A Fixation Method for Demonstrating Mycoplasmalike Organisms in Plants

Larry Dale Smith, J. L. Dale, and K. S. Kim

Graduate Assistant, Professor and Assistant Professor, respectively, Department of Plant Pathology, University of Arkansas, Fayetteville 72701.

Published with the approval of the Director, Arkansas Agricultural Experiment Station, Fayetteville.
Accepted for publication 24 October 1975.

ABSTRACT

SMITH, L. D., J. L. DALE, and K. S. KIM. 1976. A fixation method for demonstrating mycoplasmalike organisms in plants. Phytopathology 66: 531-533

A comparison of the effect of six different fixation procedures on the ultrastructural appearance of mycoplasmalike organisms (MLO's) in plants indicated that fixation with chrome-osmium consistently produced distinct electron-dense aggregate structures in the central area of the MLO's. The formation of such characteristic structures makes it possible to distinguish MLO's from normal cell components. The results suggest that the chrome-osmium fixative would be useful for the examination of field-collected plants showing yellows type symptoms where the objective is to detect the entities inhabiting such plants.

In 1967, Doi et al. (5) observed mycoplasmalike organisms (MLO's) in sieve tube elements of plants affected by various diseases, and MLO's have since been found associated with more than 70 plant diseases (10). Attempts to grow MLO's in culture have failed with the exception of *Spiroplasma citri* from the stubborn disease of citrus (14) and the spiroplasma associated with the corn stunt disease (1). All other associations with plant diseases are based on electron microscope observations of MLO's in plants showing symptoms and their absence in healthy plants.

Glutaraldehyde with osmium tetroxide postfixation is a commonly used fixative which reveals MLO's as pleomorphic bodies bounded by unit membranes and generally containing randomly dispersed ribosomes and delicate DNA-like fibrils in the central area of the organism (4). It is sometimes difficult, however, to identify MLO's in diseased plants by electron microscopy since normal cell constituents can have some of the same characteristics (11). For example, Florance and Cameron (6) demonstrated that healthy peach tissue, fixed with glutaraldehyde and osmium, can contain vesicles in expanded endoplasmic reticulum cisternae which closely resemble MLO's.

Some electron micrographs show MLO's which contain intensely electron-dense fibrils, a characteristic which could possibly serve to distinguish them from other cell components.

This study was initiated to compare the effect of various fixation procedures on the ultrastructural appearance of MLO's in infected plants and to determine whether any of these procedures might be useful for distinguishing MLO's from normal cell components. This paper reports the general characteristics of MLO's resulting from several fixation procedures with emphasis on the results obtained from chrome-osmium and the characteristics which appear to make it a useful procedure for detecting and distinguishing MLO's in plants.

MATERIALS AND METHODS

The plant species primarily utilized in this investigation were tomato (*Lycopersicon esculentum* L. 'Manapal'), periwinkle (*Vinca rosea* L.), and dodder (*Cuscuta subinclusa* Dur. & Hilg.).

The MLO that was examined most extensively was from tomato plants affected by the big bud disease (2). The MLO was perpetuated in the greenhouse by grafting diseased tomato scions to healthy tomato plants, or by transmission via dodder to periwinkle.

Specimens for fixation were veinal tissue from leaves or 3-mm shoot portions of dodder, with both healthy and diseased tissue being studied.

The six fixation procedures employed in this study were: (i) glutaraldehyde fixation with osmium tetroxide postfixation (13); (ii) chrome-osmium fixation (3); (iii) chrome-osmium fixation as in B, but treated for 12 hours with 0.5% aqueous uranyl acetate prior to dehydration; (iv) fixation with 5% potassium dichromate-potassium hydroxide solution, pH 7.2, for 1 hour at 4 C; (v) fixation with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 1 hour at 25 C; and (vi) potassium permanganate fixation (9). With some specimens the tonicity of the permanganate solution was adjusted to 14 atmospheres with NaCl.

After fixation and rinsing, all specimens were dehydrated in a graded ethanol series followed by 100% propylene oxide and then embedded in Epon 812. The specimens were sectioned on a Sorvall Porter-Blum MT-2B ultramicrotome. All sections were stained with 2% uranyl acetate for 2 hours and lead citrate for 10 minutes prior to examination by a Siemens Elmiskop 1A.

