

Anatomical Response of Resistant Alfalfa Infected with *Verticillium albo-atrum*

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We thank R. A. Haldeman, U.S. Regional Pasture Research Lab, for histological and photographic assistance and P. L. Sanders, Department of Plant Pathology, The Pennsylvania State University, for use of histological equipment.

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Accepted for publication 16 September 1992.

ABSTRACT

Pennypacker, B. W., and Leath, K. T. 1993. Anatomical response of resistant alfalfa infected with *Verticillium albo-atrum*. *Phytopathology* 83:80-85.

Two resistant clones of alfalfa (1079 and WL-5) were stubble inoculated with *Verticillium albo-atrum* and grown for two 6-wk growth cycles before being used in the histological study. During the third 6-wk growth period following inoculation, regrowth stems were sampled for histological study at weekly intervals for 6 wk. The pathogen was confined to the crown of the plants by vascular occlusions for 2 wk, after which it was present in the stems of clone WL-5. Clone 1079 confined the pathogen to the crown for an additional 2 wk. Histologically the clones responded similarly to *V. albo-atrum* until week 5. Thereafter, vascular differentiation was disrupted in clone 1079, resulting in the absence of immature, developing vessel elements and the presence of atypically narrow metaxylem vessels in most vascular bundles in the stem. Dissolution of vascular bundles

infected with *V. albo-atrum* was evident by the final week of the growth period. Confinement of *V. albo-atrum* to the crown until late in the growth period appeared to account for resistance in clone 1079. An additional resistance response was noted in stems of clone WL-5. The response consisted of hypertrophied xylem-parenchyma cells surrounding groups of infected vessel elements, eventually crushing and obliterating them. The hypertrophied cells frequently tested positive for suberin. Atypically narrow xylem-vessel elements were confined to infected vascular bundles in clone WL-5, and no vascular dissolution occurred. *V. albo-atrum* in the xylem vessels of both clones was frequently encased in a thick material that tested positive for suberin and lignin.

Additional keywords: lucerne, *Medicago sativa*, vascular wilt.

Verticillium albo-atrum Reinke & Berthier suppresses growth and flowering in resistant alfalfa (*Medicago sativa* L.) plants and can persist without inducing classic foliar symptoms (16). Infected, resistant plants are potential reservoirs of inoculum. Alfalfa, unlike most hosts of *Verticillium* species, is harvested several times during the growing season. Dissemination of the pathogen is facilitated by mowing, which not only produces aerial infection courts but also spreads the pathogen (9); *V. albo-atrum*, a

monocyclic, soilborne pathogen in most host species, becomes an aerial pathogen with an artificially induced repeating cycle in alfalfa (13). As a consequence, resistance to root infection is of minor importance in this crop, because such resistance mechanisms are continually circumvented by crop-management procedures.

The growth and physiological responses of resistant alfalfa plants to the chronic presence of *V. albo-atrum* have been studied (17). In addition, Newcombe and Robb (11), working with detached alfalfa stems, showed that an efficient vessel wall-coating response was involved in early resistance to this pathogen. No anatomical studies have been conducted on intact, resistant plants chronically infected by *V. albo-atrum*. The objective of this study

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was to determine whether anatomical mechanisms are involved in the expression of long-term resistance to *V. albo-atrum* in alfalfa.

MATERIALS AND METHODS

Plant-culture procedures and inoculation method. Two clones of alfalfa (1079 and WL-5) previously selected for resistance to *V. albo-atrum* were used in this study (17). Susceptible clones were not included, because survival of susceptible plants following inoculation is less than 2 mo. Clones were used rather than plants grown from seed to ensure genetic uniformity, allowing histological comparisons among plants over time. The plants were used in several previously reported studies, and the histological samples in this study were collected during those studies (17–19). Plants were maintained on a 6-wk harvest schedule, and the methods of plant cultivation and pathogen inoculation were described (17–19). Briefly, clonal plants were grown in 20-cm-diameter \times 90-cm-tall polyvinyl-chloride cylinders containing a 2:1 (v/v) mixture of commercial potting mix and coarse sand. The growth medium was amended with slow-release, macro- and micronutrients (13-13-13 Osmocote, Mallinckrodt, Inc., St. Louis, MO and Esmigran, Sierra Chemical Co., Milpitas, CA) to ensure adequate nutrition for long-term growth. Seven plants were grown in each container. Plants were watered to container capacity when a soil tensiometer, located 30 cm from the top of the container, registered -0.04 MPa. Plants were 6-wk old when inoculated with *V. albo-atrum* and were 18-wk old when the histological study began.

