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Electron microscopical studies on the leafroll- and corky bark-affected grapevines

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Abstract. Young shoots and leaf veins of leafroll- and corky bark-affected grapevines were examined by electron microscopy. The diseases in vines were verified by graft-indexing tests on healthy indicator plants at Davis, California. Virus particles were consistently observed in the phloem cells of either corky bark- or leafroll-affected Cabernet Franc by the electron microscope. These particles were elongated, flexuous rods, typical of closterovirus morphology, confined in the phloem tissue and they formed typical virus aggregates. Virus-like particles could be detected from both cytoplasm and nucleus of infected cells. Virus concentration was much higher in leaf vein than in shoot tissues of the vines with severe symptoms. Ultrastructural details indicated that the malformation and disorganization of mitochondria, chloroplasts and endoplasmic reticulum; as well as the accumulation of starch granules, tannic material, osmophilic globules and characteristic virus-associated small vesicles were consistently associated with symptom development. The diameter of virus particles in corky bark -affected tissues was about 7-8 nm, while that in leafroll-affected tissues was slightly larger about 10-11 nm. The virus particle aggregate in leafroll-affected cell was sometimes enclosed by an outer membrane which was not found in corky bark-affected cells.

Key words: Closterovirus; Corky bark; Electron microscopy; Grapevine; Leaf roll.

Introduction

Leafroll and corky bark are both worldwide virus -like diseases of grapevine. The effects of these diseases on grapevine include reduction of vine growth, loss of yield both qualitatively (less sugar content and flavor) and quantitatively, and even death of plants (Beukman and Gifford, 1969; Lehoczky, 1972; Lider *et al.*, 1975). Certification of nursery stock, and roguing of diseased plants and replanting are the principle control measures for these diseases. The diseases are mainly transmitted by grafting, although there were some evidences that mealybugs might vector the transmission of leafroll of grapevines (Engelbrecht and Kasdorf, 1985; Rosciglione and Castellano, 1985). The two diseases are identified mainly by indexing tests on the indicators, St. George (*Vitis rupestris*), LN-33 (Couderc 1613 × Thompson Seedless), and Cabernet Franc or Mission (*Vitis vinifera*). As shown in Table 1, two distinct symptom patterns can be distinguished by disease reaction on these indicators (Bovey *et al.*, 1980).

Leafroll disease has symptoms primarily expressed in leaf and fruit tissues. Cane symptoms other than reduced growth are not observed. On Mission, Caber-

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net Franc, Zinfandel, Carignane and LN-33, it causes distinct color changes of leaves and/or fruits. On Baco 22, vine dwarfing and leaf epinasty symptoms can be detected (Alley *et al.*, 1963; Hoefert and Gifford, 1967). The disease was first reported by Fabre in France in 1853. In early literature, the disease was also called "rougeau", "flavescence" (Vidal, 1943), or "busissure" (Cook and Goheen, 1961). By budding and grafting, Scheu first proved the transmissible nature of this disease in Germany in 1936. Castellano, *et al.* later (1983 and 1985) found the presence of both isometric and long flexuous virus particles in leafroll infected grapevines. Whereas Namba *et al.* (1979) in Japan and Faoro *et al.* (1981) in North Italy demonstrated that a closterovirus with particle size at approximately 9 × 1000 nm was associated with leafroll disease of grapevine. Mechanical transmission of the virus to herbaceous plant hosts has never been succeeded. Zee *et al.* (1987) and Iwanami *et al.* (1987) were recently able to purify the closterovirus directly from certain grapevine varieties. Whether or not there are still some other viral associations are still under investigation.

The effect of corky bark on grapevine was quite similar to that of leafroll infection. On Mondeuse, Cabernet Franc, Gamay and Petite Sirah grapevines, the disease causes color changes of leaves and/or fruit, and leaf epinasty similar to symptoms of leafroll -affected grapevines. The leaves on diseased vines usually do not abscise normally but instead, they remain on the cane long after the end of growing season. Other than leaf symptoms, corky bark also cause roughness of bark and wood-splitting on old canes and trunks of Niagara and St. George. Cane cracking, dwarf growth or even death of plants are often observed on the hybrid LN-33 (Beukman and Gifford, 1969; Beukman and Goheen, 1965). The disease was first described by Hewitt under the name of "rough bark" in California in 1954. The same disease was also described under the name "legno riccio", "rugose wood", "wood pitting", or "stem pitting" by other researchers (Graniti, 1964; Graniti and Ciccarone, 1961). Transmission of this disease by grafting was demonstrated by Graniti and Martelli in 1965. The viral nature of corky bark was since accepted by most subsequent workers. However, unlike that of leafroll disease, the exact viral association of this disease was never been successfully proved. Belli *et al.* (1980) found an isometric virus associated with corky bark disease in grapevine. However, because no disease indexing tests accompanied this work, relation of the virus to corky bark or other diseases remained to be determined. On one occasion, a closterovirus at 11-12 × 800 nm was discovered by Conti *et al.* from grapevine legno riccio-inoculated *Nicotiana clevelandii* in 1980. Unfortunately, repeat experiments attempted by the same authors were not successful. In an attempt to purify a probable viral agent from corky bark -affected grapevine, Lee *et al.* (1987) recently found both long flexuous and spherical virus particles in a partially purified sample from corky bark-affected grapevines. However, because the concentration of the virus particles was so low, the results were still far from conclusive.

