PHYTOPLASMAS ASSOCIATED WITH ASH DECLINE

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Abstract. The epidemiology and etiology of a disease of *Fraxinus velutina* (Arizona or velvet ash) called ash decline was investigated. Symptoms of ash decline include a progressive dieback of stems and branches as well as yellowing and necrosis of leaf tissue. The incidence and severity of ash decline in *F. velutina* cv. Modesto trees in the Phoenix, Arizona, U.S., metropolitan area was studied over an 8-year period. Symptom severity increased dramatically during this time period with a 30% mortality rate of trees in the study group. Etiological studies determined that phytoplasmas were associated with ash trees showing ash decline symptoms. Phytoplasmas were detected in the phloem sieve tube elements of plants using the DAPI microscopic test, and phytoplasma DNA was detected in ash tissue using a polymerase chain reaction (PCR) assay. The relationship of the phytoplasmas associated with ash decline to the ash yellows (AshY) phytoplasmas is discussed.

Key Words. Ash decline; ash yellows, phytoplasma, velvet ash.

Arizona or velvet ash (*Fraxinus velutina* Torr.) cv. Modesto is a common urban tree in the Phoenix, Arizona, U.S., metropolitan area, used in landscapes and parks to provide shade and seasonal foliar color. In the early 1980s, a progressive dieback of stems and branches was observed in urban velvet ash trees, and the condition has since been described as ash decline (Stutz et al. 1989; Bricker and Stutz 1992).

Symptoms of ash decline (AD) typically begin as a branch dieback in early July, with dieback symptoms becoming most severe by late August to early October. Leaves of affected branches initially turn a distinctive olive color followed by death of leaf tissue. Necrotic, brown leaves are retained on dead branches after normal leaf senescence in December and during the subsequent growing season and serve as an indicator of AD. Each succeeding year, an increasing area of the canopy is affected, but trees do not die immediately and may live a number of years in a debilitated condition. Leaves produced in years subsequent to initial symptom development may be chlorotic, although yellowing is not typical until late in the development of AD. Leaves of affected trees may be morphologically distinct from those on healthy trees with smaller leaf areas and entire margins (versus crenulate-serrulate margins) associated with AD. In the later stages of AD, trees may have a significant loss of the canopy area as well as peeling and cracking bark on the main trunk and branches. This damage may serve as entry points for opportunistic fungi such as *Hendersonula toruloidea* Nattrass, the causal agent of sooty canker. During this late stage, basal shoots may form on the main trunk near ground level.

Several fungi indigenous to the Phoenix metropolitan area are known to infect ash species and cause symptoms with some similarities to those of AD, including *Verticillium dahliae* Kleb. (Smith and Neely 1979) and *Phymatotrichopsis omnivora* (Duggar) Hennebert, the causal agent of cotton root rot (Streets and Bloss 1973). Phytoplasmas cause an ash disease called ash yellows (AshY) that occurs widely in the United States and Canada, affecting 12 species of *Fraxinus* (Sinclair and Griffiths 1994, 2000) including white ash (*F. americana* L.) primarily in the northeastern and midwestern United States and eastern Canada (Sinclair and Griffiths 1994, 2000; Sinclair et al. 1990) and green ash (*F. pennsylvanica* Marsh.) in the Great Plains and Rocky Mountain region (Sinclair and Griffiths 1994, 2000; Walla et al. 2000). AshY phytoplasmas have been detected in *F. velutina* with dieback symptoms in Zion National Park (in southern Utah) (Sinclair et al. 1994), and phytoplasmas have been detected in Modesto ash in Las Vegas, Nevada (Sinclair et al. 1990). A strain of AshY phytoplasmas from Utah has been found to be highly aggressive in green ash and periwinkle [*Catharanthus roseus* (L.) G. Don] (Sinclair and Griffiths 2000) and genetically distinct from tested strains obtained in the eastern and midwestern United States (Griffiths et al. 1999). Common methods used to detect phytoplasmas in plants include microscopic detection using the DNA fluorochrome 4´, 6-diamidino-2-phenylindole•2HCl (DAPI) or polymerase chain reaction (PCR) assays using universal primers (Sinclair and Griffiths 1994; Sinclair et al. 1996). AshY phytoplasmas have been identified by restriction fragment length polymorphism (RFLP) analysis of PCR products and group-specific primers (Ahrens and Seemüller 1992; Sinclair and Griffiths 1994; Sinclair et al. 1996; Griffiths et al. 1999). Phytoplasma strains associated with AshY represent a phylogenetically discrete group of strains designated as '*Candidatus* Phytoplasma fraxini' (Griffiths et al. 1999).