RESULTS

In all specimens of infected plants fixed with chrome-osmium the MLO's exhibited distinct electron-dense

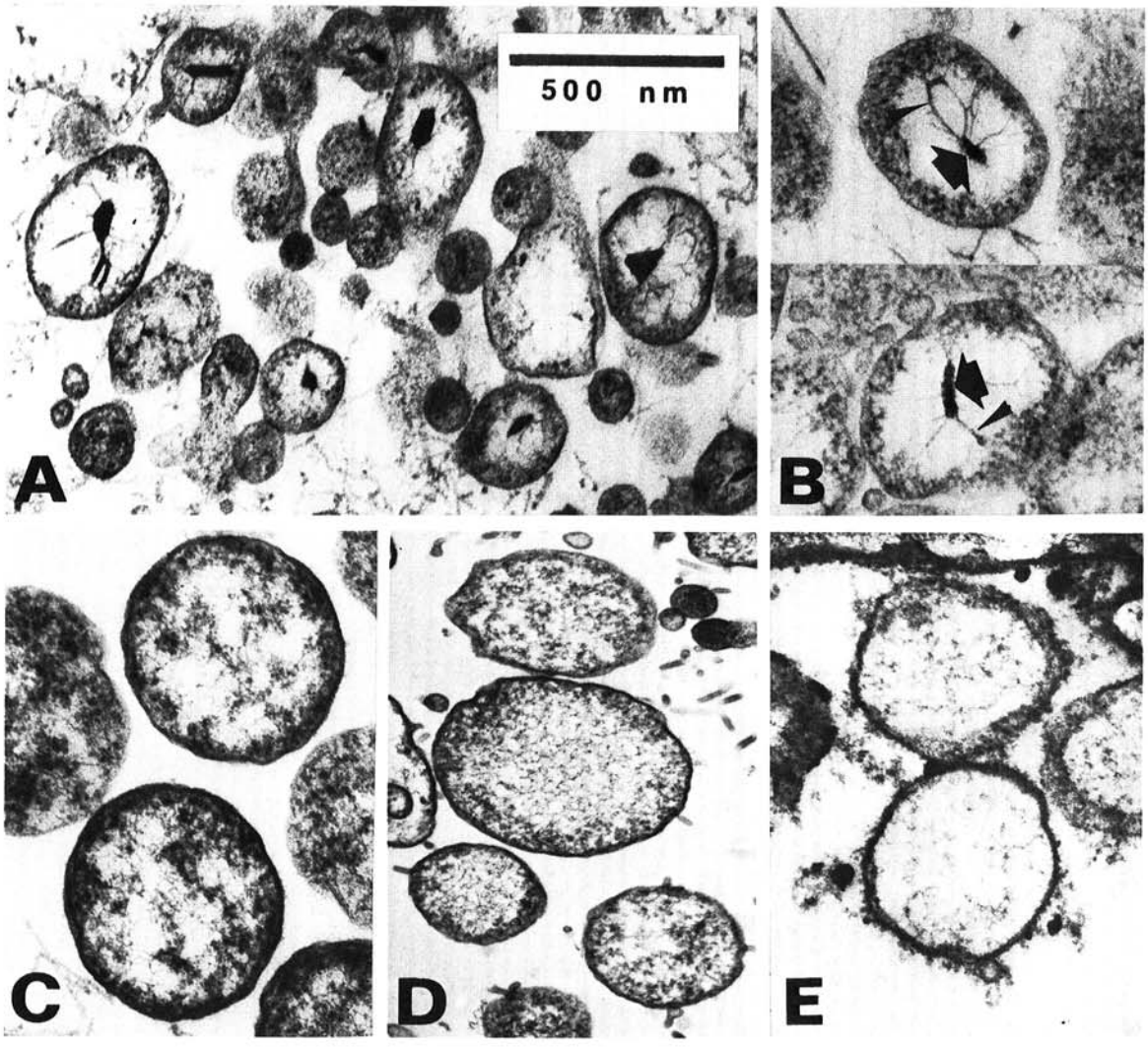

Fig. 1-(A to E). Electron micrographs illustrating comparative fixation of mycoplasmalike organisms (MLO's) and tissue resulting from different fixation procedures: **A)** MLO's of tomato big bud in tomato, fixed with chrome-osmium, showing aggregates in bodies. **B)** Individual MLO's of tomato big bud in tomato, showing dense aggregates (large arrows) and peripherally extending fibrils (small arrows). **C)** MLO's of tomato big bud in tomato, fixed with glutaraldehyde-osmium. **D)** MLO's of tomato big bud in periwinkle fixed with chrome-osmium and treated with uranyl acetate prior to dehydration. **E)** Healthy peach tissue fixed with chrome-osmium, showing vesicles of expanded endoplasmic reticulum superficially resembling MLO's, but distinguishable by lack of aggregates. All micrographs same magnification.

aggregates apparently formed by clumping of fibrillar material in their central areas (Fig. 1-A, B). This characteristic appears to make this fixative of value in distinguishing MLO's from normal cell components. The clumped aggregates were of various shapes and typically possessed fibrils extending outward toward the periphery of the organisms. The area surrounding the aggregates was typically electron-lucent and devoid of ribosomes and ground substance. The aggregates were observed in MLO's, but not in any cell organelles. Vesicles resembling those described by Florance and Cameron (6) were also found in the sieve elements of healthy peach included in the study, but the vesicles did not contain the aggregate structures when fixed with chrome-osmium (Fig. 1-E) and thus they were distinguishable from MLO's.

In specimens fixed with chrome-osmium and treated with 0.5% uranyl acetate prior to dehydration the MLO's did not contain the aggregate structures and the central areas of the organisms were homogeneous and finely fibrillar (Fig. 1-D).

