Histology of Delicious/Malling Merton 106 Trees Affected by Apple Union Necrosis and Decline

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ABSTRACT

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Tissue sections from the graft union of apple union necrosis and decline-affected *Malus domestica* trees were examined by light microscopy to determine the anatomical basis of this delayed incompatibility, which has been associated with tomato ringspot virus (TmRSV) infection of rootstock tissue. Histopathological changes in Delicious/Malling Merton 106 trees began abruptly after at least 8 yr of compatible growth. The relative number of ray and axial parenchyma cells increased greatly at the union and above it. Local areas of unlignified parenchyma developed at the union in place of xylem. This tissue was removed when bark was peeled off to expose the union, thereby leaving indentations in the xylem. Pegs of peridermlike tissue were differentiated from cells within these indentations. These three changes occurred within 1–2 yr after symptom induction. Abnormal tissue at the union interfered with mobilization or translocation of carbohydrates. These histological changes did not appear to be the result of a hypersensitive reaction of cultivar Delicious to TmRSV in rootstock tissue.

Additional key word: incompatibility.

Apple union necrosis and decline (AUND), a delayed graft union incompatibility of *Malus domestica* Borkh. that was first reported in the Northeast, is associated with tomato ringspot virus (TmRSV) infection of rootstock tissue (13,14). Recently this disease was reported in Washington State (12). Symptoms develop only after infected trees have reached bearing age, although infection may have occurred several years earlier. Dark tissue produced at the union is considered to be the result of a hypersensitive reaction of certain scion cultivars to virus-infected rootstock tissue (13,14). However, the causal role of TmRSV has not yet been verified because visible symptoms have not developed at the union of inoculated trees (4). Cultivar Delicious is the most sensitive scion and Malling Merton 106 (MM 106) is the most common rootstock among AUND-affected trees.

General aboveground symptoms of decline and the diagnostic symptoms observed at the graft union of trees affected with AUND resemble symptoms reported for other examples of graft incompatibility associated with virus infection. Prune brownline disease occurs when *Prunus domestica* is grafted on Myrobalan plum (*P. cerasifera*) or peach (*P. persica*) infected with the peach yellow bud strain of TmRSV (9). Walnut blackline occurs when English walnut (*Juglans regia*) scions are infected with a walnut isolate of cherry leafroll virus (CLRV-W) (10). These diseases also appear to be caused by hypersensitive reactions.

The objective of this study was to determine the histology of discolored tissue produced at the graft union of AUND-affected Delicious/MM 106 trees. A preliminary report has been presented (15).

MATERIALS AND METHODS

In an orchard in Shoreham, VT, four Delicious/MM 106 trees with general aboveground symptoms of decline and a necrotic graft union were selected for histological study. The rootstocks of nine randomly selected trees, including one tree used during this histological study, were previously indexed positively for TmRSV by both enzyme-linked immunosorbent assay (ELISA) and mechanical transmission to cucumber (8, and M. A. Tuttle, *unpublished*). We believe these trees were originally infected with TmRSV as nursery stock since all trees within rows displayed symptoms at approximately the same time and there were no escapes. Three graft union samples with similar symptoms and representative of the disease found in Winchester, VA (supplied by Keith Yoder of the Winchester Fruit Research Laboratory of the Virginia Polytechnic Institute) were included in the study. Tissue from VA was not tested because previous experience indicated that TmRSV can only be detected in fresh bark tissue (M. A. Tuttle, *unpublished*). A healthy Delicious/MM 106 tree at the University of Vermont Horticultural Farm, South Burlington, was selected for comparative study.

Tissue samples (approximately 2 × 2 × 1-cm blocks) were placed in formalin-acetic acid-alcohol (FAA) fixative (2) under vacuum for about 12 hr. Fixed samples were softened by either boiling them in water for up to 12 hr or soaking them for up to 2 mo in a 10% glycerol solution containing 1% detergent. Softened tissue was dehydrated in a tertiary butyl alcohol series (2), infiltrated with Paraplast Plus (Sherwood Medical, St. Louis, MO 63103) under vacuum, embedded, and sectioned at 10 μm on a rotary microtome. Tissue was further softened during sectioning by moistening the cutting face with wet filter paper for 15 sec to 5 min after every five to eight sections . This treatment usually prevented the formation of rippled or frayed sections and ribbons with successive sections alternately thicker and thinner than 10 μm.

