Use of Dienes' Stain to Detect Plant Diseases Induced by Mycoplasmalike Organisms

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ABSTRACT

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Application of a 0.2% solution of Dienes' stain to hand-cut or freezing-microtome sections indicated mycoplasmalike organisms (MLO) in the phloem of infected plants. The stain appeared to be specific for MLO diseases, including those of spiroplasma etiology, and gave no reaction in tissues of plants infected by other pathogens. The procedure is quick and could be of diagnostic value.

The most reliable method for demonstrating the presence of mycoplasmalike organisms (MLO) in diseased plants is electron microscopy of phloem tissue. This elaborate process involves the use of expensive equipment and reagents. The need for a quick, simple method of detecting MLO diseases has long been recognized and for this reason some light-microscope staining methods have been described (5,6,8,10). These often involve specialized fluorescent microscopy or time-consuming embedding procedures. This article outlines a method that is rapid and simple and requires only low-power light microscopy. It involves the use of Dienes' stain, which is widely used to detect animal mycoplasmas (7) and plant spiroplasmas in culture (2).

MATERIALS AND METHODS

Dienes' stain was prepared by dissolving 2.5 g of methylene blue, 1.25 g of azure II, 10.0 g of maltose, and 0.25 g of sodium carbonate in 100 ml distilled water (7). The stain was filtered through Whatman No. 1 filter paper and a series of dilutions (0.5–0.1%, v/v) was made in water.

Trifolium repens L. plants with clover phyllody (CP) mycoplasma were collected from two areas of grassland and were maintained in plots or in the greenhouse. Infected plants have distinctive green, leaflike flowers, unlike the healthy white flowers.

Greenhouse-grown *Vinca rosea* L. plants were originally infected with CP by leafhopper (*Euscelis plebejus* Fallen) transmission from a single CP-infected *T. repens* plant. Stocks of plants were maintained by grafting pieces of infected *V. rosea* plants onto healthy seedlings. Infected plants were identified easily by their green flowers and bushy growth form, since healthy *V. rosea* have white or pink flowers.

Rose (*Rosa chinensis* var. *viridiflora* Dipp.) and *Helenium autumnale* L. plants naturally infected with MLO and showing flower greening were maintained in the greenhouse. Stems of apple with rubbery wood and chat fruit diseases and loganberry (*Rubus loganbaccus* Bailey) canes with Rubus stunt were supplied by Long Ashton Research Station. The John Innes Institute supplied cuttings of *V. rosea* infected with *Spiroplasma citri* and corn stunt spiroplasma.

Sections of healthy and infected stems were cut by hand into distilled water, with a single-edge razor blade. The sections were transferred to stain for 10 min, after which the stain was withdrawn and replaced with distilled water. After being washed in distilled water, the sections either were mounted in water and examined immediately or were left in distilled water for several hours before examination without any obvious detrimental effects. Both longitudinal and transverse sections of stems were examined. Transverse sections were more useful because they could be cut

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more easily by hand and the phloem tissue could be more easily located in them.

It was possible, by using a freezing-microtome, to cut stem and leaf tissue into uniform 100-μm thick sections that were suitable for staining and photographing. Thinner microtome sections often lost their cell contents, which resulted in unstained phloem.

Tests also were done to see whether disease organisms other than MLO would react similarly. Sections of stem and leaf phloem from plants infected with fungi, bacteria, and viruses were examined. Table 1 gives a list of all the diseases tested.

