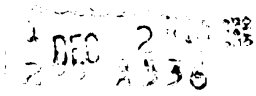

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ABSTRACT

ELECTRON MICROSCOPY OF TOBACCO TISSUE
CULTURE INOCULATED WITH TMV AND OF
VIRUS AND MYCOPLASMA-LIKE
DISEASES OF MICHIGAN
HIGHBUSH BLUEBERRY

By

James X. Hartmann

The development of infection in plant tissue culture cells inoculated with TMV was followed by electron microscopic examination of ultrathin sections. No natural openings which may serve as avenues of virus entry were located in cell walls exposed directly to the external medium. However, plasmodesmata 60-75 nm wide and irregularly shaped openings as large as 900 nm were present in cell walls between cells. Amorphous, electron dense material which occurred within cells appeared to have been secreted into the external medium. Polyribosomes were frequently associated with numerous small vesicles as early as thirty-six hours after infection. Virus was first detected in the cytoplasm as small crystalline aggregates at 48 hr after inoculation. Amorphous X-body

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inclusions were first seen 60 hr after inoculation in cells containing large virus crystals and an apparently high virus content. Infected cells appeared to have undergone division and daughter cells contained virus. Infected cells at 60, 72, 96 and 120 hr after inoculation were similar in appearance.

Virus-like particles spherical in outline and measuring 26-28 nm in diameter 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 phloem vascular tissue. Epidermal leaf cells and root xylem cells contained crystalline arrays of particles. Larger masses of virus-like particles were seen in root than leaf cells. Particles hexagonal in outline and 28-31 nm in diameter were partially purified from diseased leaves. Observation of virus-like particles <u>in situ</u> and purification from symptomatic leaves is presented as presumptive evidence for virus causation of blueberry shoestrong disease. No such particles were observed in healthy blueberry tissue.

Mycoplasma-like organisms were found in ultrathin sections of phloem sieve tube elements from stunt diseased blueberry tissues. Spherical, elongate and irregular forms, as evidenced by serial section methods, were

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present in leaf and fruit pedicel tissue. A distinct external unit membrance, presumed ribosomes and fibrillar nucleic acid were characteristic of these structures. No mycoplasma-like organisms were observed in healthy blueberry plants of the same varieties. Large, electron dense inclusions were present in companion cells adjacent to degenerate sieve tubes containing small forms of mycoplasma-like organisms. Inclusions of this type were not detected in healthy phloem tissue.

ELECTRON MICROSCOPY OF TOBACCO TISSUE CULTURE INOCULATED WITH TMV AND OF VIRUS AND MYCOPLASMA-LIKE DISEASES OF MICHIGAN HIGHBUSH BLUEBERRY

By

James X. Hartmann

A THESIS

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This dissertation is sincerely dedicated to Diana, my understanding wife and the loving mother of my two sons. She has been most patient and willing to sacrifice her time and leisure that I might complete this work.

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PART THREE

OCCURRENCE AND NATURE OF MYCOPLASMA-LIKE ORGANISMS IN STUNT DISEASE OF MICHIGAN HIGHBUSH BLUEBERRY

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PART TWO

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PART ONE

ELECTRON MICROSCOPY OF TOBACCO TISSUE CULTURE *NICOTIANA TABACUM* L. INOCULATED WITH TOBACCO MOSAIC VIRUS

Introduction

A study of the sequential events in multiplication of a plant virus in plant cell culture has not been previously accomplished at the ultrastructural level. Nakata and Hildebrandt (1967) have described some of the fine structure of tobacco callus cells derived from plants infected with tobacco mosaic virus (TMV). However, electron microscopy of events in cells following viral inoculation *in vitro*, at a specified time in their growth phase, has not been possible. A low efficiency of infection and subsequent low virus titer, usually less than 5% of that in host leaves (Kassanis, 1967), has precluded time sequence studies at the electron microscope level.

Recently a greatly improved method for inoculating tobacco callus cells with TMV has been described (Murakishi *et al.*, 1970). The growth curve of virus multiplication in this system has also been determined (Murakishi *et al.*,

1970). In the present report an attempt was made to elucidate the nature and mode of infection in this plant virus-plant cell culture system. In particular, a correlation has been sought between events observed at the ultrastructure level and the virus growth curve previously reported (Murakishi et al., 1971).

A detailed description of the cultured plant cells, such as cell wall structure, cytoplasmic content in relation to growth, and appearance of plasmodesmata, was attempted in this study. Comparative studies were made on the type and concentration of fixatives and embedding materials to prepare tissue culture cells for electron microscopy.

Literature Review

Introduction

The recent text by Esau (1968) and other review articles (Esau, 1967; Schlegel et al., 1967; Schneider, 1965) provide much information regarding electron microscopy of plant viruses in host cells. Moreover, these references include comparative studies on various viruses and host responses.

The present review more specifically concerns electron microscopy and closely related studies on tobacco mosaic virus in plant host cells. No attempt is made to

include all the reports on electron microscopy of other plant viruses, but selected papers are included if they are pertinent to the review.

I. Uptake of Virus into Cells.

Past literature has considered two possible means by which a mechanically transmitted virus can enter a plant cell. Virus may be uncoated outside the cell wall or cell membrane and enter as nucleic acid, or intact virus may enter the protoplast and then be uncoated. Best (1965) suggested that TMV ejected its nucleic acid after the virus protein coat adsorbed to the cell wall.

Direct evidence for pinocytic activity in higher plants and pinocytosis of TMV was shown by electron microscope studies (Cocking, 1965, 1966). It was concluded that rupturing of the plasma membrane was not necessary for virus entry. Examination revealed TMV adsorbed at the surface of protoplasts, particularly in small invaginations. Vesicles containing TMV were observed three hours after inoculation but were seen more frequently at seven hours.

Staining procedures designed to enhance the appearance of the protein coat of TMV particles <u>in situ</u> were developed in Cocking's laboratory. Both prolonged soaking in uranyl acetate and phosphotungstic acid were employed. Cocking and Pojnar (1968, 1969) utilized these

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staining techniques to follow uncoating of TMV. After six hours incubation of protoplasts with TMV, 30% of the sections examined showed some virus in vesicles. Due to a loss in thickness of the particles, it was concluded that some particles were being rapidly uncoated. A recent review of viruses in isolated plant protoplasts describes the above experiments in greater detail (Cocking, 1970).

Although Cocking showed that protoplasts can take up virus by pinocytosis, the question of how virus traversed the cell wall remained unanswered. It has been generally assumed that with mechanically transmissible viruses, wounding or breakage of the cell wall is required; or natural openings to the exterior, such as ectodesmata, function as avenues of entry (Mundry, 1963).

An electron microscope study on penetration of TMV into leaves following experimental inoculation has been carried out (Gerola et al., 1969). Leaves were rubbed with a TMV and carborundum suspension, and modifications of the leaf surface were studied on replicas and ultrathin sections. Scratchings and a great number of TMV particles were seen on the leaf surface. Some of the virions were oriented in a vertical position. In thin sections of epidermal cells, the cell wall showed blebs under the cuticle.

It was inferred from the above studies that rubbing favored implantation of virus on the cuticle, and the

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alterations in the cell wall provided an "easy way" of penetration of particles into epidermal cells. Crossing of the cell wall by virus was "facilitated" by the blebs that formed inside it.

A possible means of crossing the cell wall once virus is under the cuticle can be postulated from Brant's (1964, 1965, 1966) studies on ectodesmata. The number of ectodesmata demonstrable was increased by applying pressure to leaves or treating with glucose and other compounds. When treated and non-treated leaves were inoculated with TMV, there was an increase in the number of lesions on treated leaves. When ^{14}C-labeled TMV was inoculated onto treated leaves, the distribution of β tracks corresponded to the distribution of plasmodesmata.

II. Multiplication

Although Black (1950) and Goldin (1960) observed TMV and TMV inclusions in ultrathin sections of infected cells, no conclusions were drawn regarding possible stages of virus multiplication. Light microscope work in Zech's laboratory (see review by Mundry, 1963) facilitated studies at the electron microscope level on the sequence of events following inoculation with TMV. von Wettstein and Zech (1962) reported the first, and to this date the only, definitive electron microscope study of early events in plant virus infection. An accurate timing of events was

possible because a single apical cell on the leaf hair cell chain was inoculated. After infection of this cell, successive infections could proceed only toward the base of the leaf hair.

The events observed at the electron microscope level correlated with phases deduced from cytochemical studies which had implicated the nucleus as the site of viral RNA synthesis. During the first phase of TMV reproduction, thirty minutes to two hours after infection, the nucleus became enlarged and traversed by an elaborate system of cytoplasmic channels. Nuclear material was observed within the channels and appeared to have been pinched off from the nucleus and transported through the channels into the cytoplasm.

During the second phase of TMV multiplication, a pronounced increase in cytoplasmic mass took place. In the third phase, cytoplasmic strands traversed the cell vacuole. The endoplasmic reticulum appeared as closed concentric structures and was coated on the cytoplasmic side with electron dense material.

Finally, during formation of TMV crystals, twenty-three hours after inoculation, the intranuclear channels had receded. Many inclusions with virus particles were distributed in strands extending from the nucleus to the cytoplasm.

Electron microscope studies following those of von Wettstein and Zech employed infected leaf tissue. Due to the small percentage of cells infected, later stages of infection were investigated. Shalla (1964) studied virus multiplication in tomato leaflets, at various intervals after inoculation, by electron microscopy and local lesion assay methods. At forty-eight hours post inoculation, prior to the presence of assayable virus, individual TMV particles and virus aggregates were observed in the ground cytoplasm of mesophyll cells. At seventy hours, just prior to rapid increase in infectivity, virus was encountered more frequently. The most rapid synthesis occurred 80-300 hours after inoculation.

Shalla's primary conclusion was that TMV particles were assembled in the ground cytoplasm. The apparent absence of particles in any other part of the cell in early infection led to this conclusion.

The advantage of fixing infected tissue in both glutaraldehyde and osmium, instead of osmium alone as done previously, was demonstrated by Kolehmainen et al. (1965). Although more fine structure of the host cell was preserved, only the ribonucleic acid-containing core of the virus particles was visible. Their work showed possible stages in crystal development, and an interpretation of X-body structure was given.

Milne (1966a) sampled cells at defined time intervals and first saw virus fifteen hours after inoculation. Filamentous regions were always associated with infection, but the filaments did not appear to be direct precursors of virus.

A study on the distribution of TMV in tobacco plants was carried out by Esau and Cronshaw (1967a). TMV crystalline inclusions and X-bodies were seen in epidermal, mesophyll, and parenchyma cells and in tracheary and sieve tube elements. Furthermore, TMV was present in the nuclei, chloroplasts, ground cytoplasm, vacuole, and between the plasma membrane and cell wall of parenchyma cells. They concluded that distribution of TMV was not limited to a particular host tissue or location within a cell.

Although TMV did not selectively multiply in a particular type of tissue, there appeared to be areas of cells in systemically infected leaves in which TMV could not multiply. Atkinson and Matthews (1967, 1970) have observed TMV crystals in yellow-green but not in dark-green areas of mosaic leaves.

The interaction of TMV with other virus, tobacco etch, has been studied by electron microscopy (Fujisawa et al., 1967). Interference with multiplication or simultaneous multiplication occurred, depending on the time interval between the sequence of inoculations. Both

viruses occurred in the same cell when it was infected simultaneously.

The literature reviewed thus far has centered on observations of infected cells, but little information regarding the site of synthesis of viral RNA and protein was given. Autoradiography at the light and electron microscope levels has been employed to determine the site of viral RNA and protein synthesis. Smith and Schlegel (1965) and De Zoeten and Schlegel (1967) utilized uridine-^{3}H incorporation into infected leaf discs to follow synthesis of virus RNA. Host cell RNA synthesis was inhibited with actinomycin-D. Incorporation into nuclei and nucleoli of TMV and clover yellow mosaic virus (CYMV)-infected plants was found. In the case of TMV, as the time following a labeling period increased, the label appeared to have moved into the cytoplasm and into TMV crystals. Electron microscope autoradiography was particularly useful in showing incorporation in the nucleolus in CYMV infection.

TMV protein has been localized in tobacco cells during the early stages of infection. Antibodies specific to TMV were labeled with ^{125}I, and electron microscopic autoradiography used to determine the site of virus protein synthesis. The distribution of label suggested that virus protein synthesis occurred in the nucleus, and the protein then moved to the cytoplasm. The amount of label in the nucleus rapidly increased until it was four times

that of the control at forty-eight hours. Label in the cytoplasm began rapidly increasing twelve hours after inoculation, and at forty-eight hours was ten times that of the control (Langenburg and Schlegel, 1969).

Two major obstacles to sequential studies of plant virus replication referred to by Schelgel et al. (1967) are, "the hererogenous condition of plant cells in the early stages of infection and the high multiplicity required for infection." Perhaps the closest approach to studying events in a partially synchronous system was made by Nilsson-Tillgren et al. (1970). Systemic infection in which fully expanded leaves were evenly infected was obtained under certain conditions. The first sign of systemic infection in the above system was veinclearing, in which a majority of cells seemed to be in the same stages of infection. Veinclearing occurred 70-120 hours after inoculation. At 16-18 hours before veinclearing, virus particles and small crystals were found in only six per cent of the cells in small areas of the leaf. About twenty hours after veinclearing, the number of infected cells leveled off at ninety per cent.

