Histopathology of *Syngonium podophyllum* Artificially Inoculated with *Xanthomonas campestris* pv. *syngonii*

Cathy H. Zumoff and Robert S. Dickey

Research technician and professor, Department of Plant Pathology, Cornell University, Ithaca, NY 14853.
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ABSTRACT

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A histological study was made of *Syngonium podophyllum* 'White Butterfly' after inoculation with *Xanthomonas campestris* pv. *syngonii* to determine the development and effect of the pathogen in leaf, petiole, and stem tissues. The pathogen entered through the stomata of leaves sprayed with an aqueous suspension of the bacteria. The pathogen initially colonized substomatal and intercellular spaces of the spongy mesophyll and caused no apparent effects on surrounding cells even when initial water soaking was evident. As water soaking progressed, disruption of mesophyll cells occurred: Palisade and spongy parenchyma cells were surrounded by bacteria, followed by thinning and disruption of the cell walls. As the numbers of bacteria increased, they sometimes filled either large areas in the mesophyll or areas previously occupied by palisade cells. Bacteria did not occur in the vascular bundles of veins or rib veins. As inoculated leaves yellowed, dried, and shriveled, the tissues were compressed and became completely indistinguishable, and bacteria were not identifiable by light microscopy. *X. c.* pv. *syngonii* occasionally entered an uninjured petiole via stomata with subsequent development limited to substomatal and adjacent intercellular spaces. Petioles inoculated by needle-puncture with bacterial cells remained symptomless although the pathogen multiplied and eventually spread the length of the petiole and into the leaf but not the stem. The bacteria predominantly occurred in the intercellular and substomatal spaces at the periphery of the needle-punctured side; however, invasion and disruption of xylem tracheary elements occurred after needle inoculation. Evidence suggested that some of the damage to leaf and petiole tissues was mechanically produced by the bacterium. The pathogen was not isolated from or observed in stems of either leaf- or petiole-inoculated plants.

Dickey and Zumoff (3) recently reported the occurrence of a leaf blight of *Syngonium podophyllum* Schott, the symptoms of which are similar to those reported by Wehlburg (19,20), but which is caused by a new pathovar of *Xanthomonas campestris* designated *X. c.* pv. *syngonii*. Although the pathogen was originally isolated from lesions on leaves of *S. podophyllum* cultivars Cream and White Butterfly, it produces necrosis on the leaves of cultivar Green Gold as well, and growers have reported that as many as one-third of their *Syngonium* plants have been affected.

Typical symptoms on the lower leaves of naturally infected plants included lesions, from which the pathogen was isolated, that were dark, moist or dry, and sometimes papery. When infected leaves were allowed to remain on the plant, necrosis spread unevenly, and the leaves ultimately became yellow, dry, and shriveled. Spread of the pathogen from infected leaves to adjacent or nearby leaves occurs, especially when overhead water is applied to the plants (3). Symptoms initially did not occur on petioles artificially inoculated with the pathogen midway between the stem and leaf lamina; however, they do occur eventually at the petiole-lamina junction (3). The pathogen was readily isolated from the infected leaf tissues and the portions of the petiole between the point of inoculation and leaf lamina. The pathogen was not isolated from the junction of stem and inoculated petiole or from other areas of the stem. In addition, rooted cuttings that are produced from the stem sections are not infected (3). The present study was initiated to determine the histological effects of *X. c.* pv. *syngonii* on the leaf, petiole, and stem tissues of artificially inoculated *S. podophyllum* 'White Butterfly' and to elucidate the possible mode and extent of spread of the pathogen in the infected plant.

MATERIALS AND METHODS

Two groups of cuttings of *S. podophyllum* 'White Butterfly' were rooted under mist in a steam-treated mixture of soil, perlite, and peat moss. One group of rooted cuttings was kept on a greenhouse bench for 57 days before inoculation. One petiole on each of three plants was punctured with a needle containing cells from a 48-hr potato-dextrose agar (PDA) (3) culture of *X. c.* pv. *syngonii* strain LX 105, LX 114, or L 212. The petioles were inoculated midway between leaf and stem (about 45–70 mm from petiole-lamina junction). These petiole-inoculated plants were maintained in a greenhouse with an average weekly temperature of 21.1–26.1 C, were watered from overhead, and were fertilized weekly with a commercial fertilizer. The petioles that had been inoculated with strain LX 105 or LX 114 were removed 41 days after inoculation and cut into 5-mm segments, and alternate segments were processed for either bacterial isolation or histological study. At 88 days after inoculation, portions of the stem that were 5, 15, 25, and 35 mm below and 5, 25, 80, and 150 mm above the node to which the petiole inoculated with strain LX 114 had been attached were prepared for histological examination. The petiole inoculated with strain L 212 was not removed, but at 125 days after inoculation, stem sections at 5 mm below and 5, 75, 90, and 105 mm above the node of the inoculated petiole were excised and processed.

