Responses of Six Eurasian *Ulmus* Cultivars to a North American Elm Yellows Phytoplasma

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ABSTRACT

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Elms (genus *Ulmus*) of six clonal cultivars representing Eurasian species and hybrids were grafted when 2 to 3 years old with bark patches from *U. rubra* infected with an elm yellows phytoplasma or were left untreated as controls. The cultivars were *U. glabra* × *minor* 'Pioneer', *U. minor* × *parvifolia* 'Frontier', *U. parvifolia* 'Pathfinder', *U. wilsoniana* 'Prospector', and the complex hybrids 'Homestead' and 'Patriot'. Trees were evaluated for infection and symptoms 1 or 2 years after inoculation. Infection was detected via the 4',6-diamidino-2-phenylindole-2HCl (DAPI) fluorescence test in 26 of 86 grafted trees representing five cultivars. Infection of selected trees was confirmed by polymerase chain reaction (PCR) amplification of a fragment of phytoplasmal rDNA, and the phytoplasma was identified by restriction fragment length polymorphism (RFLP) analysis of the amplified DNA using restriction enzymes *AluI*, *RsaI*, and *TaqI*. Elm yellows phytoplasma was also identified by nested PCR and RFLP analysis in two of seven inoculated, healthy-appearing, DAPI-negative trees and one noninoculated control tree. All RFLP profiles were identical to that of reference strain EY1. Phytoplasma-associated symptoms, observed in five cultivars, included suppressed growth, progressive size reduction of apical shoots and leaves, chlorosis, foliar reddening, witches'-brooms, and dieback. Phytoplasma was not detected in cv. Homestead. Possible resistance of this cultivar to elm yellows phytoplasma was indicated by localized phloem necrosis in stems below inoculum patches.

Additional keywords: Dutch elm disease, *Ophiostoma novo-ulmi*, resistance

The phytoplasmal disease elm yellows occurs widely in indigenous *Ulmus* (elm) species in the eastern half of the United States, causing phloem and rootlet necrosis, foliar yellowing, and tree death that usually occurs within 1 to 2 years after onset of foliar symptoms (18,24,25). The disease is notorious for epidemics that eliminate all indigenous elms larger than seedlings in the affected localities (18,19). Elms of Eurasian species typically remain apparently unaffected (19). In Italy and

France, elm yellows affects numerous Eurasian elm species and cultivars (13,14). The elm yellows syndrome in Europe varies from slow growth and formation of witches'-brooms to foliar reddening or yellowing and decline leading to death (5,8,12-14). Only one documented record exists of natural infection of Eurasian elms in North America; elm yellows phytoplasma was identified (9) in declining *U. parvifolia* with witches'-brooms in New York State.

The disparity between European and American experience with elm yellows may have several bases. (i) Strains of the pathogen in North America may differ in virulence from those in Europe. Differences in nonribosomal DNA have been detected between American and European elm yellows phytoplasma strains (6,9); therefore, phenotypic differences may also occur. (ii) Eurasian elms that have survived in elm yellows outbreak areas may be resistant or tolerant. Resistance, as here considered, refers to a host's ability to limit colonization by phytoplasma or to suppress its reproduction. Tolerance refers to the

relative ability of a plant to continue growing while systemically colonized by phytoplasma. (iii) Vector species in Europe and America may differ and may have different elm host preferences. Only one vector species has been identified in the United States (2), and no information is available about vectors in Europe. (iv) Climatic differences may condition differences in elm yellows symptom expression between Europe and America.

Numerous elm cultivars selected or bred for resistance to the Dutch elm disease pathogen, *Ophiostoma novo-ulmi*, have been released in Europe and North America (7,21,22,26,30). Reactions of these cultivars to the elm yellows pathogen are unknown. If they are resistant to, or tolerant of, infection by the elm yellows pathogen, they may be suitable for deployment in areas where elm yellows is endemic. On the other hand, if any of these elm types is found to be susceptible to severe damage, then tree planters should consider alternative options.

Several Eurasian elm cultivars with Dutch elm disease resistance have been released from the breeding program of the U.S. National Arboretum (26-32). Our research objective was to characterize responses of six of these clones to inoculation with elm yellows phytoplasma.