Some symptoms of AshY on white ash in the eastern United States are similar to those found in velvet ash affected by AD, including chlorosis and dieback. Significant differences between symptoms of AshY in white ash and AD in velvet ash include the time of year dieback occurs (winter in white ash versus summer in velvet ash), the delay in chlorosis in tree canopies until the final stages of AD, and the low frequency of witches'-brooms associated with AD.

Our objective was to study the epidemiology and etiology of AD by (1) documenting changes in the incidence and severity of AD in the Phoenix metropolitan area,

(2) screening trees with AD for *Verticillium* wilt and cotton root rot, (3) assaying for the presence of phytoplasmas in trees with AD using the DAPI staining technique and PCR, and (4) determining whether phytoplasmas present in velvet ash are genetically similar to AshY phytoplasmas using RFLP analysis of PCR products.

METHODS
Rating and Characterization of Disease Severity
Forty-eight Modesto ash trees immediately south of the Arizona State University campus in Tempe (a suburb in the Phoenix metropolitan area) were surveyed for AD symptoms beginning in 1985. In 1988, the survey group was expanded to 108 individuals by the inclusion of 60 Modesto ash trees in residential areas to the west of the campus. The resulting group of trees was surveyed again in 1990 and 1992. The mean diameter at breast height (dbh) of trees in these areas was 44.7 ± 2.7 cm (17.6 ± 1.1 in.).

Each tree was rated for decline symptoms according to the classification system of Silverborg and Ross (1968): class 1 = apparently healthy trees; class 2 = trees with a few dead branches; class 3 = trees with foliar symptoms but with less than one-half of the foliar crown dead; class 4 = trees with foliar symptoms and more than one-half of the foliar crown dead; and class 5 = dead trees. Group observation data were pooled for each year that data were collected, and the percentage of individuals in each class was then calculated. A mean severity rating (MSR) was also calculated for each set of observations using the following formula: MSR= Σ class rating/number of individuals rated.

Screening for Fungal Pathogens
Isolation of *V. dahliae* was attempted from stems [10 to 15 cm (4 to 6 in.) in length] collected biweekly beginning on 5 June 1990 and continuing until 9 November 1990 from trees in the disease survey area. Collections were made from eight randomly selected trees in disease classes 2, 3, and 4. In addition, samples from eight apparently healthy ash trees were collected at the campus of Mesa Community College, Mesa (also a suburb in the Phoenix metropolitan area). Stem segments were surface sterilized in 0.05% sodium hypochlorite, sectioned into 1 to 2 mm (0.04 to 0.08 in.) segments, and placed on the surface of Petri plates containing potato dextrose agar (PDA). Additional isolations were made from leaves collected from 20 trees with symptoms of AD in August 1992 using a technique to isolate *Verticillium* from green ash (Ash and French 1992). Sections were made at the petiole base, surface sterilized in 70% ethanol (ETOH) for 20 sec, rinsed in sterile distilled water (SDW), placed in 10% bleach for 20 sec, rinsed in SDW, submerged in 70% ETOH for 20 sec, rinsed in SDW, and placed on alcohol medium.

Trees with symptoms of AD were also screened for evidence of *P. omnivora* infection. Root samples from 44 trees were collected on 16 November 1990, 2 July 1991, 1 September 1991, 11 September 1991, 13 September 1991, and again on 24 August 1992. Root samples were visually examined in the field and in the lab using a dissecting microscope for the presence of root rot symptoms and mycelial strands on the root's surface.

