2019 TACF Project Report:

Development of an In Vitro Excised Twig Assay to Identify Resistance to *Phytophthora cinnamomi* in Hybrid Chestnut Trees

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Introduction

Phytophthora root rot is a lethal disease of the American chestnut tree (*Castanea dentata*) that was killing trees in the southern range of this native forest tree species long before chestnut blight was reported in North America (Anagnostakis 2012, Crandall et al. 1945). Currently, the American Chestnut Foundation (TACF) has an active breeding program to develop backcross hybrid American chestnut trees ([*C. mollissima* × *C. dentata*] × *C. dentata*) with resistance to both *Phytophthora cinnamomi*, which causes Phytophthora root rot, and to *Cryphonectria parasitica*, which causes chestnut blight (www.acf.org). For the past 14 years, we have worked in collaboration with TACF to develop a seedling inoculation procedure and to conduct annual trials with this procedure to screen backcross hybrid chestnut family progenies for resistance to *P. cinnamomi*. These seedling trials have become the standard for evaluating chestnut progeny for resistance to *P. cinnamomi* and currently are the best screening method available (Jeffers et al. 2009; Jeffers et al. 2012). Results from this 14-year collaborative effort to identify resistance to *P. cinnamomi* recently were published (Westbrook et al. 2019). In addition, Zhebentyayeva and colleagues (2019) are making progress in identifying specific genes associated with resistance to *P. cinnamomi*.

Conducting seedling trials is an effective method for determining resistance, but it is a labor-intensive and time-consuming process that requires a team of individuals and a full year to obtain results. Seeds need to be collected, stratified, and planted, and then seedlings need to be grown, nurtured, inoculated, and evaluated. Consequently, the development of an alternative assay to evaluate resistance to *P. cinnamomi* in backcross hybrid chestnut trees that does not require growing seedlings would greatly accelerate the breeding process and create a more targeted breeding strategy. The successful development of another method to evaluate resistance will not replace the need to conduct seedling trials but, instead, will complement trials with seedlings and provide additional insight into hybrid tree resistance. Having two methods to evaluate resistance to *P. cinnamomi* should enhance the breeding program conducted by TACF.

Excised twigs previously have been used to study the interaction between *Phytophthora* species that cause root and crown rot on apple trees and apple scion and rootstock cultivars—including resistance of host plant cultivars, virulence of the pathogens, and seasonal variation in host susceptibility (Jeffers et al. 1981; Jeffers and Aldwinckle 1986; Utkhede and Quamme 1988). More recently, Santos et al. (2015) used excised shoot pieces and a root inoculation assay to determine resistance to *P. cinnamomi* in two F1 crosses: European chestnut × Japanese chestnut (*C. sativa* × *C. crenata*) and European chestnut × Chinese chestnut (*C. sativa* × *C. mollissima*). These authors were able to show a significant correlation

between resistance determined by an in vitro shoot assay and resistance determined by a whole plant root inoculation assay using clonal plant material for each assay. Consequently, in 2017-18, we began to experiment with excised twigs to determine if this method could be used to measure relative resistance to *P. cinnamomi* in American, Chinese, and backcross hybrid chestnut trees.

Initially, we compared lesion lengths created by *P. cinnamomi* infection on twigs from susceptible American and resistant Chinese chestnut trees. These preliminary results consistently showed a consistent difference in lesion lengths on twigs from American and Chinese chestnut trees when an excised twig assay was used (*data not shown*). Therefore, we used these two species as controls in experiments with twigs from hybrid chestnut trees. This study tested twigs from several different chestnut tree species and hybrids: American, Chinese, F1 hybrids (*C. dentata* × *C. mollissima, C. dentata* × *C. crenata,* and *C. mollissima* × *C. dentata*), and BC3F2 hybrids ([*C. mollissima* × *C. dentata*] × *C. dentata*) trees.

The primary goal of this project was to develop and validate an in vitro assay using excised dormant twigs to rapidly and consistently identify resistance to *P. cinnamomi* in backcross hybrid American chestnut trees. More specific objectives were to examine the amount of variation in lesion lengths among American, Chinese, or F1 hybrid chestnut trees with different lineages and to compare lesion lengths on twigs from BC3F2 hybrid chestnut mother trees that produced seedlings previously used in Phytophthora root rot resistance seedling inoculation trials.

Materials and Methods

Collection of dormant chestnut shoots

Dormant shoots were collected from 89 chestnut trees at The American Chestnut Foundation (TACF) Meadowview Research Farms in Meadowview, VA. Additional shoots were collected from the TACF Carolinas chapter Pryor Orchard in Edneyville, NC. The 89 chestnut trees sampled included American, Chinese, F1 hybrids, and BC3F2 backcross hybrids (Table 1); the BC3F2 hybrids were selected based on past performance of their progenies in *P. cinnamomi* seedling trials (Westbrook et al. 2019). All shoots were collected from 31 January to 04 February 2019 because the excised twig assay requires dormant shoots that have only grown for one season. Therefore, only shoots that were produced during the 2018 growing season were collected. Shoots were cut from the terminal ends of branches, resulting in lengths approximately 15 to 90 cm. Cut shoots from each tree were placed in sealed plastic bags and held at ambient outside temperatures until the bags could be stored in a cold room at 4°C until use. Cut shoots were at ambient outside temperatures for no longer than 5 days before cold room storage. Most shoots were cut from the top crown of trees using a 6-meter-long pole pruner. Some shoots also were collected at ground level.

Excised twig assay

Inoculation tube preparation. *P. cinnamomi* isolate JJ-1 was used to develop the excised twig assay. This isolate has been used extensively to screen chestnut trees for resistance to this pathogen (Westbrook et al. 2019). The excised twig assay was conducted using individual culture tubes for each single-twig replicate. Inoculation tubes were prepared using aseptic technique by pipetting 1.5 ml of sterile PAR-V8 agar (Ferguson and Jeffers 1999; Jeffers 2015) into sterile 17 × 100-mm sterile polypropylene culture tubes without caps. The agar medium was allowed to solidify overnight before pipetting 0.5 ml of *P.*

cinnamomi mycelium agar suspension (PcMAS) into each tube on top of the PAR-V8 medium. The PcMAS was made by crushing fully colonized PAR-V8 agar in a standard disposable Petri plate into 0.25- to 2-mm-wide pieces. Agar was crushed using the top of a sterile, 25-mm-diameter test tube with a flat screw-on cap. The fully colonized PAR-V8 agar was prepared by growing isolates of *P. cinnamomi* in (90 × 15-mm) Petri plates containing 15 ml of PAR-V8 medium. If plates were not fully colonized, the non-colonized portion was removed before crushing. Cultures were incubated in the dark at 25°C for 5 to 9 days. The crushed agar was mixed with sterile reverse-osmosis (RO) water at a ratio of 1:20 (i.e., 10 ml crushed agar/200 ml water). The PcMAS was constantly stirred with a magnetic stir bar to ensure a homogenous suspension during pipetting into inoculation tubes. Twigs pieces and the PcMAS were added to inoculation tubes on the same day as (day 1 of assay).

