Light and Electron Microscopy of Potato Virus M Lesions and Marginal Tissue in Red Kidney Bean

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ABSTRACT

Local lesions and marginal tissue in potato virus M (PVM)-infected *Phaseolus vulgaris* 'Red Kidney' bean were studied by light and electron microscopy. Three zones, from the margin toward the central zone of lesions, were represented by non-necrotic cells, "seminecrotic" cells which were discolored but not disintegrated, and necrotic cells. Callose, stained with aniline blue, was detectable by the fluorescent method 3 to 4 days after inoculation, and was deposited in a zone of non-necrotic cells at the lesion margin as the lesions expanded. Thickening, due to callose deposition, was observed on the inner wall of non-necrotic cells immediately adjacent to seminecrotic ones. Such thickening, as determined by electron microscopy, was structurally less complicated than that of seminecrotic cells. Seminecrotic cells surrounding the necrotic zone were characterized initially by roughening of the plasmalemma and accumulation of membrane-bounded vesicles and tubules in the area between the cell walls and the protoplast. Callose deposition formed secondary wall thickening which sealed off the plasmodesmata. It is suggested that these changes in cell wall structure are related to a series of events of localization. The cell-to-cell spread of PVM may be restricted by blocking of plasmodesmata connections initially by deposition of callose and later, more extensive secondary wall thickening involving boundary formation.

Additional key words: histochemistry and ultrastructure, virus-host interaction.

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Potato virus M (PVM) incites necrotic lesions in mechanically inoculated primary leaves of *Phaseolus vulgaris* L. 'Red Kidney' bean (14, 29). Although extensive studies on tobacco mosaic virus (TMV) infection in local lesion hosts have revealed physiological changes within and around local lesions (23, 24, 25, 26, 27, 29, 30, 35), it is desirable to accumulate more information regarding cytological and ultrastructural alterations due to virus infection to explain the blocking mechanism which effectively checks virus spread beyond necrotic lesions. Most changes which accompanied the formation of local lesions were not necessarily specific to local lesion hosts, but commonly associated with systemic infections (4, 18). Most of the histochemical and electron microscopy studies made on local lesions have been concerned with TMV (13, 15, 16, 18, 20, 22, 31, 33, 34). A study of other virus-host combinations may help to understand the processes of local lesion formation. In a previous report (29), "secondary cell wall thickening" was described as the most characteristic change in PVM lesions. In the investigation reported herein, special attention was given to callose deposition in connection with secondary cell wall thickening in PVM lesions.

MATERIALS AND METHODS.—Methods of growing Red Kidney beans and of inoculating the primary leaves with an Alberta isolate (AP-1) of PVM (28) have been described in previous reports (14, 29). Inoculated plants were maintained in a greenhouse at 17 ± 2 C. Throughout this paper, the age of lesions will be indicated by the number of days after inoculation of bean.

Measurement of 12 lesions selected randomly was made with an ocular micrometer in a Leitz microscope (X 100). An average value was obtained from measurements made at four positions coincident with the 45 degree axes of a lesion.

The fluorescent method with a slight modification was used to detect deposition of callose substance (2, 12). Samples of tissues (1.5 mm^2) were cut from leaves containing a virus lesion, from injured control leaves, or from comparable healthy leaves. The tissues were boiled for 3-4 min in water and completely decolorized by two to three washings in 95% alcohol prior to staining them in 0.01% aniline blue in 1/15 M K_3PO_4 (pH 9.5) for 2-3 hr at room temperature. Observations were made with a Leitz Orthoplan fluorescent microscope. A high-pressure mercury vapor lamp (HBO 200 w) served as the illuminator. Two exciter filters, UGI (2 mm), with maximum transmissibility at 366 nm, absorbed the visible spectrum. A barrier filter (K 430) was placed in the ocular tube of the microscope.

