Histopathology of *Xanthomonas campestris* pv. *citri* from Florida and Mexico in Wound-Inoculated Detached Leaves of *Citrus aurantifolia*: Light and Scanning Electron Microscopy

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ABSTRACT

Detached leaves of *Citrus aurantifolia* were wound-inoculated with three strains of the citrus pathogen *Xanthomonas campestris* pv. *citri* (strains F1 and F20 from Florida and XC90 from Mexico), a noncitrus pathogen *X. campestris* pv. *pruni* (strain XP1), a saprophyte *Erwinia herbicola* (strain EH1), or water. The wound response in leaves inoculated with water, strain XP1, or strain EH1 appears similar, consisting of three distinct zones of cytological change. Zones develop abnormally in leaves inoculated with *X. c. citri.* A zonal terminology based on host anatomical changes associated with normal wound repair was adopted that can also be used in infected tissue where normal host anatomy is modified. In water, strain EH1 and strain XP1 inoculations, zone one is closest to the wound and composed of disrupted preexisting cells, zone two contains preexisting cells with thickened cell walls and occluded intercellular spaces and zone three is a periderm. Strain XC90 induces large raised foliar lesions that occur when stimulation of cell division in zone three or bundle sheath, vascular parenthyma, and procambium cells results in protrusion of proliferating host cells through the epidermal surface. Strain F1, which produces large water-soaked lesions, inhibits development of zones two and three and is associated with a faintly stained matrix. Strain F20, which produces small necrotic lesions without water-soaking, causes initial slight suppression of cell division and subsequent disruption of zone three. Like strain F1, strain F20 was associated with matrix formation in intercellular spaces. Two characteristics, the intercellular matrix and the ability to trigger and/or prolong meristimatic activity, differentiate the Mexican and the Florida strains of *X. c. citri*.

Xanthomonas campestris pv. *citri* (Hasse) Dye causes citrus bacterial canker disease (CBCD), named after the characteristic raised lesions induced on leaves, fruit, and stems of susceptible citrus varieties. In 1984, isolates of *X. c. citri*, referred to as the nursery strains, were identified in Florida, with symptoms ranging from slight necrotic flecking to expanding water-soaked lesions (20). Pustule formation by these isolates is either delayed or absent. Currently, five groups of *X. c. citri* are distinguished (5). Groups A, B, C, and D produce canker symptoms but can be differentiated by host range, geographical distribution (5), and genomic finger-printing (7). The Florida nursery strains have been placed into group E. This study examines how two Florida isolates affect their host, comparing their histopathology with that of a canker-producing strain from Mexico. Although the histopathology of type A strains has been examined in some detail, concurrent comparisons among different strains of *X. c. citri* have not been reported.

Infection by *X. c. citri* is initiated primarily through wounds, with natural openings such as stomata and lenticils also serving as points of entry (15). The host's wound response is known to be a deterrent to infection and protection occurs after regions of wound-induced lignification are established (15). By using a wound-inoculation system rather than vacuum infiltration to compare strain differences, the interaction between *X. c. citri* and the host's wound response can also be examined.

MATERIALS AND METHODS

Bacterial cultures. All bacterial cultures were from the USDA Fruit Laboratory bacteria collection in Beltsville, MD. *X. c. citri* (strain XC90) was isolated from raised lesions on leaves of *Citrus aurantifolia* Swingle, from Mexico. *X. c. citri* (strain F1) was isolated from a flat, expanding water-soaked leaf lesion on Carrizo *citrange (Poncirus* Raf. × *C. sinensis* Osbeck), in Florida. *X. c. citri* (strain F20), also from Florida, is a nonaggressive isolate from a flat, nonexpanding necrotic leaf lesion in grapefruit, *C. paradisi* Macfady. Included as controls were *Erwinia herbicola* (Lohnis) Dye (strain EH1), a saprophyte isolated from lemon, *C. limonia* Osbeck, in Argentina and *X. c. pruni* (Smith) Dye (strain XP1), a pathogen isolated from apricot, *Prunus armeniaca* L.

Inoculation and incubation. Detached leaves from *C. aurantifolia* seedlings were needle inoculated according to a procedure described by Randhawa and Civerolo (19) except that leaves were washed in 1% sodium hypochlorite after the initial tap water rinse and no ethanol or detergent treatments were given. One young terminal leaf, one-half to two-thirds expanded, and two other nearly fully expanded leaves were placed in each dish. Each treatment was replicated twice.

Leaves were placed on 1.0% water agar, adaxial side up, in 100-mm-diameter plastic petri dishes and wounded with a hypodermic needle. A 10-μl drop of bacterial suspension containing 1×10^7 cells per milliliter, or sterile distilled water, was placed on each of five or six wounds on each half of the leaf. The edges of the petri dishes were sealed with Parafilm. To determine humidity effects on wound repair, water-inoculated controls were incubated on filter paper in unsealed petri dishes to maintain a lower humidity. Leaves were incubated at 25 C under 16 hr photoperiods with warm white fluorescent lights at a photon flux density of about 60 $\mu E \cdot sec^{-1} \cdot m^{-2}$.