Nilsson-Tillgren et al. (1970) also encountered the recurrent problems of low efficiency of infection and variability of the host plant response. The necessity of developing cell systems similar to those utilized in animal virology remains obvious.

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III. Crystalline Inclusions

The reader who is unfamiliar with the early light microscope studies concerning crystalline inclusions is referred to several excellent reviews (Mundry, 1963; McWhorter, 1965; Esau, 1967; and Allen, 1968). As a brief introduction to the electron microscopy of TMV crystalline inclusions, three basic types of virus inclusions have been noted. These include (a) regular crystalline bodies with external plane faces; (b) thin paracrystalline bodies with only two dimensional structure; and (c) vacuolate, ameboid, amorphous bodies (McWhorter, 1965).

Individual TMV rods were first seen by Kausche et al. in 1939. The first electron micrograph of a hexagonal crystalline TMV inclusion was presented by Rubio-Huertos (1950). Black et al. (1950) also observed TMV crystals in thin section. Steere and Williams (1953) illustrated the presence of numerous rod-shaped particles in a hexagonal crystal. The crystal was extracted from an infected cell after freeze drying.

In 1956, Rubio-Huertos found differences in the type of X-bodies and crystals of TMV depending upon the strain of TMV used. With the common strain of TMV, very few virus particles were present in X-bodies, whereas much virus was present when aucuba mosaic strain was employed.

After examination of TMV-inoculated tomato cells, Shalla (1959) stated that unless crystalline inclusions

were present in cells, rod-shaped particles would not be present. Goldin (1960) agreed with this statement after studying crystal-containing material fixed in trichloroacetic acid. The lack of appropriate fixation and embedding procedures may have accounted for the inability to detect individual virus particles in cells other than those containing crystals.

The development of infection in leaf hairs has been followed. It was found that crystals appeared after a pronounced increase in cytoplasmic material. Intranuclear channels receded at the time of crystal appearance. In strands radiating from the nucleus to the cytoplasm many inclusions with virus particles were observed (von Wettstein and Zech, 1962). Unfortunately, in this study glutaraldehyde and osmium fixation were not employed, and exact interpretation of their micrographs is difficult.

With improved fixation procedures, TMV particles were identified in the cytoplasm before crystals were visible (Shalla, 1964). Aggregation of particles in small areas was interpreted as a developmental stage in crystal formation. Kolehmainen et al. (1965) extended this observation on formation of crystals by finding single layers of virus between endoplasmic reticulum and ribosomes. It was suggested that after continual dispersion and recrystallization of virus, ribosomes and endoplasmic reticulum were eliminated.

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It wasn't until 1966 that a method for preservation of TMV crystals for electron microscopy was reported (Warmke and Edwardson, 1966a). Previously, potassium permanganate had been shown to dissolve and damage TMV, but these workers found that very short fixation periods (1 to 4 minutes) allowed excellent preservation of crystals. They saw that the three dimensional hexagonal crystals were composed of several to many monolayers. Paracrystals were interpreted as linear aggregates of particles, aligned end to end, which had resulted from breakdown of hexagonal crystals (Warmke and Edwardson, 1966b).

Besides the use of potassium permanganate for crystal preservation, Warmke and Christie (1967) found that very dilute solutions of osmium tetroxide (0.08-0.02%) preserved crystals. The images were similar to those produced by potassium permanganate, ribosomes were preserved, and clear areas around virus aggregates did not occur. Permanganate was superior in preserving membrane systems and in maintaining a larger percentage of crystals intact.

The major drawback of Warmke's procedures was that while crystals were well preserved, most host cell components were not. Esau (1968) has since published work in which a balance was achieved. Karnovsky's fixative followed by osmium tetroxide did not preserve the exact relationship of crystals, but did prevent their re-distribution while

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IV. X-Body Inclusions

The term "X-body" was coined by Goldstein (1924, '26) after detailed cytological studies of TMV-infected cells. Iwanowski (1903) had earlier described similar granular, vacuolated, amoeba-like inclusions which ranged in size from 5-30μ.

Light microscope studies (Zech, 1952, 1954; Hirai and Wildman, 1963) indicated that X-bodies appeared in two days, vacuolate X-bodies in four days, incomplete virus crystals in four days, and complete crystals in six days. Much additional light microscope work followed these studies. The fine structure of X-bodies remained undetermined until the development of appropriate fixation and staining techniques for electron microscopy.

In 1964, Shalla used Dalton's chrome osmium fixative to prepare TMV-infected tomato leaflets. Heavily staining filaments approximately four times the width of TMV particles were present in ultrathin sections. The filaments were found in the cytoplasm and interpreted as possible developmental forms of TMV, and the principal components of X-bodies.

Kolehmainen et al. (1965) found unordered or well-oriented masses of tubes in the cytoplasm of infected tobacco leaves. They noted that the thick filaments described by Shalla (1964) were actually three or more aggregated microtubules. They did not associate the tubules with X-bodies. Electron dense globules of highly variable size were observed in the cytoplasm. It was suggested that X-bodies consisted of aggregates of these globules and vacuoles containing a few TMV rods.

Filamentous regions have been detected as early as fifteen hours after TMV infection of tobacco leaf palisade cells (Milne, 1966a). The filaments were always associated with infection, but did not appear to be direct TMV precursors. Milne (1966b) also examined TMV-infected Chenopodium amaranticolor and found filaments and virus in massively inoculated tissue. The first appearance of virus in this host at thirty hours post inoculation coincided with appearance of filaments. Milne (1967) suggested from staining reactions that the filaments contained lipid or nucleic acid.

The fine structure of X-bodies has been elucidated by comparative light and electron microscope studies. Esau and Cronshaw (1967b) confirmed previous suggestions that X-bodies consisted of filaments associated with endoplasmic reticulum and ribosomes. The terms X-component or X-tubules were coined for reference to the filaments.

It was suggested that these filaments may be the non-infectious virus protein or X-protein that has been found in extracts from TMV-infected tobacco. A series of developmental forms was suggested. Filaments composed of groups of tubules progressed to individual tubules which were then assembled in ordered arrangement.

Ideas regarding the development of filaments (X-component or X-tubules) were later extended (Esau, 1968). In the earliest stages the material appeared as aggregates of granules. Older aggregates consisted of mixtures of granules and of flexuous bands or composites of tubules. It was assumed the granular aggregates were composed of protein sub-units which became polymerized into tubules.

Nilsson-Tillgren et al. (1969) described the earliest forms of X-component differently. Early inclusions were seen as differentiations of the endoplasmic reticulum which appeared shortly before virus particles. These differentiations were considered sites for virus assembly. Transitional stages and final X-body inclusions, occurring only two hours apart, were reported. From the electron micrographs shown, the intermediate stage was not readily apparent.

At this point it should be noted that Esau (1968) and other workers assumed without proof that the X-component was protein in nature. In 1967 Esau stated "it seems to be an acceptable assumption that the tubules

constitute virus-related noninfectious protein." Perhaps this assumption was made because Sheffield had shown (1934) that X-bodies of the aucuba strain of TMV stained strongly for protein. Further, Takahashi and Ishii (1953) had previously isolated protein from TMV-infected plants that was identical to coat protein.

The staining reaction of X-bodies has been interpreted as characteristic for lipid or nucleic acid (Milne, 1967). More recently (Warmke, 1969) it has been shown that amorphous inclusions of aucuba mosaic are markedly different from X-bodies produced by the common strain of TMV. The amorphous inclusions Sheffield studied may not have been X-bodies in the strict sense, and thus formulation of ideas regarding the composition of X-bodies perhaps should not be based on her work. The exact chemical composition of the filaments (X-component) within X-bodies associated with the common strain of TMV requires demonstration.

Recently, Granett and Shalla (1970) attempted to determine if any correlation existed between the frequency of virus coat or X-protein and X-bodies. A definite correlation would confirm Esau's (1968) proposal. Although the U-1(SB) strain of TMV produced more X-bodies than a U-1(D) strain, no consistent differences were found between these common isolates in X-protein yields. This was presented as strong evidence against Esau's proposal that

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X-bodies have X-protein as their chief component. However, a U-5 strain of TMV which rarely produced X-bodies yielded no X-protein. Thus the nature and role of X-component in virus synthesis remains unsolved.

V. Plastids and TMV

TMV particles were associated with chloroplasts isolated from infected cells as early as 1940 by Kausche and Ruska. Black and Morgan (1950) visualized TMV in infected cells and reported virus masses in close association and occasionally inside chloroplasts. However, the formalin-acetoalcohol mixture they utilized did not provide well-defined micrographs. Disintegration of chloroplasts was observed, but it was not known if this was due to preparation techniques or virus infection.

Thus it was not until 1964, when preparative techniques had vastly improved, that Shalla could definitely state that vacuoles in chloroplasts commonly contained virus particles. The formation of these vacuoles was accompanied by severe distortion of the chloroplasts. Presence of virus in the chloroplasts does not require synthesis or assembly there since the vacuoles could have arisen by envelopment of ground cytoplasm by projections of the chloroplast. Milne (1966a) confirmed Shalla's (1964) observation, but again did not imply that virus was synthesized in chloroplasts.

Shalla and Amici (1967) could find no distribution of ferritin-tagged antibody against TMV over chloroplasts in situ or those that had been isolated. It was suggested that TMV antigen was in too low a concentration to be detected by this method.

A distinctly different relationship of TMV to chloroplasts was reported by Esau (1968). Aggregates of a common strain of TMV were observed in the chloroplasts and were not bounded by membranes. Unlike those observed by Milne and Shalla, these virus particles had the appearance of being produced in the plastids.

The contrasting reports of TMV formation in chloroplasts versus their being engulfed there were clarified by Shalla (1968). The intracellular behavior of TMV depends on the strain employed. Unlike the U-1 strain, clusters of particles of the U-5 strain were present in the chloroplasts. The U-5 strain, like the strain Esau had employed, appeared to be formed in the chloroplasts since no membrane surrounded the aggregates.

Modifications in the ultrastructure of tomato plant cells produced by TMV infection have been studied (Arnott et al., 1969). No virus was detected within plastids, but three types of abnormal plastids were found in yellow areas of infected leaves. These were classified as (a) supergranal, which were uncommonly large, contained fewer large dark grana, had more lamellae per granum, and

lacked ribosome-like particles in the stroma; (b) thylakoidal type with large membranous sacs and osmiophilic globules associated with thylakoidal membranes; and (c) reticulate, which included those with simple membranous sacs twisted into a membranous reticulum.

Modifications in chloroplast structure accompany necrosis in hypersensitive tobacco leaves held at temperatures which cause systemic infection (Carroll, 1970). In mesophyll cells undergoing necrosis, chloroplasts were invested with osmiophilic globules, had distorted lamellar systems, accumulated starch, and lost their plastid envelopes.

Studies have also been done on the relationship of TMV to plastids in etiolated leaves. When bean leaves were etiolated and inoculated with a bean strain of TMV, no virus was recorded in the proplastids. But dense inclusions within the proplastids were associated with massive virus formation (Fujisawa and Matsui, 1969).

An interesting study of TMV-infected etiolated leaves has indicated that conditions in the chloroplast in the presence of light may not allow the common strains of TMV to survive there. Pratt (1970) covered the upper half of TMV-inoculated tobacco plants. After three to five weeks virus was seen in plastids of etiolated leaves, and no membrane surrounded the aggregates. When an etiolated, infected plant was allowed to green in the

light, virus particles were not seen in chloroplasts, even when there was a heavy concentration of virus in the cytoplasm.

VI. Nucleus and TMV

The nucleus has been interpreted as the site of virus RNA synthesis by several investigators (see review by Schlegel et al., 1967). A particular strain of TMV has been visualized in the nucleus (Esau and Cronshaw, 1967). The relationship of TMV to the nucleus of a dividing cell has also been investigated. Virus was usually left outside the nuclear membrane when it reformed (Esau and Gill, 1969).

The presence of TMV in the nucleus can apparently be induced by doubly infecting a cell with cucumber mosaic virus and TMV. With TMV alone, no virus was detected in the nucleus (Honda and Matsui, 1969).

Electron microscopic studies on other plant viruses have shown that of all host cell organelles, virus particles are found most frequently in nuclei (Esau, 1968). However, with TMV, much light microscopic and autoradiographic evidence has incicated that virus RNA is synthesized in the nucleolus and then transported to the cytoplasm.

Materials and Methods

Cells and virus

Healthy callus tissue cells were established from stem pith of tobacco (Nicotiana tabacum L. var. Havana 38). A common strain of TMV was increased in White Burley tobacco plants and purified from leaf extracts by differential centrifugation. Methods of maintenance of the cells and inoculation with virus have been described (Murakishi et al., 1970). Briefly, 100-300 mg of cells which had grown for six days in modified Murashige and Skoog (MS) medium were inoculated with 150 µg/ml of TMV per ml of medium. Forty-ml test tubes containing 3 ml of the virus-cell mixture were vibrated for 20 sec on a Vortex mixer, the contents empied into a 50-mm funnel lined with coarse filter paper, and the cells transferred to MS agar medium after draining for ten minutes.

