

Development and testing of an *in vitro* screen for Phytophthora root rot resistance

Year 1 Progress Report and Proposed Year 2 Work

Principal investigator: Scott Merkle

Performing institution: University of Georgia

Awardee institution: University of Georgia Research Foundation, Inc.

Duration of project: 2 years (first year to develop and test screen on known resistant and susceptible material; second year to screen material from putatively resistant parents)

Long-term goal: To develop and optimize a rapid *in vitro* screen for Phytophthora root rot resistance for chestnut plantlets

Short-term goal 1: Develop and test a +/- PRR resistance screen using susceptible American chestnut plantlets and resistant Chinese chestnut plantlets

Short-term goal 2: Test the ability of the *in vitro* screen to detect intermediate levels of PRR resistance in hybrid backcross chestnut material and identify promising clones for field testing

Short term goal 3: Test the ability of the *in vitro* screen to detect PRR resistance in transgenic chestnuts engineered with candidate PRR resistance genes from the FHI project

Short term goal 4: Validate results of *in vitro* screen with *ex vitro* tests in soil

Year 1 Progress

Introduction

Although it has taken us longer than one year to get to this point, we have accomplished our first three short-term goals, as described in detail below. In the process, we ended up substantially changing our approach from what was described in the proposal, using what we believe to be a much more efficient and quantitative approach. The main changes from the proposed approach were that: (1) We used unrooted chestnut shoots for the *in vitro* screen, rather than whole plantlets with roots. This change allowed us to skip the rooting step and the difficult step of removing rooted shoots from gelled germination medium and reestablishing them in a new agarose-water matrix. (2) We quantified resistance/susceptibility of the clones by measuring the length of the stem lesion caused by *P. cinnamomi* from the point of infection at the interface of the medium surface and the shoot after a standard length of time. This change allowed us to measure a continuous index of resistance/susceptibility (lesion length) rather than using the discontinuous (and somewhat subjective) numerical rating scale we originally proposed. (3) We combined testing of wildtype American chestnut, Chinese chestnut, BC3F3 hybrid backcross chestnut and transgenic American chestnut clones into the same experiments. This change allowed us to make more rapid progress and obtain replicated data by testing some of the same clones in multiple experiments, rather than testing different material in different experiments. Below is a summary of the experiments we have conducted to date and their results.

Materials and methods

Plant material

Chestnut genotypes assayed for resistance to *P. cinnamomi* included clones derived from open-pollinated seeds collected from TACF Backcross-3 (BC3) trees from Dr. Joe James' Chestnut Return Farm in Seneca, SC. These clones have demonstrated *P. cinnamomi* resistance in tests and were therefore likely to have inherited some level of resistance to the pathogen. We also tested Backcross 3-F3 (BC3F3) lines derived from nuts collected from TACF's Research Farm in Meadowview, VA that may carry some resistance to *P. cinnamomi*, based on the presence of DNA markers for resistance inherited from the Chinese chestnut parent (personal communication, Jared Westbrook, TACF). Finally, we tested some transgenic American chestnut lines transformed with a pair of candidate genes for *P. cinnamomi* resistance, RPH1 and NPR3/4, both of which were cloned from Chinese chestnut, engineered into a multigene vector (pFHI-23RN), and transformed into the TG-8A American chestnut embryogenic culture line as described in Kong et al. (2014). The wildtype American chestnut line, TG-8A, is an embryogenic culture line derived from a seed collected from a large surviving American chestnut growing near Thoroughfare Gap, VA. The untransformed TG-8A was used as the susceptible control, since, like all American chestnuts tested to date, it carries no resistance to *P. cinnamomi*. Chinese chestnuts are naturally resistant to *P. cinnamomi*; hence we used the Chinese chestnut cultivar "Qing" (shoot cultures supplied by Dr. William Powell; SUNY-ESF) as a resistant control. We later incorporated the Chinese chestnut genotype "Myrick" as a second resistant control, as it has been used for that purpose in Dr. James' field tests.