The other group of rooted cuttings was kept on a greenhouse bench for 80–90 days before inoculation. Several leaves on each plant were petiole- or spray-inoculated with strain L 212 as previously described by Dickey and Zumoff (3). The petioles were puncture-inoculated about 2.5 cm from the petiole-lamina junction, or the leaves were sprayed with an aqueous suspension of cells at a concentration of about 10^8 colony-forming units (cfu)/ml prepared from a 24-hr PDA slant culture grown at 27 C. These plants were maintained on a greenhouse bench as described above except the average weekly temperature ranged from 21.7 to 23.3 C. Selected leaf and petiole samples were excised for histological study 1–10 days after initial water soaking and necrosis developed in the leaf (= 18–28 days after inoculation). A small portion of the sample or tissue from very near the sample usually was used for bacterial isolation. Segments and samples of stem, petiole, and leaf also were processed for uninoculated, symptomless White Butterfly plants from each group of rooted cuttings.

Tissue pieces approximately 5 × 7 mm were fixed in Formalin-acetic acid-alcohol (FAA) (15), dehydrated in a tertiary butyl alcohol series (10), infiltrated and embedded in Paraplast (Sherwood Medical Industries, St. Louis, MO), sectioned on a rotary microtome at 10 μm, and stained with Harris hematoxylin

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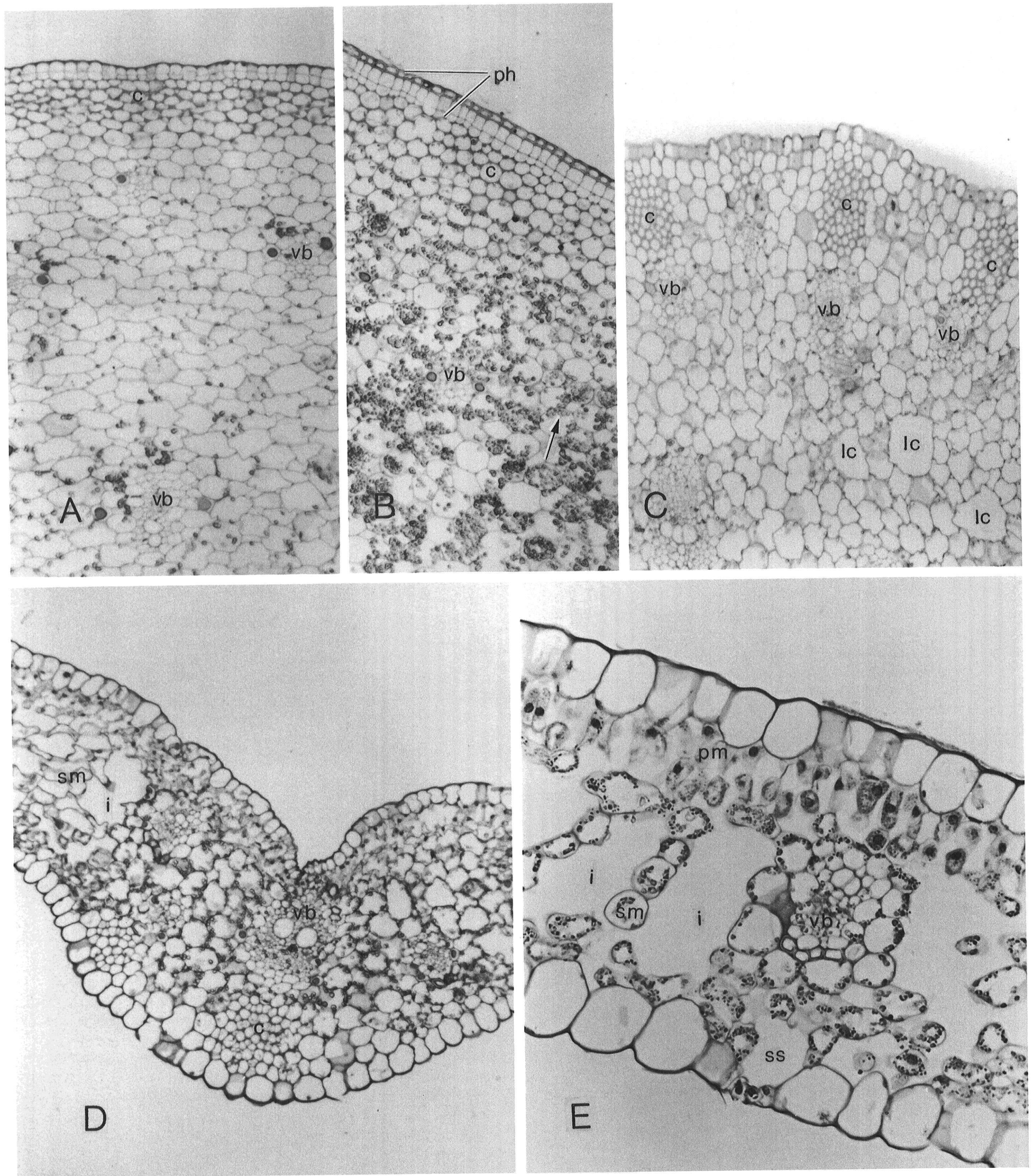

