Sampling and Histological Procedures for Diagnosis of Ash Yellows

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ABSTRACT

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The DAPI (4′,6-diamidino-2-phenylindole·2HCl) fluorescence test was superior to Dienes' stain for histological detection of mycoplasmalike organisms (MLOs), which cause ash yellows, in ash (*Fraxinus* spp.). MLOs were detected by the DAPI test more than twice as often in roots (2–6 mm diam) as in twigs of *F. americana* from which one twig and one root per tree were tested. Repetitive sampling of the root systems of 30 *F. americana* revealed that, within limits of the DAPI technique, the testing of one root per tree would give a false-negative result for the tree as a whole in fewer than 10% of cases. Therefore, given independent probabilities of false-negative results, the testing of two roots from a tree would result in less than 1% chance of erroneously declaring the tree free from MLO infection. MLOs were detected by the DAPI test in the roots of 68% of 108 *F. americana* that were growing slowly or showing dieback but lacked witches'-brooms or deliquescent branches. Deliquescent branching was a strong indicator but not a definitive diagnostic symptom of MLO infection.

Mycoplasmalike organisms (MLOs) are the apparent cause of ash yellows (AshY), a disease of *Fraxinus* spp. characterized by loss of vigor, dieback, and premature death (7,8). Ash in advanced stages of decline because of AshY often develop witches'-brooms on the lower bole or at the root collar (5,8). The brooms are diagnostic for MLO infection, but only a minority of affected trees have brooms at a given time.

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Therefore, field diagnosis of AshY in individual trees is usually uncertain. Matteoni and Sinclair (8) reported that deliquescent branching also is a diagnostic symptom of AshY. Because their records were made on sites where AshY was severe, however, it was unknown whether this symptom might occur on white ash in the absence of this disease.

AshY was discovered in midwestern states in 1986 (16), but disease surveys were hampered because observers could not confidently diagnose this disease except in trees bearing witches'-brooms. Histological techniques, such as the use of Dienes' stain (3) and the DAPI (4′,6-diamidino-2-phenylindole·2HCl) fluorescence test (9), were available for diagnosis, but sampling procedures that minimize false-negative results had not been developed. Matteoni and Sinclair (8) used Dienes' stain for diagnosis of AshY. They reported a high level of agreement between the results of transmission electron microscopy and the Dienes' stain test for detection of MLOs in witches'-brooms and deliquescent branches. We initially used Dienes' stain for evaluation of ash that lacked witches'-brooms or deliquescent branches. Strong positive reactions such as those illustrated (8) were unambiguous, but weak reactions could not be interpreted reliably. The DAPI fluorescence test, recently used for studies of several plant diseases caused by MLOs (2,4,6,10,11), offered the promise of more reliable diagnoses, because DAPI binds to DNA and fluoresces under UV radiation. If fluorescent particles can be seen in mature sieve cells treated with DAPI, this is prima facie evidence of the presence of microorganisms (5,8).

The objectives of the present work were: 1) to ascertain the reliability of the DAPI fluorescence technique in comparison to Dienes' stain for histological diagnosis of AshY, 2) to develop and verify procedures for sampling individual trees as well as populations of ash for histological diagnosis of AshY, and 3) to test the reliability of deliquescent branching, slow growth, and dieback as diagnostic criteria of AshY. Preliminary findings were reported in an abstract (15).

MATERIALS AND METHODS

DAPI fluorescence test. Aqueous DAPI (Sigma Chemical Co., St. Louis, MO) was initially used at a concentration of 0.1 μg/g based on published reports (9,10), but we found that 0.2 μg/g was superior for staining large twig sections, and 0.4 μg/g for large root sections. The latter concentration was adopted for routine work with root samples.

Segments (2–6 × 7–12 mm) of roots and twigs were fixed and stored in 2.5% glutaraldehyde in 0.1 M PO_4 buffer at 1 C for up to 6 mo. For sectioning, segments were rinsed in this buffer and mounted on the stage of a freezing microtome. Tangential or oblique longitudinal sections were cut from full-length segments of barely frozen tissue of the innermost secondary phloem of roots or stems at a nominal thickness of 15 μm, flooded with DAPI solution for 30 min, and mounted on microslides in 50% aqueous Karo white corn syrup. One slide bearing four to six sections selected from one segment was prepared per root or twig. Sections were examined with an epifluorescence microscope fitted with an exciter filter that allowed peak transmission at 360 nm (longwave cutoff at 400 nm) and a barrier filter with a shortwave cutoff at 420 nm. Prepared slides could be stored in darkness at room temperature for several days with no change in appearance of the sections.