Electron microscopy

The following procedure was adopted after completion of light and electron microscope observations of cells prepared in various ways (see results): Aggregates of cells 2-5 mm in diameter were placed for three hrs in 2.5% glutaraldehyde containing 4% sucrose in a 0.1M phosphate buffer at pH 7.2. After a brief rinse in the buffer-sucrose solution, the specimens were postfixed in 2% osmium

containing 4% sucrose in 0.1 M phosphate buffer (pH 7.2) for 2 hours. All fixation steps were done at 4°. The fixed materials were rinsed in buffer-sucrose solution, dehydrated in a graded ethanol series, and embedded in ERL epoxy resins (Spurr, 1970). Ultrathin sections were cut on a Porter-Blum ultramicrotome, stained on the grids with an alcoholic solution of uranyl acetate for 30 min followed by aqueous lead citrate for 5 min, and examined with a Philips 300 transmission electron microscope.

Results

Effects of Fixation and Embedding Procedures on Plant Tissue Culture Cells

Numerous preparative procedures were tested for their ability to preserve the highly vacuolate nature of tissue culture cells and the intracellular localization of virus. The procedure used by Sutton and Street (1968) for plant tissue culture preserved some cells well, particularly younger cells rich in cytoplasm. However, often over fifty per cent of the cells in a given sample appeared collapsed or crushed.

We found that the use of Spurr's low viscosity resin medium (Spurr, 1970) allowed preservation of the vacuolate and delicate nature of nearly all cells in a given sample. Minimum crushing or physical damage to cells occurred when this resin was employed (Fig. 1). In

contrast, when araldite or epon plastics were employed, cells often were irregular in outline, and cytoplasm was lost from some cells (Fig. 2). These differences were also observed in the light microscope after final preparation.

Very short fixation periods in permanganate or dilute osmium solutions preserved TMV crystals in excellent three dimensional relationship (Warmke and Edwardson, 1966a; Warmke and Christie, 1967). When these solutions were tested on crystal-containing tissue culture cells, the cytoplasmic detail was not sufficiently preserved to warrant their use in our study.

Features Common to Both Virus-Inoculated and Control Cells

The ultrastructural detail of cells was examined at twelve hour intervals after inoculation with TMV or similar inoculation with buffer. An extremely small amount of cytoplasm, commonly in the form of thin strands, was present in inoculated cells (Fig. 3). These cells had grown for seven days in suspension culture. Their appearance contrasted sharply with that of cells sixty hours after inoculation (Fig. 1). Even though cultured cells were always highly vacuolate, the amount of cytoplasm continually increased from 0-60 hr after inoculation.

The plastids in light grown cells lacked a well developed lamellar system and contained large starch grains.

There were no observable differences in the plastids, nuclei, nucleoli, or mitochondria of infected versus control cells.

Non-virus crystals were present in the cytoplasm of both inoculated and control cells (Fig. 4). They typically measured 0.2 to 0.5 μm across with a 6 to 10 nm repeating lattice structure. Square or hexagonal in outline, they were sometimes present within a membrane-bound body. Large amounts of electron dense, amorphous globules of unknown composition and origin were found inside and outside inoculated and control cells (Fig. 2). The globules were often associated with the tonoplast. Both an extremely dense type and a more loosely aggregated or less electron dense type of material were evident. The material appeared to be secreted or somehow eliminated from the cells as deduced from micrographs showing the material at the cell wall surface (Fig. 5).

Studies on Possible Mode of Virus Entry

Cells at all stages of growth were examined for the presence of natural openings in the cell wall exposed directly to the external medium. No such openings were observed; the exterior cell wall of plant tissue culture cells was seen as a barrier to virus entrance. The vibration procedure used during virus inoculation appeared to increase the frequency of displacement of the parietal

cytoplasm and its incompassing membrance away from the cell wall (Fig. 3). TMV was never observed inside the resultant area between the cell wall and the protoplast.

Although no natural openings were detected in the wall exposed directly to the external culture medium, plasmodesmata and large irregularly shaped openings were found in recently deposited and older walls between cells (Figs. 6a,b,c,d,e). The plasmodesmata were either single or branched and often occurred at thinner areas of the cell walls (Figs. 6b,c). The inner tubule (desmotubule) observed in the plasmodesmatal canals (Fig. 6a) measured 25-40 nm in diameter. The overall width of the plasmodesmata, as usually delimited by the plasmalemma, varied 60-75 nm. Irregularly shaped openings observed in newly deposited walls were as large as 900nm (Fig. 6d). TMV particles measured 12 to 15 nm in width in thin sections.

The vibration of cells during the inoculation procedure often gave the outer cell wall an abraded appearance (Fig. 7). Abrasions of this type were not usually present on walls of nonvibrated cells. The cellulose fibrils appeared separated from the wall, but commonly only an exterior layer was removed. Some cell walls of younger cells consisted of a loose network of cellulosic microfibrils (Fig. 8). TMV was detected at the cell wall and apparently attached to it after inoculation; thus far virus particles have not been seen traversing the wall.

Observable cracks and partial splitting of the wall were not common and could have been produced during preparation of cells for electron microscopy.

Observations on Virus Replication

Masses of free polyribosomes, in close proximity to smooth membranous vesicles, were present in cells 36 hr after inoculation with TMV and may represent an early stage in virus replication (Fig. 9). However, polyribosomes could also be found in 36 hr contril cells, but usually not in discrete masses associated with vesicles as in the infected cells (Fig. 10).

Virus particles were first detected 48 hr following inoculation. Particles were most often seen as small arrays in the cytoplasm (Fig. 11), as contrasted to larger crystals of virus which were more common at 60 hours. The particle aggregates at 48 hours were of relatively small size as observed in serial sections. Often, individual particles or small groups of particles were emeshed in the parietal cytoplasm. TMV was found adjacent to but never in plasmodesmata (Fig. 11).

Two outstanding characteristics of infected cells at 60 hr post inoculation were the presence of amorphous, non-membrane bound bodies and large TMV crystals (Fig. 12). The amorphous inclusions were similar in structure to X-bodies previously described by Esau and Cronshaw (1967).

Within amorphous inclusions, aggregates of tubules were the predominant components, but ribosomes and TMV particles were included. Crystals of TMV (Fig. 13) were not membrane bound. Crystals of TMV were also present by light microscopy during this period. In cells sampled 60 hr after inoculation, polyribosomes were often associated with areas containing virus and numerous small vesicles (Fig. 12). Although TMV aggregates and crystals were not membrane bound, virus seemed to have an affinity for membranous material, resulting in a bristled appearance of TMV on membrane surfaces (Fig. 14). Cells at 60 hours post inoculation appeared to be entering their peak growth phase and peak accumulation of virus. Cells had apparently undergone division in some cases (Fig. 15), and it was evident that newly formed daughter cells were infected. Small aggregates of virus similar to those observed at 48 hr post inoculation were present in these newly divided cells (Fig. 16). No marked differences in host organelles of control cells (Fig. 17) versus infected cells (Fig. 12) were observed.

Little additional information could be obtained from examination of infected cells after 60 hours. There was a gradual dimunition of cytoplasm and an increased virus accumulation. Between 60 and 120 hours after infection, infected cells were essentially similar in appearance.

Both contained large amounts of virus and X-bodies. Tubules within the X-bodies of cells at 120 hours were slightly thicker and more aggregated than those at 60 hours.

Discussion

In their study of tissue culture cells derived from TMV-infected plants, Nakata and Hildebrandt (1967) encountered difficulties in preparation of cells for electron microscopy. They described the existence of two different types of cell cytoplasm, but suggested this may have been due to preparation. The present study indicates that the latter explanation was more probable.

Because of the large size (average 110 μm x 230 μm) of plant tissue culture cells, their vacuolate nature, and their disorganized growth, they can be easily damaged during fixation and embedding. The altered morphology shown in Fig. 2 resulted primarily from transfers and infiltration of cells in a viscous resin such as that described by Sutton and Street (1967). It was found that these problems could be overcome (Fig. 1) by utilizing Spurr's low viscosity resin (1970).

The non-virus crystals detected in tobacco tissue culture (Fig. 4) have been previously reported in cultured plant cells (Matsushima et al., 1969). Their production was dependent on the presence of indolacetic acid and kinetin in the culture medium. Kolehmainen et al. (1965)

stated they were especially frequent in leaf cells containing TMV crystals. This finding was not extended to tissue culture cells; the electron-dense globules found in infected and control cells (Fig. 2) are very similar to those previously reported in TMV-infected tobacco leaf tissue (Kolehmainen et al., 1965). It was suggested they corresponded to X-bodies. Esau and Cronshaw (1967) have since elucidated the fine structure of X-bodies associated with TMV infection. Moreover, we found the dense globules in the same frequency in infected and control cells, and thus showed they are not related to X-bodies produced by virus infection.

The presence of the electron dense globules outside the cell wall and apparently egressing through the cell wall (Fig. 5) may be significant. Ectodesmata in the plant were first recognized because of excretions on the leaf surface and have since been implicated as virus entry points in leaf and root cells (Brants, 1964, '65, '66; Thomas and Fulton, 1968). Although we did not detect natural external openings by electron microscopy, other methods involving use of special strains may be successful.

In view of the increased efficiency of infection due to vibration of cells (Murakishi et al., 1971), other means of virus entry are likely. Abrasion of the cell wall (Fig. 7) may enable TMV to enter through the remaining

cell wall. The loose, fibrillar construction of the cell wall at some points may allow passage of TMV either intact or as RNA (Fig. 8).

Various authors have been unsuccessful in demonstrating the existence of plasmodesmata in plant tissue culture cells (Kassanis et al., 1958; Brants, 1961). A previous report has indicated their presence (Spencer and Kimmins, 1969), but their morphology was not evident in the micrographs presented. We previously suggested that dissociation of cell clumps into smaller aggregates or single cells exposes plasmodesmata directly to the external medium. These openings could then allow TMV to enter the protoplast. Although the diameter of plasmodesmata we observed (60-70 nm) is much smaller than the 300-500 nm width reported by Spencer and Kimmins (1969), the 12-15 nm width of TMV could be accomodated.

The significance of polyribosome masses in TMV-infected tissue culture cells (Fig. 9) remains to be determined. A statistical analysis of numerous sections, comparing infected and control cells, is necessary since healthy meristematic cells are also rich in polysomes (Bonnet and Newcombe, 1965). However, recent reports have provided biochemical evidence for the existence of polyribosomes containing TMV RNA (Kiho, 1970). The latter is significant because uncoating and translation of the viral genome may occur as virus polyribosomes are formed.

Although early events in virus multiplication prior to 48 hours after inoculation were not observed, a wider sampling of cells may have revealed earlier stages of infection. We examined numerous samples 36 hours after inoculation, but never detected virus. Virus may continue to spread and be distributed among cells during this time, and thus be in too low a concentration in each cell to be detected until 48 hours. Evidence of partial synchrony and even increase in virus concentration was indicated by the nearly simultaneous appearance of crystals in adjacent cells, as seen in the light microscope at 48-60 hours after inocuation. Infectivity assays of infected tissue culture cells showed virus replication before 48 hours (Murakishi et al., 1971). Studies in progress on the kinetics of virus synthesis by means of gel electrophoresis allow a much earlier (12-24 hours) detection of TMV RNA in our tissue culture-virus system (L.E. Pelcher, unpublished information).

Summary

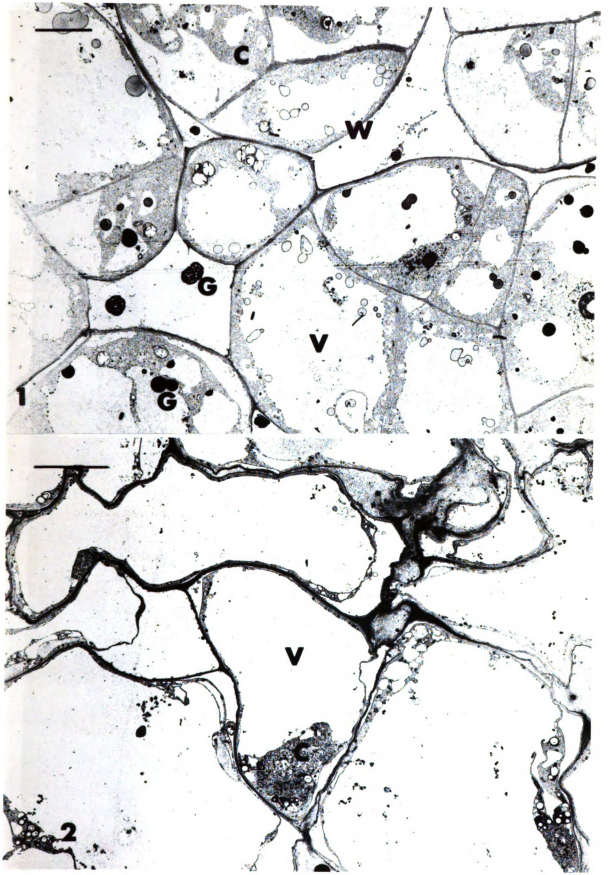
The development of infection in plant tissue culture cells has been studied by electron microscopy. No natural openings in the cell wall exposed to the external medium were detected. However, plasmodesmata and irregularly shaped openings were present in cell walls between cells and may serve as entry points for virus. Polyribosomes

which may represent TMV-RNA bound to host cell ribosomes were present in infected cells and particularly in association with membranous vesicles. Virus could first be detected 48 hours after inoculation and appeared as small aggregates in the cytoplasm. At 60 hours after inoculation X-body inclusions were present in cells containing large virus crystals. Infected cells examined at 72, 96, and 120 hours after infection were similar in appearance. Large virus crystals and X-bodies were prominent. Comparisons of infected and control cells indicated that no obvious pathological effects on host cell organelles occurred as a result of infection.