MATERIALS AND METHODS

Elm materials and experimental design. The cultivars evaluated were *U. glabra* × *minor* 'Pioneer', *U. minor* × *parvifolia* 'Frontier', *U. parvifolia* 'Pathfinder', *U. wilsoniana* 'Prospector', and the complex hybrid clones 'Homestead' and 'Patriot'. Homestead is five-eighths *U. pumila* and three-eighths *U. minor* (28). Patriot is one-half *U. wilsoniana* and one-fourth each *U. minor* and *U. pumila* (16,27). The tests were performed at Ithaca, NY, where elm yellows is common in native elms. Trees utilized in the tests were propagated by rooting softwood cuttings either at the U.S. National Arboretum or in commercial nurseries. The plants were 1 to 2 years in age and 0.2 to 2 m in height when acquired. Therefore, comparisons between inoculated, diseased

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plants and noninoculated controls were made within cultivars, and symptoms were compared between cultivars, but growth comparisons between cultivars were not made. Each cultivar was initially (1997) represented by 30 to 32 trees. Additional groups of Frontier (20 trees) and Prospector (18 trees) were used beginning in 1998, because the first group of Frontier was severely damaged by voles and the first group of Prospector initially had such small stems that successful grafting was in doubt. Plants within each group were similar in size and age. All trees were grown in greenhouses until May 1998, when they were transplanted into a field plot at 0.7-by-1 m spacing.

Phytoplasma inoculum and inoculation. In July 1997 and again in July 1998, one *U. rubra* (red elm) growing at Ithaca and displaying foliar symptoms typical of elm yellows (18) was selected as a source of inoculum. Low branches similar in diameter to the stems of trees to be tested were collected and checked for discoloration of the cambial surface of the bark. Branches with light yellow discoloration were cut into segments 30 to 40 cm long. The proximal ends of segments were placed in a bucket of water. Segments were used immediately or after storage for 1 to 2 days at 4°C. A total of 15 or 16 trees per cultivar were inoculated in 1997 when their stems were 8 to 15 mm in diameter by grafting two red elm bark patches, 20 to 35 by 5 to 8 mm in area (patch size varying with stem diameter), into the main stem and wrapping with grafting tape. Equal numbers of control trees per cultivar were left untreated. A further 13 Frontier and 12 Prospector trees were inoculated a year later, and the respective control groups were augmented with 7 and 6 trees, respectively. Identity of the elm yellows pathogen in inoculum material was verified by polymerase chain reaction-restriction fragment length polymorphism (PCR-

RFLP) analyses of DNA samples from trees that responded to inoculation, as described below.

Observations, measurements, and analyses. Grafts were examined a year after inoculation. Tree mortality was recorded annually for 3 years after inoculation. Trees that died from transplanting shock or associated freeze damage or that sustained severe vole damage within the first year were deleted from the trial, leaving a total of 86 inoculated trees and 76 untreated controls (Table 1). Other symptoms were recorded in late summer for 2 years, beginning a year after the first inoculation. Symptoms associated with phytoplasmal infection were identified and verified as follows. First, each plant was classified in one of four categories: H = apparently healthy; Q = stunted or abnormal in form or foliar markings, but lacking symptoms like those of known phytoplasmal diseases; EYQ = expressing abnormalities such as premature foliar yellowing or reddening, short apical shoots, or general stunting, indicative of, but not diagnostic for phytoplasmal infection; and EY = expressing symptoms typical of elm yellows (4,5), such as witches'-brooms, attenuation of apical shoots, or phloem necrosis. Apical attenuation is progressive reduction in internode length and leaf size on a shoot (Fig. 1C). To assess inner phloem for discoloration or necrosis, a lenticular sample of bark (approximately 30 by 5 mm) was removed from the stem below a bark patch on each tree in categories EY and EYQ and from all grafted nonsymptomatic trees of Prospector and Homestead. The cambial face of the sample was examined for discoloration and methyl salicylate (wintergreen) odor, which are symptoms of elm yellows in elms native to North America. Tree height and the longest current-season shoot on each tree were measured each year. Lengths of longest current-season shoots

on diseased (positive DAPI test, below) and control trees were compared within cultivars by one-tail *t* tests (23).

In September a year after inoculation, every plant in categories Q, EYQ, and EY was assayed by the 4',6-diamidino-2-phenylindole-2HCl (DAPI) fluorescence test to detect DNA in phloem sieve tubes (17,20). Four or more arbitrarily selected trees in category H per cultivar group were also tested. Longitudinal sections of root segments, twig segments, or both, 6 to 8 by 1 to 3 mm in size, were examined. Twigs were selected for elm yellows-like symptoms, if such were present. Test results from roots and twigs of a subset of sampled trees were found to be similar (*data not shown*). Therefore, the test was applied only to twigs of most trees.

