Flexuous Rods and Vesicles in Leaf and Petiole Phloem of Little-Cherry Diseased Prunus spp.

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ABSTRACT

Two abnormal structures in phloem parenchyma and companion cells of leaf midribs and petioles were consistently correlated with little-cherry disease of sweet cherry and oriental flowering cherry. These structures were elongated, flexuous rods (about 12.0-12.5 nm in diameter, usually arranged in large aggregates) and small vesicles (about 75 nm in diameter) mostly spherical or ellipsoid, bounded by a double membrane, and containing fibrous strands radiating from an electron-dense center. The vesicles were often intermingled with the flexuous rods, and were characteristically attached to the inner surfaces of membranes which lined large vacuoles in the cytoplasm.

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Little-cherry (LC) disease of sweet cherry (*Prunus avium* L.), which was first recognized in 1933 in the Kootenay Lake district of British Columbia, Canada (2), was responsible for the virtual destruction of the sweet cherry industry in southeast British Columbia within 15 years. The disease manifests symptoms only in the fruit, which are about one-half the size of unaffected fruits, are angular and often pyramidal in shape, and lack the sweetness and flavor of normal, ripe cherries. Since 1947, when Foster and Lott (7) transmitted the disease by bud-grafting to healthy trees, it has been considered a virus disease. There are no known herbaceous hosts, and no known efficient vectors. Wilde (18) reported a low incidence of transmission with leafhoppers, but we believe that this failed to account for the rapid spread of LC.

In 1969, the disease appeared in the Okanagan Valley of B.C., 195 km to the west of the Kootenay district, and subsequent surveys have traced its spread to several areas within the valley, with incidence higher in some years than in others, but of sufficient importance to stimulate concern and further research on the disease. Electron-microscopic examination of leaf and fruit tissues has demonstrated the presence of a number of ultrastructural abnormalities associated with the disease, but only two of these have been consistently present in all diseased-tree sources. These two structures are briefly described here.

MATERIALS AND METHODS.—Samples were taken from midribs of leaves, from leaf petioles, and from fruit stems, of LC-diseased sweet cherry trees in four locations of the Kootenay district; others were from LC-infected orchards in the Okanagan Valley; still others from LC-diseased buds from Kootenay, grafted to healthy trees and maintained in Vancouver; and from oriental flowering cherry, *P. serrulata* Lindl. 'Shirofugen' and 'Kwanzan', both of which are known reservoirs of LC (15, 19). Surveys have shown Shirofugen in B.C. to be 100% infected with LC (A. J. Hansen, *personal communication*). The disease in the sweet cherry was diagnosed through fruit symptoms, and in most cases also by indexing on the indicator cultivar, Sam (20). Healthy controls were trees at the Research Station, Summerland, B.C., and at the Post-Entry Quarantine Station, Sidney, B.C., most of which originated from the Irrigation Experiment Station, Prosser, Wash., and were regularly indexed for, and found to be free of, LC as well as of other known stone-fruit viruses.