Figure 1. Tobacco tissue culture cells 60 hr after inoculation. Note the regular appearance of the cell wall (W) outline. Cells were embedded in ERL epoxy resins. Amorphous globules (G) within and external to cells. (C) cytoplasm. Bar represents 4 µm.

Figure 2. Tobacco tissue culture cells embedded in Araldite resin* 60 hr after inoculation. Cell wall outlines are irregular and cells appear crushed. (C) cytoplasm; (V) vacuole. Bar represents 10 µm.

*All figures are of cells prepared as described in materials and methods except figure two as indicated.


C
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1
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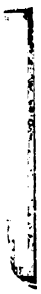


Figure 3. Cells grown in suspension culture for seven days and prepared immediately after inoculation. Thin strands of parietal (C) are displaced from cell walls (arrows). Bar represents 10 µm.

Figure 4. A non-virus crystal found in both inoculated and control cells. Bar represents 0.2 µm.

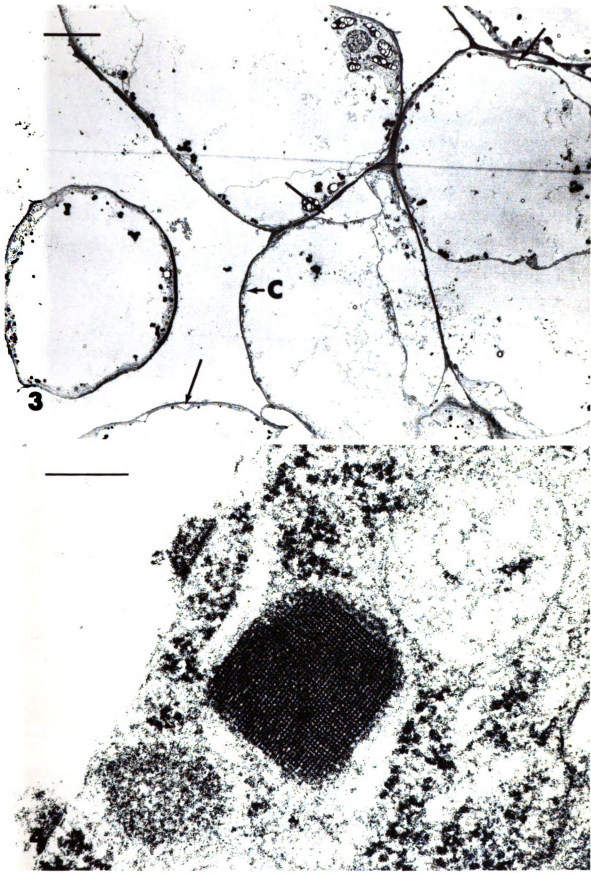
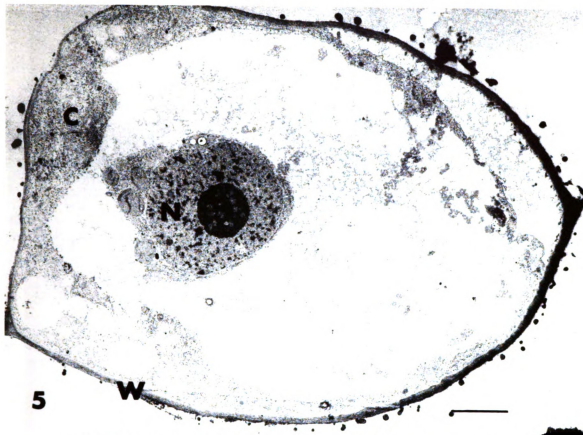

C
3

Figure 5. Tissue culture cell showing amorphous globules (G) outside the cell wall (W). (C) cytoplasm; (N) nucleus. Bar represents 5 μm.

Figure 6a. Cross section of a plasmodesmata (P) in a cell wall (W) between two cells. (R) ribosomes. Bar represents 0.2 μm.



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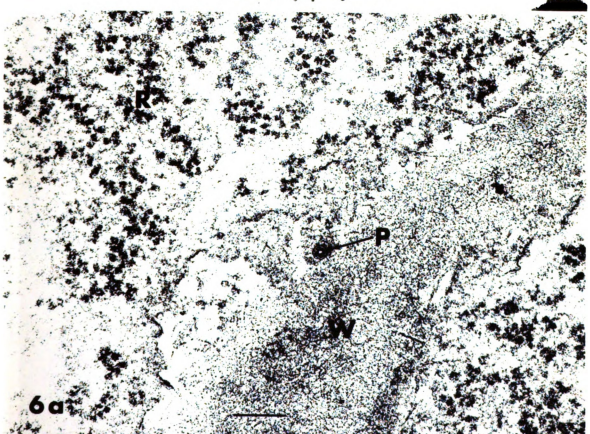
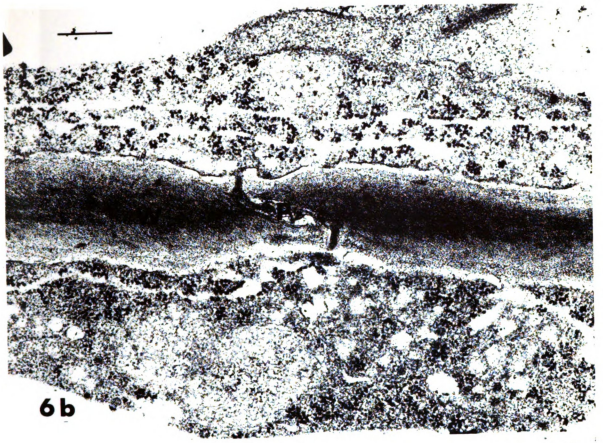


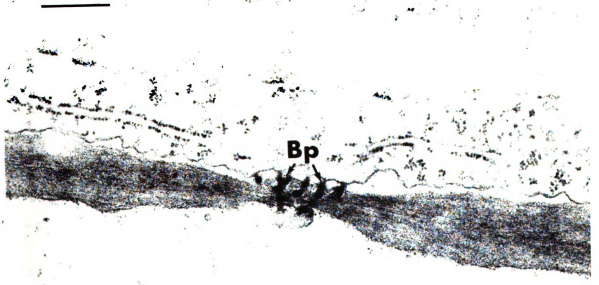
6a

Figure 6b. Plasmodesmata (P) in a wall (W) between two tissue culture cells. Cytoplasm is present in the middle of the plasmodesmatal canal. Bar represents 0.3 μm.

Figure 6c. A branched plasmodesmata (Bp) located in a thin cell wall area between two tissue culture cells. Bar represents 0.5 μm.



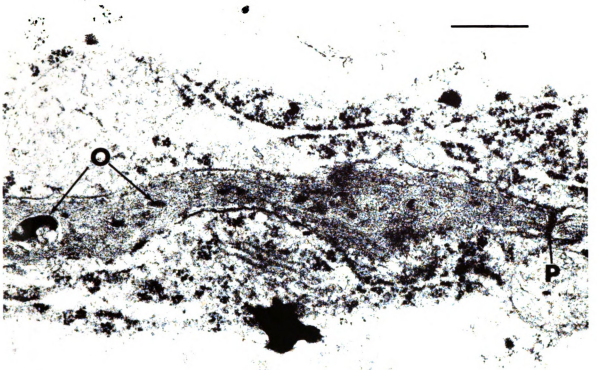
6b



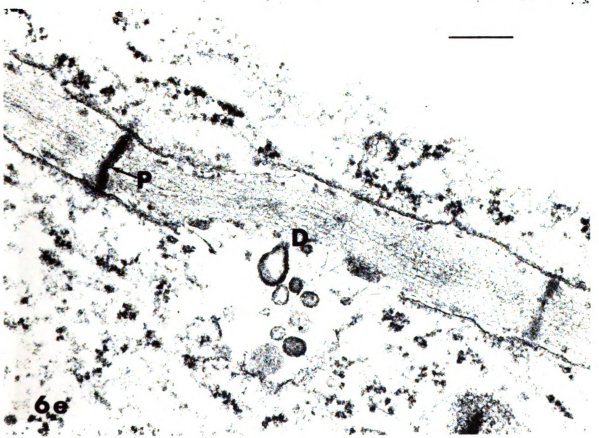
6c

Figure 6d. Large irregular openings (O) in a newly deposited cell wall between tissue culture cells. (P) plasmodesmata. Bar represents 0.5 µm.

Figure 6e. Plasmodesmata (P) occurring in a cell wall which is being deposited. Bar represents 0.5 µm.



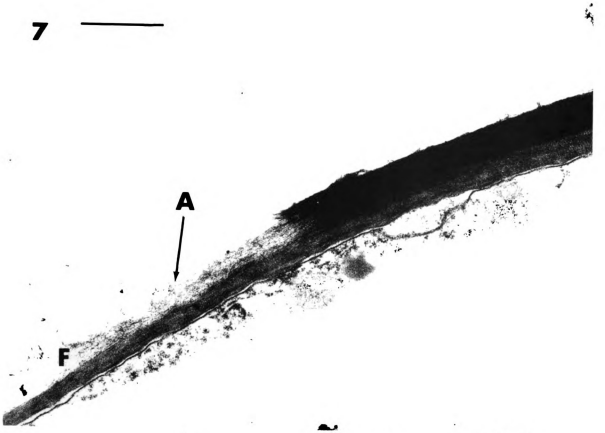
6d



6e

Figure 7. A cell wall showing an abraded appearance (A) on the external surface possibly due to vibration during an inoculation procedure. (F) cellulose fibrils. Bar represents 2 µm.

Figure 8. A loose network of cellulose fibrils in the cell wall from a young, 36 hr, tissue culture cell. Bar represents 0.5 µm.


7
A
F

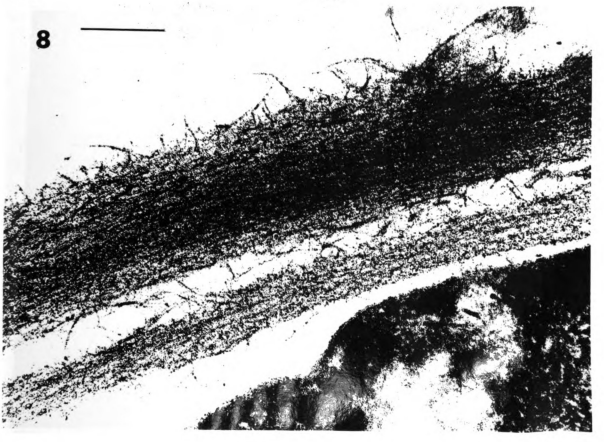

8

Figure 9a. Polyribosomes (Pr) in a cell 36 hr after inoculation with TMV. Note the presence of smooth membranous vesicles. Bar represents 1 μm.

Figure 9b. Higher magnification of polyribosomes showing individual ribosomes presumably linked by RNA. Bar represents 167 nm.

Figure 10. Polyribosomes in a control cell 36 hr after inoculation with buffer. Note the lack of association with membranous vesicles. Bar represents 1 μm.

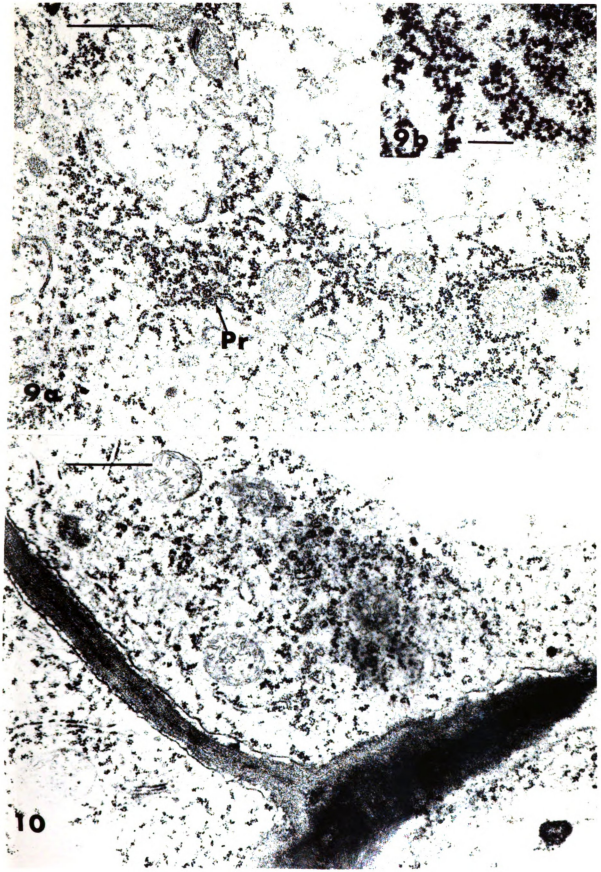

9b
Pr
9a
10

Figure 11. Virus particles (V) in a cell 48 hr after inoculation. Branched plasmodesmata (P). (C) cytoplasm. Bar represents 0.5 µm.

Figure 12. Infected cell 60 hr after inoculation containing a virus crystal (Vc) and X-body (X). (N) nucleus; (Pl) plastid; (S) starch (M) mitochondria (V) vacuole. Bar represents 2 µm.

