Rapid Compression Technique for Detecting Mycoplasmalike Organisms in Leaf Midrib Sieve Tubes by Fluorescence Microscopy

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ABSTRACT

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Phloem tissue from herbaceous plants infected with mycoplasmalike organisms (MLOs) was removed from small pieces of compressed and crushed leaf midribs, treated 20 min in fixative, rinsed 5 min in buffer, stained 3–5 min in 4′-6-diamidino-2-phenylindole (DAPI) solution, compressed in DAPI on a slide, and then examined by fluorescence microscopy for presence of MLOs. Causal MLOs were well fixed and stained and were readily discernible in phloem sieve tubes by this procedure. With this method it was possible to distinguish among plants infected with *Spiroplasma citri* or MLOs. MLOs were also detectable in unfixed phloem tissue placed and compressed directly in DAPI on a slide, but the MLOs lacked clarity. It is believed that this compression technique, either with or without tissue fixation, would be useful for rapid screening of herbaceous plants for presence of MLOs.

Additional key words: aster yellows, blueberry stunt MLO.

Various techniques have been used for the visualization and demonstration of mycoplasmalike organisms (MLOs) and spiroplasmas in diseased plants. The choice of procedure depends on the desired level of characterization of the organism involved, availability of equipment, and rapidity desired from the technique. Electron microscopy is the only method by which the characteristic ultrastructure of MLOs can be observed, but it is time-consuming and expensive. In recent years, more simple techniques have been developed for confirming MLOs in plant tissue, using either of two DNA-binding fluorochromes, 4′-6-diamidino-2-phenylindole (DAPI) or the benzimidizole derivative, Hoechst 33258, and fluorescence microscopy. Russell et al (12) first used this technique to monitor animal tissue culture cells for mycoplasma contamination. Seemuller (17), de Leeuw et al (10), and Cazelles (1) were among the first to use these DNA-specific dyes and fluorescence microscopy to demonstrate mycoplasmas in plant tissue.

Tissues of various plant parts examined by fluorescence microscopy have been prepared by freehand sectioning (4,7,8,10,13), sectioning by rotary and freezing microtomes (2,3,5,6,14–20), or microtome sectioning of paraffin-embedded material followed by deparaffinization and rehydration before staining (3,11). In some instances, the phloem of the vascular system to be examined has been separated from other tissue by enzymatic digestion with pectinase (1,3). Most workers have used glutaraldehyde for tissue fixation; however, Deutsch and Nienhaus (4) used a mixture of glutaraldehyde and paraformaldehyde. Cousin and Jouy (3) reported that fixation with glutaraldehyde and Carnoy's fluid was satisfactory, whereas fixatives containing formalin were not suitable. Hiruki and da Rocha (8) reported that different types of buffer, pH, and ionic strength had little effect on the efficacy of DAPI staining of MLOs and that comparable amounts of fluorescence were obtained with aqueous DAPI solution.

Although they give equally satisfactory results, DAPI appears to have been used more extensively for plant mycoplasma detection than has Hoechst 33258. In general, most protocols for DAPI use 2-hr treatment of specimens with a glutaraldehyde-containing fixative, either before or after sectioning or enzyme digestion of tissue, followed by a buffer rinse, and then 20-min treatment with DAPI, followed by mounting of sections or tissue in buffer on a slide and examination by fluorescence microscopy. In some instances, the specimens, after rinsing in buffer, have been mounted directly in DAPI on a slide before examination (1,14). In contrast to these procedures, de Leeuw et al (10) treated hand-cut sections of unfixed stem tissue of *Aster tripolium* L. affected with witches'-broom for 30 min in 2.5% aqueous DAPI before examination by fluorescence microscopy. They reported that a marked degree of fluorescence was present in the phloem of diseased plants but no fluorescence was observed in phloem from healthy plants. They did not observe individual MLOs by this procedure but later confirmed their presence by electron microscopy.

Many of the techniques used for demonstrating MLOs in plants with DNA-binding dyes and fluorescence microscopy have given satisfactory results and are being used for testing plants for mycoplasma infection. Most of these methods are relatively rapid, but they usually take 2 hr or more for completion, primarily because of the time interval used for fixation. This paper describes a rapid compression technique for detection of MLOs in herbaceous plants by fluorescence microscopy.

MATERIALS AND METHODS

Reagents used in the procedure were Karnovsky's fixative (9) and DAPI (Polysciences, Inc., Warrington, PA 18976) at a concentration of 1 μg/ml in 0.1 M phosphate buffer, pH 7.0. Excised leaf midrib sections, 2–3 mm in length, were individually placed on a clean glass slide. During observation under a stereoscopic microscope at ×15, the tissue sections were firmly compressed and crushed with a small spatula, and the readily distinguishable vascular system was removed with fine forceps and placed in approximately 0.3 ml of Karnovsky's fixative in small (18-mm inside diameter) beveled-edge watch glasses. In an initial test, fixation periods were 15, 30, and 45 min. Fixation times did not give differing results, but to ensure adequate fixation, in subsequent tests, a fixation period of 20 min was used. In some tests, no fixation was used. After fixation, the vascular tissue was rinsed 5 min in phosphate buffer and then placed in DAPI solution for 3–5 min. The tissue, containing mostly phloem sieve tubes and some xylem, was then mounted in a drop of DAPI on a glass slide, teased apart with fine forceps, and further compressed with a spatula. In some instances, the fixed tissue was transferred directly from the buffer rinse in DAPI on a slide. When no fixation was

