Cytopathology and Properties of Cherry Leaf Roll Virus Associated With Walnut Blackline Disease

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ABSTRACT

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A virus, associated with walnut blackline disease was purified from the herbaceous host *Phaseolus vulgaris* 'Pinto' and compared with cherry leaf roll virus (CLRV). Virus particles were isometric and approximately 26 nm in diameter. Virus preparations were unstable, but generally consisted of a middle (102–116S) and a bottom (129–133S) nucleoprotein component. The buoyant density was 1.465 g/cm^3 at 24 C. In both walnuts and herbaceous hosts, virus particles occurred in a linear row within cell wall protrusions, paracrystalline arrays within phloem elements and in spiral arrangements in apparent replication complexes. This virus presents one of the few cases in which virus particles and host cytopathology can be studied within the infected tree (walnut). On the basis of its properties and cytopathology the virus associated with walnut blackline disease is considered to be the walnut strain of CLRV (CLRV-W).

Walnut blackline (WBL) disease has become a major threat to walnut production (18,19). The disorder involved gradual girdling of the tree by a narrow necrotic strip of cambium and phloem tissues at the rootstock-scion union with subsequent tree decline (25). Early studies attributed this disorder to various noninfectious factors (2,7,8,16,23,24). Recent reports indicate that WBL disease is associated with a graft-transmissible virus (19). Several symptomatologically different virus isolates were obtained from walnuts. The virus has been serologically identified as a strain of cherry leaf roll virus (CLRV) and designated as CLRV-W (19).

The purpose of this study is to compare CLRV-W associated with walnut blackline disease with CLRV. Furthermore, the early cytopathology, both in herbaceous hosts as well as in walnuts, is described.

MATERIALS AND METHODS

Virus. Of the 11 single-lesion virus isolates recovered from walnut blackline affected trees (19), the walnut virus isolate designated as 5-48-1 was used in this study. The herbaceous hosts used in this study were *Nicotiana tabacum* L. 'Havana 425,' *Cucumis sativus* L. 'National Pickling,' and *Phaseolus vulgaris* L. 'Pinto.' These were grown and mechanically inoculated as previously described (17).

Virus purification. For comparative purposes CLRV-W 5-48-1 was isolated and partially purified. Although the dilution end point, the temperature inactivation point, and the longevity in vitro of CLRV-W 5-48-1 was similar to that of CLRV (1), the virus was extremely labile during purification. Published purification methods for CLRV proved unsatisfactory. The most useful method for CLRV-W 5-48-1 involved the following: infected tissues were frozen in liquid nitrogen, triturated with extraction buffer (0.02 M phosphate, pH 6.2, containing 0.02 M diethyldithiocarbamate [DIECA] and 0.02 M 2-mercaptoethanol) in a Waring Blendor, and pressed through four layers of cheesecloth. Extracted plant sap was acidified with 1 N HCl to pH 5.0, and stirred at 25 C for 1 hr. Extracts were centrifuged at 8,000 rpm for 15 min (Sorvall GSA rotor) and pellets were discarded. The supernatant was mixed with sodium EDTA-treated bentonite (4) (40–50 mg/300 ml of sap) and stored overnight at 4 C. Bentonite was removed by centrifugation at 8,000 rpm for 15 min (Sorvall GSA rotor). After clarification, the virus was concentrated by centrifugation at 30,000 rpm for 2 hr at 4 C (Beckman 30 rotor) followed by low-speed centrifugation of the resuspended pellets (extraction buffer without antioxidants) at 5,000 rpm for 10 min (Sorvall SS-34 rotor). The second high-speed centrifugation with a 30% sucrose (w/v) cushion was at 40,000 rpm for 90 min at 4 C in a Beckman 40 rotor followed by low-speed centrifugation as in the first cycle. All purification steps were monitored by infectivity tests.