The *V. albo-atrum* isolate was used in previous studies (17,18) and was stored on silica gel in a freezer at -25 C. The pathogen was grown on prune-yeast-extract agar (25) for 2 wk at approximately 25 C. Spores were collected in sterile water, and their concentration was adjusted to 3.65×10^6 spores per milliliter. Plants were harvested and then inoculated by placing a 20- μ l drop of spore suspension on each freshly cut stub. All stubs on the plant were inoculated. Control plants were treated similarly with water. Inoculated plants were kept in a mist chamber for 24 h after inoculation. The plants were returned to the greenhouse, were grown for 12 wk, and were harvested twice before being used in the histological study.

During the next growth period, the aerial portion of one plant was removed from each container weekly for 6 wk and was used for growth-parameter analysis and histological study. The experiment used a randomized complete-block design with five replications; all five replications were used in the growth analysis (17,18). In addition, plants from two of the replications were used in the histological study. The entire experiment was repeated. Environmental conditions and results of the growth-parameter analyses were reported previously (17,18).

Histological sampling. Several stems and the crown of one plant per container per week were sampled for 6 wk for histological study. The leaves were removed and used in the growth-parameter analysis as previously described (17,18), and the stems and crown were diagrammed, were surface sterilized in 0.0525% sodium hypochlorite for 5 min, and were aseptically cut into serial sections. Selected sections were fixed in Rawlin's formalin-acetic acid-alcohol solution No. 1 (FAA) (22) for histological study, and the remaining sections were placed on 2% water agar for isolation of the pathogen. Plates were incubated at 25 C for 10 days and were examined microscopically for conidiophores of *V. albo-atrum*. The location of the pathogen was recorded on the corresponding diagram.

Specimens fixed in FAA were dehydrated using the standard tertiary butyl-alcohol schedule (10) and were embedded in Paraplast-Plus (Curtin Matheson Scientific, Cleveland, OH). Adjacent 5-mm stem pieces were embedded so that longitudinal and transverse sections could be examined for each stem-area sampled. Sections were cut at 10 μ m on a rotary microtome, were mounted on chemically cleaned slides with Haupt's adhesive, and were stained with Johansen's quadruple stain (10). Histochemical tests for lignin (phloroglucinol-HCl), gums (orceinol),

suberin (Sudan IV), starch (IKI), and pectin (iron-absorption method) were carried out on selected sections (23).

RESULTS

Symptoms. The first symptoms appeared on regrowth stems of clone 1079 during week 5 and consisted of a few leaves showing V-shaped chlorosis. The chlorotic leaves abscised during week 6. Symptoms did not appear on clone WL-5 until week 6 and consisted of occasional leaves with the V-shaped chlorosis characteristic of Verticillium wilt.

Pathogen isolation. *V. albo-atrum* was isolated from all inoculated plants included in the histological study and was not isolated from noninoculated plants. The pathogen was isolated from crown tissue of both clones throughout the study. It was not detected, however, in the stems of clone 1079 until week 5, when it was isolated in a discontinuous pattern (18). All sampled stems of clone 1079 were completely colonized by *V. albo-atrum* by week 6. The pathogen was detected in stems of clone WL-5 in weeks 3, 5, and 6 of the growth period. Complete colonization of all sampled stems was not noted in clone WL-5, unlike clone 1079. As with clone 1079, the discontinuous pattern of isolation in clone WL-5 implicated spore translocation in the vascular spread of the pathogen (14,18).

