

カブモザイクウイルスと重複感染したダイコンにおけるキ ュウリモザイクウイルス濃度の増大について(2)

電子顕微鏡的,免疫組織化学的観察

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Increase in Cucumber Mosaic Virus Concentration in Japanese Radish Plants Co-infected with Turnip Mosaic Virus (II) Electron Microscopic and Immunohistochemical Observations

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Abstract

In Japanese radish plants infected with both cucumber mosaic virus (CMV) and turnip mosaic virus (TuMV), the concentration of CMV was significantly higher than in plants inoculated only with CMV. The distribution of viruses or viral antigens in inoculated plants was examined by electron microscopy and enzyme-immunohistochemistry. These observations indicated that the increase of CMV concentration was mainly due to an increase in the number of CMV-infected cells. Co-infection with TuMV had little effect on the cell-to-cell transport of CMV but significantly enhanced the long-distance transport of this virus.

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Key words: multiple infection, cucumber mosaic virus, turnip mosaic virus, long-distance transport, immunohistochemistry.

INTRODUCTION

In a previous report¹⁴⁾, we showed the concentration of cucumber mosaic virus (CMV) increased in Japanese radish plants (*Raphanus sativus* L.) co-infected with turnip mosaic virus (TuMV) as assayed by indirect ELISA. The increase was most significant in upper systemically infected leaves. Also, the symptoms on plants infected with both viruses were severer than on plants infected with TuMV alone. Japanese radish plants inoculated only with CMV did not display any stymptoms. There are reports that multiple infections with two unrelated viruses sometimes lead to an increase in the concentration of one virus above that found in plants infected with only one of the components of the mixtures. In some instances the increase resulted from enhanced multiplication^{4,5)}, in others from enhanced transport of virus¹⁾, or for both of these reasons¹³⁾. In this study, CMV and TuMV were inoculated to radish plants, either alone or together, and the infected plants were later examined by electron microscopy and enzyme-immunohistochemistry. The aim was to investigate whether TuMV enhanced the multiplication or the transport of CMV in radish plants infected with both viruses. In this paper, we report the enhanced long-distance transport of CMV in radish plants co-infected with TuMV.

MATERIALS AND METHODS

Viruses and plants. The virus isolates of CMV and TuMV were the same as used previously¹⁴⁾. They were propagated in tobacco (*Nicotiana tabacum* L. cv. Ky57) and turnip

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(*Brassica campestris* L. cv. Yoriikabu), respectively. Purified preparations of CMV and TuMV were, respectively, adjusted to final concentrations of 1 mg/ml and 0.1 mg/ml with 0.01 M phosphate buffer (PB) (pH 8.0) and inoculated to the cotyledons of young Japanese radish (cv. Miyasigesobutori) seedlings. Inoculated plants were grown in a growth chamber at 25 C with a 16 hr photoperiod.

Purification of viruses. CMV was purified from infected tobacco leaves according to the method of Lot *et al.*¹¹⁾, while TuMV was purified from infected turnip leaves by the method of Choi *et al.*³⁾

Electron microscopy. Small pieces of leaf tissue were prefixed with 5% glutaraldehyde in 0.1 M PB pH 7.2 for 16 hr at 4 C. Ribosomes were digested by the treatment with RNase⁶⁾ (2 µg/ml in 2×SSC, SSC=0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) for 16 hr at 25 C, after rinsing with PB and 2×SSC. They were postfixed with 2% osmium tetroxide for 2 hr at room temperature, and dehydrated with a graded acetone series and embedded in Spurr's medium. Ultrathin sections were cut with glassknives (Reichert OmU 4) and examined in an electron microscope (JEM100B) at 80 kV, after staining with uranyl acetate and lead citrate.

