Phloem-Limited Prokaryotes in Sieve Elements Isolated by Enzyme Treatment of Diseased Plant Tissues

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ABSTRACT

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Sieve elements of periwinkle plants (*Catharanthus roseus* L.) infected with *Spiroplasma citri*, mycoplasmalike organisms (MLO), or clover club leaf (CCL) agent were separated from other cell types by partial digestion of plant tissue with macerating enzymes and viewed by dark-field microscopy. Sieve elements separated from plants infected with *S. citri* contained minute helical, motile filamentous microorganisms typical of spiroplasmas. In sieve elements from plants infected with the MLO associated with pear decline, aster yellows, or tomato big bud disease, no helical spiroplasmas were seen, but pleomorphic microorganisms with predominantly filamentous, usually branched, form were present. Sieve elements from CCL-diseased periwinkle contained numerous slender rod-shaped bacteria. Morphology of these phloem-limited prokaryotes could be studied further when the microorganisms were released from the isolated sieve elements by slight osmotic shock. Sieve elements separated from healthy periwinkle plants contained a few organelles but no structures resembling spiroplasmas, MLO, or phloem-limited bacteria. The method offers a means for studying the morphology and other properties of viable cells of uncultivated as well as cultivable pathogens in sieve elements.

Additional key words: detection, diagnosis.

Phloem-limited prokaryotes, including spiroplasmas, mycoplasmalike organisms (MLO), and bacteria, are known or presumed to cause hundreds of yellows, stunting, and scorch diseases of plants (3,12,17). The few successful attempts to cultivate these prokaryotes in vitro have yielded only spiroplasmas (1,3–5,8,13,18). Morphology of most phloem-limited prokaryotes, and verification of their presence in plants, have therefore generally been described or interpreted through electron microscopy of killed and fixed organisms in a relatively limited number of ultrathin sections that represent a very small proportion of an infected plant part. To permit observation of a larger proportion of plant tissue, and to permit the study of unkilled, unfixed organisms, we developed a method for separating sieve elements from other plant cells by partial digestion of infected tissues with macerating enzymes. The phloem-limited prokaryotes within the sieve elements were then examined by using dark-field microscopy.

In this paper, we describe a method for enzymatic digestion of infected plant tissues and compare the morphologies within sieve elements of some selected phloem-limited prokaryotes that cause distinct diseases in Madagascar periwinkle, *Catharanthus* (=*Vinca*) *roseus* L. A brief report of this work has been presented (15).

MATERIALS AND METHODS

Diseased plants. Periwinkle plants infected with *Spiroplasma citri* were kindly provided by G. Oldfield (USDA, Riverside, CA); aster yellows-diseased periwinkle has been maintained in our USDA Beltsville greenhouse for several years; tomato big bud-infected periwinkle was kindly provided by J. Dale (University of Arkansas, Fayetteville); periwinkle infected with the pear decline agent was provided by J. Kloepper (University of California, Berkeley); periwinkle infected with clover club leaf (CCL) agent was kindly provided by L. M. Black (University of Illinois, Champaign-Urbana). The disease agents were maintained in periwinkle plants inoculated by grafting. All plants inoculated by grafting for experiments were derived from cuttings from a single healthy periwinkle plant. Plants infected with *S. citri* or with the pear decline agent were incubated in a growth chamber at 27 C (night), 30 C (day). Plants with the remaining diseases were incubated at 24 C (night), 27 C (day). All plants were exposed to a light period of 14 hr at about 10^4 lux.