Sampling and processing. Samples about 2 mm square were cut with a razor blade 1, 2, 3, 4, 5, 7, 10, and 14 days postinoculation and included the centrally located wound. Seven-day samples included surrounding lesion and adjacent symptomless tissue from nonwounded controls. Experiments were repeated at least three times. Tissue was fixed at 4 C in 2% glutaraldehyde and 1.5% acrolein in 0.05 M Na_2HPO_4-$KH_2PO_4(PO_4)$, pH 7.0. Samples were postfixed in 1% OsO_4, dehydrated in ethyl alcohol, and embedded in Spurr's epoxy resin for light microscopy. Observations were made from samples taken from five to seven wound sites from each replicate in all experiments. Thick (5–7 μm) sections, containing the wound and surrounding tissue from a minimum of five tissue blocks per treatment were placed in Haupt's adhesive and 4% Formalin, dried, and either stained in 1% brilliant green (10 min) or

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in 0.01% methylene blue (1 hr) to differentially stain cellulose (blue), lignin (green), and pectin (violet) (8). Sections were rinsed in water and mounted in glycerin.

Leaf samples for SEM were dehydrated in ethanol, critically point dried, mounted on double-sided tape, and attached with silver paint to SEM stubs. Samples were coated with gold in a Polaron Model #E5100 Series II Sputter Coater (Polaron Instruments, Line Lexington, PA).

RESULTS

Leaf symptoms. Leaves in all experiments remained turgid and green during the 2-wk sampling period. On control leaves, small necrotic lesions developed at wound sites exposed to droplets of distilled water after 3 days (Fig. 1A). Leaf maturity did not influence lesion size and the necrotic lesions did not expand significantly during the duration of the experiment.

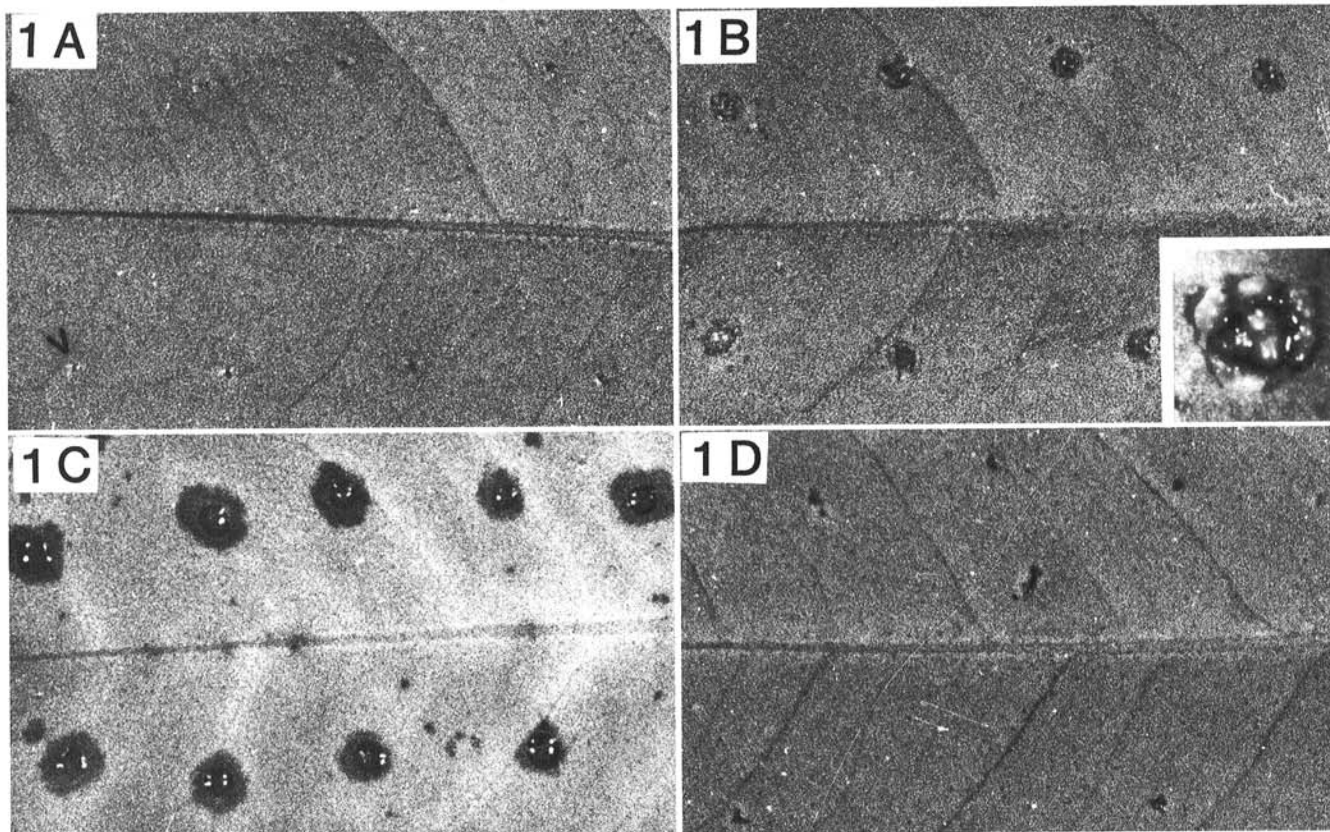

Fig. 1. Detached leaves of *Citrus aurantifolia* that had been wound-inoculated 14 days before harvest. **A,** Water inoculated leaves. Pinprick lesions (arrow) with wound callus. **B,** Strain XC90. Raised blisterlike lesions. Inset, Enlargement of foliar blister. **C,** Strain F1. Expanding water-soaked lesions with drops of liquid over wound-sites. **D,** Strain F20. Small lesions without water soaking.

