Identification of Viruses and Mycoplasmas in Maize by Use of Light Microscopy

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ABSTRACT

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Maize tissues infected with maize dwarf mosaic virus (MDMV) strain A or B, maize stripe virus (MStpV), maize mosaic virus (MMV), maize rayado fino virus (MRFV), maize chlorotic dwarf virus (MCDV), maize bushy stunt mycoplasma (MBSM), or corn stunt spiroplasma (CSS) were studied by light microscopy. Intracellular inclusions in stained epidermal strips were uniquely associated with infection by MDMV-A, MDMV-B, MStpV, or MMV. For MStpV identification, paradermal sections of the leaf sheath were better sources than such sections of the leaf blade. Both irregular and granular inclusions were found in parenchyma and phloem cells of leaf sheaths, leaf veins, and roots infected with MRFV. The phloem-inhabiting pathogens MCDV, MBSM, and CSS were found in longitudinal sections of the midrib and root. Stomata in epidermal strips of leaves infected with CSS showed a unique type of deformity. Light microscopy offers a rapid technique for detection and identification of maize viruses and mycoplasmas.

Maize (*Zea mays* L.) is an important crop worldwide. Sweet corn, hybrid seed corn, and field corn are grown throughout southern Florida. In the early 1980s, a severe outbreak of viruses and viruslike diseases in maize occurred in southern Florida. A number of factors, such as changes in pathogen populations through introduction of susceptible cultivars and introduction of pathogens by means of vectors and/or infected plant materials, have been speculated to have caused the outbreak of disease complexes (3).

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To date, maize dwarf mosaic virus (MDMV) strains A and B, maize stripe virus (MStpV), maize mosaic virus (MMV), maize rayado fino virus (MRFV), corn stunt spiroplasma (CSS), and maize bushy stunt mycoplasma (MBSM) have been identified from the Florida disease complexes by means of pathogen morphology and physicochemical properties, vector transmission, host ranges, and serological relationships (3,14). Although not currently found in Florida, maize chlorotic dwarf virus (MCDV) is a disease of major economic importance in the Southeast (4). Most of the techniques used to identify these diseases are time-consuming, complicated, and expensive. Techniques developed by Christie and Edwardson (5,6) have made possible the visualization of viral inclusions with the light microscope. These techniques have been used successfully for virus identification in many plant species, but little work has been done on viruses and mycoplasmas in maize.

We report here a rapid and inexpensive assay for maize plants infected with these pathogens that uses the light microscope to detect characteristic inclusion bodies and cell deformities.

MATERIALS AND METHODS

Pure strains A and B of MDMV were supplied by R. Louie (USDA-ARS, Wooster, OH), and MMV, MStpV, MRFV, CSS (*Spiroplasma kunkelii* Whitcomb et al), and MBSM were isolated from the field in Florida; all were maintained in sweet corn cv. Guardian in greenhouses in Fort Lauderdale, Florida. MCDV was obtained from E. Rosenkranz (Mississippi State University) and maintained at Union City, Tennessee.

Colonies of *Dalbulus maidis* (DeLong & Wolcott) and *Peregrinus maidis* (Ashmead) were reared on Guardian sweet corn with 12 hr of light per day in an insect-rearing room at 24 ± 1 C. *D. maidis* was used to perpetuate CSS, MBSM, and MRFV, and *P. maidis* was used to perpetuate MMV and MStpV (3,14). *Graminella nigrifons* (Forbes) was maintained on oats and used to transmit MCDV in sweet corn.

Strips from the lower epidermis of the leaf, freehand razor-cut longitudinal sections of roots, razor-cut paradermal sections of leaf sheath, and sandpaper-abraded leaf tissues were used fresh or fixed in ethylene glycol monomethyl ether. Tissues were stained with Luxol brilliant green BL-calcomine orange 2RS (O-G), a general stain for protein, or with azure A (5,6), a nucleic acid-specific stain, and mounted in LR White resin (12). Slides were examined with a Nikon Fluophot microscope equipped with a

Nikon FX-35A camera. Epidermal strips and sections from leaves, sheaths, and roots of healthy plants were used as controls.

