EFFECTS OF INOCULATION TIMING ON SYMPTOM DEVELOPMENT IN *ULMUS AMERICANA* L.

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Abstract.—Field inoculation trials are an important component of screening American elms (*Ulmus americana*) for levels of resistance to Dutch elm disease. A major concern in screening is variability in disease ratings from year to year. Previous studies have demonstrated that timing of inoculation can have a significant impact on disease susceptibility. In this study, trees were inoculated in the main stem using a drill method of inoculation. A recently collected isolate of *Ophiostoma novo-ulmi* with known pathogenicity was used for inoculations. Three different inoculation times were examined: early (May 26), mid (June 23), and late (August 4) season. Trees were assessed for wilt symptoms at 2, 4, 6, and 8 weeks post inoculation using a disease severity scale of 1-6. The trees in the early season inoculation group had the highest mean disease severity ratings at 4, 6, and 8 weeks post inoculation (WPI), while the late season inoculation group had the lowest disease rating at every time point as well as the smallest area under the disease progress curve. Scientists evaluating American elms for resistance to Dutch elm disease should avoid late season inoculations due to reduced disease susceptibility.

Introduction

American elm, *Ulmus americana* L., populations have been decimated by the introduction of *Ophiostoma ulmi* (Buisman) Melin & Nannf. and *O. novo-ulmi* Brasier. Due to the significant losses, there is interest in selecting, developing, and releasing American elm cultivars with higher levels of resistance compared to susceptible genotypes. Before being released to the public, cultivars generally undergo repeated testing to determine their relative resistance to *O. novo-ulmi*. In order to test genotypes for resistance, artificial inoculations are frequently used (Mittempergher and Santini 2004, Smalley and Guries 1993, Solla et al. 2005a).

Previous studies have demonstrated that a number of variables can impact disease development in artificially inoculated elms (Solla and Gil 2002, Solla et al. 2005b, Sutherland et al. 1997, Tchernoff 1965). The variable examined in this study is the timing of inoculation. There have been multiple studies which have examined the impact of timing of inoculation on disease development in American elms (Pomerleau 1965, Smalley 1963, Smalley and Kais 1966, Smalley and Lester 1983, Takai and Kondo 1979). However, the studies were conducted more than 30 years ago, and it would be advantageous to determine if utilizing current isolates would result in differences from previous findings. Brasier (1996) and Plourde and Bernier (2014) examined pathogenicity of *Ophiostoma novo-ulmi* isolates from North America, but the most recent isolate examined in either study was from the mid-1990s.

The goal of this study is to determine if timing of inoculation significantly impacts disease development in artificially inoculated American elm trees using an isolate recently collected from a diseased elm. If differences exist in disease severity based on different inoculation times, which has been evident in previous studies, consideration should be given to utilize inoculation times

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that maximize disease severity in order to effectively determine the levels of resistance within a given genotype. By inoculating during times of greatest susceptibility, there should be greater continuity in results between trials performed in different years and locations.

Materials and Methods

Twenty-four *Ulmus americana* trees were used for this study, 16 trees from a Minnesota seed source and 8 trees from an Ontario, Canada, seed source. Seed from both locations were generated through open pollination. Trees were transplanted in a nursery field at the University of Minnesota, St. Paul campus, on July 6, 2014. During the growing season, the trees were watered as needed and received 4.9 ml of Osmocote® Plus (15-9-12) (Everris NA Inc., Dublin, OH) every 3 months to ensure adequate access to nutrients. At the time of inoculation, trees were 3-4 m tall and approximately 2-4 cm d.b.h.

A Minnesota isolate of *Ophiostoma novo-ulmi*, with known pathogenicity, was used for inoculations. After 7 days of growth on selective media for *Ophiostoma* described in Harrington (1981), three 0.5-cm^2 pieces of colonized media were added to 100 ml of liquid media described in Stennes (1981). Cultures were placed on a shaker at 150 rpm and allowed to grow for 3 days at room temperature. Spore suspension concentrations were determined using a hemocytometer and adjusted to 1×10^6 spores/ml (Buiteveld et al. 2015). This process was repeated for each inoculation.

There were three treatments based on when they were to be inoculated: early, mid, and late season. Each treatment contained eight trees, five randomly selected trees from the Minnesota, seed source and three randomly selected trees from the Ontario seed source. Due to limited plant material, the mid inoculation treatment had six trees from the Minnesota seed source and only two from the Ontario seed source. For each treatment, six trees were inoculated with a spore suspension and one tree from each of the two seed sources were inoculated with sterile water to serve as controls. The early season inoculation group was inoculated on May 26, 2016 (40 days after budbreak), the midseason inoculation group on June 23, 2016 (68 days after budbreak), and the late season inoculation group on August 4, 2016 (110 days after budbreak). Inoculations were made using a drill method modified from a study by Townsend et al. (2005). Briefly, trees were inoculated by drilling a 4 mm deep hole with a 2.4 mm diameter drill bit 0.5 m above the ground on the main stem (Fig. 1). Twenty-five µm of the spore suspension containing 1×10^6 spores/ml were injected into the hole using a micropipette and sealed with Parafilm M® (Bemis Co., Neenah, WI) to avoid desiccation.

