Detection of Ash Yellows Mycoplasmalike Organisms in Different Tree Organs and in Chemically Preserved Specimens by a DNA Probe vs. DAPI

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ABSTRACT

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DNA of mycoplasmalike organisms (MLOs) was detected by dot hybridizations with a biotin-labeled cloned DNA probe, pBB115, in samples obtained from each of six naturally infected mature white ash (*Fraxinus americana*) on each of 11 dates over 14 mo. Hybridization signals were most consistent and intense with samples from the innermost phloem at the trunk base, less so with samples from fine roots, and least so with samples from coarse roots (2–6 mm in diameter), twigs, or leaves. The DNA probe detected MLOs as consistently as the DAPI (4′,6-diamidino-2-phenylindole·2HCl) fluorescence test in white ash but not in velvet ash (*F. velutina*), in which MLO populations were lower. Plant tissues destined for DNA extraction were effectively preserved in 5% (w/v) aqueous borax or by drying at 42 C as possible alternatives to freezing.

Cloned nucleic acid hybridization probes have been used to detect mycoplasmalike organisms (MLOs) and spiroplasmas in various plants (1–7, 12–17,19). Disease-specific probes have been used to detect MLOs in some naturally infected plants (3,9,13). Sampling procedures that optimize MLO detection by DNA probes have not been reported, nor have DNA hybridizations been compared with the more convenient DAPI (4′,6-diamidino-2-phenylindole·2HCl) fluorescence test (20) for practical nonspecific detection of MLO DNA. If the DAPI test were found to be as sensitive as DNA hybridizations, it would remain the method of choice for nonspecific detection. Other microorganisms or DNA viruses in phloem could theoretically cause misinterpretation of results, but such errors have not been reported.

MLOs cause ash yellows (AshY), which occurs from the Great Plains eastward in the United States and also in at least two southwestern localities (21,23). The principal symptoms are rootlet mortality, slow growth, branch dieback, and witches'-brooms (10,11,18). Until recently, diagnosis of AshY was based on symptoms and nonspecific detection of MLOs by means of the DAPI test (22). Now, cloned, biotin-labeled DNA probes capable of hybridizing nonspecifically or specifically with the DNA of AshY MLOs are available (5,8,9,17). These probes are expected to be useful for sensitive detection and identification of AshY MLOs in naturally infected trees.

In research involving DNA hybridizations for MLO detection, it is customary to store plant samples frozen until DNA can be extracted. In field research, it is sometimes impossible to freeze plant specimens soon after collection. Alternative means of preserving specimens for MLO detection would be useful.

This study was undertaken to learn whether the organ sampled or the time of year affects detection of AshY MLOs by DNA hybridization, compare dot hybridizations using a biotinylated probe with the DAPI test for nonspecific detection of MLOs in ash, and identify methods other than freezing for preservation of field-collected samples before DNA extraction.

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MATERIALS AND METHODS

MLO detection in various plant organs in different seasons. Six mature white ash (*Fraxinus americana* L.) with branch dieback, which had previously been diagnosed as MLO-infected on the basis of the DAPI test, were sampled at monthly intervals from September 1988 through November 1989, except for January–March. The trees were growing on one site near Ithaca, NY. Samples obtained from each tree on each date of sampling included bark patches from the base of the trunk, roots 2–6 mm in diameter (coarse roots), and fine roots. During April–November, one leafy twig (bare twigs before foliation and after leaf fall) from a branch low in the canopy was also collected. The bark patches were cut with a chisel or arch punch to obtain tissues from the cambium outward if the bark slipped readily (during May–August) or bark plus outer sapwood if the bark did not separate from the wood. Samples were placed on ice for transportation to the laboratory. Portions were then removed for DAPI tests as described below, and the remainder of each sample was frozen at −20 C until subsamples were taken for nucleic acid extraction.

