Scanning Electron Microscopy of Mycoplasmalike Organisms After Freeze Fracture of Plant Tissues Affected with Clover Phyllody and Aster Yellows

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ABSTRACT

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Scanning electron microscopy (SEM) of freeze-fractured root and petiole tissues of aster yellows and clover phyllody-affected aster (*Callistephus chinensis*) plants revealed the presence of mycoplasmalike organisms (MLO). The identification of MLO was based on the presence of polymorphic bodies such as filamentous branching forms, 'dumbells', and budding spherical bodies in the phloem cells, presumbably the sieve elements; these were not observed in healthy plants. The sizes and polymorphic forms of the MLO found in aster yellows- and clover phyllody-affected plants were not significantly different. The advantages and disadvantages of freeze-fracture SEM are discussed.

Mycoplasmalike organisms (MLO) have been implicated as causal agents of many plant diseases (5). In infected plants, MLO have been found mainly in the sieve elements of the vascular tissues. They are recognized in thin-section electron micrographs by a variety of spherical and filamentous, often branching, forms, a bounding trilaminar membrane, and a content of fibrils and granules presumed to be DNA and ribosomes, respectively.

We show in this paper that the location of MLO in the relatively empty sieve elements is particularly favorable for examination by freeze-fracture techniques in the scanning electron microscope (SEM). Although secondary electron SEM does not at present have the degree of resolution that makes thin-section transmission electron microscopy (TEM) so valuable for revealing the internal structures of mycoplasma cells, it is an excellent tool for examination of the variety of MLO cell forms. It allows rapid examination of a large number of phloem cells, and, moreover, a large part of the interior of each phloem cell, compared with the small-volume sampling of thin-section TEM. The value of SEM in determining the surface morphology, size, and shape of some animal mycoplasmas in suspensions has been demonstrated (4) but it has not previously been possible to examine mycoplasma or MLO in situ by SEM.

In the present work we report employing the freeze-fracture and SEM technique for the visualization of MLO in aster plants affected with clover phyllody and aster yellows diseases.

MATERIALS AND METHODS

Infected plants.—Aster (*Callistephus chinenis* Nees) plants affected with either clover phyllody or aster yellows were obtained by inoculation via the leafhopper vector *Macrosteles fascifrons* Stål. as described earlier (7, 8). Roots and petioles of young leaves of aster plants showing pronounced symptoms were used in this study (3-4 wk after inoculation in the case of aster yellows, and 6-7 wk in the case of clover phyllody). Healthy plants of the same age as diseased plants were used as controls.

Freeze fracture and electron microscopy.—Root and petiole tissues, 3-5 mm in length, were fixed in 6% glutaraldehyde and postfixed in 2% OsO_4 as described earlier (9). Then they were stained in 2% aqueous uranyl acetate for 2 hr at 37 C with subsequent dehydration through a 10% incremental ethanol series to absolute ethanol. (Although this staining procedure is not necessary for SEM viewing, all samples were treated identically so that they could be compared by SEM and thin-section TEM). At this point, the specimens were grouped into two sets, one for SEM viewing, the other for comparative thin-section TEM. For SEM viewing, samples were prepared by the technique of Sybers and Ashraf (11) as modified by Humphreys et al. (3): tissue pieces prepared as described above were taken from the absolute ethanol and frozen on copper support disks. Then they were fractured in any chosen plane with a scalpel or razor blade (this can be done with the copper disk mounted just below the liquid nitrogen surface and the tissue viewed through a dissecting microscope). Then the fractured tissues were thawed in absolute ethanol, rinsed several times in ethanol, placed in the pressure chamber of a SAMDRI PVT-3 critical-point drier (Biodynamics Research Corp., Rockville, MD 20852), infiltrated with liquid CO_2, critical-point dried, mounted, and coated in a vacuum on a rotary stage, first with about 5-nm carbon and then with 20- to 25-nm gold, and examined in a Cambridge MK IIA SEM at 20 KV. Coating thicknesses were determined using an Edwards FTM2 film-thickness monitor (Edwards, Crawley,

