Detection of Mycoplasmalike Organisms in Infected Blueberry Cultivars by the DAPI Technique

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ABSTRACT

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Coded samples from stunt-infected and healthy blueberry plants were evaluated for the presence of mycoplasmalike organisms (MLOs) by comparing two histological techniques. By the DAPI (4',6-diamidino-2-phenylindole) technique with root tissue, MLOs were directly detected, whereas by the aniline blue (AB) technique with petiole samples, MLOs were indirectly monitored by detecting the resulting excess callose in sieve tubes. With DAPI, 24 of 25 infected and 25 of 25 healthy root samples were correctly evaluated. With AB, 19 of 25 infected and 10 of 25 healthy petiole samples were correctly evaluated. MLOs were observed by electron microscopy in root samples from one stunt-infected, DAPI-positive plant but not in root samples from one known healthy, DAPI-negative blueberry plant. Root samples from clones of several symptomless highbush blueberry cultivars were classified as MLO-positive by the DAPI technique. Petiole and stem samples from these same plants were rated negative by the DAPI test.

Blueberry stunt, caused by a mycoplasmalike organism (MLO) (3), is a widespread, serious disease of cultivated highbush blueberry (*Vaccinium corymbosum* L.) in the eastern United States. Aniline blue (AB) has been used for indirect detection of MLOs in blueberry. Excess callose that forms in sieve tubes of stunt-infected blueberry plants fluoresces when stained by AB (9). The DNA-specific fluorescence stain (DAPI (4',6-diamidino-2-phenylindole) was originally used for rapid diagnosis of mycoplasma contamination in animal tissue culture systems (13). It has also been used successfully to detect plant MLOs in infected crops including pear and apple (14). Our study was undertaken to determine the applicability of the DAPI technique for detecting blueberry stunt MLOs in roots and to compare it with the AB technique, as described previously (9), in petioles from the same plants.

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MATERIALS AND METHODS

Cuttings of Cabot blueberry known to be infected with blueberry stunt disease were obtained under permit from Michigan State University (MSU) and North Carolina State University (NCSU) and were grown in a screened, insecticide-treated greenhouse at Oregon State University (OSU). Bluecrop and Earliblue plants propagated from sources indexing negative for blueberry stunt disease by graft analysis on the susceptible blueberry cultivar Cabot were kindly supplied by R. D. Milholland of NCSU. Blueberry plants to be evaluated for stunt disease were grown in pots in an insecticide-treated screenhouse at OSU and sampled in November 1983 as needed.

Two histological techniques were used for detecting blueberry stunt. For the DAPI technique, samples about 2 × 5 mm taken from the main root, stem, and petiole were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 (histological buffer), at 4 C. Before sectioning, the pieces were washed in histological buffer for at least 3 min, then radial longitudinal sections about 25 μm thick were cut with a freezing microtome and processed by a slight modification of the method described by Seemüller (16). Sections were stained on slides for at least 1 min with a 1-μg/ml solution of the DNA-specific compound DAPI (Sigma Chemical Co., St. Louis, MO) in histological buffer. Sections mounted in the DAPI solution were covered with coverslips ringed with nail polish. Young sieve tubes in these sections were subsequently examined for DNA-specific fluorescence. Young sieve tubes were selected for evaluation because previous experience with apple and pear tissue (U. Schaper, *unpublished*) has shown that MLOs tend to reach their highest concentrations in young rather than mature sieve tubes. Fluorescence was observed with a Universal Epifluorescope (Carl Zeiss, Inc., Thornwood, NY) with an HBO 50W mercury source, an excitation filter at 365 nm, an intermediate dichroic beam splitter filter, and a barrier filter that passes light of wavelengths greater than 420 nm.

For the AB technique, petiole sections about 5 mm long from the same plants were fixed as mentioned before. Transverse sections about 25 μm were cut with a freezing microtome, stained according to methods described by Hiruki et al (10), and examined for excess callose—especially in the sieve tubes of the younger portion of the phloem.