For each tree and sample date, two or three specimens of each of the following types were fixed in 2.5% aqueous glutaraldehyde for the DAPI test: petiole segments 6–8 mm long from two leaves, if available; twig segments 6–8 mm long and including nodes; blocks, approximately 2 × 2 × 8 mm, of innermost phloem from the base of the trunk; and root segments 2–4 mm in diameter and 6–8 mm long. DAPI tests on longitudinal sections of phloem were performed as described previously (22). Fine roots were not tested with DAPI.

DNA extractions from 0.3-g samples of plant tissue, dot hybridizations with a biotin-labeled probe at 42 C on nitrocellulose filters, and detection of hybridized probe by means of the BluGENE detection system (Gibco Laboratories, Grand Island, NY) were performed as described previously (5–7,16,17). Amounts of total DNA spotted on the filters were not standardized. Probe pBB115 from

the tomato bigbud MLO (17), which detects many MLOs, was used for all samples, because probes derived from AshY MLOs (9) were not available when this work began. DNA samples from all six field trees on a given sample date could not be hybridized on one filter; therefore, two filters per sample date were used, each bearing all of the samples from three trees. Positive and negative standards on every filter consisted of nucleic acid from diseased periwinkle plants containing strain AshY1 of the ash yellows MLO and diseased and healthy ash trees growing in pots in a screened greenhouse. The relative intensities of detection signals indicating hybridization of probe with DNA from plant samples were scored on a 0–5 scale.

Comparative sensitivities of a biotinylated probe and DAPI for MLO detection. Diagnostic comparisons with probe pBB115 and DAPI were performed on three groups of ash growing on sites where ash yellows had been diagnosed previously. Two groups of white ash (hereinafter called enigmatic trees) were selected because they displayed symptoms like those of ash yellows but had been scored negative in DAPI tests for MLO infection. One group, growing in a woodlot, consisted of 12 trees, 85–90 yr old, that showed abnormally slow twig growth and, in some cases, branch dieback. The other group consisted of 16 10- to 15-yr-old trees in a field. The young trees were growing abnormally slowly and/or displayed deliquescent branching (18) on one or more low limbs. Samples from two additional trees known to have ash yellows were processed as positive controls with each group. The DAPI test was performed on roots 2–4 mm in diameter because these are the most satisfactory sample type for the test (22) (Table 1). DNA samples from inner phloem from the base of the trunk were probed, because that tissue had been found to give the most consistent positive results (Table 1).

The third group consisted of 16 velvet ash (*F. velutina* Torr.) in Zion National Park, UT. Fluorescent microscopic inspection of DAPI-treated root sections from these trees indicated the presence of DNA, presumed to be that of MLOs, in phloem sieve tubes of all of them. The populations of MLOs were believed to be relatively low, however, because the characteristic fluorescent particles were dispersed and often difficult to detect. Trunk samples from 12 trees and fine roots from four trees were collected for DNA extraction and hybridization as described above.

Preservation of plant samples for DNA extraction. The specific objective was to find a treatment that would preserve specimens for both the DAPI test and DNA extraction for probing. Drying and treatment with chemicals that could be purchased in a grocery store or pharmacy were considered. In preliminary work with MLO-infected periwinkle, we infused and stored samples of symptomatic leaves and dwarfed stems with various formulations and concentrations of methyl, ethyl, or isopropyl alcohol; NaOCl (0.5% aqueous, w/v); NaCl (20% aqueous, w/v); or borax (5% aqueous, w/v) in glass vials. Other samples were dried for 5 hr at 42 C or were killed in 50% ethanol and then dried. For standards, one sample was stored at −20 C and another fixed and stored in 2.5% aqueous glutaraldehyde. After 2 wk, DNA was extracted from all samples, spotted on nitrocellulose filters, and probed in the usual manner.