The ultrastructure of both healthy tissues and those with 4-, 8-, and 12-day lesions was examined. The tissues were fixed in 3% Formalin-glutaraldehyde (1:1) mixture in 0.1 M phosphate buffer (pH 7.0) under mild vacuum overnight at 4 C. The samples were rinsed in 0.1 M phosphate buffer (pH 7.0), fixed in 2% OsO_4 in phosphate buffer (0.1 M, pH 7.0) for 2 hr, then stained with 0.2% lead citrate for 2 min at room temperature. Observations were made in a Philips 200 electron microscope (60 kv).

RESULTS.—*Local lesion size.*—A few minute, water-soaked lesions appeared 3 days after inoculation, and were visible only when the leaf was viewed such that light was transmitted through the leaf. The number of lesions increased rapidly between the 3rd and 4th days. Lesion size reached a maximum

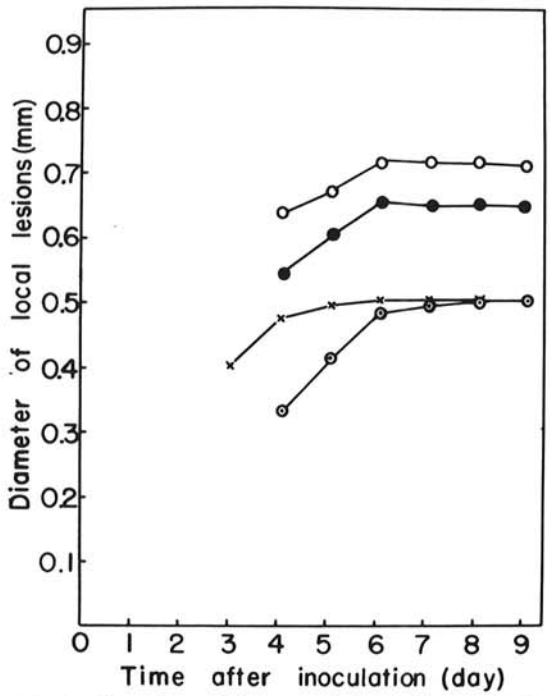

Fig. 1. The sizes of four developing lesions incited by potato virus M in the primary leaves of Red Kidney bean. The zones of necrotic cells and "seminecrotic" cells were included in the measurement of PVM lesions.

6 to 7 days after inoculation, and averaged 0.6 mm in diameter. Measurements of four selected local lesions are presented in Fig. 1. In all cases, the lesions rapidly increased in size soon after they became visible and for the next 2 to 3 days; then increase in lesion size slowed down or ceased. In some cases, small decreases in size were observed between the 7th to 12th day after inoculation, which suggested shrinkage of the lesions due to necrotic degeneration.

Detection of callose.—Three zones, from the margin toward the central zone of lesions, were distinguished in light microscopy. They were tentatively named non-necrotic cells (resistant zone), "seminecrotic" cells, and necrotic cells (Fig. 2). When a stained tissue containing a PVM lesion was observed under a fluorescent microscope, a bright-yellow fluorescent band was detected in the boundary between the seminecrotic cells which were discolored to various degrees and the non-necrotic cells which normally constituted a band of ca. 60 to 80 cells in total number (Fig. 3, 5, 7). Unstained local lesions and stained noninoculated leaf tissue did not contain a comparable fluorescent band. The fluorescence in epidermal cells was observed only in the inner wall of non-necrotic cells immediately adjacent to the seminecrotic ones (Fig. 5, 7). A notable difference in the thickness of the cell wall was made apparent by comparing the fluorescent and nonfluorescent portions of the cell (Fig. 4, 5, 6, 7). Callose deposition was not detected in necrotic cells.

Since callose formation was considered to be a quick cellular response to injury, leaves were examined daily for callose formation in response to inoculation with PVM and to mechanical and chemical injury. The primary leaves of 12-day-old Red Kidney bean were (i) inoculated with PVM; (ii) punctured with a fine needle; (iii) punctured with a heated needle; or (iv) injected by means of a syringe (needle size, 25G 5/8) with a minute quantity (0.01 ml) of 85% phenol. The leaves were sampled after 1, 2, 3, 4, and 5 days by collecting 20 samples (2-5 mm^2) per treatment.

