CHALLENGE INOCULATIONS TO TEST FOR DUTCH ELM DISEASE TOLERANCE: A SUMMARY OF METHODS USED BY VARIOUS RESEARCHERS

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Abstract.—A variety of methods have been used by different research groups to "challenge" inoculate American elms (*Ulmus americana*) with the purpose of determining whether some clones may be resistant to the Dutch elm disease fungus. The methods used by seven research groups are described, along with observations on complications and benefits associated with each. The response of test trees to challenge is affected by many factors, including the age of parent material, size/maturity of test material, vigor of the plant being inoculated, portion of the plant inoculated, season/time of year, source of inoculum, amount of inoculum, and method of delivery. The testing goal must be kept in mind when choosing methods, and the details of what methods were used must be described when reporting results. Inclusion of susceptible and resistant controls is critically important, as it allows calibration of response between different studies.

Introduction

Over the decades, many researchers have used a variety of methods to challenge elm plant material with the Dutch elm disease (DED) pathogens, *Ophiostoma ulmi* (Buisman) and *O. novo-ulmi* (Brasier). The methods have evolved over time, with variations in the methodology even within a particular working group. During the elm workshop, Raymond P. Guries, Garrett L. Beier, Susan E. Bentz, and James M. Slavicek participated in a panel discussion to share information about their standard methods. Alden "Denny" Townsend provided comments in advance. The summary presented here is a combination of information presented by the panel and also captured from related literature. This summary contains an overview of the methodologies used by several working groups, along with some of the complications and benefits associated with each. A discussion section highlights common themes and factors to consider when choosing a challenge protocol.

Note that the terms "resistance" and "tolerance" are both used in this summary, based on that term the particular work group uses. In a human medical sense, the general public tends to interpret "resistance" as meaning that the trees cannot become infected by the pathogen. The definition in plant pathology, however, is the ability to exclude or overcome, completely or to some degree, the effect of a pathogen (Agrios 2005). Townsend (2000) prefers the term "tolerance" because it implies that the pathogen is able to infect the tree but the result in no long-term deleterious effects.

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Overview of Methods

Wisconsin Methods

Beginning in the 1960s and continuing through the 1990s, researchers at the University of Wisconsin were highly active in testing American and other elms for resistance to DED. They used field trials and then also developed a greenhouse screening method (Smalley and Guries 1993).

The field trial approach involved producing rooted cuttings (ramets) of the trees to be tested and then outplanting them to field plots for 3 to 6 years prior to testing. The test trees were challenged by using a hypodermic needle to inject a suspension of 10^6 spore/ml into 0.1-mm drill holes in small branches in the crown. The choice to use small holes on small branches was intended to mimic the type of wound to which bark beetles would introduce the pathogen. Trees were evaluated after 2 to 3 months and again after 1 year post-inoculation. One observation from the years of field testing was that the period of susceptibility was quite variable and was affected by soil moisture, air temperature, age of the plant material, and other factors. Resistance appeared to increase with age while the length of the susceptible period decreased (Smalley and Guries 1993). Field testing required a large area of land and a long period of time; results were highly variable.

Wisconsin also developed a greenhouse methodology to reduce the costs of land and labor, speed up the screening process, and standardize conditions (Green et al. 1984). Root cuttings produce multiple shoots that can be harvested as softwood cuttings after 2 weeks, then rooted and planted into a controlled climate greenhouse. These cuttings generally produce large, vigorous, uniform plants that can be challenge inoculated after 2 to 3 months in the greenhouse. The inoculation method used was a drip hole drilled into the stem, which was then filled to runoff via hypodermic needle with a calibrated dosage (10^6 spores/ml) of mixed spores of *Ophiostoma ulmi* and *O. novo-ulmi.* After 1 to 2 months, the stems of the most susceptible clones exhibited complete mortality, but crown damage was not primarily used for evaluation. Instead, the stems of inoculated shoots were peeled and the extent of discoloration, as measured by height and width of the lesion in the xylem, was recorded as a measure of susceptibility. This approach compressed the testing period to less than 1 year. It was a very aggressive challenge inoculation, but it did allow differentiation in response. Researchers found that rankings of response by clones, families, and accessions were generally similar between greenhouse and field testing approaches. Field trials were still conducted but with fewer clones as the most susceptible were generally not included.