Radial, tangential, and transverse sections were afixed to slides with Haupt's adhesive (5), then overlaid with mercerized cotton thread to further secure the sections. Next, the sections were deparaffinized in xylene and coated with a 0.5% solution of parlodion in 50:50 ethanol-diethyl ether (5). Sections were stained with periodic acid-Schiff's (PAS) reagent and hematoxylin, safranin and fast green, safranin and hematoxylin, or PAS. Sections were stained before the Haupt's adhesive had dried to minimize loosening and cracking of xylem tissue. Histochemical tests were used to determine presence of suberin (Sudan black B) (5) and lignin (saturated solution of phloroglucinol in 50–70% HCl). Sections stained with Sudan black B were also stained with safranin and hematoxylin to increase contrast, and mounted directly in blue-label Karo corn syrup (Best Foods, CPC International, Inc.) (6). Sections with permanent stains were cleared with xylene and mounted in Permount (Fisher Scientific). Before staining, some

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sections were placed in 5% sodium hypochlorite for 5 min to remove cellular debris and clarify cell walls.

Stained sections were examined with a Leitz Dialux microscope and photographed on Kodak Panatomic-X film. A Hoya X1 green filter was used for prints of tissue stained with PAS and hematoxylin to maximize contrast. A Ladd graphic data digitizer was used to determine the relative area of vascular ray tissue in normal xylem and abnormal xylem produced after the onset of AUND union symptoms.

Starch in unfixed wood and bark tissue across the graft union was stained with IKI (90 mM KI and 11.8 mM I_2) to determine if abnormal tissue at the union interfered with the flow of carbohydrates as indicated by areas of starch accumulation (darkly stained tissue).

RESULTS

Union morphology. Removal of the abnormally thick bark at the union of Delicious/MM 106 trees often revealed a dark line, either immediately or within 1 min, in the wood. This line was comprised of pegs of dark tissue embedded in pits or in a smooth suture (Fig. 1). On several trees, short grooves were observed that extended from the pits into the scion or replaced the pits and necrotic tissue.

Histology of healthy tissue. Transverse, radial, and tangential sections from a symptomless Delicious/MM 106 tree were examined to determine the histology of healthy apple vascular tissue. Fusiform initials were not arranged in horizontal tiers in tangential sections, therefore cambial tissue was nonstoried. Secondary phloem contained sieve-tube elements, companion cells, ray and axial parenchyma, and fiber-sclereids. Secondary xylem contained vessel elements, ray and axial parenchyma, and fibers. Vessels, about 0.05 mm in diameter, occurred singly and were distributed almost evenly throughout each growth ring. Vertical or axial parenchyma cells were isolated from each other and were not associated with vessels. The structure of rays was primarily multiseriate and heterogeneous, with the center part composed of radially elongated procumbent cells and the edge composed of a row of nearly isodiametric cells called upright cells.

Histology of the graft union area in AUND-affected Delicious/MM 106 trees. The history of secondary xylem development is revealed through examination of xylem tissue because annual rings are formed and anatomical changes do not occur between development and decomposition. Histological changes began abruptly at the union of Delicious/MM 106 trees after at least 8 yr of normal, compatible growth, as determined from annual rings. The relative number of ray and axial parenchyma cells increased greatly in an area extending from the union to about 2–3 mm above the union spatially, and thus chronologically, before the development of dark pegs (Fig. 2). Consequently, the number of water-conducting vessels and thick-walled, supportive fibers was reduced drastically. Xylem formed at the union prior to this change resembled xylem from a healthy apple tree. About 40% of the total area of normal apple wood was comprised of parenchyma (Fig. 3), whereas about 94% of abnormal xylem at the union was parenchyma (Fig. 4). Crystals were observed in distended xylem parenchyma. A gradient in the proportion of cell types occurred between the abnormal tissue at the union and normal scion and rootstock tissue: the gradient extended 6 cm above the union and 1 cm below the union. A large increase in the proportion of parenchyma cells to sieve elements was seen in scion phloem. AUND-affected trees did not tend to revert to forming a normal proportion of cell types in xylem or phloem at the union.