PCR and RFLP analyses were used to detect and identify phytoplasmal rDNA in extracts from 15 DAPI-tested trees representing four categories: DAPI-positive with elm yellows symptoms; DAPI-negative, apparently healthy (H); DAPI-negative with symptoms scored as Q; and DAPI-negative with symptoms scored as EYQ. Leaves sufficient to yield at least 2 g of midribs for DNA extraction were collected from each tree in September 1999. Midribs were excised, weighed, ground in liquid nitrogen, and stored at -80°C. DNA was extracted using the DNeasy Plant Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, with DNA elution performed using 1 ml of Buffer AE. DNA was concentrated using a standard procedure with sodium acetate and ethanol (1). PCRs and visualization of amplified products were performed using standard procedures (1,10,11), beginning with 1 µl of undiluted sample or 1 µl of 1:5 dilution as template for phytoplasma-universal primer pair P1/P7 (15). To obtain sufficient amplified DNA for digestion with restriction enzymes, 1

Table 1. Incidence of various symptoms in six elm cultivars as related to elm yellows phytoplasma infection^a

Cultivar	Total trees ^c		Number of trees with symptom ^b													
			WB		Attenu ^d		Stunting		Yellowing		Reddening		PN		Dead ^e	
	Inf/inoc	Cont	Inf	Non	Inf	Non	Inf	Non	Inf	Non	Inf	Non	Inf	Non	Inf	Non
Frontier	4/16	10	...	...	4	...	...	...	3	...	4	...	...	...	...	...
Homestead	0/14	11	...	...	...	...	...	3	...	1	...	1	...	...	...	...
Pathfinder	3/11	6	3	...	1	...	1	3	...	...	3	1	...	...	1	5
Patriot	6/9	11	4	...	...	...	3	1	3	...	...	...	...	...	3	...
Pioneer	13 ^g /16	15 ^g	1	...	3	...	9	1	7	...	5	...	...	...	12	2
Prospector	2 ^g /20	23	...	...	...	...	1	2	1	...	...	...	1	...	1	...
Totals	28/86	76	8	0	8	0	13	10	14	1	12	2	1	0	17	7

^a Data represent trees that survived the first year after planting in a field plot. Infection was detected by means of the 4',6-diamidino-2-phenylindole-2HCl fluorescence test, polymerase chain reaction (PCR), or both. Noninfected inoculated trees and nongrafted controls are grouped as noninfected (Non).

^b Symptoms: WB = witches'-broom, Attenu = apical attenuation, Stunting = general stunting, Yellowing = foliar yellowing, Reddening = foliar reddening, PN = phloem necrosis.

^c Inf = infected. Inoc = inoculated. Cont = noninoculated controls.

^d Attenu = attenuation: progressive reduction in internode length and leaf size on shoots.

^e Death in the second and third years after inoculation.

^f Frontier normally produces red pigment in young leaves and has red foliage in autumn; phytoplasma-infected plants had more prominent red color in leaves on apical shoots in summer and displayed red foliage earlier in autumn than did noninfected plants.

^g Includes one asymptomatic tree in which phytoplasmal DNA was detected only by PCR.

µl of each undiluted P1/P7 amplicon (1.8 kb) was used as template with primer pair R16F2/R2 (11) in nested PCR. The products obtained from the nested PCRs (1.24 kb) were digested separately with restriction enzymes *AluI*, *RsaI*, and *TaqI*

and analyzed using published methods (10). Positive and negative controls were used in each assay. The positive control was DNA from a periwinkle plant infected with phytoplasma reference strain EY1 (4,9).

RESULTS

Graft unions and phytoplasma infection. Nearly all of the living inoculated trees had at least one living bark patch a year after inoculation, indicating compatibility of the red elm patch donors with the cultivars. Positive DAPI reaction in phloem sieve tubes was found in 26 of 86 inoculated trees that survived more than a year after outplanting (Table 1). Every DAPI-positive tree had symptoms in category EY or EYQ, and only one tree in these categories was scored negative in the DAPI test. None of 8 inoculated trees in category Q or 28 in category H were scored positive in the DAPI test. All DAPI test results for Homestead were negative.

Phytoplasma DNA was detected by direct or nested PCR in 7 of 15 trees tested, including 4 of 4 DAPI-positive trees in symptom category EY, 2 of 5 inoculated visually healthy DAPI-negative trees, and 1 nongrafted visually healthy control. All RFLP profiles were identical to those of reference strain EY1 (6,10,11). Phytoplasma DNA was not detected in samples from five DAPI-negative trees in categories Q or EYQ.

