# Electron Microscopy of Viruslike Particles From Shoestring-Diseased Highbush Blueberry, Vaccinium corymbosum L.

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ABSTRACT

Viruslike particles measuring 26-28 nm in diam were found in ultrathin sections of leaf and root tissues from highbush blueberry affected by shoestring disease. Leaf epidermal, palisade, and spongy mesophyll cells contained characteristic particles. Particles were found in xylem but not in phloem vascular tissue. Epidermal leaf cells and root xylem cells contained crystalline arrays of particles. Larger masses of viruslike particles were seen in root than in leaf cells. Particles hexagonal in outline and 28-31 nm in diam were partially purified from diseased leaves. No such particles were either observed in, or isolated from, healthy blueberry tissue. Observation of viruslike particles in situ and their isolation from leaves exhibiting typical shoestring disease symptoms is presumptive evidence that they are the incitant of blueberry shoestring disease.

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Blueberry plants with shoestring disease exhibit red streaks and vein banding on affected young shoots and leaves, respectively. Streaks on the shoots often become masked as the wood matures, but leaves remain narrow or straplike, with curled edges. The disease can be graft-transmitted (14), but the natural method of transmission and nature of the causal agent is unknown. Although we have successfully transmitted the disease between blueberry plants by grafting, repeated attempts at sap, dodder, and graft transmission to herbaceous hosts have failed. The objective of this study was to determine the presence and distribution of viruslike particles (VLP) in shoestring-diseased tissues. This report also concerns preliminary results on the purification and electron microscopy of VLP from diseased tissues.

MATERIALS AND METHODS.—*Source materials.*—Plants of highbush blueberry, *Vaccinium corymbosum* L. 'Jersey', exhibiting characteristic symptoms of shoestring disease were field-collected and grown in a greenhouse or outdoors in a peat-soil mixture.

Plants free from shoestring infection were obtained by rooting softwood cuttings in sterilized sphagnum peat. Cuttings were made from Jersey plants grown in fields where plants with disease symptoms were systematically rogued.

*Ultrathin sections.*—Strips of leaves and smooth bark and thin slices of small roots from diseased and healthy blueberry plants were fixed for 3 hr in cold phosphate-buffered (0.1 M, pH 7.2) 5% glutaraldehyde solution, rinsed in buffer, fixed with a 2% solution (w/v) of osmium tetroxide in the same buffer, and dehydrated through a graded ethanol series. Samples were kept in 100% ethanol for at least 48 hr with two changes to assure complete dehydration. The specimens were then infiltrated with epoxy resins according to Spurr (11). Spurr's standard procedure using formula A was modified by extending the infiltration over a period of 5 days. Ultrathin sections were stained with uranyl acetate in a methanol-ethanol (1:1) solution for 30 min, followed by an alkaline solution of lead citrate for 5-10 min. Specimens were examined with a Philips Model 300 transmission electron microscope.

*Purification of viruslike particles.*—Young leaves with severe symptoms of shoestring disease were frozen overnight, then homogenized for 3 min in two vol of cold extraction solution (see RESULTS for details) with a Waring Blendor. Root pieces were rinsed in water, blotted dry, ground in liquid nitrogen with a mortar and pestle, then homogenized in a Waring Blendor with two vol of extraction solution for 8-10 min. The homogenates from each of the above were squeezed through four layers of