DAPI Testing
Stem samples from 16 trees in each of the disease classes 1 to 4 were collected on 14 September 1990, 2 October 1990, and 24 August 1992 for the DAPI fluorescence test (Sinclair et al. 1989). Stem samples were collected from symptomless branches and from branches with chlorotic leaves, leaf tip and edge necrosis, "olive" dieback, or from witches'-brooms (shoot proliferations at the base of a tree near the soil line) when present. Root samples were harvested 16 November 1990, 18 February 1991, 2 July 1991, and 6 September 1991.

DNA Isolation
A modification of the procedure of Rogers and Bendich (1985) was used to extract DNA from ash tissue. Ash leaf midrib samples [~0.5 g (0.02 oz] were ground in liquid nitrogen, and the resulting powder was transferred to two 1.7 mL (0.05 fl oz) Eppendorf tubes. Isolation buffer [0.8 mL (0.02 fl oz.)] [2% CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl, 0.2% mercaptoethanol, 20 mM EDTA, 100 mM Tris-Cl (pH 8.0)] was added, and the sample was incubated at 65°C (149°F) for 30 min with an occasional gentle agitation. An equal volume of chloroform-isoamyl alcohol (24:1) was added to each tube, and the solution was gently mixed. The homogenate was then centrifuged at 14,000 rpm at room temperature for 10 min. The supernatant was transferred to a new tube, 2/3 volume of isopropanol was added, and the solution was recentrifuged at 4,000 rpm for 3 min at room temperature. The aqueous layer was removed and the pellet was briefly air dried at room temperature and then resuspended in 0.5 ml of TE [10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)] buffer. The nucleic acid solution was digested with 10 µg/ml of RNase A for 15 to 30 min at 37°C (98.6°F). Following incubation, 7.5 M ammonium acetate was added to a final concentration of 2.5 M, and a 0.5 volume of isopropanol was added to the solution and gently mixed. The solution was centrifuged for 10 min at 4°C (39.2°F) at 4,000 rpm. The pellet was dried under vacuum and dissolved in 100 µl TE resulting in a final concentration of approximately 17 µg/ml of DNA, as determined by spectrophotometry. The extracted DNA was stored at –20°C (–4°F) until used as template for PCR.

DNA extracts from healthy periwinkle, periwinkle infected with ash yellows (AshY) phytoplasmas, white ash

infected with AshY phytoplasmas, and healthy white ash were provided by Wayne Sinclair (Cornell University, Ithaca, NY) for comparison with the AD pathogen.

DNA Amplification

Phytoplasma-specific primers described by Ahrens and Seemüller (1992) from a 558 bp fragment of the 16S rRNA gene were used to amplify phytoplasmal DNA by PCR. Each 50 µl reaction mixture contained 100 to 200 ng of plant DNA extract, 0.5 µM of each primer, 100 µM of each dNTP, 5 µl of 10× PCR buffer [100 mM Tris-Cl (pH 8.3), 500 mM KCL, 15 mM $MgCl_2$, and 0.1% (w/v) gelatin], and 1 U of Taq DNA polymerase (Stratagene, La Jolla, CA). The mixture was subjected to 3 min initial denaturation at 95°C (203°F) prior to 35 PCR cycles [denaturation at 95°C for 1 min, annealing at 55°C (131°F) for 1 min, and extension at 72°C (161.6°F) for 1 min (8 min for the last cycle)]. PCR product (15 µl) was electrophoresed in 1.5% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide (EtBr), and visualized by UV transillumination. Molecular weights were determined by comparing the PCR product to a 100 bp ladder (Gibco BRL, Life Technologies, Inc., Grand Island, NY).

Restriction Enzyme Analysis

PCR product (35 µl) was diluted in 165 µl dd H_2O, and DNA was extracted with 1 volume of chloroform and centrifuged for 2 min (14,000 rpm). The supernatant was transferred to a fresh tube, and 15 µl of 3 M sodium acetate and 2 volumes of 95% ETOH were added before storage overnight at –20°C (–4°F). The mixture was centrifuged for 10 min [14,000 rpm at 4°C (39.2°F)], the supernatant was removed, the pellet was washed with 70% ETOH, the suspension was centrifuged for 2 min, and the pellet was rehydrated with 17 µl distilled H_2O. Two microliters of 10× assay buffer [25 mM Tris HCl (pH 7.7), 10 mM $MgCl_2$, 10 mM BME, 50 mM NaCl, 100 µg/ml BSA] and 1 µl (= 2 units) of undiluted *Alu*I restriction endonuclease (Stratagene, La Jolla, CA) were added to the PCR product, and the product was digested for 2.5 hr at 37°C (98.6°F). The restriction products (15 µl) were separated by electro-phoresis on a 10% polyacrylamide gel in TAE buffer, stained with EtBr. and visualized as previously described.