Deposition of callose was detected 1 day after chemical or mechanical injury. Fluorescence, characteristic of PVM lesions, was not detected in inoculated leaves before the lesions developed, although a few scattered fluorescent flecks due to Carborundum injury were noted. Small scattered fluorescent spots were seen in some 3- to 4-day lesions. Some 4-day lesions were strongly fluorescent. At this stage, the average lesion size was small, and fluorescence appeared to have covered the lesion, because callose was formed, in addition to epidermal cells, in the non-necrotic spongy cells situated under seminecrotic palisade cells. However, in 8-day lesions (Fig. 3), such fluorescence in the lesions disappeared and a band of strong fluorescence predominated at the peripheral zone of the lesions. Fluorescence was more intense in small lesions than in large lesions regardless of the treatment.

Electron microscopy.—The 4-day lesions had only seminecrotic cells surrounded by non-necrotic cells. The 8- and 12-day lesions contained necrotic cells in a central zone in addition to those found in the 4-day lesions. A 12-day lesion differed from an 8-day lesion by a discernible expansion of the necrotic area which resulted from further necrotization of seminecrotic cells. Therefore, 8-day lesions were used to describe ultrastructural changes in development of the lesions.

Callose appeared first as isolated deposits in apparent association with short endoplasmic reticulum and dictyosomes in the inner portion of non-necrotic cells immediately adjacent to seminecrotic cells (Fig. 9, 10), and later as deposits rather uniform in texture that often increased cell wall thickness 3 to 5 times the thickness of walls of normal cells (Fig. 11). Non-necrotic cells were characterized by increased ribosome density (Fig. 8) and by an extensive system of endoplasmic reticulum (Fig. 8, 9), dictyosomes (Fig. 8), and frequent fission of chloroplasts. Chloroplasts in the non-necrotic cells immediately adjacent to seminecrotic cells appeared to be more ovoid in shape, and contained more starch grains than those of healthy cells. PVM particles were not detected in non-necrotic cells.

The zone of seminecrotic cells was four to six cells wide, and manifested degrees of cell wall thickening. Outer cells of the zone were less thickened than were inner cells, but thickening was always associated with boundary formation (Fig. 14). Callose deposition was rather difficult to detect as such in seminecrotic cells, though its deposition in cells was observed in close proximity to plasmodesmata in the cell wall with progressive