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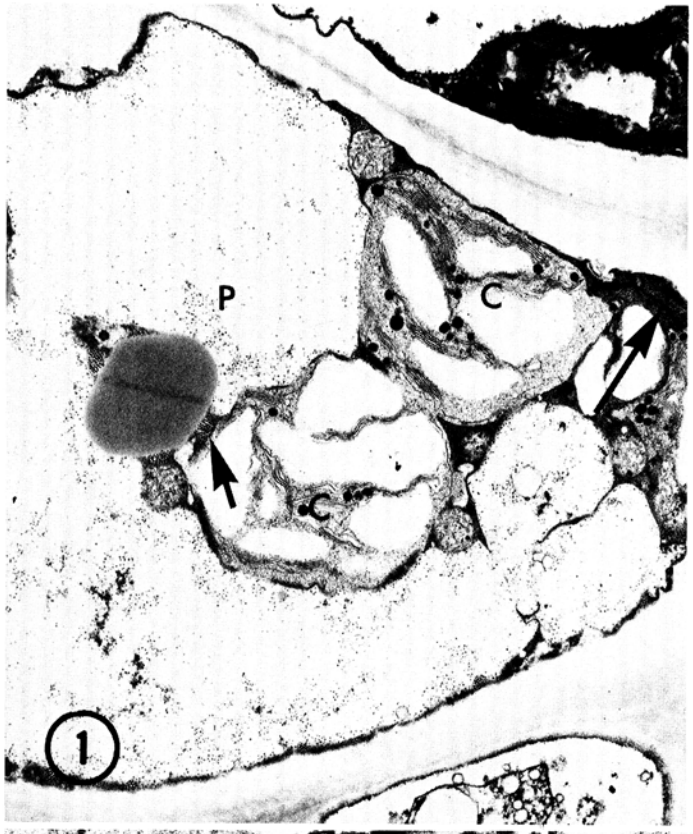
**Fig. 1-4. 1)** Epidermal cell from blueberry leaf affected by shoestring disease. Viruslike particles are present as scattered particles and as small crystalline aggregations (arrows) in the vacuole and cytoplasm. Chloroplasts are starch-filled and partially degenerated. P = viruslike particles; C = chloroplasts (× 14,000). **2)** Portion of cytoplasm of a palisade parenchyma cell in an affected leaf. Viruslike particles occur enclosed in small vesicles and scattered throughout the cytoplasm. P = viruslike particles; VE = vesicles (× 24,000). **3)** Viruslike particles associated with chloroplasts in affected palisade parenchyma cell. Note particles adhering the outer chloroplast membranes. Cross-sectioned tubules are present in the cytoplasm between chloroplasts. P = viruslike particles; T = tubules (× 90,000). **4)** Longitudinally sectioned tubules in palisade cell of affected leaf (× 72,000).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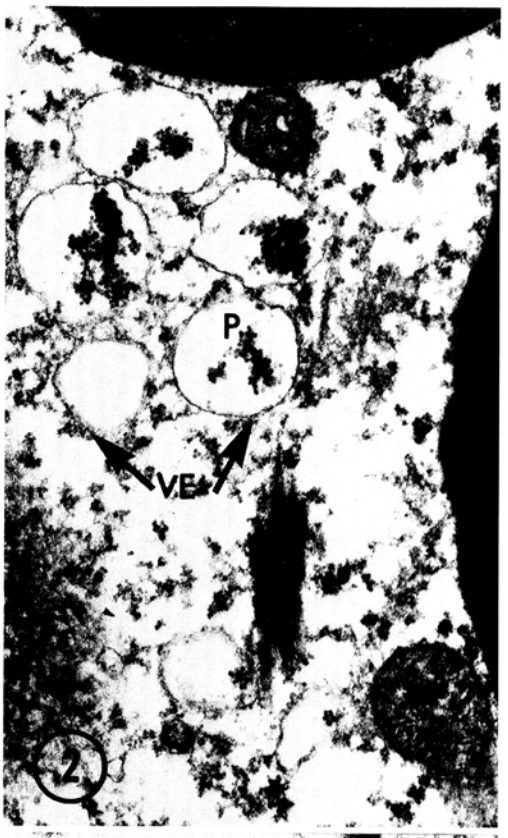
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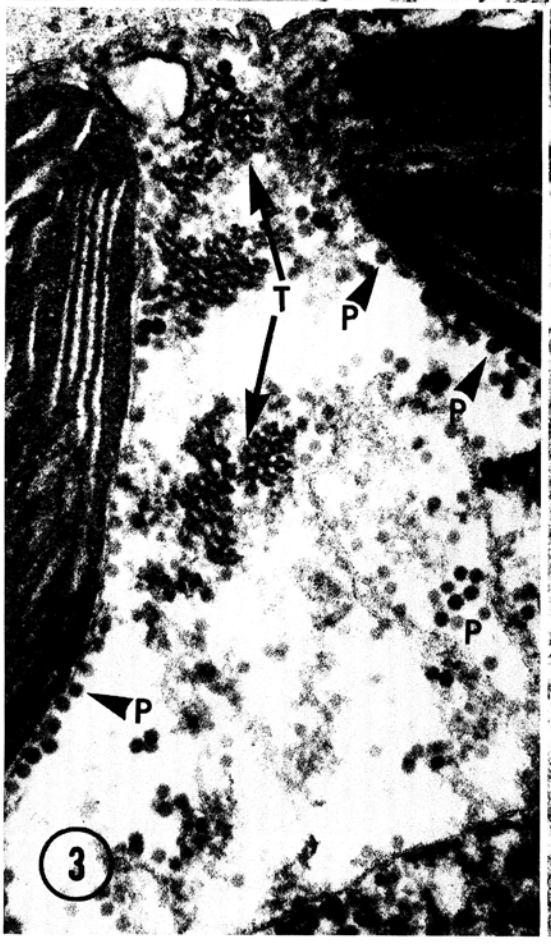
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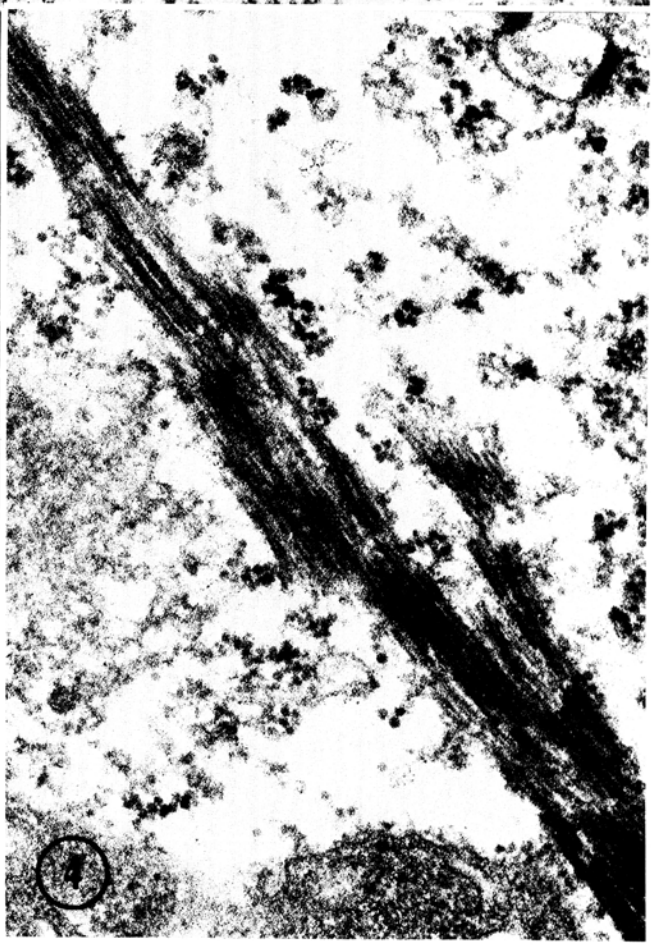
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cheesecloth, and the resultant filtrate was centrifuged for 20 min at 10,300 *g* and 4 C. The clarified supernatant fluid was subsequently centrifuged at 89,000 *g* for 2.5 hr in a Spinco Model L2 ultracentrifuge. The resultant pellets were dispersed in 0.1 M, pH 6.8, phosphate buffer at the rate of 1.0 ml/30-50 g of initial leaf or root material. Samples from these solutions were placed on Formvar-coated grids and negatively stained with a 2% aqueous solution of sodium phosphotungstic acid neutralized with 1 N KOH.

