

Anatomical Response of a Susceptible Alfalfa Clone Infected with *Verticillium albo-atrum*

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ABSTRACT

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Alfalfa plants, cloned from a *Verticillium* wilt-susceptible plant, were stubble inoculated with *Verticillium albo-atrum* and sampled periodically. Stem samples were embedded in paraffin and examined microscopically. Infected plants had discontinuous alterations in xylem vessel element development which were characterized by atypically narrow metaxylem vessel elements interspersed with undifferentiated cambial derivatives. The lack of cambial derivative differentiation caused mature xylem vessels to be interrupted by groups of immature cells. Protoxylem and some metaxylem

vessel elements were obliterated by hypertrophied xylem parenchyma. Xylem vessel lumens and pit chambers were frequently plugged with gum and pectin, and the interior cell walls were often coated with gum, suberin, and occasionally pectin. Such deposits probably impede lateral water flow and, together with the disruption in xylem vessel element differentiation, the blockage of mature vessels by immature parenchyma, and the destruction of metaxylem vessel elements, result in xylem malfunction.

Additional key words: lucerne, *Medicago sativa*, *Verticillium* wilt.

Verticillium wilt caused in alfalfa (*Medicago sativa* L.) by *Verticillium albo-atrum* (Reinke and Berth.) has become a major problem since it was first reported in the United States in 1976 (4). The disease is characterized by V-shaped chlorosis of leaflet tips followed by leaflet desiccation and abscission. Internal water stress has been implicated in symptom expression in tomato (19), potato (6), and hops (21) infected with *V. albo-atrum*, and Talboys (23) has recently summarized the various mechanisms of vascular occlusion. The importance of alfalfa as a forage crop has prompted investigations into the mechanisms of wilting in this host. Krátká and Kudela (11) reported increases in IAA-oxidase and pectinase activity in susceptible plants infected with *V. albo-atrum*. Heale and Gupta (7) proposed gel blockage of xylem vessels caused by high levels of exopectin lyase as a wilt mechanism. In addition, toxin activity was reported by Carr (2), Panton (13), and Stoddart and Carr (18). The present study details the anatomical response of a susceptible alfalfa genotype to *V. albo-atrum* and suggests an anatomical mechanism of pathogenesis.

MATERIALS AND METHODS

The heterogeneous nature of alfalfa made it necessary to use clones rather than seed to produce a genetically uniform plant population. Plant uniformity was needed to ensure valid histological comparisons between treatments and sampling dates.

The plant cloned was a selection from Vertus (clone 774) which showed typical symptoms when inoculated with *V. albo-atrum*. Stem cuttings were rooted in perlite, then transplanted into 10-cm-diameter (400 cc) clay pots filled with Terra-Lite Reddi-Earth Peat-Lite mix (W. R. Grace & Co., Cambridge, MA). Plants were grown in a greenhouse under natural light and were cut back to 10 cm after 6 wk of growth to encourage production of multiple stems.

Plants were allowed to grow an additional 6 wk prior to inoculation in June.

Six plants were inoculated by cutting back all stems to 10 cm with a sterile razor blade and then placing a 5- μ l drop of *V. albo-atrum* spore suspension (520,000 spores per milliliter) on each stub. Inoculum was prepared from 36-day-old cultures of *V. albo-atrum* grown on prune-lactose-yeast agar (22). An equal number of plants was treated with sterile water. Following inoculation, all plants were placed under mist for 24 hr and then returned to the greenhouse. Temperature and humidity were monitored with a hygrothermograph. Temperature in the greenhouse ranged between 15 and 41 C.

Plants were sampled at weekly intervals for 6 wk, at which time severe symptoms of *Verticillium* wilt were present. Each sampling included two inoculated stems and one control stem. The stems were excised, traced on lined paper, and then surface sterilized in 10% bleach (5.25% sodium hypochlorite) for 5 min. Stems were then aseptically divided into serially numbered 5-mm pieces. Two consecutive pieces were fixed in Rawlins' (15) formalin-acetoalcohol solution No. 1 (FAA) for histological study, and the next four pieces were placed on 2% water agar for isolation of *V. albo-atrum*. This sampling sequence was repeated for the entire length of each stem. Stem sections on water agar were examined microscopically after 7 days for the presence of the pathogen, and positive sections were recorded on the appropriate stem diagram.

Specimens in FAA were dehydrated by using the standard tertiary butyl alcohol schedule (10) and embedded in Paraplast-Plus (Curtin Matheson Scientific, Cleveland, OH). Adjacent specimens were prepared so that longitudinal and transverse sections were available from each stem area sampled. Sections were cut at 10 μ m on a rotary microtome, mounted on chemically cleaned slides with Haupt's adhesive, and stained with Johansen's quadruple stain (10). Histochemical tests for pectin (iron absorption method), gums (phloroglucinol and orcinol), lignin (phloroglucinol-HCl), and suberin (Sudan IV) were carried out on selected specimens (16). Sections were also examined under polarized light to detect cellulose breakdown (16).

