Cytology and Histology

Graft Union Histology and Distribution of Tomato Ringspot Virus in Infected McIntosh/Malling Merton 106 Apple Trees

Margaret A. Tuttle and Alan R. Gotlieb

Former graduate research assistant, Botany Department, and associate professor, Plant and Soil Science Department, University of Vermont, Burlington 05405.

Portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the MS degree. Vermont Agricultural Experiment Station Research Report 36.

Accepted for publication 14 September 1984 (submitted for electronic processing).

ABSTRACT

Tuttle, M. A., and Gotlieb, A. R. 1985. Graft union histology and distribution of tomato ringspot virus in infected McIntosh/Malling Merton 106 apple trees. Phytopathology 75:347-351.

Tissues from the graft union of *Malus domestica* 'McIntosh'/Malling Merton 106 (MM 106) trees infected with tomato ringspot virus (TmRSV) were sectioned, stained, and examined through a light microscope. After at least 8 yr of compatible growth, the orientation of vessels and fibers just above the union became inclined rightward with respect to the main stem axis. The angle of inclination increased gradually, reaching almost 90 degrees after about 5 yr. Three to 5 yr after the initiation of counterclockwise spiral grain, a zone of solid parenchyma and a xylem indentation developed at the union. Most trees also had a scion overgrowth or inverted shoulder. These trees did not produce pegs of dark tissue, which characterizes apple union necrosis and decline in other cultivars. Abnormal tissue at the union interfered with mobilization or translocation of carbohydrates. TmRSV was readily detected with enzyme-linked immunosorbent assay in MM 106 tissue. Rootstock bark tissue was more reliable than rootstock sprout leaf tissue for indexing trees. TmRSV was detected in three of 21 McIntosh scions. Tobacco ringspot virus was not detected in rootstock tissue.

Additional key words: ELISA.

The diagnostic symptom of apple union necrosis and decline (AUND) is the formation of pegs of dark tissue in xylem indentations or in pits at the graft union of tomato ringspot virus (TmRSV)-infected *Malus domestica* Borkh. trees (10,12–14). Different symptoms develop at the union of several scion cultivars and TmRSV-infected Malling Merton 106 (MM 106) rootstocks (8). These symptoms include pitting, invagination, horizontal cracking, and union breakage (cultivars Paulared and Spartan); invagination and union breakage (cultivars McIntosh, Spartan, Stayman, and Tydeman's Early); scion overgrowth (cultivar Rome Beauty); and slight pitting or vertical grooving (cultivar Empire).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

Although TmRSV is not detectable with enzyme-linked immunosorbent assay (ELISA) in leaf tissue from Delicious, Paulared, Rome Beauty, and Tydeman's Early, it is consistently detectable in MM 106 and Stayman and inconsistently detectable in Empire, McIntosh, and Spartan leaf tissue (4). During our preliminary studies (7), TmRSV was detected in bark tissue from McIntosh/MM 106 trees. In Canada (9), tobacco ringspot virus (TbRSV) was detected in rootstock leaves from trees with necrotic unions, but in New York (8), only TmRSV has been detected in AUND-affected Delicious/MM 106 trees.

The objectives of this study were: to examine the histological changes that had occurred at the graft union of McIntosh and TmRSV-infected MM 106 trees; to determine whether TmRSV can be consistently detected with enzyme-linked immunosorbent assay (ELISA) in scion bark tissue; and to determine whether rootstock tissue is infected with TbRSV.

MATERIALS AND METHODS

Histological study. Four approximately 14-yr-old McIntosh/MM 106 trees growing adjacent to AUND-affected Delicious/MM 106 trees in Shoreham, VT, were selected for histological work. Symptoms were first noted in Delicious in 1977. We believe these trees were infected with TmRSV in the nursery because this orchard did not display the aggregate distribution typical of natural infection by a nematode-transmitted virus. Healthy samples were obtained from a tree on the Horticultural Farm at the University of Vermont.

Tissue was prepared for light microscopy by following previously reported procedures (14). All sections were stained with periodic acid-Schiff's reagent and hematoxylin. Starch in wood and bark tissue 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.