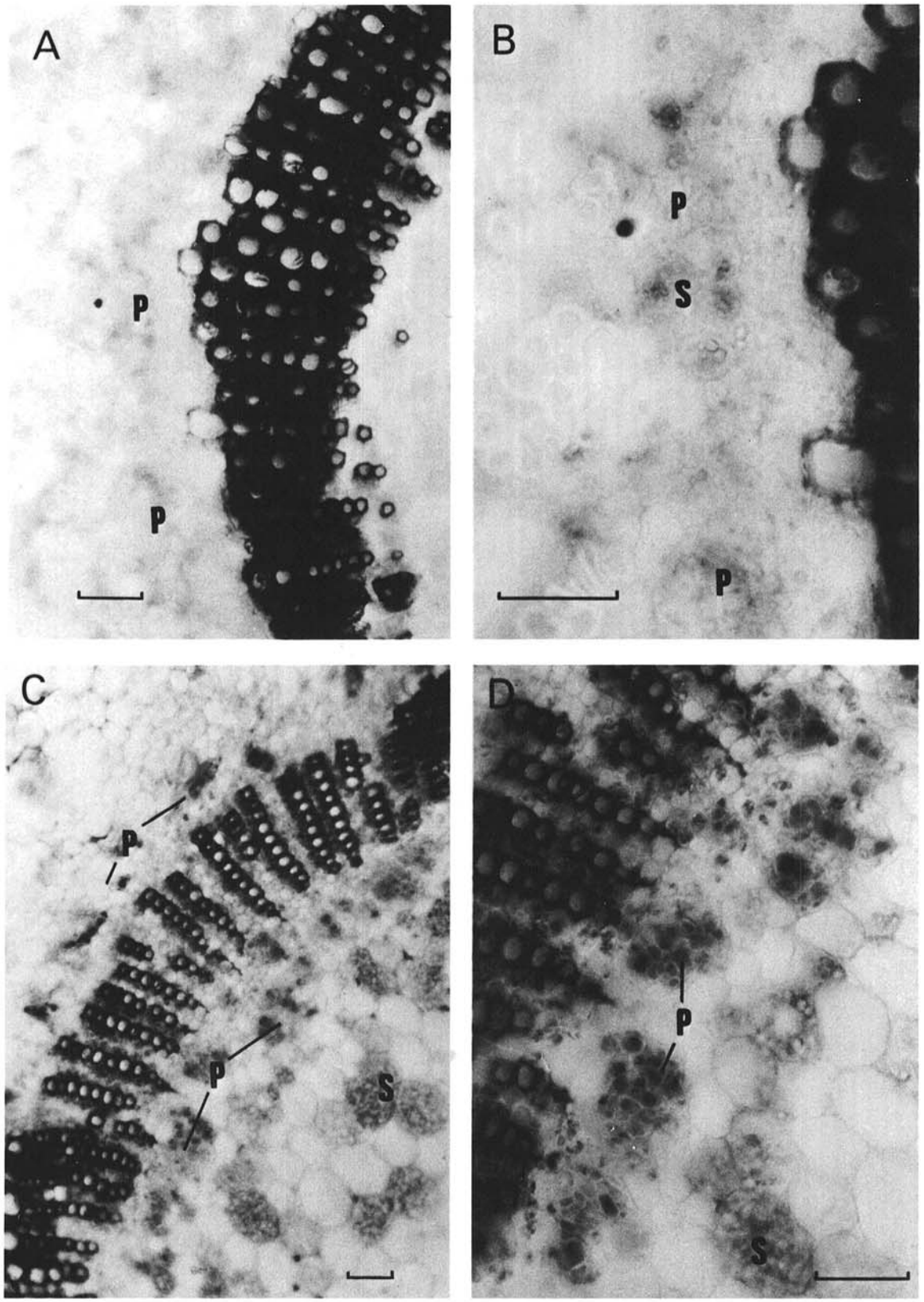

Fig. 1. Light micrographs of transverse sections of *Vinca rosea* L. stems treated with Dienes' stain. **A and B** show a section of healthy plant in which the phloem **(P)** remains unstained. **C and D** show a section of plant containing clover phyllody mycoplasmas in which the phloem tissue stains blue. Starch grains **(S)** appear yellow in stained sections. Scale bar represents 30 μm.

