Relatedness of Mycoplasmalike Organisms Associated with Ash Yellows and Lilac Witches'-Broom

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ABSTRACT

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The mycoplasmal diseases ash yellows (AshY) and lilac witches'-broom (LWB) were diagnosed on the basis of symptoms and the DAPI (4',6-diamidino-2-phenylindole·2HCl) fluorescence test in 12 taxa of *Fraxinus* and 35 taxa of *Syringa*, respectively, at the Arnold Arboretum, Jamaica Plain, MA. AshY and LWB also occurred together in other arboreta in the United States and Canada. Ash mycoplasmalike organisms (MLOs) were transmitted to lilacs, and lilac MLOs were transmitted to ash by dodder (*Cuscuta* spp.). Ash and lilac MLOs caused identical symptoms in ash, lilac, periwinkle (*Catharanthus roseus*), and carrot (*Daucus carota*). DNA from diseased lilac or ash from diverse locations and from periwinkle infected with AshY or LWB MLOs hybridized with a biotin-labeled cloned DNA probe that detects AshY MLOs specifically. DNA from healthy ash, lilac, or periwinkle or from periwinkle infected with two other plant MLOs did not hybridize with the AshY-specific probes. Thus, the lilac and ash MLOs are similar and possibly identical.

Mycoplasmalike organisms (MLOs) cause ash yellows (AshY) in several taxa of *Fraxinus* (7,10,14,18) and lilac witches'-broom (LWB) in several taxa of *Syringa* (8,9). During a study of LWB at the Arnold Arboretum, Jamaica Plain, MA, symptoms of AshY (14,17,18) were observed in the ash collection, which is intermixed with and adjacent to the lilac collection. The coincidence of mycoplasmal diseases in adjoining and related hosts (*Fraxinus* and *Syringa* in family Oleaceae) indicated a possible relationship between the causal MLOs. The recent development of cloned DNA probes specific for the detection and identification of AshY MLOs (4) provided a direct means of assessing the relatedness of the MLOs associated with ash and lilacs.

This report presents evidence for the coincidence of mycoplasmal diseases in ash and lilac, the transmissibility of MLOs between ash and lilac, and the relatedness of ash and lilac MLOs as determined by the use of cloned DNA probes. Part of the findings were reported previously (7).

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MATERIALS AND METHODS

MLO infection in ash and lilacs. The ash collection at the Arnold Arboretum consists of 128 specimens within 58 taxa of *Fraxinus*. Most of the ash are located on a 0.9-ha site adjacent to the lilac collection. The 1.7-ha lilac collection at the time of the study consisted of about 500 specimens within 358 taxa of *Syringa*. Some ash and lilac specimens are intermixed and are only 4–5 m apart.

The ash and lilacs were examined for symptoms of AshY and LWB, respectively. The symptoms of both diseases have been described (8,9,14,17,18). Shoot and root samples were harvested and examined for the presence of MLOs in the phloem by the DAPI (4',6-diamidino-2-phenylindole·2HCl) fluorescence test. The sampling, sectioning, and DAPI staining procedures have been described (8,17).

Transmission of MLOs. Attempts were made to transmit AshY MLOs from Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) to healthy lilacs and LWB MLOs from lilacs or periwinkles to healthy ash by means of dodder (*Cuscuta subinclusa* Dur. & Hilg.). The transmission of ash MLOs by dodder has been reported (10). Periwinkle cultivars Little Pinkie or Twinkles, infected with an ash yellows MLO strain designated AshY1, were propagated by cuttings. This strain has been used in other studies (4,13). Cuttings and potted specimens of MLO-infected *S.* × *josiflexa* Preston ex Pringle 'Royalty' and *S.* × *prestoniae* McKelv. 'Isabella' were obtained from T. Cole, Dominion Arboretum, Ottawa, Canada. The cuttings were rooted to maintain a supply of diseased lilacs. Attempts were also made to transmit AshY and LWB MLOs from periwinkle to healthy carrot (*Daucus carota* L.).

