Search for Causative Agents of the Sugarbeet Yellow Wilt in Chile[1]

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Introduction

Bennett and co-workers reported the findings of their investigation of the yellow wilt disease of sugarbeet in Argentina (4)[4] in 1946 and in Chile (3) in 1967. They concluded that (a) yellow wilt apparently was caused by a single virus, designated *Chlorogenus patagoniensis* n. sp. (or *Beta virus* 6); (b) there were two distinct sets of symptom expression: one the "yellowing phase" and the other the "wilting phase"; (c) the causative agent could be transmitted by grafting diseased to healthy tissue, by each of two species of dodder, *Cuscuta subinclusa* and *C. campestris,* and by a leafhopper, *Paratanus exitiosus;* and (d) the disease occurs in the field on sugarbeet, table beet, fodder beet, and Swiss chard in addition to 14 other host plants tested in the greenhouse and 2 tested in the field. Despite their extensive studies, many problems, such as visualization of the causative agent; its fate in host plants and in insect vectors; isolation, characterization and identification of the agent; and effective methods of controlling the disease remained unsolved.

At the time when the studies described above were made, the existence of a new group of plant pathogens, the mycoplasmalike organisms (MLO) (7, 19) had not yet been described. The MLO, now considered to be the etiological agents of a number of yellows-type plant diseases, are transmitted by leafhopper, planthopper, or psyllid vectors, as well as by grafting and dodders (25, 33). The MLO are sensitive to tetracycline derivatives. Based on this information, Ehrenfeld (11) demonstrated that the yellow wilt disease of sugarbeet was suppressed, but not eliminated, by chlorotetracycline treatments and suggested that the agent of yellow wilt was not a virus, but a MLO entity. Subsequently, Urbina-Vidal and Ehrenfeld (reported at the 1st International Congress for Bacteriology, Jerusalem, Israel, Sept. 3, 1973), using electron microscopy thin sectioning techniques, ob-

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[4]Numbers in parentheses refer to literature cited.

served microorganisms in yellow wilt-affected sugarbeet plants, morphologically resembling known MLO (25, 33). Further studies to visualize the presumptive etiological agents of yellow wilt in the diseased sugarbeet plants were carried out, based on a working hypothesis that the yellow wilt disease may be caused by a complex of several agents. The present report describes the coexistence of at least two possible etiological agents of the sugarbeet yellow wilt disease in Chile.

Materials and Methods

Sugarbeet (*Beta vulgaris* L.) seeds were seeded directly in a field of the La Platina Experimental Station, Santiago, Chile, in October 1972. About 50 days later symptoms of yellow wilt began to appear. During the next 9 months, the apparent incidence of infection increased gradually, finally reaching about 95% in September, 1973 (Fig. 1). During that period, nearly all of the affected plants developed symptoms of the yellowing phase of the disease including vein clearing and yellowing. In the later stages of disease development during that period many plants exhibited typical "witches broom" clusters with dwarfing and numerous axilary buds (Fig. 2), and some plants died. Symptoms of the wilting phase developed on a low percentage of the affected plants, but usually in combination with symptoms of the yellowing phase.

Portions of leaves and petioles were excised from the infected (Figure 2) as well as from normal-appearing plants and prefixed with 3% glutaraldehyde in 0.05 M sodium cacodylate buffered solution (pH 7.2) in the field at the end of September. Specimens in the fixative were processed for electron microscopy examination 5 days later. After rinsing in the cold buffered solution with 5% sucrose overnight, they were postfixed with 2% osmium tetroxide in the same buffered solution for 6 hours at 4°C. The fixed specimens were dehydrated at 4°C by passing through a graded ethanol series of 50, 75, 90, and 95% for 15 minutes each, and at 100% for 60 minutes. Subsequently the specimens were passed twice through propylene oxide for 60 minutes, and embedded in a resin mixture of Epon 812 and Araldite 506 (27). Ultrathin sections were cut with diamond knives on a Porter-Blum MT-2 microtome, and stained with 8% uranyl magnesium acetate in distilled water for 3-10 minutes at room temperature, followed by 0.4% lead citrate in 0.1 N sodium hydroxide for 20-60 seconds. The sections were studied with a Siemens Elmiskop I electron microscope, modified to a model IA, at 80KV.

Results

Two types of possible etiological agents of the yellow wilt were detected in the diseased plants, but were never observed in the normal-appearing materials. One was a mycoplasmalike organism (MLO) and

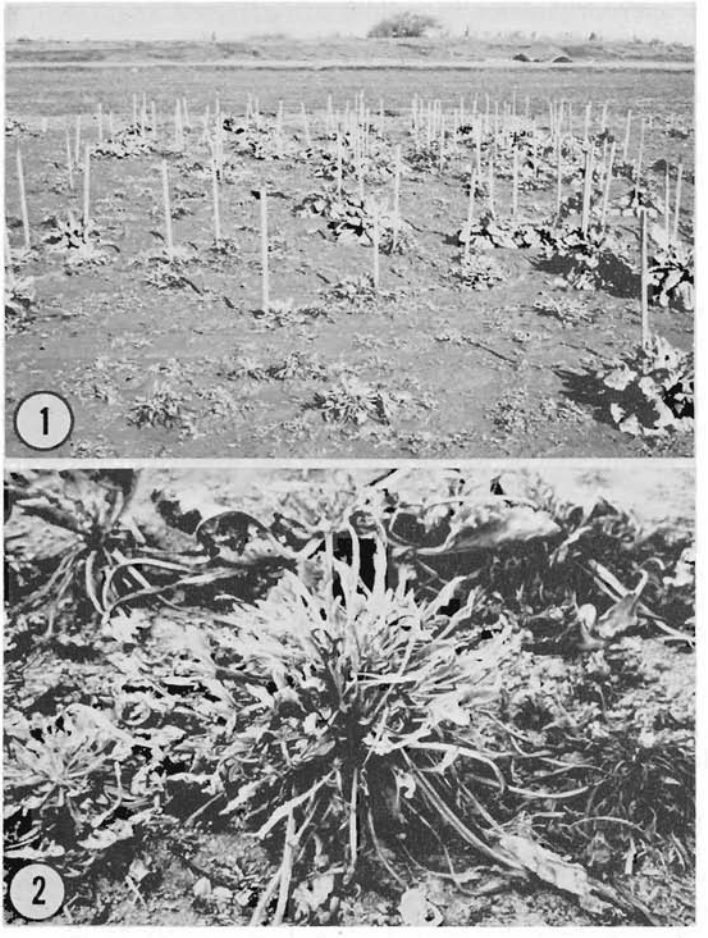


Figure 1.—Sugarbeet field in early September at the La Platina Experimental Station; 11 months old. More than 95% of the plants showed yellow wilt symptoms.

Figure 2.—Diseased plants showing advanced stages of the typical yellowing phase, including witches' broom clusters, dwarfing, and leaf necrosis.

the other was an elongated, flexuous viruslike particle associated with characteristic inclusions.