Separation of sieve elements. Tissues were collected from branches showing early stage symptoms (~20–30 days after grafting). Midribs including petioles were cut from fully expanded young leaves, surface sterilized with 20% Clorox (approximately 1% sodium hypochlorite) for 5 min, and then rinsed twice in sterile distilled water. The midribs were then aseptically cut into segments about 1 cm long. Each segment was longitudinally sliced aseptically by hand into thin sections (~100 μm thick) using surface-sterilized styrofoam to support the segments. The sections were immediately transferred into petri plates containing the macerating enzymes in solution. This solution consisted of 0.8% cellulase (Yakult Pharmaceutical Industry Co., Nishinomiya, Japan); 0.2% macerozyme (Yakult Pharmaceutical Industry Co.); 1 mM calcium chloride; and 0.6 M mannitol. The enzyme solution was freshly prepared, adjusted to pH 5.5, and filter-sterilized (pore size 0.2 μm) before use. After incubation in the enzyme solution for 60–90 min at 22–25 C, the tissue was partially digested, leaving the vascular tissues as bundles. These vascular bundles were aseptically separated from the surrounding macerated parenchymatous cell mass with fine forceps and transferred to a new batch of enzyme solution for further digestion for another 30–60 min. After digestion, the phloem tissue, consisting of layers of sieve elements, was carefully stripped from the xylem tissue using sterile sharp forceps and transferred to a buffer solution (pH 7.5 for tissue infected with *S. citri*, pH 7.2 for the others). This solution consisted of 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1.0 mM calcium chloride, and 0.5 M mannitol. After incubation for 60 min, the specimens were observed immediately by dark-field microscopy or were stored at 4 C until later examination.

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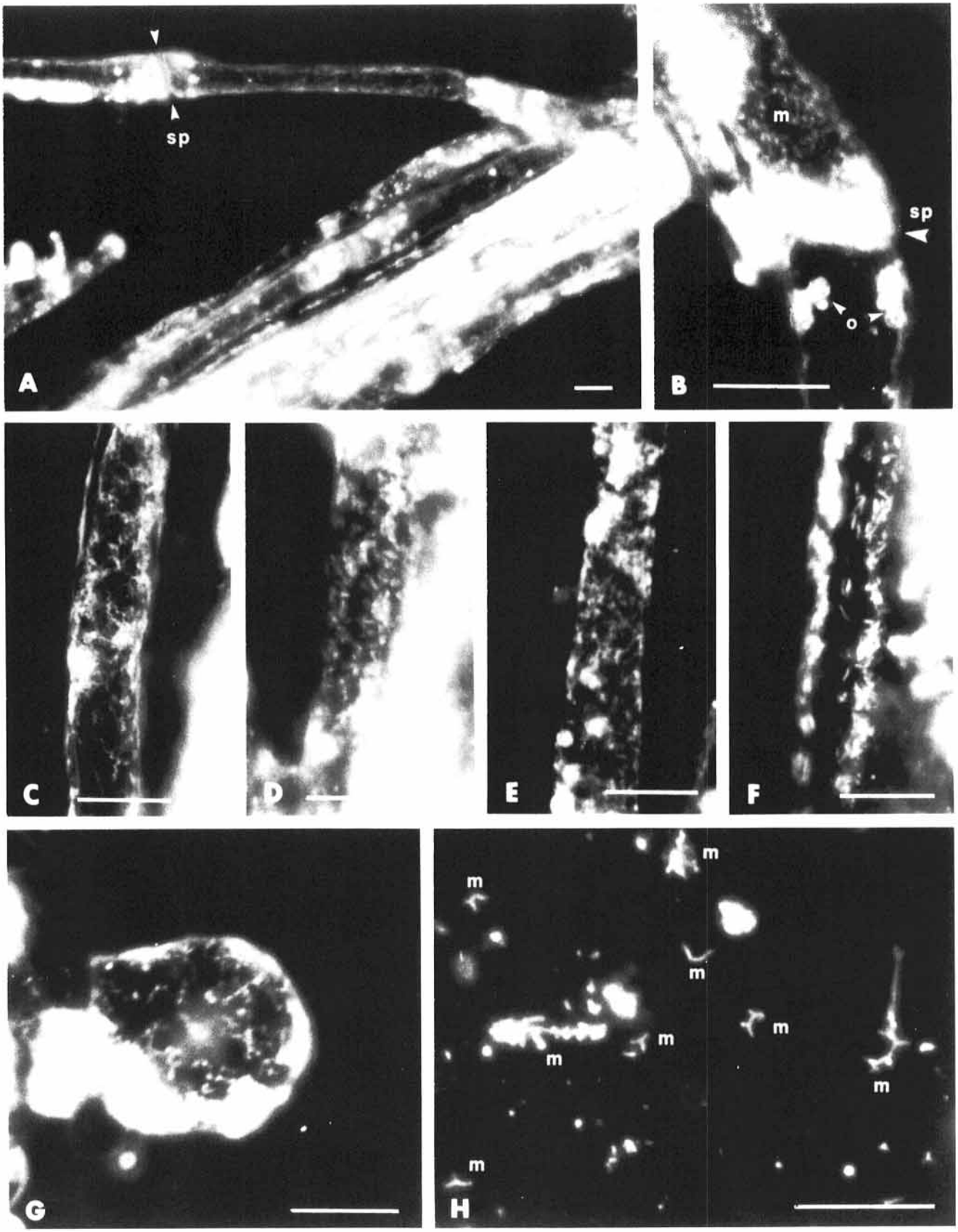