For a comparison of fresh vs. fixed specimens, one twig was collected in April from each of 20 white ash saplings that had become infected with the AshY agent by bark-patch grafting. One segment from each twig was sectioned, treated with DAPI, and examined without fixation. Another segment from the same twig was fixed and stored in glutaraldehyde solution as described above for several days before being sectioned, treated with DAPI, and examined.

Comparison of DAPI test with Dienes' stain. Twigs were collected from 73 white ash, including 13 apparently healthy seedlings and saplings, 9 trees with witches'-brooms (diagnostic for AshY), and 51 trees that were growing slowly and showing deformities such as curved twigs or deliquescent branching. One or more segments 7–10 mm long, each containing a node, were cut from at least one twig per tree and sectioned as described above. The sections from each segment were divided into two groups, one treated with Dienes' stain and the other with DAPI. At least one slide bearing four to six sections was prepared for each stain treatment per tree, and one person examined all sections. Each tree was scored as positive, negative, or ambiguous for MLO infection based on Dienes' stain reaction and, separately, on DAPI reaction in phloem sieve tubes. Dienes' stain was used as described previously (8) except that sections were cut at a nominal thickness of 15 μm.

Development of sampling technique. White ash ranging from apparently healthy to severely debilitated were selected for testing with DAPI to compare diagnostic usefulness of twig and root samples and to learn whether one sample per tree would be sufficient for an estimate of AshY incidence. The 117 selected trees were located along roadsides, field edges, and stream banks near Ithaca, NY, where AshY is common and severe. Many of these trees had been subjected to addition of fill over roots or erosion of soil around roots. Thus, the slow growth or poor condition of some trees could have been due to injuries, poor site conditions, infectious disease, or combinations of these factors. Trees with witches'-brooms were excluded, because in previous work they always contained MLOs (8). One twig and one root were collected from each tree during June–October. The twig was taken from a low branch showing deliquescent branching, curved twigs, or slow growth, if such was available. Two 1-cm lengths were cut to include woody nodes, and these segments were fixed and stored as described above. Stout twig segments (e.g., 6–8 mm diam) were split or quartered longitudinally before fixation. The first root of the desired diameter (2–6 mm) that could be found by excavating distally along large roots was collected, stored on ice for up to 8 hr, washed, and sampled by removing three segments 8–12 mm long. Sections from one twig segment and one root segment per tree were processed for the DAPI test and scored for MLO infection as described above. A second root or twig segment was used if sections from the first one were unsatisfactory for scoring. In April 1987, the 37 trees for which the root and twig samples were both scored negative or for which data were incomplete because of unsatisfactory root specimens (11 trees) were revisited and sampled as before, and the new samples were processed as above.

Two sets of 15 white ash were selected for multiple sampling of root systems to learn how many roots should be tested with DAPI to obtain a reliable result, either positive or negative, for a single tree. All trees in one set lacked witches'-brooms but were known to have AshY, based on a previous positive DAPI test of one root. All members of the second set were slowly growing trees suspected of having AshY but for which one root each had tested negative with DAPI. Nine additional samples were collected from scattered points on the root system of each tree and processed for the DAPI test as described above. For trees growing where their roots did not mingle with roots of other trees of the same species, sampling was expedited by pulling up small roots 60–100 cm apart in an arc under the branches by means of a stout steel hook. Small roots of ash are distinctive in color and form and thus were not confused with those of other species.

Assessment of symptoms in relation to DAPI reaction. A sample consisting of 227 white ash, growing near Ithaca, was constructed so as to include at least 100 trees with and at least 100 without deliquescent branching. These trees included the 117 studied for development of sampling procedures plus 110 in a woodlot. Each tree was classified in one of four groups, listed in descending order of priority for classification: deliquescent (one or more branches with deliquescent habit, regardless of other symptoms), dieback (displaying dead twigs or branches in the middle to upper crown), slow (apical twigs growing less than 25 cm per year), or normal (branches of normal form and apical twigs growing at least 25 cm per year). The "normal" classification did not include a presumption of freedom from MLO infection, because prior studies had revealed MLOs in some white ash growing more than 20 cm per year (8). One root from each tree was tested with DAPI, and the incidence of positive DAPI reactions was related to symptom classification.

RESULTS AND DISCUSSION

DAPI fluorescence test. Plant nuclei and particles in diseased sieve tubes fluoresced blue-white (Fig. 1), whereas normal sieve tubes were devoid of fluorescence and usually were invisible. The fluorescent particles in sieve tubes, interpreted as MLOs, tended to aggregate at sieve plates and often were so numerous they caused bright fluorescence of one to several entire sieve cells along a sieve tube. Examination of one section per root was often adequate to detect these particles, but all sections on a slide were examined before declaring a negative result. Xylem, phloem fibers, and stone cells in phloem autofluoresced shades of blue, and this fluorescence was somewhat muted in comparison to that of DNA. Substances in the phloem parenchyma and cortex of twigs often autofluoresced white.