In follow-up tests with the most promising treatments, leaves of MLO-infected periwinkle, root segments (up to 5 mm diameter) of healthy and MLO-infected white ash, and innermost phloem from the butts of healthy and MLO-infected white ash were preserved by infusion with NaCl or borax as described above, or drying at 42 C for 72 hr in darkness. A fourth set of samples was frozen at −20 C, and a fifth set was infused with 2.5% aqueous glutaraldehyde to provide a standard for the DAPI test. After 1 and 13 wk, DNA was extracted from 0.3-g portions of five replicates of each sample, and dot hybridizations were performed with probe pBB115. The relative intensity of the detection signal for MLO DNA was scored on a 0–5 scale. Other root segments and blocks of conductive phloem from each treatment were sectioned, treated with DAPI, and examined as described previously (22). Treatments were evaluated for integrity of preserved tissues during sectioning and ease of detection of the DAPI-DNA complex in phloem sieve tubes.

RESULTS

MLO detection in various plant organs at different seasons. MLO DNA was detected by dot hybridizations in at least one trunk or root sample from every tree in every month of sampling, September–December 1988 and April–November 1989 (Table 1). MLO DNA was most frequently detected in trunk phloem and next most frequently detected in fine roots. Detection of MLO DNA in leaves, twigs, and coarse roots was inconsistent.

DNA samples from different organs varied significantly ($P < 0.001$ in ANOVA) in intensity of detection signals for MLO DNA (Table 2). Signals were most intense with samples from the inner phloem at the base of the trunk, often being comparable to signals from the DNA of periwinkle plants containing ash yellows MLOs (Fig. 1). The detection signal with DNA from fine roots was second in intensity to that from the trunk base (Table 2). Hybridization signals with samples from leaves and twigs of the naturally infected trees, as well as the diseased potted ash used as positive standards, were often weak or absent (e.g., Fig. 1). The experiments were not designed to allow comparisons of hybridization signal intensity from month to month.

Faint false detection signals often developed in hybridizations with DNA extracted from healthy ash or periwinkle. These signals were never scored higher than 1 on the 0–5 scale (Table 2) and usually did not interfere with interpretation of test results. MLO detection was declared for samples giving hybridization signals more intense than any from a healthy standard on the same filter. Weak false detection signals were recorded for healthy ash samples 3 and 4 of July, as shown in Figure 1.

MLO detection in coarse roots by the DAPI test was consistent, except for one tree in November 1988. Samples from the base of the trunk tested positive, except for two trees in May and one in June 1989. MLO detection in leaves and twigs by DAPI was inconsistent (Table 1).

Comparative sensitivities of a biotinylated probe and DAPI. Probe pBB115 did not detect MLO DNA in phloem samples from the butts of any of the 12

Table 1. Number of trees, of six, in which mycoplasmalike organisms were detected with DNA probes and/or DAPI, as related to the plant part sampled and month of the year[w]

Collection date[x]	Leaves		Twigs		Phloem of trunk		Coarse roots		Fine roots[y]
	DAPI	Probe	DAPI	Probe	DAPI	Probe	DAPI	Probe	Probe
11 April 1989	...	...	...	1	6	5	6	2	3
9 May 1989	...	...	1	4	4	6	6	2	6
9 June 1989	0	1	1	0	5	6	6	2	4
12 July 1989	1	2	1	3	6	6	6	5	6
8 August 1989	2	1	0	1	6	6	6	0	6
12 September 1989	0	4	1	5	6	6	6	4	6
11 October 1989	3[z]	0[z]	2	4	6	6	6	2	4
13 November 1989	...	...	3	1	6	6	6	2	6
Average	0.8	1.6	1.3	2.4	5.6	5.9	6.0	2.4	5.1

[w] DAPI (4′,6-diamidino-2-phenylindole·2HCl) and the nonspecific probe pBB115 were used as described previously (16,17,23).

[x] Leaves were not available in April, May, or November. Twig samples of April for DAPI test were lost.

[y] Fine roots were not tested with DAPI.

[z] Leaves were available on only three trees in October.

85- to 90-yr-old enigmatic ash but did detect MLO DNA in the two comparable trees, used as infected standards, that had been scored positive in the DAPI test.