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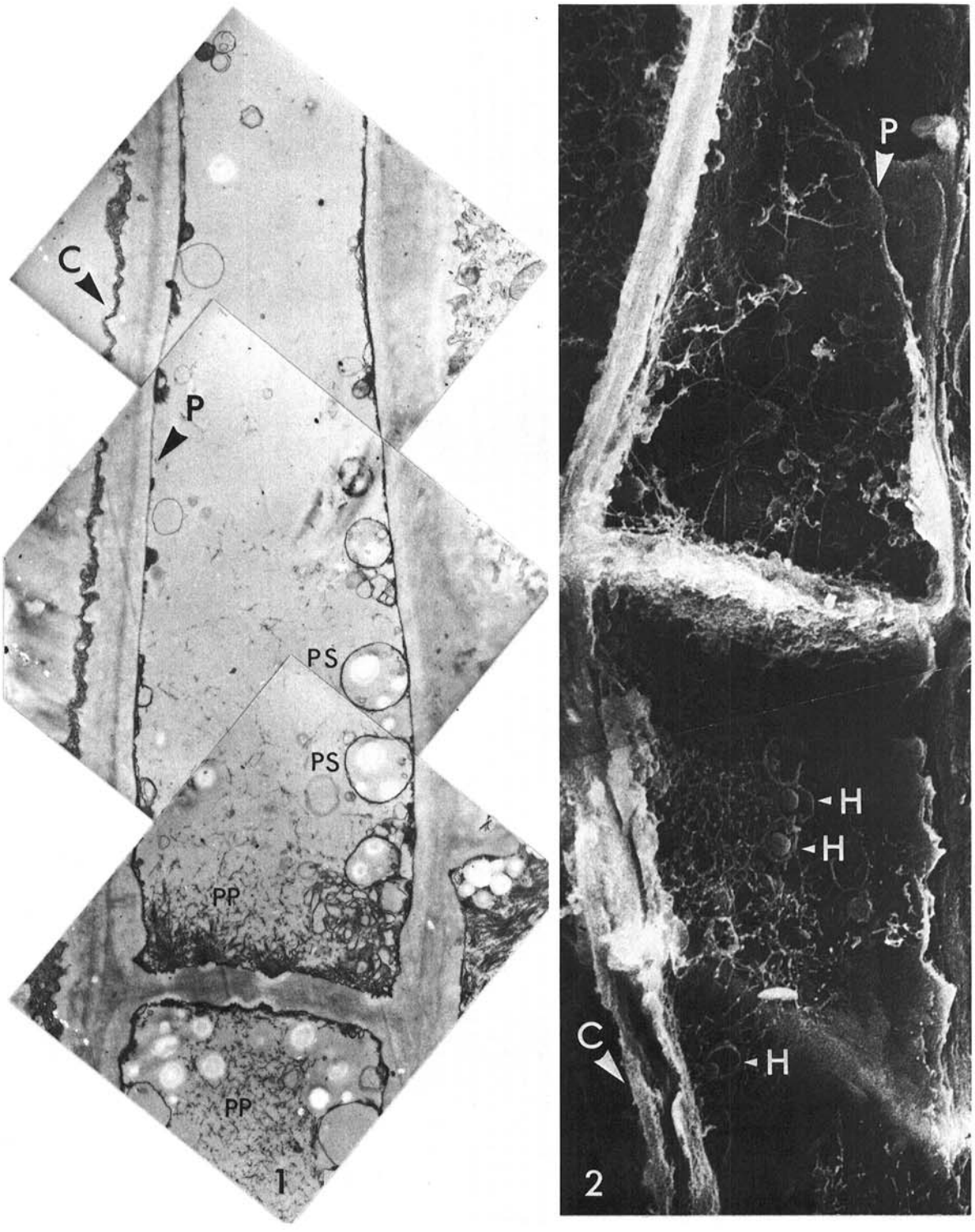

Fig. 1-2. 1) Thin-section transmission electron micrograph of phloem cells of healthy aster petiole. Plasma membrane of the phloem cell (P). This cell contains plastids with starch grains (PS) and also P-protein fibrils (PP). A thin layer of cytoplasm (C) of an adjacent vacuolated cell also can be seen (× 8,000). 2) Scanning electron micrograph of phloem cells of healthy aster root showing a fracture through the phloem cell plasma membrane (P) and through the cytoplasmic layer of an adjacent cell (C). Fibrous P-protein and vacuoles correspond well with structures seen in thin sections (Fig. 1). Fractured plastids or vacuoles reveal a hollow interior (H), often with inner bodies that may be starch grains (compare Fig. 1) (× 8,000).

England). For TEM, the ethanol-dehydrated samples were continued through propylene oxide-Spurr's resin dilutions, to a final embeddment in Spurr's medium (10). Sections were cut on a Reichert OMU2 ultramicrotome, stained 12 min with lead citrate, and viewed in a Philips 300 electron microscope at 60 KV.