National Arboretum Methods

The methodology of researchers at the Agricultural Research Service (ARS) National Arboretum during the 1990s was field trials with multiple ramets of the test cultivars. Townsend noted[2] that ARS researchers tried the University of Wisconsin methods of inoculation (small wounds in upper crown), but were only able to evoke minor symptoms, so instead they used a main stem wound. This method allowed sufficient foliar symptoms and branch dieback to differentiate the clones. In some studies, material was greenhouse grown for a year then outplanted to the field site for 2 or 3 years prior to inoculation (Townsend et al. 1995). Inoculations were made into a 2.4-mm hole in the lower one-third of the main trunk. The spore suspension consisted of 3×10^6 spores/ml of a mixture of *O. ulmi* and *O. novo-ulmi*. Crown symptoms were recorded 4 weeks after inoculation and dieback was recorded after 1 year. Inoculation earlier in the season resulting in greater crown symptoms. In another study, test cultivars were outplanted to the field site 9 years prior to inoculation, and evaluations were conducted 4 weeks, 1 year, and 2 years after inoculation (Townsend et al. 2005). In some

studies conducted in Ohio, ARS scientists found that trees younger than 3 years had "juvenile resistance".[2]

Forest Service Methods

The inoculation methods currently being used by the U.S. Forest Service Northern Research Station in Delaware, Ohio, are a continuation of methods similar to those previously used by ARS. Ramets of the test material are outplanted to field plots and grown for 3 to 7 years prior to challenge inoculation. The point of inoculation is a hole drilled at an angle downward into the main stem of the tree, approximately 1 foot above soil level. The hole accommodates all of the calibrated spore suspension, so all trees received equal dosage. Small branches are pruned from the main stem for several feet above the point of inoculation so that the spores are not translocated into side branches.

As an example of the specific methodology used in one recent experiment, American elm trees were inoculated with a 50/50 mixture of *O. ulmi* and *O. novo-ulmi* spores on June 7 and 8, 2016. The inoculum was prepared a week in advance as follows: frozen cultures of *O. ulmi* (strain PG442) and *O. novo-ulmi* (strain H961) were thawed and spread on separate potato dextrose agar plates, 50 µl/plate, and nine plates/isolate. The plates were kept dark and at room temperature. Fungal spores were harvested after 11 days of growth by addition of sterile deionized water to the plate surface. The surface was scraped gently with a bent glass rod and the spores of each isolate were removed to a separate sterile 50-ml conical tube. Fungal spore concentrations for each isolate were determined using a hemocytometer. The final 50/50 concentration of spores was adjusted to a volume appropriate for the inoculation of trees. Trees in field plots received either 6×10^5 or 1.2×10^6 spores; potted elms were inoculated with a total of 2.8×10^4 spores. A 0.5 cm diameter brad point bit was used to drill a 1.3 cm deep hole 30 cm from the base of trees located in field plots, and the fungal spores were pipetted into the hole. A 0.2-cm-diameter bit was used to drill a 0.6 cm deep hole 15 cm from the base of potted trees and the fungal spores were pipetted into the hole (Pinchot et al., in press).

Minnesota Methods

University of Minnesota researchers have been focused on screening "survivor elms" for resistance. To generate ramets, dormant shoots are collected and grafted onto established seedling rootstocks to obtain vigorous scionwood. Softwood cuttings are then collected and placed in a peat/perlite mix to promote rooting. After softwood cuttings have developed sufficient roots, they are transplanted to a larger container and subsequently planted in the field for field trials. For most studies, inoculations take place after the test trees have reached a minimum diameter at breast height (d.b.h.) of 2.5 cm. Inoculations are made by using a 2.38-mm drill bit to make a downward slanted hole in the stem, approximately 4 mm deep. Depending on the size of the tree, the hole is filled with 25 to 60 µl of an *Ophiostoma novo-ulmi* spore suspension at 1×10^6 spores/ml. Inoculations are timed to occur at about 40 days following budbreak (Tchernoff 1965), which is usually late May or early June. Based on field trials in 2015 and 2016, it was found that trees were highly susceptible when inoculated approximately 270 growing degree days (base 50) after budbreak. Growing degree days are calculated by averaging the daily maximum temperature and minimum temperature and subtracting the base temperature. If the average of the maximum and minimum temperature is not greater than the base temperature, there are no growing degree days accumulated on that day. Trees are evaluated