MLO DNA was detected by dot hybridization in DNA from trunk phloem of one of the 16 enigmatic ash and both infected standards 10 to 15 yr old. The roots of the enigmatic tree were then resampled for another DAPI test, which was again scored negative. Phloem from the butt of the tree was then tested with both DAPI and the DNA hybridization probe, and MLOs were detected by both methods.

When the results for all three studies with white ash were combined, dot hybridizations and the DAPI procedure both gave positive results with 11 MLO-infected trees, and both gave negative results with 28 enigmatic trees.

MLO DNA was detected by dot hybridization in only one of the 16 infected velvet ash. This detection occurred in a sample extracted from fine roots. Although DAPI tests on root phloem of all these trees were positive, similar tests on trunk phloem were negative, indicating that MLOs were scarce in, or perhaps absent from, the trunks.

Preservation of plant DNA for probing. In the preliminary tests, DNA that hybridized with probe pBB115 was obtained from all preserved periwinkle specimens except those that were dried after killing in 50% ethanol. The intensity of detection signal for hybridization varied with treatment, however. The order of treatments, from strongest to weakest detection signal was: freezing > 5% borax or 20% NaCl > drying or 2.5% glutaraldehyde or alcoholic solutions.

In further tests of borax and NaCl solutions in comparison with freezing and drying, hybridization results after 1 and 13 wk of storage were similar: all four treatments preserved MLO DNA sufficient for relatively strong detection signals. These were strongest with tissues that had been frozen or preserved in 5% borax solution (Fig. 2). Healthy control tissues gave no hybridization signals.

After 13 wk, healthy and diseased specimens of roots and trunk phloem from each treatment were sectioned on a freezing stage, treated with DAPI, and examined with a fluorescence microscope. Borax, NaCl, and glutaraldehyde all fixed organelles and allowed visualization of fluorescent particles in diseased sieve tubes. Glutaraldehyde was the most satisfactory fixative. Tissues preserved in borax solution were adequately fixed and could be sectioned readily, but borax, in comparison to glutaraldehyde, caused the cell walls to diffract more light and enhanced the autofluorescence of intracellular structures in parenchyma. Therefore, if sections were more than one or two cells thick, it was difficult to see fluorescent particles in sieve tubes. Tissues preserved in NaCl were mushy and difficult to section. Previously frozen tissue could be sectioned adequately, but smeared DNA from the unfixed nuclei sometimes interfered with interpretation. Dried tissues, even when rehydrated before mounting on the freezing stage, remained too brittle to be sectioned adequately.

Table 2. Relative intensities of hybridization signals from nucleic acid samples extracted from various organs of MLO-infected white ash in eight successive months[y]

Month	Leaves	Twigs	Trunk phloem	Coarse roots	Fine roots	Healthy controls
April	ND[z]	0.3	1.3	0.3	0.5	0.0
May	ND	1.3	3.0	0.8	2.1	0.5
June	0.2	0.0	2.3	0.3	1.3	0.3
July	1.2	1.8	4.2	1.8	2.5	1.0
August	0.3	0.3	2.8	0.0	1.8	0.0
September	1.3	1.5	3.5	1.3	2.0	0.2
October	0.3	2.0	3.2	1.2	1.8	0.5
November	ND	0.8	3.8	1.2	2.2	0.5
Mean	0.7 bc	1.0 bc	3.0 a	0.9 bc	1.8 b	0.4 c

[y] Each datum is the average of scores for six samples from different trees. Hybridization signals were scored on a 0–5 scale where 0 = no signal and 5 = an intensely dark spot such as in columns 1 and 14 of Figure 1. The same trees were sampled each month. Values across rows (sample sources) but not within columns (months) may be compared, because probe solutions varied from month to month. Significant variation ($P < 0.001$ in ANOVA) in strength of hybridization signal was associated with the source of DNA. Means followed by the same letter do not differ at $P = 0.01$, according to Duncan's new multiple range test.