Fig. 1. Portions of transverse sections of uninoculated *Syngonium podophyllum* 'White Butterfly.' **A,** Peripheral tissues of young stem showing continuous layer of collenchyma (c) and vascular bundles (vb) scattered throughout parenchyma cells (×138). **B,** Peripheral tissues of mature stem. Note region (ph) of a phellem with periclinally dividing cells of phelloderm and phellogen layers immediately below the phellem. Several of the numerous spherical inclusions characteristically present in ground parenchyma are indicated by an arrow (×117). **C,** Peripheral region of the petiole. Note groups of collenchyma cells (c) with collateral vascular bundles (vb) located interior to each group with an intervening zone of parenchyma cells and the arrangement and formation of large cavities (lc) in the parenchyma (×79). **D,** Rib vein area of leaf showing arrangement of collenchyma cells (c), vascular bundle (vb), spongy mesophyll (sm), and intercellular space (i) (×103). **E,** Leaf tissue with vein. Note prominent substomal space (ss), vein vascular bundle (vb), somewhat disrupted palisade mesophyll (pm), large intercellular spaces (i) separated by single-layer strands of spongy mesophyll (sm) (×256).

(0.5 hr) and orange G according to a modified schedule of Johansen's (10) or with Johansen's quadruple stain (10). Sudan IV was used to determine the presence of suberin and cuticle (15). Production of lignin and gum was determined by a modification of the phloroglucinol method (10), in which sections were mounted in a saturated solution of phloroglucinol in 18% HCl (B. Pennypacker, *personal communication*). The sections were examined with a Leitz Ortholux microscope. Photographs were taken with a Zeiss Photomicroscope II with bright field optics.

The sections or samples that were used for isolation of bacteria were rinsed and vortexed two times in sterile deionized water (SDW), dried, and comminuted in 1.5 ml of SDW. After 1–1.5 hr, loopfuls of the suspensions were streaked on nutrient broth-yeast extract agar (16) that was incubated at 27 C or room temperature and examined at 3, 7, and 10 days for growth of characteristic *X. c.* pv. *syngonii* colonies.

RESULTS

Histology of uninfected plants. Results of our studies of stem, petiole, and leaf structures of *S. podophyllum* are briefly described because previous investigations (2,5–7) have been limited to specific or selected tissues or structures of *Syngonium* species. This information is essential to determine the locations and effects of the pathogen in host tissue.

The epidermis of stems was devoid of stomata (Fig. 1A), and the walls eventually contained both lignin and suberin characteristic for phellem. Phellogen and phelloderm layers occurred immediately interior to the phellem of mature stems and were adjoined by two to four continuous layers of collenchyma that contained cellulose but lacked suberin or lignin (Fig. 1B). The bulk of the tissue underlying the collenchyma consisted of parenchyma cells that often contained orange spheres with a central mass of dark blue needle-shaped particles when stained with hematoxylin-orange G. Idioblasts containing raphides and druses were scattered among the parenchyma cells. The vascular bundles near the periphery were small and incompletely developed, but the collateral and amphivasal bundles in the central cylinder were large and fully formed (Fig. 1A and B).