Many sections that had fluorescent particles in sieve tubes also contained autofluorescent sieve tubes. This autofluorescence varied from dull white to shades of tan. Often the blue-white fluorescence of the DNA-DAPI complex could be seen within autofluorescent sieve tubes, increasing their luminosity. Autofluorescent sieve tubes were seen in 45% of the 159 specimens that tested positive with DAPI but in only 9% of the specimens that tested negative. Therefore, the autofluorescence was interpreted as a histological symptom of AshY. As ash apparently free from MLO infection may sometimes be found with autofluorescent sieve tubes, however, we consider this symptom to be only

indicative of, not diagnostic for, AshY.

Fixed specimens were superior to fresh ones for the DAPI test, because DNA from nuclei was often smeared extensively in unfixed sections. This smearing sometimes made it difficult to ascertain the original location of fluorescent particles. Nuclei in fixed sections retained their integrity, and cell outlines were clearer than in unfixed sections, which facilitated the identification of cell types.

Comparison of DAPI test with Dienes' stain. The DAPI test gave more conservative and definitive results than did Dienes' stain. Of the 73 ash tested, 38 were declared positive and 20 negative for MLO infection in both tests. All of the remaining 15 ash were negative in the DAPI test, whereas five were declared positive (weak positive reaction) and 10 ambiguous with Dienes' stain. DAPI and Dienes' stain always gave the same result when the Dienes' stain reaction was strong, as in sections from witches'-brooms. Weak Dienes' stain reactions could not be interpreted reliably, because they occurred not only in sections from diseased trees but sometimes in sections from trees known to be free from MLOs. Healthy-appearing trees that were growing rapidly were sometimes scored ambiguous with Dienes' stain but were always scored negative with DAPI. In sections scored ambiguous with Dienes' stain, diffuse light blue pigment often occurred throughout the phloem, dark blue color sometimes appeared in scattered parenchyma cells, and blue particles often occurred in sieve tubes. Immature tissues near meristems often stained light blue, whether or not a strong positive reaction developed in mature sieve tubes. In sections treated with DAPI, fluorescent particles other than the organelles of parenchyma cells were usually seen only in phloem sieve tubes, and ambiguous results were rare.

Sampling scheme. Of the 117 trees from which twig and root specimens were tested with DAPI, 75 (64%) were initially scored positive for MLO infection in sieve tubes of the root or the twig, or both. Thirty-one trees were scored negative, and 11 were undetermined because of unsatisfactory specimens. The 37 trees that were negative or undetermined on first examination were resampled (one twig and one root, as before) during April–May 1987. MLOs were detected in one tree that had initially been scored negative and in five that had been undetermined. The proportion of trees scored as MLO-infected was therefore 69% in the final data set (Table 1). Thus, repeated sampling of trees initially scored negative or undetermined resulted in only a 5% improvement in diagnostic accuracy. Considering only the trees initially scored negative, resampling resulted in only a 1% increase in diagnostic accuracy.

MLOs were detected more than twice as often in root samples as in twigs from the same trees (Table 1). The DAPI test was positive for both twigs and roots of 22 trees, for twigs only of 5 trees, and for roots only of 54 trees. In work not reported here, we noted that the incidence and intensity of DAPI fluorescence in twigs of diseased trees diminished during the winter, although autofluorescence of sieve tubes persisted.

Our findings for MLO detection are in accord with those of Schaper and Seemüller (11,12) and Seemüller et al (13,14) for apple proliferation and pear decline. They used graft transmission as well as the DAPI test to show that MLOs in the aboveground parts of trees degenerated and became increasingly difficult to detect during late winter, whereas MLOs in roots were readily detectable at any time. Apparently the roots of apple, pear, and ash infected with MLOs are the principal site of overwintering of the MLOs, and these parasites spread up into branches and twigs during the growing season, as was suggested for MLOs in elm by Braun and Sinclair (1). In ash, some twigs may be missed during the annual recolonization of the aboveground parts. Incomplete recolonization or low titer of MLOs in twigs could explain the lower incidence of detection of MLOs in twigs than in roots.

Frequency of MLO detection in individual trees in relation to sampling intensity. For the 15 trees known to be infected with MLOs, 9.8 of 10 root samples per tree were positive, on average, when tested with DAPI. No infected tree had fewer than nine roots positive. All of the 15 slowly growing but previously DAPI-negative trees remained negative when 10 root specimens per tree were tested. Within the limits of the DAPI technique, therefore, testing one root per tree would give a false-negative result for the tree as a whole in fewer than 10% of cases. If two roots from a given tree were tested, and the associated probabilities of false results were independent, the tree would erroneously be scored negative in fewer than 1% of cases.