Twig assay overview. The assay was conducted by placing surface disinfested 70-mm-length twig pieces into inoculation tubes containing actively growing *P. cinnamomi* mycelium. Inoculated twigs were incubated for 14 days in the dark at 20°C and then lesion lengths were measured.

Twig inoculation. Enough dormant shoots for each tree were removed from cold storage to cut 20 70-mm-long twig pieces. Shoots were disinfested in 0.6% sodium hypochlorite plus 0.025% Tween 80 in RO water for 5 minutes and then rinsed in three successive containers of RO water to remove disinfesting solution. Disinfested shoots were cut aseptically into 70-mm pieces (i.e., twigs). Twigs were cut from the distal end of the shoot toward the terminal bud. Whole shoots were cut into twigs restricting the diameter of the base of twigs to a range between 4 to 7 mm. Freshly cut twigs immediately were placed into inoculation tubes. Twig pieces were oriented in the inoculation tubes with buds pointing upward—i.e., with the distal end sitting on the PAR-V8 agar and immersed in the PcMAS suspension. When all twig pieces were inoculated, the tubes were placed upright in racks into clear plastic boxes with non-airtight lids (32 × 26 × 10 cm; Box 395C; Pioneer Plastics, Dixon, KY). Each box held 130 inoculation tubes, and boxes were randomly assigned a position in a low-temperature incubator set to 20°C. All boxes for a trial were placed in the same incubator.

Trials and replicates. Two independent trials were conducted. Trial 1 was started approximately 14 days after shoots were collected, and Trial 2 was started approximately 38 days after shoots were collected. In each trial, all 89 trees were tested by inoculating 20 replicates twigs plus two non-inoculated control twigs for each tree. Therefore, a total of 1,958 twigs were used in each trial. Twigs from as many as 16 trees were inoculated in one day; therefore, a 6-day period was required to assay all 89 tree samples. The 89 trees were randomly assigned an assay start date during this 6-day period.

Lesion length measurement. After 14 days of incubation and lesion development, twigs were removed from inoculation tubes, surface disinfested, and wiped with 70% ethanol to remove attached mycelium of *P. cinnamomi*. Lesions on individual twigs were measured by removing a strip (2 to 3 mm wide) of periderm to expose the internal lesion in the phloem and cambium layers. If the lesion margin could not be seen clearly, the lesions were visualized using an iron sulfate-hydrogen peroxide staining method (described below). Internal lesion lengths were measured to the nearest millimeter with a Vernier caliper. Lesions were measured from the base of the twigs to the highest section of the lesion margin that covered at least 25% of the twig circumference. Lesions on twigs pieces were measured over a 6-day period to correspond with the 6-day assay period start dates. Means, standard deviations, and medians were calculated for each tree in each trial. In addition, the same calculations were made for the two trials combined.

Inoculation with *P. cambivora*. In a preliminary trial to explore the potential utility of the excised twig assay, trees with excess shoots were assayed using isolate VA2 Midwest of *P. cambivora*, another species of *Phytophthora* that was documented to be pathogenic on American chestnut (Sharpe 2017). Six American, five Chinese, four F1, and one BC3F2 chestnut trees were assayed in both trials at the same time as inoculations with the *P. cinnamomi*. Ten replicate twigs from each tree were inoculated in each trial. Lesions lengths were measured as described above, and means and standard deviations were calculated for each trial and for the trials combined.

Iron sulfate-hydrogen peroxide staining method

On some twigs, lesion lengths were difficult to determine visually because there was not much discoloration of the colonized phloem and cambium tissues. Therefore, an iron sulfate-hydrogen peroxide staining method was developed to better visualize colonized tissue and measure actual lesion lengths. To visualize internal lesions with this method, twig pieces with stripped periderms were placed in 0.18 M iron (II) sulfate heptahydrate (5 g/100 ml water) adjusted to an approximate pH of 3.0 with 2.0 M hydrochloric acid (300 µl/100 ml) measured with pH paper. Twigs were submerged in the iron solution for a minimum of 2 sec and maximum of 60 sec. Twigs were then transferred to a water rinse to remove excess iron solution (1 liter of RO water) for a minimum of 2 sec and a maximum of 3 min. Rinse water was replaced when water developed a cloudy orange tint (from oxidized iron). Twigs were shaken to remove excess rinse water and placed in 3.0% hydrogen peroxide. The 3.0% hydrogen peroxide was adjusted to an approximate pH of 3.0 with 2.0 M hydrochloric acid (300 µl/200 ml) measured with pH paper. Twigs were submerged in the hydrogen peroxide solution for a minimum of 2 sec and a maximum of 15 sec. Lesion lengths were measured immediately after removal from the hydrogen peroxide solution. After staining, internal lesions turned dark brown to black and were easily measured. The portions of phloem and cambium tissues that were not colonized by *Phytophthora* spp. were not noticeably stained and showed minimal color change compared to colonized tissues.

Verification of twig infection

Eleven trees were selected to verify that lesion lengths corresponded with colonization by *P. cinnamomi*: seven trees in Trial 1 (two American, two Chinese, one F1 hybrid, and two BC3F2 hybrids) and four trees in Trial 2 (one each of American, Chinese, F1 hybrid, and BC3F2 hybrid) (Table 2). Inoculated twigs were prepared for lesion length measurements as described above except that the periderm strips from inoculated twigs were embedded in PAR-V8 medium adjusted to 0.75% agar to visualize hyphae growing out of periderm tissue. Agar plates were incubated for 2 days in the dark at 25°C. The length of periderm from which hyphae grew was measured.

Excised twig assay for four other species of *Phytophthora*—Preliminary experiment

A preliminary experiment was conducted to determine if the excised twig assay could be used to evaluate resistance to other species of *Phytophthora* spp. pathogenic to American chestnut (Sharpe 2017). Four species of *Phytophthora* were compared—including two isolates of *P. cambivora* and one isolate each of *P. cinnamomi*, *P. cryptogea*, and *P. heveae* (Table 3). Twigs used in this experiment were from excess shoots not used in the main experiment, which resulted in twigs being inoculated 60 days after collection. For each isolate of the four species of *Phytophthora*, 78 twigs from 10 American trees were inoculated: EM-31 (8 twigs), SM-68 (8 twigs), AN-35 (9 twigs), EM-50 (10 twigs), AN-32 (7 twigs), AN-86 (9 twigs), SM-17 (7 twigs), SM-68 (6 twigs), AN-74 (7 twigs), and EM-102 (7 twigs). Twigs were inoculated and lesion lengths were measured as described above.

Results

Staining twigs with Iron sulfate-hydrogen peroxide provided consistent and accurate visualization of internal lesions

The visual appearance of lesions caused by *P. cinnamomi* and the other four species of *Phytophthora* in cambium and phloem tissues of infected twigs varied among and within chestnut species. Lesions on twigs from resistant Chinese chestnut trees generally were the most conspicuous with some lesions exhibiting a clear lesion margin with contrasting color compared to non-necrotic tissue (Figure 5). Lesions on twigs from susceptible American chestnut trees generally were less conspicuous and usually lacked a clear lesion margin. Lesion appearance on twigs from F1 and BC3F2 hybrid trees varied widely across a spectrum of clearly visible to inconspicuous.