[2] Personal communication from Alden Townsend, retired, USDA Agricultural Research Service, U.S. National Arboretum, 3501 New York Ave. NE, Washington, DC 20002.

biweekly for 10 to 14 weeks for percentage of the crown exhibiting permanent wilt following inoculation. Additionally, evaluations are made 1 year post inoculation. Researchers have observed that variation in the timing of inoculations can result in differing levels of susceptibility, with late May inoculations resulting in more severe symptoms.

Greenhouse trials have also been conducted to screen genotypes for resistance. Currently, trees are being screened in the greenhouse and then later in the field to determine if correlations exist between greenhouse screening results and field screening results. Plant material utilized in greenhouse screening experiments are considerably smaller than those in field trials. Generally, plants are inoculated 1 year after being grafted or rooted. Inoculations are made in the same method as described above, except for the size of the inoculation hole and the amount of inoculum injected. Due to the small size of the plant material, 15 to 25 µl of spore suspension at 1×10^6 spores/ml are injected into a 1.59 mm diameter hole approximately 3 to 4 mm deep.

Other Methods Not Addressed During Panel

The methodology used by the National Park Service in Washington, D.C., in the 1960s and 1970s was twig inoculation. Wester (1972) described propagation by grafting buds from select large elm trees onto 2-year-old elm seedlings, then growing the budded plant material for 4 years prior to challenge inoculation. Strong shoots that were 2 years old were selected for inoculation, and inoculations took place about 15 cm below the current growth, during the month of June. Small holes, 1 to 2 mm diameter, were drilled approximately 1 mm deep into the tissue, then flooded with a heavy suspension of *O. ulmi* spores and sealed. Trees were evaluated for wilt symptoms for two seasons after inoculation.

European methods (Tchernoff 1965) involve active sucking of spore suspension into cut xylem elements. For older trees, a utility knife is used to slash the stem, and then while the knife is still in the wound, at least 4 drops of a spore suspension are placed on the wound so that they can be "sucked" into the xylem by vascular tension. On younger trees, a small surgical chisel (with a 2 mm wide point) is used to create a wound into which at least one drop of spores is allowed to be "sucked" in.

Takai and Kondo (1979) compared four inoculation methods, including introduction of spores into drill and 6.5-mm chisel wounds in the lower stem and introduction of spores to a scalpel slit on an upper branch, and pressure injection of spores into a drill hole on an upper branch. The two basal stem methods resulted in more severe and rapid disease development. The two upper branch methods were more similar to the overland transmission by bark beetles. Takai et al. (1979) also infected young elm stems by caging naturally infested native elm bark beetles on the stem, resulting in an inoculation method more representative of natural conditions.

Discussion

Many factors influence the response of test trees to challenge: age of parent material, size/maturity of test material, vigor of the plant being inoculated, portion of the plant inoculated, season/time of year, source of inoculum, amount of inoculum and method of delivery (Takai and Kondo 1979, Tchernoff 1965, and all of the authors involved in writing this summary). There are different methods, but there is no right method. It is important to specify details on what methods were used in testing, including the source of inoculum and timing of testing. Inclusion of susceptible and resistant controls is critically important, as it allows calibration of response between different studies.

The goal of the testing must be kept in mind. More severe testing may eliminate some sources of resistance or tolerance, such as unattractiveness to beetles, which under natural conditions could result in trees being less likely to become infected. Some of these more discrete sources of resistance may be valuable to persistence of American elms in natural forests, even if they are not strong enough to "guarantee" an elm is DED resistant for an urban planting. When publishing results, it is important to interpret the implications of testing methods so that the public does not infer some degree of resistance or tolerance as "immunity."