Virus distribution study. Trees from the same McIntosh/MM 106 block in Shoreham, VT, were used for ELISA indexing. Methods reported by Clark and Adams (3) for the direct, or double antibody sandwich, enzyme-linked immunosorbent assay (ELISA) were followed with minor changes. Labeled and unlabeled γ-globulin for TbRSV were obtained from J. F. Peterson (Macdonald College, Montreal, Canada). TmRSV antibody was prepared in rabbits by injecting antigen obtained from infected MM 106 rootstocks in Shoreham. In gel double diffusion tests, the apple isolate was identical to the grape isolate of TmRSV supplied by D. Gonsalves (Geneva Experiment Station, New York). The day before sampling, wells of polystyrene microtiter plates (Dynatech Laboratory, Inc., Alexandria, VA 22314) were coated with unlabeled γ-globulin at 1 μg/ml for TmRSV and TbRSV. Plates were incubated at 37 C overnight, then at 4 C until test samples were applied. Leaf extracts were used at 1:5 and 1:10 (w:v) dilutions and bark extracts were used at dilutions of approximately 1:9 and 1:18. Controls used in each plate included sample buffer (PBS-Tween [3] with 2% PVP), extracts of healthy and virus-infected cucumber leaves, and extracts of healthy rootstock bark, rootstock sprout leaves, or scion bark. Alkaline phosphatase-labeled γ-globulin conjugates were used at 1:400 and 1:200 (v:v) dilutions for TmRSV and TbRSV, respectively. Test reactions usually were allowed to incubate for 60 min before being measured photometrically at 405 nm with a Dynatech Microelisa Minireader. Bark extracts with a test reaction absorbance ($A_{405\ nm}$) of at least 0.15 and greater than twice the value for healthy bark were considered positive (6).

The graft union was exposed by making a narrow wedge-shaped cut and peeling the bark back. Samples 12 mm in diameter were obtained with a cork borer. Each test sample consisted of one bark sample per tree. A disk of moist bright yellow tissue, comprised of cambium and youngest phloem, was removed from the inner surface of each bark sample. These disks were about 1 mm thick and weighed about 0.1 g. Each disk was cut immediately into small pieces and ground in cold sample buffer with a chilled mortar and pestle in ice. Extracts were squeezed through cheesecloth and plated while in the orchard. One aliquot from each sample was plated routinely on the same plate in the laboratory about 6 hr later to determine if sensitivity was lost during storage. All extracts were maintained on ice until used. Young terminal leaves from rootstock sprouts and scions were collected in plastic bags and transported in an ice-filled chest to the laboratory. Leaf tissue was ground with a chilled mortar and pestle, then plated the day of collection to avoid a loss in sensitivity.

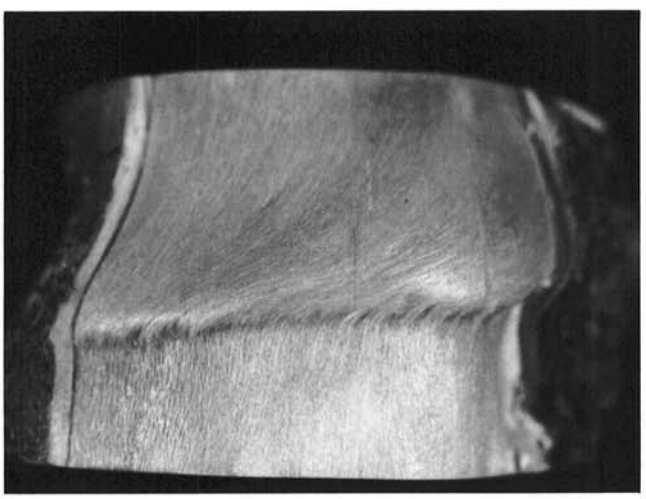


Fig. 1. Graft union of a tomato ringspot virus-infected McIntosh/MM 106 apple tree with bark removed to reveal an inverted shoulder with scalloped edge and spiral grain.

RESULTS

Union morphology. Removal of bark at the union of TmRSV-infected McIntosh/MM 106 trees revealed symptoms ranging from a shallow xylem indentation with no scion overgrowth (inverted shoulder) to a deep indentation with a large inverted shoulder (Fig. 1). Spiral grain with a rightward (counterclockwise) inclination was observed just above the union. Xylem indentations often appeared discolored or water-soaked because of shadowing by the inverted shoulder.

Histology of healthy tissue. Secondary vascular tissue from a healthy tree was examined. The union could not be located because of tissue continuity between scion and rootstock. Secondary phloem contained sieve-tube elements, companion cells, ray and axial parenchyma, and fiber-sclereids. Secondary xylem was characterized by diffuse apotracheal parenchyma; multiseriate, heterogeneous rays; and singular vessels with a diameter of about 0.05 mm.