Enzyme immunohistochemistry. Small pieces of tissues were fixed with 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 12 hr at 4 C. After rinsing with PB, they were decolorized with ethanol and infiltrated polyethyleneglycol (PEG #1,000) at 40 C for 2 hr, and then embedded in PEG⁸⁾. Sections 12 µm thick were cut with a cryostat (CLINISTAT, Harris) at -4 C. The PEG in the sections was melted on slides at 40 C, and then removed with 99.5% ethanol. After drying for about 30 min at 40 C, they were immersed in 99.5% ethanol (10 min), 50% ethanol (5 min), and TBS (0.05 M Tris-HCl, 0.85% NaCl, pH 7.6, 2×10 min). Anti-viral antibodies (anti-CMV or anti-TuMV IgG, 2 µg/ml in BSA-TBS; BSA-TBS=0.2% BSA, 0.1% NaN₃-TBS) were incubated on the sections for 1 hr at 37 C in a moist chamber. After rinsing with TBS-Tween (0.05% Tween 20-TBS, 4×5 min) and TBS (5 min), they were incubated with a second antibody (alkalinephosphatase labeled anti-rabbit goat IgG, TAGO, diluted 100-fold with BSA-TBS) for 1 hr at 37 C. After rinsing as before, they were incubated for 5–10 min at room temperature with a substrate solution made by adding 9.8 ml 0.1 M Tris-HCl (pH 8.2) to 2 mg naphthol AS-MX phosphate (Sigma) dissolved in 0.2 ml dimethylformamide and then immediately before use, adding to this solution Fast Red TR Salt (Sigma) to 1 mg/ml. The sections were then rinsed with distilled water to stop the reaction, mounted in 40% glycerol and examined using a light microscope (Olympus BH-2).

RESULTS

Electron microscopic observations on infected cells

Single infection with CMV. Since the size and shape of CMV particles are similar to those of ribosomes in the cytoplasm of infected radish leaf cells, it is rather difficult to discriminate between them. However, digestion of the ribosomes with RNase allowed us to discriminate the CMV particles very clearly. CMV particles were found scattered in the cytoplasm of cells in both the inoculated (Plate I-A) and the upper systemically infected leaves (Plate I-B). However relatively few of cells in the systemically infected leaves contained CMV particles. Tonoplast-associated vesicles containing fibrils were also observed in cells with CMV particles.

Single infection with TuMV. TuMV particles and cytoplasmic cylindrical inclusions (CI) were often observed in the cytoplasm of epidermal, mesophyll, and phloem cells of both the inoculated and the upper systemically infected leaves. In systemically infected leaves with mosaic symptoms, TuMV particles and CI were observed mainly in cells from the light-green areas. TuMV particles were often associated with the CI and occasionally found aggregated together. Vesicles containing fibrils were often observed in the cytoplasm.

Dual infection with CMV and TuMV. In the cotyledons inoculated with viruses

it was found that during the early stage of infection, some cells contained only CMV, some contained only TuMV, and some contained both viruses. However many cells were infected with both viruses at a later stage (Plate II-A, B). In the upper systemically infected leaves with mosaic symptoms, most of the cells in the light-green areas contained CMV particles, TuMV particles and CI (Plate I-C). In cells of the dark-green areas, CMV particles were often observed, but TuMV was not. The CMV particles were mainly scattered in the cytoplasm and were rarely observed in the vacuoles of infected cells. Tonoplast-associated vesicles were also observed. Sometimes, electron-dense structures containing CMV particles were also observed. These structures were seen in specimens treated either with (Plate II-A, B), or without RNase (Plate II-C). We postulate that these structures are the sites of virus assembly.

Detection of viral antigens by enzyme-immunohistochemistry

Detection of CMV. Table 1 summarises the results from examinations for CMV of radish plants whose cotyledons had been inoculated with CMV, either alone, or together with TuMV. In plants inoculated only with CMV, the virus was first detected in the inoculated cotyledons at 2 days post inoculation (p.i.) (Plate III-A), in the petioles of cotyledons at 5 days p.i., and in the upper systemically infected leaves of some plants at 6 days p.i. (Plate III-F). In some plants, CMV could be detected in neither upper leaves nor the petioles of cotyledons.

In plants inoculated with both viruses, CMV was first detected in the inoculated leaves at 2 days p.i., as same as in singly inoculated plants. Then, the virus was detected in both the petioles (Plate III-C), stems (Plate III-E) and upper leaves of most plants at 4 days p.i., which was 1–2 days earlier than for plants inoculated only with CMV. At 8 days p.i., CMV was distributed throughout the upper leaves (Plate IV-A). The frequencies of CMV detection were always significantly higher for these plants than for those inoculated only with CMV. In plants inoculated with both viruses the results from examinations of the cotyledon petioles for CMV were always as high as those from examinations of their upper leaves.