The two diseases described seem to occur wherever grapes are grown (Goheen and Hewitt, 1964; Lehoczky, 1972). In most of grapevine grown countries, they were generally considered to be most important disease problems subjected to stringent quarantine control. During the past, much effort has been given to investigation of both diseases; but the etiological and pathological bases of both diseases remained mostly to be determined. Although purification of the likely related closterovirus from leafroll affected plant material has been succeeded and limited supply of antiserum for the immuno-detection of this virus on grapevine is now available (Zee *et al.*, 1987), direct proof of the obtained viral agent as the major cause of leafroll disease by Koch's postulates was far from complete and field indexing tests remained to be the main way of detecting and distinguishing both diseases. Field indexing is time and space consuming. Test results are affected by sensitivity of the indicator plants, the survival rate of the grafting buds, the virulence of the disease agent, and the changing environment (Alley *et al.*, 1963; Goheen and Hewitt, 1964). Furthermore, symptom expression is often complicated by the complex infection of the tested vines. Because a viral nature of both diseases has been well accepted in the past, the main objective of this study was a search for virus or viruses in diseased tissues and an examination of pathological effects occurring during disease development. During the course of this study, long flexuous virus-like particles, typical of closterovirus morphology were consistently detected from certain diseased material. The possible involvement of closterovirus in

Table 1. *Symptom expression of leafroll and corky bark on grapevine virus indicator plants*

Indicator plants	Leafroll	Corky bark
St. George (*Vitis rupestris*)	no symptoms	corky bark, wood grooving, rough bark
Cabernet Franc (*Vitis vinifera*)	red leaves	red leaves
Mission (*Vitis vinifera*)	red leaves	red leaves
LN-33 (Couderc 1613 × Thompson Seedless)	red leaves	red leaves, vine dwarfing and wood grooving

Table 2. *Indicator plants from field indexing experiments at Davis, California, expressing leafroll, or corky bark reaction, that were used in the electron microscopic studies*

Disease condition[1]	Indicator plants
Leafroll	Cabernet Franc #1 & #2; Zinfandel
Corky bark	Cabernet Franc #3, #4 & #5; LN-33 #1, #2 & #3; St. George

[1]Disease condition determined by indexing on Cabernet Franc, Mission, LN-33, and St. George indicator plants.

leafroll and corky bark diseases are herein discussed.

Materials and Methods

Healthy and diseased grapevine leaf and shoot tissues collected directly from the 1982 grapevine virus disease indexing field and the grapevine virus collection plot at the University of California at Davis, were used for the electron microscopical studies (Table 2). Midveins of grape leaves and bark tissues of young shoots were sampled from either healthy or diseased vines during the summer of 1983. These tissues were cut into approximately 2 × 5 mm pieces, fixed in 2% glutaraldehyde (in 0.1 M phosphate buffer at pH 7.0) at 4°C, post fixed in 2% osmium tetraoxide (in the same buffer), dehydrated in a graded acetone series (10 to 100%) and finally embedded in epoxy resin mixtures (Shalla and Shepherd, 1981). Before ultrathin sectioning, all the embedded materials were trimmed under a dissecting microscope in order to position the vascular tissues. These tissues were then ultrathin-sectioned in a direction perpendicular to their long axis. The ultrathin sections were stained with uranyl acetate and lead citrate and examined by an AE-1 transmission electron microscope.

Results

Leafroll

Virus particles were detected from all examined leafroll-affected Cabernet Franc grapevine. The virus was a long flexuous rod with a diameter of approximately 10-11 nm (Fig. 1). It formed in aggregates and was detected mainly from phloem parenchyma cells that were immediately adjacent to mature sieve tube elements. Concentration of the virus in diseased plants seemed to be very low. In each vascular bundle, generally, only 3-6 cells contained the virus particles, while the majority of cells remained free from virus colonization. The virus aggregates, in different sizes, were either enveloped by a membranous structure (Fig. 1), or simply submerged in cytoplasmic matrix (Fig. 2). The virus aggregates appeared to be arranged either parallel with each other along their long axis or randomly directed. Sections of aggregates have primarily three different distinctive patterns: (a) region in which hexagonal cross sections of virus particles were arranged in order and the center to center distance of the particles was approximately 13 nm, (b) region in which virus particles appeared nearly parallel with each other, and (c) regions in which no orderly arrangement was evident. High magnification indicated that the hexagonal arrays contained an outer light-colored layer and a dense colored core (about 75 A). Particles which contain only the dense core were sometimes observed in the infected cells (Fig. 1). Both fibrous inclusion (a virus aggregate in which particles arrange without a precise order) and banded inclusions (a virus aggregate in which virus particles arise from an orderly arrangement and usually appear as stacked

layers) that are characteristic for a closterovirus were found in the infected cells (Fig. 1). In leaf tissues expressing strong symptoms, virus concentration in infected cells was very high. The virus aggregates at the final stage of infection might occupy most spaces of a cell.