RESULTS AND DISCUSSION

Maize dwarf mosaic virus strains A and B. Epidermal strips were taken at various stages of infection with MDMV and stained with the O-G combination. In early infections, 7–10 days after inoculation, dark-staining cylindrical inclusions, similar to those previously described in potyvirus-infected cells (5), were seen perpendicular to the cell wall. These inclusions did not stain with azure A. Seen at later stages of infection were cylindrical inclusions consisting of laminated aggregates and scrolls in the cytoplasm (Fig. 1A). At high magnification, plates appeared as straight lines that could still be seen when the focus was raised or lowered, whereas scrolls appeared as dots that moved in a straight line when the focus was changed. These inclusions did not stain with azure A. Some inclusions were scattered throughout the cyto-

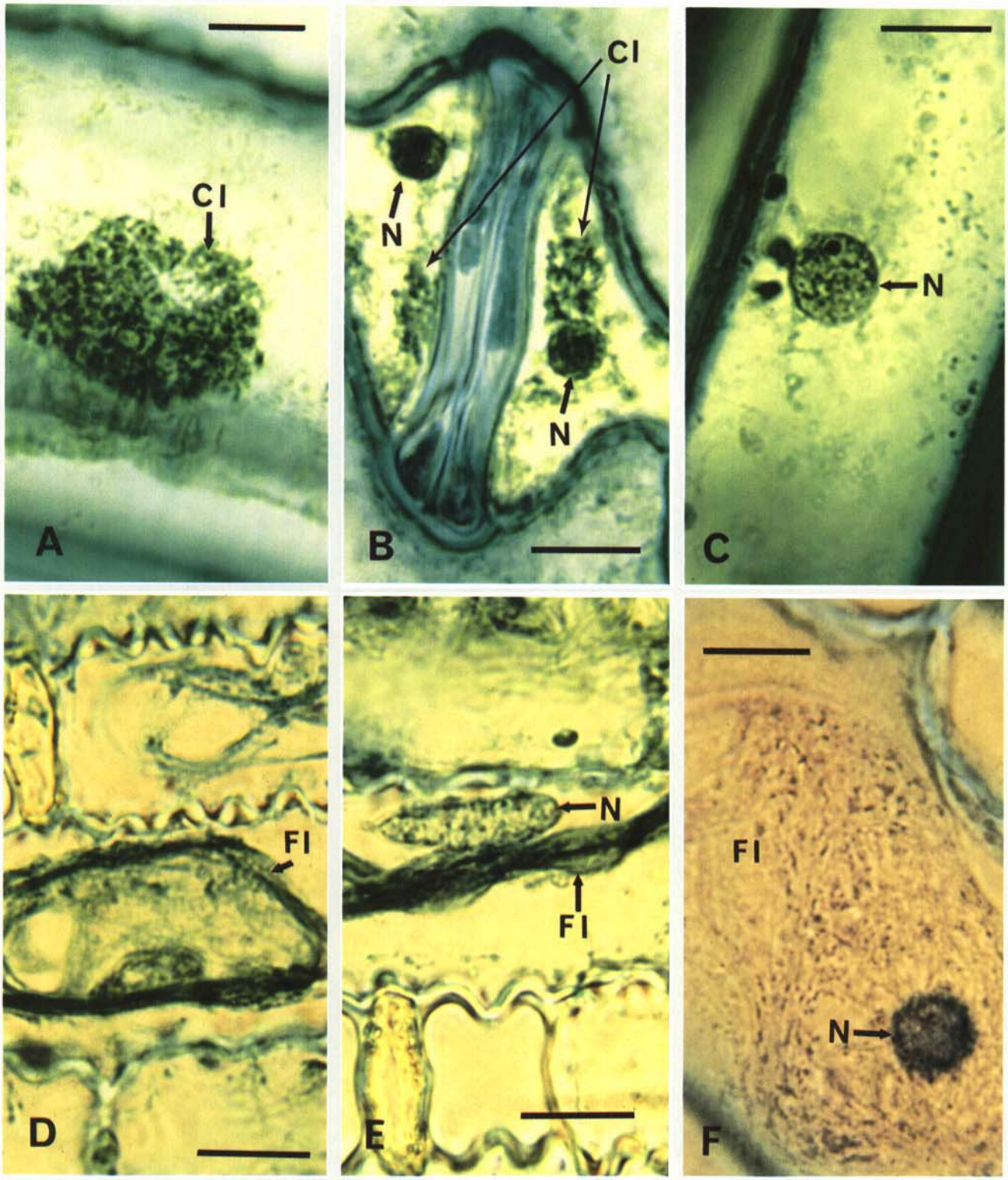

Fig. 1. (A) Cytoplasmic inclusions (CI) of maize dwarf mosaic virus strain A (MDMV-A) in epidermal cell stained with O-G. **(B)** Cytoplasmic inclusions (CI) of MDMV-A in subsidiary cells of epidermal tissues stained with O-G. N = nucleus. **(C)** Healthy maize cells stained with O-G. N = nucleus. **(D)** Encircled fibrous inclusion (FI) of maize stripe virus (MStpV) in epidermal cells stained with O-G. **(E)** Interwoven fibrous inclusion (FI) of MStpV in epidermal cells stained with O-G. N = nucleus. **(F)** Fibrous inclusion (FI) of MStpV stained faintly with azure A. N = nucleus. All scale bars = 10 μm.

plasm and others were in clusters that frequently were larger than the nucleus. There was rarely more than one inclusion per cell. Inclusions were found more often in midrib epidermis than in blade epidermis (Fig. 1B); smaller ones could be seen in subsidiary cells of the stomata. No inclusions were observed in healthy tissues (Fig. 1C). Variations in strains of MDMV may account for differences in the frequency of cluster formations.

Maize stripe virus. Massive intracellular inclusions were visible in epidermal strips of MStpV-infected leaves and leaf sheath tissues stained with O-G. Leaf samples with symptoms of MStpV infection collected in the field contained similar inclusions in the cytoplasm. Stained with O-G, the inclusions appeared as whorls of dark green fibrous material in the cytoplasm (Figs. 1D and E). Frequently, the fibers encircled the nucleus and almost filled the cytoplasm. Most leaves had some areas with no