RESULTS

Thin stem sections of *V. rosea* plants of flowering age have a complete ring of xylem surrounded by a ring of phloem. The external phloem is separated from the xylem by a very narrow but distinct band of cambium. Some stems also have a ring of internal phloem. No color differentiation was observed among tissues subjected to concentrated Dienes' stain. In all sections of *V. rosea* treated with 0.5–0.1% dilutions of Dienes' stain, however, the xylem was colored bright turquoise blue and cells in the cortex stained pale purplish blue. Moreover, the phloem of healthy stem sections remained unstained (Fig. 1A and B) but phloem of infected stem sections contained many regularly distributed areas that stained a distinct blue (Fig. 1C). These areas could be resolved under high magnification as groups of phloem cells with blue-stained contents (Fig. 1D).

Application of 0.2% solution of Dienes' stain was most useful and made it possible to distinguish between MLO-infected and healthy tissue for both woody and nonwoody plants. CP could be detected in the phloem of leaves as well as in stems.

All MLO-infected plants showed similar results, but none of the plants containing other groups of pathogens showed blue coloration in the phloem of leaves or stems (Table 1).

Electron micrographs of the phloem from plants giving positive results with Dienes' stain showed numerous MLO in the phloem sieve elements.

TABLE 1. Reaction to Dienes' stain in sections of stems or leaves of plants with different diseases

Host	Disease	Etiological agent	Blue staining reaction in phloem
Vinca rosea	None	···	−
V. rosea	Clover phyllody	MLO	+
V. rosea	Citrus stubborn	*Spiroplasma citri*	+
V. rosea	Corn stunt	Spiroplasma	+
Clover	None	···	−
Clover	Clover phyllody	MLO	+
Apple	None	···	−
Apple	Rubbery wood	MLO	+
Apple	Chat fruit	Possibly MLO	+ (young shoots only)
Rose	None	···	−
Rose	Flower greening	MLO	+
Helenium	Flower greening	MLO	+
Loganberry	Rubus stunt	MLO	+
Melon	Watermelon mosaic	Virus	−
French bean	Tobacco mosaic	Virus (systemic)	−
Tobacco	Henbane mosaic	Virus (systemic)	−
Tomato	Bacterial spot	Bacterium (*Xanthomonas* vesicatoria)	−
Tomato	Bacterial wilt	Bacterium (*Pseudomonas solanacearum*);	−
Tomato	Potato blight	Fungus (*Phytophthora infestans*)	−
Grapevine	Downy mildew	Fungus (*Plasmopara viticola*)	−
Tomato	Grey mold	Fungus (*Botrytis cinerea*)	
Apple	Powdery mildew	Fungus (*Podosphaera leucotricha*)	−
French bean	Bean rust	Fungus (*Uromyces phaseolus*)	−

DISCUSSION

Dienes' stain was developed as a specific stain for animal mycoplasma colonies on agar (3). Mycoplasmas actively take up the stain, and the typical "fried-egg" growth form of the colonies can be identified more easily. Bacteria decolorize the stain (7). Plant MLO are similar in many respects to animal mycoplasmas and are phloem-restricted (1,4,9).

Our work suggests that plant MLO and animal mycoplasmas may react similarly with Dienes' stain and that the stain distinguishes between healthy and infected tissues. Electron microscopy confirmed the presence of MLO in tissue giving positive results with Dienes' stain and supports the idea that the color reaction results, at least in part, from specific staining of MLO within sieve elements. Because the stain does not appear to react positively with the phloem of plants infected with other disease organisms, it may be specific for diseases caused by MLO.

The technique described here is very rapid and requires only inexpensive reagents and minimal laboratory equipment. We used this method to follow the route of infection of CP in grafted *V. rosea* plants. Infection was detected before the plants reached flowering age and therefore before any symptoms were apparent. The stain also has been used to show the absence of MLO after tetracycline treatment of *V. rosea* (J. Deeley, *unpublished*).

The method can be used to detect disease during the early stages of infection and also may be useful as a diagnostic test on diseases of unknown etiology.

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