Histology of graft union area of McIntosh/MM 106 trees. The grain of scion and rootstock was aligned with the longitudinal axis of the main stem for at least 8 yr (determined by observation of annual rings); then, the orientation of vessels and fibers just above the graft union inclined to the right. The angle of inclination increased gradually to approximately 90 degrees in about 5 yr. As a result of this spiral grain, radial sections of scion tissue resembled transverse sections (Figs. 2 and 3). Adjacent fibers and vessel elements were oriented horizontally and vertically (Fig. 3); therefore, reorientation occurred abruptly. Phloem sieve elements and companion cells also were inclined to the right above the union (Fig. 2). These trees did not tend to reverse spiral grain with time.

About 4 yr after initiation of spiral grain, a zone of parenchyma and a xylem indentation developed at the union (Figs. 2 and 4).

The longitudinal axis of reoriented xylem fibers and vessels was either parallel to the tangential plane (thus, cells were cut longitudinally in tangential sections [Fig. 5]) or between parallel and perpendicular to this plane (thus, cells were cut obliquely [Figs. 5-7]). Both orientations were observed above the zone of parenchyma in sections of tissue at an advanced stage of symptom development (Fig. 4). The orientation of individual ray parenchyma cells apparently was not affected during the development of spiral grain, whereas the orientation of whole rays was affected (Fig. 5).

An accumulation of starch was detected with IKI in scion xylem tissue just above the union of a tree sampled in early December. Starch would be evenly distributed in the main stem of a healthy tree.

TmRSV distribution in MM 106. The rootstocks of four McIntosh/MM 106 trees were assayed during preliminary studies. Bark and sprout leaf tissue from rootstocks of two trees tested positively; leaf tissue from the third tree also tested positively, whereas leaf tissue from the fourth tree tested negatively.

The rootstocks of 43 of 45 McIntosh/MM 106 trees with union symptoms were shown to be infected with TmRSV by indexing one bark sample and one pooled sprout leaf sample from each tree. These tests were conducted between late June and early October of 1982. TmRSV was detected in both bark and leaf tissue of 19 (42%) rootstocks, only in bark tissue of 21 (47%) rootstocks, and only in leaf tissue of 3 (7%) rootstocks. Several rootstocks were reindexed.

Leaf tissue from 15 rootstocks with only a positive bark ELISA result was resampled and tested. Only three samples tested positively in the second trial. Both bark and leaf tissue from one of the two rootstocks that had tested negatively the first time tested positively the second time almost 4 mo later. Bark tissue from two of the three rootstocks with only a positive leaf result was reindexed at different locations around the circumference of the main stem, and both rootstocks tested positively in the second or third trial.

Individual sprouts were tested from two rootstocks with consistently positive bark tests. Leaves from all five sprouts of one rootstock and six of nine sprouts of the other rootstock were consistently negative.