Production of shoot cultures and preparation of shoots for screening

We derived shoots of American and hybrid chestnuts from somatic embryos produced from embryogenic cultures following the protocol of Andrade and Merkle (2005) using a shoot induction media series based on that used by Oakes et al. (2013). All media had a woody plant medium (WPM) base. Mature somatic embryos were placed on bud induction medium (M1) that contained 20 mg/L BAP. Somatic embryos were treated on M1 for 1-3 months. Once adventitious buds formed, embryos with buds were placed on shoot development medium (M2), containing 2 mg/L BAP and 10 mg/L KIBA, for 30 days. The resulting shoots were cultured on shoot elongation medium (M3), which had 2 g/L ethanesulfonic acid (MES), 2 g/L of polyvinylpyrrolidone (PVP) and 50 µg/L BAP, for an additional 30 days before transfer to pre-rooting medium (PR) for shoot multiplication (Fig. 1). PR contained 2 g/L MES, 2 g/L of PVP, 25 µg/L BAP and 25 µg/L meta-Topolin. Shoots were cut into nodal segments and placed horizontally on 100 ml of PR medium in GA7 vessels (Magenta), and incubated in a lighted incubator with a 16-hour photoperiod at 100 µmoles/m²/sec¹ and 22° C. Shoots were allowed to elongate on PR medium until they reached 2-5 cm in height before being used in the resistance assays.

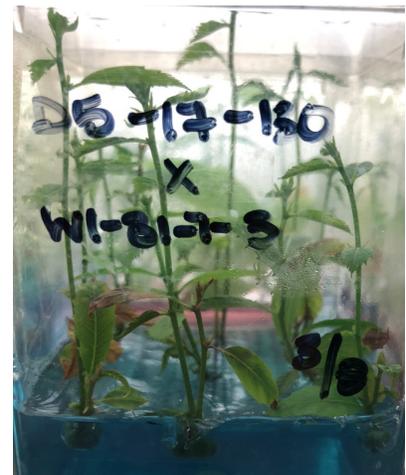


Fig. 1: *In vitro* shoot culture of a BC3F3 line used in Experiments 2 and 3.

Culture and preparation of P. cinnamomi

A *P. cinnamomi* isolated from roots of infected chestnut trees was provided by Dr. Steve Jeffers (Clemson University) and was grown on V8A-10% medium, which contained 100 ml V8 juice, 1 g CaCO₃, 15g Bacto Agar, and 900 ml deionized water per liter (Jeffers and Martin 1986). The stock culture of *P. cinnamomi* was stored in glass vials containing 10% clarified V8 agar (cV8A) and maintained in an incubator at 15° C. Using a sterile loop, an agar plug containing mycelia was extracted from the stock culture and placed on a V8A-10% plate. The culture was incubated in the dark at 25°C for 5-14 days prior to use (Fig. 2).

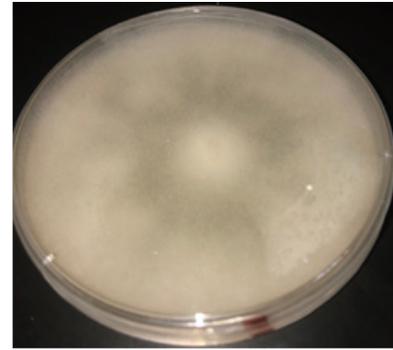


Fig. 2: Plate of *Phytophthora cinnamomi* at day 14

Inoculation of shoots with P. cinnamomi

Individual shoots between 2 and 5 cm long were excised from the cultures, and any basal callus and the lowest 1/3 of the leaves were removed from the stem. Shoots were placed in individual 15 x 2.5 cm glass test tubes with 25 ml of 7% agarose-water. Test tubes were inoculated with a 3 mm plug of *P. cinnamomi* taken from the actively growing edge of a colony growing on the Petri plate with a cork borer (Fig. 3). Test tubes were incubated in a lighted growth room at 75 $\mu\text{mol}/\text{m}^2/\text{sec}^1$ and 22° C. The shoots were scored every 3-4 days for PRR infection by measuring the length of the dark lesions on stems, measured from the base of the stem. These dark lesions contrasted sharply with the green portion of the shoot (Fig. 4) However, when measuring lesion lengths, there were some inconsistencies with the degree of necrosis on each stem. Any dark coloration that appeared was counted as necrotic tissue. However, if a stem had dark tissue and visibly lighter brown areas, only the darkest portion was measured.



Fig. 3: Shoot challenged by *P. cinnamomi* inoculum plug (red circle)



Fig. 4: Developing lesions on shoots as a result of *P. cinnamomi* inoculum. Note transition from green stem to dark lesion (red circle).

Experiments

Since there was some variation in the genotypes tested in each of the three experiments and there were other slight variations in how the experiments were conducted, each experiment is briefly described here.