To accurately visualize internal lesions that could not be distinguished with the naked eye, a method was developed to stain tissues colonized by species of *Phytophthora*. This method sequentially exposed cambium and phloem tissues to dissolved iron (II) sulfate heptahydrate and then to hydrogen peroxide. When a twig with exposed cambium and phloem tissues was submerged in iron (II) sulfate heptahydrate solution, tissues colonized by *Phytophthora* spp. stained a light blue-gray color whereas the color of healthy, non-colonized tissues remained unchanged. After staining in iron (II) sulfate heptahydrate, twigs were placed in a hydrogen peroxide solution. Within seconds, the colonized phloem and cambium tissues turned from light blue gray to black, which clearly exposed the lesions (Figure 4). After the lesions turned black, the extent of lesion development, which previously was inconspicuous, could be measured accurately.

To validate the consistency and accuracy of the iron sulfate-hydrogen peroxide staining method, lesion lengths on twigs from 11 trees (seven in Trial 1 and four in Trial 2) were measured before and after staining, and the periderm strip was placed in isolation medium to document the extent of *P. cinnamomi* colonization (Table 2). There was no significant difference between mean lesion lengths measured before or after staining for all trees tested. Lesions lengths based on hypha growth from the colonized periderm strip consistently had a greater mean length that was significantly greater than the lesions lengths measured on the twigs—either before or after staining (Table 2). This result was expected because the twig periderms were plated in agar medium and incubated for two days at 25°C until hyphae of *P. cinnamomi* could be seen growing out into the agar medium. Therefore, the pathogen had two additional days to move distally in the periderm. Successful growth of *P. cinnamomi* hyphae from twig periderms also confirmed that the lesions were caused by pathogen colonization.

Lesion lengths varied among tree species and hybrids

Once all lesions could be accurately visualized, twigs from the 89 trees were uniformly inoculated and lesion lengths were measured following a standard procedure (Table 1; Figures 1 and 2). Lesion lengths varied within each tree species and among the different chestnut species and hybrids. Variation among replicates for individual trees, based on standard deviations (n = 20), was relatively consistent among trees from all chestnut species and between trials. Standard deviations ranged from 3 to 17 with most between 3 and 10 (Table 1). However, standard deviations did appear to be correlated with overall mean lesion length—with greater lesion lengths having greater standard deviations (see data for *All trees* in Table 1). Therefore, means for both trials combined were compared using a Kruskal-Wallis rank sum test (Figure 2). There was a significant and obvious difference between lesion lengths on twigs from

American and Chinese trees; lesions on twigs from Chinese trees shorter than lesion lengths on twigs from American trees. There also was a significant difference among the lesion lengths on twigs from American, Chinese, and F1 hybrids—with lesions lengths on F1 hybrids intermediate between American and Chinese. Lastly, there was a significant difference between lesion lengths from BC3F2-Resistant trees and BC3F2-Susceptible trees—with lesions on the susceptible hybrids slightly longer than those on the resistant hybrids.

Diameter of twig pieces had no effect on lesion length

To determine if the diameter of twig pieces influenced pathogen colonization and lesion length, 20 twig piece diameters were measured for each of 27 trees—including seven American, six Chinese, five F1 hybrids, and nine BC3F2 hybrids (23 trees from Trial 1 and four trees from Trial 2; Table 4). The numbers of twigs in each diameter class varied considerably with most twigs having a base diameter of 5 or 6 mm; twigs with these two diameters accounted for 74% of the 537 twigs measured for this experiment Table 4). However, there was no statistically significant difference among lesion lengths on twig pieces with base diameters of 4, 5, 6, and 7 mm (Table 4).

Lesion length increased slightly with time in storage

Lesion lengths increased on twigs from 58 (65%) trees and decreased on twigs from 31 (35%) trees between Trial 1 and 2 (Table 1); however, this increase was affected by tree species. Lesion lengths on twigs from all American trees were greater in Trial 2 compared with those in Trial 1, but lesion lengths in Trial 2 compared to Trial 1 were greater on 50% of Chinese trees, 42% of F1 hybrids, 65% of BC3F2-Susceptible hybrids, and 74% of BC3F2-Resistant hybrids (Table 1). Trial 1 was started approximately 14 days after shoot collection, and Trial 2 was started approximately 38 days after shoot collection. The increase in lesion length on twigs from American trees with additional time in storage is supported by the experiment comparing different *Phytophthora* spp. (Table 3). Twigs in this experiment were inoculated 60 days after collection, and the mean lesion length caused by *P. cinnamomi* was greater than the lesion lengths in Trials 1 and 2. Therefore, lesion lengths appear to increase somewhat the longer shoots are stored.

Visual appearance of lesions differed across all tree species and hybrids

Lesion appearance varied within tree species and BC3F2 hybrid tree groups. The severity of necrosis did not appear to be correlated with an increased lesion length. Lesion appearances could be put into the following categories: (1) clearly visible necrosis and appearance of a dark border at the lesion margin—generally seen on the shortest lesions; (2) clearly visible necrosis and no dark border; (3) complete brown necrosis of entire twig piece; (4) barely or partially visible necrosis; and (5) inconspicuous necrosis requiring iron sulfate-hydrogen peroxide staining for visualization (Figure 5). The majority of trees displayed lesions with appearances in categories 2, 4, and 5 (*data not shown*). Lesion appearance does not appear to correlate with lesion length (*data not statistically analyzed*). Only lesions with appearances in categories 1 and 2 consistently resulted in lesions less than 20 mm in length.

Different species of *Phytophthora* exhibited differential lesion lengths on excised twigs

The trees that were assayed using the *P. cambivora* VA2 Midwest isolate during Trial 1 and Trial 2 showed remarkably similar lesion lengths to those caused by with *P. cinnamomi* JJ-1 (Table 1; Trials 1 +2 combined). Six American, five Chinese, four F1 hybrids, and one BC3F2 hybrid trees were assayed in Trial

1 and Trial 2 at the same time as inoculations with *P. cinnamomi*. This result is not totally unexpected because the *P. cambivora* VA2 Midwest isolate was identified to be the most virulent isolate of *P. cambivora* tested on American and Chinese chestnut seedlings in a greenhouse experiment where root rot severity was being evaluated (Sharpe 2017).

In a preliminary experiment, twigs from 10 American chestnut trees were inoculated with isolates of four species of *Phytophthora* following the standard excised twig assay procedure (Table 3, Figure 6). *P. cinnamomi*, *P. cryptogea*, and *P.* cambivora isolate VA2 Midwest caused similar lesion lengths that were significantly greater than those caused by *P. cambivora* isolate NC 38364 and *P. heveae*.

Discussion

In this study, we developed a standard procedure for uniformly and consistently inoculating excised twigs cut from dormant shoots produced in the previous growing season. The lengths of lesions that developed on inoculated twigs then could be measured to determine relative resistance in trees with different genotypes. The diameters of excised twigs did not affect lesion development—at least in the range of 4 to 7 mm. However, the duration of time shoots were in cold storage did affect lesion development. Lesions lengths were greater on twigs cut from dormant shoots that were stored for longer periods of time—i.e., lesion length increased as time in cold storage increased. However, the increase in lesion length was consistent among tree species.