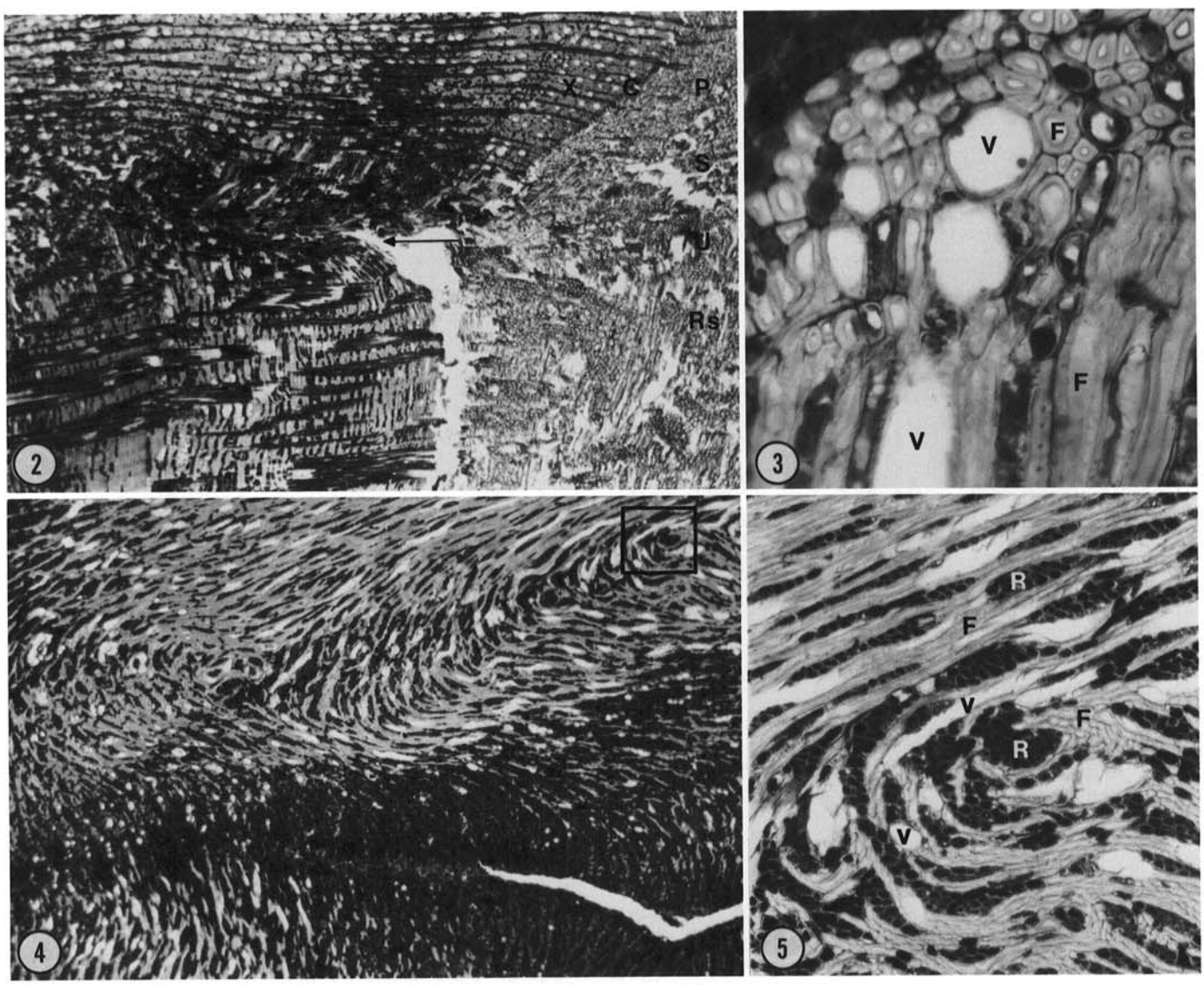
The test sensitivity for all infected bark sample extracts decreased during storage on ice, whereas the test sensitivity for all infected cucumber leaf extracts changed negligibly under the same conditions. $A_{405\ nm}$ values for aliquots plated about 6 hr after sampling averaged 52% lower than aliquots plated at the time of sampling in the orchard.

TmRSV distribution in McIntosh. Scion leaf and bark tissue from TmRSV-infected trees were tested with ELISA. Leaf tissue from four McIntosh scions were negative. TmRSV was detected above the McIntosh/MM 106 union in three of 37 bark samples from 21 scions. One positive sample came from about 1 cm above a union displaying severe symptoms. A sample from about 12 cm above this positive sample was negative. This scion indexed negatively at two different locations around the circumference of the main stem. The other two scions with a positive ELISA result also indexed negatively at different locations.

Testing for TbRSV infection. TbRSV was not detected in rootstock bark or leaf tissue from 12 McIntosh/MM 106 trees displaying union symptoms.

DISCUSSION

Histological changes observed at the graft union of TmRSV-infected McIntosh/MM 106 trees had not been reported in apparently healthy trees and these changes did not include symptoms previously described for AUND, a delayed graft incompatibility associated with TmRSV infection of rootstock tissue (14). Although infection probably occurred in the nursery, anatomical changes did not develop in McIntosh/MM 106 trees or nearby Delicious/MM 106 trees (14) until after fruiting had begun.



Figs. 2–5. Portions of sections through the graft union of tomato ringspot virus-infected McIntosh/MM 106 apple trees. **2,** Radial section showing inverted shoulder, spiral grain, xylem indentation (arrow), and zone of parenchyma (Z). Note the location of xylem (X), phloem (P), cambial zone (C), scion (S), rootstock (Rs), and union (U) (×27). **3,** Radial section showing fibers (F) and vessels (V) at the point of reorientation (×250). **4,** Tangential section from the same tree as the section in Figs. 2 and 3, showing zone of parenchyma and scion vessels and fibers reoriented into several configurations (×27). **5,** Enlargement of the boxed area of Fig. 4 showing abrupt change in direction of spiral grain. Note the orientation of vessels (V), fibers (F),and ray parenchyma (R) (×98).