Histological response. *Clone 1079.* The anatomy of noninoculated plants closely resembled anatomy previously reported for alfalfa (8,15). Immature, enlarging vessel elements were present adjacent to the vascular cambium in all vascular bundles (Fig. 1). Xylem-vessel elements had helical, scalariform, and opposite pitting, depending on their developmental stage, and simple perforation plates. Xylem parenchyma and fiber-tracheids also were present in the xylem tissue. Older stems had well-developed multiseriate rays composed of radially elongated parenchyma in the vascular cylinder of the stem base. Scattered xylem vessels in the crown were plugged with chromophyllic material, and the plugged vessels were associated with vascular bundles present when the cutting was propagated.

V. albo-atrum was confined to the xylem-vessel elements of the crown and the inoculated stub for the first 4 wk of the growth period. Extensive vascular deposits ranging from complete plugging of the cell lumen to globular deposits on the cell walls were present in the xylem vessels of the crown. In many cases, hyphae were present in the plugged vessels, but hyphae also were noted in apparently nonoccluded vessels. In the latter case, contiguous vessels had vascular deposits varying from a coating on the vessel wall to complete occlusion of the lumen. Xylem vessels in the stem stubs, which were the inoculation sites 3 mo earlier, were plugged, and the pathogen had ramified throughout the stub tissue. Both thick-walled resting hyphae and thin-walled hyphae were present.