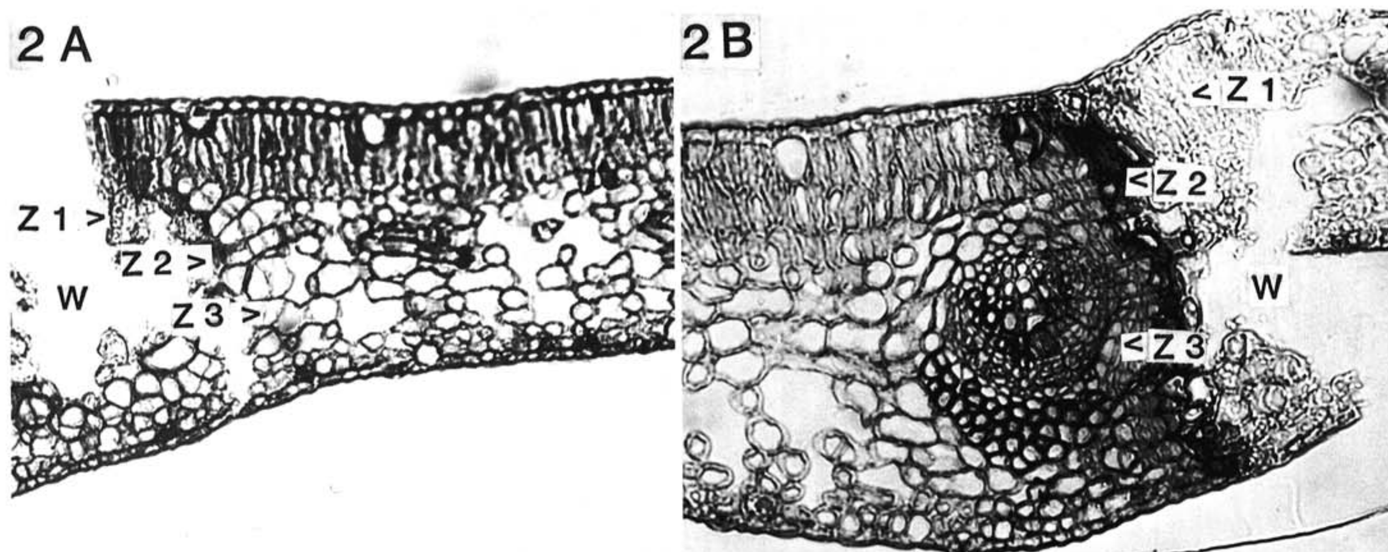

Fig. 2. Healthy wound repair in leaves of *Citrus aurantifolia* wound-inoculated (W) with distilled water (×220). **A,** Three days. Cells in zone one (Z1) are stained erratically and contain disrupted cytoplasm. Zone one is only one to two cells wide. Cells in zone two (Z2) contain thick, densely stained walls. There are regular divisions across the enlarged cells in zone three (Z3). **B,** Five days. Zone one is faintly stained, zone two is resolved into a distinct dense-staining region, and the cambium of zone three is fully developed and associated with vascular tissue.

Leaves inoculated with strain XC90 showed slight swelling and chlorotic halos surrounding the wound 2 or 3 days after inoculation. The epidermis ruptured 1–3 days later and a blister formed and continued to enlarge (Fig. 1B). Additional eruptions occurred around the wound during the next 9 days. Lesions formed most rapidly and were larger on leaves removed from near the stem apex than on more fully expanded leaves three to five nodes further from the apex.

Strain F1 produced expanding water-soaked lesions surrounded by chlorotic halos. Water-soaking appeared 48–72 hr after inoculation and large drops of liquid appeared over the wound site 1 or 2 days later (Fig. 1C). Leaves nearest the stem apex showed the most prominent lesion development in four out of six tests.

Leaves inoculated with strain F20 developed callus around the wound without water-soaking after 48–72 hr. A drop of liquid appeared over the wound and tissue adjacent to the wound appeared swollen. Small lesions without water-soaking developed after 7 days (Fig. 1D). Leaf maturity did not influence symptom development and lesions did not enlarge during the 2-wk sampling period. These symptoms could not be distinguished from those on wounded healthy leaves.

Leaves inoculated with *E. herbicola* showed small necrotic lesions after 4 days. No expansion of lesions occurred during the next 10 days. After 72 hr, small necrotic lesions surrounded wound sites of leaves inoculated with strain *X. c. pruni*. Lesions were similar to those on healthy controls and leaves inoculated with *E. herbicola*, although chlorotic halos developed. Lesions did not expand and water-soaking did not occur.

Histology of healthy wound repair. Twenty-four hours after wounding, cytoplasm was disrupted both in wounded and adjacent intact cells. Disrupted cells were only lightly stained with brilliant green and methylene blue. Walls of cells farther from the wound were stained more intensely. Brilliant green stained cell walls green and methylene blue stained walls blue and middle lamellae pink to violet. Cytoplasm throughout the sections was brown in both stain preparations. Brown stain reactions resulted from osmium fixation and appeared in sections not treated with light microscopy stains.

After 48 hr, hypertrophy, sporadic cell division, and irregular wall thickening appeared in mesophyll cells separating faint-staining and normal staining cells. Three to five days after wounding, there were three distinct zones traced from the wound-site through an area of cellular change into normal tissue (Fig. 2). Transitional cells between zones contained characteristics of