DAPI reaction as related to symptoms. Positive DAPI reactions occurred in the root phloem of 88% of the 105 trees with deliquescent branching, 82% of the 38 trees with dieback but lacking deliquescent branches, and 61% of the 70 slowly growing trees showing neither of the above symptoms. Four of 14 normal-

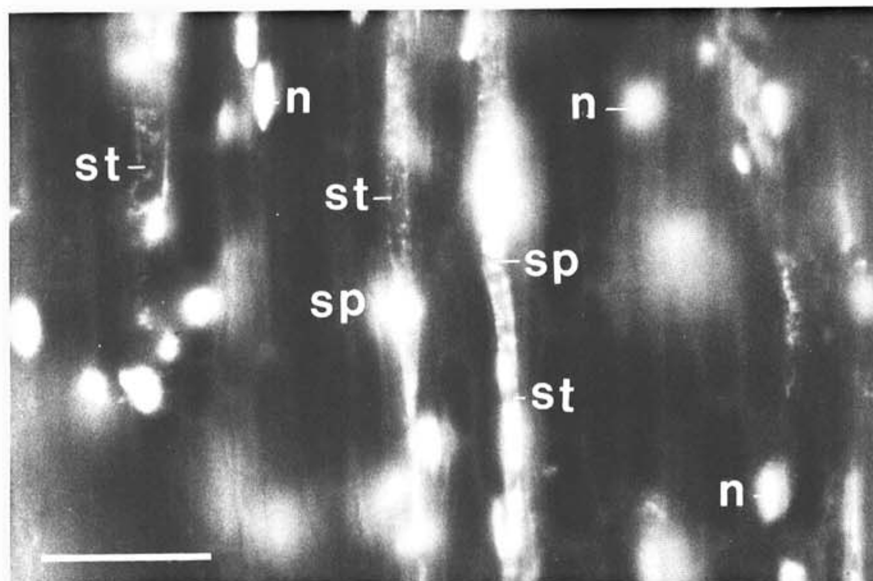

Fig. 1. Radial section of secondary phloem of an MLO-infected ash root about 4 mm in diameter, treated with DAPI and observed with an epifluorescence microscope. Fluorescent particles assumed to be MLOs are visible in sieve tubes (st) and have aggregated at some sieve plates (sp). Nuclei (n) of phloem parenchyma cells appear as elliptical fluorescent spots above and below the plane of focus. Bar = 50 μm.

Table 1. Detection of MLOs in roots and twigs of white ash trees in three health classes by means of the DAPI test

Health class[a]	Total trees	Trees with MLOs detected in: Root	Twig	Root or twig
Normal	9	2	2	3 (33%)
Slow	54	33[b]	9	34 (63%)
Dieback	54	41[b]	18	44 (81%)
All	117	76[b]	29	81 (69%)

[a] Normal = apical twigs growing at least 25 cm per year, no dieback of twigs in middle or upper crown; slow = apical twigs growing less than 25 cm per year, no dieback of twigs in middle or upper crown; dieback = dieback in addition to normal death of shaded branches.

[b] Difference in frequency of MLO detection between roots and twigs significant at $P = 0.01$ by chi-square analysis.

appearing trees were also scored positive. These findings are similar to those of Matteoni and Sinclair (8), who used a combination of direct testing and symptom interpretation to estimate that 57% of the ash they studied on plots in central and southeastern New York were infected with MLOs. MLOs in phloem of normal-appearing white ash may represent strains of low virulence, early stages of colonization by strains of normal virulence, or strains infecting tolerant trees.

Contrary to the suggestion of Matteoni and Sinclair (8), deliquescent branching is not definitively diagnostic for ash yellows, because 12% of the trees in the deliquescent category tested negative for MLO infection. As shown earlier in this paper, it is unlikely that more than one of the deliquescent trees would have tested positive if resampled. In survey work to be reported elsewhere, we have noted deliquescent branching, dieback, and slow growth in ash on various sites where no witches'-brooms or histological evidence of MLO infection was found. Apparently, deliquescent branching may be induced by various factors that damage apical buds or suppress growth for several years.

Recommendations for sampling for diagnosis of AshY. When the DAPI test is used to detect MLO infection in ash, a given tree may be accurately assessed (<1% chance of false-negative result) by collecting and examining two small roots from different parts of the root system. If the frequency of infection in an array of trees is to be assessed to less than 5% error, only one small root per tree need be examined.

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