The relative susceptibility of clone 774 to *V. albo-atrum* was determined in a pathogenicity test with alfalfa cultivar Saranac AR as the standard of susceptibility. Clone 774 was vegetatively

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propagated, and Saranac AR was grown from seed to ensure a representative sample of the cultivar. Thirty-five 5-wk-old plants of each were inoculated as previously described using a spore suspension of 2,950,000 spores per milliliter. The plants were grown in the greenhouse, under natural light supplemented with metal halide lamps to give a photoperiod of 12 hr, during December and January and were monitored for symptoms. Temperature and relative humidity were recorded on a hygrothermograph. The temperature ranged between 17 and 30 C. Additional histological specimens were collected as previously described.

RESULTS

Symptoms. Symptoms first appeared in infected plants 14 days after inoculation and consisted of leaflet chlorosis and occasional leaflet abscission. Severe symptoms were present 5 wk after inoculation and included stunting and leaflet and auxiliary shoot death. Plants inoculated in December were dead 6 wk after inoculation. Symptom expression was inhibited after 5 wk in plants inoculated in June due to excessive greenhouse temperature.

Isolations. *Verticillium albo-atrum* was isolated from the entire length of all inoculated stems beginning 14 days after inoculation. The fungus was never isolated from the control plants.

Susceptibility of clone 774. The pathogenicity test indicated that clone 774 was as susceptible as Saranac AR to *V. albo-atrum*. Symptom expression was identical and occurred at approximately the same interval following inoculation.

Anatomy of uninoculated plants. The anatomy of uninoculated stems of alfalfa clone 774 closely resembled that previously reported by Grove and Carlson (5). The epidermis, which included numerous stomata, surrounded a cortex composed of isodiametric chlorenchyma. Collenchyma cells were usually present in the cortex at the four corners of the relatively square stem. An endodermis delineated the vascular region which was composed of discrete vascular bundles (Fig. 1). Layers of perivascular fibers were often present between the phloem and the endodermis. The xylem, separated from the phloem by the vascular cambium, contained vessel elements with annular, helical, reticulate, and scalariformly pitted secondary walls and generally simple perforation plates. Immature xylem vessel elements were located adjacent to the cambium (Fig. 2). Protoxylem vessel elements were frequently obliterated by the stretching which occurred as the stem elongated and by the subsequent formation of tyloses. The pith, which borders the vascular bundles, contained large isodiametric parenchyma.

Anatomy of inoculated plants. Anatomical changes associated with the presence of *V. albo-atrum* in the stem were visible in several vascular bundles 2 wk after inoculation. Affected vascular bundles (Fig. 3) generally lacked the immature, enlarging xylem vessel elements (Fig. 2) usually found adjacent to the vascular cambium. In several instances, atypically small cells with the secondary thickenings and wall pitting of xylem vessel elements were present in that area (Fig. 3). Xylem vessel elements had brown wall deposits and frequently were occluded with materials which were positive for gum and occasionally for pectin (Fig. 3). Older xylem parenchyma cells were often hypertrophied and their enlargement eventually caused the obliteration of some metaxylem vessel elements (Fig. 3). Distortion in xylem vessel element shape was noted in some vascular bundles (Fig. 4) and could sometimes be attributed to pressure from adjacent hypertrophied cells. Little or no reduction in cellulose was detected in these cells when they were examined for birefringence under polarized light.

On one occasion, we observed a leaf trace bundle (Fig. 4) that exhibited hypertrophy of the xylem parenchyma, obliteration of xylem vessel elements, and disruption in the formation of new vessel elements. The leaflets associated with this leaf trace bundle had undergone abscission.

Mycelia and conidia were found throughout the stem although rarely in large quantities and only in vascular bundles showing anatomical changes. The number of vascular bundles showing anatomical changes and the severity of those changes fluctuated

throughout the stem, possibly reflecting the degree of pathogen colonization.

Host response to the presence of *V. albo-atrum* intensified with the passage of time. Crushing of metaxylem vessel elements by hypertrophied xylem parenchyma increased and progressed centrifugally, reducing the number of xylem vessel elements in affected bundles (Fig. 5). Extracellular material, which was positive when tested for gum and pectin, accumulated between the pith and the area of crushed cells (Fig. 5) and frequently between xylem vessel elements (Fig. 6). Atypically small xylem vessel elements became more numerous (Fig. 6). Their presence, along with the absence of large-lumened immature xylem vessels, reflected a disruption in xylem-vessel element differentiation that prevented cell enlargement but not secondary wall development. Undifferentiated cambial derivatives frequently surrounded the small vessel elements (Fig. 7). Examination of longitudinal sections showed that the small-lumened vessel elements were frequently isolated and failed to join into xylem vessels (Fig. 8).

The undifferentiated cambial derivatives replaced normal xylem vessel elements in some vessels causing interruption in the continuity of the vessel. Vessels blocked in this manner were probably nonfunctional. Other xylem vessels were blocked by elongated plugs which tested positive for gum and occasionally pectin. Xylem vessel element walls and pit chambers were often lined with gum and sometimes with suberin and pectin (Figs. 9 and 10). Tyloses were observed in the protoxylem vessel elements where they were part of the aging process and rarely in any other xylem vessels.

During the later stages of the disease, the vascular bundles showed various combinations of the aforementioned anatomical responses. In addition, collapse and distortion of the vascular cambium and hypertrophy and some dissolution of the phloem cells were noted (Fig. 11).