In virus-infected cells, electron dense round bodies (EDRBs) that consisted mainly of clusters of granular materials were usually scattered throughout the cytoplasm. The outer membrane of these structures seemed to originate from endoplasmic reticulum (Fig. 2). These EDRBs morphologically resembled the viroplasm-like structure reported from beet yellow stunt virus-infected sugarbeet leaves (Hoefert *et al.*, 1970). Vesicles which contain fine network fibril substance were also frequently associated with virus aggregates in the leafroll-affected leaf tissues (Fig. 2).

Degenerative changes of phloem parenchyma cells were consistently associated with the appearance of red-leaf symptoms on Cabernet Franc. Compared to the healthy controls (Fig. 3), disorganization and disruption of cell organelles and formation of virus associated structures (i.e. the viroplasms and vesicles as mentioned above) were only detectable from diseased vines (Fig. 2). In infected tissues, chloroplast and mitochrondria degeneration were common in cells either with or without virus colonization. Membrane integrity of these organelles appeared to be badly affected or even totally disrupted. Less thylakoid development, accumulation of starch and small vesicles, and the deposition of osmophilic substances were usually found in chloroplast. Plastids, on the other hand, became amorphous and lost the sharpness of their membrane structures. In mitochondria, cristae deformations were generally observed. In some virus-infected cells, membranous structures of all organelles were disrupted. Ribosomes dissociated from the endoplasmic reticulum and scattered throughout the cytoplasm. The disrupted membranes of these organelles appeared as a mass in those cells in which large virus aggregation was not yet evident (Fig. 4). It seemed that these membranous substances then enclose virus particles and form the large aggregates.

Similar virus particles were also detected from young shoots of infected vines. Compared to those observed in leaf tissues, virus concentration in shoots was fairly low. In each individual vascular bundle, virus colonization could be detected from no more than one phloem parenchyma cell. Numbers of virus particles detected from these infected cells was also much fewer than those in leaves. Virus clusters in shoot tissues seemed to be mostly rounded or oval shaped, usually bounded by an outer membrane, and were detected only within nucleus (Fig. 5). The nuclear matrix of these infected cells became heavily stained. In contrast to leaf tissues, no virus particles were detected from the cytoplasm of shoot phloem parenchyma cells.