We successfully identified differences in resistance to *P. cinnamomi* using the excised twig assay. Twigs from Chinese and F1 hybrid chestnut trees developed significantly shorter lesions than twigs from American chestnut trees. This demonstrated that *P. cinnamomi* colonized the phloem and cambium tissues in American chestnut twigs more extensively than it colonized phloem and cambium tissues in Chinese and F1 hybrid chestnut twigs—presumably because Chinese and F1 hybrid chestnut trees are more resistant than American chestnut trees. The excised twig assay also showed a difference in relative resistance between BC3F2 hybrid trees that produced seedlings that previously were scored as resistant (BCF2-R) and susceptible (BC3F2-S) to *P. cinnamomi* in seedling root rot trials (Westbrook et al. 2019). Twigs from BC3F2-R trees had significantly shorter lesions than twigs from BC3F2-S trees, but this difference was small—about 4 mm based the means of all trees in both trials. Interestingly, overall mean lesion lengths on twigs from BC3F2 hybrids trees were similar to the overall mean lesion length on twigs from the American chestnut trees; however, there was considerable variability in lesion lengths within each tree species. Therefore, the excised twig assay did not identify BC3F2-R trees as more resistant and BC3F2-S trees as more susceptible than American chestnut trees.

The main application for this newly developed excised twig assay would be to infer resistance to *P. cinnamomi* through nondestructive testing in BC3F2 mother and father tree populations. To do this, a correlation must be established between lesion lengths in parent trees and the survival of progeny using the current “gold standard” of Phytophthora root rot seedling trials. Recently, Zhebentyayeva and colleagues (2019) identified several major quantitative trait loci (QTLs) associated with resistance to *P. cinnamomi* based on results using seedling root rot trials. We did not find a clear positive correlation between resistance based on survival in seedling trials and resistance based on lesion length in excised twigs. In addition, Dr. Jared Westbrook has done some preliminary examination of our lesion length data and he corroborated this lack of correlation. Dr. Westbrook found the major resistance QTL intervals in only two of six of the BC3F2 hybrids that had the lowest lesion length, and he found the QTL intervals in one or two of nine of the BC3F2 hybrids that had the longest lesion lengths.

Therefore, it appears that lesion length is governed by the genes that are causing resistance in the seedling assay, but this should not be surprising. First of all, it is important to remember that root rot assays are testing resistance in seedlings produced by male and female parents and that the excised twig assay is measuring resistance of only one of these parents. Secondly, in the excised twig assay, we are inoculating shoots and not roots, and these two organs have different morphologies and physiologies. Lastly, by inoculating excised twigs, we have circumvented all of the primary front-line defense mechanisms that occur in the outer tissues of plants—the bark and periderm on shoots and the epidermis and cortex of roots. Therefore, what we are measuring in the excised twig assay, is resistance to colonization of inner tissues by *P. cinnamomi* once the primary defenses have been breached. These secondary resistance mechanisms are likely governed by genes that are not located in the major QTL intervals identified by Zhebentyayeva et al. (2019). In fact, these authors speculated that resistance to *P. cinnamomi* may be the result of a combination of major QTLs and minor effect genes, so, perhaps, lesion length is regulated by some of these minor effect genes.

The real value of this assay is not to identify resistant species of chestnut but should be to identify individual parent trees that are likely to be more resistant to *P. cinnamomi*. Then, two parent hybrid trees can be crossed to produce progeny with increased resistance from minor genes. Likewise, parent trees with short lesion lengths could be crossed with trees known to have the major QTLs to produce seedlings with improved resistance to *P. cinnamomi*. Experiments can be conducted in the future using controlled crosses to validate or refute these hypotheses.

An interesting take away from this project is that visual appearance of lesions could be used to identify resistance instead of measurements. The trees with the shortest lesion lengths usually had the most visible and necrotic lesions with a very clear lesion border. In fact, some of these short lesions even had a dark line where the lesion stopped. This suggests a reaction similar to a hypersensitive response. Another interesting take away from this project is that the excised twig assay may be useful for comparing relative virulence of different species of *Phytophthora* or different isolates within a species. In a preliminary experiment, we inoculated excised twigs with isolates of four species of *Phytophthora*, and lesion lengths ranked the isolates and species in the same order as a root rot assay conducted previously (Sharpe 2017).

For the excised twig assay to be effective, lesions had to be visible or made visible by some means. Therefore, we also developed an iron sulfate heptahydrate-hydrogen peroxide staining method to clearly visualize lesions produced on twigs from some of the chestnut trees because twigs from these trees had lesions that were inconspicuous and could not be distinguished from healthy tissue. Consequently, lesion lengths could not be accurately measured without staining. We demonstrated that this staining procedure had no negative effect on accurately measuring lesion lengths in excised twigs.

Our hypothesis to explain the success of the iron (II) sulfate heptahydrate-hydrogen peroxide staining method is based on the ancient technique of making iron gall ink (Gerhard et al. 2019). Black iron gall ink is made by combining tannins from wasp galls on oak trees, which are high in tannin, with iron (II) sulfate to create a blue-black ink, which contain ferrous tannate complexes. When the ink is put on paper, it becomes oxidized by air, and the ink turns a darker black because the ferrous tannate complexes are oxidized and form darker ferric tannate complexes. We hypothesize that the same mechanism is occurring to stain lesions in chestnut twigs because American chestnut trees are known to contain high amounts tannins (Lord 2004). Exposing the tannins in the inner twig tissues to iron (II) sulfate heptahydrate produces a reaction like that in the iron gall ink. To instantly oxidize the stained

tissues, twigs were exposed to hydrogen peroxide. We speculate that lesions were stained due to higher amounts of tannin or other phenolic compounds (Crozier et al. 2006) in the tissues colonized by *P. cinnamomi* compared to non-colonized twig tissues. Tannin concentrations in healthy and infected tissue also could be similar, but the tannins in infected tissue became more accessible for staining because of cell wall degradation by *P. cinnamomi* and cell disruption when removing periderm strips. The degree of tissue damage and necrosis appeared to influence lesion appearance after staining. Lesions that were easily seen before staining become much darker after staining compared to lesions that were fainter before staining.

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Table 1: Mean internal lesion lengths for all 89 TACF trees tested with the excised twig assay from Trial 1, Trial 2, and the two trials combined (Trials 1+2). Results for individual trees are ordered within tree species from shortest to longest lesion lengths in Trial 1.