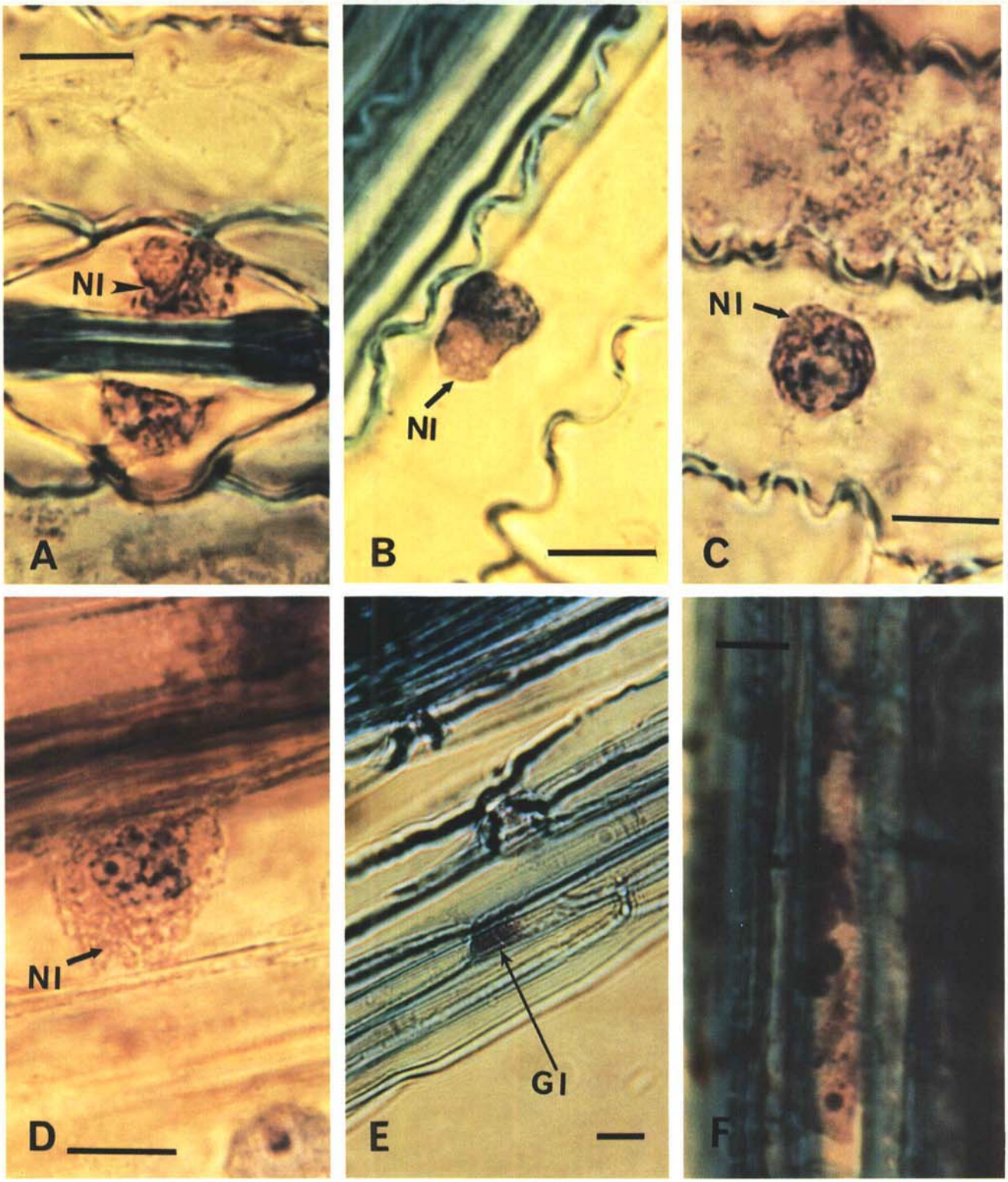

Fig. 2. (A) Nuclear inclusions (NI) of maize mosaic virus (MMV) in subsidiary cells stained with azure A. Scale bar = 10 μm. **(B)** Nuclear inclusion (NI) of MMV in epidermal cell stained with azure A. Scale bar = 10 μm. **(C)** Nuclear inclusion (NI) of MMV surrounding nucleus in epidermal cells stained with azure A. Scale bar = 10 μm. **(D)** Nuclear inclusion (NI) of MMV surrounding nucleus in phloem cells of root tissue stained with azure A. Scale bar = 10 μm. **(E)** Granular inclusion (GI) of maize rayado fino virus in phloem cells of leaf vein stained with azure A. Scale bar = 10 μm. **(F)** Phloem cell infected with maize chlorotic dwarf virus, filled with dark-staining material (azure A). Scale bar = 1 μm.

inclusions, some areas where every cell contained a large inclusion, and other areas with gradations between these extremes.

As with most other maize viruses examined by these methods, inclusions were more frequent in the midrib epidermis than in the leaf blade epidermis. Inclusions were not confined to the epidermis and could be seen in parenchyma cells clinging to epidermal strips or in cross sections of the midrib. Often there were areas, especially on the leaf blade, where the fibrous materials seemed to be loosely organized. Compared with leaf tissues, leaf sheath tissues contained higher concentrations of inclusions. These tissues were easily obtained by cutting paradermally to the surface of the leaf sheath. No inclusion bodies were found in tissues from healthy plants.

Azure A stained the same inclusions, but only faintly (Fig. 1F). Because the inclusions stain deeply with O-G and only faintly with azure A, they are probably composed mainly of protein and might be the noncapsid protein previously reported (2,8). Bradfute and Tsai (2) directly observed noncapsid protein crystals by phase-contrast light microscopy in sap and from leaves infected with MStpV. Although little is known about the nature of the fibrous inclusions, their presence is clearly associated with MStpV.