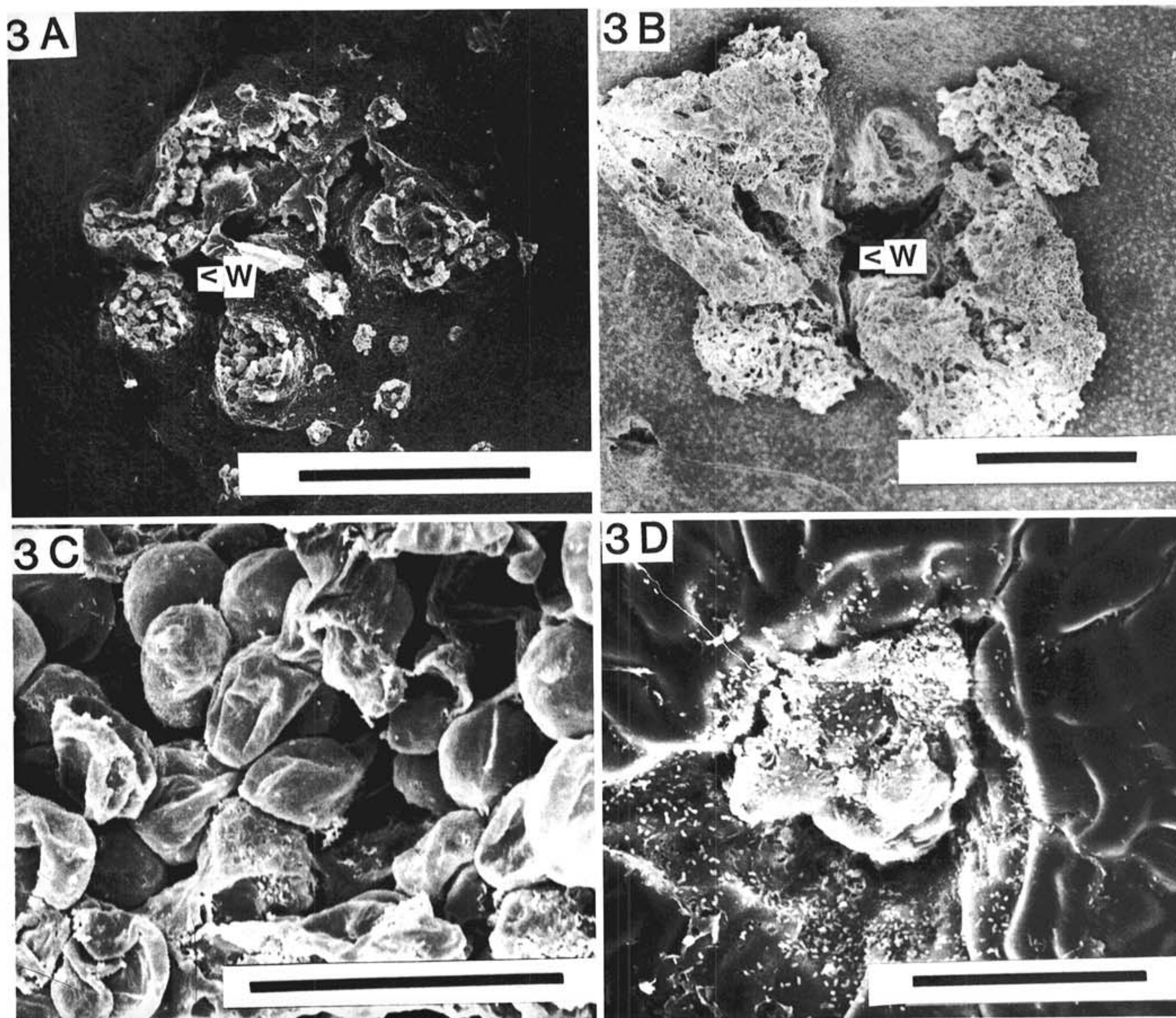

Fig. 3. Scanning electron micrographs of pustule development in leaves infected with strain XC90. **A,** Early eruptions originating from many points around the wound (W), 6 days after inoculation. **B,** Extensive cell proliferation protruding through epidermis around a wound-site, 15 days after inoculation. **C,** Collapsed host cells on a pustule surface overlying intact proliferating cells, 17 days after inoculation. **D,** Higher magnification view of the 6-day-old pustule in A, showing a high concentration of bacteria (A and B bar = 500μm, C and D = 50μm).

contiguous zones. Zone one extended 1–15 rows of cells from the wound and contained cells with faintly stained or unstained walls, and disrupted cytoplasm. Zone two was composed of one to three rows of cells with densely stained thick walls and little or no cytoplasm. Thick cell walls stained green with methylene blue. Cells in zone two showed degeneration after 7 days and appeared empty and misshapen with an occluding substance in intercellular spaces that methylene blue stained green-brown in some samples and dense blue-brown in others. At 3 days, the third zone consisted of one or two rows of hypertrophic cells with large vacuoles and peripheral cytoplasm that showed hyperplastic activity (Fig. 2A). Zone three was frequently, but not always, associated with vascular tissue. Cell enlargement and subsequent division in vascular tissue was initiated in bundle sheath cells and procambium was incorporated into the dividing layer. Regular divisions of hypertrophic cells transformed zone three into a periderm three to nine cells wide. Phellem cells developed zone two characteristics as cell walls thickened and stained green with methylene blue; intercellular spaces between zone two and phellem filled with occluding substances (Fig. 2B). Cells in the cambium contained abundant brown-staining cytoplasm and small vacuoles. The cambium region did not appear to increase in width after 5–7 days.

Ten to 14 days after wounding, zone two collapsed. Zone one cells remained intact under high humidity conditions and collapsed under low humidity. Under both conditions, zones one and two apparently corresponded to the necrosis visible at the wound site.

Strain XC90. *Surface morphology.* Pustules first appeared 3–7 days after inoculation. SEM revealed a cluster of cells protruding beyond the leaf surface at several points close to the wound (Fig.

