were stored at 4 C until used. Cuttings were rooted in vermiculite and under mist. Rooted cuttings were transplanted to 15-cm-diameter plastic pots containing a steam-sterilized potting mixture of vermiculite and peat moss (1:1, v/v) plus fertilizer, lime, and calcium nitrate. The rooted cuttings were grown in a greenhouse without mist where temperatures ranged 23–30 C and supplemental lighting for 16 hr per day maintained the vegetative growth of plants. Some cuttings and plants were grown in a high humidity chamber at 25 ± 5 C with continuous mist and supplemental lighting.

A strain (311sr) of *E. carotovora* subsp. *carotovora,* originally isolated from chrysanthemum and resistant to 2,000 μg/ml of streptomycin sulfate (ICN Pharmaceuticals, Inc., Cleveland, OH 44128), was used for the inoculations. The pathogen was grown for 24 hr at 27 C on nutrient agar (NA) slants with streptomycin sulfate (2,000 μg/ml). Suspensions of bacteria were prepared by washing cells from cultures with sterile distilled water. The concentration of the suspension was determined by optical density at 620 nm with a Beckman Spectronic 20 colorimeter and dilutions to the desired concentrations were made with sterile distilled water.

Unrooted cuttings were inoculated at the base by submerging the proximal 1.5 cm of the stem in a bacterial suspension (10^9 cells per milliliter) for 5 min after aseptic removal of 0.5 cm of the stem base. The freshly cut stem base of uninoculated cuttings was submerged in sterile water. The inoculated and uninoculated cuttings were placed in the high humidity chamber for 2 days to allow basal pith maceration. The method of vascular inoculation via the petiole of cuttings and plants (~20 cm in height) as previously described (7) was modified by submerging the basal 1.5 cm of cuttings in sterile water for 10 min to facilitate bacterial movement in the stem and then inserting a fine sewing needle smeared with bacterial cells into the adaxial side of the petiole of the first fully expanded leaf of cuttings and plants. A sterile needle was inserted into the petiole of uninoculated chrysanthemums. The inoculated and uninoculated cuttings and plants were placed in the high humidity chamber for 2 days. Apical inoculation of plants was accomplished by aseptically removing 4 cm of the stem apex and immediately applying 5 μl of a bacterial suspension (10^9 cells per milliliter) to the cut surface with an automatic pipette. The inoculated and uninoculated plants, with sterile water placed on the cut surface, were returned to the greenhouse without mist for 2 wk.

Alternate, 5-mm-long sections of stem tissue were assayed for the presence of the pathogen or prepared for light and scanning electron microscopy. The stem tissue to be assayed was located immediately proximal to the inoculated petiole in petiole-inoculated cuttings, immediately distal to the area of pith maceration in basally inoculated cuttings, and immediately proximal to the apex in apically inoculated plants. In addition, sections immediately proximal to the inoculated petiole in vascularly inoculated plants were included in the scanning electron microscope study. The presence of the pathogen was determined by placing individual sections in separate tubes of nutrient broth containing streptomycin sulfate (1,500 μg/ml). A loopful of liquid from each tube showing turbidity after 3, 5, and 8 days incubation at 27 C was streaked onto NA containing streptomycin sulfate (1,000 μg/ml) and incubated at 27 C. The bacterial colonies were examined after 3 days for morphological resemblance to strain 311sr and cells from representative colonies were tested for cytochrome oxidase activity (Patho Tec-CO test papers, General Diagnostics Division, Warner-Chilcott, Morris Plains, NJ 07950). Bacteria with colony morphology identical to that of *E. carotovora* subsp. *carotovora* 311sr and lacking cytochrome oxidase activity were considered to be the pathogen.