RESULTS.—*Appearance and distribution of viruslike particles in plant tissues.*—1) *Leaf interveinal tissue.*—Epidermal cells from diseased leaves contained an abundance of 26- to 28-nm viruslike particles (VLP). Small crystalline arrays and numerous unordered particles were evident (Fig. 1). Of the leaf cells examined, epidermal cells appeared most severely affected by the disease. Chloroplasts containing abnormally high accumulations of starch and exhibiting degeneration of the cytoplasm and necrosis of some epidermal cells were found.

In palisade parenchyma cells, VLP commonly were found in small (6- to 8-$\mu$m) vacuoles, which were more numerous in infected than in uninfected palisade cells (Fig. 2). The central vacuole of these cells was filled with material that appeared to be tannin. Many loose particles in the cytoplasm were associated with fine fibrillar material. The chloroplasts of palisade cells appeared normal, but VLP were often observed in contact with the outer chloroplast membrane (Fig. 3).

Long tubules or aggregates of rodlike material were observed in diseased palisade cells (Fig. 4). They were present in both nuclei and the cytoplasm, and measured up to 2,600 nm in length. Their exact length could not be determined in thin-sections. In cross section (Fig. 3), the measured 25-27 nm and contained a small 2- to 5-nm electron-transparent core. The tubules were somewhat angular in cross section. These tubules were not observed in healthy palisade cells or in spongy mesophyll of healthy or diseased tissues, although VLP were occasionally observed in the latter.