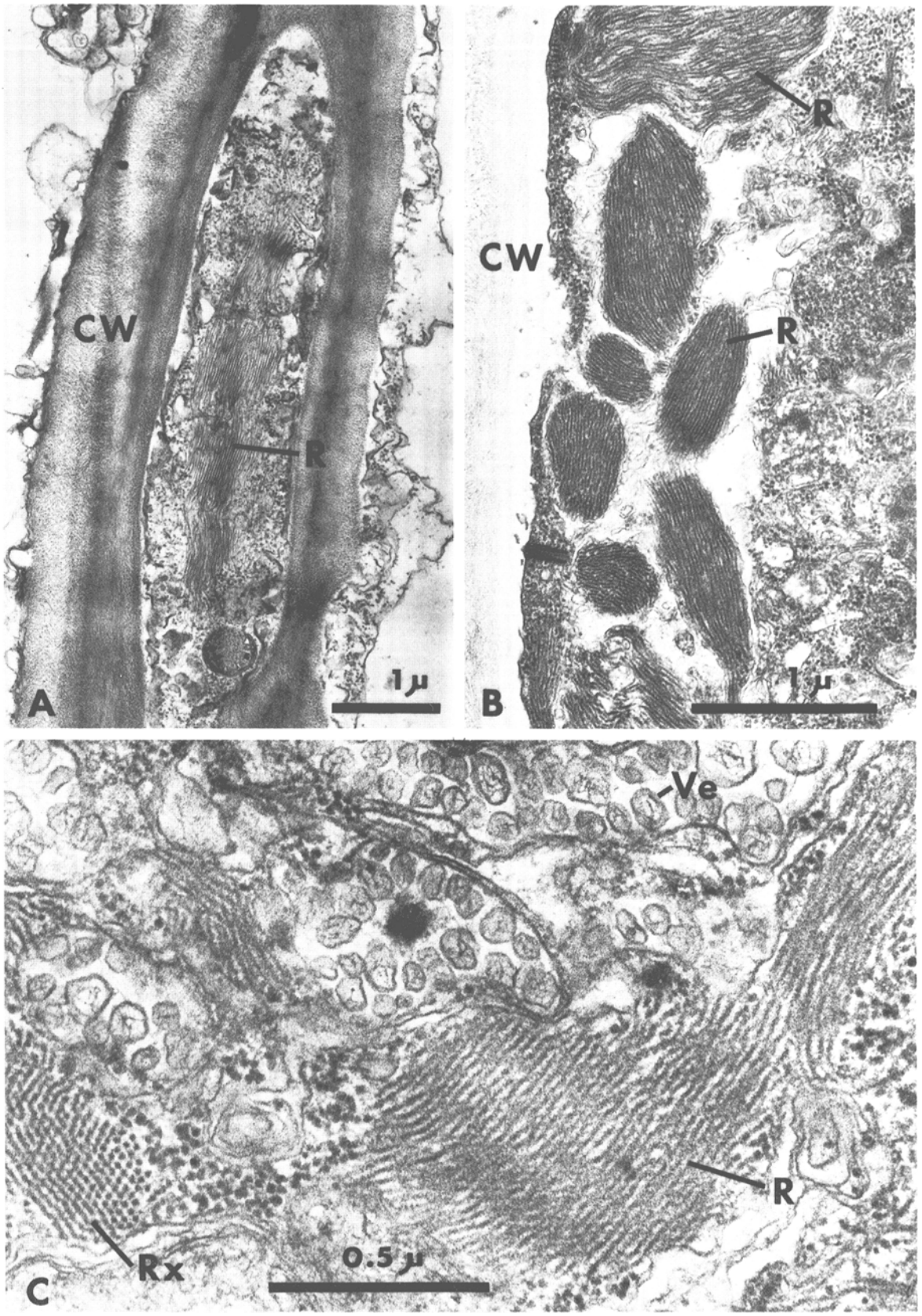
The samples, 2-3 mm^2 were fixed in 5% glutaraldehyde in phosphate buffer, pH 7.2, for 90 minutes, and rinsed with distilled water once before, and three times after, postfixation for 1 hour with Palade's OsO_4 solution, pH 7.2. After dehydration in graded dilutions of ethanol, they were treated with propylene oxide, and embedded in Epon 812. The ultrathin sections were stained with uranyl acetate and lead citrate, and electron micrographs were made with a Philips EM 200 or EM 300 electron microscope.

RESULTS.—In the phloem parenchyma and companion cells, we observed elongated, flexuous rods, sometimes singly and scattered in the cytoplasm and in vacuoles, but more often in large parallel aggregates. These sometimes extended as a single body through the cell [Fig. 1-A, (R)], but more often were found in a number of discrete groups (Fig. 1-B). The aggregates were only occasionally surrounded by a membrane, and they often appeared to be situated within a clear space or vacuole in the cytoplasm. The lengths of the individual rods were difficult to determine, although occasionally end-to-end groupings of the rods with sharp edges indicated that they were about 900 nm long. Their diameter was 12-13 nm, as measured both in longitudinal and in cross-section, and at higher magnifications [Fig. 1-C, (RX)] showed them to be rods, rather than tubules, often fibrillar in nature.