RESULTS
Rating and Characterization of Disease Severity

Results of the ash decline survey indicated an increase in severity of symptoms during the 8-year period from 1985 to 1992 (Figure 1). In 1985, 75% of the tree population were symptomless or had less than 50% of the crown affected. In contrast, by 1992 only 14% of the trees were in these three categories, and 30% of the trees in the survey group had died or been removed.

The mean severity ratings (MSR) calculated for the trees included in the disease severity survey reflected the change in disease severity reported above (Figure 2). The MSR increased throughout the survey time period starting at 2.89 in 1985 and increasing to 4.10 by 1992.

Screening for Fungal Pathogens

Verticillium was not isolated from any ash trees when twig samples were plated on PDA or alcohol agar, and no vascular discoloration was observed in symptomatic trees. There was no evidence of root rot symptoms or the presence of *P. omnivora* in any of the ash root samples collected.

DAPI Testing

A total of 200 samples were collected from 64 ash trees (16 in each of the disease classes 1 to 4) and tested using the DAPI technique. DNA of microorganisms was detected in
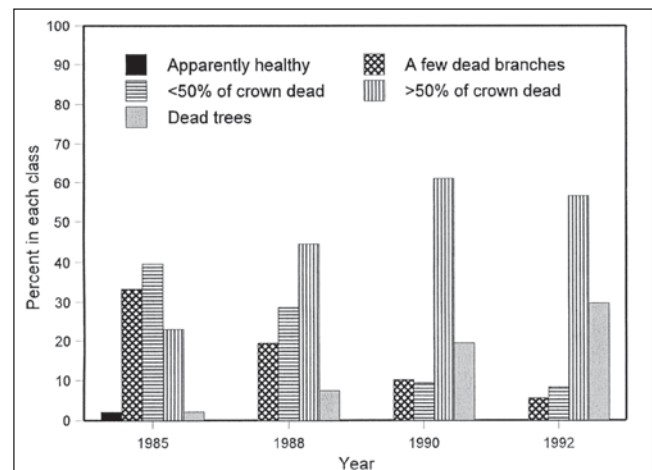

Figure 1. Percentage of *Fraxinus velutina* trees with different ash decline severity levels, 1985–1992.
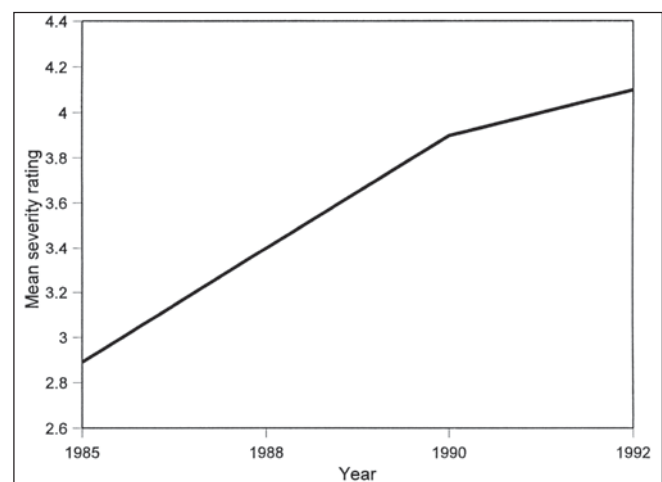

Figure 2. Mean ash decline severity ratings for *Fraxinus velutina* trees, 1985–1992.