2) *Leaf vascular tissue.*—Young leaf veins typically contained VLP only in a few xylem parenchyma cells (Fig. 5). Usually the particles were confined to small clear areas at the periphery of the cells (Fig. 5, inset). Amorphous, electron-dense material presumed to be tannins was a constant feature of these cells. Control cells of the same type contained the electron-dense material, but no VLP. No VLP were detected in phloem tissue or mature xylem conductive cells.

3) *Root tissues.*—Samples of root tissue were taken from plants with leaf and bark symptoms but with no macroscopic symptoms of root infection. VLP were present in varying amounts in nearly all cytoplasm-containing cells of the xylem tissue examined (Fig. 6). The percentage of root xylem cells containing particles appeared much greater than that of leaf xylem cells. Furthermore, individual cells contained particles in large masses (Fig. 7) not found in leaf cells.

Small clear areas at the cell periphery, which contained loosely arranged particles, were very similar to those observed in leaf xylem cells. In other root xylem cells there were numerous small vacuoles containing loose arrays of VLP. The latter were similar to those observed in leaf palisade cells (Fig. 2). VLP have not been found in root cell types other than xylem. Plasmodesmata did not contain VLP, although particles were found in close proximity to connections of sufficient diameter to accommodate them.

*Partial purification of viruslike particles.*—Attempts at purification on using either chloroform or chloroform-butanol extraction methods (12) were unsuccessful. Homogenization of blueberry leaf tissue in 0.1 M phosphate or Tris[tris(hydroxymethyl) amino methane]-HCl buffers resulted in a marked drop in pH of these buffers, and the homogenate became brown in color. We found that potassium phosphate buffer (0.1 M, pH 6.8) with 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.02 M 2-mercaptoethanol, and enough nicotine alkaloid (Eastman Kodak 95%) to raise the pH to 6.5 after grinding (usually 1 ml/100 ml of buffer) prevented both rapid browning and lowering of the pH in the homogenate. This "extraction solution" was successfully used in partial purification of VLP from leaf tissue.

Negatively stained particles from partially purified leaf preparations measured 28-31 nm in contrast to the 26- to 28-nm particles in thin-sections (Fig. 8). Reasons for the size difference between negatively stained and thin-sectioned viruslike particles have been enumerated (4). Although particles round to hexagonal in outline were seen in both sectioned and partially purified preparations, the polyhedral nature was more evident in the latter.

Partially purified material was inoculated onto Carborundum-dusted leaves and roots on numerous plants from fifteen different genera, and also onto Jersey blueberry seedlings. After inoculations with six partially purified preparations and a 1- to 2-month incubation period, no symptoms of virus infection were detected.