Detection of TuMV. Table 2 shows the results from examinations for TuMV. This

Table 1. Detection of CMV by the enzyme-immunohistochemistry in radish plants infected with either both CMV and TuMV or CMV alone

Viruses ^{a)} inoculated	Parts of plants	Days after inoculation					
		3	4	5	6	7	8
CMV	Petioles of cotyledons	0/5 ^{b)}	0/4	4/8	2/4	3/4	9/14
	Upper leaves	0/2	0/4	0/9	1/4	1/4	2/14
CMV+ TuMV	Petioles of cotyledons	0/4	3/5	7/8	—	—	5/6
	Upper leaves	0/4	3/5	9/9	—	—	6/7

a) Viruses were inoculated to the leaf blades of the cotyledons.

b) No. of plants in which CMV was detected/No. of plants examined.

Table 2. Detection of TuMV by the enzyme-immunohistochemistry in radish plants infected with either both CMV and TuMV or TuMV alone

Viruses ^{a)} inoculated	Parts of plants	Days after inoculation				
		2	3	4	5	8
TuMV	Petioles of cotyledons	2/2 ^{b)}	4/4	—	4/4	—
	Upper leaves	—	4/4	—	4/4	—
TuMV+ CMV	Petioles of cotyledons	2/2	4/4	5/5	8/8	6/6
	Upper leaves	—	4/4	5/5	9/9	7/7

a) Viruses were inoculated to the leaf blades of the cotyledons.

b) No. of plants in which TuMV was detected/No. of plants examined.

virus was detected both in the inoculated leaves and the phloem of the cotyledon petioles at 2 days p.i. (Plate III-B). At 3 days p.i., it was also detected in the upper leaves of all plants (Plate III-D), and virus was widely distributed in the upper leaves at 8 days p.i. (Plate IV-B). No differences were observed between the rates of spread of TuMV in plants inoculated with TuMV alone and those inoculated with both viruses.

Spread of CMV in inoculated stems

To investigate the cell-to-cell transport of CMV in tissues inoculated with this virus, either alone or together with TuMV, the surfaces of stems were rubbed with virus preparations and then later examined for spread of CMV into the cortex tissue. There was no difference between the rates of spread of CMV into the cortex tissue of plants inoculated with either one (Plate IV-D, F) or both viruses (Plate IV-C, E). These rates were also similar to the rates in plants that had been pre-infected with TuMV (before 3 days). However it should be noted that the concentration of CMV in the phloem tissue was higher in plants inoculated with both viruses (Plate IV-C, E). And frequencies of CMV detection in upper leaves were significantly higher in co-inoculated plants.

Distribution of viral antigens in systemically infected leaves showing mosaic symptoms

CMV was distributed in both the light- and dark-green tissues of leaves showing mosaic symptoms, while TuMV was distributed mainly in light-green tissues. This was in agreement with the results of the electron microscopic observations.

DISCUSSION

In the leaves of plants infected with both viruses, many cells contained both CMV and TuMV (virion and CI), and tonoplast-associated vesicles found in CMV infected cells⁷⁾ were also often observed. These observations indicated that both of these viruses can multiply in the same cell of radish plants, as has been previously reported for some other multiple infections with two unrelated viruses^{2,4,9,10,13)}. It was obvious that in plants inoculated only with CMV, the virus was able to multiply not only in the inoculated cotyledons but also in upper systemically infected leaves. Although a few cells in plants inoculated with both viruses contained more CMV particles than the cells containing CMV in plants inoculated with this virus alone, similar numbers of virus particles were observed in the most CMV infected cells. Therefore the difference in CMV concentration in leaf extracts from the two groups of plants as measured by indirect ELISA¹⁴⁾ was due to, not the amount of CMV per infected cell, but to the number of the cells that became infected with CMV.