Mycoplasmalike organism (MLO)

Pleomorphic bodies whose morphology resembled that of microorganisms belonging to Mycoplasmatales which have been isolated from animals (1, 13), and MLO which have been observed in more than

50 yellows-type diseased plants (16-18, 25, 33), were detected in the phloem tissues of the yellow wilt-affected plants. The MLO were observed either in mature sieve tube elements, devoid of cytoplasm and filled with sieve tube sap (Fig. 3), or in the cytoplasm of phloem parenchyma cells (Fig. 4).

These bodies, bounded by a trilaminar membrane (unit membrane), contained ribosomes and deoxyribonucleic acid (DNA)-like strands and lacked a nuclear envelope and a cell wall (Figs. 3 to 6). They varied considerably in size and electron opacity. Wide variations of the morphological profiles, such as spherical, ovoidal, amoeboidal, long elliptical, horseshoe-shaped, and doughnut-shaped forms, were observed (Figs. 3 to 6). The majority of MLO varied in size from approximately 100 nm to 800 nm (Figs. 3 and 4). However, some of them, particularly those in the phloem parenchyma cells, had fine, long cytoplasmic protrusions containing electron-lucent cytoplasm (Fig. 6).

The number of MLO in each host cell varied considerably (Figs. 3 and 4). Some sieve tube elements, as well as phloem parenchyma cells, were tightly packed with numerous MLO, while others contained a small number of MLO scattered in the elements. In the cytoplasm of phloem parenchyma cells, "colony-type" accumulations (17) of the MLO were often seen (Fig. 4). In general, MLO around the sieve plates were more crowded on one side of the plate than on the other (Fig. 3). MLO, partially squeezed through the sieve pores, were observed often. This may indicate that the MLO move from one sieve element to a neighboring one via the sieve pores.

Morphological configuration indicative of binary fission, budding, extrusion of the protoplasm and tightly lined bodies, which were similar to those considered earlier to be undergoing reproduction in sieve tube elements (16) or in phloem parenchyma cells (17), were also seen in the phloem of yellow wilt-affected sugarbeet plants.

Elongated viruslike particles

Elongated, flexuous viruslike particles were observed in the cytoplasm of mesophyll cells and phloem parenchyma cells of leaves obtained from the diseased plants, but not in the normal-appearing materials. Morphological characteristics of the particles differed from that of microtubules and phloem protein (P-protein) fibrils, which are normal cytoplasmic constituents (Fig. 7). Although measurements of elongated, flexuous particles in thin sections did not reveal accurately their length, they appeared to be more than 650 nm in length and 10-12 nm in diameter. The particles often appeared in more or less parallel arrangements, oriented perpendicularly to cell walls (Fig. 7 to 9).

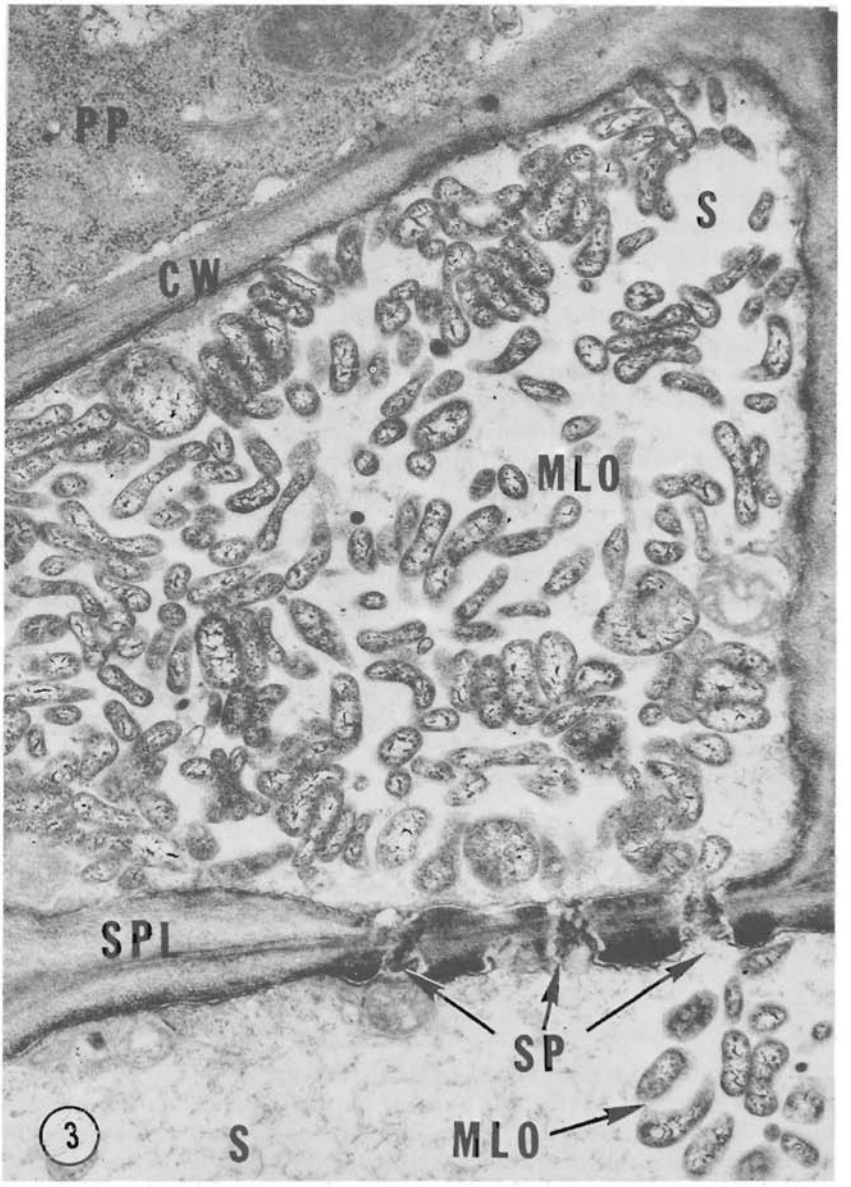


Figure 3.—Portion of phloem tissue from a yellow wilt-infected plant. Pleomorphic mycoplasmalike organisms (MLO), varying in shape and size, are present in the sieve tube elements (S). Note some MLO apparently passing through the sieve pores (SP). CW: cell wall; PP: Phloem parenchyma cell; SPL: Sieve plate. X 12,950.