In every cell where rods were present, there were also vesicles in various arrangements. The most common form consisted of large, circular vacuoles [Fig. 2-A, (V)] bounded by a membrane about 8.2 nm thick. We are uncertain whether this membrane is a single-layer type, or a double-layer type with closely apposed laminations. The vesicles were attached to the inner side of the membrane and protruded into the vacuolar space [Fig. 2-A, (Ve)]. The vesicles were not always in a single row, but were often densely crowded, so that the vacuole appeared to contain clusters of vesicles [Fig. 2-B, (Ve)]. Another common arrangement [Fig. 2-A, B, (arrows)] was the reverse, in which the vesicles appeared to be attached to the outer side of the membrane of small vacuoles, while the interior of that vacuole was filled with flexuous rods. Since in this arrangement the smaller vacuoles were always inside a larger vacuole, it is probable that the smaller vacuoles were actually involutions of the membrane of the larger vacuole, and that in fact the vesicles were still attached to the inner side of the membrane of the larger vacuole. Frequently, the entire lumen of the cell was filled with masses of vesicles, and their attachment to membranes was difficult to detect (Fig. 2-B). In many cells, the elongated flexuous rods were closely intermingled with the vesicles (Fig. 1-C).

The vesicles (Fig. 2-C) varied in diameter from about 50-80 nm, with a few up to 100 nm, and a mean of about 75 nm. In serial sections, they were roughly spherical or slightly ellipsoid, and were bounded by double membranes, each of which was about 3.5-3.7 nm thick. The vesicles were separated by an electron-translucent space of about 2 nm, so that the total thickness of the double membrane exceeded 9 nm. Inside the vesicles there were electron-dense filamentous strands usually radiating from a central electron-dense spot of varying size (Fig. 2-C). These strands resemble DNA strands in plastids and mitochondria. Occasionally, the strands were not present, and the central spot was considerably enlarged. Aside from these two internal structures, the rest of the vesicle appeared uniformly structureless or slightly granular.