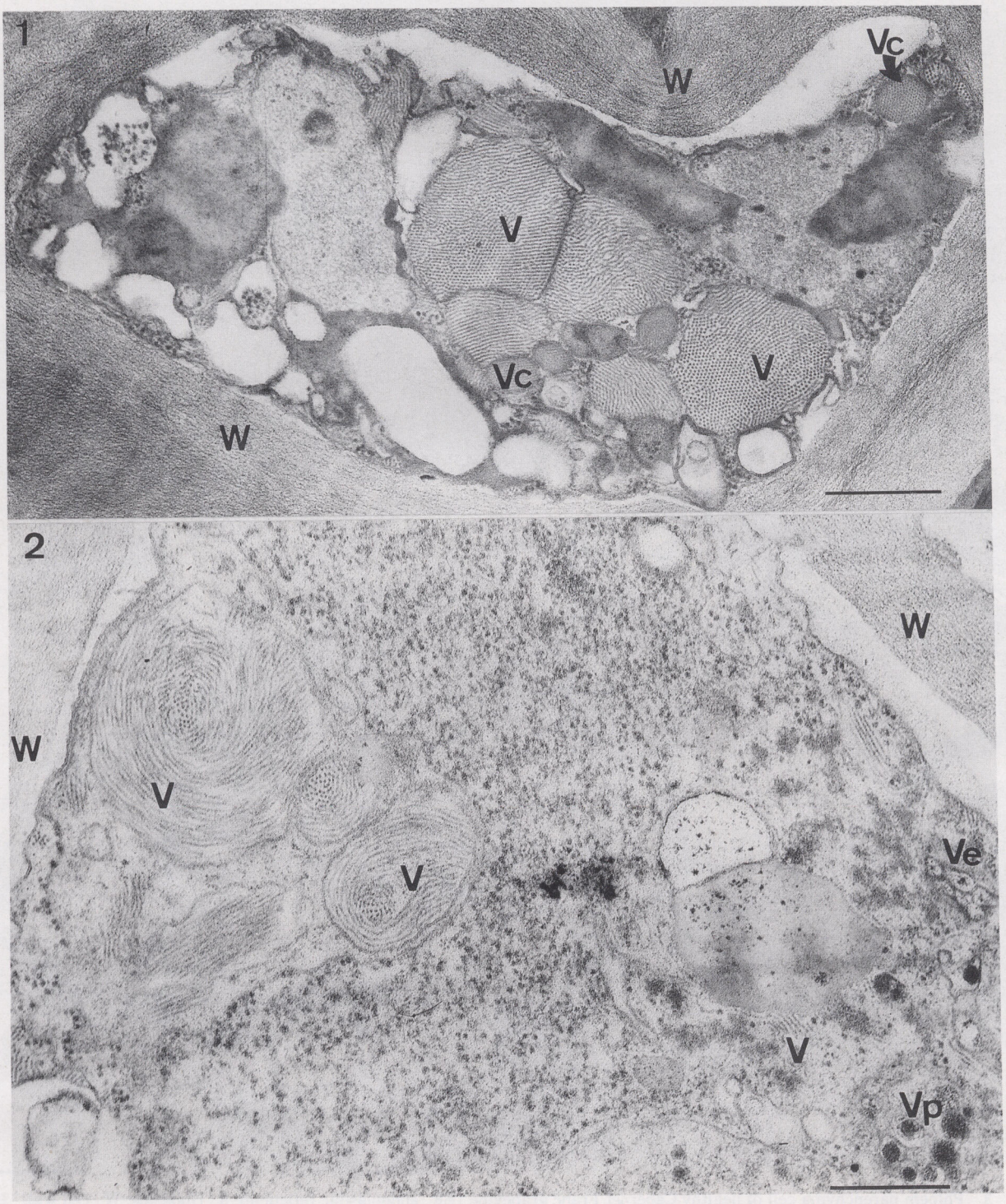
Corky Bark

Virus particles detected in corky bark-affected Cabernet Franc have a morphology similar to that of leafroll, a long flexuous rod that appeared to aggregate (Fig. 6). Diameter of the particles was about 7-8 nm, which appeared to be slightly smaller than those in leafroll-affected vines. Virus particles were only detected from phloem tissues of infected leaves. In infected cells, the virus existed in high concentration. Unlike the particles seen in leafroll-affected leaves, virus particles detected from corky bark-affected vines formed aggregates that were not enclosed by a membrane. The virus clusters were detected either in cytoplasm or within the nucleus (Fig. 7) of the infected cell. Abundant vesicles and EDRBs (viroplasm-like structures) were often observed in infected cells (Figs. 6 and 8). These viroplasm-like structures, similar to those of leafroll virus, were usually associated with dissociated endoplasmic reticulum remains and the scattered ribosomes. In some infected cells, virus particles were generally scattered among these viroplasmal structures or the fine fibril containing vesicles (Figs. 8 and 9). At an advanced stages of infection, the entire content of a host cell was totally disorganized and the cell became packed with virus particles or virus-associated structures.

The degenerative effect of the virus infection on cell morphology resembled those seen in leafroll-affected vines. In addition to Cabernet Franc, morphological alteration of phloem parenchyma cells of diseased leaves was observed on St. George and LN-33 (Fig. 10), although no definitive viral structures were ever detected from the later varieties.

Discussion

In the proceeded electron microscopic study, long flexuous rod virus particles, each with certain similar-



Figs. 1-2. Phloem parenchyma cell in leafroll-affected Cabernet Franc leaf tissue. Virus (V) containing bleb-like structures and virus cores (Vc) were detected from the infected cell shown in Fig. 1. Fig. 2 shows the vesicles and viroplasm-like electron-dense round bodies (Vp) found in another cell. (W, cell wall. Bar = 500 nm)