TACF Tree No.	Trial 1[z]		Trial 2[z]		Combined Trials 1+2[y]	
	Mean (mm)	SD	Mean (mm)	SD	Mean (mm)	SD
American Chestnut Trees (*Castanea dentata*)						
AN-35	32.9	8.8	43.3	5.5	38.1	9.0
AN-32	33.1	6.8	37.5	7.4	35.3	7.3
SM-17	34.3	4.4	37.3	5.5	35.8	5.1
EM-102	35.7	3.8	36.5	3.6	36.1 cam[x] = 38.1	3.7 cam = 6.6
AN-14	36.6	3.6	39.5	4.2	38.0 cam = 45.3	4.1 cam = 9.4
AN-74	38.8	3.1	41.2	4.3	40.0	3.9
AN-86	39.4	7.6	40.8	6.3	40.1 cam = 40.4	7.0 cam = 4.9
SM-150	39.9	5.5	42.7	4.4	41.3 cam = 41.8	5.1 cam = 4.4
SM-87	39.9	4.9	40.9	4.5	40.4	4.7
EM-50	41.4	6.1	46.7	3.5	44.1	5.6
EM-31	42.0	4.2	48.4	4.3	45.2	5.3
SM-68	42.7	4.0	40.1	3.0	41.4 cam = 39.2	3.7 cam = 3.8
SM-174	55.2	11.4	62.7	9.6	58.9	11.1
SM-26	56.5	12.2	62.8	6.6	59.7	10.2
SM-1	63.8	7.5	66.5	8.0	65.1 cam = 64.6	7.7 cam = 7.4
All trees	**42.1** median[w] = 40	**11.1**	**45.8** median = 43	**11.1**	**43.9** median = 41	**11.3**
Chinese Chestnut Trees (*Castanea mollissima*)						
M1-28	12.1	3.7	12.9	3.1	12.5	3.4
M1-32	16.2	3.6	17.8	4.3	17.0	4.0
BC-2	16.7	6.8	15.7	4.9	16.2 cam = 17.7	5.9 cam = 7.9
NO-1106	17.9	4.1	22.6	4.7	20.2	4.9
NO-1150	19.1	2.5	19.0	2.4	19.0	2.4
BX-310	19.4	4.3	23.7	2.6	21.5 cam = 29.9	4.1 cam = 5.4
AD-199	20.0	5.0	18.6	7.3	19.3	6.2
M1-889	20.2	4.5	24.1	4.2	22.2	4.7
TN-10	20.6	5.1	20.2	3.2	20.4 cam = 20.3	4.2 cam = 4.0
AD-218	20.8	5.6	21.8	4.3	21.3	5.0
AD-227	21.0	5.2	20.0	2.4	20.5	4.0
BF-25	22.5	2.7	20.6	2.9	21.5	2.9
CP-70	23.8	4.0	21.1	4.1	22.5	4.2
GR-119	24.0	3.7	24.2	2.7	24.1 cam = 26.7	3.2 cam = 3.5
NO-1159	24.0	6.3	22.1	3.5	23.1 cam = 21.9	5.1 cam = 10.1
NO-1117	24.7	5.4	27.4	5.3	26.1	5.5
All trees	**20.2** media n= 20	**5.6**	**20.7** median = 21	**5.2**	**20.4** median = 21	**5.4**

F1 Chestnut Trees: American (Am), Chinese (Ch), Japanese (Ja)						
B6-7/10 (Am × Ja)	15.4	2.7	19.6	6.0	17.5	5.1
B12-13/9 (Am × Ch)	16.2	3.3	22.6	4.0	19.4	4.9
M1-975 (Ch × Am)	20.6	3.3	25.1	6.3	22.9 cam = 23.0	5.5 cam = 2.9
B1-2/4 (Ame × Chi)	21.4	4.1	24.4	3.5	22.9	4.0
M1-1170 (Ch × Am)	21.7	3.6	27.1	4.1	24.4	4.7
M1-263 (Ch × Am)	22.2	6.2	18.0	5.1	20.1	6.0
M1-479 (Ch × Am)	24.3	3.0	23.2	4.2	23.7	3.6
M1-164 (Ch × Am)	25.5	4.5	26.8	5.6	26.1	5.1
M1-730** (Ch × Am)	25.6	3.0	21.6	4.1	23.6	4.1
M1-43 (Ch × Am)	27.2	4.7	25.8	4.8	26.5	4.8
M1-645 (Ch × Am)	27.9	5.1	26.6	4.9	27.2	5.0
M1-368 (Ch × Am)	28.4	10.0	36.0	9.8	32.2	10.5
3-50 (Am × Ch)	30.5	4.5	35.1	7.7	32.8	6.6
M1-152 (Ch × Am)	31.1	7.0	30.2	5.8	30.6 cam = 39.3	6.3 cam = 5.1
B2-3/7 (Am × Ch)	31.5	5.5	30.7	4.7	31.1 cam = 42.3	5.1 cam = 7.0
M1-1075 (Ch × Am)	32.4	6.3	31.2	5.2	31.8	5.7
M1-501 (Ch × Am)	33.0	5.8	29.0	8.5	31.0	7.5
M1-1238 (Ch × Am)	33.1	13.3	28.1	6.9	30.6 cam = 36.6	10.7 cam = 13.8
M1-674 (Ch × Am)	42.7	14.5	34.8	17.0	38.7	16.1
All trees	**26.9** median = 26	**9.1**	**27.1** median = 27	**8.4**	**27.0** median = 26	**8.8**
BC3F2 Hybrid Mother Trees with Susceptible[v] Progeny ([*C. mollissima* × *C. dentata*] × *C. dentata*)						
W3-5-81	26.3	4.9	33.2	5.8	29.8	6.3
W4-12-124	31.4	7.4	32.8	10.7	32.1	9.1
W9-12-18	32.5	5.6	31.7	8.3	32.1	7.0
W7-32-19	35.4	9.7	52.9	12.0	44.1	14.0
W7-12-125	36.3	5.7	44.2	12.1	40.2	10.2
W2-20-147	36.3	7.1	54.4	8.6	45.3	12.1
W1-12-141	40.7	7.7	50.2	6.5	45.4	8.5
W7-14-122	45.0	5.3	44.9	4.8	45.0	5.0
W7-15-145	47.3	11.9	43.5	8.3	45.4	10.3
W6-12-29	47.7	11.1	65.8	6.4	56.7	12.8
W8-13-127	48.0	11.7	56.9	13.4	52.4	13.2
W8-13-108	49.4	12.3	44.6	6.0	47.0	9.9
W4-16-36	49.7	13.0	43.9	14.6	46.8	13.9
W8-13-24	50.3	6.9	51.1	9.1	50.7	8.0
W5-16-50	51.8	17.8	35.7	5.8	43.7	15.4
W3-5-111	52.0	5.5	48.3	11.0	50.1	8.8
W8-12-121	52.6	10.5	57.0	7.4	54.8	9.2
W3-5-116	53.9	10.2	65.4	8.1	59.6	10.8
W1-7-124	62.0	10.1	59.9	10.7	60.9	10.3
W3-15-150	64.1	10.7	66.7	6.2	65.4	8.7