The epidermis of the petiole consisted of cuticularized cells devoid of trichomes (Fig. 1C). Stomata were occasionally visible and appeared to be neither sunken nor raised. The substomal spaces communicated readily with the small intercellular spaces in the immediate vicinity. Small individual groups of collenchyma cells, which occurred around the periphery, were separated from the epidermis by one or a few layers of parenchyma cells. Small vascular bundles were located immediately interior to each group of collenchyma cells with an intervening zone of parenchyma cells. The parenchyma cells usually did not contain the inclusions that occurred in stem parenchyma, and raphides and druses were not as common as in stem tissue. An unusual feature of cell arrangement in the petiole was the presence of large, seemingly empty ovoid cavities in the parenchyma that separated the incompletely formed peripheral vascular bundles from the well-developed collateral central vascular bundles. The cavities were arranged in an irregular double layer around the entire section, and each was separated by at least one and usually several layers of parenchyma cells. The functional significance of these cavities was not clear, although

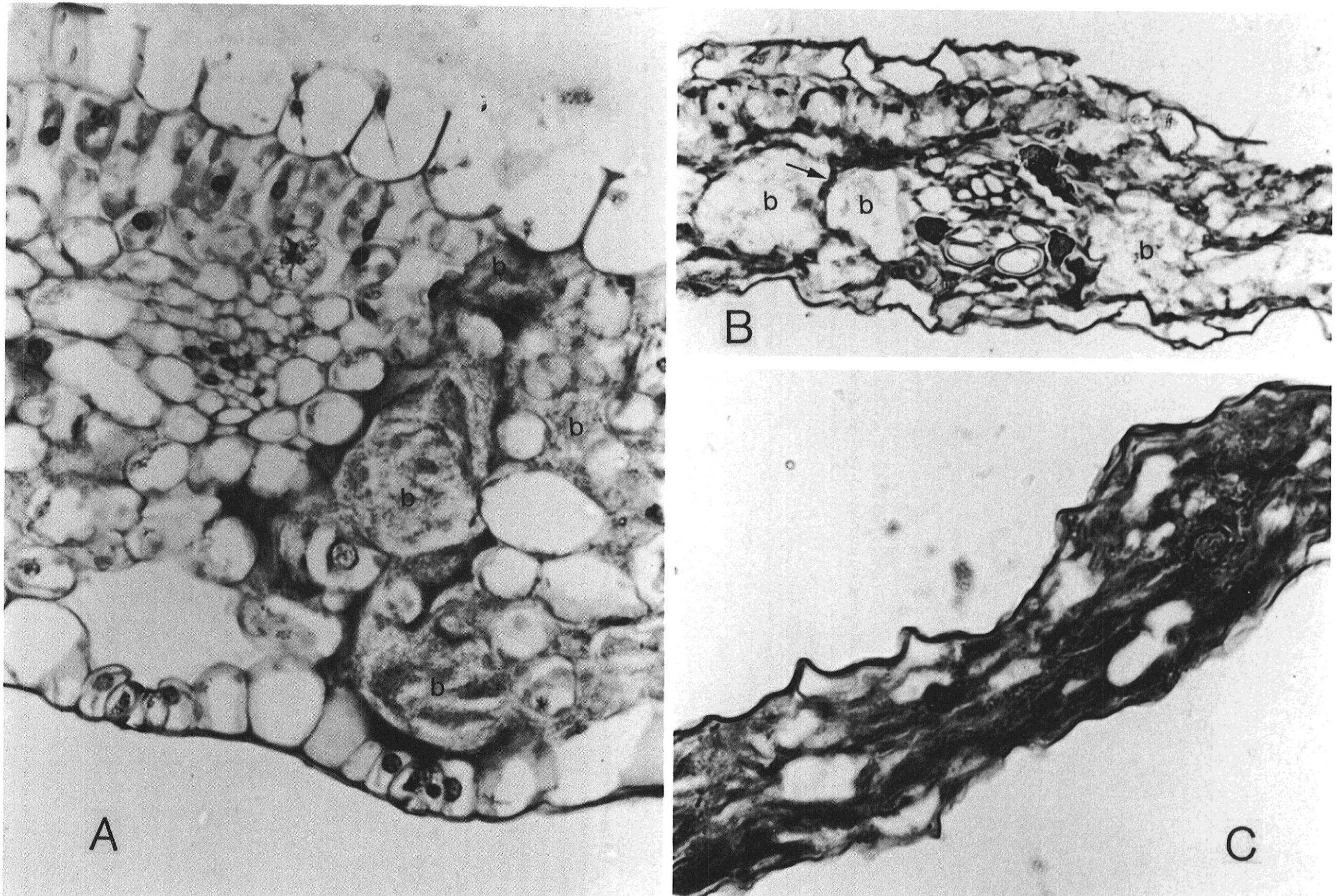

Fig. 2. Portions of transverse sections of *Syngonium podophyllum* 'White Butterfly' leaves spray-inoculated with *Xanthomonas campestris* pv. *syngonii* strain L 212. **A,** Water-soaked tissue showing colonization by masses of bacteria (b) in a substomal and intercellular space, an area in the palisade mesophyll, and surrounding the cells of the spongy mesophyll (×399). **B,** Portion of necrotic leaf tissue in which epidermal and palisade mesophyll cells are distorted and large areas of spongy mesophyll that contain clumps of bacteria (b) are divided by strands of unidentified material (arrow), presumably cell remnants. Note that vascular tissues of vein are distinguishable (×249). **C,** Portion of dry, necrotic leaf tissue showing compressed, indistinguishable internal tissue and absence of identifiable bacteria (×399).

they somewhat resembled the aerenchymatous arrangement of parenchyma most commonly found in aquatic plants (4).

The adaxial epidermis of leaves consisted of more uniform cells and fewer stomata than the abaxial epidermis (Fig. 1D and E). Trichomes were absent. The palisade mesophyll usually was formed of one layer of cells. The loosely organized spongy mesophyll often consisted of single layered strands of cells that extended between the palisade layer and the abaxial epidermis and were separated by large intercellular and substomal spaces. Small veins in the spongy mesophyll were composed of vascular bundles surrounded by parenchymatous bundle sheaths without extensions (Fig. 1E). The rib veins consisted of an epidermis, compact parenchyma cells, one or few vascular bundles and groups of collenchyma cells, and absence of large cavities (Fig. 1D). Druses and raphides were sometimes seen in leaf mesophyll as reported by Genua and Hillson (7).