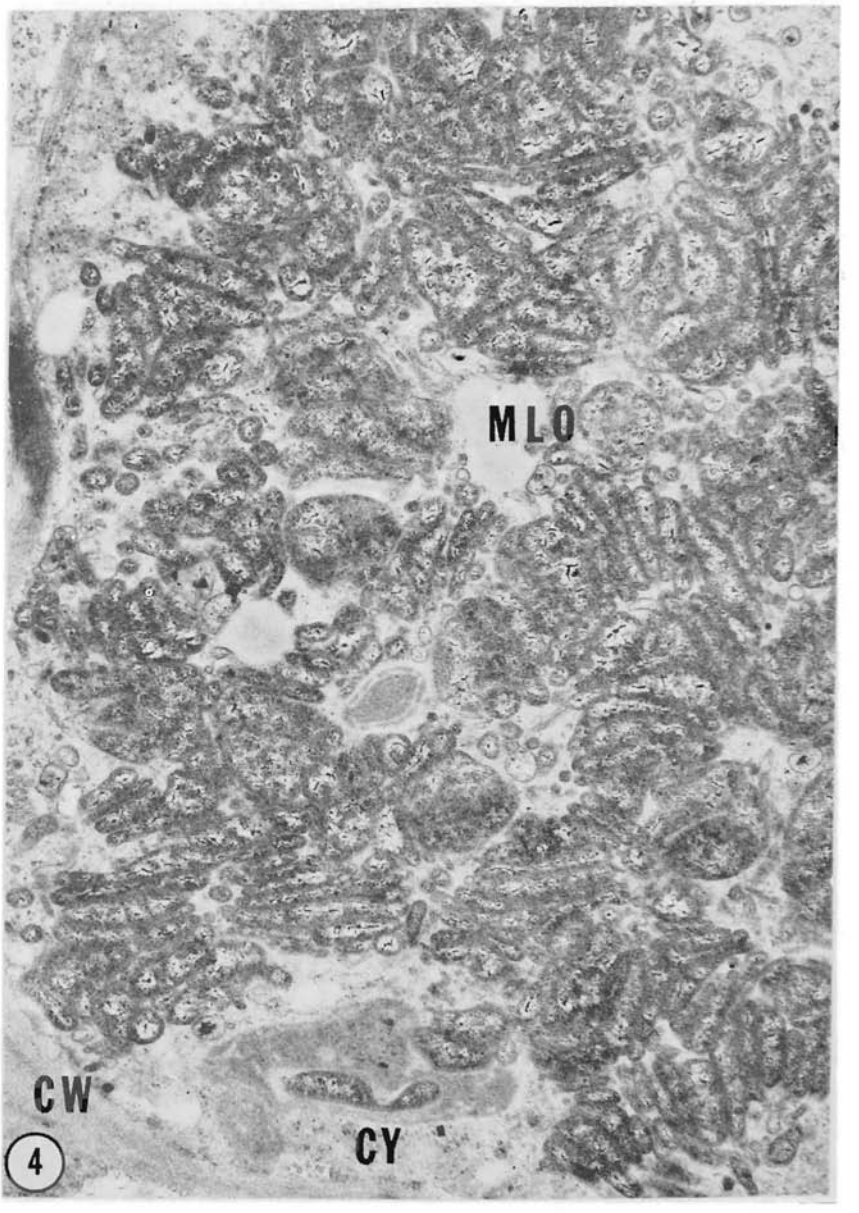
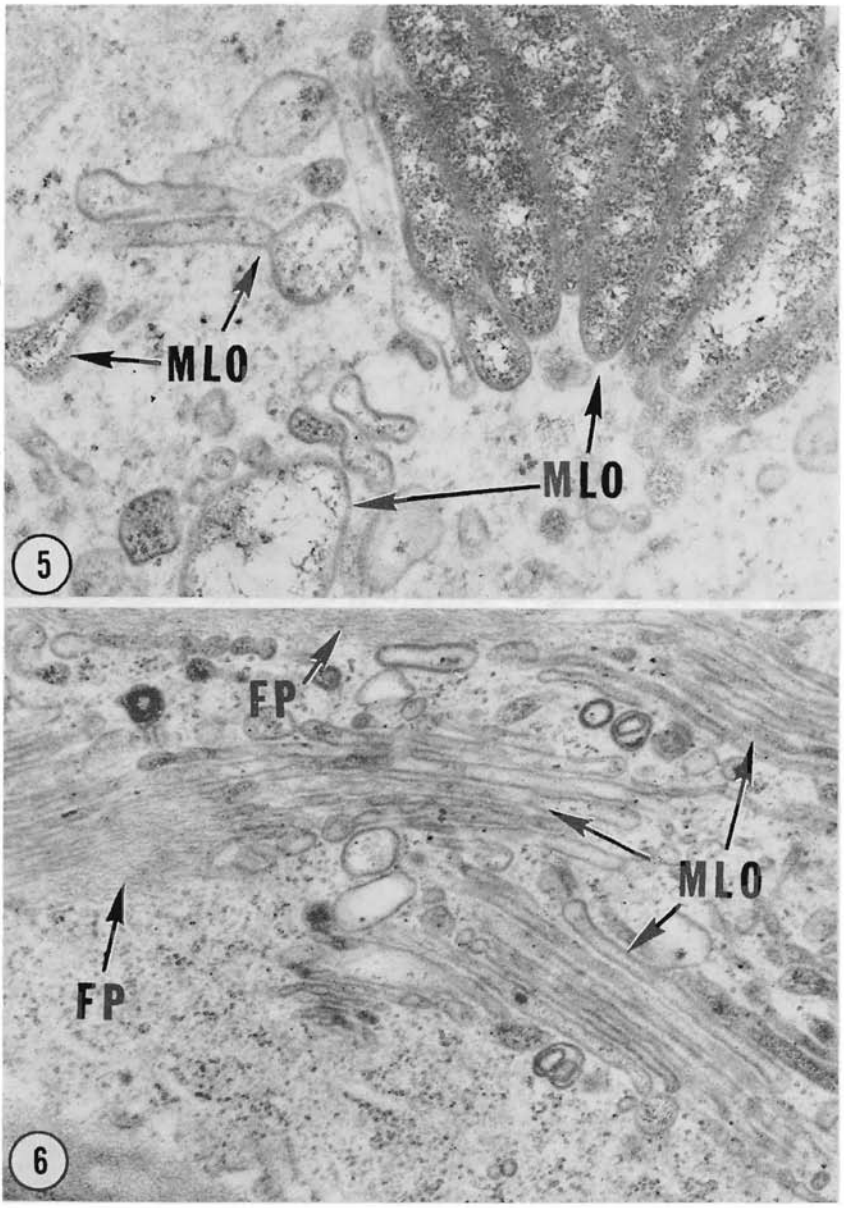


Figure 4.—Portion of a phloem parenchyma cell from a yellow wilt-diseased plant. Note colony type accumulation of intracytoplasmic mycoplasmalike organisms (MLO). CY: Cytoplasm; CW: Cell wall. X 7,000.



Figures 5 and 6.—Intracytoplasmic MLO in a phloem parenchyma cell of a diseased plant. Note the varying morphology and electron opacity of the MLO (Figure 5, X 48,250), and the long electron lucent extrusions of MLO, closely associated with filamentous particles (FP) (Figure 6, X 35,000).

