ELM YELLOWS: A WIDESPREAD AND OVERLOOKED KILLER OF ELM TREES ACROSS THE UNITED STATES

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Abstract.—The elm yellows phytoplasma (*Candidatus* Phytoplasma ulmi) is a geographically widespread pathogen that poses a significant threat to most native wild elms in North America (*Ulmus americana, U. rubra, U. alata, U. crassifolia,* and *U. serotina*) (Mäurer et al. 1993), as well as to the success of American elm research programs focused on breeding Dutch elm disease tolerance. Despite the advancements of American elm research programs in creating Dutch elm disease-tolerant varieties, elm yellows research has lagged and threatens to undermine the success of breeding programs. Here, we have three goals: 1) to present a general background on elm yellows; 2) to discuss the identification and management of a 2016 elm yellows outbreak in an American elm research plantation in Delaware, OH; and 3) to summarize recent research advancements as well as tools toward identification and management. To date, 9 of 47 trees from the American elm research plantation in Delaware, OH, tested have been confirmed to be infected with phytoplasmas similar to those known to cause elm yellows in other locations. False positives were frequent and improved methods for detecting and identifying the phytoplasma are needed.

Introduction

Elm yellows (EY) is one of the most destructive diseases of elms behind Dutch elm disease (DED), caused by the fungal pathogens *Ophiostoma ulmi* (Buisman) Melin & Nannf. and *O. novo-ulmi* Brasier (Lee et al. 2004, Marcone 2016). Unlike the DED fungal pathogens, which stimulate a defensive response in the tree that clogs xylem tissue, elm yellows is caused by phloem-obligate bacteria called phytoplasmas (in the class Mollicutes), which multiply in the phloem, sieve elements, and disrupt nutrient translocation. Phytoplasmas are classified into groups based on the nucleotide sequence of the 16S rDNA gene. Elm yellows can be caused by a number of phytoplasma groups, including the elm yellows (Group16SrV-A) (Lee et al. 2004), Illinois elm yellows (Group16SrVI-C) (Jacobs et al. 2003), and the aster yellows (Group16SrI) (Lee et al. 1995).

The primary vectors for EY are vascular-feeding insects (Order Hemiptera, including Cicadellidae and Cercopidae families) (Baker 1948). It has been suggested that the phytoplasma may be transmitted between elm trees via root grafts (Sinclair 2000), but experimentation has not been conducted to substantiate this claim. Elms infected with the pathogen exhibit rootlet necrosis followed by degeneration of phloem in the lower trunk, foliar chlorosis, and epinasty (Sinclair 2000). Infected vascular tissue exposed by peeling bark off of a fresh sample may exhibit a butterscotch color as well as the emission of a methyl salicylate (wintergreen) odor (Sinclair 2000).

Likely introduced into North America in the 1800s (Baker 1948, Marcone 2016), EY was first described in Ohio by Swingle (1938) as causing severe decline in American elm (*Ulmus americana* L.) and red elm (*U. rubra* Muhl.). Its initial presence and spread was likely

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underestimated because of the simultaneous occurrence of DED. It is now widespread across much of eastern North America, from Mississippi to southeastern Ontario (Matteroni and Sinclair 1985). Elm yellows has also been reported in parts of Europe: France, Italy, and Serbia (Jović et al. 2011, Marcone 2016, Mittempergher 2000).

Research focusing on breeding DED-tolerant American elms (see Flower et al. 2017, this proceedings) is advancing and efforts are currently underway to transition these trees into the natural environment (Knight et al. 2017, this proceedings). One significant setback on these transition studies are EY outbreaks. An enhanced understanding of the EY pathogen and its vectors is needed, not only for the sake of better understanding of this largely overlooked pathogen, but also to advance DED-resistance work. The objective of this study is to provide a synopsis of identification and mitigation activities conducted in response to an elm yellows outbreak in an American elm plantation in Delaware, OH, during the summer of 2016.

Methods And Materials

Study Site

This work took place in late July 2016 at two plantations in Delaware, OH, named East (4 acres) and Main (5.5 acres). The two plantations are situated approximately 100 yards from each other. General yellowing of established American elm canopies was observed across a portion of the two plantations as well as individual trees in varying stages of dying. Symptomatic trees exhibited EY characteristics including: 1) premature canopy yellowing that was evenly distributed across the canopy; 2) wilt of foliage throughout the canopy; 3) phloem discoloration; and 4) a strong odor of methyl salicylate on a subset of trees. These symptoms developed over the course of 3 weeks.

Genetic Approaches for Identifying the Disturbance Agent

Leaf samples were collected from the upper canopy and phloem samples were taken from branches and twigs of symptomatic and asymptomatic trees. Insects were collected near infected trees using sweep nets and yellow sticky traps. Leaf and phloem tissue were used for identification and DNA analysis. DNA was extracted from the phloem and leaf material using Qiagen DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). We used three PCR-based tests for identifying the causal agent: 1) a real-time polymerase chain reaction (rt-PCR) primers designed specifically for *Ca.* Phytoplasma ulmi (Herath et al. 2010); 2), a semi-nested PCR technique by using the phytoplasma universal primer pair P1/P7 that was specifically designed for phytoplasma 16S-23S rRNA genes (Schneider et. al. 1995). The second semi-nested PCR reaction was run using P7 and the reverse complement of the universal phytoplasma primer R16R2 (5'-CGGGGTTTGTACACACCGCCCGTC-3') (Gundersen, 1996). The PCR products were run on a 1.2 percent agarose gel made with 1x Tris-Borate-EDTA (TBE) buffer using ethidium bromide to view the amplified DNA. Based on other phytoplasma sequences, the predicted product size for the second primer pair is 480 base pair.