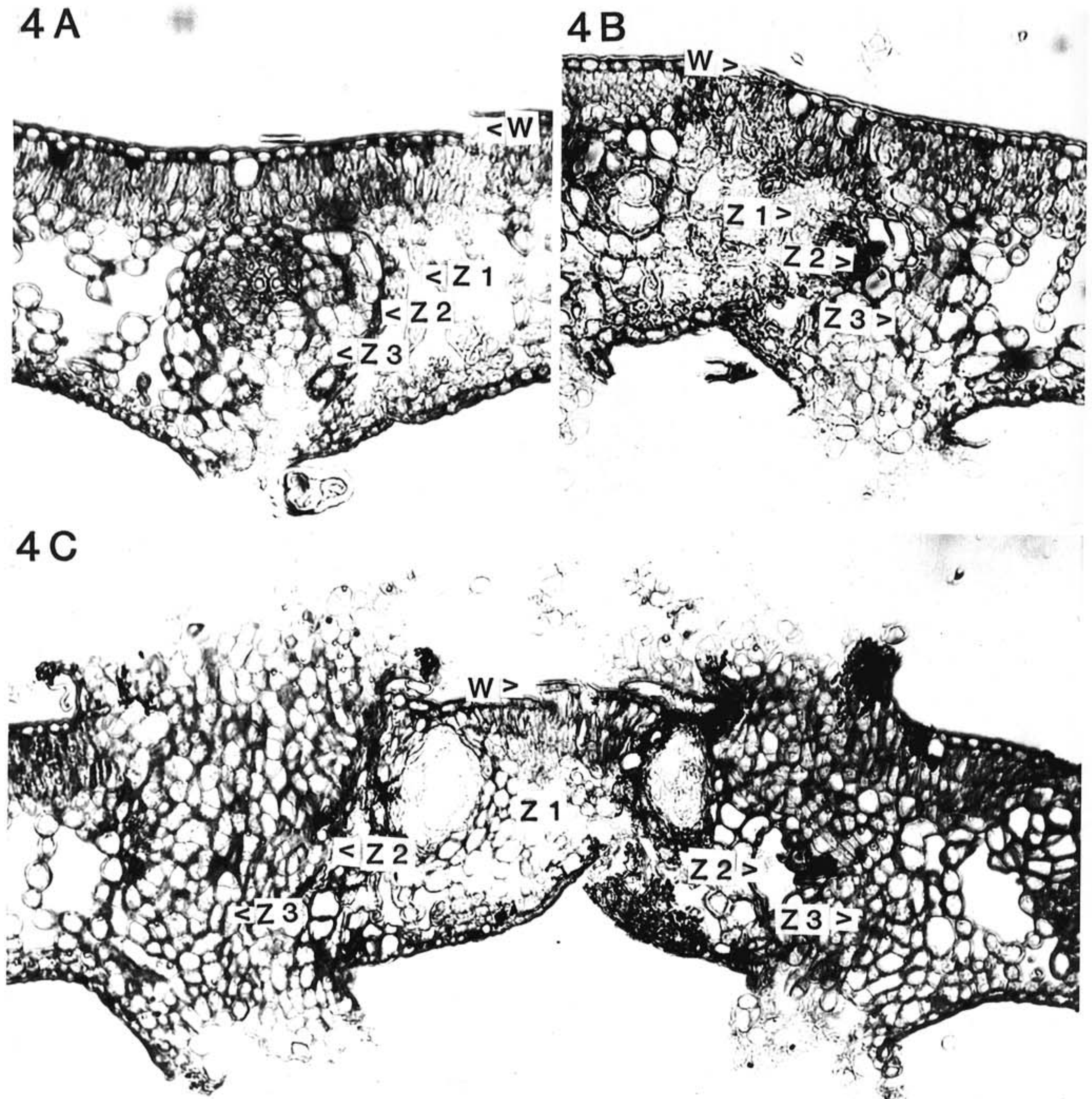

Fig. 4. Leaves inoculated at wound sites (W) with strain XC90 (×270). **A,** Three days. The wound is associated with a vascular bundle, and the epidermis has ruptured above proliferating zone three (Z3) cells. **B,** Seven days. Zone three (Z3) cambium is fully developed. Irregular cell proliferation has caused rupture of the lower epidermis and blister formation. The upper epidermal area appears like healthy controls. **C,** Ten days. Zone one (Z1) is composed of intact cells and collapsed cells among dense-staining particles. Zone two (Z2) is breaking down, and extensive cell division in zone three (Z3) has ruptured both epidermal surfaces on either side of the wound.

3A). Within 10 days, small individual pustules coalesced, and the wound was surrounded by a large blister (Fig. 3B). Blisters were composed of both intact and collapsed host cells (Fig. 3C) with associated bacteria (Fig. 3D).

Histology. Inoculations with strain XC90 appeared similar to water controls for the first 24–48 hr postinoculation as the three zones of wound repair were initiated. Within 72 hr, the orderly transformation of hyperplastic cells into a cambium was modified by numerous divisions and stimulation of additional cell division in adjacent vascular tissue (Fig. 4A). Before rupture, the epidermis swelled above clusters of dividing zone three cells. Similar cell clusters occurred above vascular bundles separated from the primary eruptions by 10 or more cell layers of undividing cells.

Seven days after wounding, zone one cells began collapsing at zone two, and a dense brown osmophilic substance was visible within the debris. Degeneration of zone two cells occurred as in healthy controls. Zone three appeared as a mass of undifferentiated dividing cells filling the interepidermal area and protruding through epidermal ruptures to form a pustule (Fig. 4C). The proliferating area was composed of small meristimatic cells containing abundant cytoplasm and small vacuoles and of larger cells with large vacuoles and peripheral cytoplasm (Fig. 4C). Remnants of zone three were detected in the proliferating cells as a cambiumlike layer twisting in and out of the plane of section (Fig. 4B). Unlike healthy wound repair, division occurred in newly derived cells as well as cambial initials. Cells erupting above epidermal surfaces began separating and a brown matrix similar to that observed at the zone one to two transition was observed between diverging blister cells and beneath epidermal cells bordering the rupture. Cells at the top of the blister were often collapsed and, like zone one cells, did not retain stain in cell walls.