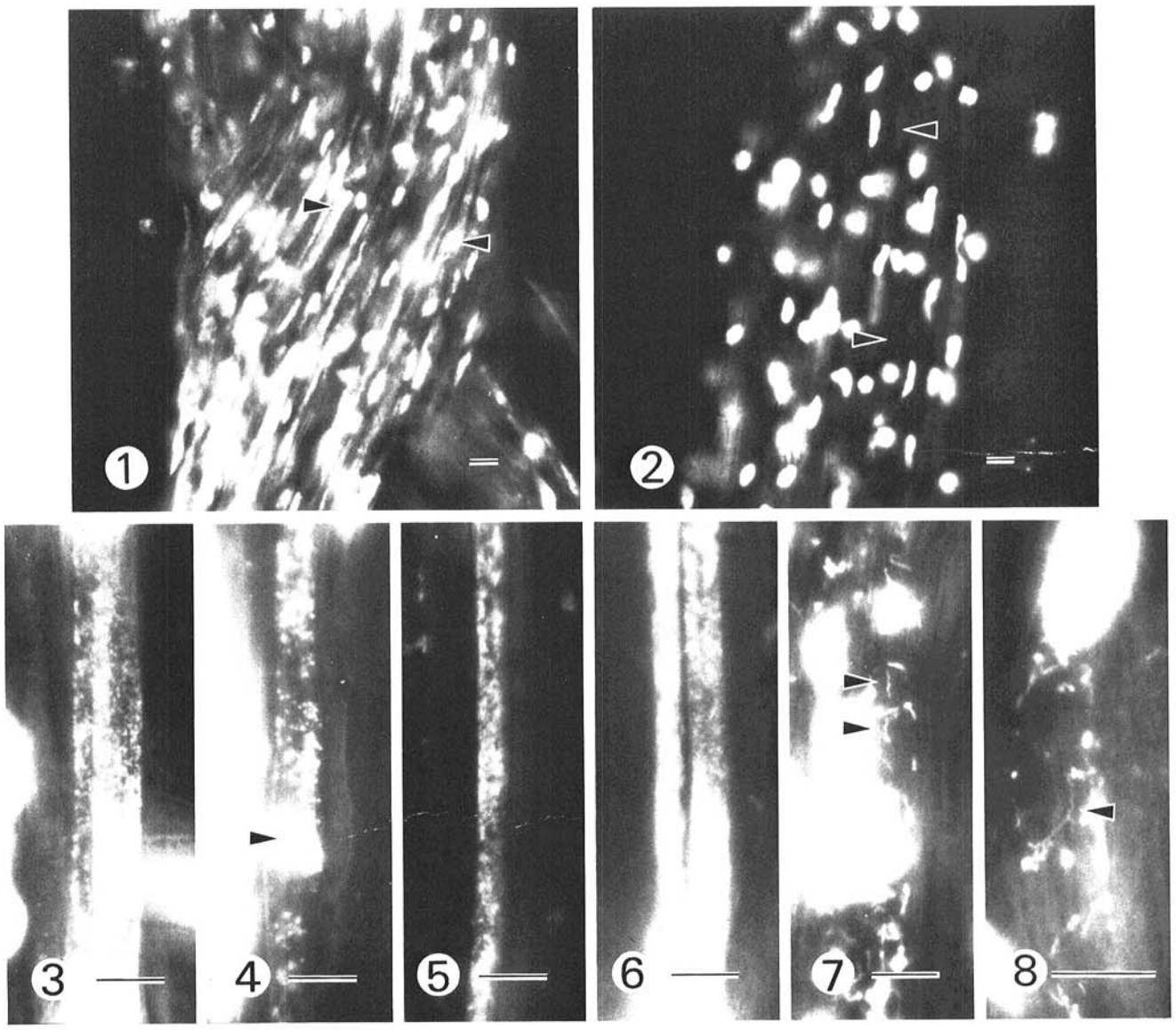
used, the extracted vascular tissue was placed directly in DAPI on a slide, compressed, and examined.

Slides were examined with a Leitz Orthoplan epifluorescence microscope with 200-W mercury lamp, fitted with a BG 38 suppression filter and filter block A containing excitation filter BP 340-380, dichromatic mirror RKP 400, and suppression filter LP 430. Photographs were made using Kodak Panatomic-X film.