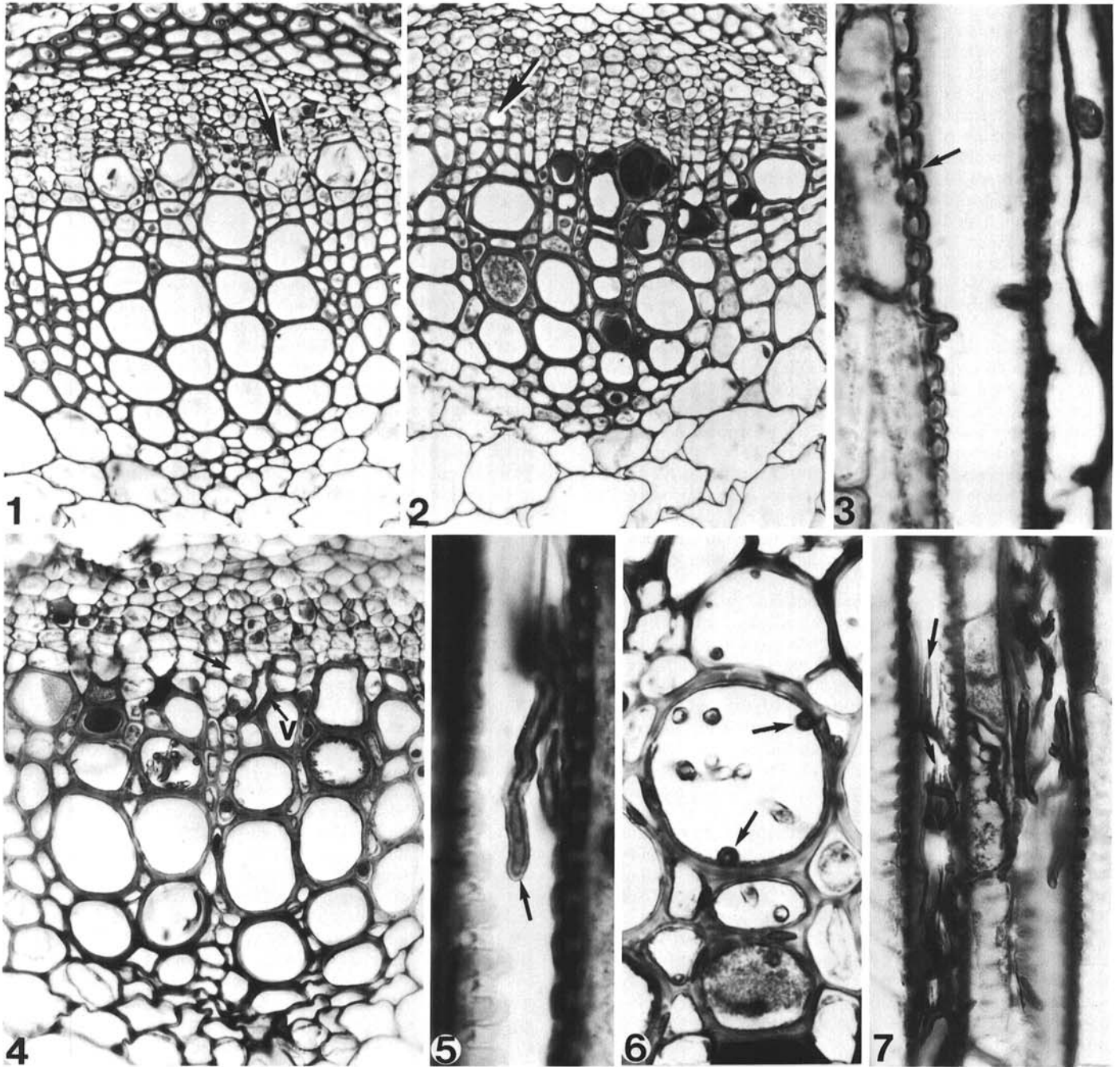
By week 5, *V. albo-atrum* was present in several vascular bundles of the stem. Small-diameter cells, with secondary wall thickenings associated with metaxylem vessels (Fig. 2), were adjacent to the vascular cambium in infected vascular bundles, replacing the immature, enlarging vessel elements usually found in healthy vascular bundles. Xylem-vessel plugging was infrequent in these vascular bundles. Vessel walls, however, were coated with a material that extended into the pit chambers, effectively occluding them (Fig. 3). Vascular plugging was more prevalent when large quantities of hyphae were present; xylem vessels containing only a few strands of hyphae were free of occlusions. Spores were embedded in some of the vascular plugs. Cambial derivatives occasionally were hypertrophied, with the adjacent vessel element consequently distorted (Fig. 4) and sometimes collapsed. Hyphae were frequently coated with a brownish material (Figs. 5 and 6) that often extended from the main body of the hyphae in thin strands (Fig. 7). The strands always pointed in the same direction, probably indicating the transpiration stream. The hyphal-coating material varied in thickness and was distinct from the thick wall associated with resting hyphae.

Stems of infected plants sampled during week 6 had less vascular occlusion than previously sampled stems, but frequently, *V. albo-*

atrum continued to be encased in thick, brownish deposits that often extended in strands from the hyphae. The absence of enlarging, immature vessel elements in all vascular bundles persisted, and the atypically narrow-lumened metaxylem vessels, previously mentioned, were more prevalent. Xylem-vessel elements in several vascular bundles were distorted and sometimes were disintegrating. In some cases, entire vascular bundles, including cambial derivatives, xylem parenchyma, and vessel elements, were disintegrating (Fig. 8). Similar anatomical responses to *V. albo-atrum* were seen when the experiment was repeated.

Clone WL-5. The response of clone WL-5 paralleled that of clone 1079 until week 5, when hypertrophied xylem and ray

parenchyma (Fig. 9) in the crown separated several vascular bundles with plugged vessel elements from the centrifugal xylem tissue. A similar response was noted in the stem. Xylem parenchyma surrounding groups of occluded vessel elements were hypertrophied (Fig. 10) and over a vertical distance of 40 μm , appeared to obliterate the plugged vessels (Figs. 11 and 12). Examination of longitudinal sections through similar areas of hypertrophied xylem parenchyma (Fig. 13) revealed the presence of hyphae in the vessels being crushed by the expanding xylem-parenchyma cells (Fig. 10). The encirclement and subsequent crushing of groups of infected vessel elements by hypertrophied xylem parenchyma was never noted in stems of clone 1079.