The dodder was raised from seed and grown on healthy periwinkles. Detached tips (2–4 cm long) of dodder strands were entwined around young shoots of MLO-infected periwinkle, ash, or lilac (donor plants) and allowed to parasitize the donor plants for 4–6 wk. The tips of most new dodder shoots were pinched out to curtail proliferation of the strands.

For each donor plant parasitized by dodder, the tips of one to four strands of dodder were entwined around young shoots of healthy periwinkle, carrot, ash, or lilac (recipient plants). All recipient plants were raised from seed. Seeds for the lilac recipient plants were collected from specimens known to be susceptible to LWB including: *S.* × *henryi* Schneid. 'Lutece', *S.* × *josiflexa* 'Royalty', *S.* × *prestoniae* 'Maybelle Farnum', and *S. villosa* Vahl (8). Although lilac MLOs are not seed-transmissible (8), selected recipient plants were tested with DAPI to verify the absence of MLOs before the transmission attempts.

The dodder was connected between donor and recipient plants for 4–6 wk, after which all dodder was removed from the recipient plants. Healthy donor plants parasitized by dodder and healthy recipient plants parasitized by dodder originating from healthy donor plants served as controls. All test plants were maintained in a screened greenhouse. The ash and lilacs received a dormant period of 2–3 mo at 4 C.

DNA hybridizations. Samples of ash, lilac, and periwinkle, healthy or infected as determined by the DAPI test, were

collected for nucleic acid extraction. Trunk samples were cut from the following: *F. americana* L. and *S.* × *prestoniae* 'Hiawatha' at Ithaca, NY; *F. americana* at Verbank, NY; and *F. bungeana* DC. and lilacs *S.* × *henryi* 'Lutece', *S.* × *josiflexa* 'Royalty', and *S.* × *prestoniae* 'Dorcas' and 'Maybelle Farnum' at the Arnold Arboretum. Ash infected with MLOs transmitted by dodder from lilacs (see Results) were also sampled. Healthy *F. americana* and *S. vulgaris* L. served as ash and lilac controls, respectively, because samples of healthy *F. bungeana* or lilacs Hiawatha, Lutece, Dorcas, Maybelle Farnum, and Royalty were not available.

Periwinkles containing MLO strain AshY1 and strain EY1 of the elm yellows pathogen were available from previous work (1,13). Periwinkles infected with lilac MLOs were obtained by dodder transmission from rooted cuttings of LWB-infected Royalty, Isabella, and Hiawatha. Periwinkle containing a Canadian strain (CX) of MLOs associated with X-disease of *Prunus* (19) was provided by L. N. Chiykowski, Agriculture Canada, Ottawa. Healthy Twinkles periwinkle served as a control.

The MLO strains AshY1, EY1, and CX served as standards. Strain AshY1 is normally detected by the AshY-specific probe AA13I and the nonspecific probe pBB115. Strain CX is normally detected only by the nonspecific probe, and strain EY1 is not detected by either probe.

Nucleic acid was extracted from 0.3-g plant samples by a phenol-chloroform procedure (11). Denatured nucleic acid in 3-μl volumes of buffer (6× SSC, composed of 0.9 M aqueous NaCl and 0.09M Na citrate) were spotted on nitrocellulose membranes. The membranes were baked for 2 hr at 80 C and stored in a desiccator until dot hybridizations were performed with biotin-labeled probes (2,3,11). For samples that had given weak hybridization signals in preliminary tests, nucleic acid was extracted from duplicate 0.3-g samples and combined before spotting on the membranes. Four replicate membranes were prepared for the set of samples in each of two experiments.

Dot hybridizations were performed at 42 C with probes AA13I and pBB115. Probe AA13I is a 950-bp fragment of chromosomal DNA from strain AshY1 and was developed for specific identification of ash yellows MLOs (4). This probe has hybridized with nucleic acid from ash trees, periwinkle, and red clover (*Trifolium pratense* L.) containing AshY MLOs (4) (W. Sinclair, *unpublished*) but not with nucleic acid from periwinkle infected with any of 10 other MLOs or with *Spiroplasma citri* Saglio et al (4).