Serology. Walnut virus isolate 5-48-1 was compared serologically with the golden elderberry strain of cherry leaf roll virus (CLRV-GE) (10) and two isolates of the walnut strain of cherry leaf roll virus (CLRV-W) (19) in agar gel double diffusion tests. Antigen sources were expressed sap from *Cucumis sativus* L. or *Chenopodium quinoa* Wild. plants.

Electron microscopy. Systemically infected leaf tissues from herbaceous hosts were vacuum infiltrated with either 5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) or with Karnovsky's fixative in Millonig's phosphate buffer. Tissues were washed and postfixed with 2% OsO_4 in the respective buffers for 3 hr at 25 C. Specimens were dehydrated either in a graded series of acetone or ethanol followed by propylene oxide. During dehydration, tissues were postfixed in a saturated solution of uranyl acetate for 12 hr in 70% of the corresponding solvent. Materials were embedded in Spurr's low-viscosity resin or Epon/Araldite.

Systemically infected walnut leaf tissues showing ringspot-type symptoms were fixed for 18 hr at 4 C with Karnovsky's fixative in 0.1 M cacodylate buffer containing 100 g/ml L-cysteine. Specimens were washed three times with buffer and antioxidant, followed by two rinses with buffer alone. Osmication, postfixation with uranyl acetate, and dehydration were done as described. Tissues were embedded in modified Spurr's resin (21). All sections were cut with a diamond knife on a Porter-Blum MT2 ultratome, stained with lead citrate, and examined with either a Zeiss EM-9 or AEI electron microscope. Partially purified virus preparations were stained with uranyl acetate at pH 4.0.

RESULTS

Partially purified virus. Virions in partially purified preparations had a mean diameter of 26 nm in negative stain (Fig. 1). The preparations contained some phytoferritin (11) and evidence of

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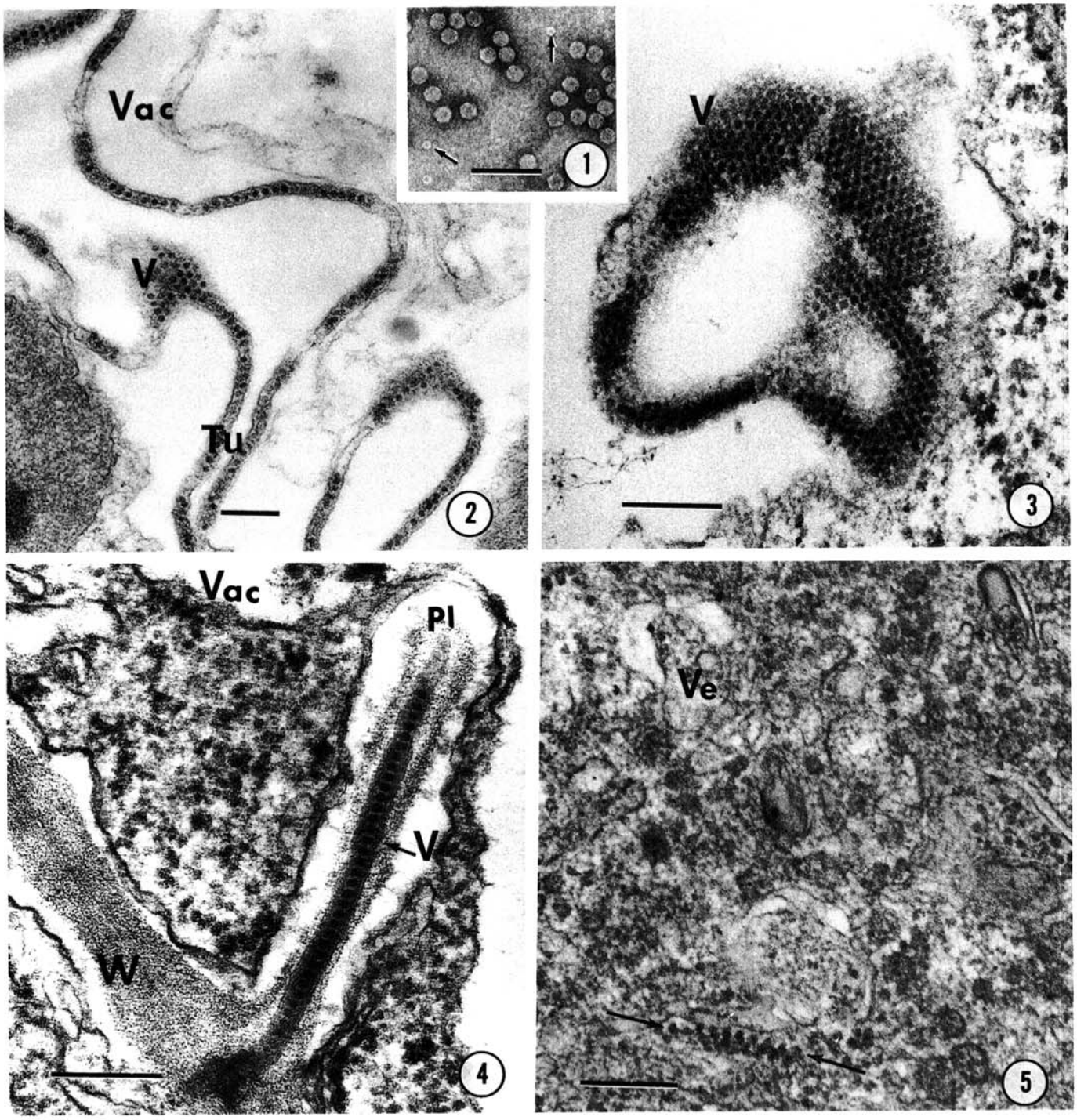
virus breakdown. Virus yields were 5–15 mg/kg, and $A_{260/280}$ ratios ranged between 1.7 and 1.8.