Fig. 1-(A to C). Sections of phloem parenchyma cells in leaf midribs from little cherry-diseased trees. CW = cell wall; R = elongated, flexuous rods; RX = rods in cross-section; Ve = vesicles. **A)** × 17,794, tip of cell showing an aggregate of rods, from a sweet cherry tree in the Okanagan Valley of British Columbia. **B)** × 30,380, groups of aggregates of rods, from Vancouver tree budded with LC-infected Kootenay sweet cherry source. **C)** × 72,695, enlarged view of aggregated rods in cross-section, and fibrillar appearance in longitudinal section. Note close association of rods and vesicles.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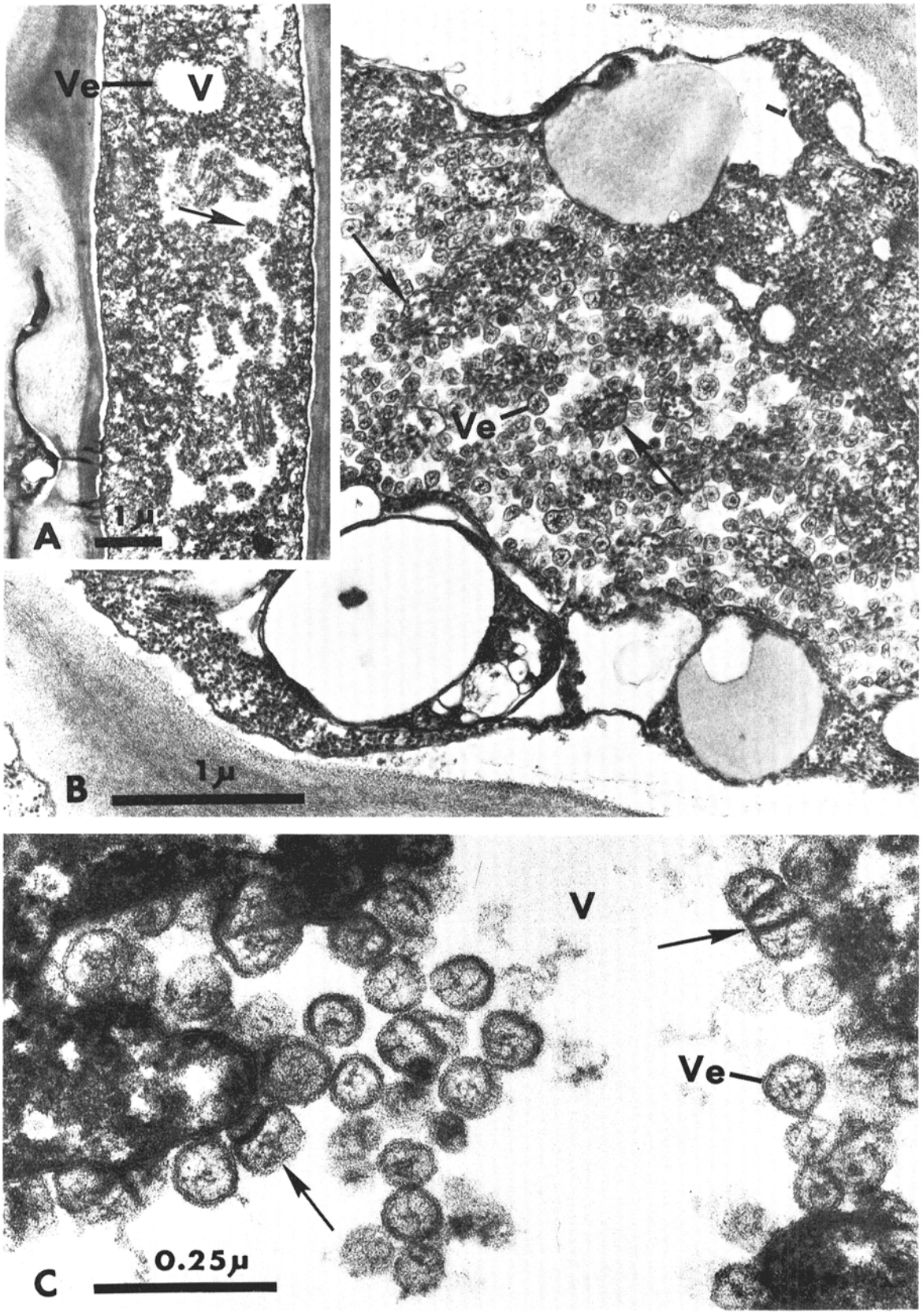
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Fig. 2-(A to C). Sections of phloem parenchyma cells in leaf midribs from LC-diseased trees. CW = cell wall; V = vacuole; Ve = vesicles. **A)** × 10,850, vesicles arranged within the vacuoles, the membrane of which may be convoluted (arrow), so that vesicles appear to be attached to the outer side rather than the inner side of the vacuolar membrane. **B)** × 31,465, vesicles filling the greater part of the cell lumen, and attachment of vesicles to vacuolar membrane seen only where the latter is convoluted (arrows). Note rods in the interior of these vacuolar convolutions. **C)** × 120,435, enlargement of vesicles protruding into vacuole. Note double membrane around vesicles, electron-dense fibers radiating from dense central area, and diad and triad of vesicles (arrows) seen only in Shiro-fugen flowering cherry.

In Shiro-fugen cells only, we found vesicles in arrangements of diads and triads [Fig. 2-C, (arrows)] that suggested either a close compression of the vesicles, or a division process. These have not yet been found in the LC-infected sweet cherry material.