Experiment 1: In the first experiment, TG-8A-23RN shoots from two independent transgenic events (TG-8A-23RN-3 and TG8A-23RN-15) were tested, as well as the BC3F3 line “D3-21-53 x W3-30-6-1” (D3xW3). The tested transgenic and BC3F3 genotypes had 10 inoculated replicates (test tubes), and 5 non-inoculated shoots. Each resistant and susceptible control line included 6 inoculated shoots and 6 non-inoculated shoots. Selected shoots were inserted into gelled agarose-water close to the wall of the test tube with their leaves splayed outward. V8 plugs were placed fungus-side down on the surface of the gel on the opposite tube wall. For this experiment, *P. cinnamomi* plates were prepared 14 days prior to the inoculation date and incubated at 20-25°C. The mycelium formed dense mats, which made cleanly cut plugs difficult to obtain.

Experiment 2: In this experiment, three B3 lines “W6-31-120-1” (JJ1), “W6-31-120-4” (JJ2), and “W6-32-41-5” (JJ3) were tested, as well as BC3F3 “D5-17-130 X W1-31-7-3” (D5xW1). The Chinese chestnut genotype “Myrick” TG-8A and Qing were also included. For the tested B3 and BC3F3 genotypes, 15 shoots were inoculated, and 5 were left as controls. Each control line had 10 inoculated repetitions and 5 that were non-inoculated. As in Experiment 1, shoots were placed close to the wall of the test tube, but in this experiment, the inoculum plug was placed on the medium in the center of the tube. Another difference from Experiment 1 was that the *P. cinnamomi* plates were prepared only 5 days prior to inoculation date and incubated at 20-25°C. The mycelia were less dense and did not occupy the entire plate, making the plugs much easier to manipulate.

Experiment 3: In this experiment, the same B3 and BC3F3 lines from experiment 2 were tested. In addition to these lines, we also tested an additional BC3F3 line “W1-31-60 X W6-31-33-8” (W1xW6) As in Experiment 2, tested B3 and BC3F3 genotypes had 15 inoculated shoots and 5 controls. Each control line had 10 inoculated repetitions and 5 that were non-inoculated. For this experiment, the amount of inoculum used to initiate the *P. cinnamomi* plates was much larger, so the mycelium filled the plates more quickly than in the two previous experiments during their incubation in the dark at 20-25°C. As in Experiment 2, plugs were harvested from the plates at day 5, when hyphae had reached the edge of the plate but were not matted. However, the relative positions of the shoot and plug in the test tubes in this experiment were returned to the arrangement in Experiment 1, in which the shoot and plug were placed on opposite walls of the test tube.

Experimental design and statistical analysis

Information on replications and controls appears under each experimental description above. To ensure that environmental (light, temperature) variation within the incubator did not affect the results of the study, test tubes in each experiment were randomly arranged in racks on the incubator shelf (Fig. 5). Differences in stem lesion length among the chestnut genotypes in each experiment were analyzed for each scoring date with analysis of variance, and means separation was accomplished with Tukey’s test using the R statistical package (R Core Team 2013).



Fig. 5: Racks of test tubes containing chestnut shoots and *P. cinnamomi* plugs in the growth room.

RESULTS

Experiment 1

P. cinnamomi mycelia expanded slowly from the plugs and, in some cases, never reached the shoot before the experiment was over, and thus never had the chance to infect the shoot to form a lesion. These tubes were dropped from the experiment, leaving a low number of tubes for analysis for some genotypes. Analysis of variance results indicated that the lesion lengths varied significantly among genotypes at all dates beginning 9 days following inoculation ($P < 0.01$). As illustrated in Fig. 7, stem lesion lengths on Chinese chestnut cultivar (“Qing”) were much shorter than those on pure American chestnut genotype (TG-8A) at all dates after day 7, indicating that the Chinese chestnut cultivar has higher resistance to *P. cinnamomi* than the American chestnut genotype. Tukey’s test results indicated that the difference in average lesion length became statistically significant ($P < 0.05$) within 9 days following inoculation and remained statistically different for the remainder of the experiment (Fig. 6). None of the tested transgenic or BC3F3 genotypes had lesion lengths that were statistically different from the susceptible American chestnut control (TG-8A), indicating that they had no higher resistance to *P. cinnamomi* than wildtype American chestnut (Fig. 7). The average “days to max” (average days required for lesions to reach maximum length) for Experiment 1 ranged from 21-25 days (data not shown). By comparing the average days required to reach maximum length across all genotypes, it was evident that all genotypes were subject to consistent inoculation intensity.