phloem sieve tube elements but was at extremely low titer and difficult to detect. Repeated sampling of each individual was often necessary in order to detect if an individual tree was infected. A positive DAPI test was obtained in the majority of the tested trees with [$\bar{x}$ = 77.9%; classes 2–4) and without (68.7%) AD symptoms (Table 1). All apparently symptomless trees that had positive DAPI tests began to develop typical AD symptoms within 1 to 2 years of the positive test. There were no significant differences between the various disease rating classes with respect to the percentage of trees with at least one positive sample, as evidenced by repeated measures analysis of variance (ANOVA) using the SAS computer package (F = 1.83, df = 4, P = 0.05). When repeatedly sampling trees within the same disease class, not all samples were positive. The percentage of positive samples within a disease class was consistently lower than the percentage of trees with at least one positive DAPI test. As an example, when testing apparently healthy trees, less than one-half of the samples tested from trees with at least one positive DAPI test were positive.

The percentage of positive DAPI tests was also affected by the type of tissue sampled, with the lowest percentage of positive samples from root tissue (27% positive) and from witches'-broom shoot proliferations found growing near the soil line (39% positive). The greatest percentage of positive DAPI tests was obtained from stem samples that had leaves with tip and edge necrosis (80%) or chlorosis (78%). Stem samples with healthy leaves also had a high percentage (73%) of positive DAPI tests.

DNA Amplification

A 558 bp DNA fragment was amplified in reaction mixtures containing nucleic acids extracted from velvet ash with AD symptoms, white ash infected with AshY phytoplasmas (Figure 3), and periwinkle infected with AshY phytoplasmas (data not shown). This DNA fragment was the same size as the DNA fragment obtained by Ahrens and Seemüller for a wide variety of phytoplasmas (Ahrens and Seemüller 1992). No PCR products were amplified from DNA extracted from healthy periwinkle and healthy white ash (data not shown).

A PCR product was obtained from 66% of the 47 ash trees with AD symptoms tested and from six of the eight apparently healthy trees included in the study. Phytoplasmas had previously been detected in samples from each of these trees using the DAPI test.

Restriction Enzyme Analysis

Gel electrophoresis of the *Alu*I digest of the 558 bp PCR product amplified from velvet ash with AD symptoms consistently resulted in a restriction pattern containing two DNA fragments, 296 bp and 191 bp in size (Figure 4). The

Table 1. Percentage of *Fraxinus velutina* trees and the percentage of samples testing positive with DAPI for microorganisms, for each disease class.

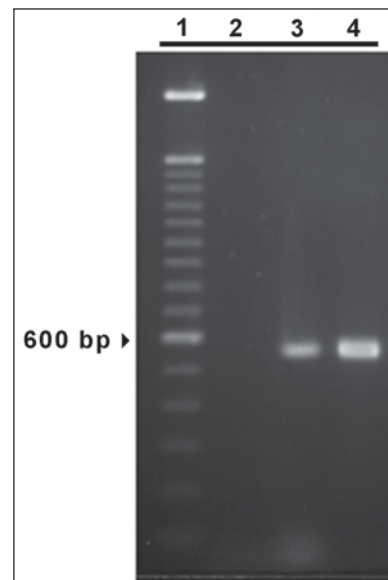| Class	Percentage of trees	Percentage of samples
1	68.7%	48.8%
2	75.0%	60.4%
3	76.5%	61.8%
4	82.3%	59.2%

Figure 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplifications, after 35 cycles, of phytoplasmal 16S rDNA fragment in *Fraxinus* spp. trees suffering from ash decline. Lane 1 = 100 bp molecular weight standard, lane 2 = water control, lane 3 = AshY phytoplasmas from *F. americana*, and lane 4 = AD phytoplasmas from *F. velutina*.
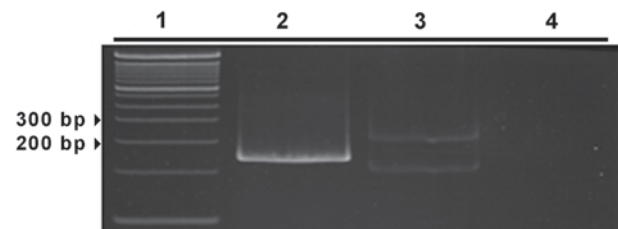

Figure 4. Polyacrylamide gel electrophoresis of *Alu*I digests of polymerase chain reaction (PCR) products obtained after 35 cycles from phytoplasmal 16S rDNA fragment. Lane 1 = 100 bp molecular weight standard, lane 2 = AshY phytoplasmas from *Fraxinus americana*, lane 3 = AD phytoplasmas from *F. velutina*, and lane 4 = water control.