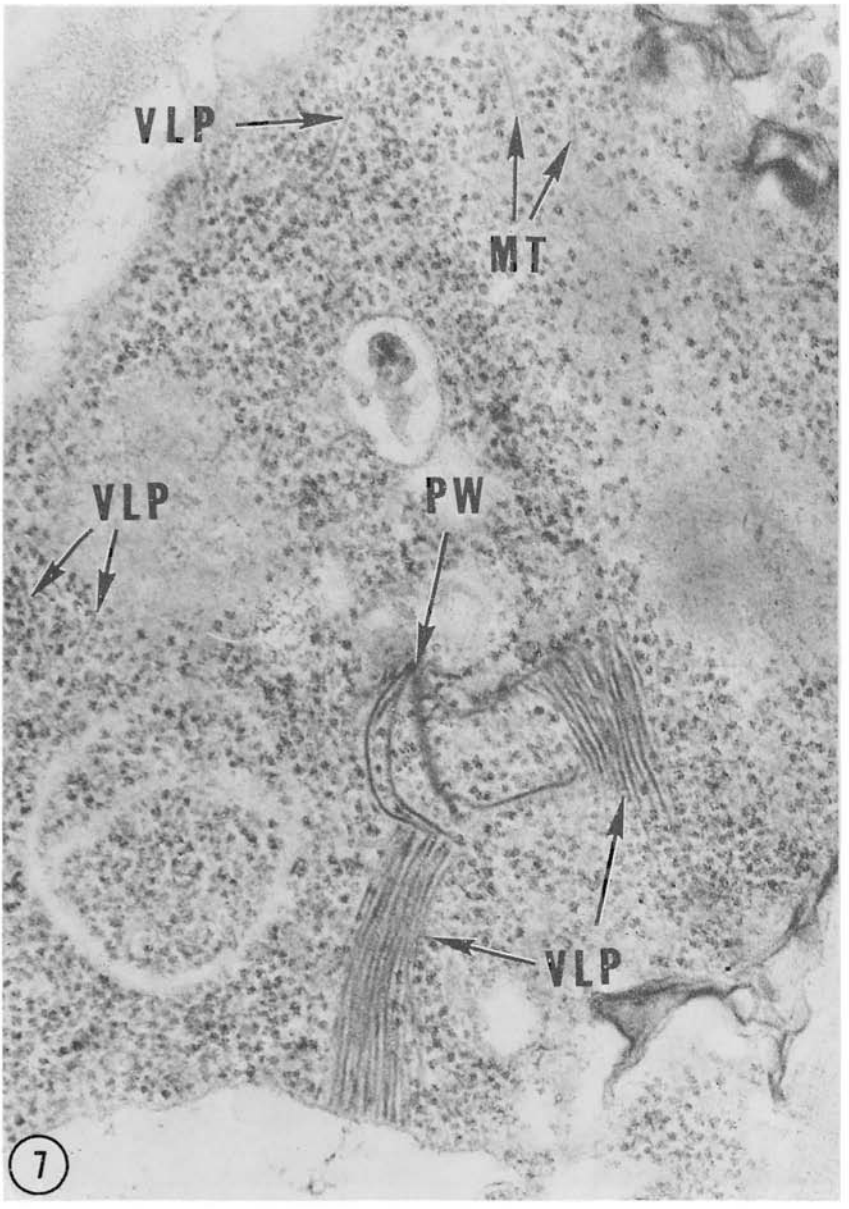
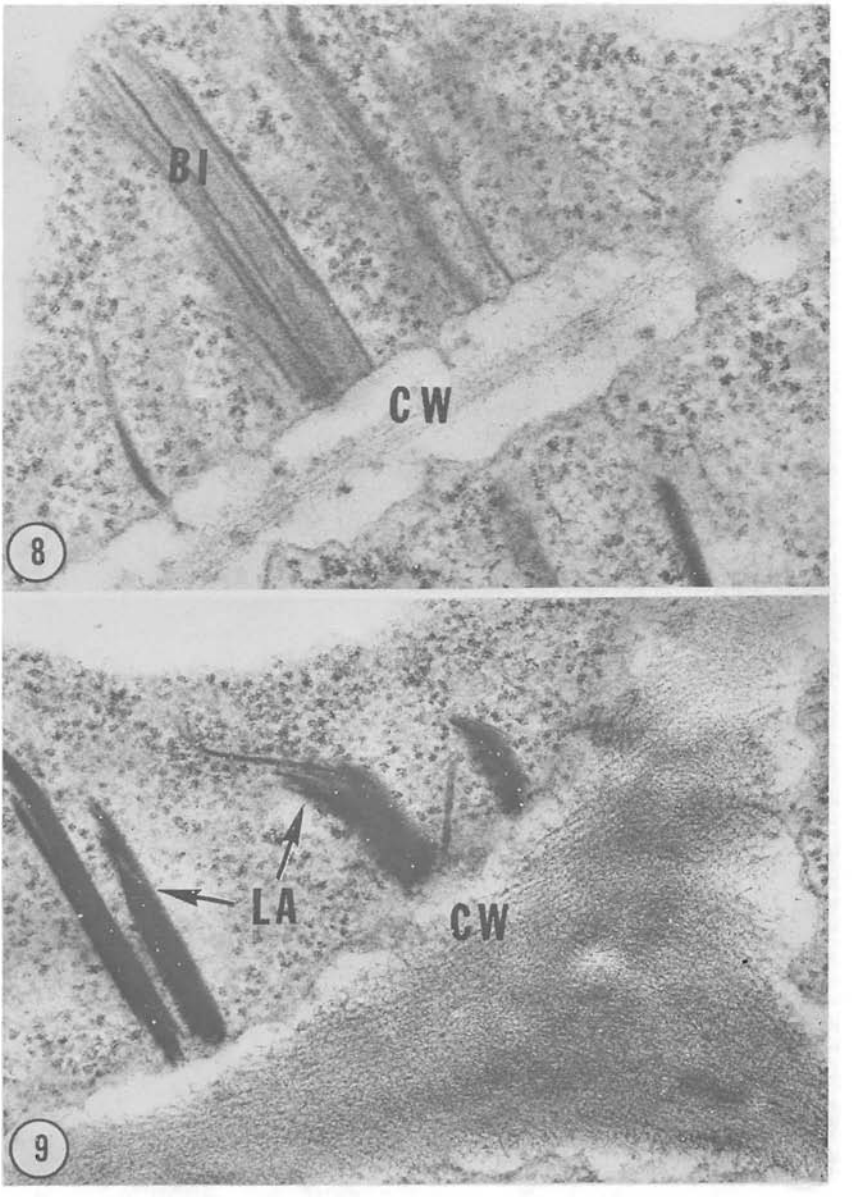


Figure 7.—Portion of a mesophyll cell of a yellow wilt-diseased plant, containing elongated viruslike particles (VLP) associated with a pinwheel inclusion (PW). Note VLP in more or less parallel arrangement and free VLP. MT: Microtubules. X 60,500.



Figures 8 and 9.—Portions of mesophyll cells obtained from a yellow wilt-diseased plant. Note characteristic bundle inclusions (Bi) (Figure 8, X 66,000) and laminated aggregates (LA) (Figure 9, X 50,100), oriented perpendicularly to cell wall (CW).