Extensive aggregation of virus particles occurred in 10–40% sucrose density gradients in 0.02 M phosphate buffer (pH 7.2), centrifuged 2–3 hr at 22,000 rpm in a Beckman SW25 rotor. Phytoferritin (53–65S) remained near the meniscus, whereas virus particles migrated to the bottom of the tubes within the first 60 min of centrifugation. Glutaraldehyde fixation (0.5% glutaraldehyde) of partially purified virus alleviated the problem to some extent since two very small, but distinct, peaks could then be resolved. Aggregation was not prevented by substitution of the phosphate resuspension buffer with either borate buffer (0.1 M, pH 8.5) or citrate buffer (0.01 m pH 6.0).

Partially purified virus, centrifuged to equilibrium in cesium chloride, formed a sharp peak at a density of 1.465 g/cm^3 (24 C). Overnight dialysis against 0.01 M sodium phosphate (pH 7.2) followed by infectivity assay indicated that the material in the band was not infectious. In negatively stained preparations, few (if any)

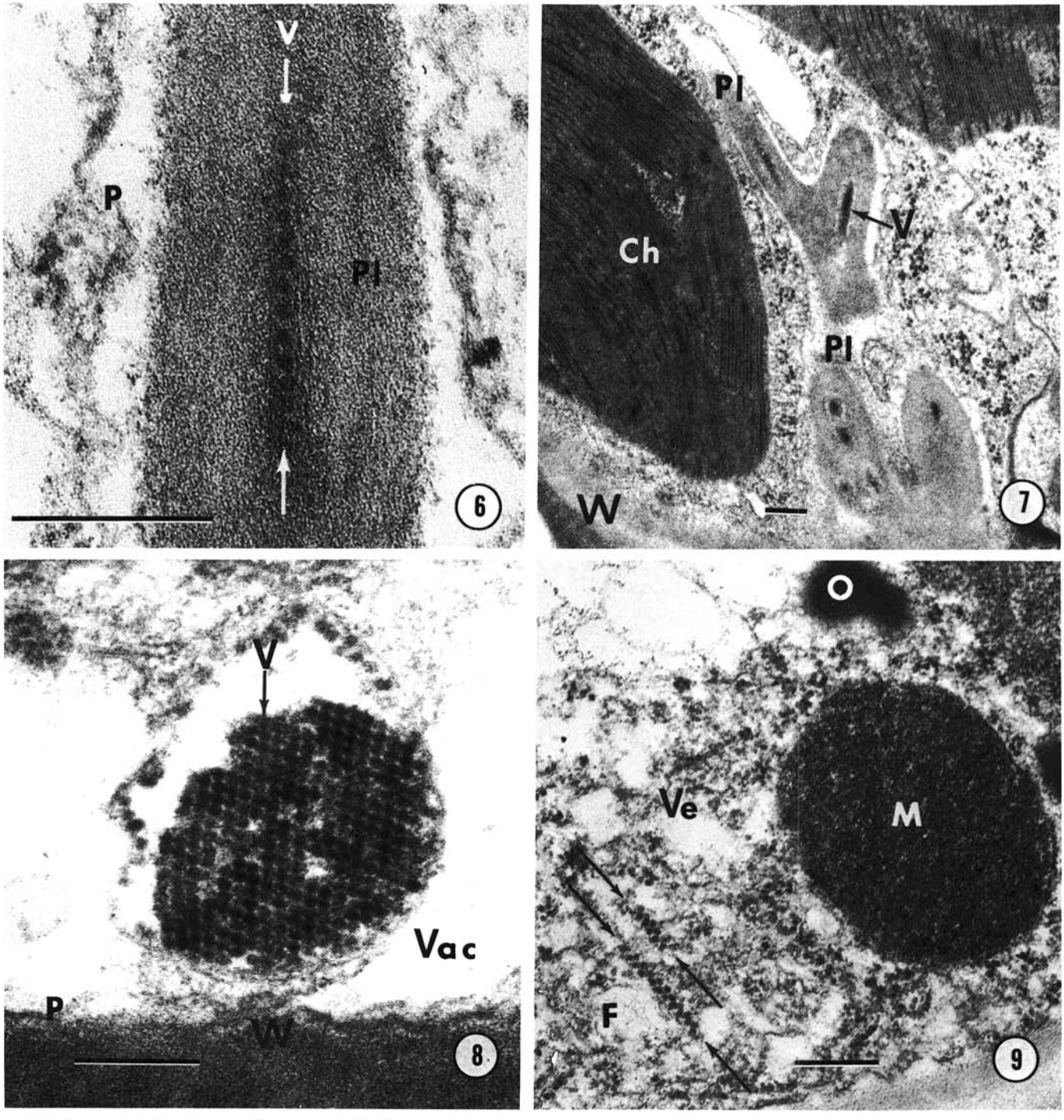


Figs. 1–5. Cherry leaf roll virus (associated with walnut blackline disease) partially purified and in thin tissue sections. **1,** Glutaraldehyde-fixed preparation of isolate CLRV-W 5-48-1 negatively stained with uranyl/acetate (pH 4), showing isometric particles (26 nm). Note the presence of phytoferritin (arrows) (×115,400). Bar = 100 nm. **2,** Leaf tissue of *Cucumis sativus* infected with isolate CLRV-W 5-48-1. Note the virus (V) in tubules (Tu) extending into the vacuole (Vac) (×56,000). **3,** Paracrystalline array of virus (V) particles in the vacuole of a parenchyma cell of infected *C. sativus* L. tissue (×84,000). **4,** Leaf tissue of *Nicotiana tabacum* 'Havana 425' infected with isolate CLRV-W 5-48-1. Note modification of the plasmodesma (Pl) containing a row of virus (V) particles (Vac = vacuole, W = wall) (×91,000). **5,** Replication center in tobacco tissue showing vesicles (Ve) and possibly virus particles or polysomes (×81,200).

intact particles were observed. There was extensive aggregation of viral breakdown products.