Histology of inoculated plants. Bacteria in infected tissues were differentiated and identified by oil immersion optics as short rods that stained dark blue with Harris hematoxylin.

The tissues from visibly unaffected areas of spray-inoculated leaves appeared normal, and bacteria were not observed. However, when the unaffected tissue was adjacent to a necrotic area, small scattered masses or clumps of bacteria enmeshed in an amorphous substance were occasionally observed in the substomatal or intercellular spaces of the spongy mesophyll.

During the initial stages of water soaking, bacteria were relatively few in numbers and were scattered in the substomal and intercellular spaces of the spongy mesophyll without any obvious changes in the surrounding cells. As water soaking progressed, numbers of bacteria increased, and occasionally a substomatal or intercellular space was filled with the pathogen. When bacteria were abundant, disruption of individual mesophyll cells and layers was more evident (Fig. 2A). Masses of bacteria sometimes either filled an area normally occupied by palisade cells or were surrounded by an orange-stained matrix that entirely filled a large area in the mesophyll. The nearby palisade and spongy parenchyma cells were observed to become surrounded by the bacteria with subsequent thinning and disruption of the cell walls.

When necrosis became apparent, the vascular bundles of veins and epidermal cells were distinguishable, although the epidermal cells were compressed and distorted (Fig. 2B). The space previously occupied by the mesophyll was either empty or contained strands of unidentified material, presumably cell remnants, that divided the area into smaller spaces that were either empty or filled with masses or clumps of bacteria. The necrotic tissues eventually became dehydrated, matted together, and completely indistinguishable and were compressed, without bacteria being visible (Fig. 2C) as the leaf became yellow, dry, and shriveled. Although the affected leaf areas were located proximal to the petiole-lamina junction following petiole-inoculation, the histopathology was the same as described for spray-inoculation.

Bacteria did not occur in the vascular bundles of veins or rib veins of spray-inoculated leaves. The rib tissues of veins usually were devoid of and unaffected by bacteria, including leaf areas