Histological techniques. Stem pieces 5 mm in length were fixed in

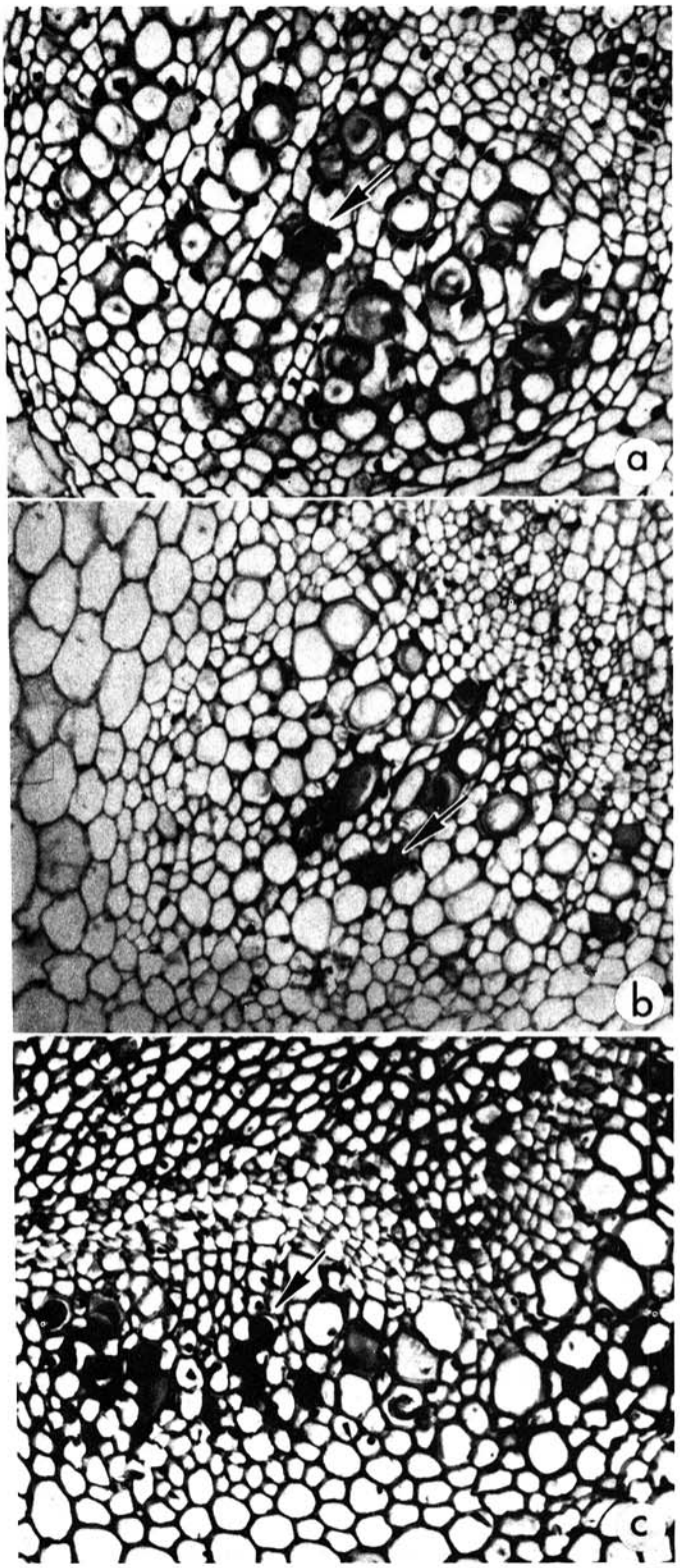

Fig. 1. Bright-field photomicrographs of transverse stem sections from cuttings and plants of chrysanthemum cultivar White Marble inoculated with *Erwinia carotovora* subsp. *carotovora* 311sr and stained with Harris' hematoxylin and orange G; a–b are from cuttings grown under continuous mist at 25 ± 5 C for 2 days prior to fixation and c is from a plant grown without mist at 23–30 C for 2 wk prior to fixation. **a,** Infected vascular bundle with bacteria (arrow) colonizing a vessel of a petiole-inoculated cutting. No hypertrophy, hyperplasia, or deterioration of stem tissue has occurred (×230). **b,** Infected vascular bundle with bacteria-filled vessels (arrow) and limited invasion of surrounding xylem parenchyma immediately above the area of pith maceration in a cutting inoculated at the base. Note the absence of hypertrophy or hyperplasia (×230). **c,** Infected, intact vascular bundle in which bacteria (arrow) occlude vessels in a plant inoculated at the apex. No hypertrophy or hyperplasia is apparent (×230).