Symptoms. Foliar and phloem symptoms and aberrant shoot form (Table 1) that developed only in inoculated trees scored positive in the DAPI test were considered to be caused by phytoplasma. Multiple trees of Frontier, Pathfinder, Patriot, and Pioneer reacted to inoculation. One Prospector plant also reacted. Symptoms associated with infection developed beginning in the year after inoculation, and trees that had such symptoms after one year also had them the next year. Symptoms included chlorosis involving the entire plant or particular branches, foliar reddening on one or more branches, attenuation of apical shoots, slow growth and stunting of the entire plant, witches'-brooms, dieback, and tree death (Fig. 1, Table 1). No new case of phytoplasma-associated symptoms appeared 2 years after inoculation. Abnormal foliar yellowing, reddening, or both occurred on one or more DAPI-positive trees of each of five cultivars but usually did not occur on all branches. General stunting occurred in scattered trees of four cultivars, but it was significantly associated with phytoplasma infection only in Pioneer ($\chi^2 = 6.60$, 1 df, $P = 0.010$), in which stunting presaged dieback and death. Of the 15 DAPI-positive trees that died in the second or third year after planting, 12 were Pioneer (Table 1). Multiple dense witches'-brooms formed on the tops of diseased Pathfinder and on scattered shoots of Patriot plants, whereas apical shoots on Frontier became attenuated. Brooms also formed on mainstems of a few Patriot and Pioneer plants following top dieback.

Neither phloem discoloration nor methyl salicylate odor was detected in bark samples from Frontier, Pathfinder, Patriot, or Pioneer. The single Prospector plant that

Fig. 1. Elm yellows symptoms in Eurasian elm clones in late summer, 1 or 2 years after graft inoculation. (A, B) 'Pathfinder', diseased and healthy, respectively, 1 year after inoculation; diseased plant has foliar reddening and apical witches'-brooms with dwarfed leaves. (C, D) 'Frontier' infected 1 year, (C) with dwarfed apical shoots and leaves and (D) affected parts converted to bushy form. (E) 'Patriot' infected 2 years; plant is stunted and has brooms at branch tips. (F) 'Pioneer' infected 2 years, with stunted branches, apical dwarfing on one branch (arrow), foliar reddening, and dieback.

had foliar yellowing and a positive DAPI reaction also had phloem necrosis and associated methyl salicylate odor. The phloem of inoculated nonsymptomatic Prospector plants was normal in appearance and odor.

Ten inoculated Homestead plants had depressed areas up to 8 cm long in living bark below inoculum patches. Samples from these areas had thin longitudinal bands of necrotic phloem or bands in which necrotic flecks were visible in phloem extending downward from the patches. These bands had apparently developed annually, were confined within the 1997 and 1998 phloem layers (also in the 1999 layer in one tree), and were separated by annual layers of living phloem parenchyma. Trees with these symptoms, including one that was also stunted with yellow foliage, were scored negative in DAPI tests. Phytoplasmal DNA was not detected in the three of these trees that were tested by PCR assay.

Growth. Current-season shoot growth of trees with positive DAPI reactions was compared to that of noninoculated controls of four cultivars in one-tail *t* tests. Growth was suppressed significantly ($P < 0.05$) in infected trees of all four cultivars (Table 2).

DISCUSSION

Tolerance of elm yellows phytoplasmal infection was indicated in three of the six elm cultivars evaluated: Frontier, Pathfinder, and Patriot. Symptoms displayed by these clones were unlike the lethal elm yellows syndrome that is typical in *U. americana* and *U. rubra*. Diseased trees of these cultivars should be observed over several years to learn whether they decline progressively and, if not, whether symptoms become less severe as the trees grow. Diminishing severity with increasing tree size might be expected if the elm yellows pathogen overwinters in roots, as was suggested to occur in *U. americana* (3), and is slow to recolonize apical parts in spring.

In contrast to the three above-mentioned cultivars, all except one of the phytoplasma-affected Pioneer trees died during the second and third years after inoculation, indicating its intolerance of the elm yellows phytoplasma. No credible statement about tolerance or intolerance of

Prospector to the elm yellows phytoplasma can be made, because infection was detected in only two plants, of which one showed severe symptoms while the other was asymptomatic.