Fig. 1. Dark-field micrographs of three types of phloem-limited prokaryotes in sieve elements isolated by enzyme treatment of diseased periwinkle (*Catharanthus roseus*) plant tissues. **A,** A sheet of sieve elements infected with aster yellows agent: sieve plate (sp); **B,** portions of sieve elements infected with aster yellows agent, showing numerous mycoplasmalike organisms (MLO) in the upper sieve element; the lower element is almost devoid of MLO: organelles (o), mycoplasmalike organisms (m); **C,** *Spiroplasma citri* in an infected sieve element; note typical helical cells; **D,** sieve elements from pear decline-affected plant containing numerous branched filamentous MLO; **E,** Sieve element from tomato big bud-affected plant containing many filamentous MLO some of which are in clumps; **F,** sieve elements from clover club leaf-infected plant containing many rod-shaped bacteria; **G,** MLO in a protoplast evagination from a sieve element from a plant with pear decline; **H,** single and clumped MLO released from osmotically shocked sieve tube elements of pear decline-infected plant. Bar represents 10 μm.

In some experiments, the isolated phloem sieve cells were directly transferred to medium LD8A, which is specific for the cultivation of spiroplasmas (16).

Optical microscopy. The isolated sieve elements were layered onto a slide and examined by dark-field microscopy at ×1,000 to ×1,250. In some cases, sieve elements in buffer containing 0.5 M mannitol were transferred to buffer containing 0.4 M mannitol prior to microscopic examination.

RESULTS AND DISCUSSION

Treatment of plant tissues with macerozyme and cellulase proved to be an effective means for obtaining periwinkle sieve elements relatively free of other cell types. Partial digestion of longitudinal slices of leaf midribs and petioles permitted the sieve elements to be readily separated from other tissues. Separated sieve elements were generally present in sheets one cell thick and several cells wide that could be placed on glass slides for microscopy. Fig. 1A depicts a sheet of sieve cells from an aster yellows-diseased periwinkle plant. The microorganisms present in sieve elements from diseased plants could be seen clearly through the cell walls when viewed by dark-field microscopy.

Sieve elements separated from periwinkle plants infected with *S. citri* contained minute helical, motile filamentous microorganisms typical of spiroplasmas (Fig. 1C). Whereas clumps of helical spiroplasma cells were commonly seen in mature sieve elements, individual spiroplasma cells were predominant in young sieve elements where some normal plant cell organelles were still present. In both mature and young sieve elements, the spiroplasmas exhibited a vigorous rotational and flexional motility, similar to that reported for spiroplasmas in culture or in juice expressed from plants (6), but no translational locomotion, as reported for cultured spiroplasmas in viscous media (7,14), was observed.