[z] Not done.

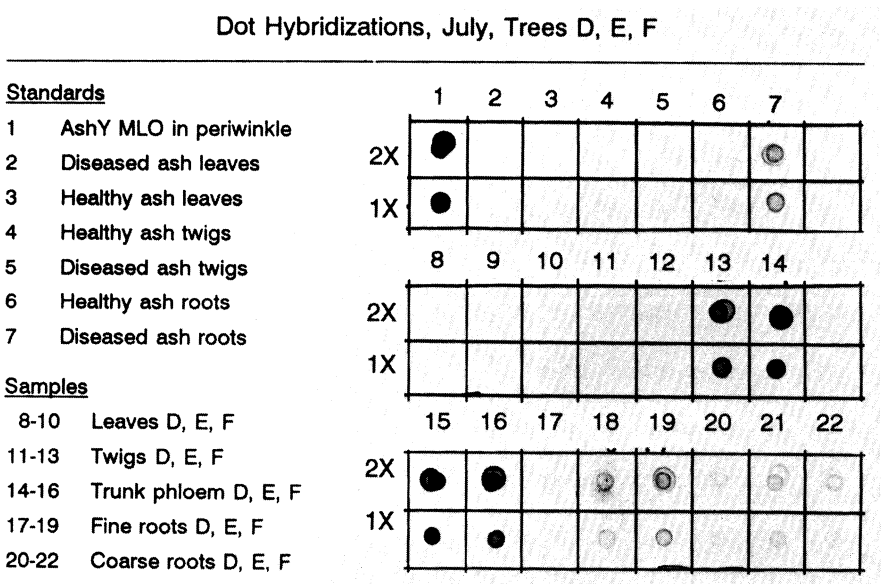

Fig. 1. A representative outcome of dot hybridization, with biotinylated probe pBB115, of DNA samples from three white ash trees, designated D, E, and F, infected with ash yellows MLOs. DNA extracts were applied in 3-μl quantities of 6× SSC buffer (0.9 M aqueous NaCl and 0.09 M Na citrate) once (1×) or twice (2×) per sample. Each nitrocellulose filter received all samples collected from three trees on one date. Standards were DNA extracts from diseased periwinkle and diseased and healthy white ash growing in a greenhouse. One twig sample and all three samples of trunk phloem gave strong hybridization signals. In this test, MLO DNA was not detected in leaf or twig samples from two standard plants known to contain ash yellows MLOs.

DISCUSSION

This study showed the usefulness and some limitations of MLO detection in naturally infected ash trees by means of DNA hybridizations with biotinylated probes. The sampling methods that were most effective for MLO detection may be applicable to many woody perennial plants.

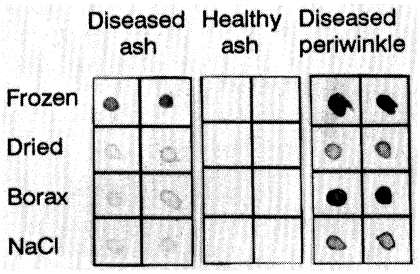

Fig. 2. Dot hybridizations with probe pBB115 of DNA from samples of ash phloem and periwinkle foliage that had been preserved by freezing at −20 C, drying at 42 C, or storage for 13 wk in solutions of borax (5% w/v) or NaCl (20% w/v).

MLO detection in naturally infected white ash. Detection, by means of dot hybridizations, of MLOs in all six diseased white ash in every month of sampling indicated that this technique is reliable with samples collected during April–November. Samples were not collected during January–March, but the detection of MLOs in April samples indicated that MLOs would have been detectable throughout the winter.

The suitability of trunk phloem for extraction of MLO DNA was based not on concentration of MLOs there but on the availability of tissue samples with a relatively high content of conductive phloem. MLOs in ash trunks are restricted to phloem sieve tubes within 600 μm of the vascular cambium (W. A. Sinclair, *unpublished*). Accordingly, we extracted DNA only from a layer less than 1 mm thick from the inner bark of trunk samples.