To evaluate the reliability of the DAPI and AB techniques, coded samples of roots and petioles of stunt-infected and healthy greenhouse-grown blueberries were examined and classified. The code was then broken and the percentage of correct determinations calculated in each case.

For electron microscopy studies, samples from roots previously rated positive or negative in the DAPI test were fixed as noted before and postfixed in 1% osmium tetroxide in the same buffer for 1 hr. Specimens were then dehydrated through a graded acetone series with uranyl acetate staining en bloc during the 70% acetone step, embedded in Spurr's medium, sectioned, mounted on grids, and stained with lead citrate according to standard procedures (12). Samples were examined for MLOs in a Philips 300 electron microscope at 60 kV.

RESULTS

Coded samples of stunt-infected and healthy blueberry plants were evaluated by the DAPI and AB techniques (Table 1). For known stunt-infected plants, evaluation was 96% correct with DAPI-

treated root samples and 76% correct with AB-treated petioles. All 25 healthy blueberry root samples were scored correctly with DAPI, whereas 40% of the healthy petiole samples were scored correctly with AB. Photomicrographs showing the fluorescence caused by DAPI and AB in stunt-infected and healthy blueberry sieve tubes are presented in Figure 1.

The DAPI technique was also used to evaluate root samples for the presence of MLOs from a number of screenhouse-grown clones of highbush blueberry cultivars, many of which had indexed free of viruses detectable by enzyme-linked immunosorbent assay (6). Plants of the following cultivars tested positive for MLOs by DAPI: Cabot, Northland, N51G, and Olympia. Angola, Jersey, and Spartan were considered doubtful positives, because only a few of the youngest sieve tubes showed a DNA-specific fluorescence that could not be distinguished from the fluorescence of plant nuclei. Twelve other cultivars were rated negative with DAPI: Berkeley-83, Bluecrop-83, Bluejay-83, Blueray-83, Bluetta-83, Collins, Coville, Dixi-83, Earliblue-83, Herbert-83, Patriot, and Pemberton. The numbers after the cultivar names identify clones in the USDA-ARS virus-tested blueberry stock program at OSU.

To compare MLO colonization in petioles and stems versus roots, one petiole piece from each of the five known infected Cabot plants (Table 1) and plants of four other symptomless cultivars with colonized roots were monitored for MLOs after DAPI staining. In four of the five known infected symptom-bearing Cabot plants, MLOs were detected in the petiole and stem samples, whereas in the petiole and stem samples of the four symptomless, root-colonized plants of the cultivars Cabot, Northland, N51G, and Olympia, no MLOs were evaluated.

During this study, we observed a previously undescribed symptom in five stunt-infected blueberry plants of the cultivar Cabot. Three of these plants were known to be infected with blueberry stunt (Table 1), and two of them were otherwise symptomless but DAPI positive. The five stunt-infected plants had enlarged stipules (about 11 × 3 mm) at petiole bases on occasional shoots but healthy

Table 1. Accuracy of classification of coded samples of blueberry stunt-infected and healthy blueberry tissues by the DAPI and aniline blue techniques

		DAPI technique		Aniline blue technique	
Cultivar and plant code	**Stunt infected**	**No. root samples correctly classified**	**Total no. evaluated**	**No. petiole samples correctly classified**	**Total no. evaluated**
Cabot (MSU-1)	Yes[a]	5	5	3	5
Cabot (MSU-2)	Yes	5	5	5	5
Cabot (NCSU-1)	Yes[a]	4	5	2	5
Cabot (NCSU-2)	Yes[a]	5	5	5	5
Cabot (NCSU-3)	Yes	5	5	4	5
Percent correct		96		76	
Bluecrop (NCSU-1)	No	5	5	2	5
Bluecrop (NCSU-2)	No	5	5	3	5
Earliblue (NCSU-1)	No	5	5	2	5
Earliblue (NCSU-2)	No	5	5	1	5
Earliblue (NCSU-3)	No	5	5	2	5
Percent correct		100		40	

[a] Enlarged stipules were noted on these plants.