Within 10–14 days, extensive degeneration in zone one resulted in a mass of collapsed cell walls and cytoplasmic debris amidst the dense brown substance observed 3 days earlier. Densely stained cell walls in the tissue at the edge of zone one represented the remains of zone two. Extensive proliferation continued to occur beyond zone two, with additional collapse in blister cells.

Strain F1. *Surface morphology.* Symptomatic leaves with large water-soaked lesions could not be distinguished from healthy controls and bacteria were rarely observed on epidermal surfaces examined by SEM.

Histology. Wound sites in leaves inoculated with strain F1 developed zone one cytoplasmic disruption within 24 hr after inoculation. By 72 hr, cell wall thickening in zone two was not evident, and irregular division of enlarged cells resulted in incomplete formation of zone three. A faint, blue-staining matrix was observed in mesophyll intercellular spaces from the wound to the edge of the sample in sections stained with methylene blue.

Five to 7 days after inoculation, tissue in locations corresponding to zones two and three had not yet developed thickened walls or a periderm (Fig. 5A and B). Mesophyll cells in zone one were asymmetrical and separated from each other. Disorganized pockets of enlarged, dividing cells were scattered throughout the mesophyll between zone one and the edge of the sample. Mesophyll cells, including dividing ones, contained large vacuoles and peripheral cytoplasm. A brown particulate

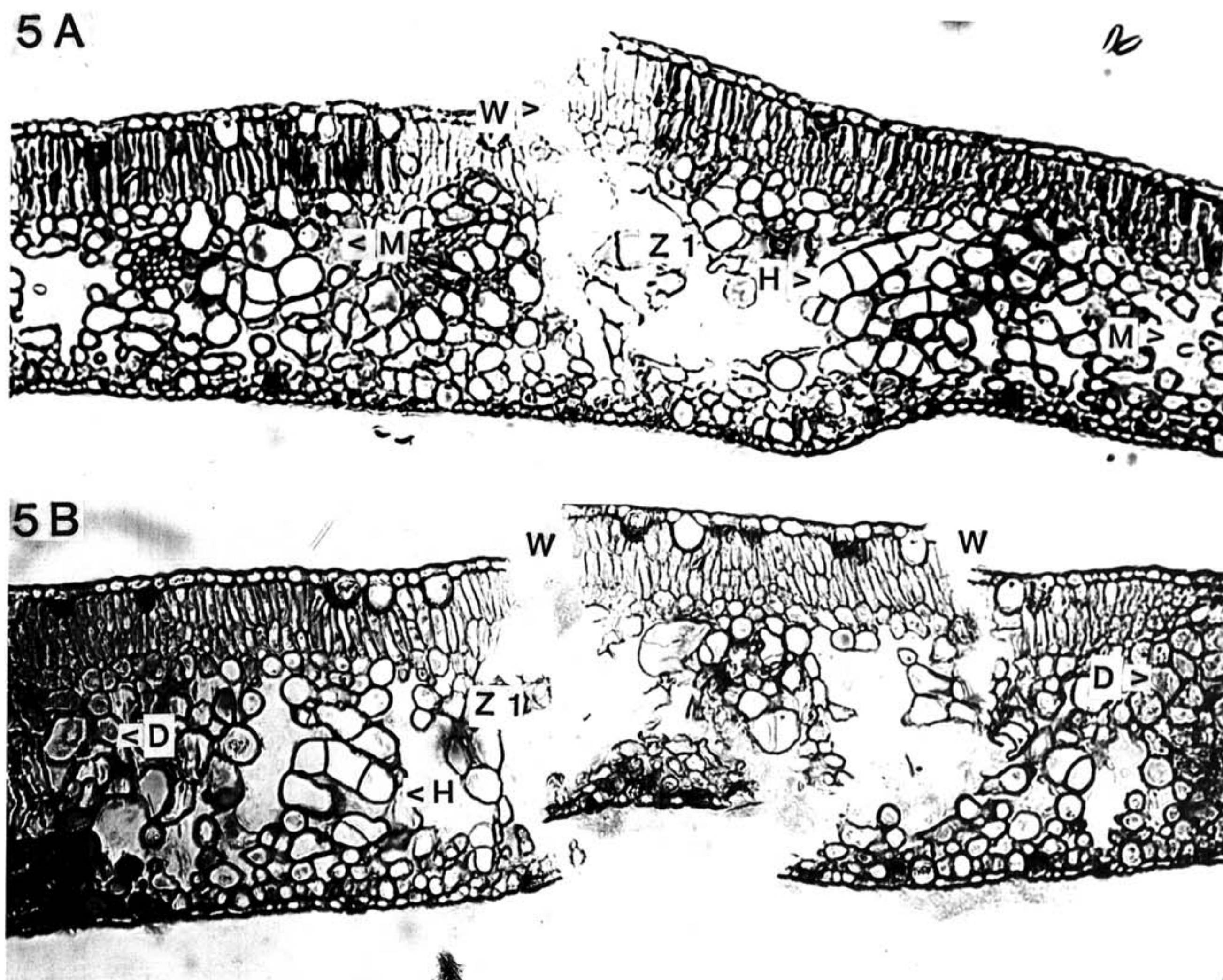

Fig. 5. Leaves inoculated at wound sites (W) with strain F1 (×240). **A,** Seven days. Zones two (Z2) and three (Z3) lack wall thickening and periderm formation. Hyperplasia, hypertrophy (H), and intercellular matrix (M) are present. **B,** Fourteen days. Scattered cell wall thickening and hypertrophy (H) are present, but zones two and three are indistinct. Blue cells (D) in the midlesion area have dense blue staining contents.

component was resolved against the homogenous blue background of the matrix observed in 72-hr samples.

