Reduction of Pith Maceration by *Erwinia chrysanthemi* in Chrysanthemum Cuttings Infected With Chrysanthemum Stunt Viroid

Ann Finer Silverglate, Robert S. Dickey, and R. K. Horst

Former research assistant, professor, and associate professor, respectively, Department of Plant Pathology, Cornell University, Ithaca, NY 14853.

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ABSTRACT

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Cuttings of *Chrysanthemum morifolium* 'Bonnie Jean,' either healthy or artificially infected with chrysanthemum stunt viroid (CSV), were dip-inoculated with *Erwinia chrysanthemi* strain 159. Infection by CSV reduced the amount of bacterial pith maceration, which was determined by measuring the length of maceration from the base of the cutting. A decrease in maceration coincided with the occurrence of viroid symptoms on the leaves and/or the detection of CSV RNA by polyacrylamide gel electrophoresis. Differences between healthy and CSV-infected stems were not noted by histological examination between 10 and 41 days after inoculation with CSV. *Erwinia chrysanthemi* was restricted to xylem vessel elements in CSV-infected cuttings 5 days after inoculation with the bacterium, whereas bacteria spread from the vessel elements to the adjacent vascular cells and into the pith tissue of healthy cuttings.

Chrysanthemum stunt disease and bacterial blight of chrysanthemum are important diseases of the florists' chrysanthemum (*Chrysanthemum morifolium* Ramat.) (4,6). Currently, major chrysanthemum propagators use culture indexing to prevent the introduction of plant material infected with the bacterial blight pathogen (*Erwinia chrysanthemi* Burkh., McFadden, and Dimock) into their foundation stock (13). A viroid indexing program has also been developed to maintain plants free of the chrysanthemum stunt viroid (CSV) (3,18).

Erwinia chrysanthemi is a soft-rotting bacterium, capable of producing pectolytic enzymes (11) and causes a degradation of vascular parenchyma and pith cells (20). The reduction of bacterial maceration in CSV-infected cuttings, correlations of the reduction with detectable viroid, and attempts to explain the maceration reduction by histological techniques have been investigated (24) and are reported herein.

MATERIALS AND METHODS

Bacteria. The culture used was *Erwinia chrysanthemi* strain 159. This strain was isolated from *Philodendron* sp., and is pathogenic for chrysanthemums. Stock culture was maintained on nutrient agar (NA) slants stored at 4 C. The strain was transferred to yeast-dextrose-calcium carbonate agar (YDC) (2) slants and grown for 18–24 hr at 30 C prior to preparation of inoculum.

Plants and cuttings. Unrooted cuttings of the florists' chrysanthemum, *C. morifolium* 'Bonnie Jean,' were obtained from the California-Florida Plant Corp., Fremont, California, rooted under mist, and potted in a steamed and fertilized vermiculite-peat (2:1,v/v) medium containing 2,270 g of ground limestone, 454 g of super phosphate, 50 g of a trace element mixture (Robert B. Peters Co., Inc., Allentown, PA 18104), 2,270 g of Osmocote 19-6-12 (Sierra Chemical Co., Milpitas, CA 95038), and 700 ml of Aqua-gro wetting agent (Aquatrols Corporation of America, Pennsauken, NJ 08110) per 0.66 m^3 of medium. Bonnie Jean was selected for its usefulness as a CSV indicator cultivar (1). Infected plants developed yellow spots. Plants were held in a greenhouse at approximately 29–30 C with supplemental fluorescent lighting (11,000 lux minimum) for 16 hr/day.

Plants were assayed for the presence of CSV and chrysanthemum chlorotic mottle viroid (ChCMV) by viroid-indexing as described below, as well as by the observation of symptoms. Indexing for the latent strain of ChCMV (ChCMV-l) was done by the cross-protection method of Horst (12). Only plants which were free of CSV, ChCMV, and ChCMV-l were retained as healthy stock plants. These were kept in a greenhouse at approximately 22 C. Additional plants were propagated by cuttings. Healthy stock plants were inoculated with CSV by the tissue implantation procedure of Dimock et al (7). Chrysanthemums known to be infected with only CSV were maintained in a greenhouse at approximately 29–30 C.