Rawlin's formalin-aceto-alcohol solution No. 1 (FAA) (8), dehydrated in a tertiary butyl alcohol series (4), infiltrated, and embedded in paraplast (Sherwood Medical Industries, St. Louis, MO 63103). The material was sectioned at 13 μm on a rotary microtome. Transverse and longitudinal sections were mounted on clean slides with Haupt's adhesive and stained with Harris' hematoxylin and orange G (4). Histochemical tests were conducted on selected tissue for pectin (iron absorption method) and gums (phloroglucinol and orcinal) (3,8). Sections were examined with a Leitz Ortholux Research microscope. Samples consisted of at least two inoculated and two uninoculated stems for each treatment.

Stem pieces also were examined with a scanning electron microscope (AMR-1000) at 10 kV accelerating voltage. These were fixed, embedded, sectioned, and stained for bacteria as for light microscopy, but were maintained in xylene rather than permanently mounted under cover slips. Sections were observed with the light microscope and areas of the glass slides containing sections to be examined with the scanning electron microscope were cut, affixed to mounts (E. Fjeld Co. Inc., Burlington, MA 01803), and spatter-coated with gold (5). Thus, the same or adjacent sections observed by light microscopy also were examined by scanning electron microscopy. The examination was limited to two inoculated and two uninoculated sections for each treatment.

RESULTS

Symptoms and location of *E. carotovora* subsp. *carotovora* in chrysanthemum plants and cuttings. Cuttings and plants inoculated at the petiole and placed under mist developed no soft rot, but exhibited a slight discoloration of the vascular bundle immediately proximal to the inoculated petiole. *E. carotovora* subsp. *carotovora* 311sr migrated in a basal direction in the stem after inoculation. In basally inoculated cuttings grown under mist, soft rot of the pith and vascular discoloration extended in an apical direction from the cutting base although streaking of the veins was apparent beyond the area of maceration. The pathogen was isolated from stem tissue in and immediately above the area of maceration. Plants inoculated apically and grown in a greenhouse without mist exhibited no symptoms; however, the pathogen remained viable in apical stem tissue. Uninoculated chrysanthemum plants and cuttings remained healthy and did not harbor the pathogen.

Light microscopy. The stems of healthy White Marble chrysanthemum plants have been described (7). The petiole inoculations introduced *E. carotovora* subsp. *carotovora* into the vascular system of the main stem via the leaf trace of cuttings. The pathogen did not invade pith tissue and incited no massive pith soft