These two abnormal structures, flexuous rods and vesicles, were consistently found in phloem parenchyma and companion cells of all sources of LC-infected trees where diagnosis had been made by both fruit symptoms and indexing on the Sam cultivar. These included 22 sweet cherry trees (eight from the Vancouver material and 14 from the field), six Shiro-fugen, and three Kwanzan trees. However, neither rods nor vesicles could be found in several sweet cherry trees from the Kootenays or in trees from the Okanagan Valley which had been diagnosed, with some uncertainty, by field symptoms only. One of the sweet cherry trees from the Okanagan Valley, tentatively diagnosed in the field as LC-infected, contained only mycoplasma in the sieve tubes, and was probably infected with Western-X disease, the fruit symptoms of which can be mistaken for LC. Of the 18 healthy indexed trees examined, all were free of both flexuous rods and vesicles. However, one tree from the Okanagan Valley that showed no LC fruit symptoms, but was completely surrounded by LC-infected trees, contained both types of ultrastructures, and was probably in an early stage of infection.

DISCUSSION.—The identity of the rods and vesicles is not yet known. There are reports of several virus diseases of plants in which there is an association of elongated, flexuous rods with vesicular bodies, including beet yellows virus (1, 3), beet yellow stunt virus (11), beet mosaic virus (10), an unknown virus of *Dianthus caryophyllus* (17), and carnation necrotic fleck virus (12). In all these cases, the elongated rods were either virus, or it was assumed (17) they were virus. The vesicles in each of these reports appear to be bounded by a single membrane, and contain electron-dense filaments radiating from a dense central area or spot. Occasionally they are found clustered in larger vacuoles, and there is some speculation (3, 13) that the vesicles may be sites of viral synthesis. Carrot mottle virus is associated with vesicle-like bodies, occurring in small numbers, and budding off the tonoplast. These vesicles are similar in structure to LC-associated vesicles, but in this case no rods or other virus-like structures have been found (14).

In plants, only the vesicles associated with carnation necrotic fleck virus (12) show similarity to the very precise arrangement of vesicles within a vacuole that is so characteristic of LC-infected tissues (Fig. 2-A). In other associations, the vesicles tend to be scattered throughout the cytoplasm. Indeed, the closest resemblance to the LC-associated vesicles is seen in connection with group-A arboviruses which induce structures, referred to as cytopathic vacuoles, 1-5 μm in diameter, and lined with membranous spherules about 50 nm in diameter (9). It has been suggested (8) that these spherules serve as sites for viral RNA synthesis.

It would be simple to assume that the elongated rods in LC-infected cells are virus particles, especially since their diameter of 12.0-12.5 nm is close to that of many known elongated flexuous viruses. However, several factors make it premature to assign this identity. We have not yet succeeded in isolating these particles from the host tissues, even by means that are commonly successful with other viruses in the same location, such as leaf-dip, and leaf-midrib, or petiole-exudate methods. Secondly, close examination of many of the rods in cross-section has failed to show any indication of a translucent central core, such as can often be detected with other flexuous viruses (4, 21).

Furthermore, the location and form in which the rods are aggregated often resembles aggregates of P-protein in later stages of development, when the individual components are fibrillar and may be from 6.0 nm to 15.0 nm in diameter (5, 6). The LC aggregates of flexuous rods also frequently show a swirling arrangement such as has been observed with P-protein aggregates (16).

We have considered, and are still pursuing, the possibility that the elongated rods are chlorotic leaf spot virus (CLSV), which is often present in sweet cherry. However, examination of phloem tissues of trees infected with CLSV alone has failed to disclose the presence of similar elongated, flexuous rods or vesicles. Moreover, Shiro-fugen, which contains both flexuous rods and vesicles, is not known to be a host of CLSV (A. J. Hansen, and M. F. Welsh, *personal communication*). Thus, the possibility still exists that the elongated, flexuous rods associated with LC may be either virus particles, or they may be aggregates of P-protein in a form and in quantities that are not found in healthy control tissues. Whatever their identity, and because no other disease of cherry has been shown to induce them, their presence together with the vesicles, appears to be a diagnostic characteristic of LC disease.

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