RFLP pattern of the *Alu*I digest of the PCR products from AshY phytoplasmas in both periwinkle and white ash was a single 240 bp fragment.

DISCUSSION

The severity of AD symptoms in ash trees in the survey area increased dramatically over the 8-year study period, with a 30% mortality rate during this period. This is the first report of high mortality rates associated with phytoplasmas in ash used as urban shade trees. The presumptive causal agent of AD appears to be phytoplasmas as evidenced by results of DAPI tests and PCR using phytoplasma-specific primers. A large percentage of the diseased ash trees had repeatable, positive DAPI tests during the study period. Phytoplasmas were detected more than twice as often in twig samples from the tree canopy (73% to 80% positive DAPI tests) than in root samples (27% positive) or witches'-brooms (39%). These results contrast with those found in white ash trees infected with the AshY phytoplasmas in which phytoplasmas were detected by DAPI testing more than twice as often in roots samples in comparison to twig samples (Sinclair et al. 1989). Detection of phytoplasmas in *F. velutina* with AD symptoms using PCR reinforces the conclusion derived from the DAPI test that a phytoplasma is the likely casual agent. Although healthy trees had positive DAPI and PCR results, all such trees developed symptoms of AD within 2 years of a positive test indicating a lag time between infection and symptom development.

The relationship of the phytoplasmas associated with AD to the AshY phytoplasma group is unclear. The *Alu*I profile obtained for the AD phytoplasmas was different from that of the AshY phytoplasmas from New York state, but this difference does not necessarily mean that the AD phytoplasmas are not part of the AshY group. Phylogenetic analysis of the 16S rDNA sequence of strains of AshY indicate that the AshY phytoplasma group is a discrete but heterogenous group (Griffiths et al. 1999). Variations were found in the RFLP profiles of AshY strains with *Alu*I, with strains separating into two groups (Griffiths et al. 1999). AshY3, a strain originally isolated from velvet ash located in southwestern Utah, was found to be genetically distinct from strains in eastern and midwestern United States, including having a different *Alu*I profile from type strain AshyY1 from New York state but the same *Alu*I profile as most of the isolates tested. Mäurer et al. (1993) also observed three different RFLP patterns in ash phytoplasma DNA obtained from different locations in North America when probed with fragments from an elm yellows phytoplasma. Based on RFLP profiles of AshY strains, Griffiths et al. (1999) postulate that there are genetically distinct populations of AshY phytoplasmas that predominate in particular regions of North America.

Although AshY occurs in urban shade trees and apparently suppresses tree growth, it has not been considered a significant problem in urban ash populations (Sinclair et al. 2000). Walla et al. (2000) found that urban plantings of green ash with AshY phytoplasma infections were in relatively good condition compared to natural and rural stands. The high mortality rates observed in the Phoenix metropolitan area could be due to the presence of an aggressive pathogen, the use of a velvet ash cultivar with low tolerance to phytoplasma infection, adverse environmental conditions, or a combination of these factors. Strains of phytoplasmas belonging to the AshY group have been shown to vary in aggressiveness, with the most aggressive strain (Strain AshY3) isolated from a velvet ash located in southwestern Utah (Sinclair and Griffiths 2000). This strain has been shown to cause death of young white and green ash after transmission by grafting. Although cultivars of white and green ash have been shown to differ in their tolerance to AshY phytoplasmas (Sinclair et al. 2000), velvet ash is generally considered tolerant of infection by the AshY phytoplasma with little effect on growth and imperceptible symptoms (Sinclair et al. 1993, 1994). It is possible the Modesto cultivar grown as shade trees in the Phoenix metropolitan area differs in its tolerance to phytoplasmas in comparison to the velvet ash trees studied previously, which were located in Zion National Park. The local environmental conditions in the Phoenix metropolitan area may also affect disease severity. Growth decline in white ash trees infected with phytoplasmas and subjected to drought was more severe than those in trees not exposed to drought stress (Han et al. 1991). Although ash shade trees in the Phoenix area are irrigated, drought stress commonly occurs because of extreme high summer temperatures and low rainfall.