Age of the plant material at time of testing has often been a topic of discussion. Solla et al. (2005) demonstrated that vascular tissue of *Ulmus minor* under 4 years old was structurally different from older plants, and, correspondingly, DED symptom expression was greater in the older plants. They cite older research papers (Caroselli and Feldman 1951, Neely 1968) that report a similar response in *Ulmus americana*. This supports the popular idea of "juvenile resistance" to DED. However, Wisconsin research (Smalley and Guries 1993) did not necessarily confirm this concept; they were able to establish a correlation between response of juvenile and mature tissue and then used this relationship to enable screening within a shorter timeframe. Minnesota researchers have also observed that young material is highly susceptible. The testing of younger tissue as a means of predicting durable resistance could use further investigation.

Field trials present additional complications. In some locations, herbivory by deer, rabbits, and voles make it difficult to obtain consistent, healthy plant material. Root grafts between adjacent trees can confound inoculation trials. Local populations of naturally occurring DED and elm yellows phytoplasma can also affect studies and testing. Disease development in individual plants is affected by the vigor of the plant, thus as weather, fertilizer, soil moisture content, and other conditions affect plant health, they also affect testing results.

Challenge inoculations do tell us that some American elm trees have a superior ability to survive infection by the DED fungus. We also must consider that challenge inoculation does not give us complete information about the ability of elms to survive long-term on the landscape.

Literature Cited

Agrios, G.N. 2005. **Plant pathology, 5th ed.** London, UK: Elsevier Academic Press. 922 p.

Caroselli, N.E.; Feldman, A.W. 1951. **Dutch elm disease in young elm seedlings.** Phytopathology. 41: 46-51.

Green, C.E.; Guries, R.P.; Smalley, E.B. 1984. **Early screening of elms for resistance to *Ceratocystis ulmi.*** Plant Disease. 69: 60-63.

Neely, D. 1968. **Twig inoculations on American elm with *Ceratocystis ulmi.*** Phytopathology. 58: 1566-1570.

Pinchot, C.C.; Flower, C.E.; Knight, K.S.; Marks, C.; Minocha, R. [et al.]. In press. **Development of new Dutch elm disease-tolerant selections for restoration of the American elm in urban and forested landscapes.** In: Sniezko, R.A.; Man, G.; Hipkins, V. [et al.], tech. cords. Gene conservation of tree species–banking on the future. Proceedings of a workshop. Gen. Tech. Rep. PNW-GTR. Portland, OR: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station.

Smalley, E.B.; Guries, R.P. 1993. **Breeding elms for resistance to Dutch elm disease.** Annual Review of Phytopathology. 31: 325-352.

Solla, A.; Martín, J.A.; Ouellette, G.B.; Gil, L. 2005. **Influence of plant age on symptom development in *Ulmus minor* following inoculation by *Ophiostoma novo-ulmi*.** Plant Disease. 89: 1035-1040.

Takai, S.; Kondo, E.S. 1979. **Seasonal development of Dutch elm disease on white elms in central Ontario, Canada. I. Following wound inoculation.** Canadian Journal of Botany. 57: 341-352.

Takai, S.; Kondo, E.S.; Thomas, J.B. 1979. **Seasonal development of Dutch elm disease on white elms in central Ontario, Canada. II. Following feeding by the North American native elm bark beetle.** Canadian Journal of Botany. 57: 353-359.

Tchernoff, V. 1965. **Methods for screening and for rapid selection of elms for resistance to Dutch elm disease.** Acta Botanica Neerlandica. 14: 409-452.

Townsend, A.M. 2000. **USDA genetic research on elms.** In: Dunn, C.P., ed. The elms: breeding, conservation and disease management. Boston, MA: Kluwer Academic Publishers: 271-278.

Townsend, A.M.; Bentz, S.E.; Douglass , L.W. 2005. **Evaluation of 19 American elm clones for tolerance to Dutch elm disease.** Journal of Environmental Horticulture. 23(1): 21-24.

Townsend, A.M.; Bentz, S.E.; Johnson, G.R. 1995. **Variation in response of selected American elm clones to *Ophiostoma ulmi*.** Journal of Environmental Horticulture. 13(3): 126-128.

Wester, H.V. 1972. **Investigation of American elm clons [sic] based on physiological vigor for crown resistance to Dutch elm disease.** Plant Disease Reporter. 56(8): 725-727.