Fig. 1. Graft union of apple union necrosis and decline (AUND)-affected Delicious/MM 106 tree with bark removed to reveal grooves and pegs of dark tissue embedded in xylem indentations.

After the development of an abnormal proportion of cell types, local areas of unlignified parenchyma developed at the union in place of xylem (Fig. 6). When bark was peeled back at the union, the unlignified parenchyma was separated from the wood, thereby leaving indentations or pits in the xylem (Fig. 1). Cambial tissue and recently formed phloem and xylem tissues bordering these areas were distorted by differential growth of the unlignified parenchyma. Cambial tissue was observed at the edge of these areas but not among unlignified parenchyma cells; therefore, these cells were not produced by the cambium. Pegs of discolored tissue developed primarily within these indentations (Fig. 6) and less frequently in the phloem or in the cambial zone.

Pegs of discolored tissue were more intensely colored than xylem or phloem in unstained and stained sections (Fig. 2). At the peg-phloem interface, files of cells extended from within the peg into the phloem, and nuclei were present in the outer layer of cells (Fig. 5). This layer of lightly stained cells divided in an organized manner resembling a lateral meristem. It was designated the generative layer to distinguish it from other lateral meristems. Other lightly stained layers, two or more cells wide, were seen within the intensely stained peg (Fig. 7); nuclei were not observed in these cells. Cells between layers frequently were crushed. A generative layer extended around the periphery of each peg and was connected with the cambial zone (Fig. 8). However, a generative layer was not evident between xylem and discolored cells of the peg. Groups of two or more pegs occasionally were united by subsequent peg development, and radially oriented cracks frequently were observed (Fig. 9).

Staining with phloroglucinol revealed scattered strands of lignified tissue within pegs. Sudan black B stained the outer edge of and concentric layers within pegs, indicating the presence of suberin (Fig. 10). The generative layer (Fig. 11) produced cell types similar to normal periderm (Fig. 12). Cells that were completely surrounded by suberized cells were necrotic (Fig. 6), whereas cells partially surrounded by the suberized cells of a recently initiated peg contained nuclei.