Figs. 1-7. 1, Cross section of a healthy alfalfa stem of clone 1079 showing a vascular bundle with newly enlarging vessel elements (arrow) ($\times 212$). 2, Cross section through the stem of alfalfa clone WL-5 infected with *Verticillium albo-atrum*. Vascular differentiation has been disrupted and narrow-lumened vessel elements (arrow) are present adjacent to the vascular cambium ($\times 250$). 3-7, Sections through stems of alfalfa clone 1079 infected with *V. albo-atrum*. 3, Longitudinal section showing the wall coating that extends into the pit lumens (arrow), effectively blocking them ($\times 950$). 4, Cross section through a vascular bundle. The hypertrophied xylem-parenchyma cells (arrow) distort the adjacent xylem-vessel element (v). Hyphae are visible in the vessel elements ($\times 350$). 5, Longitudinal section through a xylem-vessel element showing a hyphal strand encased in a thick coating (arrow) ($\times 925$). 6, Cross section showing encased hyphae adjacent to the xylem-vessel cell wall (arrows) ($\times 812$). 7, Strands of coating material (arrows) extending from the hyphae. All the strands point in the same direction, probably indicating the direction of the transpiration stream ($\times 420$).

Vessel-element plugging was more prevalent in the stems of clone WL-5 during weeks 5 and 6 than it was in clone 1079 stems, and hyphae were seldom seen in nonoccluded vessels. Hyphae were frequently encased in a thick, brown substance often extending in strands from the main hyphae. Xylem-vessel walls occasionally were coated with material that plugged the pit lumens. Disruption of vessel-element differentiation was confined to the vascular bundles showing vascular plugging rather than being present in all vascular bundles, as occurred in clone 1079. Similar anatomical responses to *V. albo-atrum* were seen in clone WL-5 when the experiment was repeated.

Histochemical tests. Results of histochemical tests were similar for both clones. Vascular plugs tested positive for pectin as determined by the iron-absorption method and for gum when tested with both phloroglucinol-HCl and orcinol. Xylem vessel wall-coating material produced a positive reaction for lignin and gum using phloroglucinol-HCl and tested positive for suberin. A reduction in pectic compounds was detected in the walls of some colonized xylem-vessel elements during week 6. Hypertrophied xylem parenchyma associated with the obliteration of infected vessel elements in clone WL-5 tested positive for suberin, as detected with Sudan IV. The hyphal wall-coating substance was frequently positive for suberin (Fig. 14) and occasionally positive for lignin and gum, as determined with phloroglucinol-HCl (Fig. 15). Starch was present in the ray parenchyma of the crowns of both clones in weeks 5 and 6 only.

DISCUSSION

Growth and physiological studies conducted on clones 1079 and WL-5 infected with *V. albo-atrum* suggested that dissimilar resistance mechanisms were operating within these clones (17,18); histological evidence obtained in this study supports this hypothesis. Both clones were able to restrict pathogen access to stem tissue during the first 2 wk of the growth period, and clone 1079 continued to restrict the pathogen until week 5 (18).