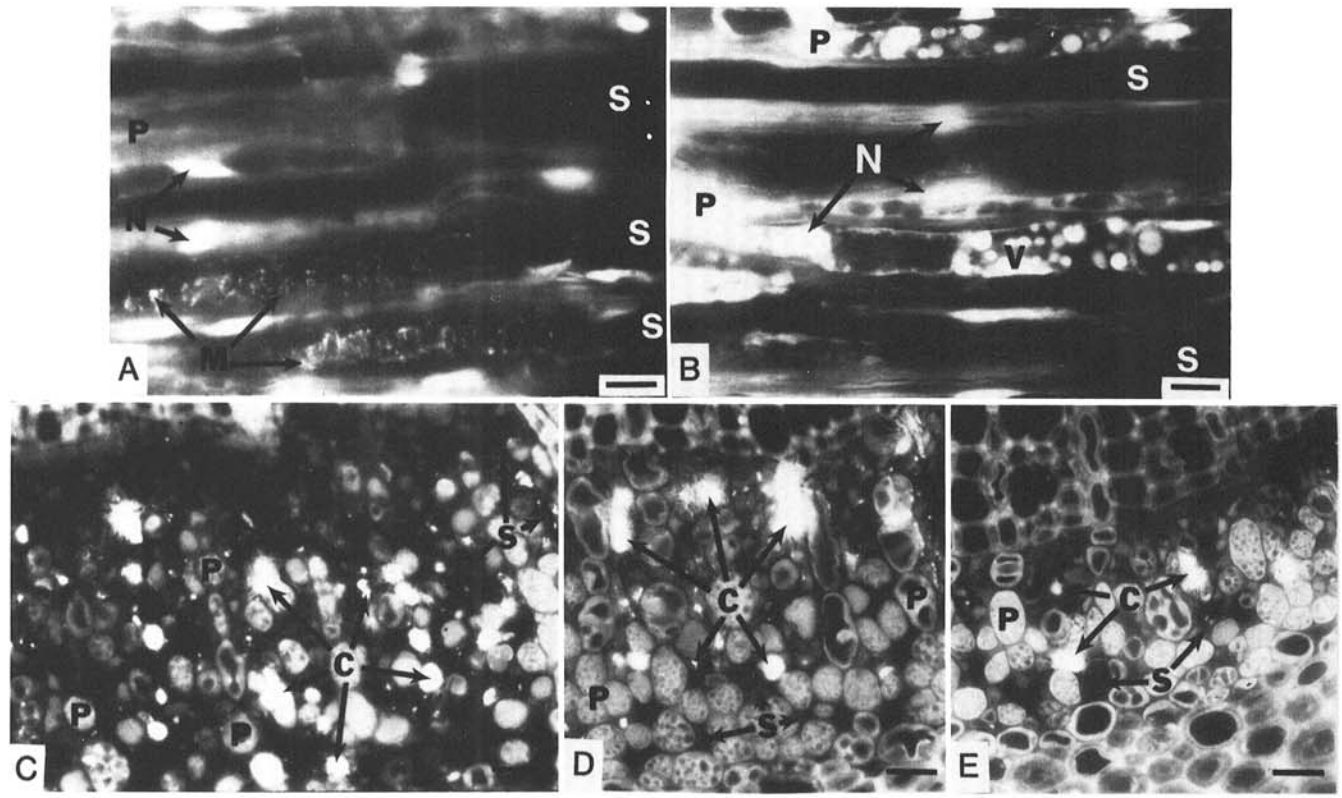

Fig. 1. Light micrographs of blueberry stunt-infected and healthy blueberry tissues. S = sieve tube, P = parenchyma cell, M = mycoplasmalike organism, N = nucleus, and V = vacuolar material. Each bar = 4,500 nm. **(A)** Longitudinal section of the phloem of a stunt-infected blueberry root (cultivar Cabot MSU-1) after DAPI staining. **(B)** Longitudinal section of the phloem of a healthy blueberry root (cultivar Bluecrop NCSU-1) after DAPI staining. **(C)** Cross section of the phloem of a stunt-infected blueberry petiole (Cabot MSU-1) after AB staining. C = callose in about 70% of the sieve tubes. **(D)** Cross section of the phloem of a healthy blueberry petiole (Bluecrop NCSU-1) after AB staining. C = callose in about 50% of the sieve tubes. **(E)** Cross section of the phloem of a healthy blueberry petiole (Bluecrop NCSU-1) after AB staining. C = callose in about 10% of the sieve tubes.