DISCUSSION.—There have been no previous

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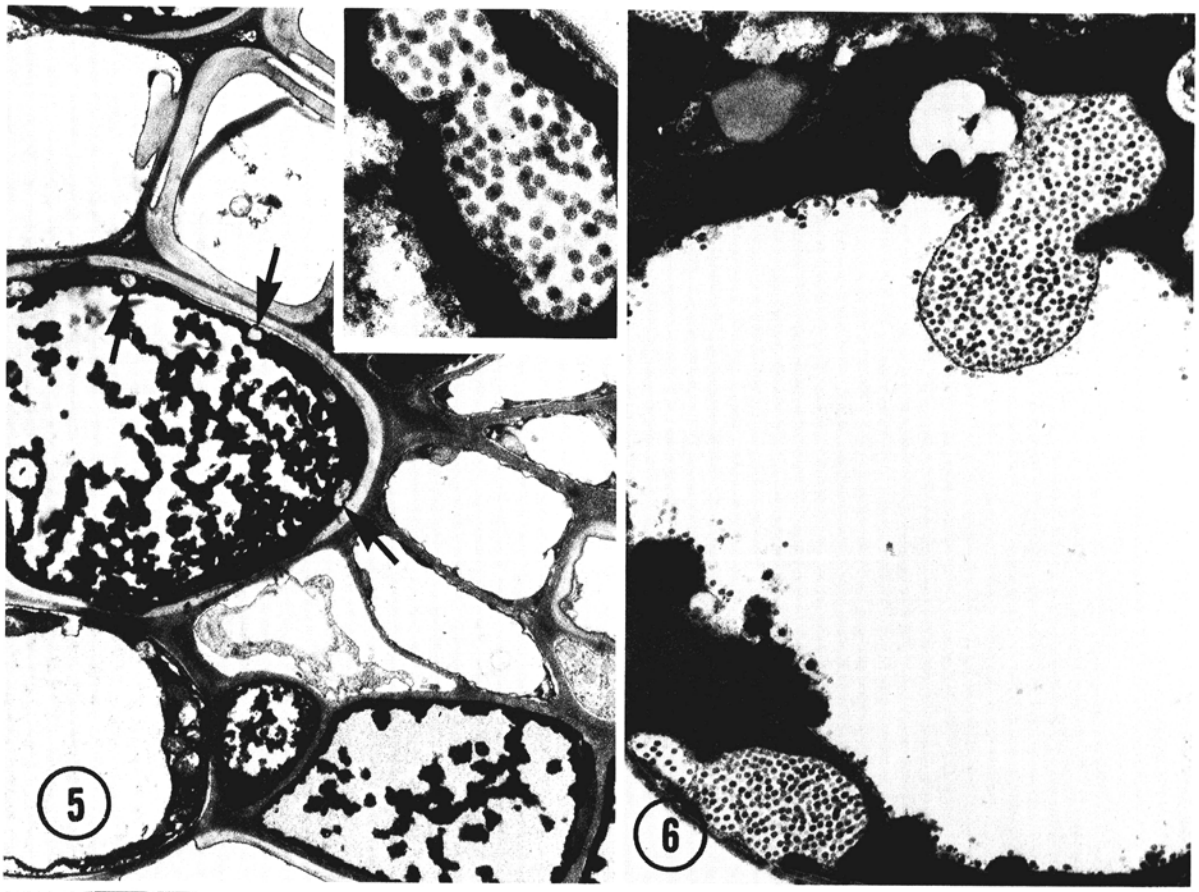
Fig. 5-8. 5) Portion of xylem in cross-sectional leaf vein from affected plant. Electron-dense material in larger parenchyma cells is presumed to be tannin. Viruslike particles occur in small, peripheral regions (arrows and insert) (× 5,500 and 80,000). 6) Portion of xylem parenchyma from roots of an affected plant. Viruslike particles are present in central vacuole and in peripheral electron transparent areas. Occasional small crystalline aggregates of VLP occur (upper left corner) (× 42,000). 7) Xylem fiber cell with large mass of viruslike particles (× 25,000). 8) Negatively stained viruslike particles partially purified from shoestring-affected blueberry leaves. Note angular nature of particles (× 200,000).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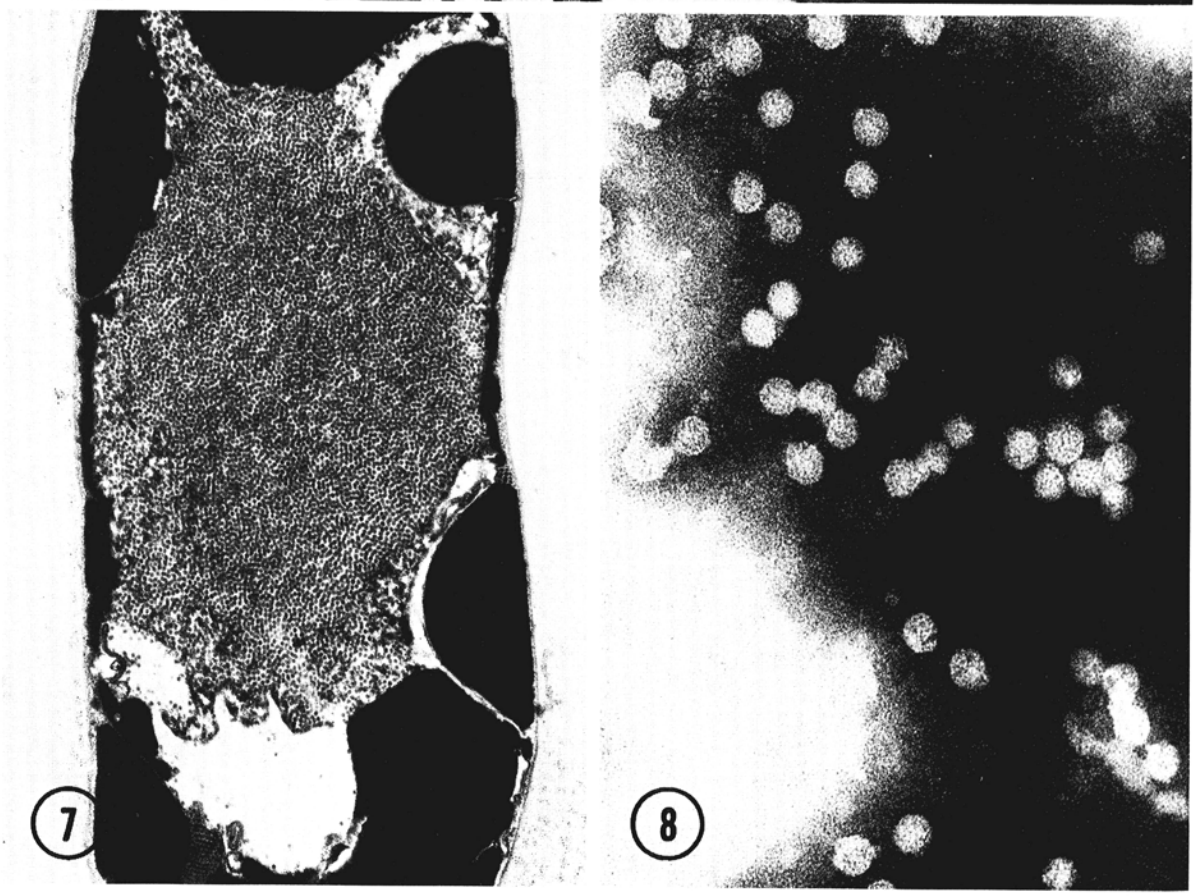
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reports of viruslike particles in association with blueberry shoestring disease, although a virus, tobacco ringspot (with dimensions similar to those of the shoestring VLP), has been associated with blueberry necrotic ringspot disease (7). Neither crude leaf sap nor partially purified leaf preparations from shoestring-affected plants reacted with tobacco ringspot or tomato ringspot antisera.