In analytical centrifugation the bulk of the virus migrated to the bottom of the cell before the rotor had reached a maximum speed of 26,000 rpm. A series of small peaks with high S values (over 400S) repeatedly appeared. Peaks with S values of 50–60S, 102–116S, and 129–133S were the slowest migrating peaks to separate from the aggregated virus peaks.

Serology. In gel-diffusion tests the walnut virus isolate 5-48-1 reacted positively with antisera prepared against two different isolates of cherry leaf roll virus, namely the golden elderberry strain (CLRV-GE) (10) antisera supplied by R. Stace-Smith, Agriculture Canada, Research Station, Vancouver, B. C., and the walnut strain (CLRV-W 8) (19) antisera supplied by Adib Rowhani, Department of Plant Pathology, University of California, Davis 95616). The walnut isolate 5-48-1 was serologically identical to three different CLRV-W isolates designated 8, 7, and 3, which differed symptomatologically. Isolate 5-48-1 was not serologically identical to the CLRV-GE isolate. The homologous reactants extended (spur formation) beyond the precipitin lines of heterologous reactants. The virus isolate 5-48-1 associated with blackline-affected walnut trees is a strain of CLRV and will hereafter be



Figs. 6–9. Virus-infected leaf tissues of *Juglans regia* English walnut showing leaf symptoms after graft inoculation. **6,** A virus (V) (arrows) containing tubule in a modified plasmodesma (Pl) in a phloem parenchyma cell (P = plasmalemma) (×170,200). **7,** Virus (V) containing tubule in a branched, modified plasmodesma (Pl) of a phloem parenchyma cell (Ch = chloroplast, W = wall) (×41,000). **8,** Small aggregate of virus particles (V) (arrow) in a phloem element (P = plasmalemma, Vac = vacuole, W = wall) (×111,000). **9,** Replication center showing fibril (F) containing vesicles (Ve) adjacent to possible virus particles or polysomes (arrows) (M = microbody, O = osmiophyllic globule) (×53,000). Bar in all figures = 0.2 μm

referred to as CLRV-W 5-48-1.

Virus-cell interaction. In thin-section profiles of herbaceous host leaf tissue, the virus particles of isolate CLRV-W 5-48-1 were isometric and ~22 nm in diameter as determined from center-to-center measurements of linear and paracrystalline arrays (Figs. 2 and 3). Although not unequivocally established to represent infectious virus, viruslike particles observed in sections will be referred to here as virus particles to conform to previous usage (9).

Cell-wall abnormalities were consistently observed within the vascular tissue of systemically infected *N. tabacum* 'Havana 425.' In parenchymatous cells, the plasmodesmata were modified to appear as cell-wall protrusions containing a linear arrangement of virus particles within the desmotubule (Fig. 4). Vesiculate areas within the cytoplasm of infected cells were observed (Fig. 5). The vesicles containing fibrils and the association of virus particles or possible polysomes with these structures resembled the replication complexes described for other viruses (3).

Virus particles were also observed in systemically infected leaf tissue excised from chlorotic ringspots from an Ashley walnut seedling that was graft inoculated with bark patches from a blackline-affected tree. The particles were 22 nm in diameter and identical to those observed in the herbaceous hosts. As in *N. tabacum,* cell-wall abnormalities of parenchymatous cells within the vascular tissue contained a single row of virus particles (Fig. 6). These protrusions were also in the form of branched structures (Fig. 7). In addition, small membrane-delimited paracrystalline arrays of virus particles occurred within the phloem elements (Fig. 8). Cells adjacent to those containing viruses were necrotic. As in herbaceous hosts, particles were difficult to identify with certainty in the cytoplasm of infected cells, but particulate material representing either virus or organized polysomes were observed in the replication complexes (20) (Fig. 9).