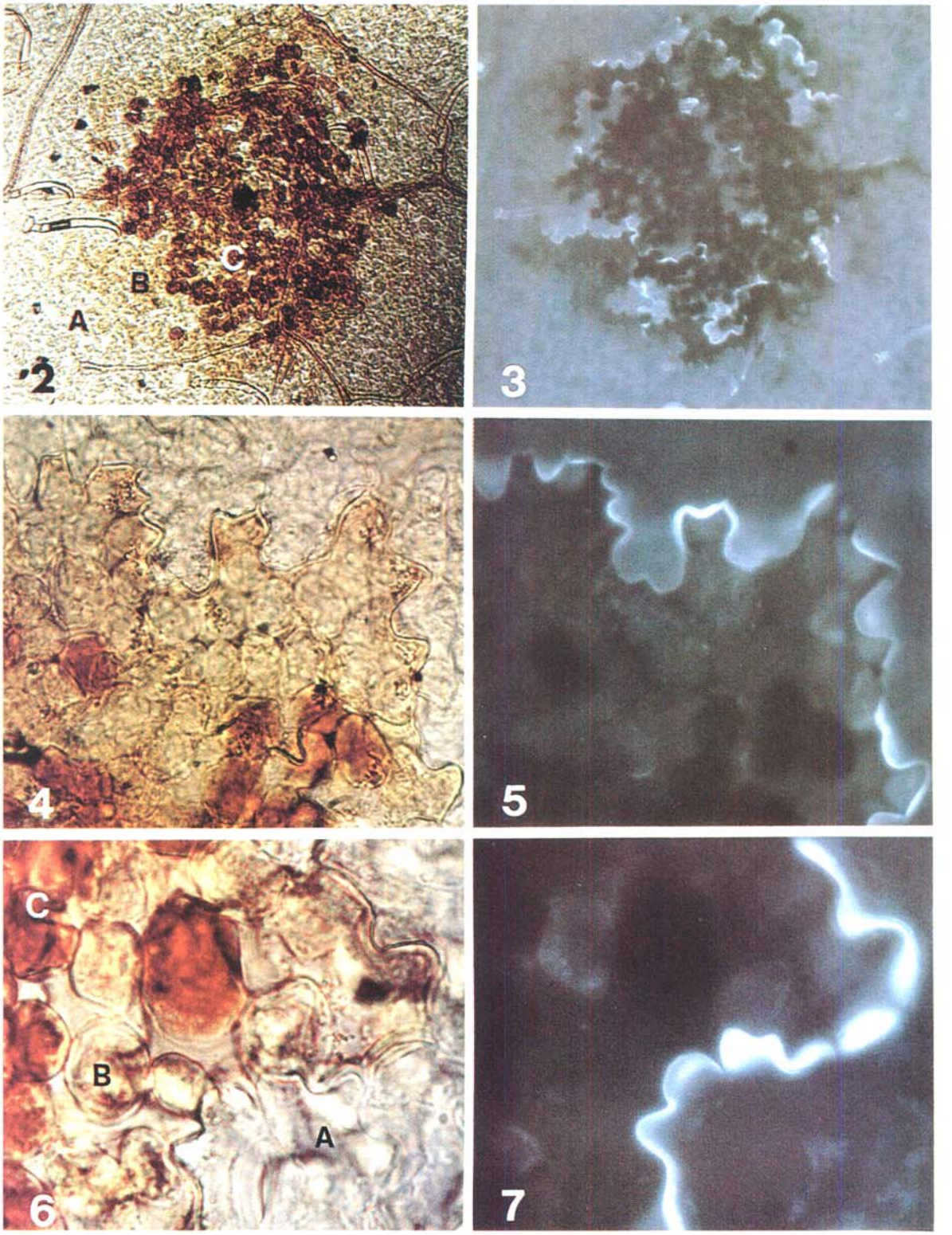

Fig. 2-7. 2) An 8-day potato virus M lesion showing three zones: (A) non-necrotic cells; (B) "seminecrotic" cells; (C) necrotic cells in the primary leaves of Red Kidney bean (X 350). **3)** Fluorescence after staining of callose deposited in the cells of (A) shown in Fig. 2 (X 350). **4)** A portion of the 8-day PVM lesion showing thickening of the cell walls at the edge of the seminecrotic zone (X 870). **5)** Fluorescence after staining of callose deposited in the area of the thickening shown in Fig. 4 (X 870). **6)** An enlarged portion of the 8-day PVM lesion showing the three distinctive zones A, B, and C (X 2,000). **7)** Fluorescence in the PVM lesion shown in Fig. 6. Note the sharp band of fluorescence which indicates that callose is deposited on the wall of non-necrotic cells immediately adjacent to seminecrotic cells (X 2,000).

thickening of the secondary wall (Fig. 12). Vesicles derived from endoplasmic reticulum increased in number and were observed near thickened walls (Fig. 12). PVM particles were detectable at this stage (Fig. 13). Changes in chloroplasts, as described for non-necrotic cells, were also evident. As thickening of the cell wall progressed, more smaller-sized mitochondria and more osmiophilic bodies of larger size were observed.