Maize mosaic virus. Epidermal strips of leaves infected with a pure culture of MMV revealed large swollen nuclei surrounded by a mass of granular material that stained about the same color as the nucleolus with either azure A or O-G (Figs. 2A, B, and C). Because azure A has better differential staining for nuclear inclusions than O-G does, we recommend that azure A be used for diagnosing MMV. These granular masses were found in roots longitudinally sectioned and stained with azure A (Fig. 2D); no granular material was found in tissues from healthy plants. A membrane usually separated the nucleus from the granular material, and most of the granular material also seemed to be enclosed within a membrane. These findings are consistent with the electron microscopic observations that MMV particles are found in high concentration between the inner and outer lamellae of the nuclear membrane (1,13). Herold et al (9) found dense masses in MMV-infected tissue with the light microscope but concluded that the material was found only in the cytoplasm around the nucleus.

Enlarged nuclei were found in all parts of the leaf epidermis, mesophyll, and root. This abnormality also can be seen in cross sections of parenchyma and vascular tissue, but epidermal strips are easier to handle. When epidermal strips are not easily obtained, sandpaper abrasion can be used to produce appropriate tissues for staining (12).

Maize rayado fino virus. No inclusions were observed in epidermal strips from MRFV-infected plants stained with either azure A or O-G. However, irregular inclusions and granular inclusions (Fig. 2E) were readily found in the parenchyma and phloem cells of leaf sheaths, leaf veins, and roots. Neither type of inclusion was observed in tissues from healthy plants. These findings agree with those of electron microscopic examinations (7,11). Azure A was ideal for staining the tissues from paradermal