All trees	**45.6** median = 45	**13.7**	**49.1** median = 48	**13.9**	**47.4** median = 46.5	**13.9**
BC3F2 Hybrid Mother Trees with Resistant[u] Progeny ([*C. mollissima* × *C. dentata*] × *C. dentata*)						
W2-15-51	17.3	3.5	17.3	3.2	17.3	3.3
W9-32-96	19.2	3.0	18.5	6.0	18.8	4.7
W1-14-58	26.8	6.3	28.2	7.1	27.5	6.7
W8-32-15	31.6	5.4	31.7	6.7	31.6	6.0
W9-32-113	33.0	5.3	31.0	16.4	32.0 cam = 33.5	12.1 cam = 10.7
W1-28-60	33.3	8.1	33.1	6.0	33.2	7.1
W1-32-69	35.2	5.8	41.5	5.8	62.7	7.8
W4-28-124	37.2	13.6	41.0	8.7	39.1	11.4
W6-32-30	39.4	5.4	55.4	9.1	47.4	10.9
W1-14-20	40.4	9.0	39.1	8.2	39.8	8.5
W5-24-75	40.7	8.9	43.8	8.2	42.2	8.6
W7-13-126	41.3	13.4	50.5	8.0	45.9	11.9
W3-32-49	48.9	8.7	59.2	6.5	54.0	9.2
W6-32-92	49.2	12.4	44.0	11.7	46.6	12.2
W1-28-98	51.0	13.7	69.2	3.6	60.1	13.5
W3-28-148	54.3	12.0	61.3	11.0	57.8	11.9
W2-14-66	57.0	7.9	58.3	11.0	57.6	9.4
W3-31-69	60.1	9.0	65.4	5.4	62.7	7.8
W6-31-92	66.4	5.2	69.7	1.3	68.0	4.1
All trees	**41.2** median = 39	**15.6**	**45.1** median = 43.5	**17.7**	**43.1** median = 41	**16.8**

[z] Lesion lengths on 20 replicate twigs for each tree were used to calculate each mean and standard deviation.

[y] Means and standard deviations for lesion lengths in Trials 1 and 2 combined (40 replicates/tree).

[x] Twigs from some trees also were inoculated with *P. cambivora* isolate VA2 Midwest (cam). Ten twigs were inoculated in each trial; therefore, means and standard deviations are for 20 twigs, and results are reported under Trial 1+2.

[w] Median values were determined from individual twig lesion lengths for all trees in each species for each trial.

[v] Hybrid mother trees that previously produced progeny susceptible to *P. cinnamomi* in a Phytophthora root rot experiment (labeled HY-S throughout this report).

[u] Hybrid mother trees that previously produced progeny relatively resistant to *P. cinnamomi* in a Phytophthora root rot experiment (labeled HY-R throughout this report).

**This tree could not be found in TACF records; it was located in the F1 section of the Price Farm.

Table 2: Mean internal lesion length measurements before and after iron sulfate-hydrogen peroxide staining, and lesion lengths based on colonization of twig periderm tissue.

Trial	TACF Tree No.	Before Staining		After Staining		Periderm[z]	
		Mean (mm)	SD	Mean (mm)	SD	Mean (mm)	SD
1	AN-32 American	33.0	7.3	31.5	5.3	47.7[y]	8.9
1	SM-150 American	39.9	5.5	42.0	5.8	47.6[y]	5.1
1	NO-1159 Chinese	24.1	6.6	24.8	5.8	36.7[y]	7.1
1	TN-10 Chinese	20.5	5.0	21.3	3.8	35.0[y]	6.5
1	M1-263 F1	23.6	9.1	22.2	6.2	31.6[y]	10.9
1	W5-16-50	51.8	17.8	53.2	14.9	57.1[y]	11.8
1	W3-5-81	26.4	5.1	29.6	7.3	54.1[y]	6.4
2	SM-26 American	62.8	6.6	63.8	5.8	70.0[y]	0.0
2	BC-2 Chinese	15.3	4.6	15.7	4.9	28.2[y]	8.2
2	M1-152 F1	30.2	5.8	31.5	5.7	46.3[y]	8.4
2	W4-12-124	30.8	9.9	34.2	11.4	50.6[y]	12.9
Grand Mean		**32.6**	**14.1**	**33.6**	**14.4**	**45.9**	**12.3**

[z] A strip of periderm tissue was removed from the entire length of the twig, embedded in 0.75% agar PAR medium, and incubated for 2 days at 25°C. The length of the strip from which *Phytophthora cinnamomi* hyphae grew was measured.

[y] For each of the 11 trees, lesion lengths before staining, after staining, and based on colonization of the periderm were compared statistically using a one-way ANOVA with a Tukey post-hoc test ($P<0.05$). Lesion lengths before and after staining were not significantly different; however, periderm lesions lengths were significantly different from lesion lengths both before and after staining.

Table 3: Lengths of lesions caused by isolates of four species of *Phytophthora* on excised twigs from 10 American chestnut trees in a preliminary experiment – see Figure 6 for data.

***Phytophthora* spp.**	**Isolate No.**	**Lesion Length for American Tree Test Group (10 American Trees)**		
		Mean[z]	**SD**	**Median**
P. cryptogea	D4-27-124	52.7 b	10.4	53.0
P. cinnamomi	JJ-1	49.2 b	9.5	48.0
P. cambivora	VA2 Midwest	48.1 b	12.1	48.5
P. heveae	28537	36.6 a	7.9	38.0
P. cambivora	NC 38364	32.5 a	11.1	35.0

[z] Means are based on lesion lengths on 78 twigs inoculated with each isolate 60 days after shoot collection; means followed by the same letter are not significantly different ($P<0.05$) using a one-way ANOVA with a Tukey post-hoc test.

Table 4: Mean lesion lengths (± standard deviations) on twigs with four different diameters.[z]

	Twig piece diameter[y]			
	4 mm	**5 mm**	**6 mm**	**7 mm**
Grand mean[x]	31.9 ±13.7	34.8 ±15.3	33.6 ±15.8	30.0 ±13.5
Total twig pieces (no.)	89	229	171	48

[z] Diameters of 537 twigs from 27 trees in the two trials—23 trees from Trial 1 and four trees from Trial 2: American (7 trees), Chinese (6 trees), F1 (5 trees), and BC3F2 (9 trees); 20 twig pieces from each tree were measured (27 × 20 = 540). Three twigs did not have one of these diameters.

[y] No statistically significant difference ($P<0.05$) was determined among lesion lengths on twig pieces with different diameters using the non-parametric Kruskal-Wallis rank sum test.

[x] Means are based on all twigs with each diameter.