In sieve elements from plants infected with pear decline, aster yellows, or tomato big bud agent, no helical spiroplasmas were seen, but pleomorphic bodies of predominantly filamentous form were present. For example, numerous filamentous, usually branched, bodies that exhibited no apparent motility were present in sieve elements isolated from periwinkle infected with the pear decline agent (Fig. 1D and G). These bodies are presumed to be MLO, since similar branched, filamentous forms were not seen in sieve elements from healthy plants, and since they correspond in shape and size to MLO described elsewhere (9,10,17,20). With slight osmotic shock, induced by transferring sieve elements into buffer containing 0.4 M mannitol, the isolated sieve elements often became swollen, developing an evagination of the protoplast, and eventually rupturing (Fig. 1G and H). This provided an advantage for studying details of MLO morphology further, especially in cases where sieve elements were packed with many MLO. Filamentous, predominantly branched MLO, similar to those observed in the case of pear decline, were found in sieve elements from plants with aster yellows and tomato big bud (Fig. 1A, B, and E).

Unlike sieve cells from spiroplasma- or MLO-infected plants, sieve elements of CCL-diseased periwinkle contained numerous slender, rod-shaped bacteria (Fig. 1F). These bacteria were present mainly in mature sieve elements. Organisms were rarely found in immature sieve cells. The bacteria were uniform in size and shape and were somewhat flexible, but not motile. Their dimensions (2.2 μm × 0.3 μm) were similar to those reported by Windsor and Black (21) for the CCL bacterium.

Sieve elements prepared from healthy periwinkle plants contained some spherical organelles 0.6–1.4 μm in diameter but contained no structures resembling spiroplasmas, filamentous MLO, or bacteria. Spherical bodies were also seen in sieve elements from MLO-diseased plants (Fig. 1B). These bodies were presumed to be plant organelles, but some may be MLO. However, spherical bodies in sieve cells from diseased plants had the same bright refractility and light yellow color as the bodies seen in sieve elements from healthy plants. They thus differed from the less refractile, blue-white filamentous MLO viewed by dark-field microscopy.

We have successfully applied the same enzyme treatment to young stem tissue from corn plants (*Zea mays* L.) with corn stunt disease. Separated sieve cells contained numerous helical, motile spiroplasmas (data not shown). Some modification of the enzyme digestion procedure may be necessary for successful application to other plant species.

This is the first report of direct observation of unkilled, unfixed spiroplasmas, MLO, and other prokaryotes in situ in the sieve tubes of infected plants (15). The freeze fracture, thick section, and scanning electron microscopy techniques have enabled important contributions to understanding the morphology of some phloem-limited prokaryotes in situ (2,6,9–12,20–22). For the most part, however, interpretations of morphology have been based on observation of relatively limited numbers of ultrathin sections. Although ultrathin section electron microscopy has yielded valuable information on the ultrastructure of the microorganisms studied, that approach (as generally applied) has significant potential for misinterpretation of morphology, as noted by several groups of researchers (17,19,20). For example, morphology of MLO generally has been interpreted on the basis of single, nonserial ultrathin sections of infected plants. However, Waters and Hunt (20) reconstructed the three-dimensional form of MLO on the basis of numerous serial ultrathin sections of a diseased plant. They demonstrated that MLO, that appear as rounded bodies in a single ultrathin cross section, may in some cases actually be filamentous forms, perhaps with complex branching. Their work underscored some of the interpretation problems encountered when only fragments of an MLO cell are observed. The examination of sieve elements prepared by our method provides a procedure that tends to minimize difficulties that commonly attend study of the morphology of MLO and other uncultured phloem-limited prokaryotes. Three major advantages of our procedure are: rapid and extensive examination of hundreds of minimally disturbed sieve elements; observation of the entire contents of each sieve element; and examination of numerous viable, unfixed, intact prokaryotic cells.

Sieve elements separated from diseased plant tissue can also constitute an inoculum source useful for in vitro cultivation of phloem-limited pathogens. The separated sieve elements provide a concentrated inoculum with minimal disturbance of internal pathogens and without inclusion of possibly inhibitory products released from other host cell types. In the present work, sieve cells separated from *S. citri*-infected periwinkle gave rise to cultures in LD8A broth and colonies on LD8A agar within 5 days and 7–10 days of incubation, respectively, at 31 C. Our procedure may aid future attempts to culture MLO and other as yet uncultivated microbes occurring in plant phloem.

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