Thin-walled and dark, thick-walled hyphae as well as spores were present in many xylem vessels (Fig. 12). Even though the upper stem was dead, the fungus was only observed in the xylem vessels. The presence of severely affected vascular bundles near those showing no anatomical change also indicated that the fungus was unable to move laterally in the host.

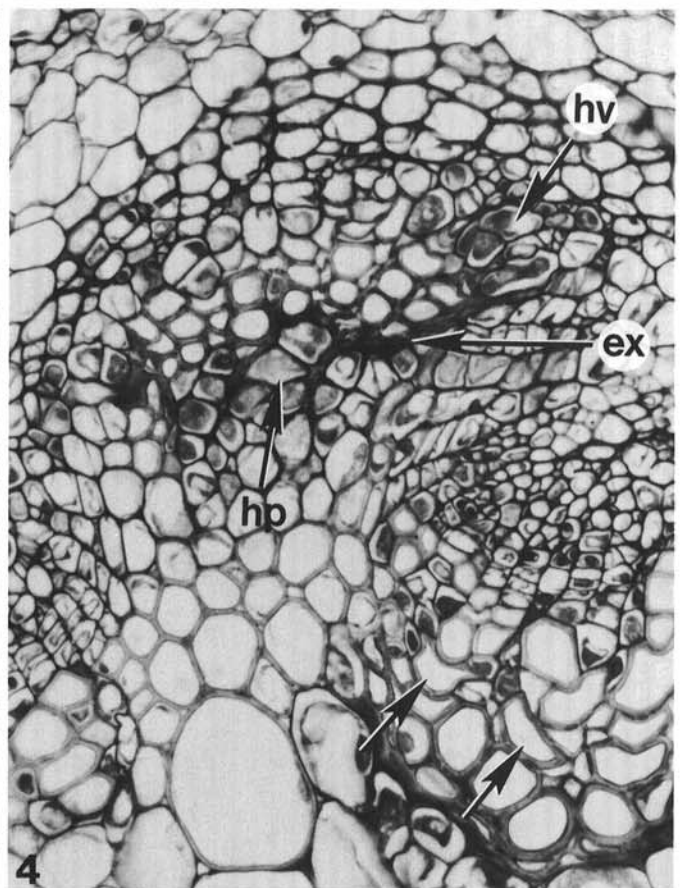
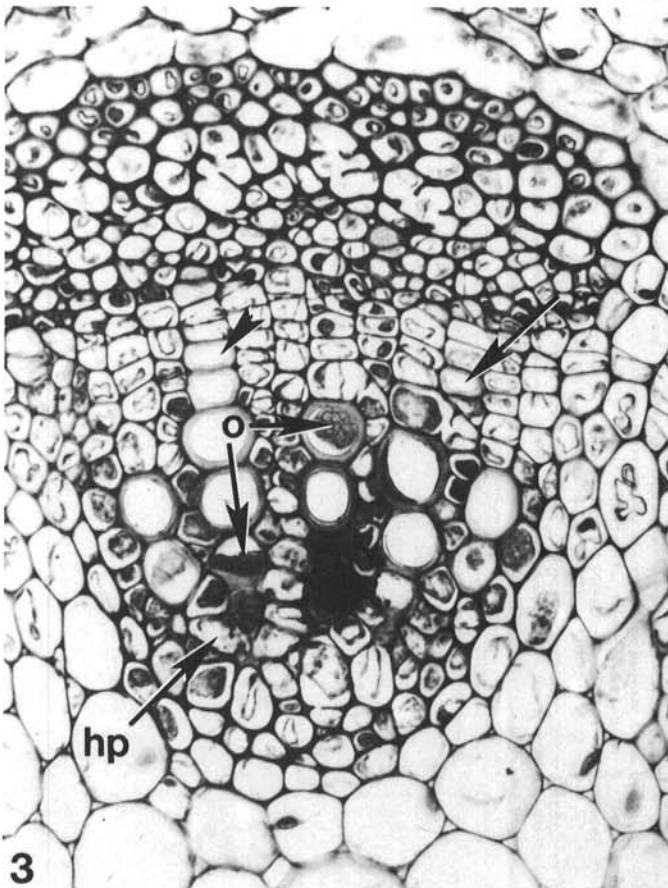
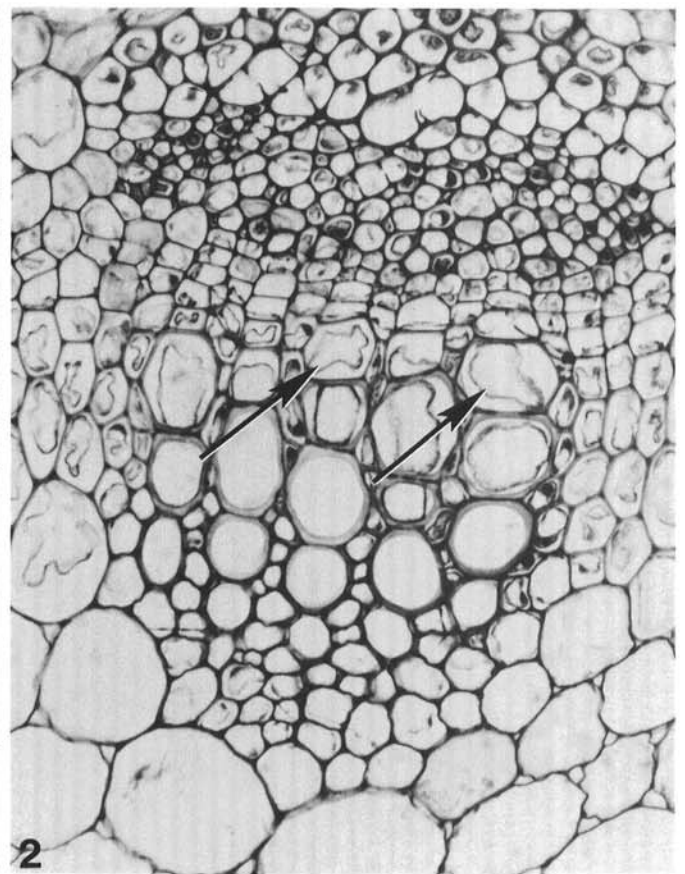