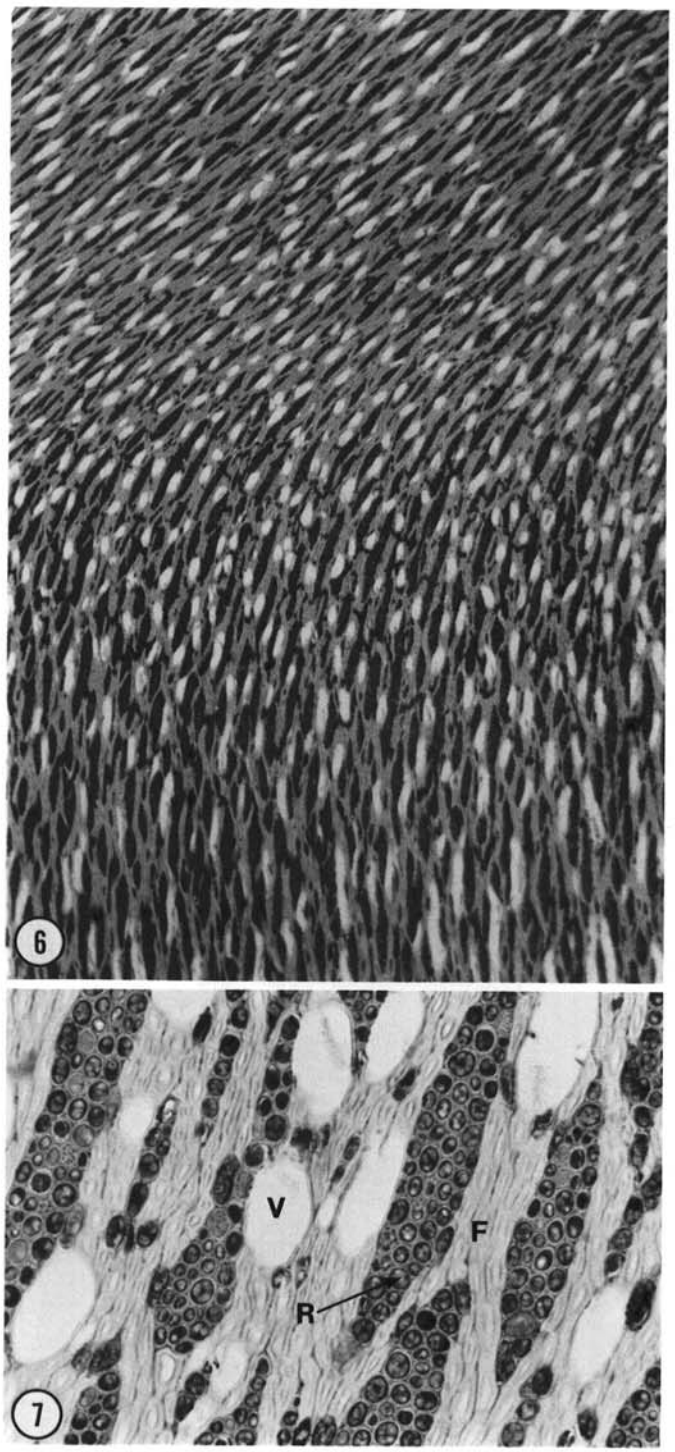
Visual symptoms had not been observed at the graft union of apple trees 1–6 yr after inoculation with TmRSV (4). These results indicate that physiological changes associated with fruiting may be necessary for symptom induction. Three anatomical changes developed in McIntosh/MM 106 trees after at least 8 yr of normal, compatible growth. The vessels and fibers just above the union inclined to the right (counterclockwise) with respect to the main stem axis. The angle of inclination increased gradually, reaching 90 degrees after about 5 yr. A scion overgrowth or inverted shoulder developed just above the union. A zone of parenchyma and a xylem indentation developed at the union about 4 yr after the initiation of spiral grain.

McIntosh/MM 106 trees infected with TmRSV may be structurally weak and appear water stressed on hot, dry days because of the zone of parenchyma at the union. Spiral grain probably did not confer structural weakness or resistance to water flow, because fibers and vessels of scion and rootstock maintained continuity in the tangential plane until the zone of parenchyma developed. Interference with mobilization or translocation of carbohydrates to the roots by abnormal tissue at the union resulted in an accumulation of starch above the union.