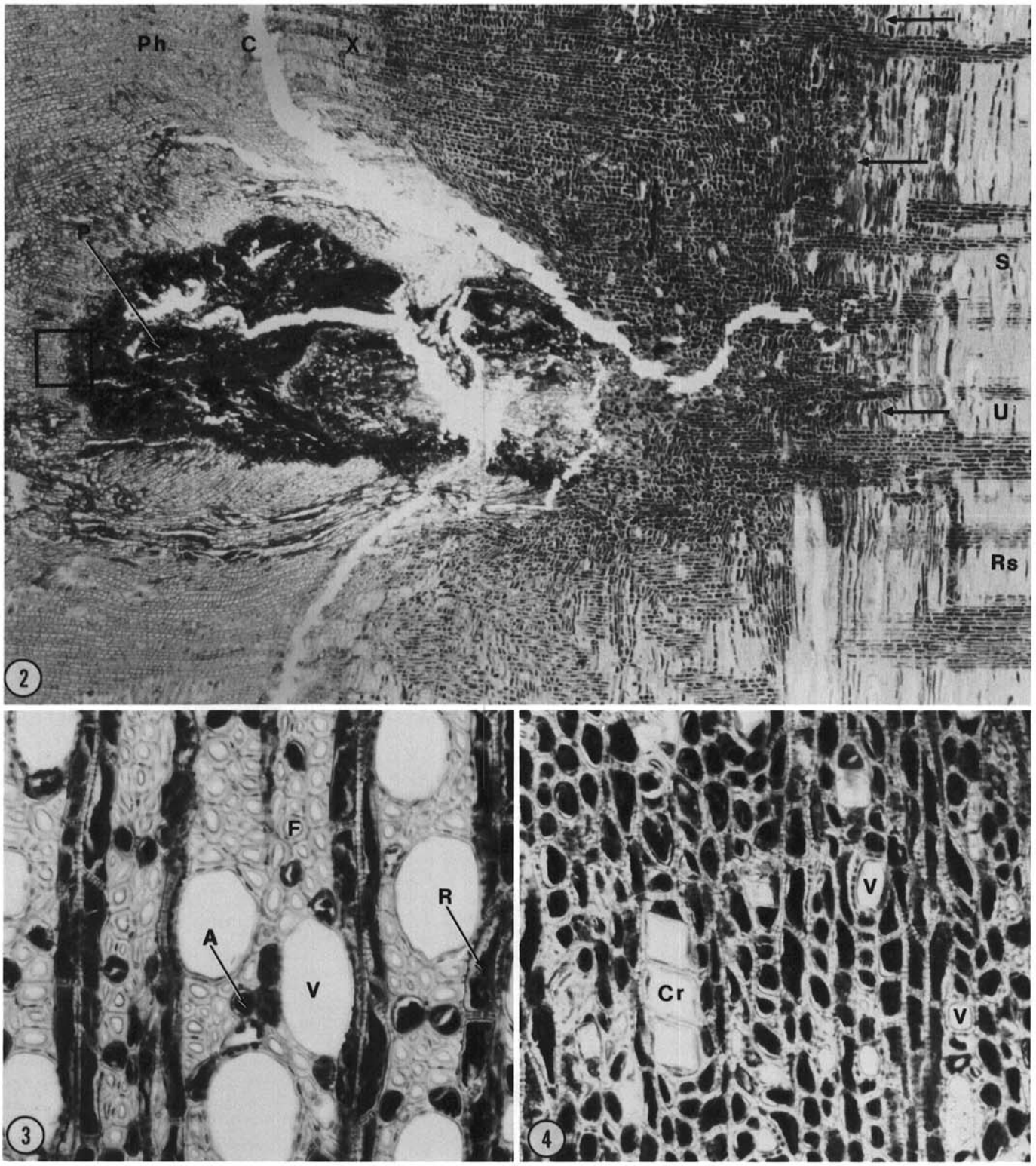
Starch was accumulated in rootstock phloem and xylem just below the union of a tree showing severe AUND sampled in late May, whereas starch was accumulated in scion phloem just above the union of a similar tree sampled in mid-August. In contrast, in late May starch was evenly distributed in a tree displaying initial union symptoms of AUND.