The particles were closely associated with various types of cytoplasmic inclusions, such as bundles (Fig. 8), laminated aggregates (Fig. 9), and pinwheels (Fig. 10). Complex inclusions consisting of pinwheel arms, bundles, and/or laminated aggregates were also seen. The morphological profiles of these inclusions strikingly resembled those observed by others in diseased plants infected with certain types of elongated viruses (2,6,8,10,14,15,20-24,26,28-32, 34).

Single particles, as well as inclusions, often appeared in the cytoplasm close to the plasmodesmata between parenchyma cells (Fig. 11), indicating that they may move from cell to cell through the plasmodesmata as pointed out recently by Weintraub *et al* (32). Occasionally the viruslike particles were aligned along the laminated aggregates (Fig. 12). At certain angles of sectioning, very fine spikes were seen on the surface of the aggregates.

In some instances, the inclusions were observed in the phloem parenchyma cell adjacent to the sieve elements in which MLO were seen (Fig. 12). Both MLO and the inclusions occasionally coexisted within the same phloem parenchyma cell (Fig. 13).

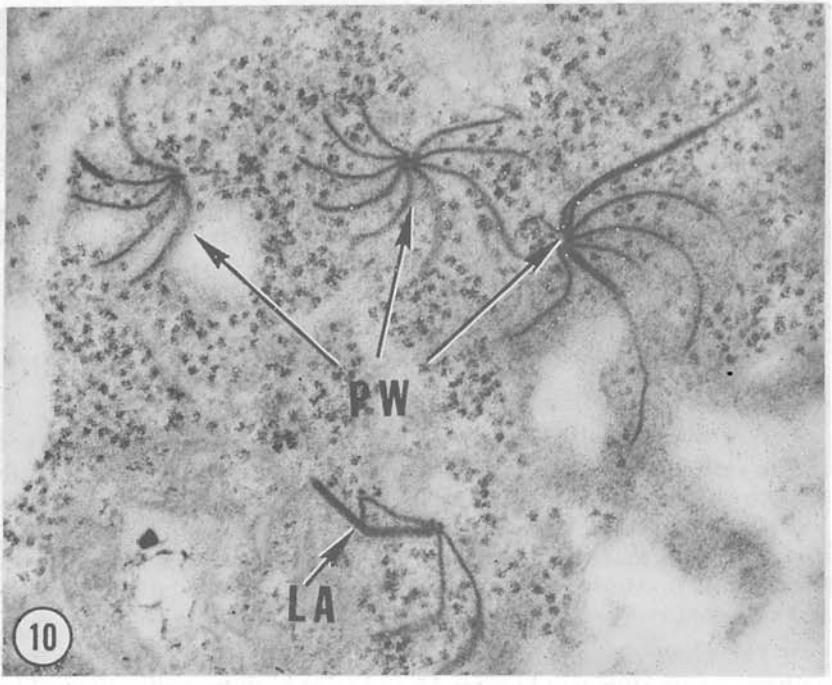


Figure 10.—Characteristic pinwheel inclusions (PW) in mesophyll cells of a yellow wilt-diseased plant. Complex inclusions consist of pinwheel arms and a laminated aggregate (LA). X 54,600.

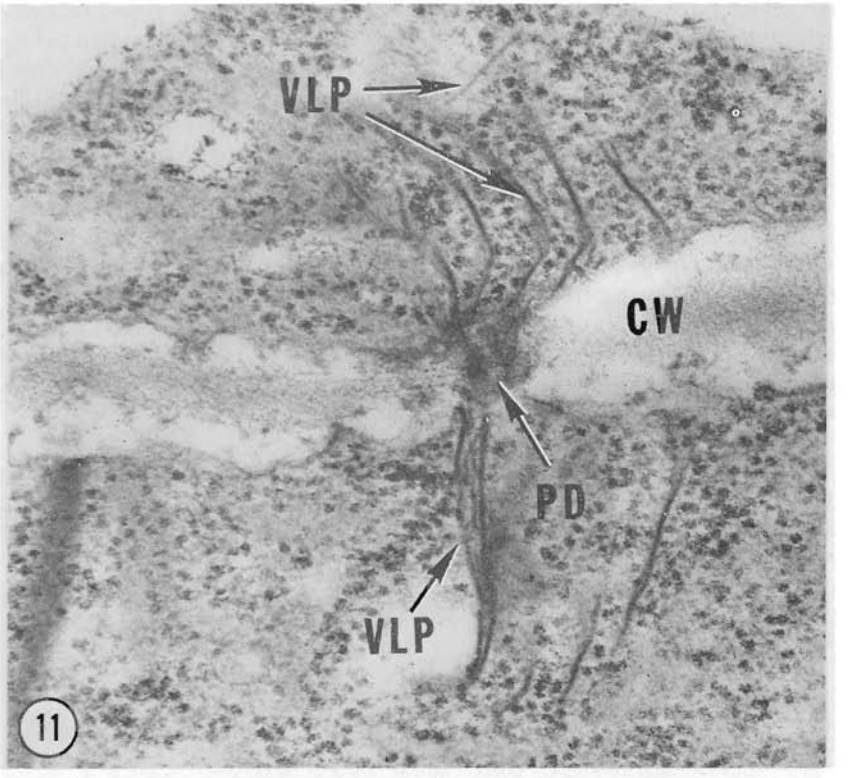


Figure 11.—Elongated viruslike particles (VLP) apparently passing through the plasmodesmata (PD) of parenchyma cells of a yellow wilt-diseased plant. CW: Cell wall. X 57,400.

Long filamentous particles

Masses of long filamentous particles were frequently observed in the cytoplasm of mesophyll, as well as phloem parenchyma, cells of both yellow wilt-infected and normal-appearing plants (Figure 14). Morphological features, size (approximately 1200-1300 nm in length) and appearance of the particles in the host cells were very similar to those of the presumed beet yellows virus particles in sugarbeet yellows-infected *Beta vulgaris,* described by Esau et al (12).

The filamentous particles that often formed large spindle-shaped aggregates are shown in Figures 15 and 16 at high magnifications. The particles also appear in irregularly aligned masses of various sizes (Fig. 14). In some instances, the particles formed highly ordered aggregates. A longitudinal section of the ordered aggregate is shown in