Feeder roots were the second most useful sample type for MLO detection in white ash by means of DNA hybridizations. MLO DNA was detected in these roots of all six diseased trees in 5 out of 8 mo (Table 1). Feeder roots were the only useful sample type for this mode of MLO detection in velvet ash. MLO titer may be relatively high near root apices because they are metabolic sinks to which substances in phloem move.

Roots of the size most useful for the DAPI test were not consistently useful for detecting MLO DNA with the biotinylated probe. The reason for this inconsistency may be that the coarse root samples included all tissues other than xylem. Conductive phloem containing MLO DNA would have constituted only a small proportion of each sample. The amount of MLO DNA extracted from these samples was often insufficient for detection.

The inconsistent detection of MLO DNA in white ash leaves and twigs by either dot hybridizations or the DAPI test was in accord with previous findings for the DAPI test (22). Apparently, the titer of MLOs varies greatly in leaves and twigs of ash trees affected by yellows.

Variation in frequency of MLO detection and in strength of hybridization signals was no doubt also related to variation in the amount of total DNA extracted from the various samples, which would have caused variable amounts in the 3-μl droplets on the filters. DNA concentration in droplets was not standardized, because other variables, such as the proportion of conductive phloem in a tissue sample, could not be controlled.

DNA probes that specifically detect AshY MLOs (9) were developed after the research here reported was underway; therefore, we used probe pBB115 throughout this work. During the testing of the AshY-specific probes, however, two of them were allowed to hybridize with a subset of the same DNA samples collected in August 1989 from the six white ash that were repeatedly sampled. The pattern of results (9) was similar to that obtained with probe pBB115.

Biotinylated DNA probe vs. DAPI test for MLO detection. Dot hybridizations and the DAPI test were equally effective for nonspecific detection of MLOs in the white ash that were chosen for comparisons, although the tissue type appropriate for optimal use of each technique differed. Coarse roots were best for the DAPI procedure, in accord with previous findings (22), whereas the inner phloem at the base of the trunk was best suited for DNA extraction for dot hybridizations.

All but one of the enigmatic white ash remain undiagnosed. DNA from trunk samples of 27 of the 28 trees that had been scored negative in the DAPI test on root phloem failed to hybridize with probe pBB115. Thus, DAPI tests and dot hybridizations with biotinylated probes are likely to give the same diagnostic results for enigmatic trees. These results still do not exclude the possibility that the enigmatic trees are infected with small populations of MLOs. If so, however, more sensitive techniques will be required to detect them. The single enigmatic tree for which there was an initial discrepancy between the results with DAPI and dot hybridization tested positive with both DAPI and the DNA probe when phloem of the butt was sampled. We did not further investigate the distribution of MLOs in this unusual tree.

The DAPI test was more effective than DNA hybridizations for MLO detection in velvet ash, apparently because MLO populations in sampled tissues were usually too small for detection of their DNA by dot hybridizations using a biotinylated probe and a streptavidin-alkaline phosphatase detection system. We have little doubt that AshY MLOs were present in the velvet ash, because in later work (21), DNA from the roots of one DAPI-positive tree hybridized with probes that specifically detect these MLOs.

Overall, the DAPI test appears more useful than dot hybridizations for nonspecific detection of MLOs, because it is at least as sensitive as DNA hybridizations while being much more rapid. One worker can diagnose 20 specimens in 4 hr by means of the DAPI test, whereas DNA extractions, dot hybridization, and probe detection for the same number of specimens require 12–13 hr.

Preserving plant samples for DNA extraction. Samples for DNA extraction can be preserved well by infusion with borax or can be preserved adequately by drying, if it is not possible to freeze them soon after collection. These alternative methods for preserving MLO-infected specimens may be useful during field studies at remote sites. None of the preservative treatments tested, however, was as useful as fixation in 2.5% glutaraldehyde for specimens that would later be examined in the DAPI test.

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