Sporadic cell wall thickening appeared after 7–14 days in asymetrical, noncontiguous mesophyll cells between zone one and the edge of the sample. Like cell division, wall thickening was not confined to a specific region in relation to the wound (Fig. 5A and B). At 10 days, pockets of misshapen cells with blue staining contents were observed at midlesion among more normally stained cells. Blue cells occurred in sections stained with either brilliant green or methylene blue.

Strain F20. *Surface morphology.* Small lesions around the wound-inoculation site were composed of wound callus. There was no evidence of water-soaking, and the epidermis was not ruptured beyond the point of wound inoculation.

Histology. Development of zones two and three proceeded as in healthy controls and collapse of zone two occurred between 7 and 10 days. However, 48 hr after inoculation, zone one cells could be discerned only with difficulty, as they remained unstained. Within 4–5 days postinoculation, many zone one ghost cells had collapsed and faint outlines of cells were observed protruding through epidermal ruptures (Fig. 6A). Within 7 days, zone one appeared empty of cells except for peripheral cells adjacent to zone two (Fig. 6B). Fourteen days after inoculation, large regions of cellular collapse containing a faintly stained blue matrix occurred within and beyond zones one, two, and three. Cells in the midlesion area were filled with dense brown staining cytoplasm and many contained a dense staining particulate substance within vacuoles.

E. herbicola. *Histology.* Zone one appeared 48 hr after inoculation and initiation of zones two and three began within 72 hr after inoculation. Zone two began degenerating 2 days later and collapsed within 10 days postinoculation. Zone three cambium formation was complete within 5 days after inoculation.

X. c. pruni. *Histology.* Zone one appeared 48 hr after inoculation, and zones two and three were initiated 24 hr later. Zone two began degenerating 5 days postinoculation and had collapsed within 10 days. Zone three adjoined vascular bundles in 7-, 10-, and 14-day samples, and the cambial region 8–10 cells wide was slightly wider than cambiums of similar age and position in water controls.

DISCUSSION

The wound response in *C. aurantifolia* forms the basis for many of the histological differences between leaves wound-inoculated