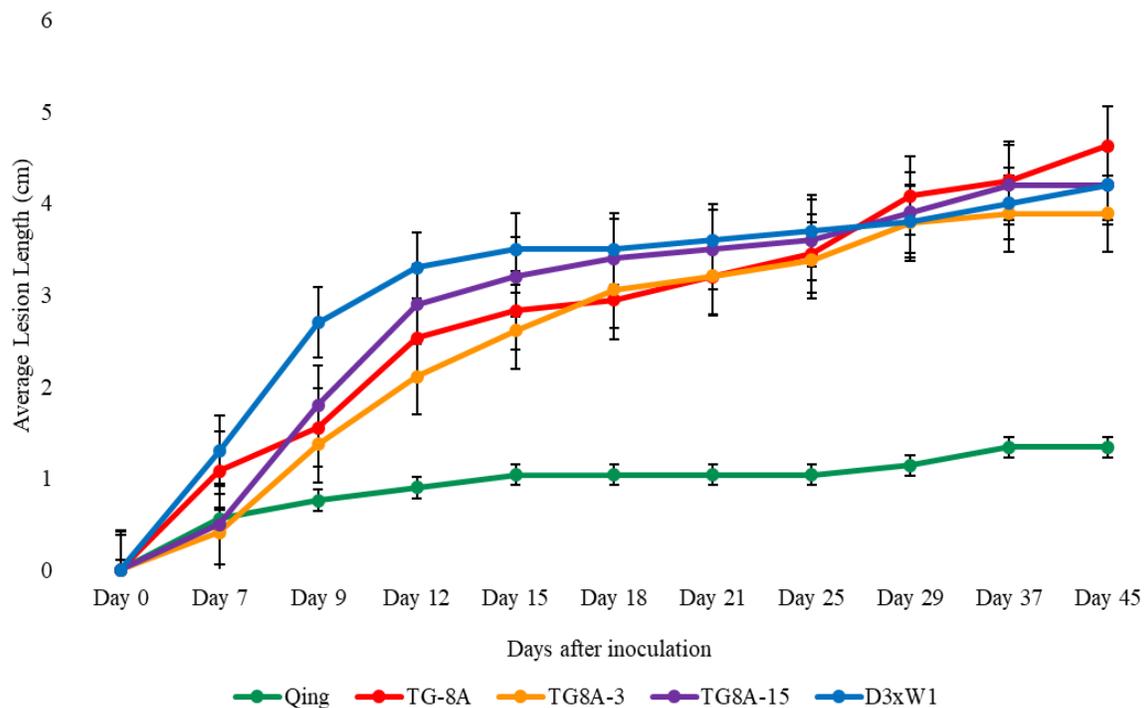


Figure 6. Experiment 1 results. Stem lesion lengths on American chestnut (TG-8A), Chinese chestnut (Qing), BC3F3 (D3xW1) and transgenic American chestnut (TG8A-3, TG8A-15) *in vitro* shoots at different lengths of time following inoculation with *P. cinnamomi*. Bars indicate standard error. See text for full names of all genotypes.

Experiment 2

Analysis of variance results indicated that lesion lengths varied significantly among genotypes between 8 and 18 days following inoculation ($P < 0.01$), but not on dates outside of that period. Tukey's test results showed that Experiment 2 data were somewhat similar to those in Experiment 1 in that stem lesion lengths on 'Qing' shoots remained smaller during this period than those of TG-8A (Fig. 8). Tukey's test results also indicated that lesion lengths on 'Qing' during this period were significantly smaller than on two of the three BC3 genotypes (JJ2 and JJ3), suggesting that the Chinese chestnut cultivar had higher resistance to *P. cinnamomi* than these B3s. However, unlike Experiment 1, there were no significant differences between 'Qing' lesion lengths and those of any of the remaining genotypes, including the third BC3, the BC3F3 (D5xW1) or the other Chinese chestnut genotype, 'Myrick'. As shown in Fig. 8, the tested lineages converged to form two distinct groupings by the final measurement date. One group ('Qing', 'Myrick' and D5xW1) appeared to be slightly more resistant than the other (TG-8A and BC3 lines). When we artificially clumped the data for all genotypes into two groups based on the plot and compared the two artificial groups using ANOVA, they were statistically different ($P < 0.05$). Within the slightly more resistant group 'Myrick' performed similarly to 'Qing' by the end of the experiment but appeared to succumb to PRR much more rapidly than 'Qing'. Additionally, the BC3F3 genotype (D5xW1) showed a similar level of resistance to *P. cinnamomi* as the two Chinese chestnuts by the end of the experiment, which is encouraging, since it may mean that this BC3F3 inherited some level of resistance from its Chinese chestnut ancestor. The shape of the lesion growth curves in Fig. 7 is quite different from those in Fig. 6, indicating a much more rapid infection of the shoots. This difference may have been due to the change that was made in the inoculation procedure (inoculated after only 5 days of incubation instead of 14, and with the plugs of inoculum placed immediately adjacent to the shoots, rather than on the opposite wall of the test tube), which resulted in a much more vigorous culture of *P. cinnamomi*. A more stringent challenge by the pathogen may have rapidly overwhelmed the shoots, quickly infecting them and even overcoming the natural resistance of Chinese chestnut. As mentioned earlier, average "days to max" for Experiment 1 had a range of 21-25 days. Average "days to max" for Experiment 2 had a range of 12-24 days. Not only was this a wider range, but its lower limit was less than the low end for Experiment 1. According to this metric, the pathogen killed the shoots almost twice as fast during the second test.