Maceration and viroid detection. Terminal shoots were removed from healthy and CSV-infected plants and cut to a length of approximately 45 mm. Stems of the shoots were inserted in steamed vermiculite and placed overnight in a continuous mist chamber maintained at 21–27 C. The follwing day shoots were cut to a length of 40 mm and inoculated by placing the base in a beaker containing 9 ml of a bacterial suspension (~10^9 cells per milliliter). Control shoots were placed in 9 ml of sterile water. After 10 min the shoots were removed from the beaker and the bases were gently rinsed with tap water. Stems of the shoots were inserted in steamed vermiculite in flats and returned to the mist chamber. After 5 days, all cuttings were split longitudinally and the length of pith maceration was measured.

The viroid indexing method was a modification by Horst and Kawamoto (*personal communication*) of the polyacrylamide gel electrophoresis (PAGE) assay procedure of Morris and Wright (16). Boiled distilled water was used whenever water is specified. All steps but the first were carried out at 4.5 C. Two grams of leaf tissue were excised from a plant with a sterile razor blade. The tissue was frozen with liquid nitrogen and was ground in a mortar. The resulting powder was placed in a 50-ml centrifuge tube containing 8 ml of extraction buffer (0.2 M H_3BO_3, 12% Na_2SO_3, 1% SDS, adjusted to pH 9). Eight milliliters each of 0.1% 8-hydroxyquinoline in water-saturated phenol and a 1:1 chloroform-butanol solution were added and the mixture was stirred and allowed to incubate for 10 min. After low-speed centrifugation (7,710 *g*) for 20

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min the upper aqueous phase was transferred to a tube containing 20 ml of cold (−20 C) ethanol plus two drops of 1 M sodium acetate (NaAc), mixed, and incubated at −20 C for at least 1 hr. Low-speed centrifugation for 15 min produced a pellet which was dissolved in 8 ml 0.2 M NaAc. This preparation was centrifuged at low speed for 8 min and the supernatant was transferred to a clean tube. Two milliliters of 1% hexadecyltrimethyl ammonium bromide (Ctab) was added while the mixture was swirled, and the precipitate was allowed to form for 10 min on ice. Following centrifugation at low-speed for 15 min, the pellet was washed with two drops of 1 M NaAc and 16 ml 70% ethanol, centrifuged again at low speed for 15 min, and the resultant pellet was suspended in 2 ml of cold 95% ethanol plus one drop of 1 M NaAc. After centrifugation for 10 min, the precipitate was dissolved in 0.2 ml of 1E buffer (one part 5X buffer [0.2 M Tris, 0.1 M NaAc, 0.005 M Na_2EDTA, pH 7.6] plus four parts water). Samples were either dialyzed overnight against 1E buffer or frozen at −20 C.

The five percent polyacrylamide gel was prepared as described by Morris and Wright (16). Gels were stored at 4 C in 1E buffer until use. Electrophoresis was performed in a BioRad Model 150 A gel electrophoresis cell with 1E buffer in both reservoirs. The power source (BioRad Model 400 power supply) was set for the constant current mode of operation at 5–6 ma per tube. The system was prerun for about 1 hr. Approximately 0.1 ml of extract was mixed with one drop 75% RNase-free sucrose solution containing 0.5 mg/ml bromphenol blue and layered on the gel surface. Electrophoresis was terminated when the dye front reached the base of the gel, about 2.5 hr.

The nucleic acids were stained with either toluidine blue O (16) or ethidium bromide (5). When toluidine blue O was used, gels were removed from tubes and soaked overnight in 0.1% Ctab. After three distilled water rinses they were stained for 3 hr in 0.7% toluidine blue O dissolved in 7% acetic acid which had been filtered. Destaining was accomplished by washing with tap water several times daily for 2–3 days.

When ethidium bromide was used, the gels were placed directly in stain (ethidium bromide, 2 mg/100 ml 0.001 M Na_2EDTA) overnight, a modification of Dickson's procedure (5). Destaining was done with either 0.001 M Na_2EDTA or tap water. The gels were viewed under ultraviolet light to detect bands.