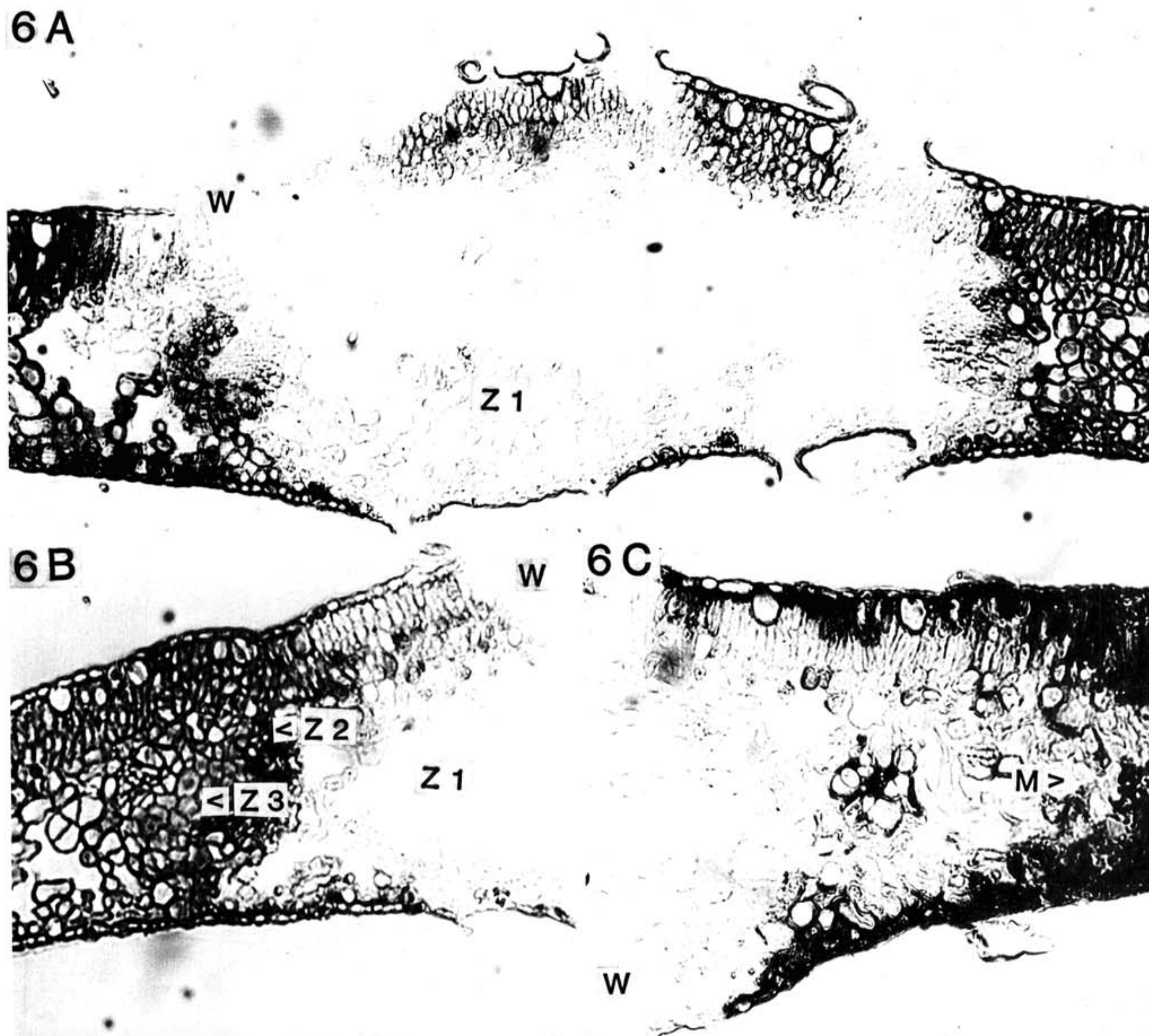

Fig. 6. Leaves inoculated at wound site (W) with strain F20 (×270). **A,** Four days. Zone one (Z1) cells are faintly stained, many too faintly for photographic resolution. Zone two has been initiated, and zone three cells are dividing. The wound-site appears swollen. **B,** Seven days. Zones two (Z2) and three (Z3) are fully developed. **C,** Two weeks. The disorganized remains of zones two (Z2) and three (Z3) and the intercellular matrix (M) are present.

with one of three strains of the citrus pathogen *X. c. citri*, the noncitrus pathogen, *X. c. pruni*, or the saprophyte, *E. herbicola*. This wound response, delineated into three distinct areas of cytological change, contains anatomical features similar to those reported in wound responses in many diverse genera (2–4,6, 15,17,18). Zone one, however, composed of preexisting cells closest to the wound, appears as a discrete zone only at high humidity. Its collapse at lower humidity may more closely resemble wound repair in leaves of *C. aurantifolia* under natural conditions. Zone two corresponds in location and appearance to impervious tissues; regions of lignification, suberization, and/or wax deposition that impede water movement by intercellular space occlusion and cell wall thickening (2,3,18). Although zone two tissues were not tested for imperviousness, their collapse after 7 days, when occlusion and thickening appeared prominent, may be an indirect measure of reduced penetrability. Similar collapse has been attributed to poor infiltration of such regions with embedding resins (18). The zone three periderm is an area of cell division, a general plant response to wounding (4,6,17,18). The zone terminology used in this paper, while based on host anatomy, was adopted because it can be used in infected tissue where normal host anatomy has been obscured by the host/pathogen interaction.

Impervious tissues and periderms combine to seal off wounds and protect internal tissues from water loss and entry of microorganisms (1,16,18). The ability to modify the wound response distinguished the three citrus pathogens from the noncitrus pathogen and saprophyte controls and probably contributed to the successful invasion by the pathogens. Because pathogenesis and wound repair were initiated simultaneously, it is unknown whether the pathogens would be able to breach the host defense once established. Previous citrus/*X. c. citri* studies indicate that once established, barriers are not easily overcome (15). Inoculations of wounded leaves of *C. natsudaidai* with a type A isolate of *X. c. citri* before barrier zone formation resulted in infections, but inoculations made after completion of lignification and cell division did not.

Two characteristics, the effect on wound repair and the presence of an intercellular matrix, appear to distinguish the Florida strains from the Mexican strain XC90. Strain XC90 stimulates wound repair, but the Florida isolates suppress it, either significantly (strain F1) or slightly (strain F20). The ability of strain XC90 to stimulate cell division was also observed in vascular cells in which masses of dividing cells appeared to originate from bundle sheath, procambium, and vascular parenchyma cells. Strains F1 and F20 apparently lack this ability to trigger and/or prolong meristematic activity in undifferentiated cells.

Matrices occurring in infections of *X. c. citri* have previously been associated with bacterial immobilization and host resistance (13). It is unlikely that the pale blue matrix observed in intercellular spaces of leaves inoculated with strains F1 and F20 represents a host defense. Bacterial invasion continues to progress despite its formation. The Florida isolates produce greater quantities of extracellular polysaccharide in culture than does strain XC90 (Civerolo, *unpublished*) and this may be reflected in vivo.

Strain F1 infections were characterized by a host cell type that was not observed in strain F20 or any other strains. Blue cells contained a dark blue color in the cytoplasm that was typically observed only in cell walls. Staining similarities between the wall and the cytoplasm may indicate cell wall damage and be observed macroscopically as the water-soaked symptom that differentiates strain F1 from strain F20.

Previous investigations into histopathology of *X. c. citri* have used type A strains, which, like strain XC90, frequently induce raised lesions (9–15). As in strain XC90 infections, type A cankers may show an association with an underlying periderm. In satsuma orange fruit, raised lesions occurred only on young fruit and were composed of proliferating wound periderm cells (9). Mature fruit did not develop a periderm and cankers were not formed. In contrast, a CBCD-A strain apparently inhibited the transformation of hypertrophic cells into wound meristems in detached *C. natsudaidai*, a situation more similar to the Florida isolates than to strain XC90 (10). Host species, leaf or fruit maturity, type of inoculation, presence of a wound and the state of wound repair, and environmental conditions such as temperature and humidity levels have all been reported to influence the histopathology of infections of *X. c. citri* (9–15). Given this high potential for variability, it is difficult to identify characteristics that might be consistently useful as taxonomic markers. However, the different histopathology displayed by the Florida strains and XC90 under identical conditions indicates that these isolates have significantly different modes of action in the host and supplements the taxonomic data on DNA fingerprinting (7) that separate the Florida and Mexican strains of *X. c. citri*.

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