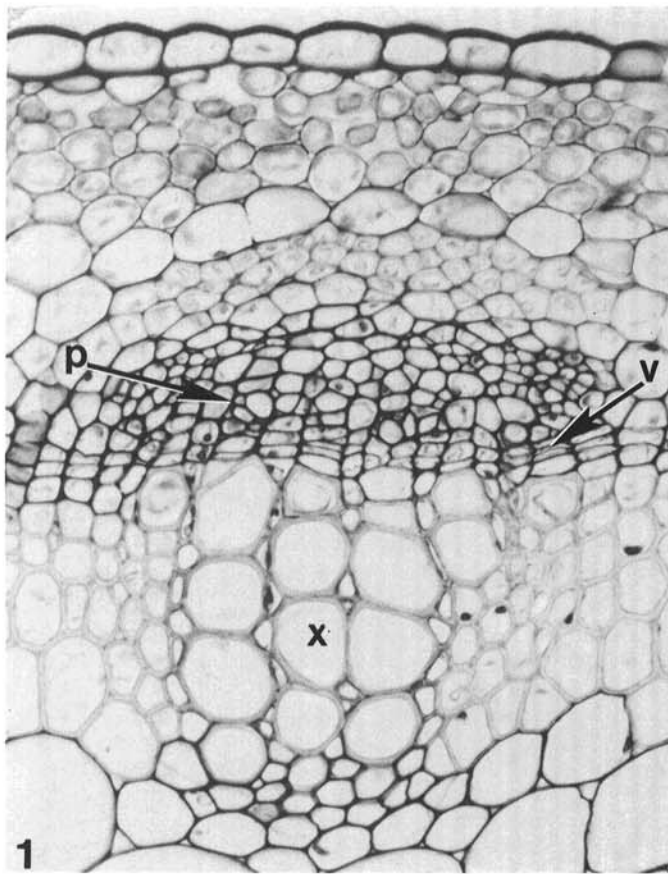
The anatomical changes noted in infected plants resulted in vascular tissue destruction which progressed both centrifugally and centripetally towards the center of the vascular bundle. Centrally located xylem vessel elements often appeared to be unaffected except for cell wall deposits and pit chamber plugs. Similar anatomical changes were noted in susceptible alfalfa plants from different germ plasm (Fig. 13).