DISCUSSION

The mean diameter of CLRV-W 5-48-1 coincides with the size reported for CLRV (13). The $A_{260/280}$ absorption ratios of partially purified virus preparations (1.7–1.8) are close to the values (1.76–1.77) reported for the CLRV strain of golden elderberry (25). The sedimentation coefficients (50–60S, 102–116S, and 129–133S) were within the range of the S values for CLRV strains from golden elderberry (10,13) and walnut (22). The top component (50–60S) of isolate CLRV-W 5-48-1 is presumably phytoferritin, since the latter has an S value of 53–65S (11) and was present in all partially purified virus preparations. Therefore, virus isolate CLRV-W 5-48-1 consists of only two nucleoprotein components similar to CLRV (1,10,13,26). Particles of isolate CLRV-W 5-48-1 broke down during equilibrium sedimentation centrifugation in CsCl as judged by the lack of intact particles and the lack of infectivity of the preparations after overnight dialysis. The density determined for the single peak of isolate CLRV-W 5-48-1 in CsCl differs slightly from that determined by the Italian workers (22), but it is close to the value of 1.46 reported by Jones and Mayo (13) for their "presumed" middle component of CLRV. Except for its extreme instability during extraction, resulting in its anomalous sedimentation behavior, CLRV-W fits the description of CLRV (1) admirably.

Ultrastructural changes induced by the CLRV-W isolate included virus particles embedded in cell-wall protrusions, paracrystalline arrays of virus particles, and replication complexes. The cytological abnormalities are characteristic of cells infected with nepoviruses (5) and also bean pod mottle virus (BPMV), a comovirus, which is beetle transmitted (6,14,15).

The cell-wall protrusions containing virus particles observed in tobacco and Pinto bean (not shown) systemically infected with virus isolate CLRV-W 5-48-1 and leaves from graft-inoculated English walnut seedlings are similar to those induced by cherry leaf roll virus (12). Although no function has been attributed to these structural abnormalities, it is interesting to speculate that they may represent a cellular response for the containment of virus spread from cell to cell. This viewpoint is based on observations that virus particles within cell-wall protrusions are neither in direct contact with, nor (apparently) liberated into, the cytoplasm. The virus particles are enclosed within the modified plasmodesmata, possibly by callose deposition, which may restrict the passage of virus particles from cell to cell. This may not be true since systemic infections are commonly observed in several herbaceous hosts as well as in walnut seedlings. Obviously the infectious entity can spread through plants from the point of inoculation. This report constitutes one of the few cases in which similar cytopathology can be observed in herbaceous hosts as well as in inoculated and subsequently infected trees.

The small aggregates of virus particles observed in the phloem of systemically infected walnut leaf tissue are similar to cowpea mosaic virus (CPMV) crystals in mature sieve tubes of cultivar Cherokee Wax bean leaves (14). It is possible that a direct relationship exists between virus aggregates in cells of the vascular system and virus migration through the phloem (12). Halk and McGuire (9) observed virus aggregates of TRSV in phloem sieve tubes of soybean and proposed that long-distance virus translocation in the phloem system causes systemic infection. The virus aggregates observed in the phloem elements of walnut leaf tissue may also represent the translocation of virus particles resulting in systemic infection. The apparent size discrepancy between embedded and purified virus is not unusual in light of the different staining characteristics of embedded virus as compared with negatively stained virus. Virus particles were not visualized in chronically diseased walnuts that showed no leaf symptoms, mainly because there was no indication of where to sample these trees.

Vascular necrosis as a cytopathological change in blackline-diseased trees was also noted in the vascular tissue of infected leaves in which virus was visualized. It follows that the vascular necrosis at the graft union could be caused by this virus. Attempts to complete Koch's postulates to unequivocally establish the causal relationship of CLRV-W and the blackline disorder of English walnuts on black walnut rootstock are presently underway.

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