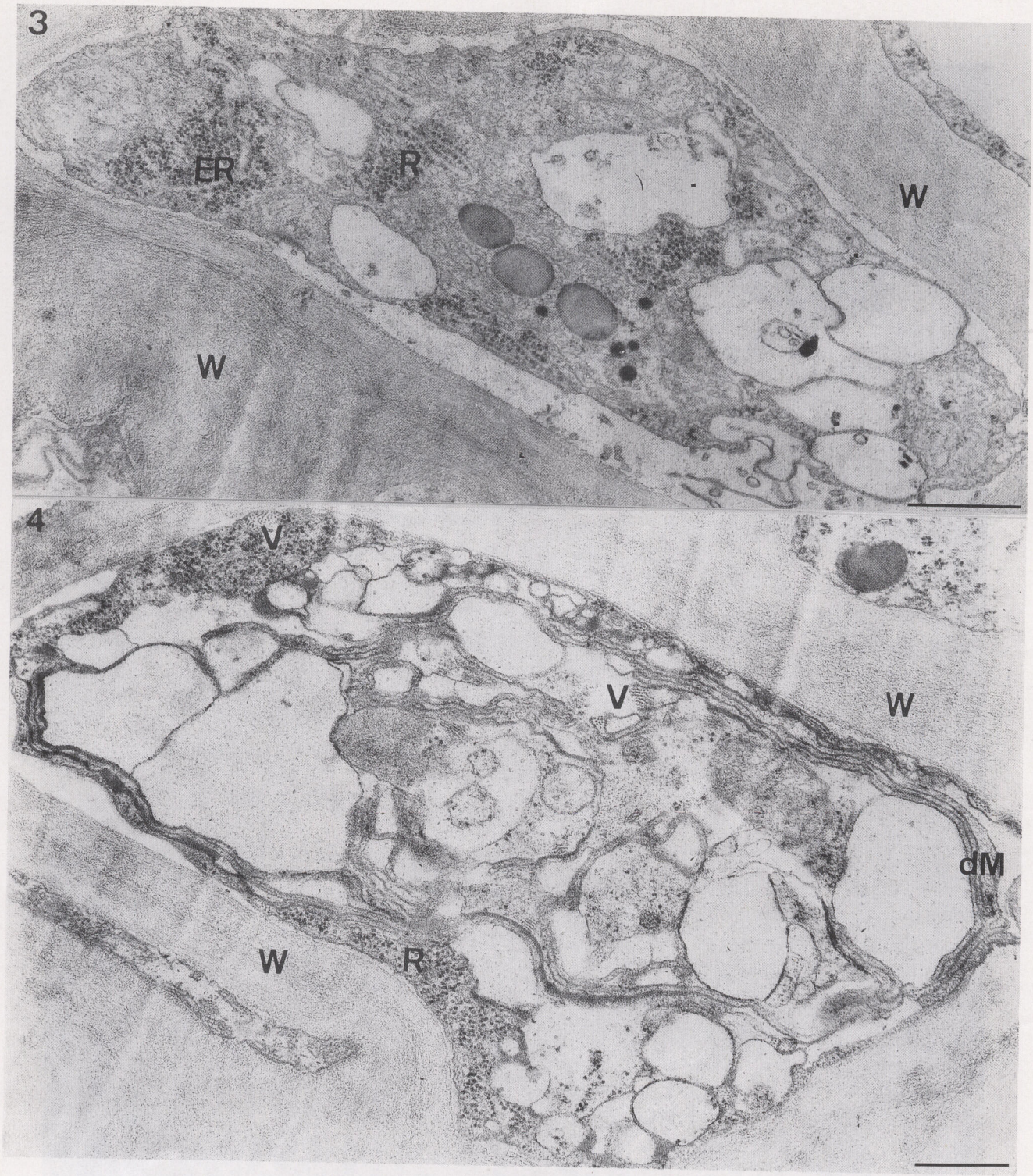


Fig. 3. Phloem parenchyma cell of healthy Cabernet Franc grapevine (ER, endoplasmic reticulum; R, ribosome; W, cell wall. Bar = 500 nm).

Fig. 4. Degenerative effect of leafroll infection on membrane of cell organelles at an advanced stage of infection. (dM, disrupted membranes; R, ribosome; V, virus particles; W, cell wall. Bar = 500 nm).

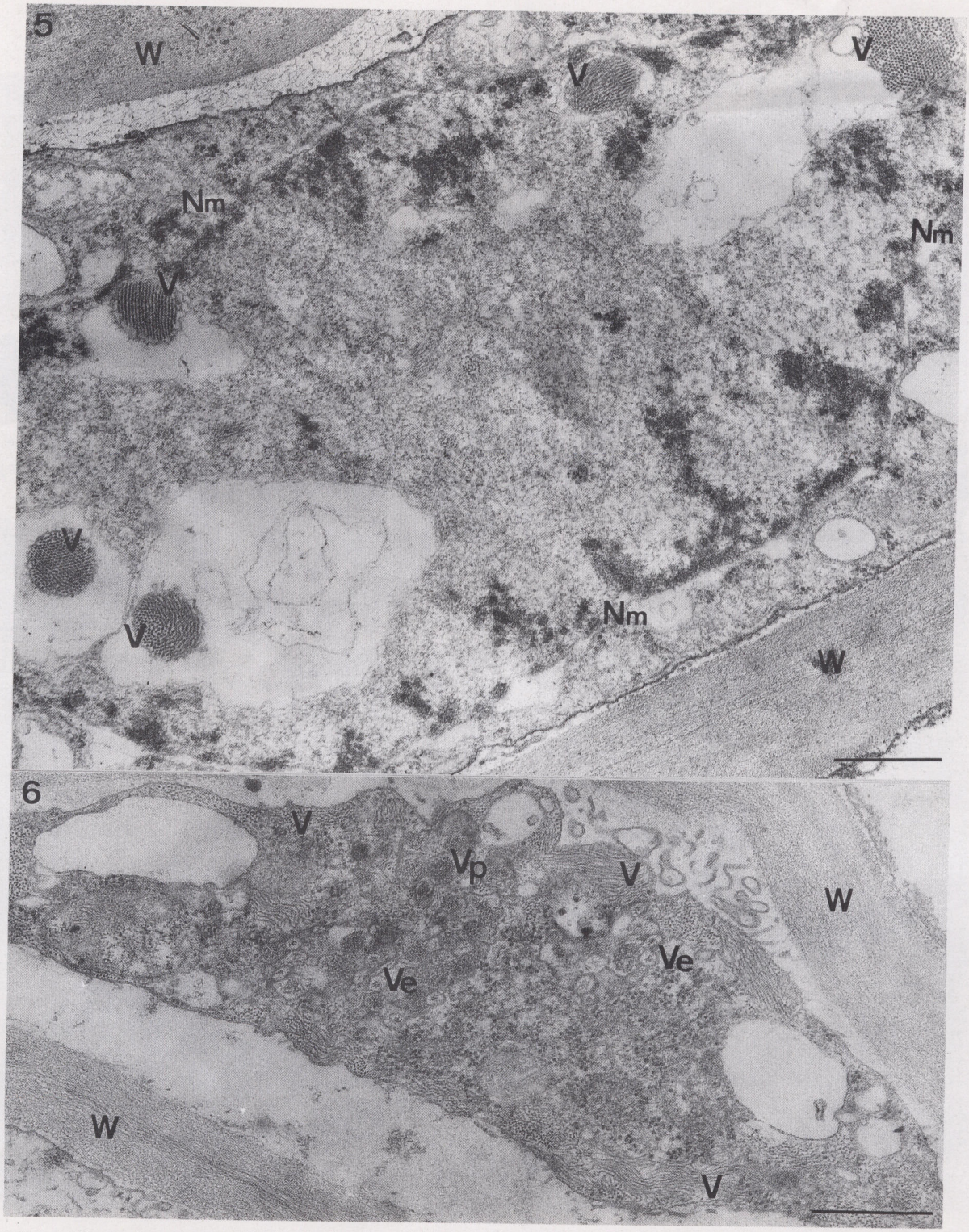


Fig. 5. Shoot phloem parenchyma cell of leafroll-affected Cabernet Franc. Virus (V) aggregates with or without an outer membrane confinement were mostly at the periphery of the nucleus (N). (Nm, nuclear membrane; W, cell wall. Bar = 500 nm).