Isolation of pathogen and histology. Cuttings (40-mm long) from healthy and CSV-infected plants were inoculated with the bacterium by slicing across the base with a scalpel blade contaminated with cells from a 24-hr YDC slant culture. Stems of the inoculated cuttings were inserted in steamed vermiculite and held at 21–27 C with continuous misting for 5 days. The stem of each surface disinfested cutting was aseptically cut into five 8-mm-long sections. The first, third, and fifth sections from the base were tested for the presence of the pathogen. Each section was rinsed in sterile water, cut longitudinally, placed in a tube of sterile water, and agitated with a vortex mixer. Loopfuls of the water were streaked on NA plates and incubated at 30 C. Plates were observed after 1 and 3 days for numbers of colonies and their morphology and pigmentation.

The second and fourth stem sections were trimmed to approximately 6 mm and were fixed in Rawlins' FAA (14) for a minimum period of 7 days and then dehydrated with a tertiary-butyl alcohol series (14). Infiltrating and embedding were done in Paraplast, melting point 56–57 C (Sherwood Medical Indust., St. Louis, MO 63103). Sections were cut at 12 μm with a rotary microtome and affixed to glass slides with Haupt's adhesive (14). Johanson's quadruple stain was used for the examination of stem tissue not infected with *E. chrysanthemi*. Solutions were prepared according to Johanson (14), but the times were modified for chrysanthemum tissue (24). Sections of stem tissue infected with *E. chrysanthemi* were stained with Harris' hematoxylin and orange G (14) which facilitates the identification of bacteria in plant tissue.

Histochemical tests were made for cellulose (IRI-H_2SO_4 method), gums (orcinol reaction, phloroglucinol test: [orcinol and phloroglucinol C.P., dihydride, ICN Pharmaceuticals, Inc., Cleveland, Ohio]), lignin (Mäule reaction, phloroglucinol test), pectate substances (iron absorption method), and suberin (Sudan IV test) (14,22). In addition, polarized light was used to observe crystalline cellulose.

TABLE 1. Effect of CSV infection on pith maceration of chrysanthemum cuttings by *Erwinia chrysanthemi* 159

Experiment	Mean amount of pith maceration (mm) from base of cutting	
	Healthy[a]	CSV-infected[b]
I	17.8[c]	5.8
II	22.6	7.2

[a]Number of cuttings: Experiment I = 19; II = 20.
[b]Number of cuttings: Experiment I = 16; II = 19.
[c]Healthy and CSV-infected means for a given experiment were significantly different ($P = 0.01$) by an unpaired Student's *t*-test.

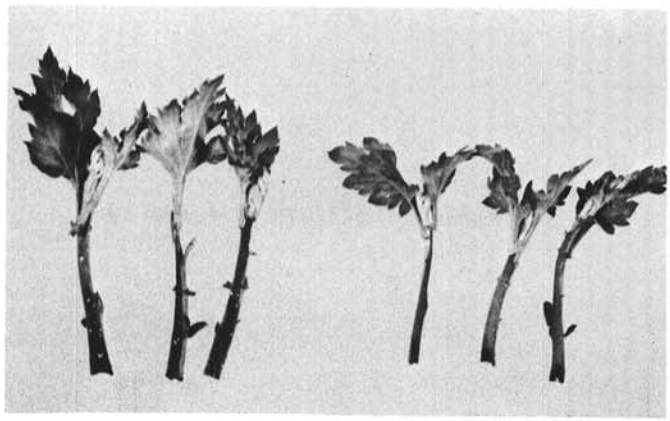

Fig. 1. Stems of chrysanthemum cultivar Bonnie Jean cuttings 5 days after inoculation with *Erwinia chrysanthemi* 159, showing different amounts of pith maceration in cuttings infected with CSV (right) and in uninfected cuttings (left).

RESULTS

Effect of CSV-infection on pith maceration of cuttings by *Erwinia chrysanthemi* strain 159. Cuttings were removed from 3-wk-old plants which originiated from either healthy or CSV-infected stock plants and were used for the bacterial maceration test.

The affected pith exhibited a dark red soft rot; in addition, vascular bundles often were discolored above the point where the pith rot terminated. Exterior stem tissues showed no symptoms. The soft rot which occurred in stems infected with CSV was approximately one-third the amount that occurred in healthy stems inoculated with *E. chrysanthemi* (Table 1). Typical results are shown in Fig. 1.