Probe pBB115 consists of plasmid pSP64 containing a 3.8-kbp fragment of chromosomal DNA from a MLO associated with tomato big bud. This probe hybridizes with the DNA of various MLOs, including those associated with AshY, aster yellows, clover phyllody, maize bushy stunt, potato witches'-broom, periwinkle littleleaf, beet leafhopper-transmitted virescence of periwinkle, and X-disease of *Prunus* in California and Ontario (CX) (12).

RESULTS

MLO infection in ash and lilacs. Ash or lilacs were rated as MLO-infected when specimens displayed symptoms of AshY (clumped foliage on shoots with short internodes, basal epicormic sprouts, and premature shoot growth from axillary buds) or LWB (witches'-brooms, deliquescent branching, and epicormic sprouts) respectively, and infection could be verified by a positive DAPI test. Some specimens without symptoms were rated as infected on the basis of the DAPI test alone. DAPI tests were interpreted as positive when they revealed sieve tubes with one or more of the following: blue-white fluorescent particles, white autofluorescence (5), or bright blue-white fluorescence of the DNA-DAPI complex (16) accompanying white autofluorescence. The reliability of DAPI for detecting MLOs in lilacs has been confirmed by transmission electron microscopy (8).

Ash yellows was identified in 13 ash specimens, including 12 taxa. AshY had previously been reported in two of the ash taxa—*F. americana* and *F. pennsylvanica* Marsh. (14).

Ten of the ash taxa are newly recognized hosts of MLOs: *F. americana* var. *americana* L. 'Ascidiata', *F. americana* × *quadrangulata* Michx., *F. angustifolia* Vahl. 'Monophylla', *F. bungeana*, *F. excelsior* L.'Aurea' and 'Diversifolia Pendula', *F. latifolia* Benth. (*F. oregona* Nutt. var. *latifolia* (Benth.) Lingelsh.), *F. ornus* L. (*F. ornus* var. *juglandifolia* Ten.), *F. potamophila* Harder, and *F. tomentosa* Michx. f. (*F. profunda* Bush).

Three MLO-infected ash were asymptomatic: *F. americana* × *quadrangulata*, *F. angustifolia* 'Monophylla', and *F. potamophila*. AshY was also identified in two native white ash located in an untended site adjacent to the ash collection.

MLOs were detected in 46 lilac specimens, including 35 taxa. Lilac hosts are: *S.* × *diversifolia* Rehd.; *S.* × *henryi* 'Lutece'; *S.* × *josiflexa* 'Guinevere', 'Nellie Bean', and 'Royalty'; *S. josikaea* Jacq. f. ex Reichenb.; *S. komarowii* Schneid.; *S. microphylla* Diels. 'Superba'; *S.* × *nanceiana* McKelv. 'Rutilant'; *S.* × *prestoniae* 'Alexanders Aristocrat', 'Charmian', 'Dawn', 'Dorcas', 'Isabella', 'Maybelle Farnum', 'Miranda', 'Olivia', 'Portia', 'Romeo', and 'Ursula'; *S. tomentella* Bur. & Franch. 'Rosea'; *S. villosa*; and *S. vulgaris* 'Bleuatre', 'Capitaine Perrault', 'Carmine', 'Charles Joly', 'Gaudichaud', 'Grand Duc Constantin', 'Le Gaulois', 'Mlle. Fernande Viger', 'Mme. Florent Stepman', 'Nana', 'Paul Hariot', 'Stadtgartner Rothpletz', and 'Verschaffelti'. Eighteen of the infected lilacs, including all of the *S. vulgaris* cultivars, did not show LWB symptoms.

The juxtaposition of MLO-infected ash and lilacs in the Arnold Arboretum was clear. Most of the infected ash and lilacs were within 200 m of each other. Four infected lilacs (*S.* × *josiflexa* 'Guinevere', *S.* × *prestoniae* 'Romeo', *S. villosa*, and an unidentified specimen) surrounded (3–7 m away) an infected *F. ornus*. Two infected *S. vulgaris* (unnamed) were 2–3 m from a recently cut white ash stump bearing MLO-infected sprouts and brooms.