Fig. 6. Leaf phloem parenchyma cell of corky bark-affected Cabernet Franc grapevine. Small vesicles (Ve), viroplasm-like bodies (Vp), virus particles (V) and degenerated cell constituents have replaced most of the normal cell contents. (W, cell wall. Bar = 500 nm).

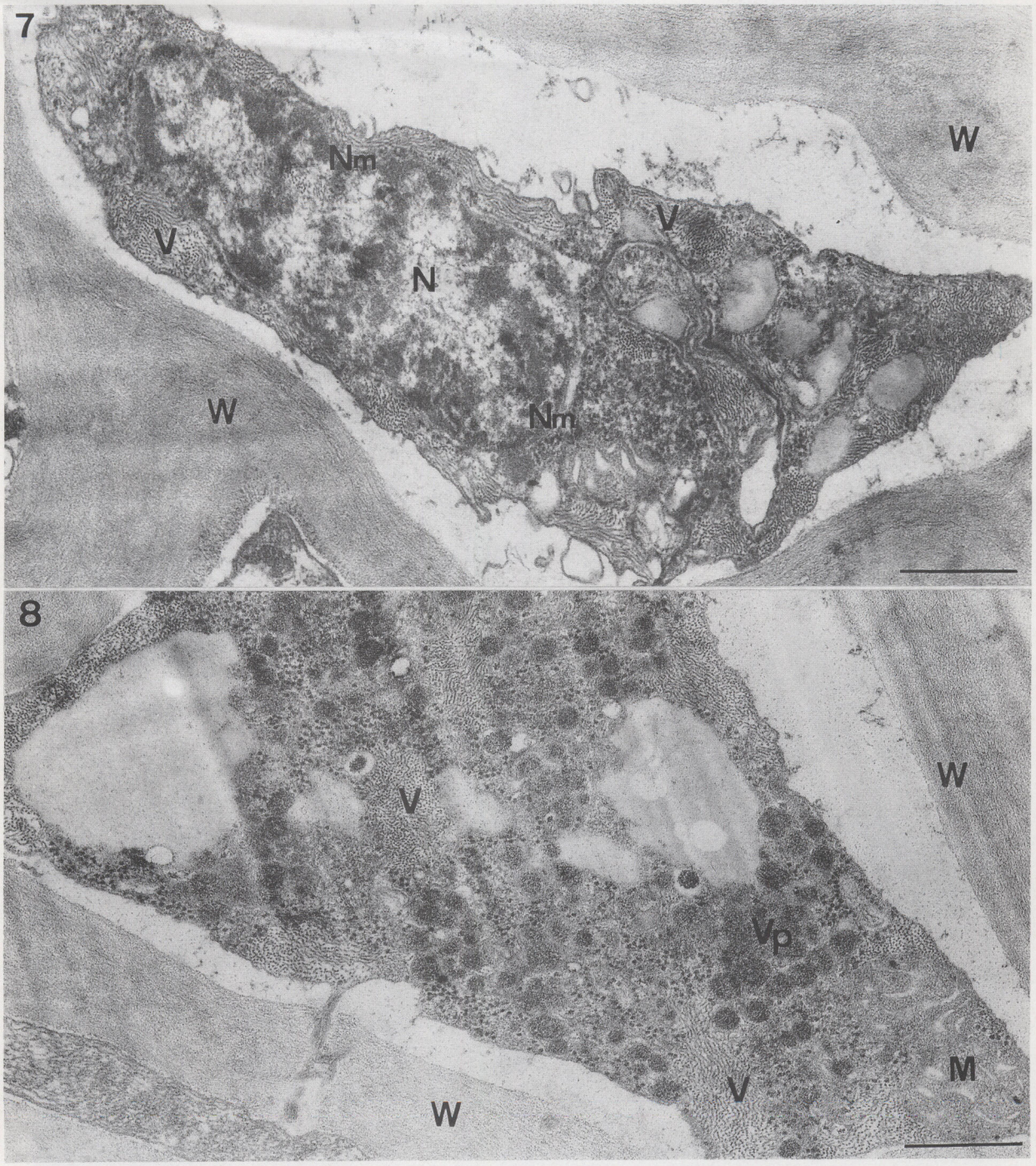


Fig. 7. Phloem parenchyma cell of corky bark-affected Cabernet Franc in which most of the cell space is occupied by the virus particles (V). Some virus particles are found in the nucleus (N). (Nm, nuclear membrane; W, cell wall. Bar = 500 nm).

Fig. 8. Viroplasm-like bodies (Vp) and virus particles (V) in corky bark-affected phloem parenchyma cell. Most cell organelles are not recognizable at this stage except for a degenerated mitochondria (M) at the lower right of the cell. (W, cell wall. Bar = 500 nm).