DISCUSSION

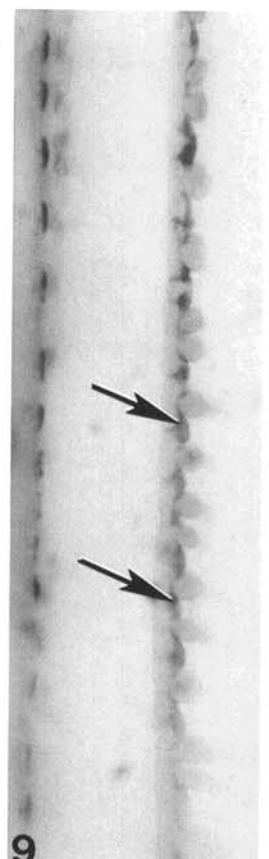
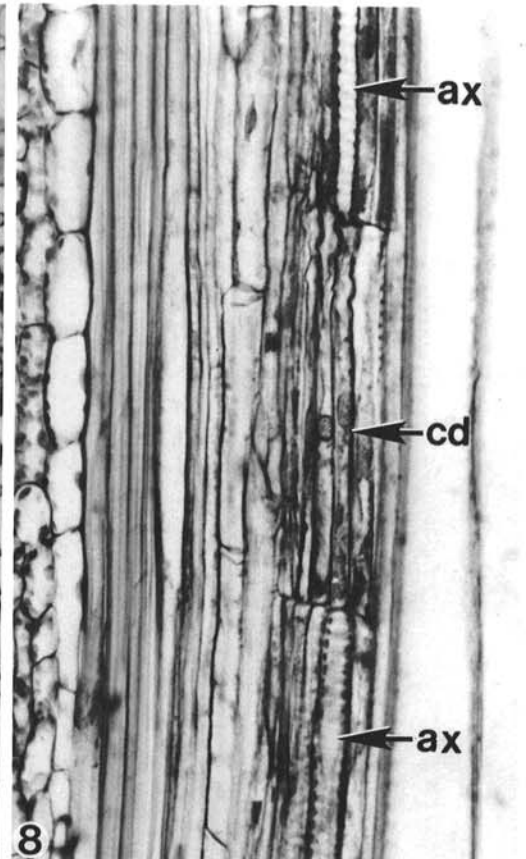
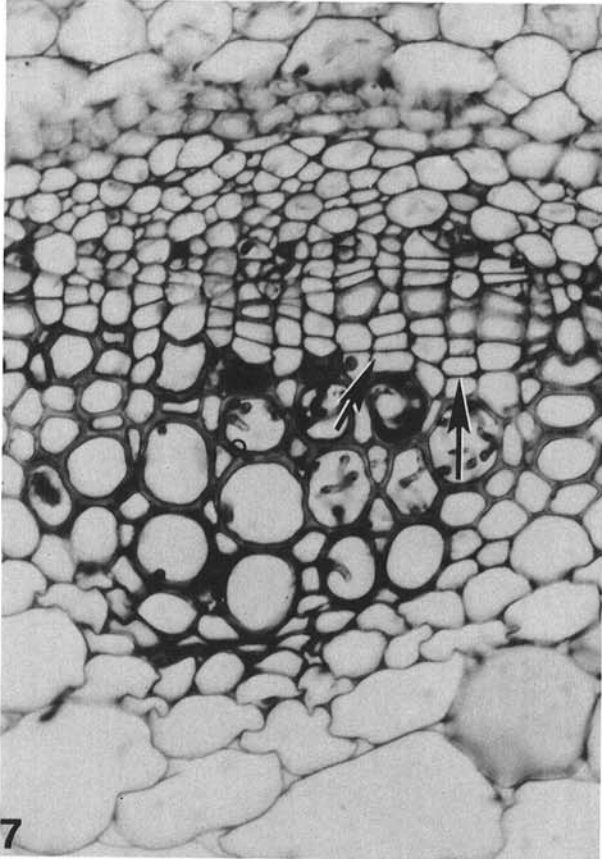
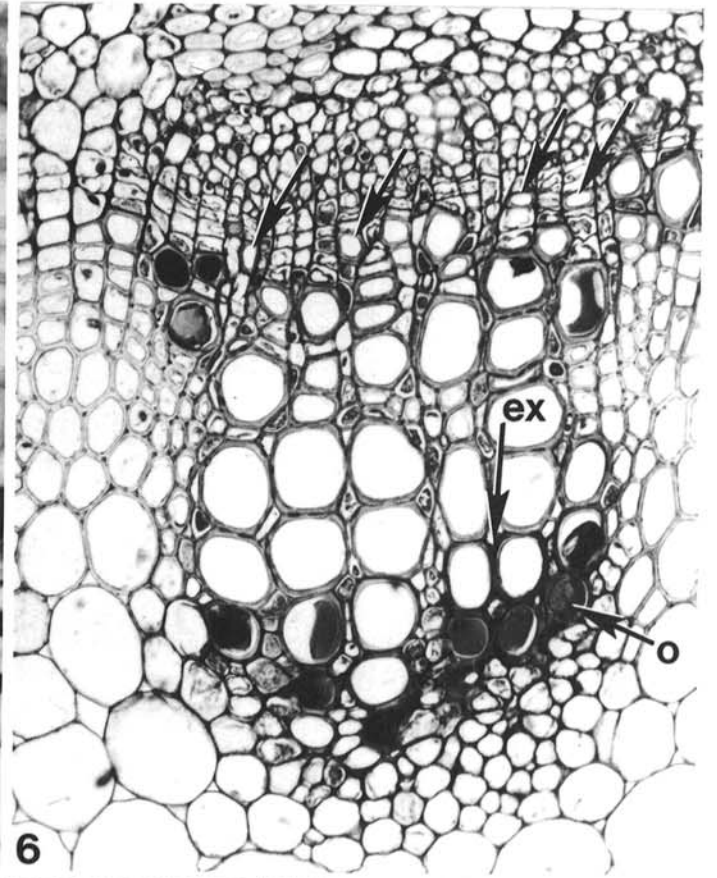
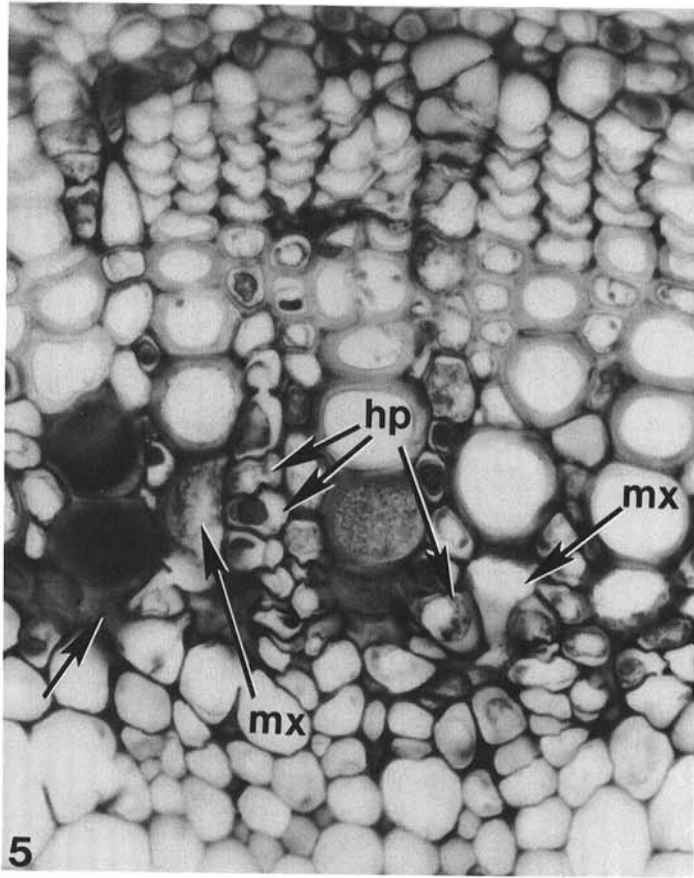
Mowing of alfalfa plants during hay production results in the formation of infection courts that allow *V. albo-atrum* access to all the vascular bundles of the stem. Stem inoculation was selected for this study because it closely parallels the natural infection that occurs during harvest.