The association of MLO-infected ash and lilacs has been observed in other arboreta. LWB was identified in lilac collections at Highland Park, Rochester, NY, the Royal Botanical Garden, Hamilton, Ontario (8), and Agriculture Canada's Central Experimental Farm at Ottawa. At all three sites, AshY was identified (by symptoms and DAPI tests) in native white ash growing within 100 m of the lilacs.

Transmission of MLOs. MLOs were transmitted by dodder from ash to lilac and from lilac to ash. It took 1–2 yr for symptoms to develop in recipient lilacs and ash. Ash MLOs were transmitted from periwinkle donors to lilac recipients in four of 16 attempts. Diseased recipient lilacs displayed premature growth from buds, reduced shoot growth, and twig dieback; DAPI tests revealed abundant MLOs in sieve tubes in shoots and roots. Lilac MLOs were transmitted from lilac donors to ash recipients in five of 10 attempts and from lilac donors to periwinkles, then from periwinkles to ash recipients in two of four attempts. Symptoms in diseased recipient ash consisted of reduced apical growth, premature growth from vegetative buds, simple or trifoliolate leaves, upright-angled branching, and a delay in the formation of terminal buds. DAPI tests of symptomatic ash and periwinkles revealed abundant MLOs in petioles, stems, and roots. No symptoms developed and no MLOs were detected in the control plants of ash, lilac, or periwinkle.

Symptoms caused by ash and lilac MLOs were the same in periwinkle (stunted growth, chlorotic and undersized leaves, proliferation of axillary shoots, and cessation of flowering) and in carrot (proliferation of stunted leaves arising from the crown).

DNA hybridizations. The nonspecific probe pBB115 detected MLO DNA in all ash, lilac, and periwinkle samples, except the healthy controls, Maybelle Farnum lilac, and the periwinkle containing elm yellows MLO (Table 1). AshY-specific probe AA13I detected MLO DNA in the following: periwinkle infected with the homologous MLO (AshY1), periwinkles infected with MLOs transmitted from lilacs Hiawatha

and Royalty, all ash samples in which MLOs had been detected by DAPI and by probe pBB115, and the MLO-infected lilacs Hiawatha, Dorcas, and Lutece. Probe AA13I did not produce a hybridization signal with nucleic acid from the following: plants with MLO strains CX or EY, Maybelle Farnum or Royalty lilac (from Arnold Arboretum) with LWB, or healthy controls (Table 1).

DISCUSSION

Mycoplasmal infection is widespread in lilacs, as LWB has been detected in 93 taxa among 19 species and hybrids of *Syringa* in nine major lilac collections in the United States and Canada (8). One likely reason for this occurrence is the transmission of MLOs during plant propagation. There is circumstantial evidence that LWB has been spread among lilac collections by the exchange of diseased plant material (6). Approximately 90% of the infected lilacs listed above originated as plants, grafts, or cuttings from sources outside the Arnold Arboretum.

Six of the infected ash (average of 37 yr old) and five of the infected lilacs (average of 61 yr old) originated from seed planted at the Arnold Arboretum. These specimens presumably became infected by MLOs transmitted by insects from ash, lilacs, or other hosts. Insect vectors of AshY are unknown (15) (W. Sinclair, *unpublished*).

One interpretation of the coincidental occurrence of AshY and LWB at several arboreta is that identical or related strains of MLOs are causing both AshY and LWB. The dodder transmissions support this interpretation, as ash and lilac MLOs were reciprocally transferred between the two hosts and caused identical symptoms in ash, lilac, periwinkle, and carrot.

The hybridization of probe AA13I with nucleic acid from MLO-infected ash and lilacs and from periwinkles containing MLOs associated with AshY and LWB confirmed that these MLOs are closely related or identical. Probe AA13I had previously hybridized only with nucleic acids of AshY MLOs (4). MLOs closely related to MLO strain AshY1 of ash yellows may be widespread in the northeastern U.S. and adjacent southeastern Canada because they were detected in samples from central and eastern New York, eastern Massachusetts, and Ottawa, Ontario.