DISCUSSION

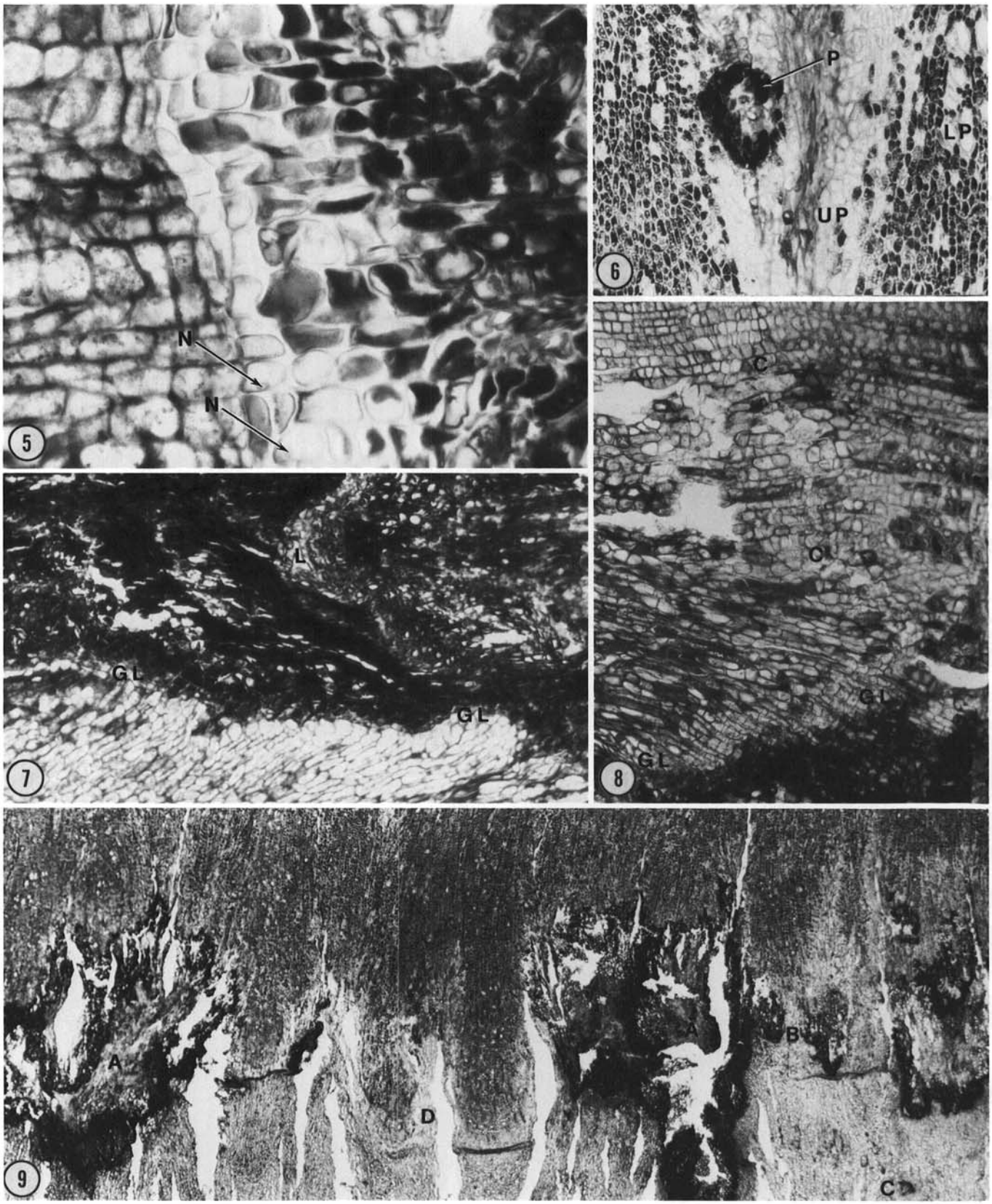
A delayed pathological reaction was observed at the graft union of Delicious/MM 106 trees apparently infected with TmRSV. After at least 8 yr of normal, compatible growth, an increase in ray and axial parenchyma, from 40 to 94% of the total area, dramatically decreased the number of fibers and vessels. This indicated a change in the vascular cambium that involved a twofold increase in the ratio of ray-to-fusiform initials, and a large increase in the relative number of fusiform initials producing cells that differentiate into axial parenchyma.

Local areas of unlignified parenchyma developed when cambial initials stopped functioning at discrete points around the circumference of the graft union. Pegs of peridermlike tissue developed primarily within these areas and less frequently in the phloem or in the cambial zone. AUND pegs are referred to as

peridermlike tissue because their formation resembles the formation of periderm. A phellogenlike (PL) layer of nucleated cells at the periphery of each peg produced unlignified parenchyma cells centrifugally and suberized cells centripetally. Pegs were comprised of alternate layers of suberized and unsuberized cells. This suggests cyclic periods of activity. In contrast with formation of normal periderm, AUND pegs developed as mirror images of normal periderm relative to the center of the tree. Also, initial PL layers differentiated from phloem parenchyma, unlignified xylem parenchyma, and cambial cells, as compared to phellogen, which