Relationship between viroid detectability and maceration decrease. Ten days after being potted, 20 healthy plants were implanted with tissue plugs, either from healthy stocks or from CSV-infected plants. The inoculations were made 35 mm below the terminal shoot apex. All plants were maintained at approximately 29–30 C with 16 hr per day of supplemental lighting. Removal of the terminal shoot at 10 days after inoculation caused the production of side shoots. At 10, 20, 26, 34, and 41 days after inoculation one apical or side shoot from each plant was removed from above the point of inoculation. Two grams of leaf blade tissue per cutting were used to index for the viroid. Healthy plants were indexed at 10, 20, and 41 days only. Five-millimeter-long stem pieces were removed from the base of each cutting and placed in fixative for histological preparation. The remaining portion of the shoot (stem plus petioles) was cut 40 mm below the apex and dip-inoculated for a bacterial maceration assay. Although the leaf blades were removed from the cuttings taken from the non-CSV-

inoculated plants on all sampling dates, the tissue was not always indexed.

The plants inoculated with CSV began to show symptoms 27 days after inoculation. The occurrence of symptoms and detection of viroid at 34 and 41 days after inoculation are given in Table 2. The bacterial pith maceration was significantly reduced whenever either CSV was detected in the leaf tissue by PAGE assay or symptom expression was evident (Fig. 2). Generally, a small reduction in maceration was obtained in cuttings taken from CSV-inoculated plants, but the reduction was greatest if the cuttings were exhibiting symptoms and contained a detectable CSV-titer. Visible symptoms (yellow spots) appeared either shortly before or at the same time as a viroid titer was detectable by PAGE assay (Table 2).

Recovery of *E. chrysanthemi* strain 159 from inoculated cuttings. *E. chrysanthemi* was isolated consistently from the first and third sections of CSV-infected and uninfected cuttings. Bacteria were not isolated from the fifth sections. These results indicate that the bacteria placed on the base of infected and healthy cuttings moved approximately the same vertical distance and were able to survive for at least 5 days within the cuttings.

Histological examinations. Anatomical differences were not observed between healthy stems and comparable CSV-infected stems on each sampling date. The anatomy of typical young stems of chrysanthemum cultivar Bonnie Jean, seen in transverse section, differed only slightly from that of cultivar Giant #4 Indianapolis White as described by Pennypacker et al (20). In stems of Bonnie Jean, the pith began at the base of the vascular bundles, with few or no extraxylary fibers separating the two. Sections of older stems exhibited bundle caps of mature fibers, large regions of interfasicular vascular tissue, and extensive extraxylary fibers.

In stems of healthy cuttings, the bacteria apparently moved upward in the pith and macerated the tissue. Bacteria also moved within the xylem vessel elements because bacteria were found within vessels above the region of detectable pith rot. A disruption of the vessels was caused by the bacteria, by destroying vessel support tissue, the xylem parenchyma (20). The bacteria then spread from the vascular bundles and macerated the nearby pith cells (Fig. 3A). Maceration of vascular bundles and pith was extensive 5 days after inoculation whereas other tissues appeared to be unaffected.

In contrast, bacteria in CSV-infected cuttings remained largely confined to the vessel elements which they initially colonized. A small amount of pith maceration occurred at the cutting base, but did not continue to extend apically. The bacteria were seen within the lumina of the vessel elements, but few cells of the xylem parenchyma, cambium, phloem or pith were degraded after 5 days (Fig. 3B).

Histochemical tests showed no discernable differences between CSV-infected and CSV-free stem sections.

DISCUSSION

The amount of pith maceration of chrysanthemum cultivar Bonnie Jean cuttings by *Erwinia chrysanthemi* 159 was found to be reduced by prior CSV infection (Table 1). The reduction is apparent in CSV-infected plants approximately 30 days after inoculation with the viroid (Fig. 2). This period of time coincides with CSV symptom expression (yellow spotting) and represents a period of viroid multiplication since viroid titer rises sufficiently to become detectable by PAGE assay. This time-course relationship of symptom development and detection ability indicates that the viroid is detectable when moderate to intense spotting symptoms occur (Table 2). It would appear that a certain threshold of viroid molecules within the cutting is necessary for the three events observed: symptom development, detection of viroid by PAGE assay, and a decrease in bacterial pith maceration.

The reaction reported herein differs from the cross-protection phenomenon (21,23) which involves closely related organisms (ie strains of a single virus). Strains of potato spindle tuber viroid may cross protect (10), and Horst (12) has shown similar data for ChCMV, but viroids have not been reported to cause a reduction of symptoms incited by other classes of agents. Viruses, however, have been found to reduce (17) or increase (9,15) the severity of fungal infections.