Figure 1.—A drill was used to make a wound 0.5 m above the ground for inoculations. Photo by Benjamin Held, used with permission.

Figure 2.—A representative tree in the early inoculated group at 3 weeks post inoculation displaying permanent wilt in a majority of the crown. Photo by Garrett Beier, used with permission.

Disease symptoms were assessed every 2 weeks following inoculation. Disease ratings were based on the percentage of the crown exhibiting permanent wilt (Fig. 2). Ratings were made on a 1–6 ordinal scale: 1=0 percent wilt; 2=1 to 25 percent wilt; 3=26 to 50 percent wilt; 4=51 to 75 percent wilt; 5=75 to 99 percent wilt; and 6=100 percent wilt. Area under the disease progress curve (AUDPC) was measured using the mean disease severity ratings at 2, 4, 6, and 8 weeks post inoculation for each treatment. Calculating AUDPC is a useful method to determine disease intensity over time (Campbell and Madden 1990, Shaner and Finney 1977).

Analysis was performed using the statistical package R version 3.2.2 (R Development Core Team, Vienna, Austria). Data on disease severity was measured using an ordinal scale and often lacked normal distribution based on Shapiro-Wilk Normality Test results. To account for repeated measures, the F1 LD F1 macro from the nparLD package (Noguchi et al. 2012) was used to calculate an analysis of variance-type statistic (ATS), which is a nonparametric method to test treatment, time, and treatment x time interaction effects. The use of ATS for nonparametric analysis of repeated measures is described in Shah and Madden (2004). Since the treatment effect was found to be significant, treatments were compared at each time point using the Kruskal-Wallis test followed by Dunn's multiple comparisons test with a Benjamini and Hochberg (1995) *p*-value adjustment. Area under the disease progress curve data was analyzed using ANOVA followed by the Fisher's LSD test with a Benjamini and Hochberg (1995) *p*-value adjustment.

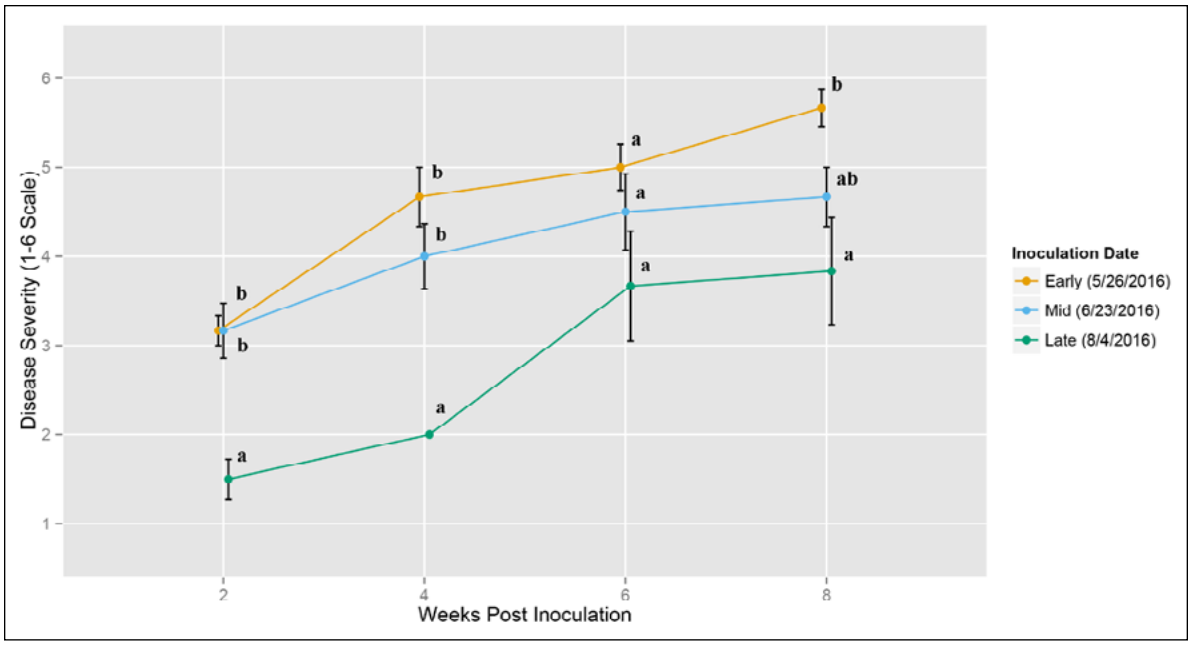

Figure 3.—Effect of timing of inoculation in *Ulmus americana* on biweekly disease severity ratings. Points represent the mean of six trees and bars represent the standard error of the mean. Groups containing the same letter within a column are not significantly different according to Dunn's multiple comparison test with a Benjamini and Hochberg p-value adjustment (α=0.05).