CONCLUSION

The results of this study appear to strongly establish the causal agent of Arizona ash decline as being a phytoplasma. The exact relationship, however, of the ash decline phytoplasma to the AshY phytoplasma group, especially AshY3, remains unclear. To address this situation AshY group-specific primers will be used to produce PCR products for ash decline and AshY phytoplasmas. Comparison of resulting DNA sequences to one another and to other phytoplasmas in the GenBank database will be done to establish their phylogenetic relationships.

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Résumé. L'épidémiologie et l'étiologie de la maladie appelée dépérissement du frêne a été étudiée chez le *Fraxinus velutina*. Les symptômes du dépérissement du frêne incluent une mortalité progressive des rameaux et des branches tout comme un jaunissement et une nécrose des tissus foliaires. L'incidence et la sévérité du dépérissement du frêne chez le *F. velutina* cv. Modesto dans la zone métropolitaine de Phoenix en Arizona ont été étudiés durant huit ans. La sévérité des symptômes a augmenté dramatiquement durant cette période avec un taux de mortalité de 30% dans le groupe d'arbres étudiés. Les études étiologiques ont déterminé que des phytoplasmes étaient associés avec les frênes qui présentaient des symptômes du dépérissement du frêne. Les phytoplasmes ont été détectés dans des éléments du phloème des végétaux au moyen d'un test microscopique DAPI et l'ADN du phytoplasme a été détectée dans les tissus du frêne au moyen d'un essai par réaction en chaîne de polymérase. La relation entre les phytoplasmes associés au dépérissement du frêne avec celle des jaunissements du frêne est discutée.

Zusammenfassung. Die Epimediologie und Etiologie einer Krankheit von *Fraxinus velutina*, die "das Eschen-sterben" genannt wird, wurde hier untersucht. Die Symptome vom Eschensterben schließen ein progressives Absterben von Stämmen und Ästen genauso ein, wie ein Vergilben und Nekrotisieren von Blattgewebe. Das Vor-kommen und das Ausmaß von Eschensterben bei *Fraxinus velutina* cv. Modesto-Bäumen im innerstädtischen Bereich von Phoenix, Arizona wurde über einen Zeitraum von 8 Jahren beobachtet. Das Ausmaß der Symptome stieg dramatisch im Untersuchungszeitraum mit einer Todesrate von 30% aller untersuchten Bäume. Die etiologischen Untersuchungen ergaben, dass Phytoplasmas mit befallenen Eschen in Verbindung steht. In den Phloemkanälen wurde Phytoplasmas bei den mikroskopischen Untersuchungen mit dem DAPI-Test identifiziert und Phytoplasma-DNA wurde im Eschengewebe bei der PCR-Untersuchung gefunden. Die Beziehung von Phytoplasmas mit dem Eschensterben zum Eschenvergilben wird hier diskutiert.

Resumen. Se investigó la epidemiología y etiología de una enfermedad de *Fraxinus velutina* (fresno de Arizona) llamada "declinación del fresno". Los síntomas de la enfermedad incluyeron la muerte regresiva de tallos y ramas como también el amarillamiento y necrosis del tejido foliar. Se estudió la incidencia y severidad de la declinación del fresno en *F. velutina* en árboles de la zona metropolitana de Modesto en Phoenix, Arizona en un período de 8 años. La severidad de los síntomas incrementó dramáticamente durante este período de tiempo, con una tasa de mortalidad del 30% de los árboles en el grupo de estudio. Los estudios etiológicos determinaron que los fitoplasmas estaban asociados con los árboles de fresno con síntomas de declinación. Los fitoplasmas fueron detectados en los elementos conductores del floema de las plantas usando pruebas microscópicas DAPI, y el fitoplasma DNA fue detectado en el tejido de los fresnos usando un ensayo de reacción en cadena de polimerasa (PCR). Se discute la relación de los fitoplasmas asociados con la declinación y el amarillamiento del fresno.