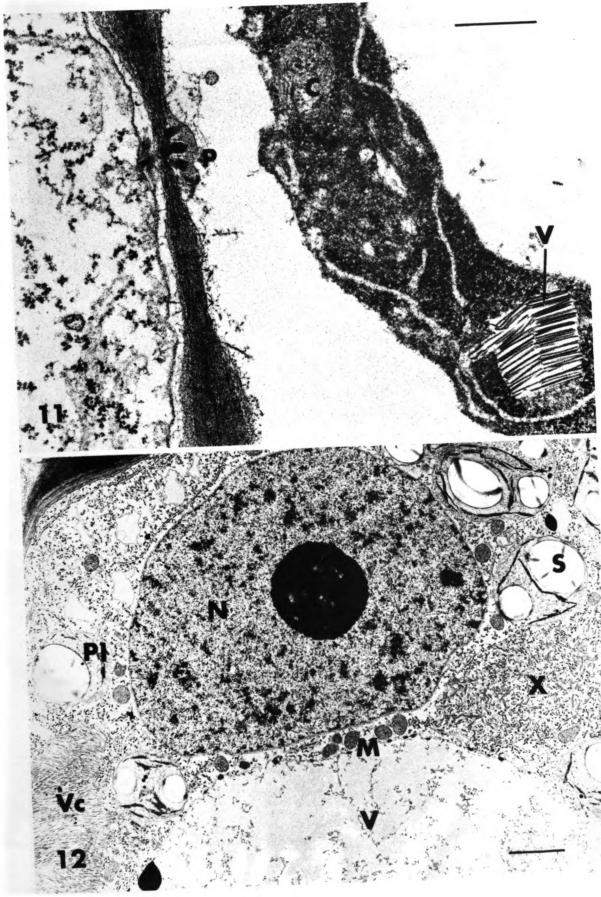
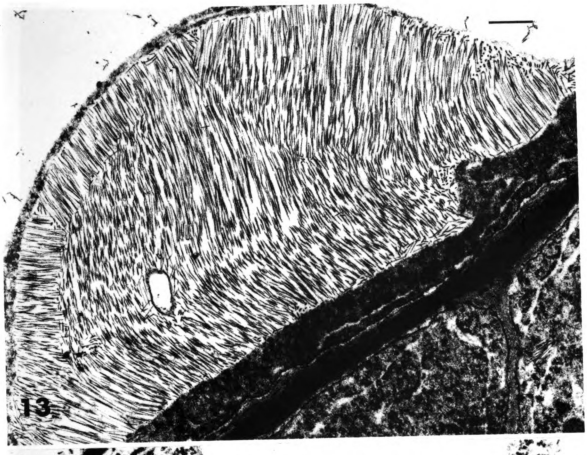

V
11
N
S
Pl
X
M
Vc
V
12

Figure 13. Crystal of tobacco mosaic virus in a tissue culture cell. Bar represents 0.5 μm.

Figure 14. Rods of tobacco mosaic virus (V) associated with membranous material (M) giving it a bristled appearance. (Pr) polyribosomes; (M) mitochondria. Bar represents 0.5 μm.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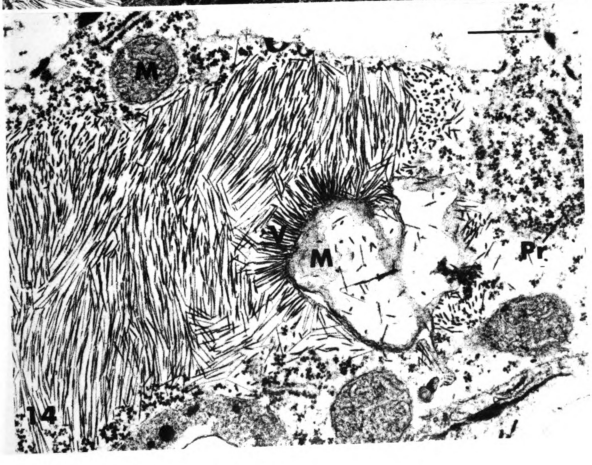
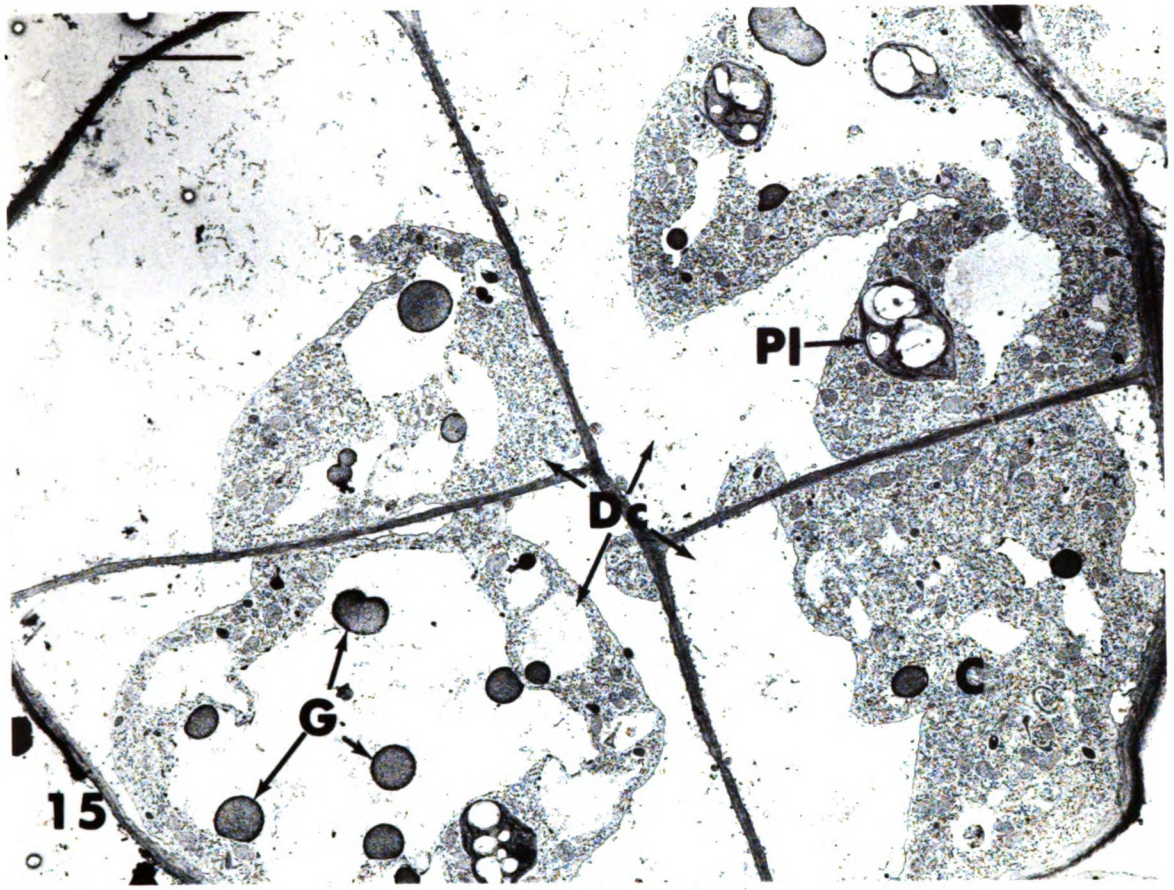

M
M
Pr
14

Figure 15. Tissue culture cell 60 hr after inoculation; it had undergone division into four daughter cells (Dc). (G) amorphous globules; (C) cytoplasm (Pl) plastid. Bar represents 5 µm.

Figure 16. Daughter cell at 60 hr showing small arrays of virus particles (V) in the cytoplasm (C). Bar represents 2 µm.



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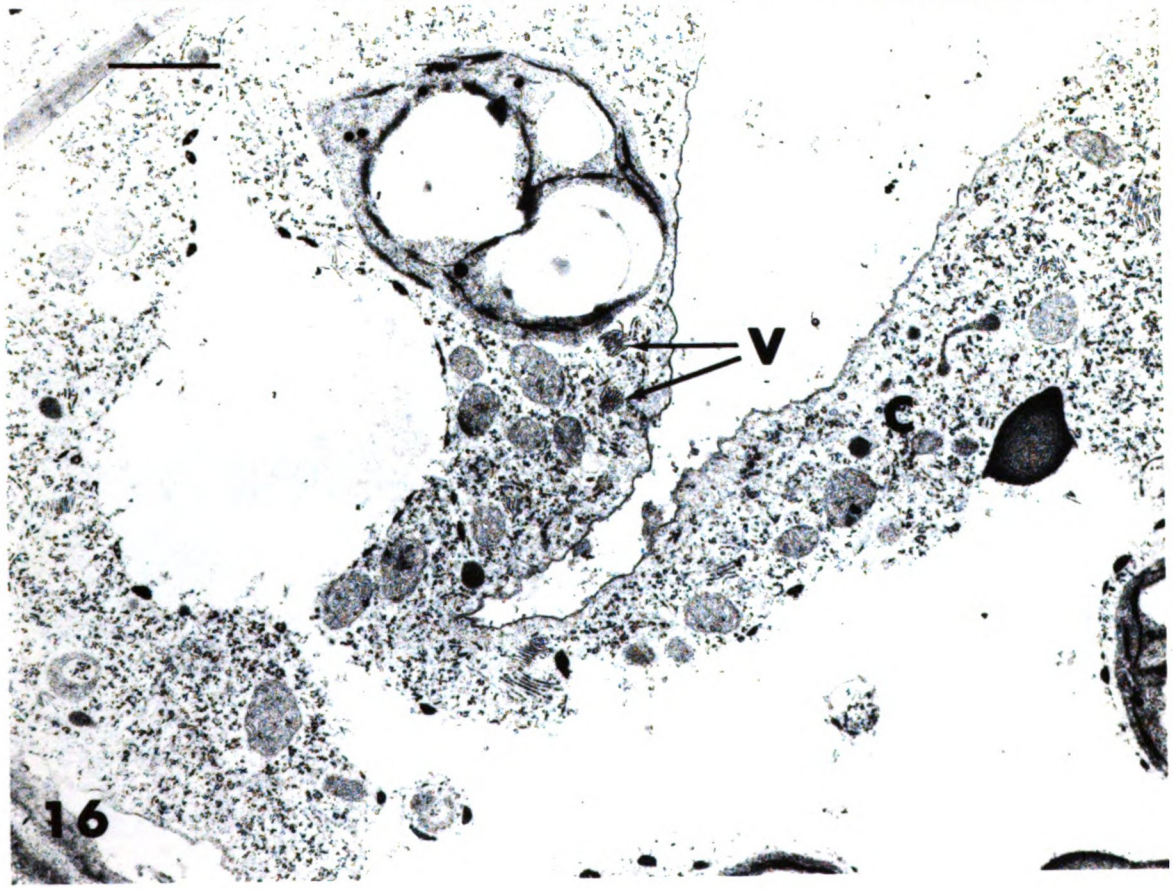
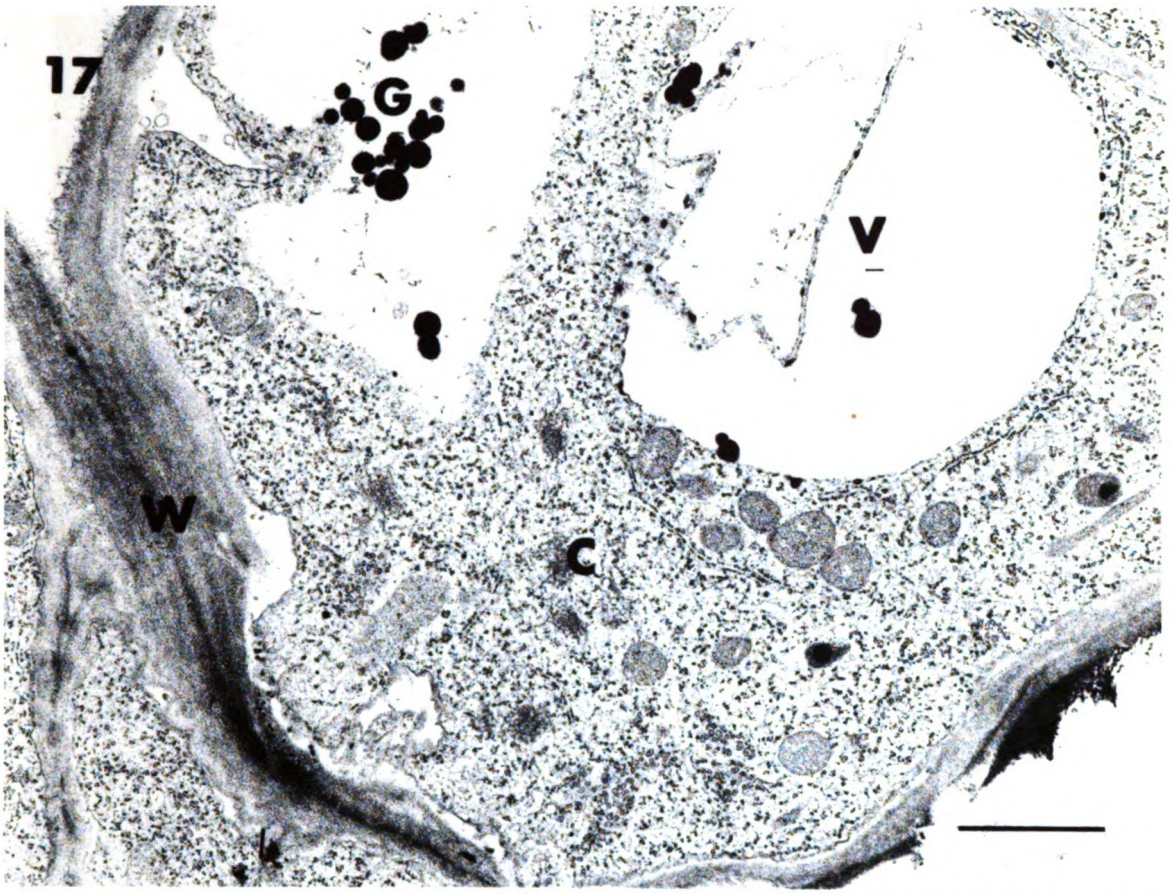
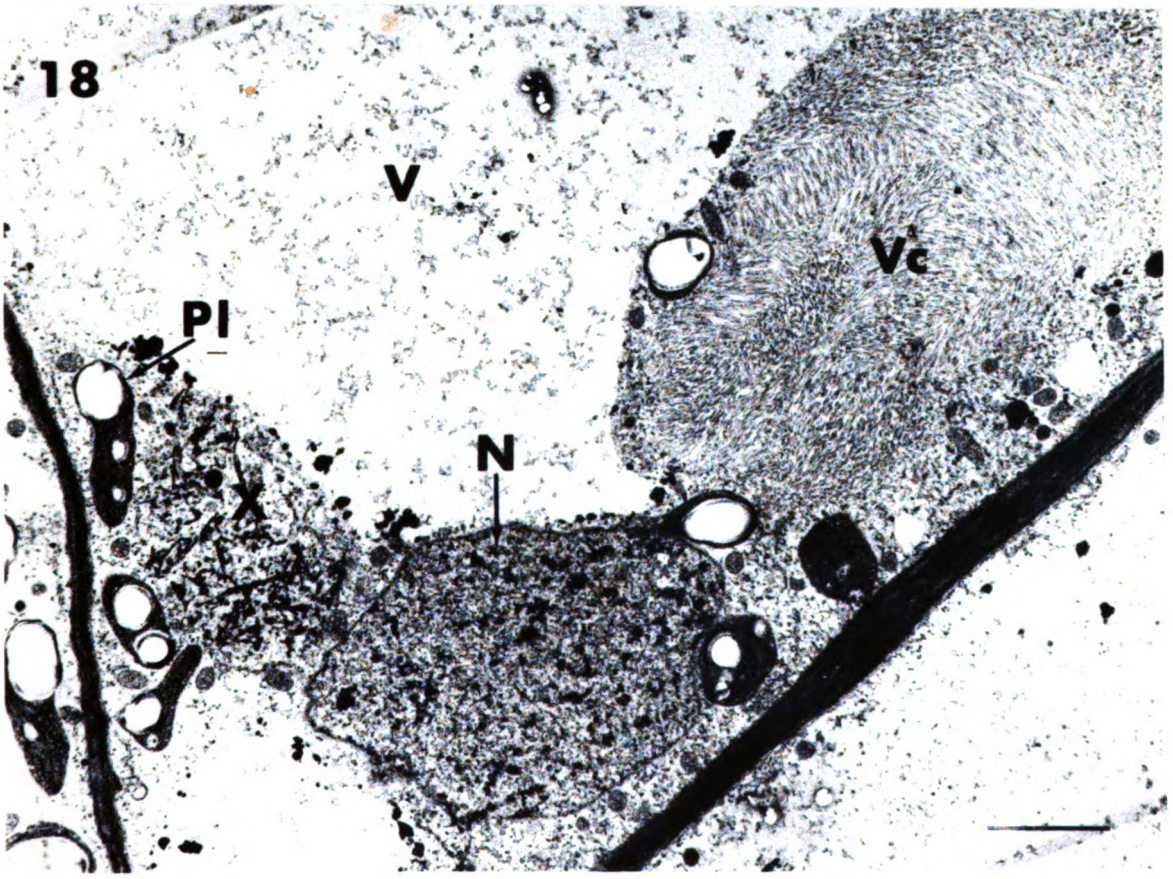