The vessels and fibers of nearly 200 species of broadleaved and coniferous trees are oriented helically in the trunk (1); therefore, spiral grain is not uncommon. However, in contrast with the pronounced inclination observed at the union of McIntosh and MM 106, the inclination normally is gradual and the longitudinal axis of both inclined and straight cells is parallel to the tangential plane. Twenty-two degrees was the maximum angle from the vertical axis measured during an extensive study involving more than 20 species of conifers (1). An abnormality closely resembling spiral grain in McIntosh/MM 106 was observed in Jonathan/Florence and Gallia Beauty/Florence apple trees displaying stem pitting below the union (2). Jonathan also is susceptible to AUND (5).

A correlation may exist between viral distribution and the type of histological change at the union of TmRSV-infected trees. TmRSV apparently is restricted to rootstock tissue in Delicious/MM 106 trees (4,5,7,10,11), and peridermlike tissue was produced at the union (14). TmRSV was detected in scion tissue from three of 21 McIntosh/MM 106 trees, and spiral grain developed at the union. TmRSV is consistently detectable in several cultivars propagated on TmRSV-infected MM 106 rootstocks that remain symptomless (4). This correlation may reveal a causal relationship between location of viral activity and type of histological change that results.

Because virus distribution is erratic in rootstock sprouts, assay of rootstock bark tissue was more reliable for detecting TmRSV-infected trees. Other researchers have reported similar observations (7). However, removing bark damages a tree, occasionally the union is buried when trees are planted, and assaying bark samples requires more time and labor because bark samples must be processed in the orchard to avoid a loss of sensitivity. Consequently, we recommend using leaf tissue from trees with sprouts, and bark tissue from trees without sprouts. Negative leaf or bark ELISA results should be confirmed by testing or retesting bark tissue.



Figs. 6–7. Portions of sections through the graft union of tomato ringspot virus-infected McIntosh/MM 106 apple trees. **6,** Tangential section showing spiral grain in an intermediate stage of development (×31). **7,** Enlargement of the union in Fig. 6 showing ray parenchyma (R) in a normal, tangential view and vessels (V) and fibers (F) in an oblique transverse view (×210).

LITERATURE CITED

1. Bannan, M. W. 1966. Spiral grain and anticlinal divisions in the cambium of conifers. Can. J. Bot. 44:1515-1538.
2. Choe, H. T., and Howlett, F. S. 1965. Morphological and anatomical structure of various budded apple varieties resulting from stem pitting. Proc. Am. Soc. Hortic. Sci. 87:29-40.
3. Clark, M. F., and Adams. A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
4. Cummins, J. N., and Gonsalves, D. 1982. Recovery of tomato ringspot virus from inoculated apple trees. J. Am. Soc. Hortic. Sci. 107:798-800.
5. Cummins, J. N., Uyemoto, J. K., and Stouffer, R. F. 1978. 'Union necrosis and decline': A newly recognized disease of apple trees, associated with tomato ringspot virus. Proc. N. Y. State Hortic. Soc. 123:125-128.
6. Gonsalves, D. 1979. Detection of tomato ringspot virus in grapevines: A comparison of *Chenopodium quinoa* and enzyme-linked immunosorbent assay (ELISA). Plant Dis. Rep. 63:962-965.
7. Lister, R. M., Allen, W. R., Gonsalves, D., Gotlieb, A. R., Powell, C. A., and Stouffer, R. F. 1980. Detection of tomato ringspot virus in apple and peach by ELISA. Acta Phytopathol. 15:47-55.
8. Rosenberger, D. A., Harrison, M. B., and Gonsalves, D. 1983. Incidence of apple union necrosis, tomato ringspot virus, and *Xiphinema* vector species in Hudson Valley orchards. Plant Dis. 67:356-360.
9. Rousselle, G. L., and Peterson, J. F. 1979. Association of tobacco

ringspot virus with a union incompatibility of apple. (Abstr.) Phytoprotection 60:169.
10. Stouffer, R. F., Hickey, K. D., and Welsh, M. F. 1977. Apple union necrosis and decline. Plant Dis. Rep. 61:20-24.
11. Stouffer, R. F., and Uyemoto, J. K. 1976. Association of tomato ringspot virus with apple union necrosis and decline. Acta Hortic. 67:203-208.
12. Tuttle, M. A. 1983. Histological study of apple union necrosis associated with tomato ringspot virus infection. M.S. thesis. University of Vermont, Burlington. 136 pp.
13. Tuttle, M. A., and Gotlieb, A. R. 1982. Histological study of graft union necrosis in apple associated with TmRSV infection. (Abstr.) Phytopathology 72:267.
14. Tuttle, M. A.,and Gotlieb, A. R. 1984. Histology of Delicious/Malling Merton 106 apple trees affected by apple union necrosis and decline. Phytopathology 75:342-347.