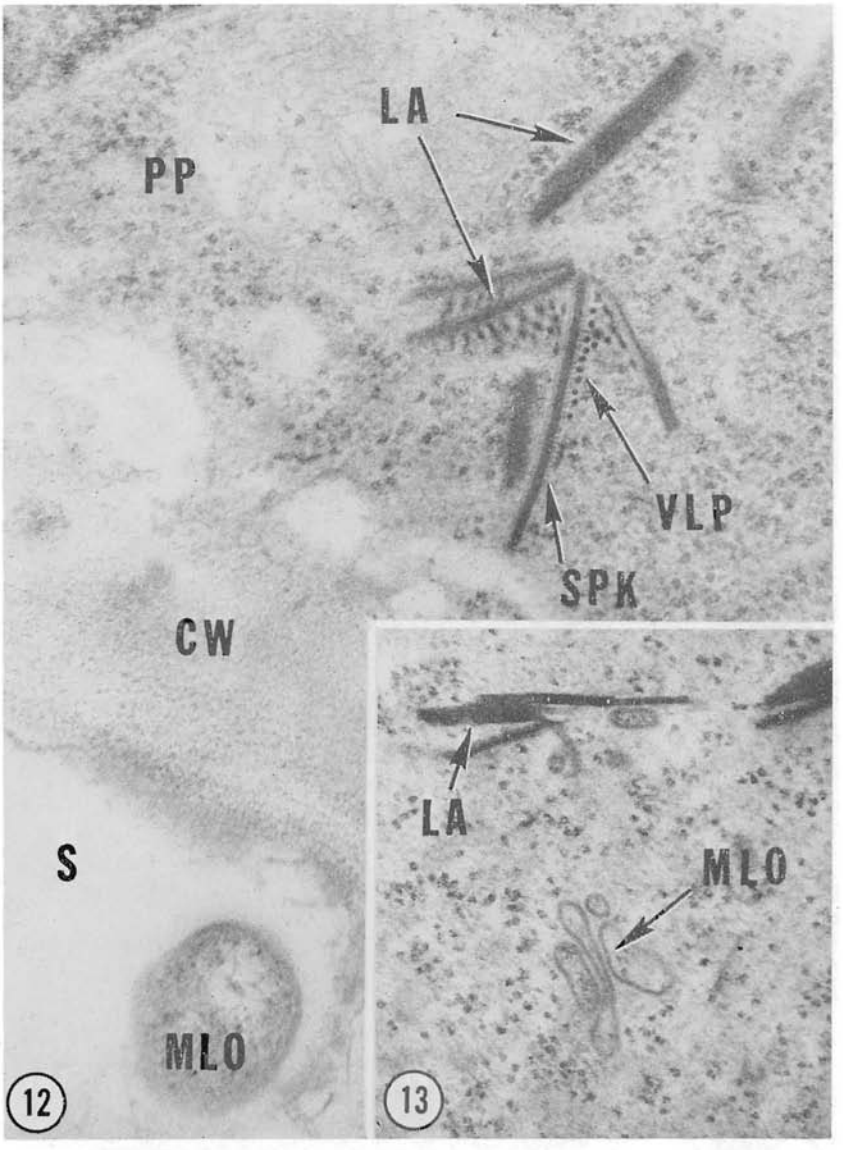


Figure 12.—Portion of phloem tissue from a yellow wilt-infected plant. Note MLO in the sieve tube element (S) and elongated viruslike particles (VLP), associated with the laminated aggregates (LA), in a phloem parenchyma cell (PP). CW: Cell wall; SPK: Fine spikes. X 68,250.

Figure 13.—Coexistence of mycoplasmalike organisms (MLO) and the laminated aggregates (LA) in the phloem parenchyma cell of a yellow wilt-infected plant. X 41,750.

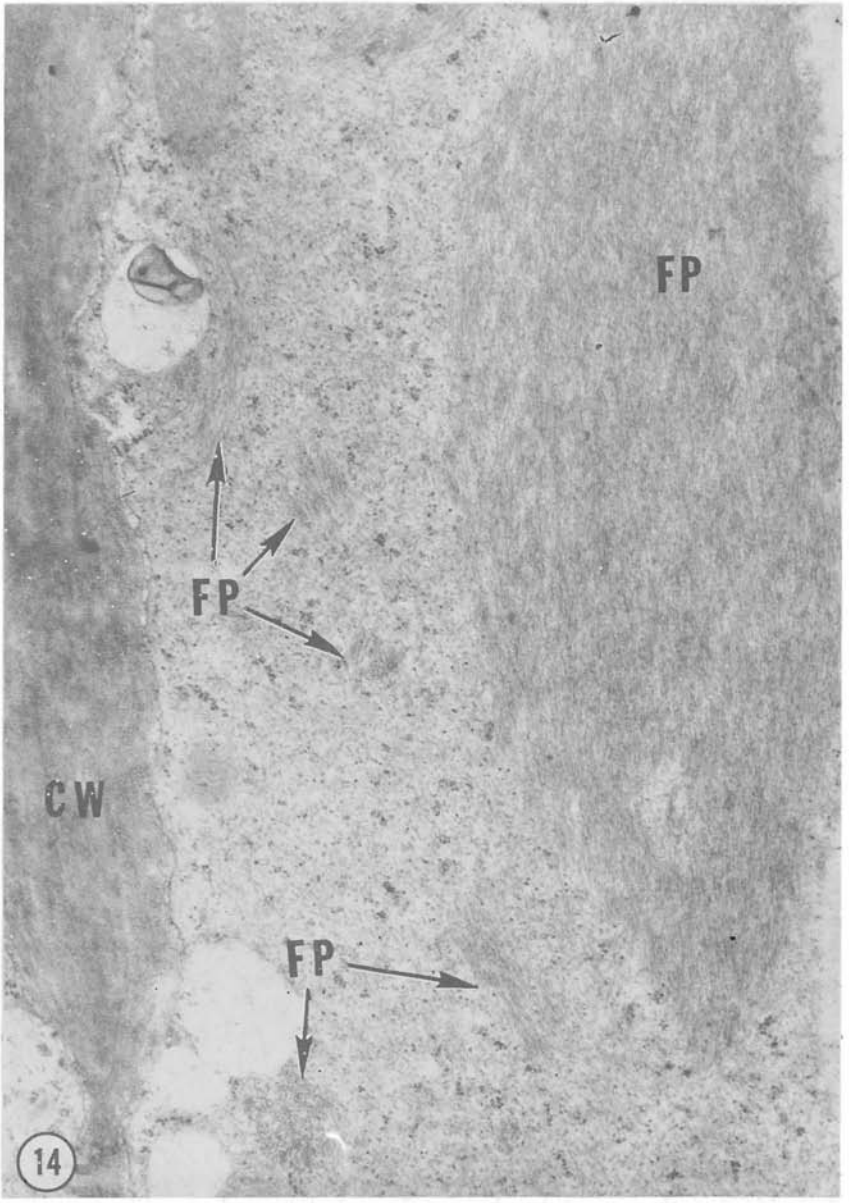
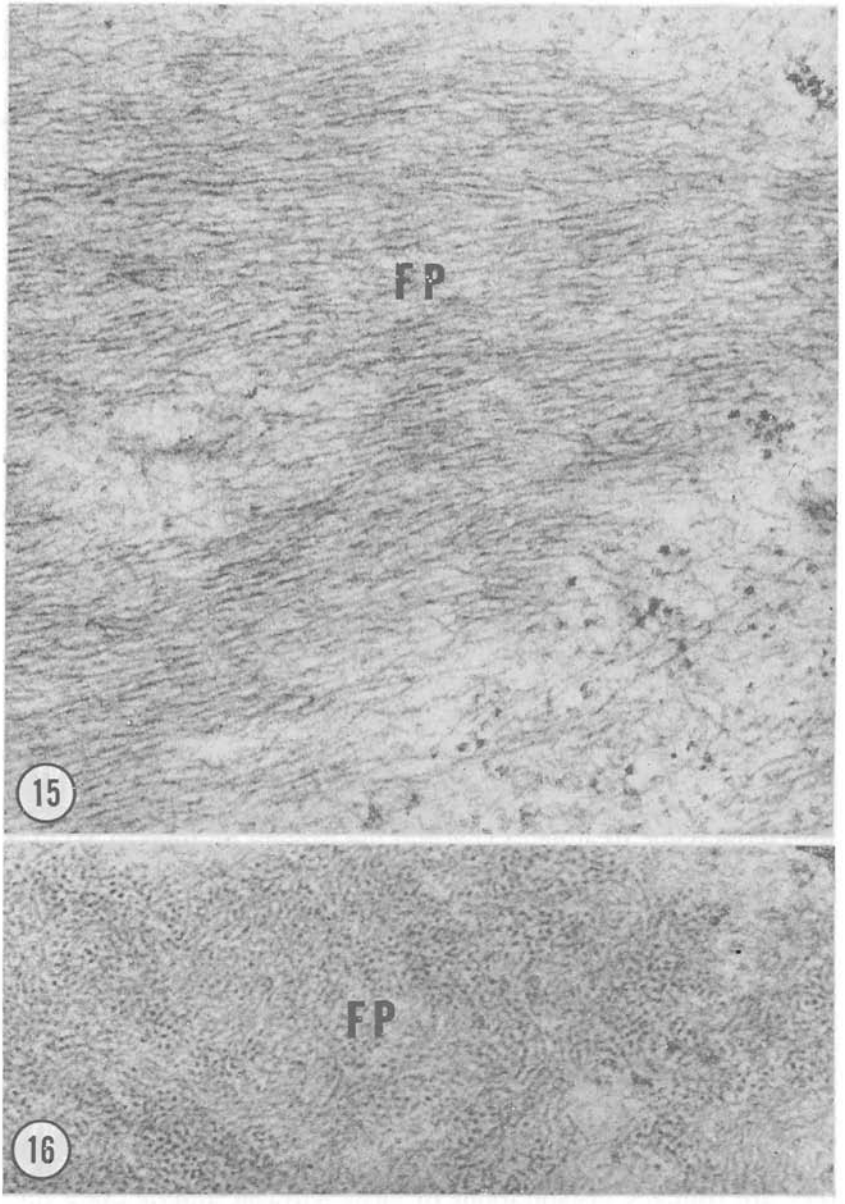