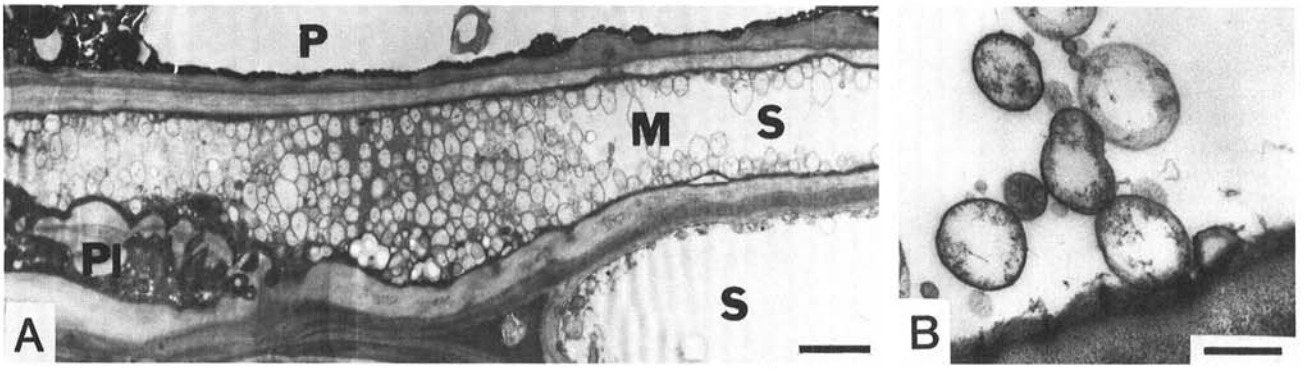

Fig. 2. Electron micrographs of sieve tubes in the youngest portion of the phloem of a stunt-infected blueberry root (cultivar Cabot MSU-1). S = sieve tube, P = parenchyma cell, M = mycoplasmalike organism (MLO), and Pl = sieve plate. **(A)** Longitudinal section of a sieve tube highly colonized with MLOs. Bar = 2,100 nm. **(B)** Enlargement of MLOs showing characteristic trilaminar membranes, DNA strands, and ribosomes. Bar = 350 nm.

Cabot plants did not. Enlarged stipule formation is a characteristic symptom in some apple cultivars infected with MLO-induced apple proliferation (2).

Ultrathin sections of root phloem of one infected Cabot plant examined with the electron microscope contained MLOs in sieve tubes of the youngest portion of the phloem (Fig. 2). No MLOs were found by electron microscopy in comparable tissues from a single plant of the DAPI-negative cultivar Bluecrop-83.

DISCUSSION

The DAPI technique for observing the characteristic light blue fluorescence in sieve tubes containing MLOs was found reliable for distinguishing between blueberry stunt-infected and healthy blueberry plants in our laboratory. DAPI-stained DNA in MLOs fluoresces as discrete foci in infected sieve tubes. This distinctive fluorescence pattern enables the experienced observer to rate MLO colonization in a sample from none to light to heavy. In a laboratory where this test is performed routinely, an experienced worker could probably cut, stain, and evaluate 30–40 tissue samples in a normal working day.

Blueberry roots are the most suitable organ to examine for MLOs by the DAPI technique. If a given root is colonized, usually a high MLO population can be detected in its sieve tubes. For symptomless plants, it is possible to fail to detect the blueberry stunt agent by only examining stem samples. As noted earlier, we were not able to detect MLOs in shoot and petiole samples of the four symptomless cultivars that had organisms in their root systems. These results are in total agreement with data obtained by the DAPI test with symptomless but MLO-infected apple and pear trees, in which obvious root colonization but hardly any stem colonization occurred (17). If blueberry cultivars have distinctive stunt symptoms, it apparently does not make much difference if root or stem samples are examined for MLO colonization. For checking symptomless blueberry plants, we strongly recommend examining root samples to reduce the amount of possibly false negative evaluation. We did not follow the colonization of MLOs in blueberry roots and shoots by the DAPI technique throughout the year, but we suspect that this colonization behavior is similar to that of MLOs that have been more extensively studied in apple and pear. In apple proliferation and pear decline, the MLO population in stems degenerates during the winter and may be replenished from infected roots the following spring, whereas roots are a suitable tissue for MLO detection throughout the year (18).