The lack of infectivity of isolated particles does not preclude them as incitants of shoestring. The virus may not be mechanically transmissible, and may be vector-dependent. Furthermore, tannins present in woody perennials are known to inhibit or inactivate viruses (2).

The VLP observed in infected tissues were distinguishable from other host cell components such as ribosomes, according to criteria enumerated by Jensen (6). Their ordered arrangement into crystals (Fig. 1, 6) is strong presumptive evidence of their viral nature (15). The distinct polyhedral nature of the particles extracted from shoestring-diseased tissue (Fig. 8) is characteristic of the icosahedral symmetry of viruses (3), and the size of isolated particles was in close agreement to particles in situ. No other type of VLP was detected in shoestring-diseased material or in control tissues.

Leaf palisade cells afforded the best observations on various structures associated with shoestring disease (Fig. 2, 3, 4). The relationship of the VLP to the numerous vesicles in affected cells (Fig. 2) is uncertain at this time. An increase in the number of cytoplasmic vacuoles in cowpea mesophyll cells infected with southern bean mosaic virus has been reported (15). Russo et al. (10) indicated that localization of artichoke mottled crinkle virus in small vacuoles came about when virus moved from the cytoplasm into the central vacuole, and that the membrane of the smaller vacuoles was derived from the tonoplast. However, in shoestring-affected cells, the vacuoles were present in the cytoplasm and did not appear to arise from the central vacuole as it was uniformly filled with tannins.

The long tubules associated with VLP in palisade cell cytoplasm and nuclei (Fig. 4) closely resemble the X-components associated with tobacco mosaic virus infections (1). Similar virus-associated tubules have been reported for beet mosaic (8) and alfalfa mosaic (5) viruses. Although the origin and function of these tubules are unknown, they appear to be limited to infested leaf palisade cells.

The finding of VLP in xylem but not in phloem tissue is similar to the distribution of virions observed in artichoke mottle crinkle disease (9). The large masses of VLP in root xylem cells (Fig. 7) and the presence of virions in a high percentage of cells examined indicates a systemic root infection. The scarcity of infected cells in leaf xylem tissue (Fig. 5) in comparison to root xylem may indicate a higher concentration of VLP in root tissues. Tomlinson (13) found blueberry necrotic ringspot virus more concentrated in extracts from cucumber roots than from tops. The presence of large amounts of VLP in root tissue, and the less acidic nature of root tissue (2), suggest its use in future transmission attempts.

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