Figure 14.—Portion of mesophyll cell obtained from a normal-appearing sugarbeet plant. Note a large spindle-shaped aggregate, as well as small irregular masses, of long filamentous particles (FP). CW: Cell wall. X 18,750.



Figures 15 and 16.—Portion of large spindle aggregate of the long filamentous particles (FP) in the phloem parenchyma cells of a yellow wilt-diseased plant. Note loosely aligned particles in longitudinal section (Figure 15, X 49,000) and transverse section (Figure 16, X 49,000).

in Figure 17 and the transverse section in Figure 18. No enclosing membrane was seen around either large or small filamentous masses (Figures 14, 15, 17, and 18).

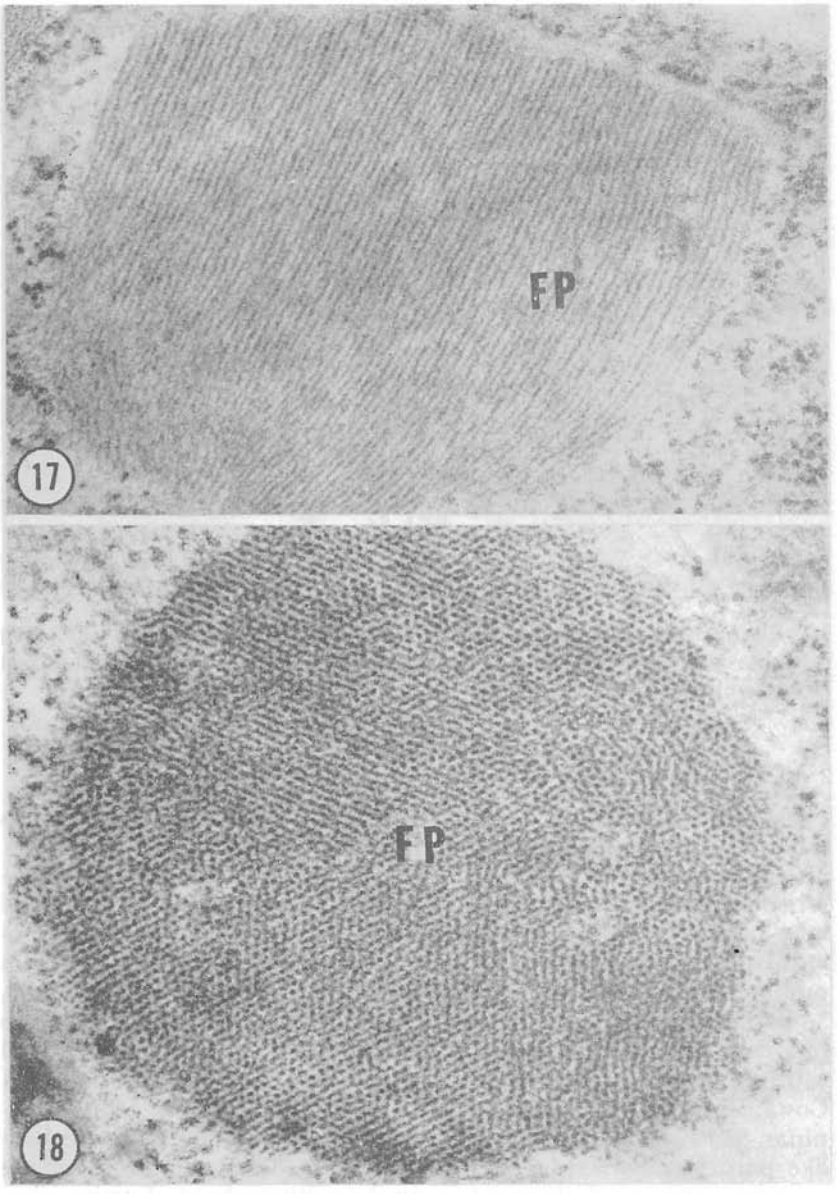
In the yellow wilt-infected plants, filamentous aggregates were often observed in phloem parenchyma cells which contained intracytoplasmic MLO (Figure 19). Occasionally the filamentous particles also were present in host cells, closely associated with the long cytoplasmic protrusions of the MLO (Figure 6) or with spherical MLO.