The results from immunohistochemistry clearly indicated that TuMV infection enhanced the long-distance transport of CMV in radish seedlings but did not affect the cell-to-cell transport of the virus. There was no difference between the treatments in the amount of CMV in the inoculated cotyledons, but very large differences were observed in the times and frequencies of CMV detection in the petioles of inoculated cotyledons and in the upper systemically infected leaves. The higher concentration of CMV in the upper leaves of radish plants co-infected with TuMV was due to an increase in the number of cells that became infected with CMV as a result of enhanced long-distance transport. We did not establish which steps of the CMV long-distance transport process were enhanced by co-infection with TuMV. It is thought that the long-distance transport of CMV occurs mainly via the phloem tissue. A lower concentration of CMV in the phloem would lead to lower accumulation of CMV in the petioles and the upper leaves. In fact we found a lower concentration of CMV in the phloem of plants not inoculated with TuMV. It is possible that transport of CMV from the mesophyll to the phloem was retarded in plants inoculated only with this virus. Alternatively, many CMV particles (or some other transportable form of the virus) may have been degraded in the phloem. Another possibility is that facet(s) of TuMV multiplication changed the physiological conditions of cells

so that either the transport of CMV was enhanced, the degradation of CMV was prevented, or that some TuMV-coded protein(s) assisted in functions involving the transport of CMV. It is known that viral coded non-structural proteins are responsible for the cell-to-cell transport of some viruses^{12,15}. In our experiments, no functional defectiveness of the cell-to-cell transport of CMV was observed. The mechanism of long-distance transport of viruses might be different from that of cell-to-cell transport. It is not certain whether the non-structural proteins involved in cell-to-cell transport are also required for long-distance transport.

We found that enzyme-immunohistochemistry technique using alkalinephosphatase labeled conjugate was useful for detecting the virus antigens *in situ*. This method should be generally applicable, and helpful for the detection of other viral antigens in plant and vector tissues in the future.

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和 文 摘 要

石本万寿広・佐野義孝・小島 誠：カブモザイクウイルスと重複感染したダイコンにおけるキュウリモザイクウイルス濃度の増大について (II) 電子顕微鏡的、免疫組織化学的観察

TuMV と重複感染したダイコンにおける CMV 濃度増大の要因を明らかにするために、感染細胞の電子顕微鏡観察を行うとともに免疫組織染色法により組織内のウイルス抗原の分布を調べた。その結果、重複感染細胞と単独感染細胞では細胞当りの CMV 量に著しい差は認められなかったが、上位葉で CMV が検出される時期ならびに頻度に大きな差が認められた。このことから、重複感染株における高い CMV 濃度は TuMV との重複感染により CMV の維管束を介した長距離の移行が促進され、それにより CMV 感染細胞数が増加した結果であると考えられた。

Explanation of plates

- Plate I.** Electronmicrographs of Japanese radish leaf cells infected with CMV, either alone or together with TuMV.
- A. CMV particles at 8 days post-inoculation (p.i.) scattered in the cytoplasm of a cotyledon inoculated with CMV. $\times 43,200$
 - B. CMV particles scattered in the cytoplasm of an upper systemically infected leaf cell (8 days p.i.). $\times 43,200$
 - C. A cell from the co-inoculated upper systemically infected leaf at 8 days p.i. CMV particles, TuMV particles and CI are seen together in one cell. $\times 38,400$
- Plate II.** Electronmicrographs of Japanese radish leaf cells infected with both CMV and TuMV 8 days post-inoculation.
- A. A cell of the co-inoculated cotyledon. CMV particles are seen associated with some electron-dense structures (arrows). $\times 43,200$
 - B. Enlarged view of A. $\times 72,000$
 - C. The electron-dense structure of the specimen without RNase treatment. $\times 76,800$
- Plate III.** Micrographs of the enzyme-immunohistochemically-stained tissues inoculated with CMV and TuMV, both alone and together.
- A. Distribution of CMV antigen at 2 days p.i. in a cotyledon inoculated with CMV.
 - B. Distribution of TuMV antigen in the phloem cells of a cotyledon petiole inoculated with TuMV at 2 days p.i.
 - C, E. Distribution of CMV antigen in a petiole of the cotyledon (C) and a stem (E) inoculated with both CMV and TuMV at 4 days p.i.
 - D. Distribution of TuMV antigen in an upper systemically infected leaf inoculated with TuMV at 3 days p.i.
 - F. Distribution of CMV antigen in an upper systemically infected leaf inoculated with CMV alone at 6 days p.i.
- Plate IV.** Micrographs of the enzyme-immunohistochemically-stained tissues inoculated with CMV, either alone or together with TuMV.
- A, B. Distribution of CMV antigen (A) and TuMV antigen (B) in an upper systemically infected leaf inoculated with both viruses at 8 days p.i.
 - C, D. Distribution of CMV antigen in stems inoculated with CMV, either alone (D) or together with TuMV (C).
 - E, F. Enlarged view of the phloem of C and D, respectively.