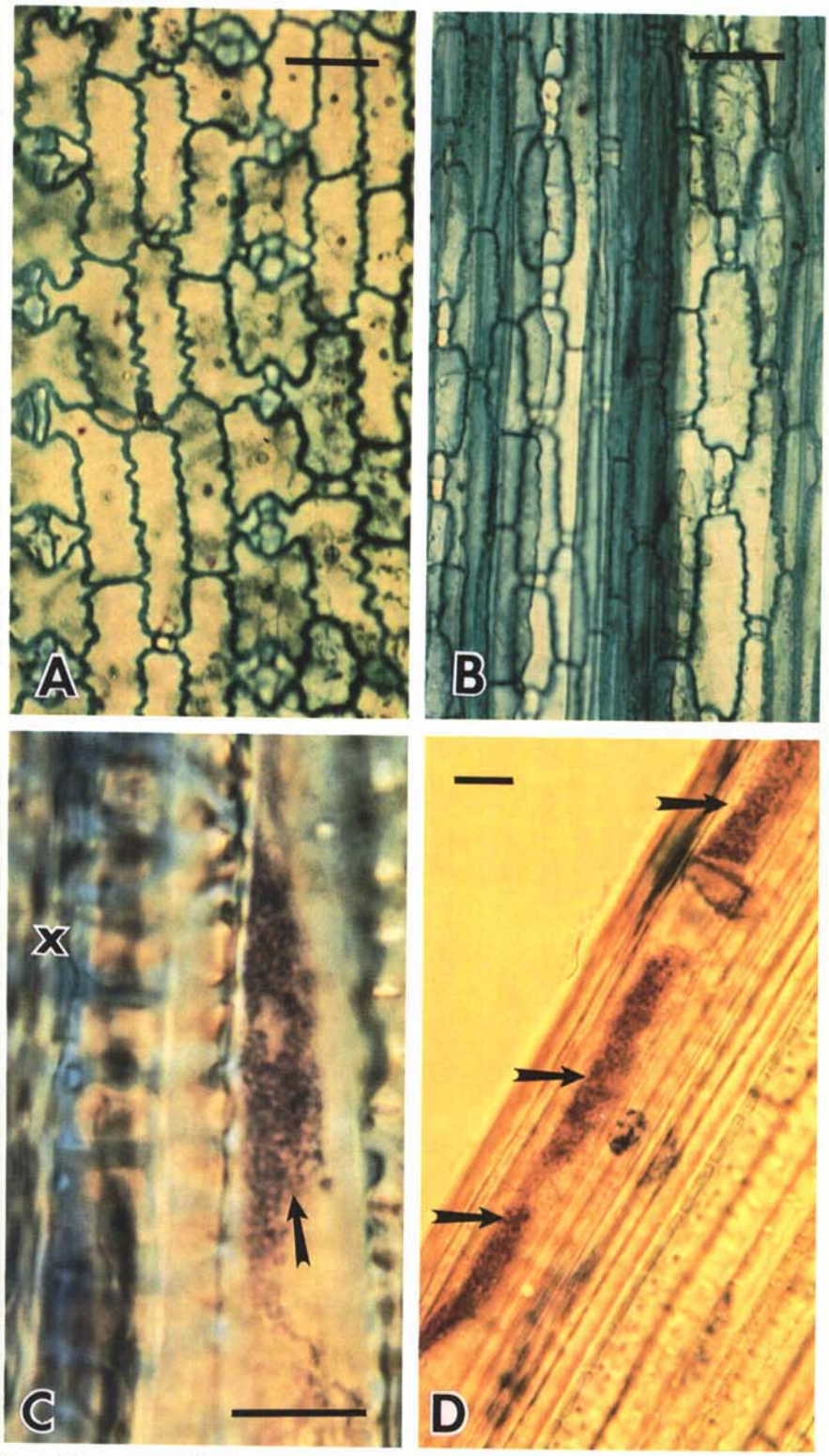

Fig. 3. (A) Low-magnification view of epidermis infected with corn stunt spiroplasma (CSS), with stomata showing rounded guard cells and subsidiary cells fused into adjoining epidermal cells; O-G stain. Scale bar = 5 μm. **(B)** Low-magnification view of epidermis infected with CSS, showing severely deformed stomata; O-G stain. Scale bar = 5 μm. **(C)** Granular material (arrow) of maize bushy stunt mycoplasma (MBSM) in phloem cell of root tissue stained with azure A. X = xylem. Scale bar = 10 μm. **(D)** Granular materials (arrows) of MBSM in phloem cell of root tissue stained with azure A. Scale bar = 10 μm.

sections of the inner side of leaf sheaths and the tissues of longitudinal sections of leaf veins and roots.

Maize chlorotic dwarf virus. Epidermal strips of MCDV-infected leaves showed no inclusion bodies or deformities; longitudinal or cross sections of the leaf midrib were necessary to show microscopic differences. The phloem of leaves infected with MCDV contained material that stained darkly with azure A (Fig. 2F) or O-G. In late stages of infection, some sieve tube elements collapsed completely, a finding consistent with that of Choudhury (4).

Maize bushy stunt mycoplasma and corn stunt spiroplasma. Epidermal strips of leaves infected with MBSM and CSS and stained with either azure A or O-G did not show any inclusions resembling those of any other virus or viruslike disease. However, gross deformities of the stomata were associated with CSS with either stain. Guard cells were rounded instead of elongated and were arranged side by side between subsidiary cells (Fig. 3A) rather than parallel to the veins, as in healthy