Specimens fixed in glutaraldehyde and postfixed with osmium tetroxide showed MLO's which were pleomorphic and bounded by unit membranes (Fig. 1-C). Most contained ribosomelike particles and had a delicate DNA-like fibrillar network.

The other fixatives utilized in this study were not satisfactory for distinguishing MLO's from normal cell components. The potassium dichromate fixation resulted in distortion of MLO's and cell organelles. Specimens fixed with potassium permanganate showed well-

contrasted cytomembranes, but exhibited swelling and distortion of MLO's and cell organelles. Adjusting the tonicity of the permanganate solution resulted in reduced swelling and distortion, but yielded no other desirable characteristics. The MLO's fixed with osmium tetroxide alone resembled those fixed with glutaraldehyde and postfixed with osmium tetroxide, but overall fixation of cell organelles was not as good.

Mycoplasmalike organisms were not observed in healthy plants utilized in the investigation.

DISCUSSION

The difficulties in distinguishing MLO's from normal cell constituents in thin-sectioned material have been recognized for some time (6, 11). Many yellows-affected field specimens have been collected by the authors and investigated by electron microscopy to determine whether they contained MLO's. In many instances, membrane-bound bodies were observed in the sieve elements, but they were difficult to distinguish as MLO's or host cell constituents. This was particularly true in fully matured or older tissues.

In this study, large aggregate structures resulting from chrome-osmium fixation were consistently observed in the MLO's (Fig. 1-A, B) and not in plastids, mitochondria, or vesicles derived from a membrane system of the sieve cell. Although not illustrated, the same distinct aggregate structures were consistently observed in other MLO's in several hosts. We suggest, therefore, that the chrome-osmium fixative is useful for the initial examination of field-collected plants showing yellows type symptoms where the objective is to detect the intracellular entities of such plants.