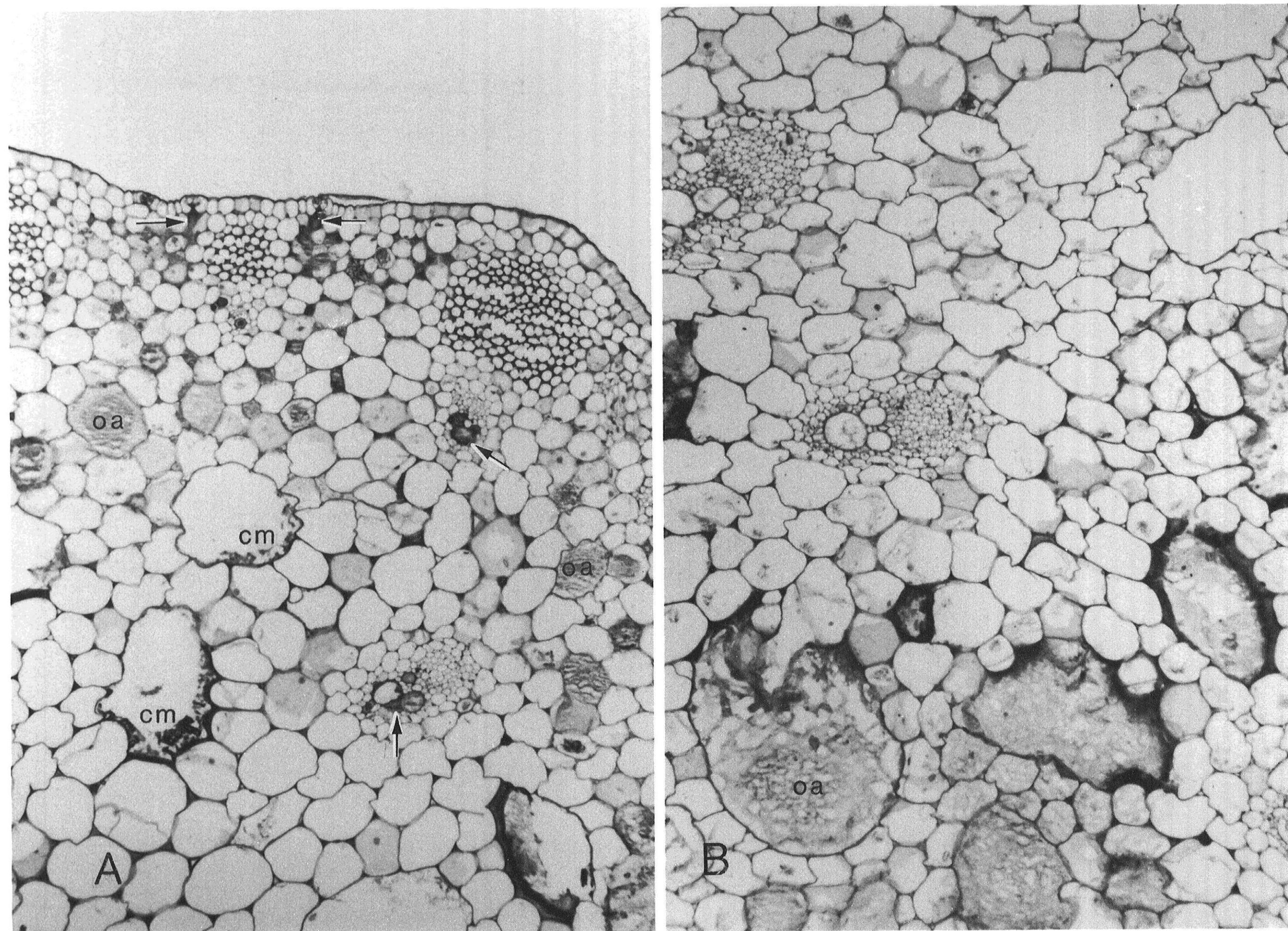

Fig. 3. Portions of transverse sections of *Syngonium podophyllum* 'White Butterly' petiole artificially inoculated by needle-puncture with *Xanthomonas campestris* pv. *syngonii* strain LX 114. **A,** Peripheral region showing distribution of bacteria in substomatal and nearby intercellular spaces and xylem tracheary elements (arrows) and large cavities that contain either clumped material which stained dark blue (cm) or amorphous material which stained orange (oa) in which bacteria were enmeshed (×98). **B,** Central region with several large cavities filled with orange-stained amorphous material and enmeshed bacteria (oa) (×249).

where the rib vein served as a boundary that separated unaffected from infected tissue. Bacteria also did not occur in veins or other tissues of sections of petiole removed from the region of the lamina-petiole junction and usually the areas extending to attachment of the petiole to the stem. However, a discoloration of the adaxial surface was observed on one petiole approximately 60 mm from the lamina-petiole junction. Ingress of bacteria apparently occurred through a few stomata. The substomatal spaces contained masses of bacteria and nearby intercellular spaces were filled with bacteria. Although the bacterial masses were limited to the periphery of the petiole, adjacent large cavities in the ground tissue contained clumps of bacteria-like rods enmeshed in an amorphous substance that resembled the clumps seen in the leaf. Disruption of parenchyma cells or vascular bundles was not observed and except for bacteria in a few widely scattered locations, all tissues appeared normal. Bacteria were not observed in petiole sections from beyond the area of external discoloration.