E. chrysanthemi, a soft-rotting bacterium, possesses pectolytic enzymes which may break down the middle lamellae of parenchymatous cells, resulting in cell death and the loss of tissue integrity (11). Pennypacker et al (19,20) have shown that the bacteria's major enzymatic effect is upon the succulent tissues of chrysanthemum (the pith and vascular parenchyma) whereas heavily lignified or suberized walls are more resistant (20). The pathology of the chrysanthemum stunt disease has not yet been studied. However, it has been the experience of growers and investigators that along with the overall stunting, a "hardening" of the plant occurs. Succulence of tissues is lost and the taking of cuttings becomes difficult because stem tissues split and crack under the pressure required to remove the cuttings. It might then be conjectured that as the viroid multiplied and moved throughout the inoculated plant the ensuing loss of succulence might be sufficient

TABLE 2. Relation of symptom expression and detection of CSV 34 and 41 days after inoculation of chrysanthemum cultivar Bonnie Jean with CSV

Days after inoculation			
34		41	
Leaf symptom[a]	Viroid detection[b]	Leaf symptom	Viroid detection
0	−	0	−
0	−	1	−
0	−	1	−
0	−	1	−
0	−	2	+
0	−	2	+
1	+	3	+
2	+	3	+
2	+	3	+
3	+	3	+

[a]Leaf symptoms: 0 = no symptoms, 1 = few spots on one or two leaves, 2 = spots on several leaves, 3 = many spots on all leaves.
[b]Detection of CSV by indexing: + = viroid detected, − = viroid not detected.

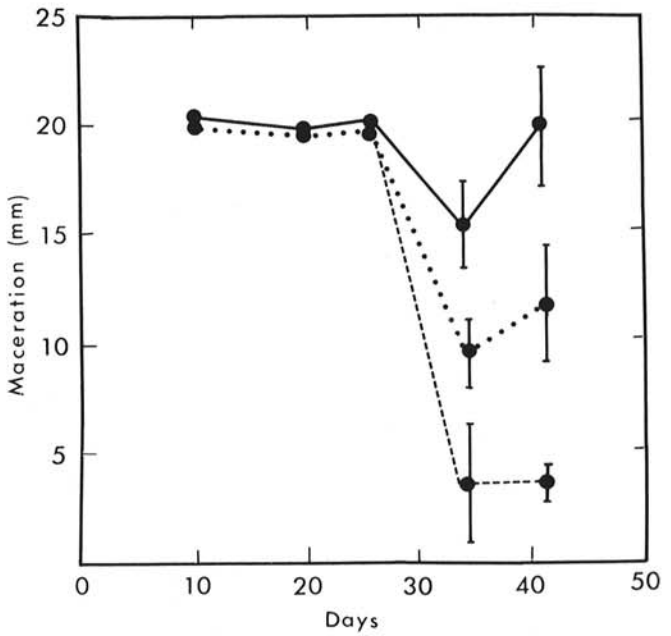

Fig. 2. Pith maceration in stems of chrysanthemum cultivar Bonnie Jean cuttings by *Erwinia chrysanthemi* 159 in non-CSV-inoculated plants (solid line) and CSV-inoculated plants (viroid detected, broken line; viroid not detected, dotted line) at 10, 20, 26, 34, and 41 days after inoculation; the vertical bars represent standard deviations.

to prevent or reduce the bacterial enzymatic degradation which normally results in soft-rot.

Sections of stems, fixed 84 days after inoculation with CSV were prepared by B. W. Pennypacker and P. E. Nelson (Pennsylvania State University, University Park, PA). These sections showed numerous extraxylary fibers and mature bundle caps as well as thickened cell walls of parenchymatous cells. However, differences in gross anatomy which would account for the reduction in bacterial activity were not revealed in our study of stem sections from CSV-infected and healthy chrysanthemums of comparable ages. The major changes in stem structure which occur do not appear to occur early enough to coincide with the correlation of detectable viroid and decreased pith maceration (Fig. 2). If the loss of succulence of CSV-infected stems is involved, it may be difficult to determine the factor(s) responsible for the reduction in maceration. Further histochemical tests might be useful toward this end, but it is difficult to apply them to such a situation. While the chance of detection of the loss or gain of small amounts of wall components would be slight, these changes could markedly affect the macerative ability of the bacteria.