The anatomy of alfalfa (5) allows the fungus to gain access to the vascular bundles of the crown which are directly connected to the inoculated stem. However, histological evidence presented in this paper indicated that *V. albo-atrum* has virtually no ability to move between vascular bundles. A similar conclusion was reached by Huang et al (8) based on the distribution pattern of infected seed produced on a systemically infected alfalfa plant. The limited degradation of cellulose in the later stages of pathogenesis that we observed agreed with reports by Talboys (20) and Heale and Gupta (7) and may be a major factor restricting fungal movement between vascular bundles.

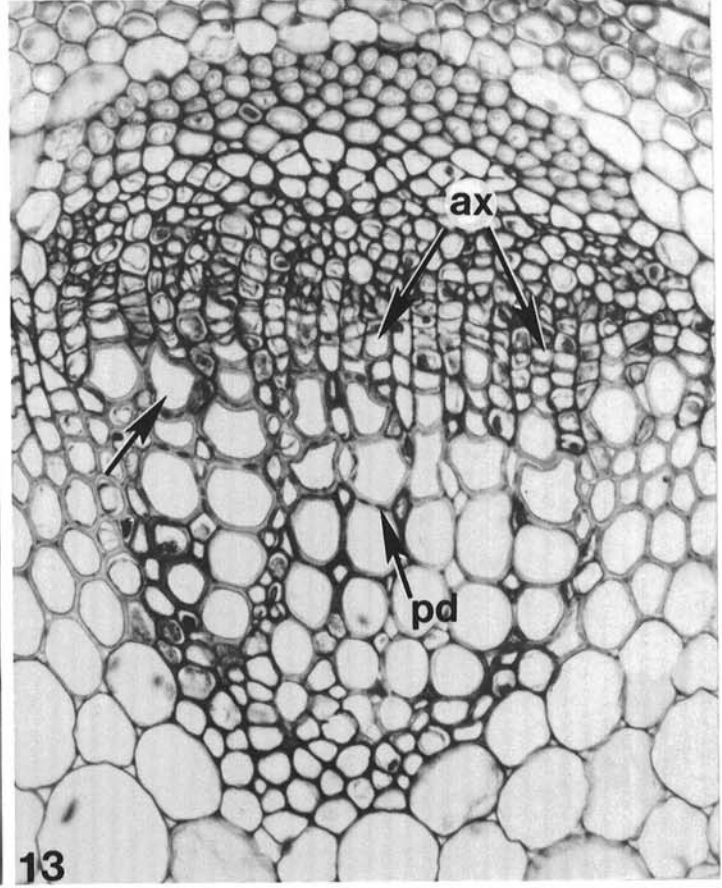
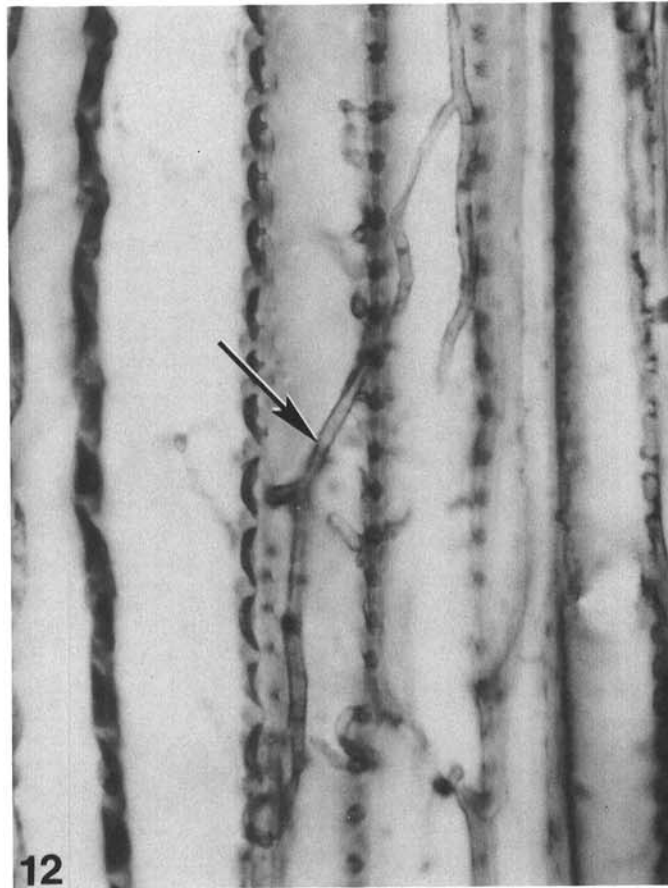
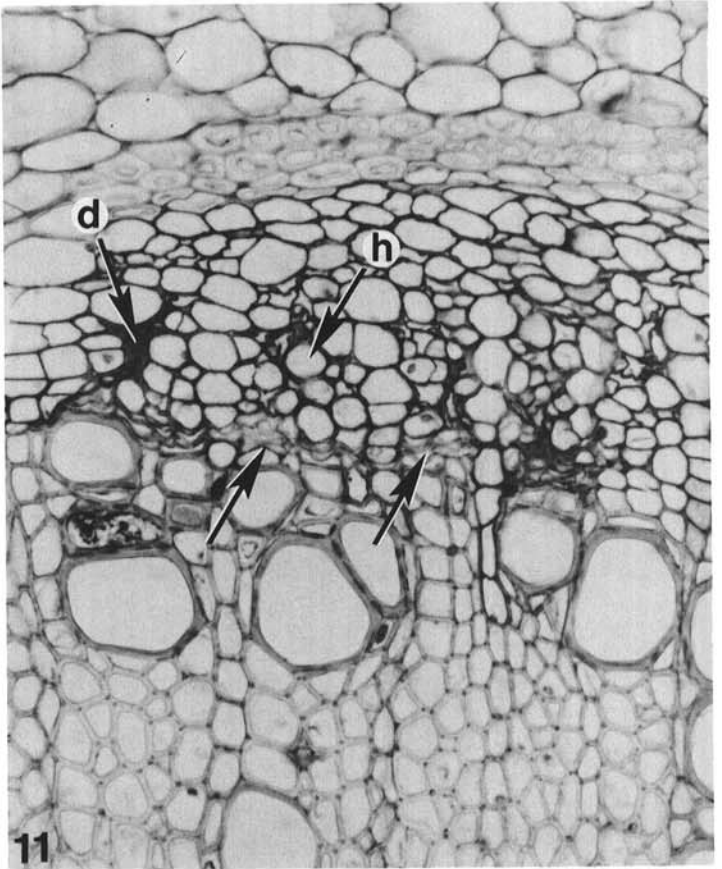
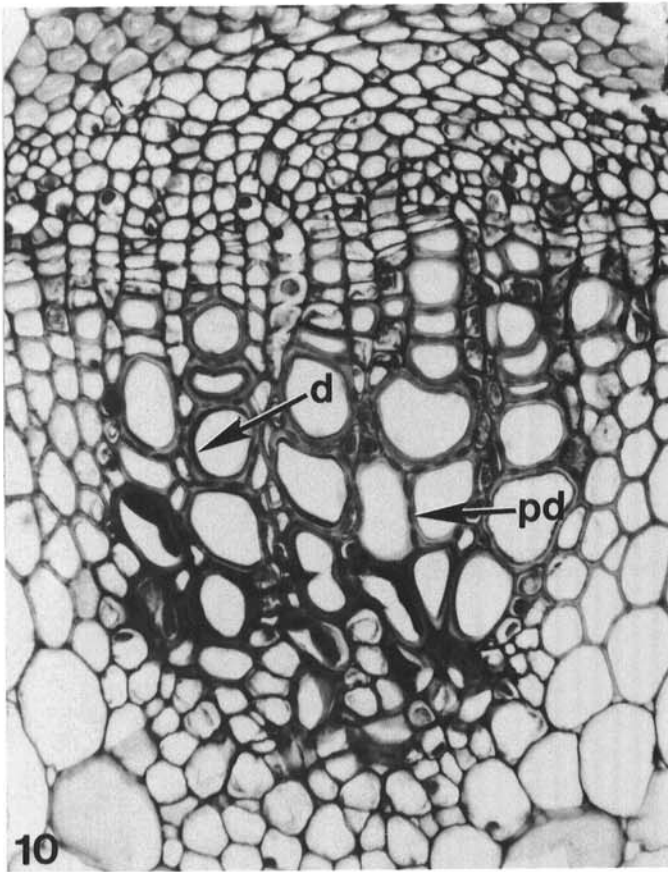
Xylem hyperplasia was reported by Talboys (21) in resistant hops infected by *V. albo-atrum* and effectively compensated for functional xylem vessel reduction caused by vascular plugging. The alterations in xylem differentiation we reported differ from xylem hyperplasia by resulting in a reduction in functioning xylem vessels. The atypically narrow xylem vessels and the absence of large immature vessel elements we observed are the consequence of a



Figs. 1-4. Portions of transverse sections through stems of alfalfa clone 774. Stem sections in Figs. 3 and 4 are infected with *Verticillium albo-atrum*. **1.** Portion of a healthy stem showing a vascular bundle with phloem (p), vascular cambium (v), and xylem vessel elements (x) ($\times 357$). **2.** Portion of a healthy stem with immature xylem vessel elements (arrows) adjacent to the vascular cambium ($\times 325$). **3.** Vascular bundle 2 wk after infection. Note the atypically small xylem vessel elements (arrows), vascular occlusions (o), and hypertrophied xylem parenchyma (hp) ($\times 342$). **4.** Leaf trace bundle 2 wk after infection showing hypertrophy of xylem parenchyma (hp), hypertrophy of vascular cambium (hv), and accumulation of extracellular material (ex). Note the distortion in the shape of the xylem vessel elements of the adjacent vascular bundle (arrows) ($\times 278$).