The distribution and effects of bacteria in artificially inoculated petioles were, in some ways, unique. Inoculated petioles remained symptomless although the pathogen multiplied and spread throughout the petiole. In petioles inoculated midway between leaf and stem, the bacteria were more numerous toward the leaf and tended to be concentrated on the same side of the petiole as the site of inoculation. The epidermal and collenchyma cells were not adversely affected by the bacteria. Substomatal spaces were filled with bacteria (Fig. 3A) that occasionally were observed to be extruded from the stomatal pore. Intercellular spaces, especially near the periphery of the petiole, frequently were enlarged and filled with bacteria. Disruption of parenchyma cells was uncommon, although bacteria sometimes filled a parenchyma cell or the space normally occupied by several parenchyma cells. The space developed when bacteria surrounded several cells and the walls apparently were crushed together, possibly caused by mechanical action of the bacterial mass. The large cavities of the ground tissue often were distorted and contained a mixture of bacteria and orange-stained amorphous substance or rod-like bodies clumped together in a dark blue-stained material (Fig. 3A and B). Histochemically, the amorphous materials adhering to the lumen wall were possibly gums. Bacteria filled one or more xylem tracheids of one to several of the peripheral and large central vascular bundles. In some bundles the individual tracheary elements were disrupted with the formation of a large lumen that contained scattered bacteria and remnants of tracheid walls. The bacteria in an infected vascular bundle in the petiole rarely spread to or affected the surrounding nonvascular ground tissue. However, when the infected vascular bundles reached the lamina-petiole junction and extended into the leaf or a rib vein, the destruction of the internal vascular and ground tissue of the rib veins was extensive. The pathogen subsequently moved into the leaf tissues adjoining the petiole to initiate the production of typical leaf symptoms.

Bacteria were not observed in the tissues of any stem samples removed at, below, or above the nodes to which the inoculated petioles were attached, although bacteria were observed in the portions of the petiole proximal to the stem.

Isolation of bacteria. All sections of uninoculated stem, petiole, and leaf tissues appeared free of bacteria and other microorganisms. Bacteria resembling *X. c.* pv. *syngonii* were not isolated from uninoculated plant samples. The pathogen was isolated from water-soaked and moist or dry necrotic areas of spray- and petiole-inoculated leaves. The pathogen occasionally was isolated from visibly unaffected leaf tissue located near a necrotic area. Bacteria were not isolated from petioles of spray-inoculated leaves, except in areas where surface discoloration was observed. The pathogen was isolated from the entire length of inoculated petioles when leaf symptoms had developed. The pathogen was not isolated from any of the stem sections.

DISCUSSION

In all the reports of diseases of Araceae caused by *X. campestris* pathovars (1,3,8,9,11–14,18–20), the leaf is the primary, if not the sole, organ attacked. Several authors indicated that symptoms do not spread to newly emerged leaves (12,18), suggesting that systemic infection of the plant does not occur. Other reports specifically state that stem, petiole, and midrib tissues are not infected (8,9,12), and Jindal et al (9) reported that when either stem, petiole, or midrib of *Amorphophallus campanulatus* Blume ex Decne. is artificially inoculated, no symptoms develop. This type of disease syndrome contrasts sharply with that produced by other *X. campestris* pathovars such as *X. c.* pv. *pelargonii*, which spread rapidly throughout the vascular system of *Pelargonium* spp. before moving into the surrounding tissues of the stem (17).

Our results indicated that *X. c.* pv. *syngonii* entered stomata of the leaf, developed, and spread throughout the mesophyll, caused severe necrosis, but did not become established in the vascular tissues or move into the petiole via the vascular system. Thus the pathogen apparently did not spread systemically from leaf to petiole or stem and hence to other parts of the plant. This observation agrees with previous reports for other *X. campestris* pathovars that infect plants of the family Araceae (1,3,8,9,11–14). The large intercellular and substomatal spaces of the leaf enabled the pathogen to spread readily throughout the mesophyll. In addition, the arrangement of spongy mesophyll cells in widely separated, single-layered strands (Fig. 1E) may have been conducive both for bacterial multiplication and for contact with large portions of walls of individual cells that facilitated disruption or collapse of mesophyll cells. The method by which *X. c.* pv. *syngonii* disrupts plant cells has not been investigated. The pathogen hydrolyzes various proteins (casein) and certain lipids (Tween 80) extremely slowly and weakly (3).