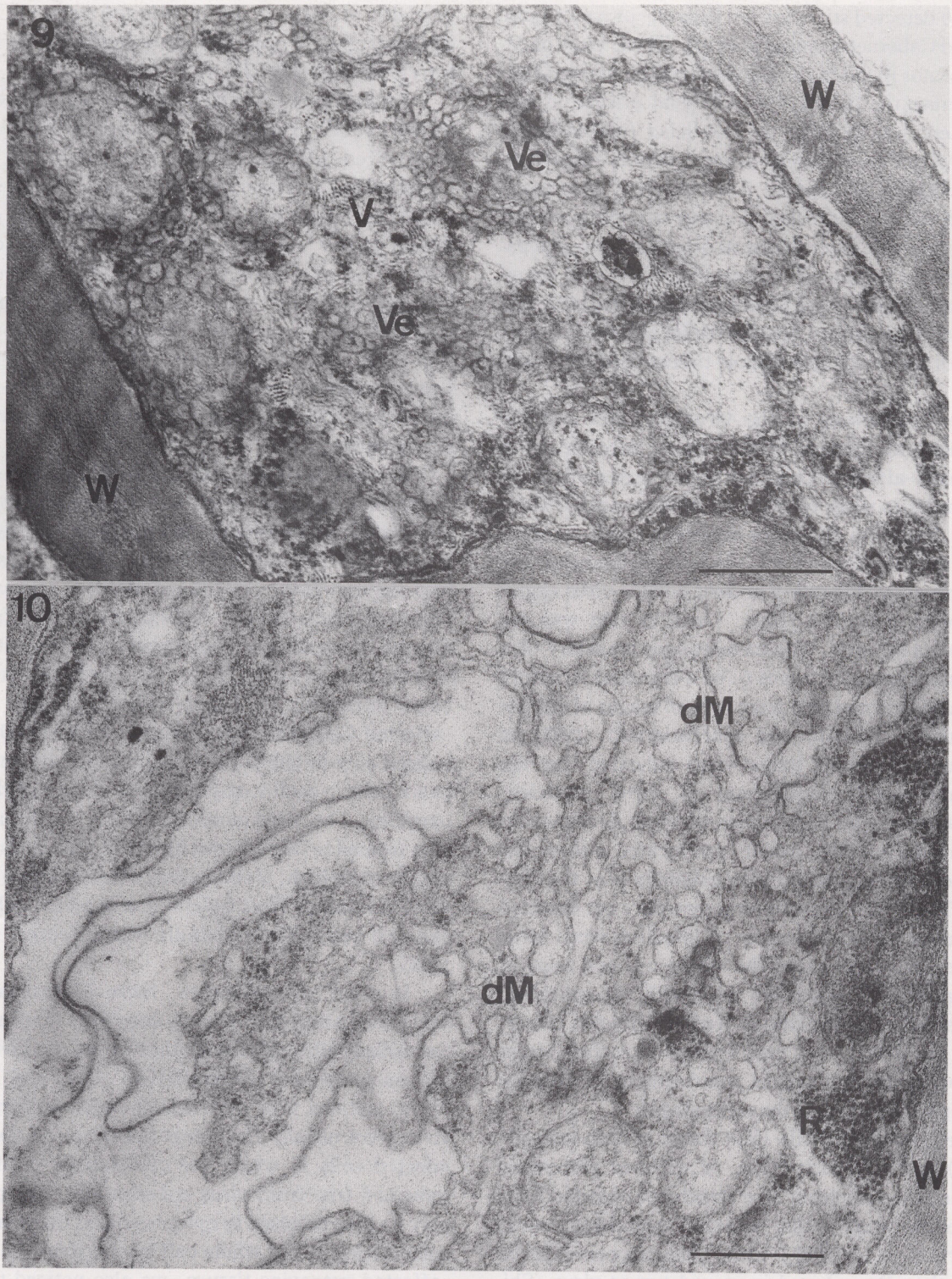


Fig. 9. Abundant vesicles (Ve) that contain fine fibril material in corky bark-affected phloem parenchyma cell of Cabernet Franc grapevine. (V, virus particles; W, cell wall. Bar = 500 nm).

Fig. 10. A phloem parenchyma cell of corky bark-affected LN-33 grapevine. Degeneration of the cell organelles is quite evident in both cultivars. (dM, degenerated membranes; R, ribosome; W, cell wall. Bar = 500 nm).

ity, were detected strictly from leafroll- or corky bark -affected Cabernet Franc grapevine. These virus particles were not found in comparable healthy plants (Fig. 3). Because the identity of individual disease in this experiment was verified by paralleled field-indexing tests, the risk of confusing several viruses from complex infection was avoided. Association of virus particles with the two individual diseases was quite evident because no virus particles were detected from comparable healthy plants. The fact that virus was found only from certain of the plants examined might be due to low concentration of virus particles present in infected tissues and unsuitable sample tissues used for examination.

The morphology of the virus particles and the ultrastructural changes of host tissue detected from corky bark-affected Cabernet Franc appeared to be quite similar to those from plants affected with leafroll disease. The main difference between viruses associated with the two diseases was the particle diameter and the membranous boundary present outside virus aggregates found in leafroll (Figs. 1 and 2) that was not found in corky bark. The membranous boundary of a plant virus aggregate seems to be a transient structure that can be detected only from certain stages of disease development. Shikata and Maramorosch (1967) in their study on wound tumor virus indicated that the membrane-bound microcrystal of virus particles, which represent an early stage of virus development after virion assembly, usually are located a short distance from the suggested virion assembly site, the viroplasm. These membranous structure were not detected at the more advanced stages of infection when virus particles finally colonized the whole cell and none of the original cell organelles were still recognizable. This seemed to be the case in leafroll-affected tissue. Failure to detect the membranous structure from corky bark-affected tissues could simply be because the samples were not at the right stages.