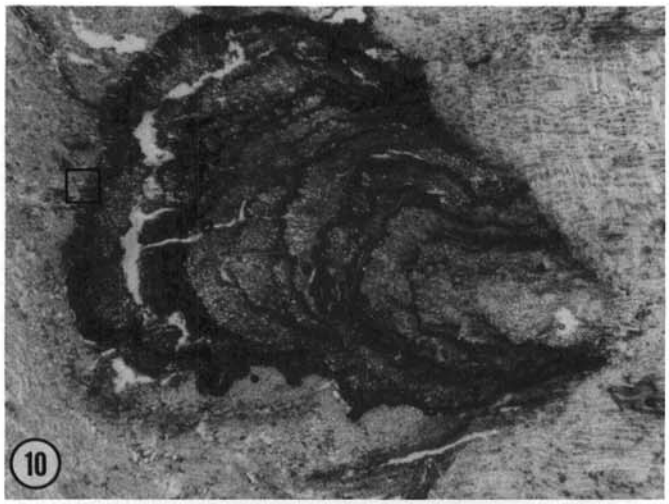
Figs. 2–4. Portions of sections through the graft union of Delicious/MM 106 trees affected by apple union necrosis and decline (AUND). **2,** Radial section showing a darkly stained peg (P) and a large increase in the relative number of parenchyma cells around the peg and above it (arrows). Note the location of xylem (X), cambial zone (C), phloem (Ph), scion (S), union (U), and rootstock (Rs) (×44). **3,** Transverse section of xylem produced before AUND symptoms developed showing a normal proportion of ray (R) and axial (A) parenchyma, vessels (V), and fibers (F) (×300). **4,** Transverse section of xylem produced after symptom induction (×297). Compare the proportion of cell types and diameter of vessels (V) to that in Fig. 3. Note the distended crystalliferous xylem parenchyma (Cr).

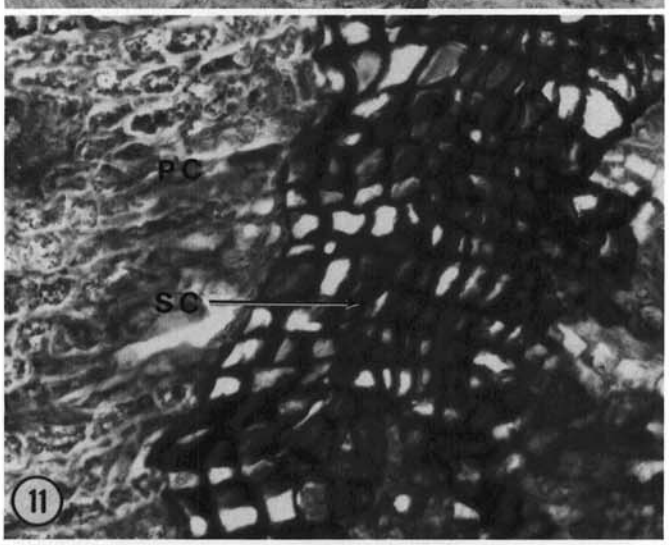


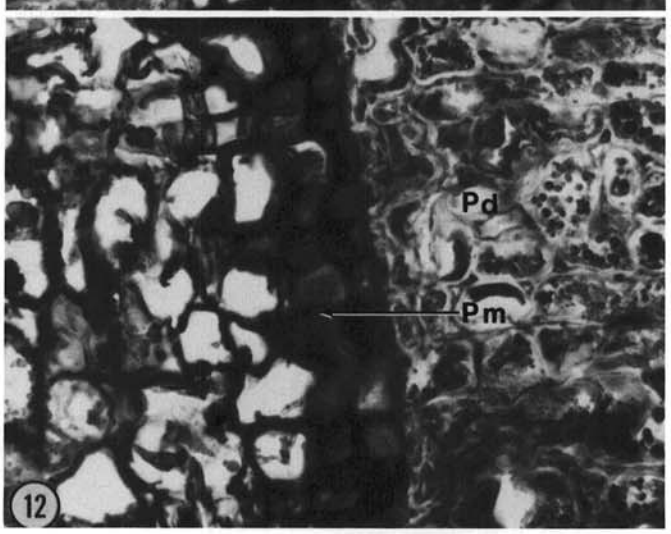
Figs. 5–9. Portions of sections through the graft union of Delicious/MM 106 trees affected by apple union necrosis and decline (AUND). **5,** Enlargement of boxed area of Fig. 2 showing nuclei (N) in the weakly stained generative layer (×481). **6,** Transverse section of a recently initiated suberized peg (P), stained with Sudan black B, surrounded by weakly stained unlignified parenchyma (UP) and lignified xylem parenchyma (LP) stained with safranin (×125). **7,** Enlargement of generative layer (GL) and a weakly stained layer (L) of uncrushed cells within the darkly stained peg shown. in Fig. 2 (×102). **8,** Radial section through AUND-affected graft union showing connection between generative layer (GL) and scion cambial zone (C) (×102). **9,** Transverse section displaying pegs at an advanced stage of development (A), pegs developing in the cambial zone (B), a peg developing in the phloem (C), and an area lacking pegs (D)(×23).

differentiates from phloem parenchyma. Subsequent PL layers differentiated from phloem parenchyma.