Stunt-diseased blueberry plants were incorrectly classified in 4% of the root samples tested with the DAPI technique, whereas no errors were made in classification of known healthy blueberry plants. With the DAPI technique for MLO detection, experience helps reduce errors as the user gains confidence in distinguishing between the very similar fluorescence that occurs with MLOs and with plant nuclei. Fluorescing nuclei can be a problem, especially in plants that are slightly diseased and are colonized only in the youngest sieve tubes. Young sieve tubes that are not yet fully developed still are nucleated. Therefore, nuclear DNA fluorescence in such sieve tubes can be mistaken as fluorescence of MLOs. Nuclei of healthy apple and pear cells occasionally are granulated when viewed by the DAPI technique (U. Schaper, *unpublished*). In nuclei of healthy blueberry cells, this granulation occurs more often than in apple and pear nuclei. Therefore, the individually fluorescing nuclear granules in blueberry cells may be misinterpreted as MLOs.

Mitochondria also can be mistaken for MLOs, but this is a minor problem. In contrast to MLO-DNA, which is always intensively stained with DAPI, plant mitochondrial DNA is only occasionally apparent in stained preparations. In such cases, the fluorescence of mitochondria is weaker than that of MLOs because the MLOs have a higher adenine-thymine (AT) content than mitochondria. DNA with high AT content enhances the binding of the AT-specific fluorochrome DAPI (15). On the other hand, the mitochondrial DNA fluorescence is quickly quenched when exposed to UV light, whereas the MLO fluorescence remains stable for at least several minutes under UV exposure. Similar observations were made with the chemically closely related DNA stain Hoechst 33258 with mammalian mitochondrial DNA (21).

Studies with the indirect AB technique resulted in incorrectly classifying 24% of the stunt-infected blueberry petioles tested and 60% of the petioles sampled from known healthy blueberries. Use of AB to classify infected and healthy blueberry root sieve tubes instead of those in petioles did not improve the accuracy of our classification. Because callose deposits tend to increase as healthy sieve tubes age (8), the AB technique would probably be most reliable when used to examine young sieve tubes. In addition, rapid fixation of tissues before AB treatment may help reduce formation of wound callose in sieve tubes (7).

The blueberry cultivar Cabot has been used or recommended as an indicator host to detect blueberry stunt by graft in suspect plants (5,11,20). The presence of DAPI-positive bodies (apparently MLOs) in sieve tubes of symptomless, presumably healthy Cabot plants from New Jersey and in several other symptomless blueberry cultivars can be explained in four ways: 1) False positive reactions in the absence of MLOs may occur. 2) Unreported, latent strains of blueberry stunt MLO may exist. 3) MLOs unrelated to the bluberry stunt MLO may occur rather commonly in some blueberry cultivars in the United States. There is evidence in Europe (1) of one additional MLO in blueberry (witches'-broom). Positive laboratory identification of MLOs associated with blueberry stunt disease would be aided by the development of accurate serological tests of the relationships among MLOs in *Vaccinium* similar to those developed for clover phyllody and aster yellows MLOs (4,19). 4) Our failure to observe stunt disease symptoms in most infected Cabot plants may be paralleled by the known latent infection of apple and pear trees by apple

proliferation and by pear decline. In infected apple and pear trees that showed no symptoms for 1 yr or more, MLOs could be found in only 3 and 5%, respectively, of all shoot tissues examined, whereas nearly every root sample was MLO-positive (17). We believe that symptomless Cabot plants with MLOs in their roots may likewise remain symptomless until conditions are such that the MLOs move into their shoot systems.

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