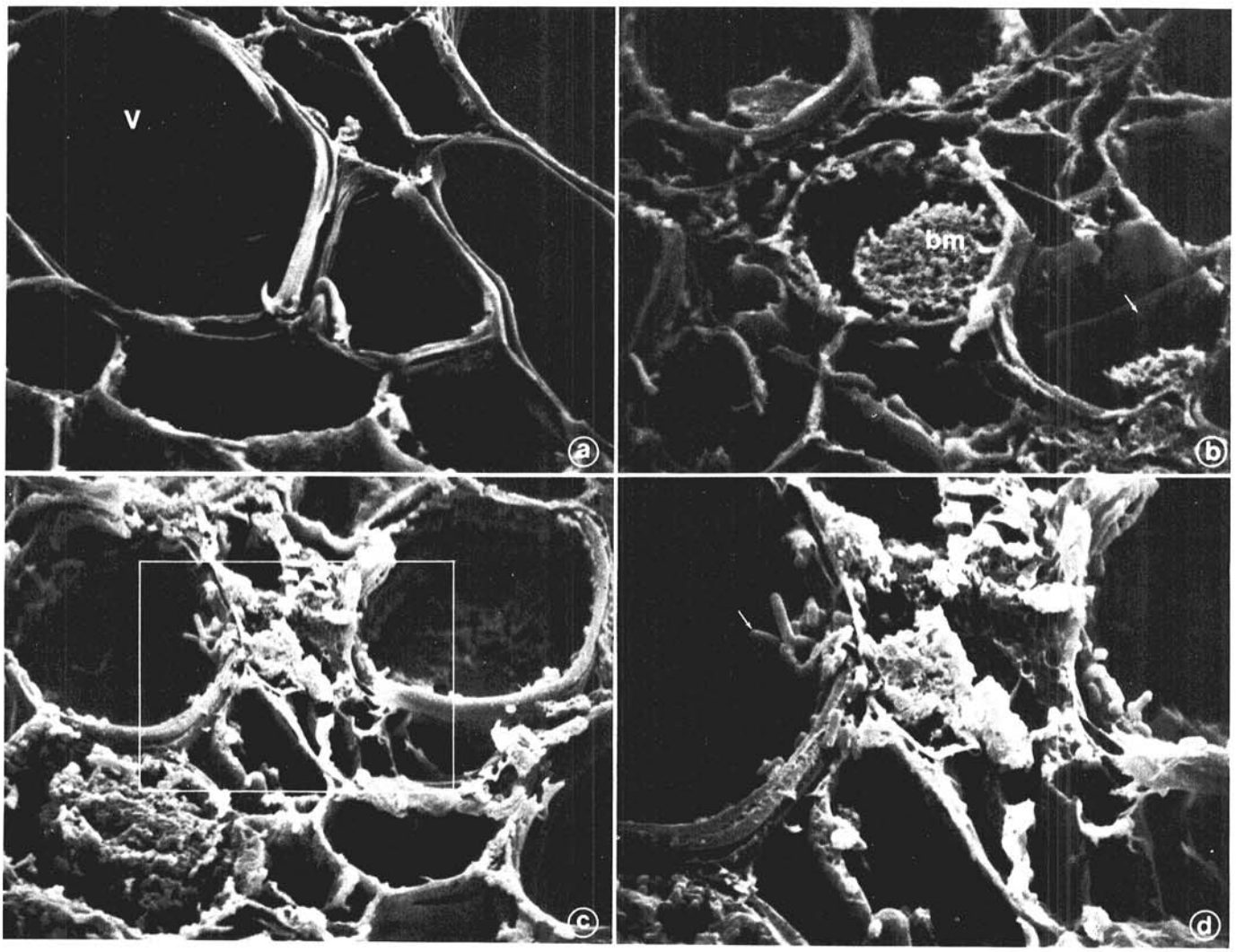

Fig. 2. Scanning electron micrographs of transverse stem sections of xylem of White Marble chrysanthemum plants uninoculated or inoculated via the petiole with *Erwinia carotovora* subsp. *carotovora* 311sr. Plants were grown under continuous mist at 25 ± 5 C for 2 days prior to fixation. **a,** Uninfected xylem with intact, empty xylem vessel elements (v) (×2,500). **b,** Infected xylem with bacterial masses (bm) in vessels and individual bacteria (arrow) attached to a vessel walls. Compare host cell wall integrity in a and b (×1,900). **c,** Infected xylem with bacterial masses in the vessels and unidentified material between vessels (×3,000). **d,** Enlargement of area in c. Bacteria (arrow) are attached to vessel walls and unidentified material (um) occurs between vessels (×5,000).

rot. Granular masses of bacteria were present in lumina of a few vessels of the vascular bundle continuous with that of the inoculated petiole; however, the pathogen was confined to these areas and did not invade neighboring xylem parenchyma (Fig. 1a). Bacteria colonized vessels in all vascular bundles in cuttings inoculated at the base and in some cases invaded xylem paranchyma to a limited extent (Fig. 1b). In apically inoculated plants the pathogen colonized, but was confined to, vessel elements in all vascular bundles that showed no evidence of deterioration (Fig. 1c). Hypertrophy and hyperplasia around infected bundles were apparent neither in cuttings inoculated at the petiole (Fig. 1a) or base (Fig. 1b) nor in plants inoculated at the apex (Fig. 1c). Occasionally very slight hyperplasia occurred around bundles in petiole-inoculated cuttings.

In all inoculated plants and cuttings, histochemical tests revealed that pectic substances were present in the areas within and surrounding bacterial masses in vessel elements, in vascular plugs, and between vessels. Gums were observed in some vessels that contained no bacteria.

Scanning electron microscopy. Transverse stem sections, prepared as for light microscopy, were examined with scanning electron microscopy. In the xylem of uninfected vascular bundles, vessels were observed to have a polygonal outline, large empty lumina, and walls with helical and reticulate secondary thickenings. Smaller xylem parenchyma cells were interspersed among vessels. Walls of all cells were distinct and intact (Fig. 2a).