Discussion

Before the existence of mycoplasmalike organisms as pathogenic agents in plants became known, Bennett and co-workers (3) mentioned that "the diversity of symptoms of the yellow wilt of sugarbeet has led to speculation as to whether more than one causal agent is involved." However, they concluded that "results of observations and experiments strongly support the concept that all the different types of symptoms observed are the result of the action of a single agent and that this agent is a virus." They also concluded that the type of symptoms produced was determined largely by environmental conditions: the wilting phase of the disease appeared under conditions of high temperature and low humidity, whereas the yellowing phase appeared under conditions of lower temperature and higher humidity.

The characteristic symptom expressions of the yellowing phase described by Bennett *et al* (3, 4) strikingly resemble those of yellows type diseases, now known to be associated with or caused by MLO (16-18, 25, 33). Furthermore, successful suppression of the disease with tetracycline treatments (11) and the present findings of MLO in yellow wilt-diseased *Beta vulgaris* plants strongly support a new concept that at least symptom expressions of the yellowing phase are caused by MLO agents. However, no MLO agents associated with any wilting diseases are known to date despite the fact that more than 50 different MLO-induced diseases have been reported. If the MLO are indeed the causative agents of the yellowing phase, a complex etiology, most likely MLO and a virus (or viruses), of the sugarbeet yellow wilt is more acceptable than the single viral etiology (3).

There exists a controversy as to whether the characteristic inclusions, such as pinwheels, bundles, and laminated aggregates, consist of certain elongated virus particles (6, 15, 24, 26, 34) or whether they are complex organizations of virus particles and/or some substances of unknown nature (2, 10, 21). Nevertheless, based on the intimate association between the viruses and the characteristic inclusions, Edwardson (9) proposed that the inclusions are diagnostic for infection by viruses of the potato virus Y group (5), whose lengths vary from 730 nm to 750 nm. Later similar inclusions induced by shorter (31), as well as longer (14), viruses were also reported. It is



Figures 17 and 18.—Highly ordered aggregates of the long filamentous particles (FP) in the mesophyll cells obtained from a yellow wilt-infected plant. Note highly aligned particles in longitudinal section (Figure 17, X 49,100) and transverse section (Figure 18, X 49,100).

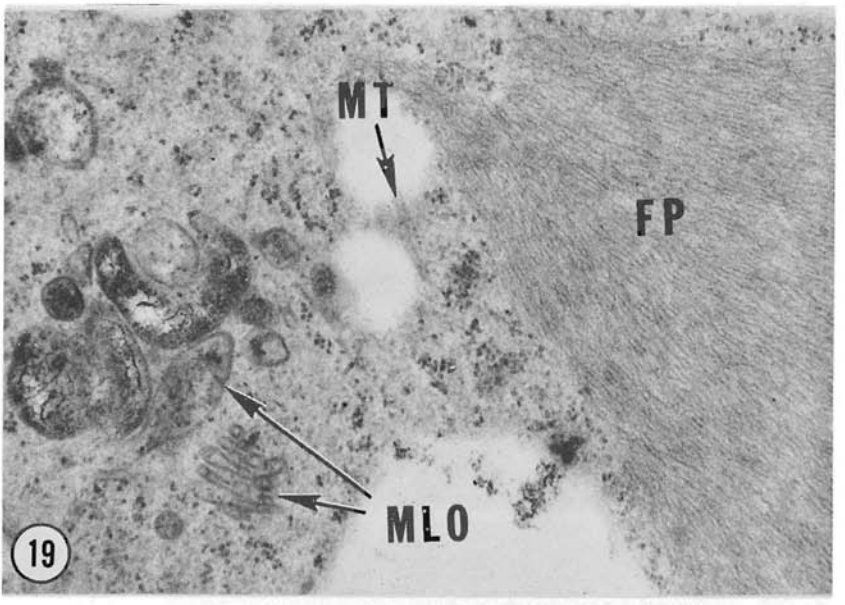


Figure 19.—Coexistence of the long filamentous particles (FP) and mycoplasma-like organisms (MLO) in a phloem parenchyma cell obtained from a yellow wilt-infected plant. MT: Microtubules. X 32,630.

generally accepted that the presence of the characteristic inclusions indicates the occurrence of infection with certain elongated viruses. Thus, the finding of the elongated viruslike particles with the characteristic inclusions suggests that the diseased sugarbeet plants examined may be infected with an inclusion-inducing virus. The inclusion-inducing elongated viruses are known to be causative agents of a number of plant diseases (2, 6, 8-10, 14, 15, 19, 20, 22-24, 26, 28-31, 34). Although no elongated viruses which induce wilting have been reported, one of the potato virus Y group is known to be associated with the sugarbeet wilting in Brazil (personal communication: A. S. Costa, Virus Section, The Agricultural Institute, C.P. 28, 13100 Campinas, Sao Paulo). At present, it is uncertain whether elongated viruslike particles are responsible in part for causing symptoms of the sugarbeet yellow wilt disease (e.g. in the wilting phase). Further electron microscopic examination of diseased plants representing the two distinct types (i.e. the yellowing phase and the wilting phase) may provide more critical information on this problem. Recently the coexistence of MLO and an elongated virus, russet crack virus, associated with pinwheel inclusions in sweet potato plants was reported (20).

The nature of the long filamentous particles observed in both yellow wilt-infected and apparently noninfected sugarbeet plants remains unknown. However, since those particles resembled the beet yellows virus (12), and since one or more types of virus yellows usually is quite prevalent in the sugarbeet crop in Chile, including La Platina (personal communication: J. O. Gaskill, Beet Sugar Development Foundation, Fort Collins, Colo.), it seems probable that the long filamentous particles observed in this study resulted from infection by at least one of the viruses of the sugarbeet virus yellows complex. Since the effects apparently were mild, as evidenced by the appearance of plants without yellow wilt symptoms, it is assumed that a mild type of virus was involved, such as a mild strain of beet yellows virus.

If the above assumption of a yellows-virus involvement is correct the findings indicate that the yellow wilt-diseased plants examined were super infected with the yellows virus. The role, if any, of that virus in the development of symptoms of yellow wilt is not known. However, it is apparent that controlled conditions of plant growth and inoculation are needed in future electron microscopy studies of the causal agent or agents of yellow wilt.

Summary

An electron microscopic search for the causative agents of the sugarbeet yellow wilt disease in Chile was carried out. Mycoplasmalike organisms (MLO), as well as elongated viruslike particles associated with characteristic inclusions (such as pinwheels, bundles, and laminated aggregates), were observed in the diseased plants, but were absent from normal-appearing plants. The presence of the MLO strongly suggests that they are largely responsible for causing the yellowing phase of the disease.

The presence of the elongated viruslike particles associated with characteristic inclusions suggests that the diseased plants were infected with a virus. Although the elongated particles may be responsible in part for yellow wilt symptom expression, further studies are needed to clarify their etiologic role.

These findings suggest the possibility of a complex disease etiology, presumably with the MLO and a viral agent rather than the single MLO or viral etiology.

Long filamentous particles, morphologically resembling the beet yellows virus, were observed in both the yellow wilt diseased and the normal-appearing plants grown in the same field. This suggests that the yellow wilt-diseased sugarbeet plants may also have been infected with a type of beet yellows virus.

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