Necrotic cells were mostly electron-opaque. Their cellular organelles were disintegrated, and were hardly visible except for some collapsed chloroplasts containing thylakoids around starch grains (Fig. 15, 16, 17). Swelling of primary cell walls between seminecrotic and necrotic cells (Fig. 15) was apparent. Necrotic cells appeared to be detached from neighboring ones along the middle lamella. Virus particles or aggregates were not visible in the necrotic cells.

DISCUSSION.—A suggestion (5) that cell-to-cell movement of virus through plasmodesmata (3, 8, 17) may be prevented by callose deposition has been supported recently by cytochemical evidence from a TMV-Pinto bean system (33, 34). In this system, the deposition of callose was detected in a band of non-necrotic cells (resistant zone) surrounding a necrotic lesion, and the rate of callose deposition was positively correlated with the ultimate size of TMV lesions. The ultrastructure of the resistant tissue has been investigated with TMV on Samsun NN tobacco (16, 22) and on Pinto bean (26). It was postulated that virus spread was halted when the events of collapse in resistant tissues preceded those of infection in tobacco (22). Deposition of membrane-bounded vesicular bodies (paramural bodies) in the resistant zone (26) or secondary wall thickening due to boundary formation of seminecrotic cells surrounding necrotic cells (29) was considered to be a contributing factor to localization of virus spread. The term "boundary formation", proposed by Esau et al. (7), was adopted in this paper and elsewhere (29) for the structure formed in the boundary between the protoplast and the cell walls, and included tubules, vesicles, and the infolding plasmalemma.

In the investigation reported here, most ultrastructural alterations of cell organelles in Red Kidney bean due to PVM infection were similar to those described previously for TMV on several local lesion hosts (13, 15, 16, 18, 20, 22, 26, 32). The significant findings in the PVM-Red Kidney bean system were two types of cell wall thickening. The first type is thickening due to the deposition of callose. Callose (β-1, 3-D-glucan) is an insoluble polysaccharide found in certain plant cells (2, 10, 19). It functions as a temporary sealing (2) or protective material (11) formed in quick response to an injury (21). The deposition of callose we observed is probably a general host reaction to cell injury (in this case, injury caused by virus infection). The thickening occurred on the inner wall of non-necrotic cells immediately adjacent to seminecrotic ones. The location of this thickening, determined by light and electron microscopy, coincided with a fluorescent zone around PVM lesions. The second type was initiated by the appearance of boundary formation on the secondary wall of seminecrotic cells. The paramural bodies detected by Spencer & Kimmins (26) for their zone 2 and some of zone 3 of TMV-infected Pinto bean tissues resemble those we observed in the seminecrotic cells. As PVM aggregates are detectable in seminecrotic cells developing secondary wall thickening, it follows then that this thickening reaction, at least in our case, is not a defense barrier, but a barrier that encloses virus particles to restrict virus spread eventually.

We confirmed for non-necrotic cells the previous finding (16, 22) that cells in the resistant zone were synthetically and metabolically in an elevated state. In particular, a close relationship was observed between the deposition of callose and a system of a short or distorted filamentous endoplasmic reticulum and dictyosomes. This observation strengthens the earlier concept that they are involved in callose formation (1, 6, 11).

It was not clear whether the deposition of callose in non-necrotic cells constitutes a stage that precedes secondary wall thickening in seminecrotic cells. However, the fact that the material deposited in the zone of seminecrotic cells does not fluoresce suggests that (i) the chemical nature of callose may be modified; and/or (ii) callose may be embedded in other cell wall constituents in such a way that it does not fluoresce or is prevented from doing so. The apparent lack of callose in bean leaf tissue infected with TMV (26), as determined by electron microscopy, may well be due to the different area of tissue examined or to the method of sampling for observation as well as to the use of different technical procedures.