In accord with its known specificity, probe AA13I did not hybridize with nucleic acid from plants containing MLOs of CX or EY, whereas the nonspecific probe pBB115 did hybridize with nucleic acid from diseased control plants containing MLO strain CX. These plants and the nonspecific probe were included to show that the DNA of different MLOs could be detected and distinguished under conditions of the experiments. Neither probe hybridized with nucleic acid from periwinkle infected with elm yellows MLOs, which was consistent with previous results and with the interpretation that elm yellows and AshY MLOs are not closely related (2,4).

The failure of probe AA13I to hybridize with nucleic acid from the Royalty and Maybelle Farnum lilacs from the Arnold Arboretum was probably attributable to the low titer of DNA in the samples rather than to an unrelatedness to MLOs of AshY. Probe pBB115, which normally gives a stronger signal than does the AshY-specific probe when hybridized with duplicate nucleic acid samples, gave only a weak hybridization signal with the Royalty sample and none with the sample of Maybelle Farnum. Low numbers of MLOs were detected in Royalty and Maybelle Farnum shoot samples by DAPI tests. Dot hybridizations with DNA probes have often failed to reveal MLO DNA in ash specimens in which MLOs were detected in apparent low numbers by DAPI tests (W. Sinclair and H. Griffiths, *unpublished*).

Table 1. Detection of DNA of mycoplasmalike organisms (MLOs) by ash yellows-specific and nonspecific hybridization probes and by DAPI[a]

Plant sample	MLO strain and original host	Locality of origin	MLO detection[b]		
			Probe		
			AA13I	pBB115	DAPI
Plants with unidentified MLOs					
Catharanthus roseus	Unknown, lilac	Ottawa, Ontario	+	+	+
	Unknown, lilac	Ithaca, NY	+	+	+
Fraxinus americana	Unknown	Ithaca, NY	+	+	+
	Unknown	Verbank, NY	+	+	+
	Unknown, lilac	Ottawa, Ontario	W	+	+
F. bungeana	Unknown	Jamaica Plain, MA	+	+	+
Syringa × *henryi* 'Lutece'	Unknown	Jamaica Plain, MA	+	+	+
S. × *josiflexa* 'Royalty'	Unknown	Jamaica Plain, MA	−	W	+
S. × *prestoniae* 'Dorcas'	Unknown	Jamaica Plain, MA	+	+	+
'Maybelle Farnum'	Unknown	Jamaica Plain, MA	−	−	+
'Hiawatha'	Unknown	Ithaca, NY	+	+	+
Plants with known MLOs					
C. roseus 'Twinkles'	AshY1, white ash[c]	Ithaca, NY	+	+	+
	CX, chokecherry[d]	Harrow, Ontario	−	+	+
	EY1, elm[d]	Ithaca, NY	−	−	+
Healthy controls					
C. roseus 'Twinkles'			−	−	−
F. americana			−	−	−
S. vulgaris[e]			−	−	−

[a] Probe AA13I hybridizes specifically with ash yellows MLOs. Probe pBB115 hybridizes nonspecifically with many MLOs but not with those causing elm yellows (12). DAPI is the DNA-binding fluorochrome 4′,6-diamidino-2-phenylindole·2HCl (16).

[b] + = Hybridization signal or positive result of DAPI test; W = weak hybridization signal; and − = no hybridization signal or negative result of DAPI test.

[c] Periwinkle plants with this MLO strain were the source of DNA probe AA13I and are used as a standard in dot-hybridization tests for specific detection of ash yellows MLOs.

[d] Periwinkle plants with MLO strains CX and EY are used as positive and negative diseased standards, respectively, in dot hybridizations using probe pBB115 for nonspecific detection of MLOs.

[e] Healthy *Fraxinus americana* and *Syringa vulgaris* were used as controls for all samples of diseased ash and lilac, respectively, because healthy material of *F. bungeana* and the hybrid lilacs was not available.