Abbreviations: Ch, chloroplast; Cx, cortex tissue; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; Ph, phloem; V, virion of TuMV; Va, vacuole; Vb, vascular bundle; Xy, xylem.

Plate I

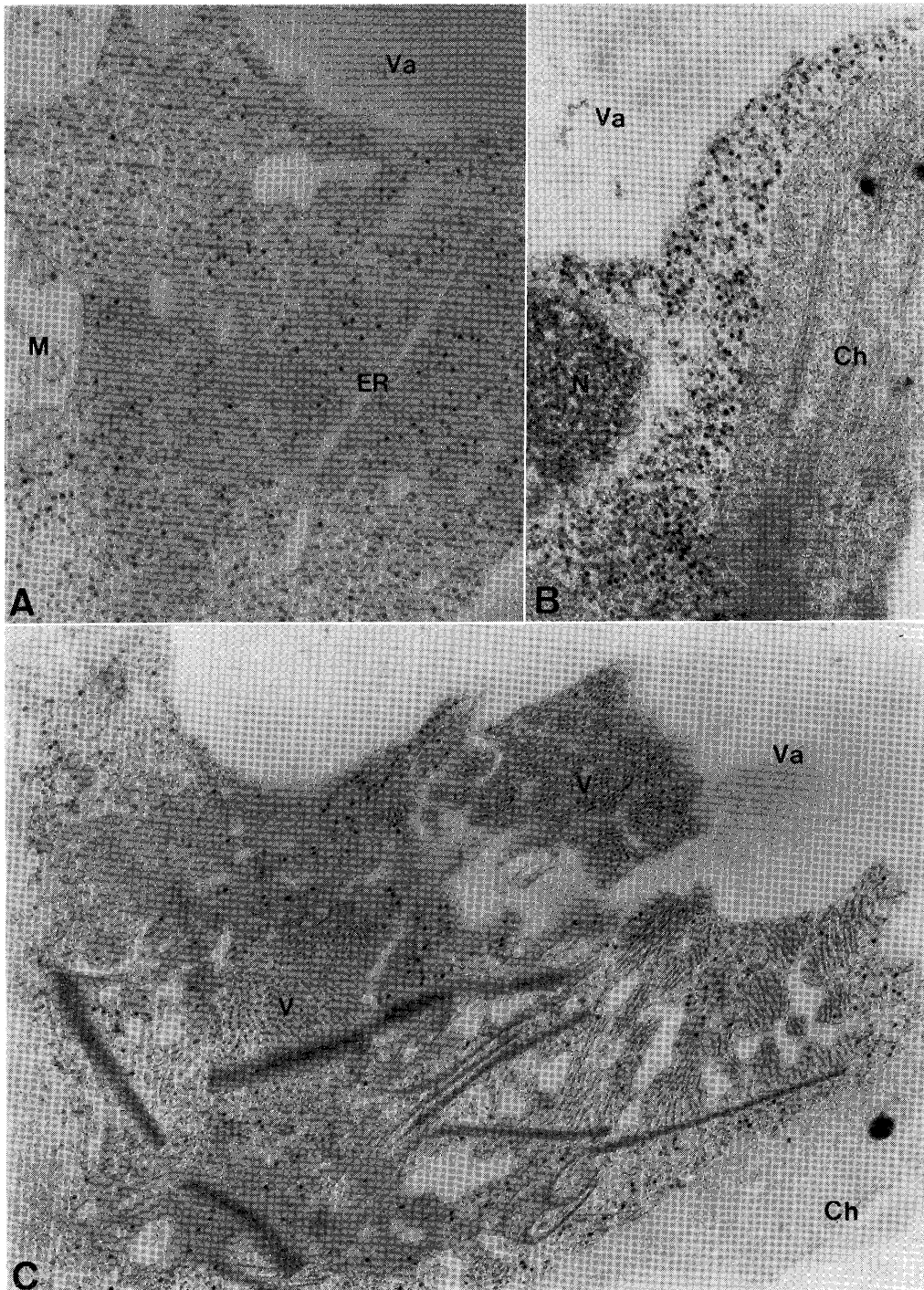