tissue or in the presence of any of the other diseases observed in our study (Figs. 1B and 2A). The stoma appeared to be permanently open, with no visible means of closure. Subsidiary cells were frequently fused to adjacent epidermal cells, with contiguous cytoplasm. In extreme cases, subsidiary cells were absent, with only a small pair of squared vestigial guard cells to mark the stomatal site (Fig. 3B). Although stomatal deformities were occasionally observed with infections by other viruses, such as MRFV, none showed this unique form, which was found in the majority of stomata of plants infected with CSS. These stomatal deformities have been found in field plants showing CSS-like symptoms in both Florida and Mexico.

With both MBSM and CSS, longitudinal sections of the midrib and root, stained with azure A, showed granular materials in the phloem. Sometimes the granular materials were concentrated in the sieve plate areas, and other times the entire cell was filled (Figs. 3C and D). No granular materials were found in tissues from healthy plants. Because azure A shows better differentiation for MBSM and CSS inclusions than does O-G, we recommend azure A for diagnosing these diseases.

Conclusion. Epidermal strips of maize leaves stained with the techniques described here show easily identifiable inclusions for MDMV, MStpV, and MMV. Longitudinal sections of leaf midribs stained with azure A show dark-staining inclusions for MCDV, MRFV, MBSM, and CSS. A unique deformity of the stomata distinguishes CSS from the other phloem-inhabiting pathogens; different techniques may be necessary to differentiate MCDV, MRFV, and MBSM.

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