Our findings reported here and elswhere (29) suggest that in the PVM-Red Kidney bean system, two types of blocking mechanisms, which are not necessarily mutually exclusive, may be in operation. One mechanism involves blocking by callose that is deposited quickly in noninfected cells immediately adjacent to infected cells. A thick layer of callose may act as a barrier by sealing the plasmodesmata

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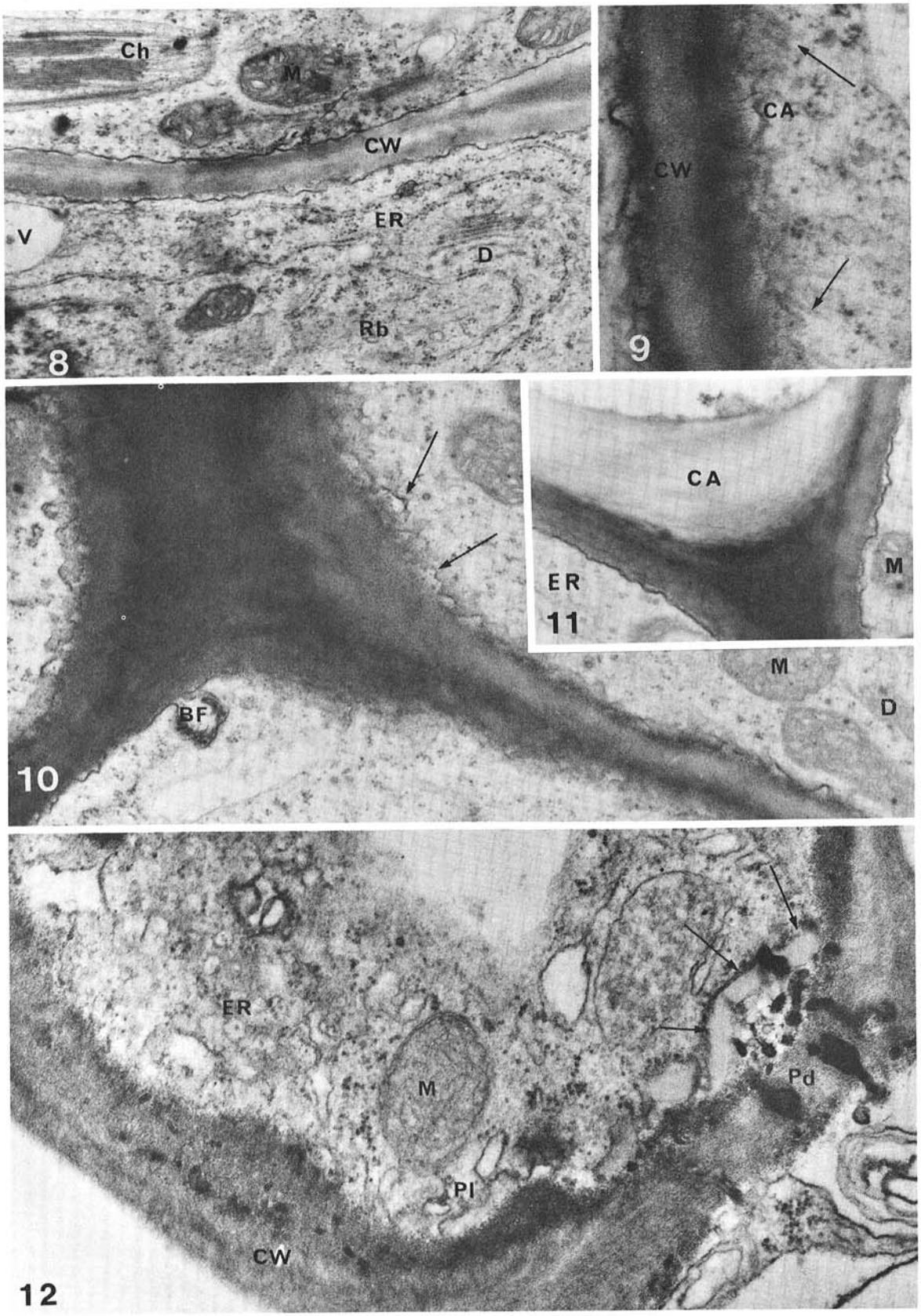
Fig. 8-12. **8)** A portion of a non-necrotic parenchymatous cell of Red Kidney bean showing chloroplast (Ch), dictyosome (D), mitochondrion (M), and vacuole (V). Note accumulation of ribosomes (Rb), and extensive system of endoplasmic reticulum (ER) near cell wall (CW) (X 26,000). **9)** Early callose (CA) deposition and presence of short, filamentous endoplasmic reticulum toward cell wall (CW) of a non-necrotic cell (arrows) (X 39,000). **10)** Roughening of secondary cell wall of non-necrotic cell, possibly caused by callose deposition (arrows). Boundary formation (BF) is in initiation in an opposite cell (X 41,000). **11)** Notable callose (CA) deposition on the secondary cell wall of a non-necrotic cell (X 26,000). **12)** A portion of a "seminecrotic" cell immediately adjacent to a necrotic cell of a PVM lesion showing thickening of secondary cell wall and callose deposition (arrows) at one end of plasmodesmata (Pd). Extensive development of vesiculated endoplasmic reticulum (ER) and invagination of plasmalemma (Pl) are evident (X 39,000).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between these two zones of cells. Callose formation is closely controlled by Ca^{++} in onion (9). Since deposition of Ca^{++} specifically in the cell walls of the resistant zone of TMV lesions was the most abnormal change in *Nicotiana glutinosa* and *N. tabacum* (31), further investigation of these points in relation to PVM infection would be of interest. A second mechanism involves blocking of virus by more complex "secondary wall thickening" which occurs in the infected cells surrounding necrotic cells. This process is initiated by boundary formation. Complete plugging and severing of intercellular cytoplasmic connections are achieved with the accumulation of tubules and vesicles (29). Cytoplasm containing virus particles in such a cell is thus enclosed in thickened walls (29, Fig. 13). The ultrastructure of this type of thickening is more complex than mere deposition of callose substance. To understand the process(es) involved in this secondary wall thickening, we need to establish more firmly the cellular factors and the specific functional roles of each organelle involved.