In an anatomical study of prune brownline disease, Mosse (11) observed peridermlike tissue at the graft union of Hale's Early peach and Myrobalan B plum. She described the developmental sequence beginning with groups of cells dying and then becoming surrounded by a phellogen. Our study provided evidence for the reverse sequence in AUND-affected trees. Phellogenlike activity began around living nucleated cells. All peridermlike pegs that completed a spherical shape enclosed dead cells. We contend that cells within the pegs died because they were isolated from nutrients by the impervious, suberized tissue.







Figs. 10–12. Portions of radial sections stained with Sudan black B to reveal suberized tissue at the union of Delicious and MM 106 affected by apple union necrosis and decline. **10,** Pegs were comprised of concentric hemispheres of dark-staining suberized tissue (×27). **11,** Enlargement of the boxed area of the peg in Fig. 10 revealed its peridermlike nature. Suberized cells (SC) were produced centripetally and parenchyma cells (PC) were produced centrifugally (×208). **12,** Normal periderm, located at the periphery of the main stem, consists of phellem (Pm) and phelloderm (Pd) (×209).

AUND symptom development may be the result of hormonal imbalance. Cambial division depends upon the presence of indole-3-acetic acid (IAA) and gibberellins (GAs) (17). IAA determines the diameter of vessels and is necessary for differentiation of cambial derivatives into xylem elements, whereas GAs promote differentiation of phloem. Their relative concentrations influence the proportion of xylem and phloem produced. In the presence of only GAs, xylem derivatives remain undifferentiated. Cambial initials in peach trees affected by stem pitting, which is associated with TmRSV infection, produce mostly xylem parenchyma (7). Concentration of GAs relative to normal tissue increases in this abnormal tissue (18). The observed increase in number of xylem parenchyma cells, decrease in diameter of vessels, local areas of unlignified parenchyma, and increase in bark thickness at the union of AUND-affected Delicious/MM 106 trees may have resulted from an increase in the proportion of GAs to IAA. In addition, normal periderm initiation is prevented or delayed by exogenous application of IAA, GAs, and several other growth hormones in *Fraxinus pennsylvanica* Marsh. (3) and *Robinia pseudoacacia* L. (1). Thus, the formation of peridermlike tissue during the development of AUND also may have resulted from a hormonal imbalance.

AUND-affected trees are structurally weak and more susceptible to drought, because the number of fibers and vessels are greatly reduced. These trees exhibit symptoms of starvation resulting from restricted carbohydrate translocation or mobilization. Starch was accumulated below the union in late May when new shoot growth should have exhausted stored reserves, whereas starch was accumulated above the union in mid-August when carbohydrates of normal trees were translocated to roots.

Various lines of evidence indicate that histological changes at the union of AUND-affected Delicious/MM 106 trees are not the result of a hypersensitive reaction as speculated by Stouffer et al (13,14). The classic description of a hypersensitive reaction is the rapid death of recently infected cells or cells surrounding an infection site preventing spread of a pathogen. However, initiation of symptom development at the union of AUND-affected trees was delayed for several years. TmRSV has never been detected in Delicious scion tissue, including bark tissue within 1 cm of the peridermlike tissue (M. A. Tuttle, *unpublished*), despite the delayed initiation of this supposed resistance mechanism. The zone of solid xylem parenchyma and pegs of peridermlike tissue at the union do not resemble the necrotic local lesions typically associated with hypersensitive reactions in herbaceous tissue. These cells did not die, instead they differentiated into abnormal tissue. Initiation of histological changes at the union of TmRSV-infected McIntosh/MM 106 trees was also delayed for several years (16). In contrast with AUND-affected Delicious/MM 106 trees, spiral grain developed in McIntosh/MM 106 trees and TmRSV was occasionally detected in scion bark tissue (16).

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