Tissue specimens examined by these procedures were from aster [*Callistephus chinensis* (L.) Nees] and periwinkle [*Catharanthus roseus* (L.) G. Don] infected with aster yellows MLO, field-collected horseweed [*Conyza canadensis* (L.) Cronq.] infected with aster yellows, periwinkle infected with the causal MLO of the blueberry stunt disease, and periwinkle infected with *Spiroplasma citri*. All specimens except the last were prepared from symptomatic fresh plant material. The periwinkle infected with *S. citri*, which was included to determine whether the compression procedure would be suitable for detection of spiroplasmas, was furnished by Jacqueline Fletcher (Oklahoma State University, Stillwater) as small pieces of leaf midrib tissue preserved in Karnovsky's fixative during shipment. Tissues from healthy plants were included as controls in the tests. As a blind test on the reliability of the compression procedure for MLO detection, 10 vascular strands removed from leaf midribs from a symptomatic periwinkle infected with the causal MLO of blueberry stunt and 10 from a healthy periwinkle were simultaneously processed and individually placed in small capsules in DAPI solution. These specimens were coded and randomized by an associate and examined as unknowns by the author.

RESULTS

The compression procedure was very satisfactory for detecting MLOs, especially when the tissue was fixed before staining. For example, with phloem tissue *en masse* at low magnification, it was very easy to distinguish between periwinkle infected with the blueberry stunt MLO (Fig. 1) and tissue from healthy periwinkle



Figs. 1–8. Micrographs of MLO- or spiroplasma-infected and healthy leaf vein tissue from periwinkle and aster prepared by compression method, either fixed or nonfixed, then stained with DAPI and examined by fluorescence microscopy showing: **1,** mass of fixed phloem tissue from periwinkle infected with MLO of blueberry stunt disease; arrows indicate typical MLO-containing sieve tubes; **2,** mass of fixed phloem tissue from healthy periwinkle showing only normally occurring nuclei; arrows indicate sieve tubes devoid of MLOs; **3,** phloem sieve tubes from fixed aster infected with aster yellows MLO; **4,** phloem sieve tube from fixed periwinkle infected with blueberry stunt MLO; arrow indicates extraneous nucleus superimposed on sieve tube; **5,** phloem sieve tube from unfixed aster infected with aster yellows MLO; **6,** phloem sieve tubes from unfixed periwinkle infected with blueberry stunt MLO; **7,** spiral forms (arrows) of *Spiroplasma citri* in phloem from fixed periwinkle; and **8,** spiral form (arrow) of *S. citri* in phloem from fixed periwinkle. Scale bars = 10 μm.

(Fig. 2). With most slide preparations, individual or small groups of phloem sieve tubes were readily observable at the periphery of the compressed material, and MLOs could easily be distinguished. This is illustrated with the aster yellows MLO in aster (Fig. 3) and the blueberry stunt MLO in periwinkle (Fig. 4). With phloem sieve tubes, which were extracted from compressed veins, placed directly in DAPI and compressed without fixation, MLOs could be distinguished in sieve tubes, but they lacked the clarity observed with fixed material. This is shown with unfixed aster tissue containing aster yellows MLO (Fig. 5) and unfixed periwinkle infected with the blueberry stunt MLO (Fig. 6).

Examination of the periwinkle tissue infected with *S. citri* (Figs. 7 and 8) showed that spiroplasmas could be seen and differentiated from MLOs in tissue processed by the compression procedure. Although fixed 2–3 days before processing, from results with the other tissue it is believed that a 20-min fixation would produce the same results.

The periwinkle and field-collected horseweed infected with aster yellows both contained MLOs, but they were not numerous. As with the other plants examined, however, it was easy to distinguish between tissue from diseased plants and that from healthy control plants. In the blind test in which coded specimens from healthy and blueberry stunt-infected periwinkles were examined, all 10 specimens of each type were rapidly and correctly identified, demonstrating that the screening procedure was reliable with this system.

DISCUSSION

Results of the present study suggest that the compression procedure described should be useful for rapidly demonstrating and confirming the presence of MLOs in tissue from herbaceous plants. With a 20-min fixation of vascular tissue, 5-min rinse in buffer, then 3–5 min in DAPI, specimens can be processed and examined within a 30-min period. In this study, this schedule produced a good fixation image, with MLOs being readily distinguishable in phloem sieve tubes. As pointed out by Hiruki and da Rocha (8) and shown in Figure 2, staining and fluorescence of phloem cell constituents other than easily recognizable nuclei was negligible. Specimens of unfixed vascular tissue mounted directly in DAPI on a slide can be examined immediately, and MLO can also be detected, but the image is less distinct than that obtained from fixed material. Use of unfixed tissue, however, would be suitable for initially screening material for further examination and characterization by electron microscopy.

Except for a fluorescence microscope, the technique requires no specialized equipment and only minimal amounts of three reagents. Because the vascular system is examined from a longitudinal aspect with the compression technique, a considerable amount of phloem tissue is viewed, compared to the amount observed with cross-sectioned material or by electron microscopy. This increases the chance of detecting MLOs in plants that may contain a low titer of the organism involved. In addition to detecting MLOs in infected plants, it was also possible to distinguish spiral forms of *S. citri* in infected plants. Due to the speed and simplicity of the procedure, it is believed that the technique could be used routinely for clinical examination of tissue from herbaceous plants suspected of being infected with MLOs or spiroplasma.

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