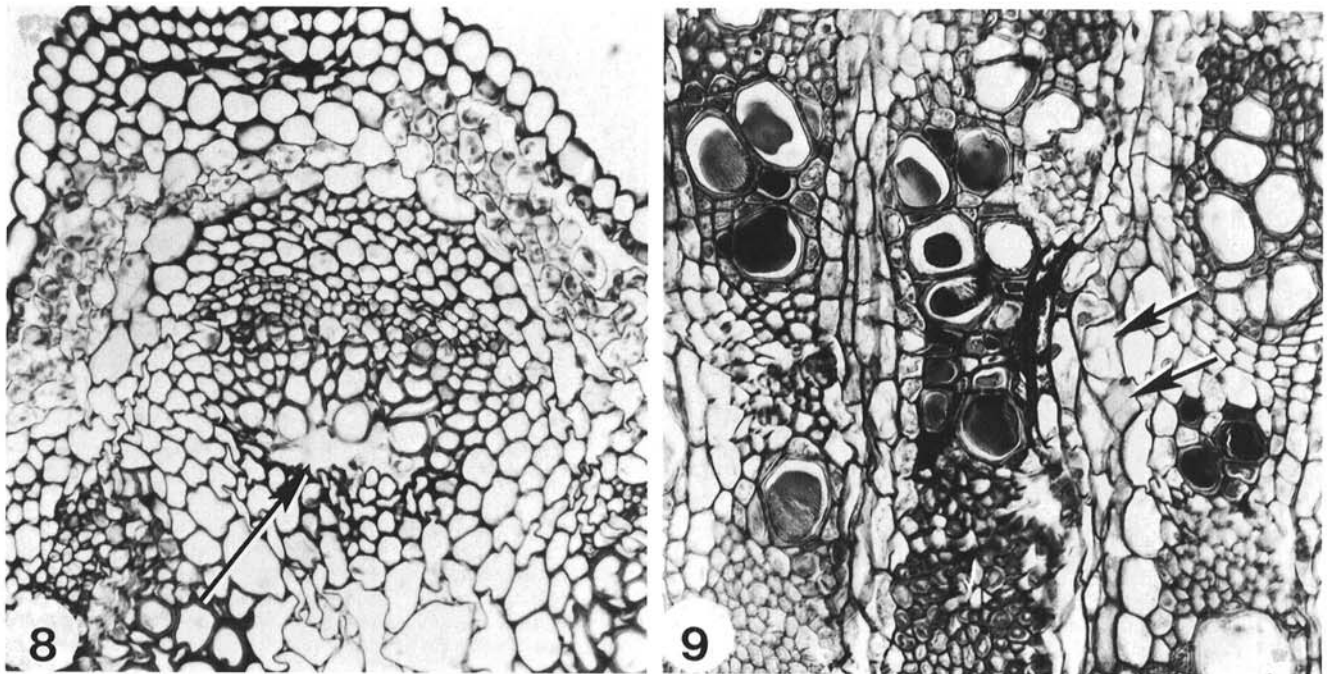
By week 5, *V. albo-atrum* was present in the vascular system of the stems of both clones. Once the stems of clone 1079 were invaded, the host's histological response to *V. albo-atrum* was very similar to that of susceptible alfalfa (15). The ultimate dissolution of infected vascular bundles undoubtedly contributed to the symptom expression noted in clone 1079. Research on

tomato infected with *V. albo-atrum* (5,12) and pea (26) and carnation (3) infected with *Fusarium oxysporum* indicated that resistant plants restrict the pathogen to the roots and lower stems via vascular occlusions. Inoculum-concentration studies using both resistant and susceptible tomato cultivars found that the initial rate of colonization is positively correlated with symptom severity (12). Alfalfa is a perennial crop harvested frequently, and it regrows primarily from crown buds. Consequently, the concept of initial colonization can be applied either, in the classic sense, to the initial entry of *V. albo-atrum* into the host via root or stem stubble or to initial pathogen entry into stem regrowth from previously colonized crown tissue. Plants in this anatomical study were involved in a previously reported growth study (18) that indicated clone 1079 reached 75% of its final height by week 4 of the growth period. The restriction of *V. albo-atrum* to the crown tissue for 66% of the 6-wk growth period and the stem growth achieved prior to pathogen appearance in the stem appear to explain the resistance of clone 1079.

In addition to vascular plugging, which restricted the pathogen to the crown for 2 wk, clone WL-5 initiated a containment response when *V. albo-atrum* invaded the stem. This additional defense response consisted of the hypertrophy of xylem-parenchyma cells surrounding groups of infected vessel elements and the subsequent crushing of those infected vessel elements. In some cases, suberin was detected in the hypertrophied cells. A similar containment response was seen in a chrysanthemum resistant to *Erwinia chrysanthemi* (21), in a susceptible carnation infected with a weakly virulent isolate of *F. oxysporum* f. sp. *dianthi*, (20), and in a resistant carnation infected with a virulent isolate of *F. o. dianthi* (2).