RESULTS AND DISCUSSION

Our first consideration, in seeking to locate and identify MLO in SEM preparations, was the identification of phloem cells in the fracture face. Phloem cells are identified easily in thin sections; the increased

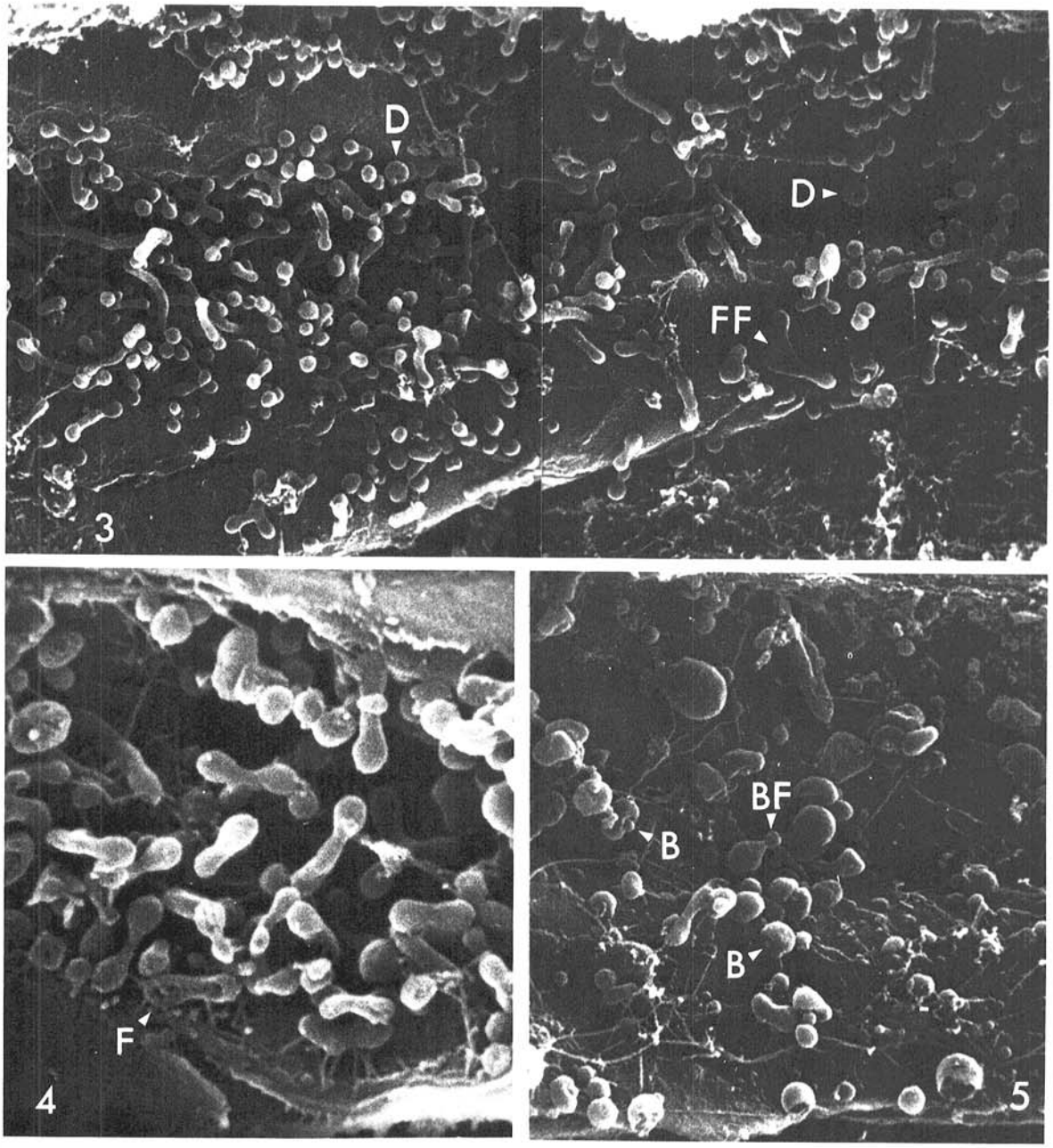

Fig. 3-5. Scanning electron micrographs of tissues after freeze fracture showing mycoplasmalike organisms (MLO). **3)** Clover phyllody affected aster root. Note filamentous forms, branching forms,and two 'dimpled' or ring forms (D) of MLO. The branching forms appear slightly flattened (FF) when lying against the plasma membrane (× 10,000). **4)** Aster yellows affected aster petiole. Note 'dumbell' forms of MLO including one (F) which lies at the fracture plane and has fractured through to reveal some internal detail (× 20,000). **5)** Aster yellows affected aster root. Note two 'dimpled' forms of MLO in each of which a smaller body (B) rests in the dimple. A 'budding' form of MLO is seen also (BF) (× 10,000).