The granular EDRBs (Vp) found in both leafroll- (Fig. 2) or corky bark- (Fig. 8) affected grapevines resembled the viroplasm-like structures reported from beet yellow stunt virus infected sugarbeet leaves (Hoefert *et al.*, 1970), while they appeared to be smaller than those found in wound tumor virus (Shikata and Maramorosch, 1967). In the study of wound tumor virus, Shikata and Moramorosch found viroplasms were commonly associated with virus progeny particles or virus cores in infected cells. In several RNA viruses, viroplasms were suggested as the site for virion assembly (Faoro *et al.*, 1981).

Similar to the results obtained from closterovirus studies, small vesicles that contain fine fibril materials were often seen in either corky bark- or leafroll -affected cells. These fine fibril materials, as suggested by Lesemann (1977), could be nucleic acids or slime materials that appear to be related to virus development. The outer membrane of the vesicles seemed to be continuous with proliferated or dilated endoplasmic reticulum that is often seen during the cell disorganization (Fig. 2). This indicated membrane fragments from breakdown cell organelles as the source of the membrane boundary of the virus aggregates after virion assembly.

In either leafroll- or corky bark-affected tissues, we also noticed that the virus-colonized cells were usually stained more heavily than ordinary cells by uranyl acetate and lead citrate (Tzeng, 1984). This resembled the chromatic cells found in citrus tristeza virus -infected citrus tissues (Schneider, 1959). In citrus tristeza virus disease, occurrence of chromatic cells was thought to be a primary symptom that indicated virus infection. In either corky bark or leafroll -affected Cabernet Franc grapevine tissue, the virus resided only in phloem tissue and generally only three to six cells in each vascular bundle contained the virus particles. The virus concentration in the diseased tissues seemed to be very low; however, this in turn might appear to be high, if compared to that of citrus tristeza virus disease on West Indian lime in which only about one cell per vascular bundle was infected (Price, 1966).

In plant virus diseases, there is increasing evidence indicating that virus is originally synthesized in the nucleus although the synthesis does not involve the protein component of the particle. Esau and Hoefert (1972) noticed that particles of beet western yellow virus were usually first observed from the nucleus of infected cells in which the cytoplasmic portion still remained free from virus colonization. The particles may be then released to cytoplasm and thus lead to the colonization of the whole cell by the virus. In leafroll -affected grapevine tissues, we also noticed the presence of virus aggregates in the nucleus of infected cells in which the cytoplasmic portion remained non -affected (Fig. 5). This might actually represent an

early stage of virus infection. In the more advanced stages of infection, disorganization of cell contents occurred and virus particles spread throughout the entire cell. The lateral movement of virus from cell to cell seemed not very likely because infected cells are always isolated from each other by cells free from viral infection. However, basipetal and/or acropetal movement of virus particles along the vascular bundle is strongly indicated by the evidence that small virus aggregates can be detected from phloem parenchyma of infected young shoot although the concentration of virus particles in these cells appeared to be fairly low (Fig. 5). The same phenomenon was also indicated by Beukman and Gifford (1969) in their study on symptom expression of corky bark disease in LN-33 grapevines.

According to Bar-Joseph (1979), the anatomical effect of group-characteristic features of closterovirus includes: (a) subcellular symptoms that are mostly confined to the phloem tissue, (b) formation of typical virus aggregates, and (c) accumulation of characteristic vesicles. The EM observation presented here indicated all these features can be detected from either leafroll- or corky bark-affected grapevines. Accumulated evidence from this study seemed to suggest that leafroll and corky bark diseases of grapevine are due to infection by members of the closterovirus group.

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葡萄捲葉病與栓皮病之電子顯微鏡研究

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利用電子顯微鏡檢視於加州大學 Davis 校區試驗農場經嫁接特定指示品種檢定證實罹患典型捲葉病或栓皮病之葡萄嫩莖與葉脈組織，發現於具捲葉或栓皮典型病徵之 Cabernet Franc 品種葡萄，韌皮部細胞中皆可偵測到有長絲狀病毒顆粒存在，此些病毒顆粒形狀與 Closterovirus 相似，僅存在於韌皮部組織，且常多數病毒聚集成典型之病毒集合體。於被感染細胞中，病毒於細胞核與細胞質中均可見之，嚴重發病植株，其葉脈中病毒濃度顯較嫩莖組織爲高，且於罹病組織細胞中，粒腺體、葉綠體及內質網膜等胞器構造之畸形、解體，澱粉粒、單寧物質及嗜鋨性顆粒等之累積，以及與病毒有關的小空泡狀構造之產生等現象皆極爲明顯。由罹病材料切片上所觀察到的病毒顆粒直徑，栓皮病約爲 7-8 nm，捲葉病則爲 10-11 nm，於捲葉病組織中，病毒顆粒集合體常見有膜狀構造包被，此於栓皮病組織中則未發現。