Figs. 5-9. Transverse and longitudinal sections through portions of alfalfa stems infected with *Verticillium albo-atrum*. **5,** Hypertrophy of xylem parenchyma cells (hp) causing the occlusion of metaxylem vessel elements (mx) and accumulation of extracellular material between xylem vessel elements and the pith (arrows) ($\times 577$). **6,** Increase in the number of atypical xylem vessel elements (arrows) and presence of xylem vessel occlusions (o) and the accumulation of extracellular material between xylem vessel elements (ex) ($\times 287$). **7,** Note the presence of undifferentiated cambial derivatives (arrows) around the atypical xylem vessels. Mycelium is visible in xylem vessel elements ($\times 391$). **8,** Longitudinal section showing atypical small xylem vessel elements (ax) interrupted by undifferentiated cambial derivatives (cd) ($\times 312$). **9,** Longitudinal section in which the pit chamber plugs are clearly evident (arrows) ($\times 560$).



Figs. 10-13. Transverse and longitudinal sections through stems of alfalfa clone 774 infected with *Verticillium albo-atrum*. **10**, Xylem vessel elements with deposits on the interior surface of the cell wall (d). Note that the pit chambers also show deposits (pd) ($\times 325$). **11**, Cross section through a stem 5 wk after inoculation. At this time, the vascular cambium shows severe distortion and dissolution (arrows). Note the hypertrophy (h) and dissolution (d) exhibited by phloem cells ($\times 283$). **12**, Xylem vessel colonized by thick-walled, dark resting hyphae (arrow) ($\times 490$). **13**, Vascular bundle from an unidentified variety of alfalfa showing severe symptoms. Note the presence of atypical xylem vessel elements (ax), distorted xylem vessel elements (arrow), and plugged xylem pit chambers (pd) ($\times 245$).

disturbance in the process of cell enlargement rather than in cell differentiation as represented by secondary wall development. Indole-3-acetic acid is involved in cell wall plasticity which is directly correlated with cell enlargement (12), and Krátká and Kudela (11) noted an increase in indole-3-acetic acid oxidase in susceptible alfalfa infected with *V. albo-atrum*. Consequently, it is possible that the atypical xylem vessel elements we observed are a result of localized reductions in indole-3-acetic acid.

The accumulation of undifferentiated cambial derivatives which accompanied formation of atypical xylem vessel elements and blocked mature xylem vessels may have been a consequence of the interruption in xylem vessel element enlargement. Pressure has been implicated in normal cambium function (3), and the failure of xylem vessel elements to enlarge may have reduced pressure on the vascular cambium, thus contributing to the proliferation of parenchyma.

The occurrence of vascular occlusion by gums, gel, and tyloses is common in vascular wilt diseases and was recently reviewed by Beckman and Talboys (1) and Talboys (23). VanderMolen et al (24) showed that vascular occlusion by gum and gels was a nonspecific response of the plant to vascular invasion, and Beckman and Talboys (1) theorized that the timely occurrence of such blockages served to limit access to the vascular system and thus functioned as a defense mechanism. Susceptible alfalfa plants are rapidly colonized by *V. albo-atrum* (14). Therefore, it is likely that the vascular plugs we observed and the gelation reported by Heale and Gupta (7) occurred too late or in too restricted a quantity to limit pathogen spread through the xylem. In addition, tyloses which were reported to restrict vascular access in infected hops (21) were only found in the naturally aging protoxylem vessels of susceptible alfalfa infected by *Verticillium*, which agrees with Isaac's observation (9).

In addition to vascular plugs, we noted extensive xylem vessel wall coating with gum, suberin, and occasionally pectin. Robb and Street (17) reported a similar phenomenon in tomato infected by *Verticillium* although the reaction was suppressed in susceptible plants. The xylem vessel wall coating and pit chamber occlusion we detected appeared extensive enough to inhibit lateral water movement in affected bundles and contribute to vascular malfunction.

Vascular response to the presence of *V. albo-atrum*, including disruption of xylem vessel element differentiation, obliteration of older metaxylem vessels by hypertrophied xylem parenchyma, and xylem vessel and pit chamber occlusion, coincided with initial symptom expression. These changes were noted in the leaf trace bundle as well as in the stem and provide evidence that vascular malfunction in the petiole may be caused by vascular changes present in leaf trace bundles before they diverge into the petiole. Similar anatomical changes were noted in other susceptible alfalfa genotypes from both field and greenhouse environments (*unpublished*). The vascular changes described in this paper constitute an anatomical mechanism which probably reduces water movement in affected vascular bundles and contributes to symptom expression.

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