resolution shows clearly the plasma membrane, as shown for the petiole of healthy aster plants in Fig. 1. Similar micrographs were obtained for the phloem cells of aster roots. The cytoplasm contains fibrils (P-protein) and spherical bodies in the size range of 200 to 1,500 nm (plastids, mitochondria, and vesicular bodies). In contrast, the identification of phloem cells in the fracture face by SEM is rather difficult. Viewed in this way most plant cells appear 'empty'. Phloem cells show a fractured plasma membrane at their circumference; most of the surrounding cells show a vacuole with a fracture through a thin layer of peripheral cytoplasm. This cytoplasmic layer is often only 50 to 100 nm thick, and both this layer and the plasma membrane of phloem cells often closely adhere to the outer cell wall. In the fracture face it is not always possible to distinguish between plasma membrane and cytoplasmic layer, or to resolve these from the cell wall (Fig. 2). In favorable cases, it is possible to examine the cell periphery to find a thicker area of fractured cytoplasm and thus to identify the cell as other than a phloem cell. In Fig. 2 the dimensions, the position in the tissue, and the content of phloem cells make identification reasonably certain. It is in cells of this type that we have looked for MLO in infected plants. Representative micrographs of such cells in infected plants containing MLO are included in Fig. 3 to 5. The branching, polymorphic forms seen in infected but not in healthy plants, leave little doubt that these are in fact MLO.

We should now consider in more detail exactly what features constitute reasonably certain identification of MLO. Spherical bodies in the size range 200 to 1,500 nm, even though numerous (Fig. 5), cannot be taken as evidence that these are MLO, since bodies in this size range are present in healthy phloem, and we cannot distinguish with certainty between these bodies and MLO by SEM. Here we have to look for some characteristic morphological detail as evidence that the bodies seen are MLO. At present definite identification of MLO can only be based on the presence of filamentous, branching forms (Fig. 3), the 'dumbells' (Fig. 4), which presumably are MLO in the process of binary fission (6), and spherical bodies with a bud (Fig. 5). No structures of this kind are found in phloem cells of healthy plants, or in the vacuoles of other cells which might be mistaken in the SEM fracture face for phloem cells. One of the less common, but probably significant forms is shown in Fig. 5 (labeled B). This is a 'dimpled' or 'doughnut' shape containing a small body in the dimple. Fig. 3 contains two dimpled forms (labeled D) one without an associated small body and one with a small body lying just to one side of the central depression. The dimpled forms with central bodies (as in Fig. 5) may correspond to the 'mushroom' type of structure found in thin-section TEM of aster yellows-affected tobacco leaves (2). Current concepts of the mycoplasma life cycle provide no adequate explanation for these forms. As seen in SEM micrographs this seems to be a type of bud formation, in which a part of the cell surface, where the bud is forming, is drawn into the main body of the parent cell. Alternatively, this could represent some kind of secretory process. After the bud, or secretory vesicle, has pinched off, this appears to leave the parent cell in dimpled form. It seems unlikely that so specific an effect could be a fixation or preparative artefact. Brown et al. (1) also reported a dimpled or doughnut form to be common in animal mycoplasmas observed on the surface of cells in tissue culture. Another slightly different form (FF in Fig. 3) is a flattened filament, seen where the MLO filament lies against the inner surface of the plasma membrane of the phloem cell.

We found no significant difference, in variety or type of forms seen, between MLO's present in tissues affected by aster yellows and clover phyllody affected tissues, or between root and petioles. The dimensions of the various forms seen in tissues of plants affected by the two diseases are in close agreement with those reported for MLO in thin sections (2, 9) provided 50 nm is subtracted from the measured diameters in SEM micrographs to allow for an estimated overall gold covering of 25 nm. Freeze-fracture SEM micrographs offer an advantage over thin-section TEM in that the entire shape of a number of MLO can be seen in each micrograph. This facilitates evaluation of the relative number of the different forms present.

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