Vessel elements in stems of both resistant clones exhibited the wall-coating response noted by Newcombe and Robb (11). The disintegration of vascular tissue in the stem of clone 1079 and the presence of similar vessel-wall coating in a susceptible alfalfa clone (15) support the conclusion that vessel-wall coating is not by itself sufficient to provide resistance (11).

Hyphae in the xylem vessels of the stems of both resistant clones frequently were encased in a thick, coating material that tested positive for both lignin and suberin. Hyphal encasement was never seen in the susceptible, alfalfa clone (15). In 1928, Fellows (6) described the encasement of hyphae of *Ophiobolus graminis* in a "protuberance" of the cell wall. He suggested the term "lignituber," because the protuberances tested positive for



Figs. 8 and 9. 8, Cross section through a 6-wk-old stem of alfalfa clone 1079 infected with *Verticillium albo-atrum* showing dissolution of a vascular bundle (arrow) ($\times 250$). 9, Cross section of crown tissue of alfalfa clone WL-5 infected with *V. albo-atrum*, showing plugged vessel elements, the hypertrophy of ray parenchyma (arrows), and the crushing of adjacent vascular tissue ($\times 200$).

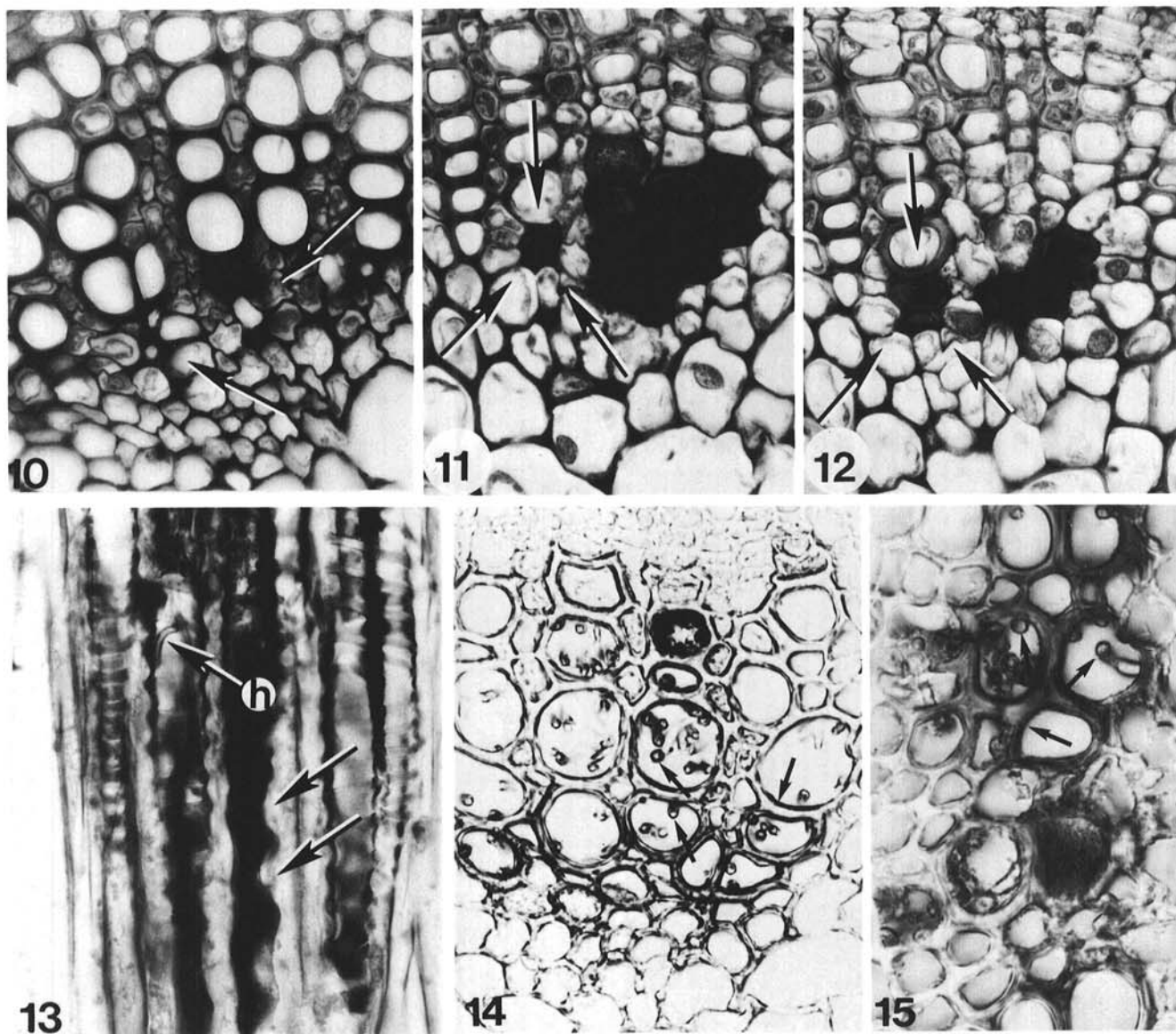
lignin. Lignitubers formed around *Verticillium dahliae* in the root hairs of tomato (7) and in root, cortex cells of chrysanthemum invaded by *V. dahliae* (1). Invasion of hop roots by *V. albo-atrum* was impeded by the formation of lignitubers around hyphae in epidermal and cortical cells (24). Similar structures, termed papillae, were a critical component of the resistance response of reed canarygrass to various foliar pathogens (27). Lignin deposition on the walls of parenchyma contiguous to infected vessel elements was implicated in resistance to the lateral movement of vascular-wilt fungi (4). In all cases, lignitubers or papillae formed in living cells and were restricted in size.