Third, the semi-nested PCR test of samples with 400-800 bp products was repeated on a larger scale. These PCR products were run on a 1.2 percent TBE gel, then post-stained in 0.0015 percent Nile Blue. Each of the 400-800 bp bands were isolated and gel-purified using GeneClean Spin kit (MPBiomedicals, Solon, OH) and sent to the Plant-Microbe Genomics Facility at Ohio State University for sequencing, using PCR primer P7 as a sequencing primer. The facility uses the 3730 DNA Analyzer from Applied Biosystems, Inc. and BigDye® Terminator Cycle Sequencing chemistry.

Management

To mitigate the damage in the plantations, management consisted of: 1) felling the symptomatic and dead trees for burning; 2) severing possible root grafts by trenching ~100 cm deep to separate the plots within each plantation; and 3) spraying the plantations with the pyrethrin insecticide Talstar® (FMC Corp., Philadelphia PA), to control vectors.

Results and Discussion

During a 3-week period in August 2016, more than 80 trees in both plantations exhibited elm yellows-like symptoms. We promptly removed trees that died as well as those that did not die but were symptomatic. Following removals and the initial wave of yellows-like symptoms in the northern portion of the East Plantation, no further canopy yellowing symptoms were identified.

The real-time PCR approach revealed that three trees tested positive for *Ca*. Phytoplasma ulmi. These three trees consisted of two samples from known EY-positive trees on the Penn State University campus, and one was from an asymptomatic tree in the East Plantation (Table 1). The semi-nested PCR approach yielded products in several trees, both symptomatic and asymptomatic, with products in the 400-800 bp range (Table 1). Despite the primers being designed for phytoplasma specificity, the presence of nine false positives, which as revealed by further sequencing, was apparent. These nine sequences taken from both symptomatic and asymptomatic trees were identified through GenBank as having similarity to sequences of various bacteria genera from soil, skin, and the cloaca of birds. Upon further investigation, it was determined that the phytoplasma PCR primers P7 and R16R2 (reverse compliment) have

Table 1.—Sample results of the symptomatic trees tested using real-time PCR, semi-nested PCR, or sequencing methods. Results are denoted by tree location (OH=Ohio, PA=Pennsylvania); symptomatic (Y=symptomatic, N=not symptomatic); real-time PCR ('+' and '-' denote positive and negative for *Ca*. Phytoplasma ulmi); Semi-nested PCR (Y=400-800 bp bands present, N=400-800 bp bands not present); Sequence (F=Failed [likely due to low DNA concentrations or a mix of different DNAs in the sequence], O=skin/soil bacteria, S=soil bacteria, V=16Sr-V phytoplasma, VI=16SrVI phytoplasma). *denotes offsite control.

			Real-time PCR			
Sample ID	Tree Location	Symptomatic	1st	2nd	Semi-nested PCR	Sequence
1, 33, 35, 36, 38, 41, 43, 45, 46,	OH	N	-	-	Y	F
2, 20, 22, 25, 28, 32, 34, 37, 39, 40, 42, 44, 47	OH	N	-	-	N	
3, 27, 48	OH	Y	-	-	N	
4	OH	Y	-	-	Y	F
5,12,26	OH	Y	-	-	Y	O
6	OH	Y	-		Y	F
7,8	PA	Y	+	+	Y	V
9	OH	N	+	+	Y	O
11*, 19*	OH	N	-		N	
10, 13, 14, 15, 16, 17, 18, 30, 31	OH	Y	-	-	Y	VI
21, 23, 24, 29	OH	N	-	-	Y	S

*denotes offsite control

identical or almost identical sequence in the 16S-23S rDNA genes of other classes of bacteria, not just Mollicutes. For some sequenced samples, more than one DNA with the appropriate size was amplified and purified together during the PCR reaction with the phytoplasma primers, so the sequence could not confirm or deny the presence of phytoplasma. Of the remaining samples yielding clear sequences, one was identified as very closely related to *Ca.* Phytoplasma ulmi, which resides within the elm yellows group (16SrV). This sample was from DNA isolated from the Penn State EY-positive trees. Nine other sequences from symptomatic trees in Delaware, OH, were identical to each other. They were identified through Genbank as most closely related to phytoplasma pathogens in the clover proliferation group (16Sr-VI), similar to the elm phytoplasma 'Arlington Heights' (Genbank Accession AF268893.1) (Table 1).

The discrepancies in identification of the elm yellows phytoplasma between the different analyses indicate that caution should be taken to avoid misidentification of the pathogen. The real-time PCR technique developed for detecting *Ca*. Phytoplasma ulmi may be producing false positives in part because of homology with some related bacterial strains. The Delaware, OH, sample (#9), that repeatedly tested positive for *Ca*. Phytoplasma ulmi using rt-PCR with EY-specific primers, was later sequenced and only found to be positive for soil-borne and bird cloaca-originating bacteria. The semi-nested PCR approach also resulted in several false-positive results from nine of the samples, which were later confirmed to be soil-borne bacteria. The results of this assessment indicate that real-time and the semi-nested PCR approaches should be viewed with healthy skepticism until new primers are designed.

Ongoing research

Because symptom identification is frequently followed by removal, many basic aspects of EY remain understudied. Efforts are currently underway to investigate the seasonal fluctuations of the pathogen within the tree and to assess the susceptibility of different DED-tolerant American elm selections to EY. There is also an ongoing effort to systematically trap insects to quantify the abundance and distribution phytoplasma within the vectors (Rosa et al. 2014). Finally, efforts are underway to reduce identification costs via nested PCR with phytoplasma primers, followed by restriction fragment length polymorphism analysis to assign phytoplasmas to recognized phylogenetic groups.

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