The results of the histological investigation of stems infected by bacteria seem to indicate a restriction or localization of bacteria in CSV-infected plants to the xylem vessel elements (Fig. 3B). The failure of the bacteria to disrupt the vessel elements with the subsequent release into the pith cells, suggests a change in vessel structure. Pennypacker et al (19) noted that plants of some chrysanthemum cultivars produce suberin in vessel walls as part of a host response to *E. chrysanthemi* infection. Vessel elements of healthy Bonnie Jean chrysanthemums lack suberin in detectable quantities (Sudan IV test) and no such changes were found to occur in the vessels of CSV-infected plants. Evidence has been reported that the agent of Pierce's disease of grapes causes a swelling of pit-closing membranes and a mechanical blockage of pits by excessive gum and gel production (8). However, there is no evidence that CSV causes gum production or pit alterations that might impede bacterial movement through plant tissue.

The survival of bacteria in CSV-infected plants is evidence against the production of specific bactericidal substance. A study of bacterial multiplication and enzyme production in the infected tissue might show that the bacteria either were unable to produce normal amounts of macerating enzymes or that the enzymes were unable to macerate the plant tissue. Alternatively, cell-free enzyme extracts could be employed for the same purpose.

The recognition of this reaction in chrysanthemum is of importance at this time despite the lack of understanding of the mechanism. The maceration assay should be used with caution to study strains of *Erwinia* spp. since infection of plants by at least one other agent can cause variation in results not due to the virulence of the strain. The problem is compounded by the symptomless state often exhibited by CSV-infected chrysanthemums.

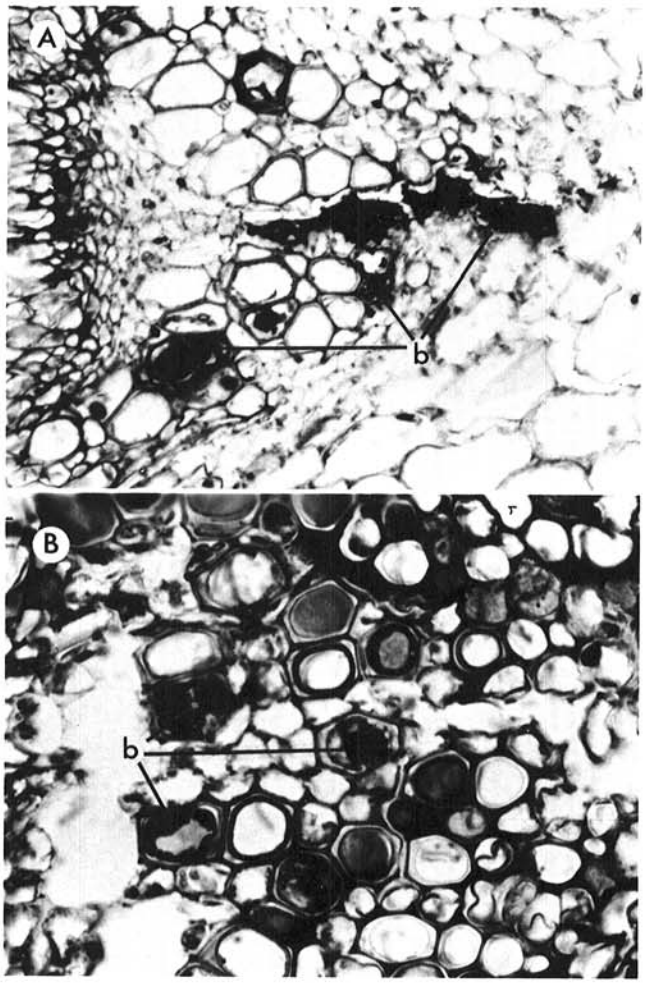

Fig. 3. Transverse stem sections of chrysanthemum cultivar Bonnie Jean cuttings infected with *Erwinia chrysanthemi* 159 (stained with Harris' hematoxylin and orange G). **A,** CSV-free stem; bacteria (b) have colonized xylem vessel elements, and have spread to nearby pith cells (× 206). **B,** CSV-infected stem; bacteria (b) remain within xylem vessel elements initially colonized without apparent change in the integrity of vessel elements and surrounding parenchyma (× 412).

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