16

Figure 17. Control cell 60 hr after inoculation with buffer. (W) cell wall; (C) cytoplasm; (G) globules; (V) vacuole. Bar represents 1 μm.

Figure 18. Infected tissue culture cell at 120 hr after virus inoculation. (N) nucleus; (Vc) virus crystal; (X) X-body; (Pl) plastid; (V) vacuole. Bar represents 3 μm.


17
G
V
W
C


18
V
Vc
Pl
N

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PART TWO

ELECTRON MICROSCOPY AND PURIFICATION OF VIRUSLIKE PARTICLES FROM SHOESTRING-DISEASED HIGHBUSH BLUEBERRY, VACCINIUM CORYMBOSUM L.

Introduction and Literature Review

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 discolored and develop abnormally. Severely affected leaves are narrow or strap-like with curled edges. The disease can be transmitted experimentally (Varney, 1957), but the natural method of transmission and nature of the causal agent is unknown. The present report concerns visualization and localization of the likely causative agent within its host tissues and purification of that agent therefrom.

Material and Methods

Source Materials

Plants of highbush blueberry Vaccinium corymbosum L. "Jersey," exhibiting characteristic symptoms of shoestring

disease were field-collected and kept 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, post-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 hrs with 2 changes to assure complete dehydration. The specimens were then infiltrated with epoxy resins according to Spurr (1970). Spurr's standard procedure using formula A was modified by extending the period of 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 300 transmission electron microscope.

Virus Purification

Young leaves with severe symptoms of shoestring disease were frozen overnight and then ground for 3 min in 2 volumes of cold (4C) extraction solution (see results for details) with a Waring blender. Root pieces were waterwashed, blotted dry, ground in liquid nitrogen with a mortar and pestle, and then ground in a Waring blender with 2 volumes of extraction solution for 8-10 min. The homogenates from each of the above were squeezed through 4 layers of cheesecloth and the resultant filtrate centrifuged for 20 min at 10,300 x g (4C). The clarified supernatant fluid was subsequently centrifuged at 89,000 x g for 150 min in a Beckman model L2 ultracentrifuge. The pellets obtained were dispersed in 0.1 M phosphate buffer pH 6.8 at the rate of 1.0 ml/30-50 gm 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 phosphotungestic acid neutralized with 1 N KOH.

Results

Appearance and Distribution of Viruslike Particles in Plant Tissues

Leaf interveinal tissue

Epidermal cells from the adaxial surface of diseased leaves contained abundant viruslike particles. In Fig. 1 small crystalline arrays and numerous unordered particles are evident. Of the leaf cells examined, epidermal cells appeared most severely affected by the disease. Chloroplasts contained abnormally high accumulations of starch and degeneration of the cytoplasm and necrosis of some epidermal cells was seen.

In palisade parenchyma cells, viruslike particles were commonly found in small (6-8 μm)vacuoles which were more numerous in infected than in uninfected palisade cells (Fig. 2). The central vacuole of these cells was filled with material resembling tannins. Many loose particles in the cytoplasm were associated with fine fibrillar material. The chloroplasts of palisade cells appeared normal but viruslike particles appeared to have an affinity for the outer chloroplast membrane (Fig. 3).

Long tubules or aggregates of rodlike material were often associated with the presence of viruslike particles (Fig. 4). Thus far these tubules have been found only

in diseased palisade cells. They were present in both the cytoplasm and nuclei and measured up to 260 nm in length. Their exact length could not be determined in thin sections as no definite ends were evident. In cross section (Fig. 3) they measured 25-27 nm with a small 2-5 nm electron transparent core which distinguished them from viruslike particles. The tubules were somewhat angular in cross section.

Spongy mesophyll cells contained very little virus and exhibited no evident effects of infection.

Leaf vascular tissue

Entire sections of young main leaf veins typically had only a few xylem parenchyma cells which contained viruslike particles (Fig. 5b). Usually the particles were confined to small clear areas at the periphery of the cells (Fig. 5a). 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. Affected cells did not differ markedly from control cells except for the presence of virus particles. No viruslike particles were detected in phloem tissue.

Root tissues

Samples of root tissue were taken from plants with leaf and bark symptoms but with no microscopic symptoms of

root infection. However, viruslike particles 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. In less dense aggregations of particles, fibrillar material was often present, and the hexagonal nature of some particles was evident (Fig. 8).

Particle containing cells differed markedly in appearance within a given sample. In Fig. 9 the cytoplasm contained mitochondria, ribosomes, presumed endoplasmic reticulum, and scattered viruslike particles. In Fig. 10, a different cell of the same type, the cytoplasm was amorphous and electron dense. Little if any cytoplasmic structure or detail was present. Viruslike particles were in small crystalline arrays, sometimes partially obscured by the surrounding electron dense material.

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 viruslike particles. The latter were similar to those observed in leaf palisade cells (Fig. 2). Thus far, no viruslike particles have been located in root cell types other than xylem. Intercellular connections were not seen

to contain viruslike particles although particles were in close proximity and the connections of sufficient diameter to accomodate them.

Partial Purification of Virus

Attempts at purification using chloroform or chloroform-butanol extraction methods were negative. Homogenization of blueberry leaf tissue in phosphate or trisaminomethane - HCl buffers resulted in a marked drop in pH of these buffers (Table 1) and the homogenate became deep brown in color. If higher initial pH and larger volume of buffer (up to four) were employed, the pH of the homogenate could be stabilized at 6.4. But with this larger dilution factor virus could not be concentrated enough to detect.

We found that potassium phosphate buffer (0.1 M pH 6.8) with 0.01 M ethylenedinitrilo tetraacetic 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 per 100 ml of buffer) prevented both rapid browning and lowering of the pH in the homogenate. This "extraction solution" was successfully used in partially purifying virus from leaf tissue.

Virus could not be purified from root tissue in readily detectable quantities. Although young rootlets

were sampled, the root tissue was extremely difficult to homogenize. Unlike leaf tissue, roots were not highly acidic (Table 1).

Identification and Measurement of Viruslike Particles Extracted From Diseased Leaves

Negatively stained particles from partially purified leaf preparations measured 28-31 nm (Fig. 11). This size range is consistent with that of particles in thin section. Reasons for the slightly larger size of negatively stained particles have been previously enumerated (Horne, 1967). 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.

Discussion

A small, polyhedral virus has been associated with another disease of blueberry, necrotic ringspot. Appearance of virus *in situ* was not reported, but 25 nm virions were purified from petunia inoculated with crude sap from diseased blueberry (Lister *et al.*, 1963). Our study is believed to be the first isolation of viruslike particles directly from the visualization within diseased blueberry tissue. Moreover, we are unaware of a previous association of viruslike particles with shoestring disease.

The infectivity of the observed particles requires demonstration before their viral nature is certain. Reproduction of the disease using isolated virions would be added proof that they represent the causal agent. However, it seems reasonable to assume the particles described herein are blueberry shoestring virions (henceforth BSV). Particles similar in size and morphology were observed in both leaf and root tissues of shoestring affected plants. They were distinguishable from other host cell components such as ribosomes according to criteria enumerated by Jensen (1969). Their ordered arrangement into crystals (Figs. 1 & 10) is strong presumptive evidence of their viral nature (Weintraub and Ragetli, 1970). The distinct polyhedral nature of the particles extracted from shoestring diseased tissue is characteristic of the icosahedral symmetry of viruses (Hall, 1964), and the size of isolated particles was in close agreement to particles in situ. No other type of viruslike particle was detected in shoestring diseased material.

Leaf palisade cells afforded the best observations on various structures associated with BSV infection (Figs. 2, 3, 4). The relationship of BSV to the numerous vesicles in affected cells (Fig. 2) is unclear at this time. An increase in the number of cytoplasmic vacuoles in cowpea mesophyll cells infected with southern bean mosaic virus has been reported (Weintraub & Ragetli, 1970;

Russo, Martelli and Quacquarelli, 1968) indicated localization of artichoke mottled crinkle virus in small vacuoles came about when virus moved from the cytoplasm into the central vacuole. The membrane of the smaller vacuoles was thus derived by the tonoplast. However, with blueberry shoestring virus the vacuoles were present in the cytoplasm and did not appear to arise from the central vacuole as it was uniformly filled with tannins.

Long tubules similar to those associated with BSV particles in palisade cell cytoplasm and nuclei (Fig. 4) have been described in connection with other isometric viruses (Martelli and Russo, 1969; Hull et al., 1970). Rodlike tubules 18-20 nm in diameter, found in association with Beet Mosaic virus, were observed encased in vesicles in the cytoplasm (Martelli and Russo, 1969). This size contrasts to the larger diameter (25-27 nm diameter) of tubules described for BSV infection. The morphology of BSV associated tubules more clearly resembles the X-components associated with TMV infection (Esau and Cronshaw, 1967). The origin and function of viral associated tubules is unknown. In the case of BSV infection they appear limited to palisade infected cells. Whether this is a particular response of this cell type to virus infection or an indication that virus is most actively synthesized in palisade cells is unsolved.