Results

There was a significant effect of timing of inoculation on disease severity rating (p<0.001). The early season inoculation group had the highest average disease rating at 4, 6, and 8 weeks post inoculation (WPI), while the late season inoculation group had the lowest average disease rating for every time point. Although trees in the early season inoculation group had a higher average disease rating compared with the midseason inoculation group at 4, 6, and 8 WPI, differences in the populations were not found to be statistically significant (p>0.05). At 2 and 4 WPI, there was a statistically significant difference between late season inoculations and the early and midseason inoculations for disease severity. At 8 WPI, the late season inoculation group had an average wilt rating of 3.8, while the early inoculation group averaged 5.7, and the difference between the groups was found to be statistically significant (p<0.05) (Fig. 3). The number of trees at 100 percent permanent wilt at 8 WPI varied depending on the timing of inoculation. For the early season group 4 of 6 trees had 100 percent wilt, for the midseason group only 1 of 6 trees had 100 percent wilt, and none of the late season inoculated trees had 100 percent wilt.

Disease progression was also affected by timing of inoculation. The late season inoculation group displayed a slower progression of disease compared with the early and midseason inoculations. Area under the disease progress curve at 8 WPI for the late season inoculation group was 10.7 and significantly less than that of the early season and midseason inoculations, 22.2 and 18.8 respectively (Table 1).

Table 1.—Effect of timing of inoculation in *Ulmus americana* on area under the disease progress curve (AUDPC) at 8 weeks post inoculation

Inoculation group	Inoculation date	Mean[a] ± SE
Early	5/26/16	22.2 ± 1.4 b
Mid	6/23/16	18.8 ± 2.0 b
Late	8/4/16	10.7 ± 1.9 a

[a] Means containing the same letter are not significantly different according Fisher's LSD test (α=0.05).

Discussion

A major concern for researchers testing elm genotypes to evaluate resistance to DED has been a lack of consistency between years and locations carried out in different trials. Findings from this study show that American elms display different susceptibility to infection with *Ophiostoma novo-ulmi* depending on the timing of inoculation. These results confirm studies completed by others (Pomerleau 1965, Smalley 1963, Smalley and Kais 1966, Smalley and Lester 1983, Takai and Kondo 1979). To maintain consistency and effectiveness in screening, it is advisable to inoculate trees when they are at their greatest susceptibility to infection. Alternatively, using the same susceptible and resistant controls across experiments could help investigators assess resistance of different genotypes inoculated at different times by using the controls as baselines. Ideally, controls used in resistance studies would be clones of a genotype in order to reduce potential variability in disease susceptibility due to genetics. One limitation to this study is seedlings were used instead of clones, which may have been an additional source of variation in disease susceptibility.

Investigators have used terms such as the greatest and highest level of susceptibility when referring to inoculation timing (Pomerleau 1965, Smalley and Kais 1966, Takai and Kondo 1979). The use of these terms is problematic, as they do not have a universal definition. Should greatest susceptibility be based on the inoculation time when the highest percentage of trees show visible wilt symptoms when later rated or when the trees display the highest mean wilt symptoms when later rated? If mean wilt symptoms are to be used, how long after inoculation should trees be rated for wilt symptoms? For the purpose of this study, the time of greatest susceptibility was considered the inoculation time that resulted in highest mean percent wilt 8 WPI. Additional studies with more inoculation time periods, such as every week, could be used to more accurately determine the time of greatest susceptibility.

A common finding amongst scientists who have performed studies to examine the effect of timing of inoculation is that results vary depending on year (Pomerleau 1965, Smalley and Kais 1966, Tchernoff 1965). Although the recommended use of calendar dates or days after budbreak allows for simplicity in inoculation timing, variation in weather from different years and locations, limits its reliability. A method to help reduce the variability caused by weather conditions would be to use growing degree days. Takai and Kondo (1979) conducted a study examining the effects of timing of inoculation on disease susceptibility. After examining disease severity and mortality they calculated the growing degree days which corresponded to the inoculation dates for the beginning and end of greatest susceptibility. A critical component of calculating growing degree days is base temperature. Takai and Kondo (1979) arbitrarily selected 5.6 °C as their base temperature. Mathematical equations are available to determine the appropriate base temperature for growing degree days (Yang et al. 1995), however, before a base temperature can be determined, the inoculation time of greatest susceptibility must be defined. For future studies investigating the effects of timing of inoculation on symptom development,

we suggest including the location of the trial, the date of budbreak, and the 8 WPI wilt rating so results from this study can be combined with that of others to more accurately determine the number of growing degree days to the time of greatest susceptibility. Factors other than timing have also been shown to affect periods of greatest susceptibility. Smalley and Kais (1966) found that plant size as well as inoculation method, branch versus trunk inoculations, impacted the duration and timing of susceptibility. Therefore, caution should be used when comparing experiments examining resistance when different methods were used.

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