The phenomenon reported here occurred in xylem-vessel elements, and hyphal encasement in the lignin- and suberin-positive material was extensive. Despite the presence of vessel-wall coating in susceptible alfalfa infected with *V. albo-atrum*, we never detected the hyphal-encasement phenomenon in susceptible plants (15); as a result, we feel it is distinct from the

vessel wall-coating reaction. The similarities between lignitubers and the hyphal encasement we noted, coupled with detection of the phenomenon in resistant clones and not in susceptible clones, causes us to conclude that hyphal encasement may be a form of lignituber and may be involved in resistance. The mechanism of formation of these elongated encasement structures is unknown.

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Figs. 10-15. 10, Cross section through a 6-wk-old stem of alfalfa clone WL-5 infected with *Verticillium albo-atrum*. The hypertrophied xylem-parenchyma cells (arrows) intrude on the vessel lumen ($\times 420$). 11 and 12, Cross sections through a 5-wk-old stem of alfalfa clone WL-5. Forty micrometers separate the section in 11 from the section in 12. Two containment reactions consisting of hypertrophy of xylem-parenchyma cells and subsequent crushing and obliteration of infected vessel elements are visible in these sections. The more prominent reaction proceeds from 11 to 12 and the less obvious reaction (arrows) proceeds in the opposite direction ($\times 455$). 13, Longitudinal section through a 6-wk-old stem of alfalfa clone WL-5. This section was less than 5 mm from the section shown in 10. The hypertrophied xylem-parenchyma cells (arrows) reduce the diameter of the infected xylem vessel. Hyphae (h) and vascular-plugging material are visible in the affected vessel ($\times 420$). 14 and 15, Cross sections through a 5-wk-old stem of alfalfa clone 1079 infected with *V. albo-atrum*. 14, Some vessel walls and hyphal-coating material (arrows) react positively to Sudan IV, indicating the presence of suberin ($\times 418$). 15, Hyphal wall-coating material (arrows) and the vessel wall-coating material react positively to phloroglucinol-HCl, indicating the presence of lignin and/or gum ($\times 525$).

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