The finding of BSV in xylem but not phloem tissue is similar to the distribution of virions observed in artichoke mottle crinkle disease (Russo et al., 1967). The large masses of BSV in root xylem cells (Fig. 7) and the presence of virions in a high percentage of cells (Fig. 6) examined indicates a systemic root infection. When diseased bushes are cut back, usually all new growth exhibits shoestring symptoms. The scarcity of infected cells in leaf xylem tissue (Fig. 5) in comparison to root xylem may indicate a higher concentration of BSV in root tissues. Tomlinson (1955) found ringspot virus more concentrated in extracts from cucumber roots than tops but did not suggest viral multiplication in the root.

Additional work is in progress on further purification and transmission to herbaceous hosts. The optimum concentration of EDTA used in purification needs determination. Wells and Sisler (1969) have shown an 88-99% loss of southern bean mosaic virus infectivity when a lower concentration (0.001 M) was employed than that used in our study (0.01 M). However, if EDTA is omitted, aggregation can cause loss of yield in virus purification (Takanami & Tomaru, 1969). Although 2-mercaptoethanal (2-ME) prevented oxidation of blueberry leaf homogenates, infectivity may have been lowered. Fulton and Barnett (1969) showed diethyldithiocarbamate stabilized prunus necrotic ringspot virus while 2-ME did not. We are now testing many of the

parameters affecting transmission of viruses from woody plants as discussed by Fulton (1966) in relation to BSV transmission to herbaceous hosts.

Thus far, all attempts using leaf material as a source for mechanical transmission have been negative. The presence of large amounts of virus in root tissue, and the less acidic nature of root tissue suggest its use in future transmission attempts.

Summary

Viruslike particles spherical in outline and measuring 26-28 nm in diameter 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 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 leaf cells. Particles hexagonal in outline and 28-31 nm in diameter were partially purified from diseased leaves. Observation of viruslike particles *in situ* and purification from symptomatic leaves is presented as presumptive evidence for virus causation of blueberry shoestring disease. No such particles were observed in healthy blueberry tissue.

TABLE 1

Relationship of Buffer Type and Volume to pH of Tissue Homogenates

Buffer Type	pH of Buffer	vol. per g. tissue[a/]	pH of Homogenate
0.1 M PO_4	7.0	2	4.2
0.1 M PO_4	7.0	3	4.5
0.1 M PO_4	7.0	4	5.4
0.1 M PO_4	8.0	2	5.4
0.1 M PO_4	8.0	4	6.4
0.05 tris. HCl	7.4	3	3.5
0.1 M PO_4 + 2 ME + EDTA	9.0	2	5.8
0.1 M PO_4 + 2 - ME + EDTA + nicotine alkaloid	6.8	2	6.5
0.1 M PO_4 + root tissue	6.8	2	6.8

a/ all leaf tissue except bottom row as indicated.

Figure 1. Leaf epidermal cell from blueberry affected by shoestring disease. Viruslike particles (V) are both unordered and in crystalline arrangements. (S) starch grain; (Nc) necrotic chloroplast. Bar represents 1 µm.

Figure 2. Viruslike particles (V) in vacuoles (Va) of shoestring diseased palisade parenchyma cell. Bar represents 500 nm.


V
S
Ic
1
V
Va
2

Figure 3. Viruslike particles (V) associated with the chloroplast envelope. Cross sectioned tubules (T) in association with viruslike particles. Bar represents 200 nm.

Figure 4. Longitudinal view of tubules (T) associated with viruslike particles in shoestring diseased leaf cells. Bar represents 200 nm.

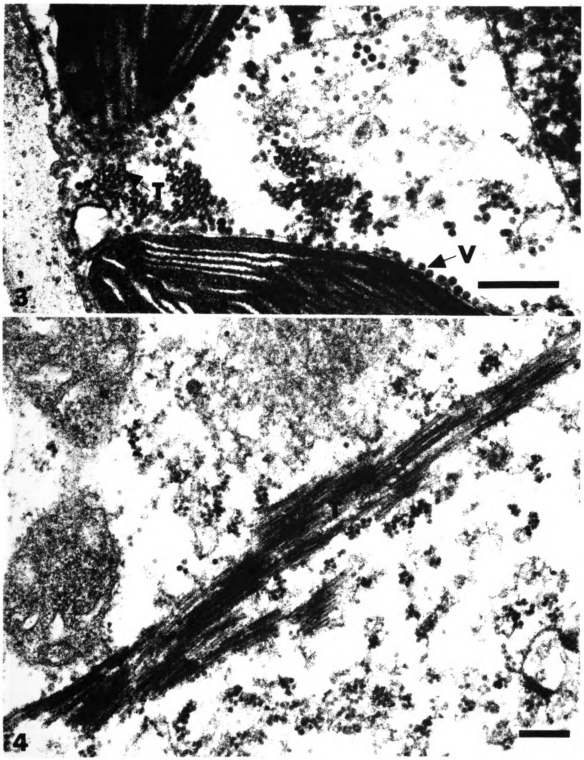
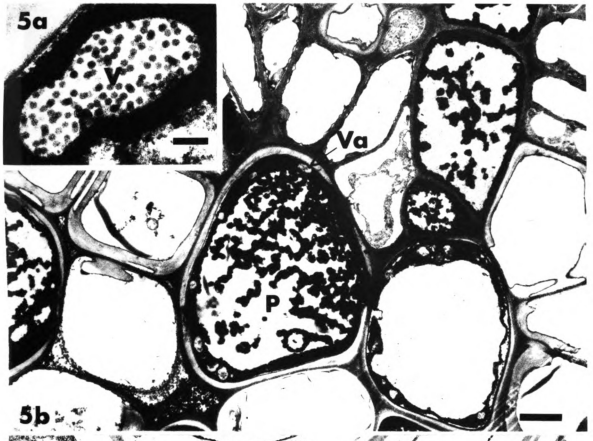

T
V
3
4

Figure 5a. Viruslike particles (V) in vacuole from leaf xylem cell. Bar represents 100 nm.

Figure 5b. Xylem area from main vein of shoestring diseased leaf. Parenchyma cells (P) with small vacuoles (Va) which contain viruslike particles. Bar represents 2 µm.

Figure 6. Xylem area from the root of a shoestring diseased plant. Viruslike particles are in nearly all cytoplasm-containing cells. Bar represents 2 µm.


5a
V
Va
P
5b

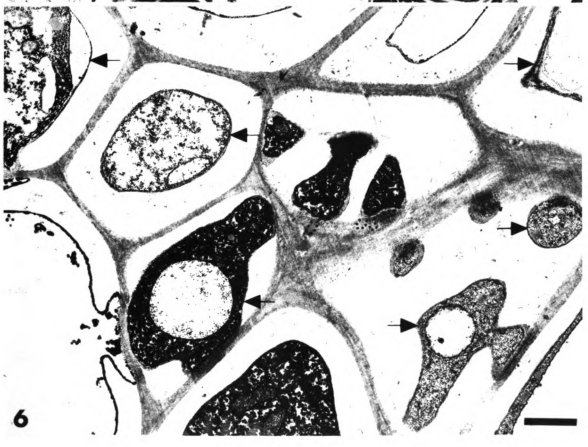
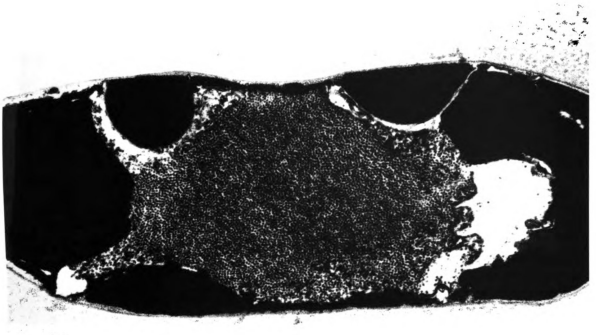
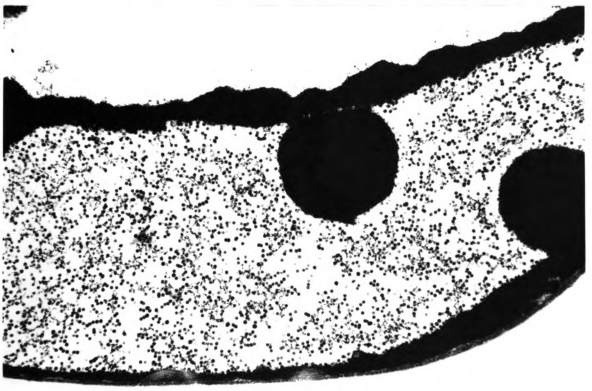

6

Figure 7. Root xylem cell with large mass of viruslike particles and surrounding electron dense material. Bar represents 500 nm.

Figure 8. Fibrillar material associated with viruslike particles, some hexagonal in outline. Bar represents 500 nm.



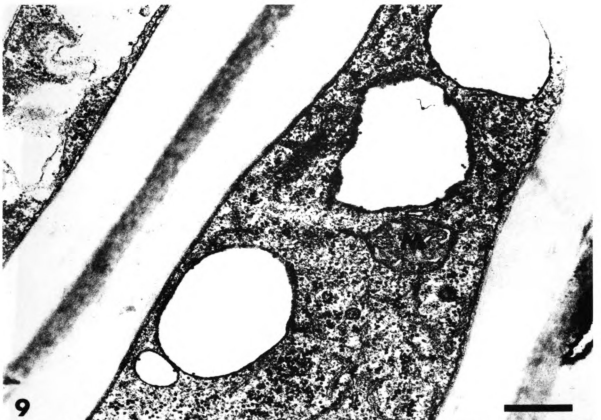
7



8

Figure 9. Root xylem cell. Viruslike particles are scattered in the cytoplasm. (M) mitochondrion. Bar represents 500 nm.

Figure 10. Root xylem cell. Cytoplasm very electron dense with no recognizable host cell organelles. (Ca) crystalline array of viruslike particles; (La) loose array of virus. Bar represents 500 nm.


9

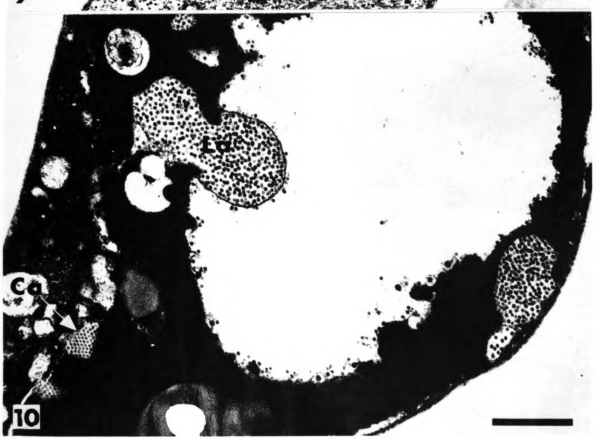
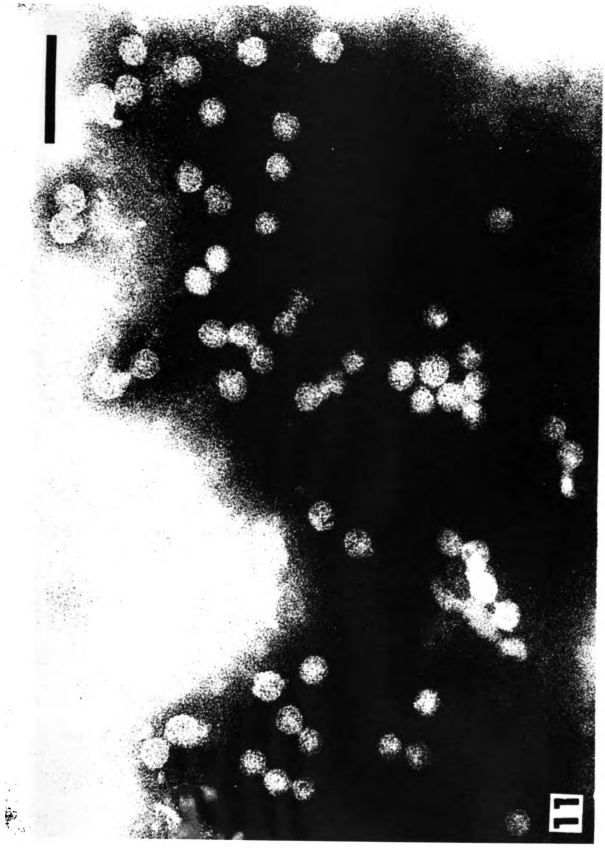

Ca
10

Figure 11. Negatively stained viruslike particles extracted from shoestring diseased blueberry leaves. Bar represents 100 nm.



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PART THREE

OCCURRENCE AND NATURE OF MYCOPLASMA-LIKE ORGANISMS IN STUNT DISEASE OF MICHIGAN HIGHBUSH BLUEBERRY

Introduction and Literature Review

Mycoplasma are the smallest free-living organisms and perhaps the most primitive on the evolutionary scale. They possess both DNA and RNA but lack a cell wall and are highly pleomorphic. They generally require sterols for growth, are resistent to penicillin, and sensitive to tetracyclines. Various reproductive forms can be found (Hayflick and Chanock, 1965).

A group of plant diseases, termed the yellows type because of their conspicuous yellowing, have long been thought to be caused by viruses. These diseases are characterized by witches' broom growth of axillary shoots, clearing of veins, stunting, phyllody, and an abnormally erect growing habit. Viral etiology was concluded because transmission was possible only by leafhoppers, grafting, or dodder, and the agents were filterable. However, numerous attempts at purification had failed (Matthews, 1970).