In infected bundles of petiole-inoculated plants, bacteria were readily identified by their rod-shaped morphology and average dimensions of 0.4×1.7 μm. The identification of bacteria in scanning electron micrographs was confirmed by comparison with light micrographs of the same or adjacent tissue sections in which bacteria were identified by staining. Bacteria were attached to the interior of vessel walls and appeared as distinct entities even when present in masses (Fig. 2b). Large masses of bacteria occupied numerous lumina while smaller masses frequently projected from one side of the interior vessels. Vessel walls had undergone degradation with breakage and separation of wall thickenings (Fig. 2b). Unidentified material that had a spongy appearance occurred between vessels in the location of histochemically identified pectic substances (Fig. 2c,d).

In infected bundles of petiole-inoculated and basally inoculated cuttings, bacterial masses were observed in vessels although vessel walls and xylem parenchyma appeared to be intact. Unidentified material with a spongy appearance was present in some lumina and corresponded in location to pectic substances identified by histochemical staining.

DISCUSSION

E. carotovora subsp. *carotovora* colonized, but was restricted to, vascular bundles in White Marble chrysanthemum plants and cuttings. Bacteria did not invade susceptible pith or initiate maceration from vascular tissue. These results corroborate histological evidence of Pennypacker et al (7) for the tolerance of White Marble to *E. carotovora* subsp. *carotovora*. Our findings also agree with the results of gross observations (10) of vascularly inoculated cuttings; *E. carotovora* subsp. *carotovora* is restricted to vascular tissue and requires wounding to gain access to susceptible pith and to produce typical symptoms.

Pennypacker et al (7) reported a host reaction involving hypertrophic, hyperplastic, suberized cells around infected vascular bundles in misted White Marble plants 48 hr after petiole inoculation with *E. carotovora* subsp. *carotovora*. The authors hypothesized that this response serves to sequester the pathogen in vascular tissue and preclude its invasion of susceptible pith. It is reported herein that this reaction was not observed in misted cuttings after petiole or basal inoculation nor in nonmisted plants after apical inoculation; however, in both cuttings and plants, the pathogen colonized and was confined to vessels. These results suggest that factors other than hypertrophy and hyperplasia may be involved in restricting bacterial spread in the samples examined. Nevertheless, other explanations for this discrepancy are possible. In this study the host response may have occurred before or after stem tissue was sampled, or suberin may be responsible for bacterial containment although the presence of suberin was not determined.

The reasons for the absence of hypertrophy and hyperplasia in cuttings and nonmisted plants in contrast to their presence in misted plants after inoculation (7) are unknown. The chrysanthemum response appears analogous to a plant wound-healing reaction in which cells around injured areas become hypertrophic and hyperplastic and elaborate suberin (2,7). Destruction of vascular tissue, which may induce hypertrophy and hyperplasia, was not evident in the chrysanthemums examined in this study, but it was present in those showing the response (7).

This investigation failed to produce evidence concerning the mechanisms of confinement and survival of *E. carotovora* subsp. *carotovora* in vascular tissue. In addition, the mode of attachment of bacteria to host walls as well as the method of pathogen aggregation into masses remain obscure. However, gels composed of plant cell wall constituents including pectin (11,12) may be involved. Gels or pectic materials frequently occur in vascular infection and have been postulated to restrict the movement and multiplication of bacterial and fungal pathogens in vascular tissue (1,11,13,14). Gels formed rapidly upon vascular inoculation of chrysanthemum with *Verticillium dahliae* (9) and pectic substances surrounded masses of cells of *E. chrysanthemi* in chrysanthemum vessels (6). In this study, observations by light microscopy suggested that cells of *E. carotovora* subsp. *carotovora* were sometimes embedded in pectic material in vessels. Moreover, in infected vascular tissue examined with a scanning electron microscope, unidentified material with a spongy appearance was present in and between some vessels in the location of pectic substances identified histochemically. Further histochemical and electron miscroscope studies are needed to elucidate the mechanisms of vascular infection and survival of *E. carotovora* subsp. *carotovora* in chrysanthemums.

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