Tolerance of phytoplasmal infection may be due to a plant's ability to suppress the parasite's reproduction, to relative insensitivity to substances that phytoplasmas induce or secrete during infection, or to both of these attributes. The latter attribute is likely to account for elm tolerance of elm yellows phytoplasma, because this organism becomes numerous and readily detectable by microscopic procedures in tolerant plants (this article; 4), while remaining undetectable or difficult to detect by these procedures in intolerant plants such as *U. americana* and *U. rubra* (3,4).

Phytoplasmal DNA was detected by nested PCR in samples from three asymptomatic plants that were scored negative in DAPI tests, indicating either cryptic infections or false positive PCR results. Previous studies have revealed phytoplasmal DNA in asymptomatic elms (8). Cryptic infections may indicate either phytoplasma populations held in check by host resistance or nascent populations in recently infected trees that will later have symptoms. One of the asymptomatic infected plants was a nongrafted control. It may have become infected naturally, because elm yellows was common in native elms in the vicinity of the test plot. Additional cryptic infections may have been detected if more asymptomatic trees had been tested by PCR analysis.

The absence of detectable infection and the localized phloem necrosis below bark patches in Homestead could be evidence of resistance. Tissue degeneration could have resulted from a defense process that killed conductive phloem and prevented systemic movement of the pathogen. Other interpretations are available, however. The necrosis may have been induced by a different biotic agent, such as a virus, or by a substance exported from diseased *U. rubra* bark patches into recipient stems.

The low incidence of infection detected in inoculated trees of most clones (32% overall) could not be ascribed to graft failures, because >75% of the bark patches fused with the recipient stems. Neither was absence of phytoplasmas in the inoculum

likely, because the scion material had appropriate symptoms and did induce elm yellows symptoms in 26 trees representing five clones. Wound trauma at graft sites furnishes a plausible explanation for the low infection rate. Many bark patches were imperfectly fitted to the stems, as indicated by callus formation at one or more edges of the patches. Trauma at tissue junctions could have led to sequestration of phytoplasmas in the sieve tubes of inoculum patches, regardless of whether a patch made union with the tree stem, because graft union depends upon interactions of parenchyma cells. Sequestration of phytoplasmas in bark patches was previously detected microscopically in experiments with ash yellows (W. Sinclair, *unpublished*). Sequestration could also result from partial incompatibility between bark patch and recipient stem. We presume that the red elm patches were not equally compatible with all six cultivars, because these clones had various species in their pedigrees.

The elm yellows reactions reported here are consistent with a previous report of symptoms in Eurasian elms inoculated with a North American strain of the elm yellows pathogen (4) and with symptoms observed in various Eurasian species and clones naturally infected in Italy (5,8,12,13). Eurasian elms vary in response from no apparent reaction to phloem necrosis and death (4,13). Elm yellows tolerance, apparent resistance, and cryptic infection of Eurasian elm clones in this study are also consistent with the survival of Eurasian species in American localities where native elm populations were decimated by elm yellows. None of these cultivars, grown in field plots at the United States Department of Agriculture laboratory at Delaware, OH, has shown symptoms of elm yellows, although the disease is frequently seen in American elms there. Up to now, elm yellows is apparently a rare health problem in Eurasian elms growing in North America. If the disease becomes problematic in Eurasian elms on this continent, as could occur due to expansion of vector feeding preferences, arrival of a new vector, or appearance of a new, more aggressive strain of the pathogen, then the present findings may serve as a basis for choosing tolerant cultivars.

Table 2. Average lengths of longest current-season shoots on elm yellows-affected and healthy elms of four cultivars, with *t* tests of the differences

Cultivar ^c	Number of trees ^a		Average length (cm) ^b		One-tail <i>t</i> test		
	DAPI-positive	Control	DAPI-positive	Control	df	<i>t</i> value	<i>P</i>
Frontier	3	7	25 ± 9	55 ± 5	8	5.45	0.016
Pathfinder	3	6	43 ± 3	80 ± 36	7	2.49	0.028
Patriot	6	11	48 ± 36	134 ± 25	15	5.23	<0.001
Pioneer	9	16	28 ± 13	73 ± 18	23	7.63	<0.001

^a Number of trees on which shoots were measured. One 4',6-diamidino-2-phenylindole-2HCl (DAPI)-positive tree of Frontier and three of Pioneer were excluded from shoot measurements because of severe dieback.

^b Average length of longest shoot ± standard deviation.

^c Frontier, Patriot, and Pioneer measured 2 years after inoculation; Pathfinder measured 1 year after inoculation.

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