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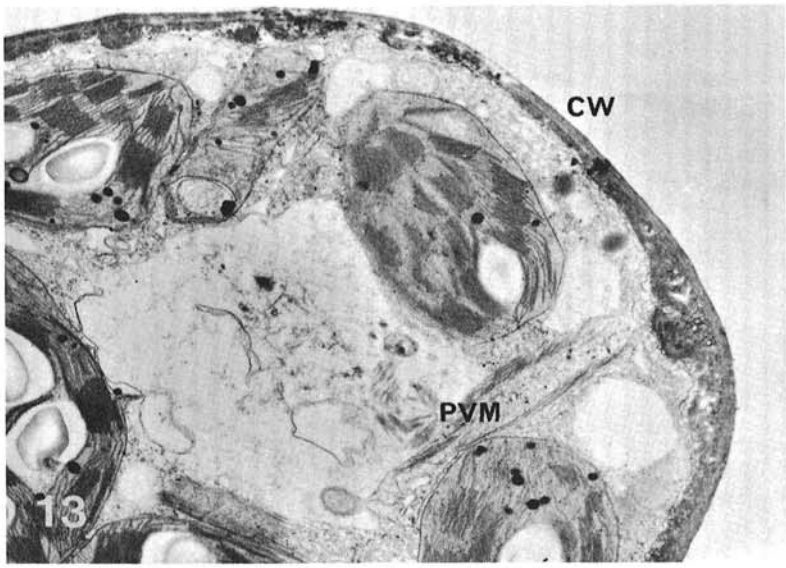
Fig. 13-17. 13) Potato virus M (PVM) particles observed in a "seminecrotic" cell of Red Kidney bean where cell wall (CW) thickening was in progress (X 15,200). **14)** Enlarged portion of a seminecrotic cell showing secondary wall thickening due to boundary formation in the area between the plasmalemma and the cell wall (X 30,400). **15)** The structure resembling abscission layer (arrow) formed between a seminecrotic and a necrotic cell. The thickness of a cell wall of the seminecrotic cell is not uniform (X 10,000). **16)** Necrotic cells stained electron-opaque. Cellular organelles were hardly discernible except collapsed chloroplasts containing thylakoids (arrows) around large starch grains (Sg) (X 21,600). **17)** A portion of cells revealing disintegration of cellular contents (X 16,200).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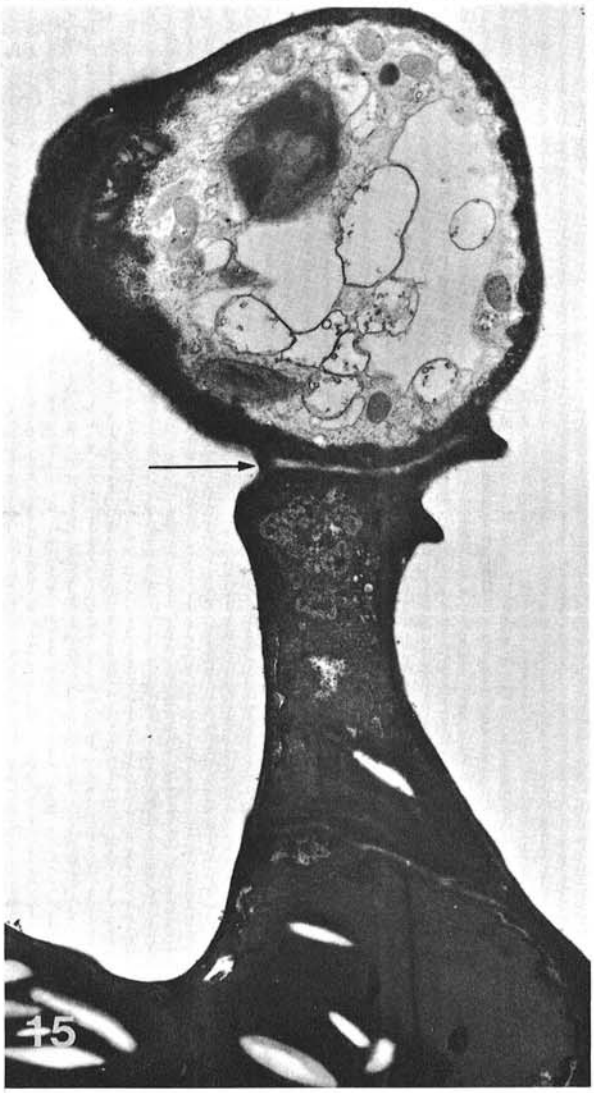
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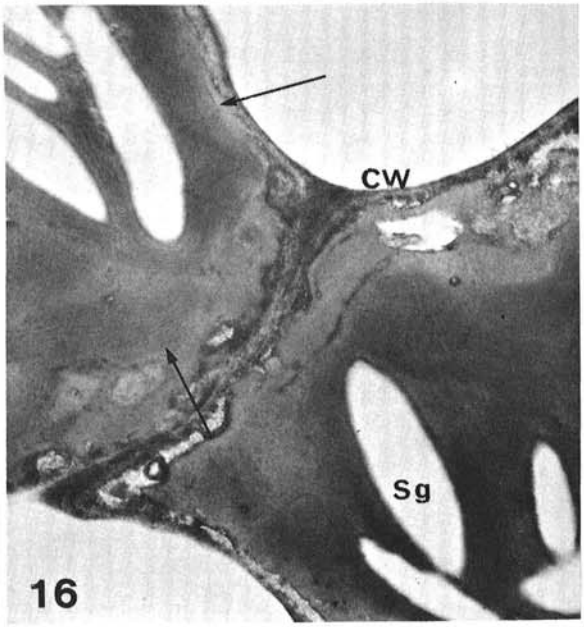
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