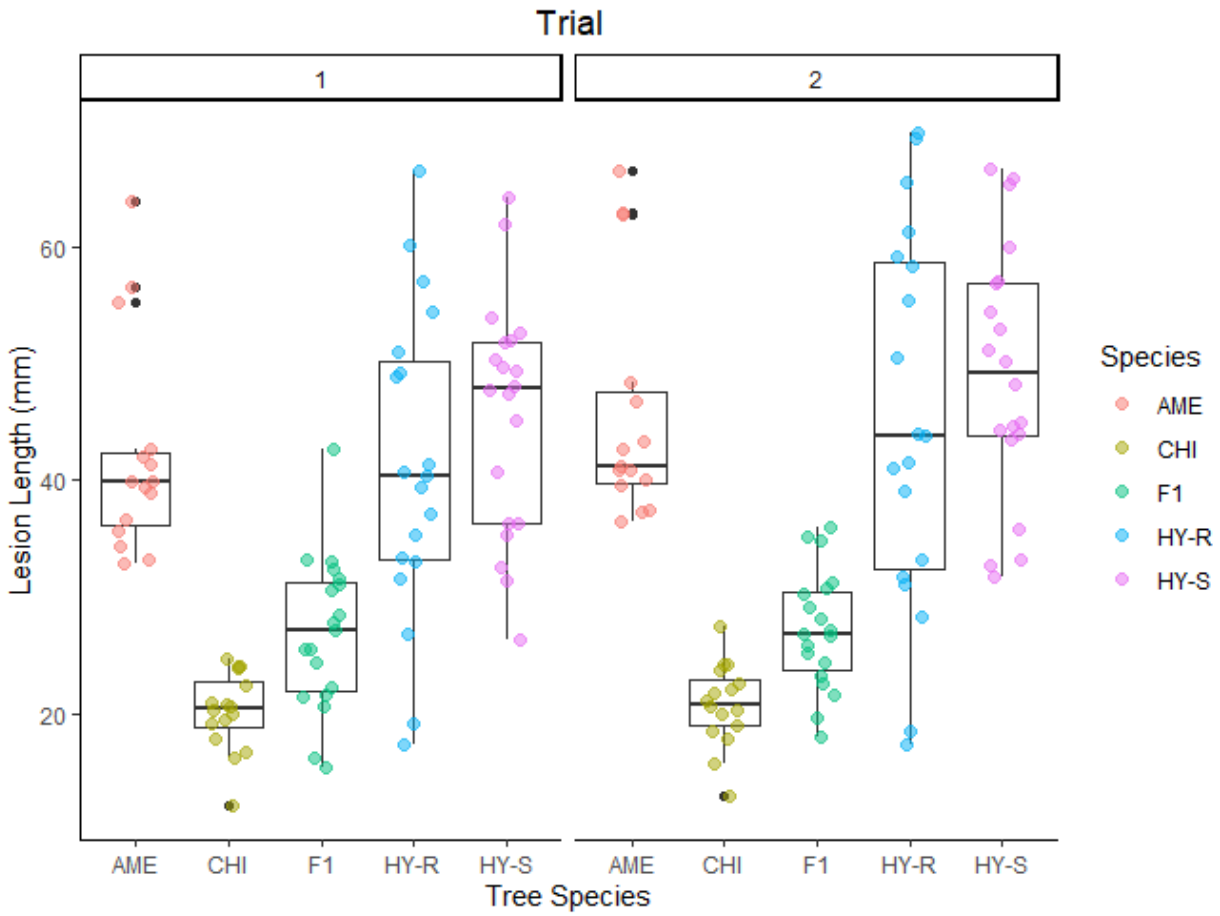

Figure 1: Boxplots and overlaid jitter plots were created from mean lesion lengths for each tree tested in Trial 1 and Trial 2. Boxplots are overlaid with jitter plots with colored dots representing mean lesion lengths on twigs from individual trees. Black dots are from boxplots. Boxplot components are explained in Figure 3. Tree species: AME = American chestnut, CHI = Chinese chestnut, HY-S = Hybrid mother trees producing susceptible progeny with a Phytophthora root rot survival scaled BLUP value of (-20) to (-13); HY-R = Hybrid mother trees producing resistant progeny with a Phytophthora root rot survival scaled BLUP value of 58 to 98.