The electron microscope finally enabled elucidation of the etiology of yellows diseases and opened a new era in plant pathology. In 1967 Doi et al. published electron micrographs of mycoplasma-like organisms (MLO) in the phloem of petunia plants with aster yellows, Paulownia trees and potato plants with witches' broom disease, and mulberry plants suffering from dwarf disease. Ishie et al. (1967) treated mulberry plants suffering from dwarf disease with antibiotics and observed suppression of symptoms.

Since these initial studies, MLO have been associated with numerous plant diseases. Further, MLO have been cultivated in vitro, inoculated onto host plants, and the disease has been reproduced. A recent review (Maramorosch, 1971) summarizes much of this work.

Stunt disease of blueberry has been considered of viral etiology since 1942 (Wilcox, 1942). The disease is graft, dodder, and leafhopper transmissible. The leafhopper, Scaphytopius magdalensis Prov., accounts for natural spread of the disease (Maramorosch, 1955).

Nearly all varieties of cultivated highbush blueberry are susceptible to stunt disease. In the 1940's, the blueberry industries of New Jersey and North Carolina were threatened with extinction by this disease. Although indigenous to New Jersey, the disease occurs in other blueberry growing regions of the U.S. and Canada. Dwarfing, excessive branching, and yellowing of the leaves are

characteristic symptoms of the disease. These symptoms would place the disease in the yellows type which is described above and shown to be caused by MLO. However, information on the causal agent has been lacking (Stretch and Varney, 1970).

Recently, Chen (1971) associated MLO with stunt disease of blueberry in New Jersey. The present paper concerns work undertaken simultaneously in Michigan and includes studies on two blueberry varieties and the distribution of MLO in plant parts. Furthermore, serial sections of MLO in situ are described in an attempt to ascertain the three dimensional nature of this group of plant pathogens.

Materials and Methods

Plants of highbush blueberry, Vaccinium corymbosum L. "Coville" and "Concord," exhibiting characteristic symptoms of stunt disease were field-collected and grown in a greenhouse or outdoors in a peat-soil mixture. To obtain plants free from stunt disease, healthy softwood cuttings were rooted and grown in sterilized sphagnum peat. Cuttings were made from plants grown in fields systematically rogued of plants with disease symptoms.

The main veins of young leaves from diseased and healthy blueberry plants were fixed for 3 hr in cold phosphate-buffered (0.1M, pH 7.2) 5% glutaraldehyde

solution, rinsed in buffer, postfixed with a 2% solution (W/V) of osmium tetroxide in the same buffer and dehydrated through a graded ethanol series. The samples were then infiltrated and embedded with epoxy resins according to Spurr (1969). Ultrathin sections were stained with uranyl acetate for 30 min, followed by lead citrate for 5-10 min. Sections were examined with a Philips 300 electron microscope.

Results

Identification of Diseased Plants

Cultivated plants exhibiting stunt disease were collected from Van Buren and Allegan counties of southwestern Michigan. No difficulty was encountered in differentiating stunt disease plants from those affected with other disorders. The reduced height and excessive branching of stunt diseased plants contrasted to that of surrounding healthy plants. Furthermore, affected branches had shortened internodes, leaves were cupped in appearance and a characteristic yellowing pattern of the foliage was observed. Leaves of affected plants exhibited premature coloring in the late summer, a final symptomatic indication of stunt disease.

Detection of Mycoplasma-like Organisms

Numerous pleomorphic bodies were detected in ultra-thin sections of leaf phloem cells from diseased plants. These structures were not found in other types of diseased leaf tissue and appeared restricted to phloem sieve tube elements (Fig. 1). Measurements ranged from 135 nm to 530 nm for spherical and 100-225 nm by 400-850 nm for elongate forms.

At higher magnifications the organisms in diseased tissue were seen to possess ribosome-like bodies, osmiophilic strands presumed to be nucleic acid and a surrounding unit membrane (Fig. 1b). These observations correlate with those given previously for MLO in plants (Doi *et al.*, 1967).

The Nature of Forms Studied in Serial Sections

It has been suggested that various reproductive forms of plant MLO may exist and growth or developmental cycles have been postulated on the basis of thin section studies (Sinha and Paliwal, 1969; Worley, 1970). It is highly probable that the existence of different forms in single thin sections is due to the sectioning of a few forms in different planes.

A most often stated observation of MLO *in situ* is that elongate forms appear to be "budding" or dividing by

fission. In sections through stunt-diseased blueberry leaf veins (Fig. 2e), apparent "budding" was found. However, consideration of serial sections before the one depicted (Fig. 2 a-d) indicated that a biconcave form cut through the center of its long axis can give the appearance of "budding." The existence of "elementary bodies" (Fig. 2b) has also been suggested from thin sections. When serial sections are studied (Fig. 2a, c) it was seen that an "elementary body" can arise by sectioning of a long sinuous form with denser cytoplasm in the area cut off.

Spherical "mature" and "immature" forms were followed through serial sections and found to be spherical to elliptical in shape (Fig. 2c, f). The long flexous forms often appeared to have been flattened spherical forms. Another interesting form appeared to have a central vacuole or clear area (Fig. 2f). This can be interpreted from serial sections as an involution of a spherical form.

Association of Inclusions With Diseased Tissue

A comparison of stunt diseased tissue with healthy phloem tissue indicated that cystalline inclusions may result from MLO infection. Large, electron dense inclusions, angular in outline were found in companion cells adjacent to degenerate sieve tubes containing small, electron dense forms of MLO (Fig. 3). Thus far inclusions of

this type have not been detected in healthy blueberry phloem tissue.

Discussion

Chen (1971) has recently reported the presence of structures resembling MLO in phloem of Jersey variety blueberry from New Jersey. The present paper confirms the association of MLO with blueberry stunt disease and extends the finding to two additional varieties. It is believed to be the first report of possible MLO causation of a yellows-type disease of Michigan and correlation of inclusions with MLO infection.

Numerous crops of Michigan are affected by yellows-type diseases, as evidenced by symptoms, but their etiology has not been determined. A preliminary survey (Hooper, unpublished results) has shown by electron microscopic observations that diseased carrot, celery, onion, lettuce, and cherry crops of Michigan harbor MLO. Whether alternate hosts exist for blueberry mycoplasma or those of other crops has not been determined and thus far no MLO have been isolated in culture from this diseased material. It is necessary to cultivate these organisms from diseased tissue as added proof they represent the causal agents.

In general, the structure of MLO in plants as deduced from serial sections of them in blueberry tissue can be stated as follows. The predominant structure

closely resembles a highly flexible, sac-like object which can be partially involuted, flattened or elongated. Sections from a particular plane of this type structure can give the appearance of various "reproductive forms" as seen in Fig. 2a-f. The lack of a cell wall probably accounts for the pleomorphic nature of MLO. It should be stressed that interpretations of various reproductive structures should be done after examination of serial sections. We do not want to imply that these structures do not exist but that their existence can't be deduced from examination of single thin sections.

The inclusions we have associated with MLO infection of blueberry have not been previously described in connection with a yellows type disease. It is possible that these bodies are a natural constituent of healthy blueberry phloem tissue and are increased in number as a result of MLO infection. A wider sampling of healthy blueberry tissue is necessary before they can be proven to have a direct relationship to a MLO disease.

Summary

Mycoplasma-like organisms were found in ultrathin sections of phloem sieve tube elements from stunt-diseased blueberry tissues. Spherical, elongate and irregular forms, as evidenced by serial section methods, were present in leaf and fruit pedicel tissue. A distinct external unit

membrane, presumed ribosomes and fibrillar nucleic acid were characteristic of these structures. No mycoplasma-like structures were observed in healthy blueberry plants of the same varieties. Large, electron dense inclusions, were present in companion cells adjacent to degenerate sieve tubes containing small forms of mycoplasma-like organisms. Inclusions of this type were not detected in healthy phloem tissue.

Figure 1. Ultrathin section of phloem tissue from stunt diseased leaf tissue.

a. Distribution of mycoplasma-like organisms (MLO) in cell types. (C) companion cell; (ST) sieve tube.

b. A single mycoplasma-like organism showing fine structure. (M) unit membrane; (R) ribosome-like body; (S) presumed nucleic acid strands.

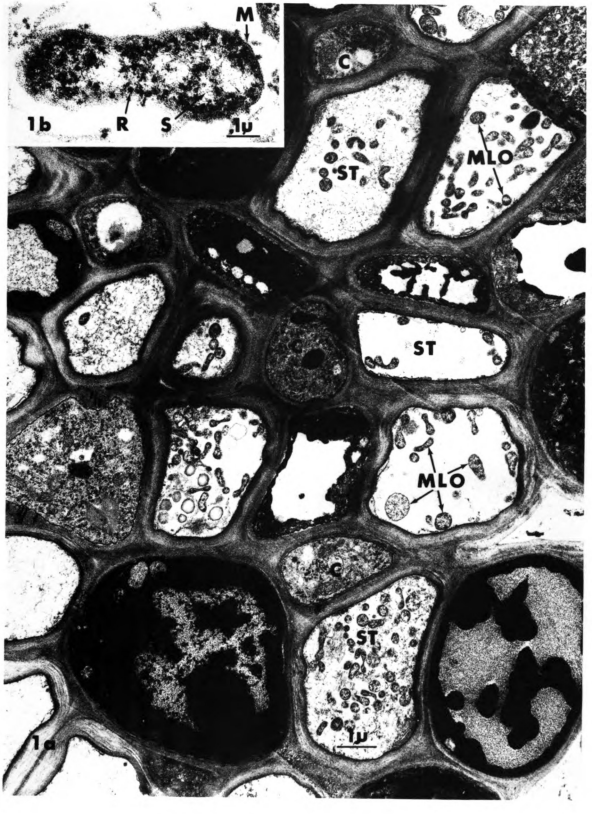
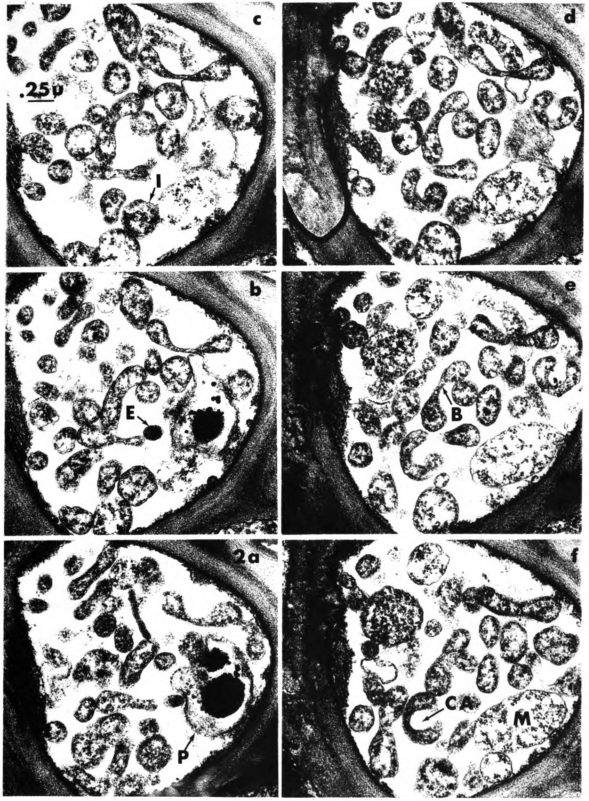

M
1b
R
S
1μ
C
ST
MLO
ST
MLO
C
ST
1a
1μ

Figure 2. Serial sections of mycoplasma-like organisms with apparent replicative forms. (B) budding form; (E) elementary body; (I) immature form; (M) mature form; (CA) clear area; (P) plastid.


c
.25μ
I
d
b
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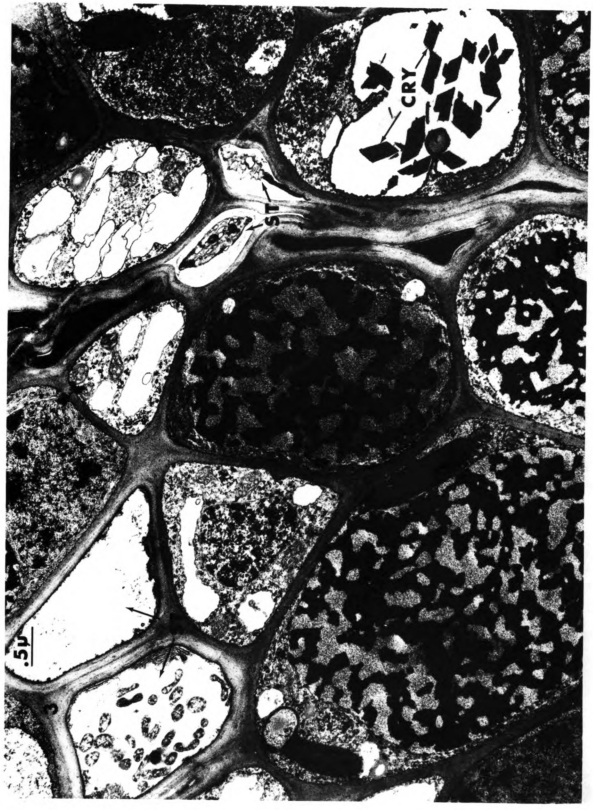
e
B
2a
P
f
C
M

Figure 3. Phloem area with companion cell containing inclusions (CRY) and adjacent degenerate sieve tube elements. (ST) sieve tube.


CRY
.5μ

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