Plate II

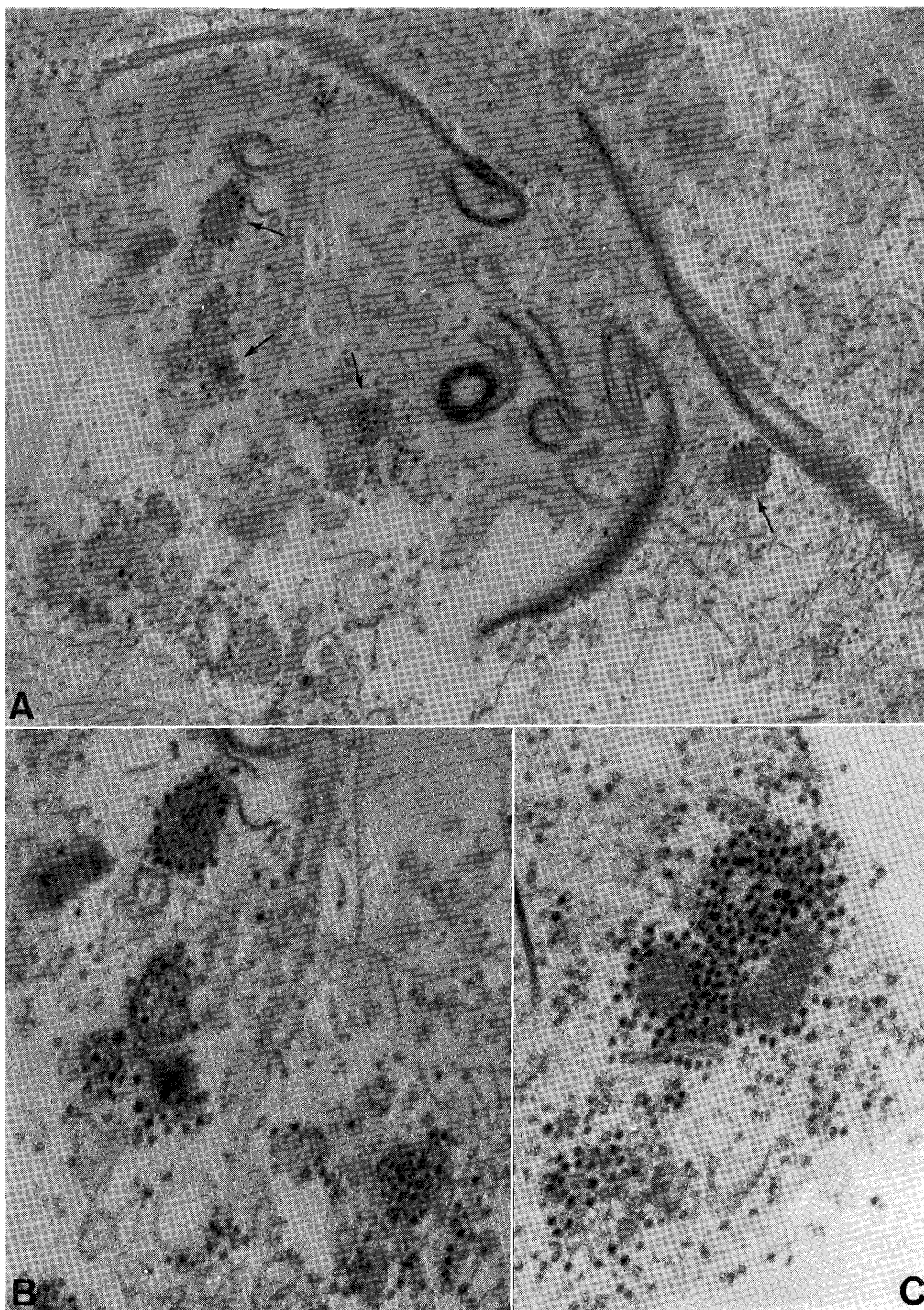


Plate III

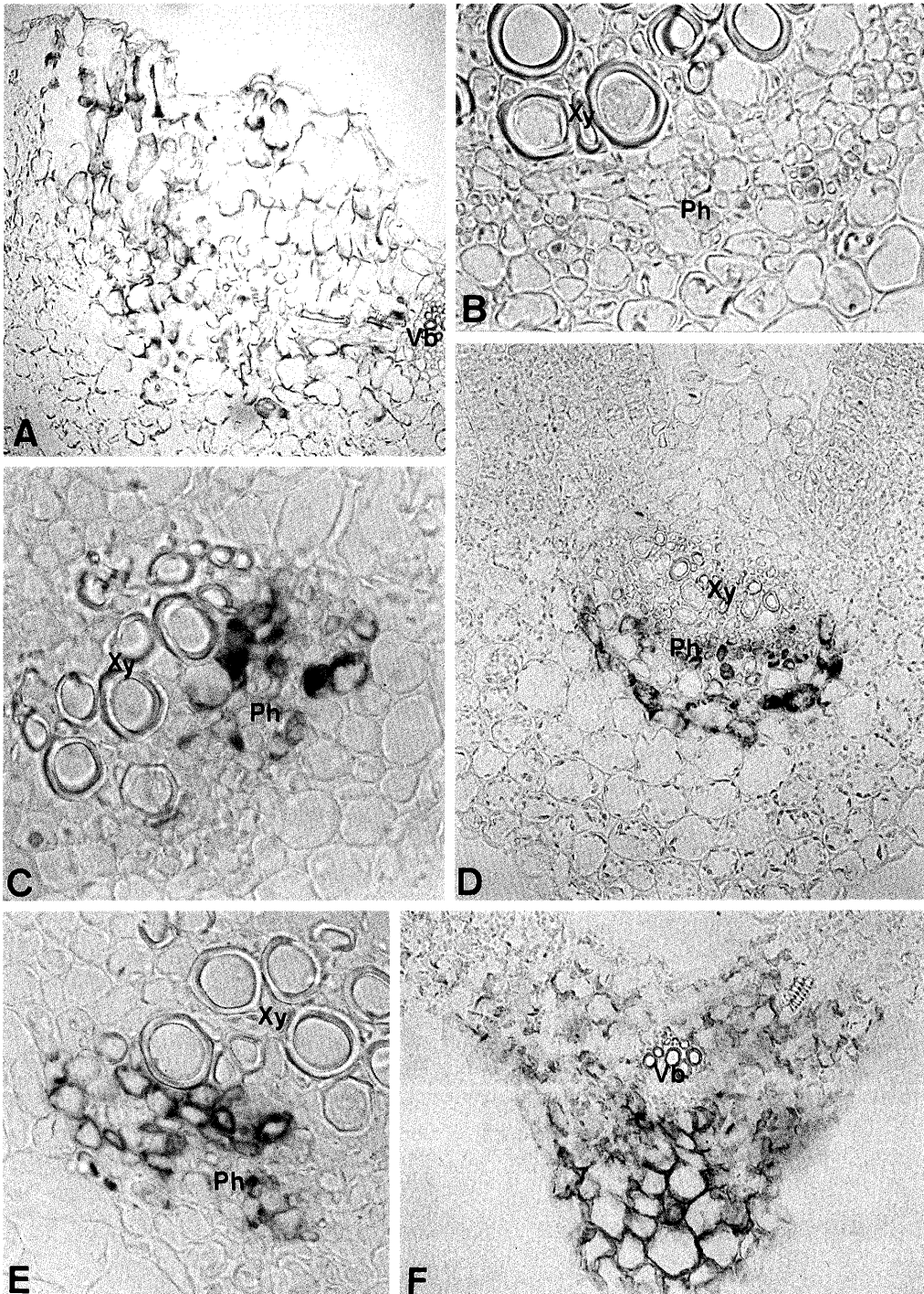


Plate IV