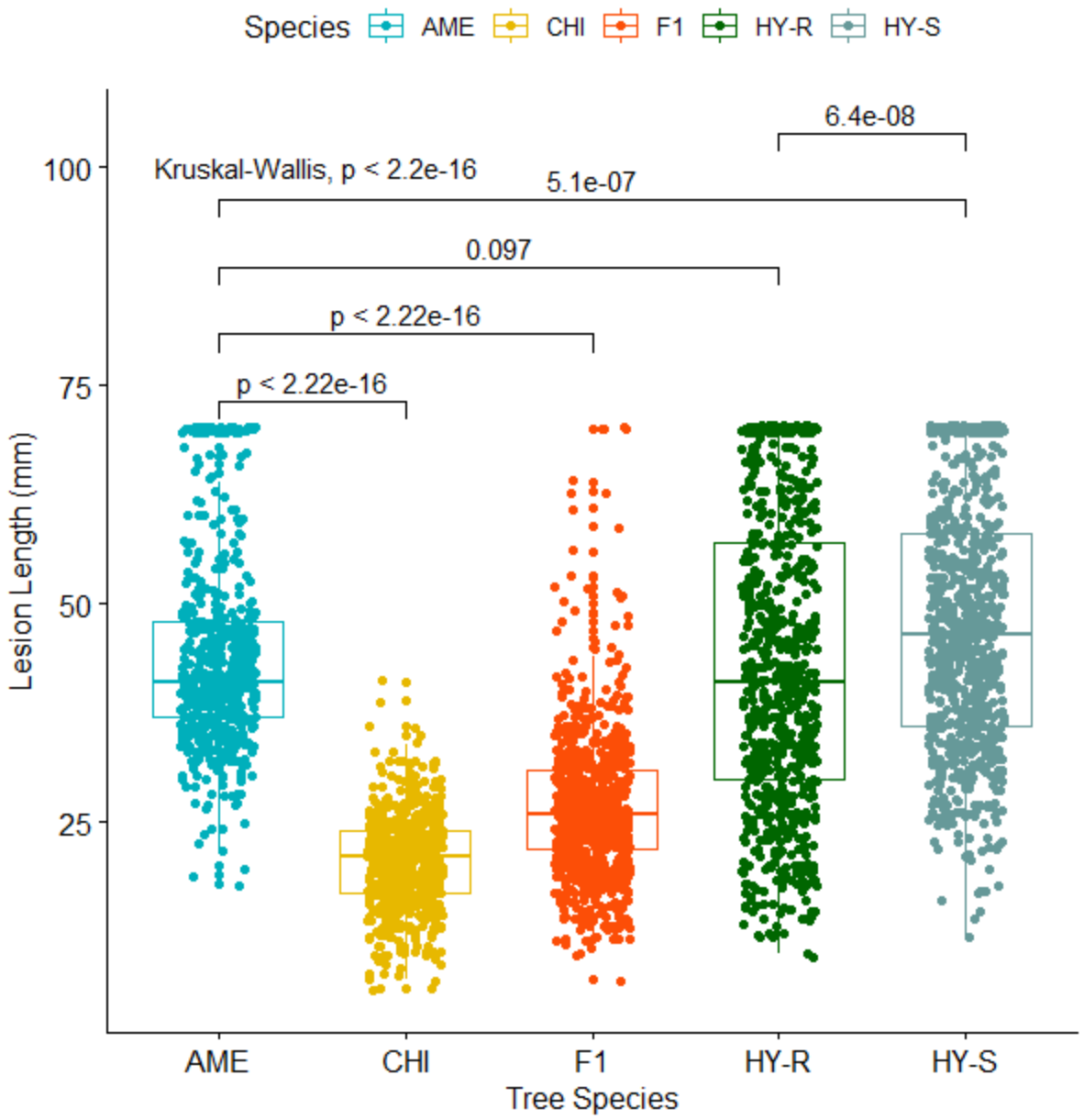

Figure 2: Lesion lengths on twigs from five tree species in Trials 1 and 2 combined. Significant differences among species was determined by using the non-parametric Kruskal-Wallis rank sum test for non-normal data. Lesion lengths for each tree species were compared to lesion lengths on American trees using a pairwise Wilcoxon rank sum test. HY-R and HY-S were also compared using the same statistical method. *P*-values < 0.05 are significantly different. Boxplots are overlaid with jitter plots; colored dots represent mean lesion lengths on twigs from individual trees. Black dots are from boxplots. Boxplot components are explained in Figure 3. Tree Species: AME=American chestnut, CHI= Chinese chestnut, HY-S = Hybrid mother trees producing susceptible progeny with a Phytophthora root rot survival scaled BLUP value of (-20) to (-13), and HY-R = Hybrid mother trees producing resistant progeny with a Phytophthora root rot survival scaled BLUP value of 58 to 98.

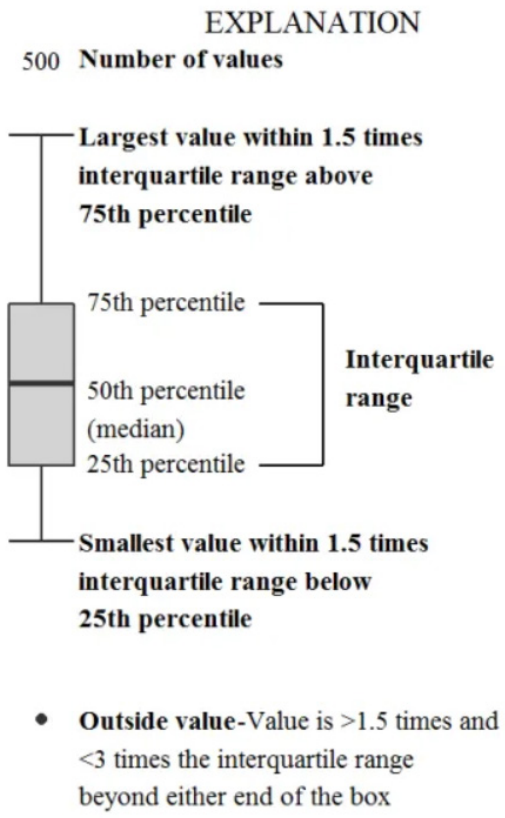

Figure 3: Legend explaining boxplot components Figures 1, 2, and 6.

Figure 4: Visual enhancement of lesions by iron sulfate-hydrogen peroxide staining. **Left:** Twigs before staining. **Right:** Twigs after staining. **Top row:** Non-inoculated control twigs.

Figure 5. Variation in the appearance of internal lesions on twigs from different trees.

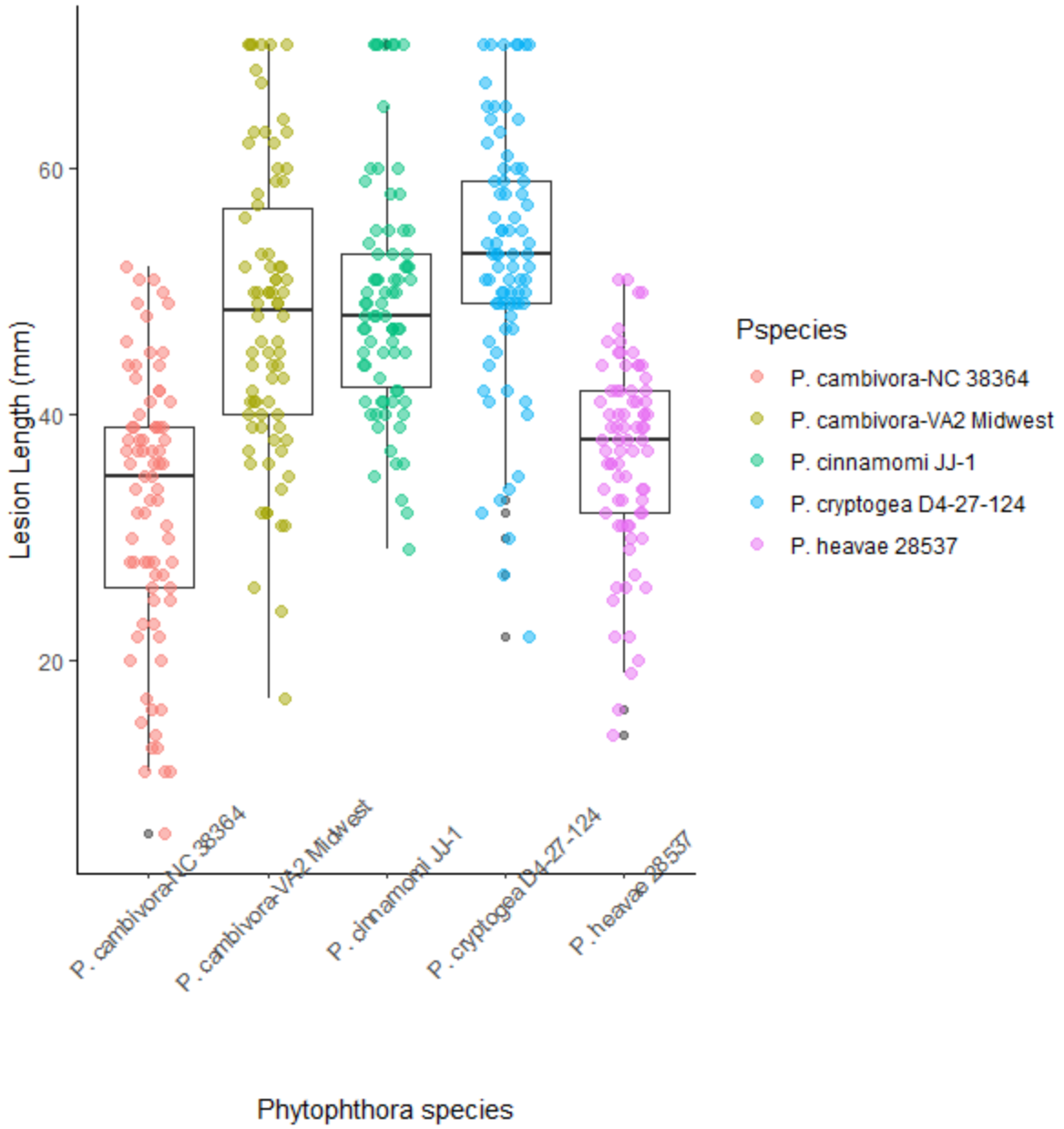

Figure 6: Lesion length comparison for isolates of four species of *Phytophthora*—preliminary assay. Boxplots are overlaid with jitter plots; colored dots represent mean lesion lengths on twigs from individual trees. Black dots are part of the boxplots. Figure 3 explains boxplot components. See Table 3 for data analysis,