The aggregate structures observed in this study are presumed to be nucleic acid. They appear to be produced in a manner similar to the formation of nucleic acid structures observed in bacterial DNA plasms by Kellenberger (8). Kellenberger (8) demonstrated that following fixation with osmium tetroxide, bacterial DNA coalesced during dehydration and produced aggregates similar to those observed in MLO's in the present study. In our study, the electron-lucent area around the aggregates in non-uranyl acetate-treated MLO's appears to result from aggregation of nucleic acids during dehydration. When MLO's were treated with uranyl acetate prior to dehydration, the DNA remained in dispersed form and no aggregate structures were produced (Fig. 1-D). This suggests that uranyl acetate treatment following chrome-osmium fixation further stabilizes the fine structure of MLO's, including nucleic acids. Hayat (7) reported a similar phenomenon in animal heart tissue and suggested that treatment with uranyl acetate prior to dehydration acted both as a stain and a fixative. In studies of intramitochondrial DNA in a number of species of plants and animals, Nass et al. (12) also observed distinct DNA structures after osmium tetroxide fixation and similarly noted that their density and size was reduced by uranyl acetate treatment prior to dehydration.

In the present investigation, it appears that MLO's fixed with chrome-osmium and treated with uranyl acetate prior to dehydration contain nucleic acid in dispersed form and thus exhibit a homogeneous central area in the organisms (Fig. 1-D). The MLO's fixed with chrome-osmium but not treated with uranyl acetate prior to dehydration contain the aggregated nucleic acid which has coalesced during dehydration. The formation of these distinct aggregate structures provides a characteristic which can be used to distinguish MLO's from normal cell constituents.

LITERATURE CITED

1. CHEN, T. A., and C. H. LIAO. 1975. Corn stunt spiroplasma: isolation, cultivation, and proof of pathogenicity. Science 188:1015-1017.
2. DALE, J. L., and L. D. SMITH. 1975. Mycoplasmalike bodies observed in tomato plants with big bud in Arkansas. Plant Dis. Rep. 59:455-458.
3. DALTON, A. J. 1955. A chrome-osmium fixative for electron microscopy. Anat. Rec. 121:281.
4. DAVIS, R. E., and R. F. WHITCOMB. 1971. Mycoplasma, rickettsiae, and chlamydiae: possible relation to yellows diseases and other disorders of plants and insects. Annu. Rev. Phytopathol. 9:119-154.
5. DOI, Y., M. TERANAKA, K. YORA, and H. ASUYAMA. 1967. Mycoplasma- or PLT group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or Paulownia witches' broom. Ann. Phytopathol. Soc. Jap. 33:259-266.
6. FLORANCE, E. R., and H. R. CAMERON. 1974. Vesicles in expanded endoplasmic reticulum cisternae structures that resemble mycoplasmalike bodies. Protoplasma 79:337-348.
7. HAYAT, M. A. 1969. Uranyl acetate as a stain and a fixative for heart tissue. Proc. 27th Ann. Meet. Electron Microsc. Soc. Am., Claitors Publishing Division, Baton Rouge, Louisiana. 412 p.
8. KELLENBERGER, E. 1962. The study of natural and artificial DNA-plasms by thin sections. Pages 233-249 *in* R. J. Harris, ed. The interpretation of ultrastructure. Vol. 1. Academic Press, New York. 438 p.
9. LUFT, J. H. 1956. Permanganate–a new fixative for electron microscopy. J. Biophys. Biochem. Cytol. 2:799.
10. MARAMOROSCH, K. 1974. Mycoplasmas and rickettsiae in relation to plant diseases. Annu. Rev. Microbiol. 28:301-324.
11. MARAMOROSCH, K., E. SHIKATA, and R. R. GRANADOS. 1968. Structures resembling mycoplasma in diseased plants and insect vectors. Trans. N. Y. Acad. Sci. 30:841-855.
12. NASS, M. M. K., S. NASS, and B. A. AFZELIUS. 1965. The general occurrence of mitochondrial DNA. Exp. Cell Res. 37:516-539.
13. SABITINI, D. D., K. G. BENSCH, and R. J. BARNETT. 1962. New fixatives for cytological and cytochemical studies. Fifth Int. Congr. Electron Microsc. 2:L-3. Academic Press, New York.
14. SAGLIO, P., M. L'HOSPITAL, D. LAFLÈCHE, G. DUPONT, J. M. BOVÉ, J. G. TULLY, and E. A. FREUNDT. 1973. Spiroplasma citri gen. and sp. n.: A mycoplasma-like organism associated with stubborn disease of citrus. Int. J. Syst. Bact. 23:191-204.