The occasional occurrence of the pathogen in small areas of petioles of spray-inoculated leaves was attributed to ingress through stomata by inoculum inadvertently sprayed on the petiole or of bacteria from the surface of the necrotic leaf that were splashed on the petiole during overhead watering. This conclusion was supported by the scattered location of infected areas along the length of the uninjured petiole, confinement of bacterial development to peripheral tissues, i.e., substomatal and adjacent intercellular spaces, and no indication of spread of the pathogen from vascular tissues.

It must be emphasized that needle-puncture inoculation of petioles is totally unnatural, but spray-inoculation of leaves approximates natural infection (3). After needle-puncture inoculation of the petiole, the pathogen developed and moved throughout the length of the petiole and into the leaf but did not spread from the petiole into the stem. The mechanism(s) that prevented spread of the pathogen to the stem tissues was not determined. Ability of the pathogen to enzymatically degrade cell walls appeared to be limited. The occurrence of crushed parenchyma cells, ruptured tracheid walls, and large distorted cavities may have been the result of mechanical damage produced when especially large numbers of the pathogen developed and exerted pressure on the walls. The material present in the large cavities of infected petioles was not readily identifiable and may have been composed of bacteria that became enmeshed with plant products. Staining with phloroglucinol indicated that gums may also have been present in the large cavities. There was no evidence of hypertrophy, hyperplasia, or other response of the plant that might confine the pathogen.

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Physiology and Biochemistry

Concurrent Loss in Tn*5* Mutants of *Pseudomonas syringae* pv. *syringae* of the Ability to Induce the Hypersensitive Response and Host Plasma Membrane K^+/H^+ Exchange in Tobacco

C. Jacyn Baker, Merelee M. Atkinson, and Alan Collmer

First and second authors, USDA-ARS, Microbiology and Plant Pathology Laboratory, Beltsville, MD 20705; third author, University of Maryland, Department of Botany, College Park 20742.

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ABSTRACT

Baker, C. J., Atkinson, M. M., and Collmer, A. 1987. Concurrent loss in Tn5 mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the hypersensitive response and host plasma membrane K^+/H^+ exchange in tobacco. Phytopathology 77:1268-1272.

Stimulation of a plasmalemma K^+ efflux/H^+ influx exchange in tobacco by phytopathogenic bacteria has been shown to correspond with development of the hypersensitive response. To further test this relationship, mutants of *Pseudomonas syringae* pv. *syringae* were tested for loss of ability to induce the hypersensitive response or stimulate the K^+/H^+ exchange. Mutants were produced by using plasmid vector pGS9 to introduce transposon Tn5 into *P. s.* pv. *syringae*. Ability to induce the hypersensitive response was assayed by infiltrating mutants, at a concentration of 5×10^7 cfu/ml, into tobacco leaves. Stimulation of the K^+/H^+ exchange was detected by incubating suspension-cultured tobacco cells with mutants at a concentration of 10^8 cfu/ml for 18 hr, then testing for an increase in pH of the medium with a pH indicator. Colonies that failed to stimulate H^+ influx were assayed quantitatively in a second assay that also confirmed the absence of K^+ efflux from suspension-cultured tobacco cells. All mutants that failed to induce one response, either the hypersensitive response or the K^+/H^+ exchange response, also failed to induce the second response. Of 1,600 mutants screened, about 1.7% were auxotrophic. Six prototrophic mutants completely failed to induce either response, and three were able to only partially induce either response; all other prototrophs induced both responses. The results demonstrate a close relationship between bacterial induction of the K^+/H^+ exchange and the hypersensitive response.

Additional key words: ion transport, *Nicotiana tabacum*.

The hypersensitive response is characterized by the rapid death of plant cells at the site of pathogen invasion. Bacterial pathogens causing this response are localized within the hypersensitive lesion and do not invade the surrounding tissue. One of the earliest detectable changes characteristic of tissues undergoing the hypersensitive response is an increased leakage of electrolytes that can be detected within 4–6 hr after inoculation (8,13). Atkinson et al (5) demonstrated that electrolyte loss during the hypersensitive response of tobacco to *Pseudomonas syringae* pv. *pisi* begins within 1–2 hr after inoculation as a specific plasmalemma K^+ efflux/H^+ influx exchange. Recently, Pavlovkin et al (22), studying membrane potential changes during a hypersensitive reaction involving cotton inoculated with *P. s.* pv. *tabaci*, also reported an early and specific loss of intracellular K^+ and proposed an increased plasmalemma permeability to H^+. Because of the fundamental roles played by K^+ and H^+ in eukaryotic cells

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