Because of the widespread and coincidental mycoplasmal infection of ash and lilacs and the transmissibility of MLOs between these two hosts, and because ash and lilac MLOs from diverse locations were detected by an AshY-specific probe in DNA hybridization tests, we conclude that the MLOs of AshY and LWB are closely related and perhaps identical.

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LITERATURE CITED

1. Braun, E. J., and Sinclair, W. A. 1979. Phloem necrosis of elms: Symptoms and histopathological observations in tolerant hosts. Phytopathology 69:354-358.
2. Davis, R. E., Lee, I.-M., Douglas, S. M., Dally, E. L., and Dewitt, N. 1989. Cloned nucleic acid hybridization probes in detection and classification of mycoplasmalike organisms (MLOs). Acta Hortic. 234:115-122.
3. Davis, R. E., Lee, I.-M., Douglas, S. M., Dally, E. L., and Dewitt, N. 1990. Development and use of cloned nucleic acid hybridization probes for disease diagnosis and detection of sequence homologies among uncultured mycoplasmalike organisms (MLOs). Zentralbl. Bakteriol. Suppl. 20:303-307.
4. Davis, R. E., Sinclair, W. A., Lee, I.-M., and Dally, E. L. 1990. Specific diagnosis of ash yellows by means of biotinylated cloned DNA probes. (Abstr.) Phytopathology 80:990.
5. Dyer, A. T., and Sinclair, W. A. 1991. Root necrosis and histological changes in surviving roots of white ash infected with mycoplasmalike organisms. Plant Dis. 75:814-819.
6. Hibben, C. R. 1987. Note on spread of lilac witches-broom disease. Lilac Newsl. 13(2):5.
7. Hibben, C. R., and Franzen, L. M. 1987. Coincidence of lilac witches'-broom and ash yellows in two arboreta. (Abstr.) Phytopathology 77:118.
8. Hibben, C. R., and Franzen, L. M. 1989. Susceptibility of lilacs to mycoplasmalike organisms. J. Environ. Hortic. 7:163-167.
9. Hibben, C. R., Lewis, C. A., and Castello, J. A. 1986. Mycoplasmalike organisms, cause of lilac witches'-broom. Plant Dis. 70:342-345.
10. Hibben, C. R., and Wolanski, B. 1971. Dodder transmission of a mycoplasma from ash witches'-broom. Phytopathology 61:151-156.
11. Lee, I.-M., and Davis, R. E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1:303-310.
12. Lee, I.-M., Davis, R. E., and Dewitt, N. D. 1990. Non-radioactive screening method for isolation of disease specific probes to diagnose plant diseases caused by mycoplasmalike organisms. Appl. Environ. Microbiol. 56:1471-1475.
13. Matteoni, J. A., and Sinclair, W. A. 1983. Stomatal closure in plants infected with mycoplasmalike organisms. Phytopathology 73:398-402.
14. Matteoni, J. A., and Sinclair, W. A. 1985. Role of the mycoplasmal disease, ash yellows, in decline of white ash in New York State. Phytopathology 75:355-360.
15. Matteoni, J. A., and Sinclair, W. A. 1988. Elm yellows and ash yellows. Pages 19-31 in: Tree Mycoplasma Diseases and Epidemiology. C. Hiruki, ed. University of Alberta Press, Edmonton.
16. Russell, W. C., Newman, C., and Williamson, D. H. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature 253:461-462.
17. Sinclair, W. A., Iuli, R. J., Dyer, A. T., and Larsen, A. O. 1989. Sampling and histological procedures for diagnosis of ash yellows. Plant Dis. 73:432-435.
18. Sinclair, W. A., Iuli, R. J., Dyer, A. T., Marshall, P. T., Matteoni, J. A., Hibben, C. R., Stanosz, G. R., and Burns, B. S. 1990. Ash yellows: Geographic range and association with decline of white ash. Plant Dis. 74:604-607.
19. Sinha, R. C., and Chiykowski, L. N. 1980. Transmission and morphological features of mycoplasmalike bodies associated with peach X-disease. Can. J. Plant Pathol. 2:119-124.