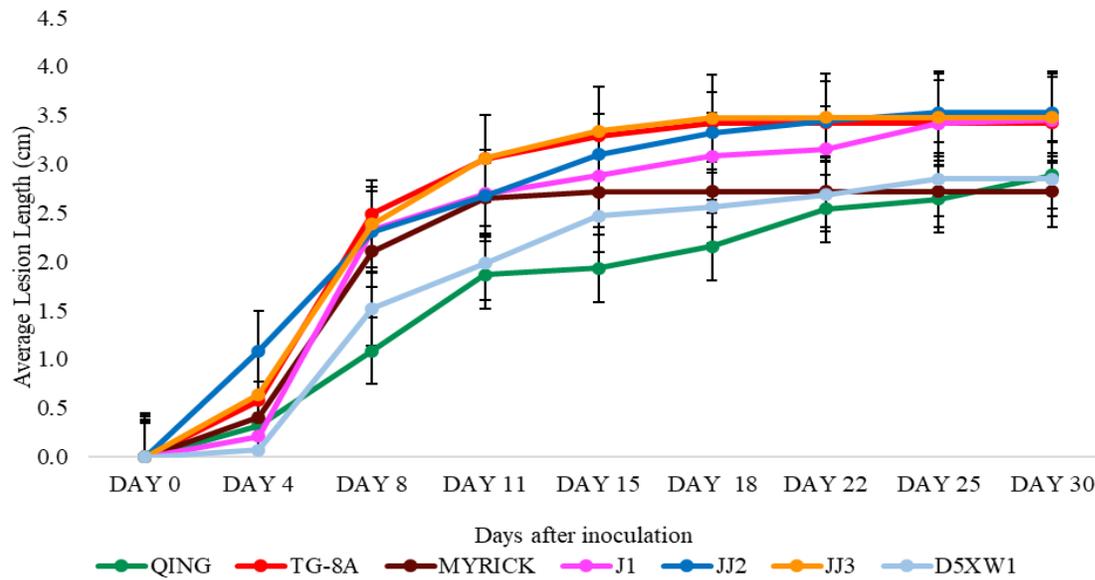


Fig. 7. Experiment 2 results. Stem lesion lengths on American chestnut (TG-8A), Chinese chestnut (Qing and Myrick), BC3 (JJ1, JJ2, JJ3) and BC3F3 (D5xW1) *in vitro* shoots at different lengths of time following inoculation with *P. cinnamomi*. Bars indicate standard error. See text for full names of genotypes.

Experiment 3

As was the case in Experiment 1, slow growth in some tubes resulted in mycelia failing to reach the shoot before the experiment concluded, and consequently, the *P. cinnamomi* never had the chance to infect the shoot to form a lesion. These tubes were dropped from the experiment, but the number of tubes never dropped below 8 replicates. Analysis of variance results indicated that lesion length varied significantly among genotypes beginning 5 days following inoculation. As illustrated in Fig. 8, and supported by Tukey’s test results, the data were similar to those in Experiment 1 in that the stem lesion lengths on ‘Qing’ shoots remained smaller on all dates after day 5 than those of TG-8A. For the third time, we verified that the Chinese chestnut cultivar has higher resistance to *P. cinnamomi* than the tested American chestnut genotype. Tukey’s test results also indicated that lesion lengths on ‘Qing’ were significantly smaller by day 14 than all three BC3s, as well as one of the BC3F3s (WxW), indicating the Chinese chestnut cultivar had higher resistance to *P. cinnamomi* than these BC3 and BC3F3 genotypes. By day 23, the average length of ‘Qing’ stem lesions was also significantly shorter than the other BC3F3 (D5xW1). However, unlike Experiment 2, there was a significant difference between ‘JJ2’ and the D5xW1, indicating that, while not statistically different from the fully susceptible American wild type, the ‘D5xW1’ cross showed significantly less susceptibility than the second BC3.

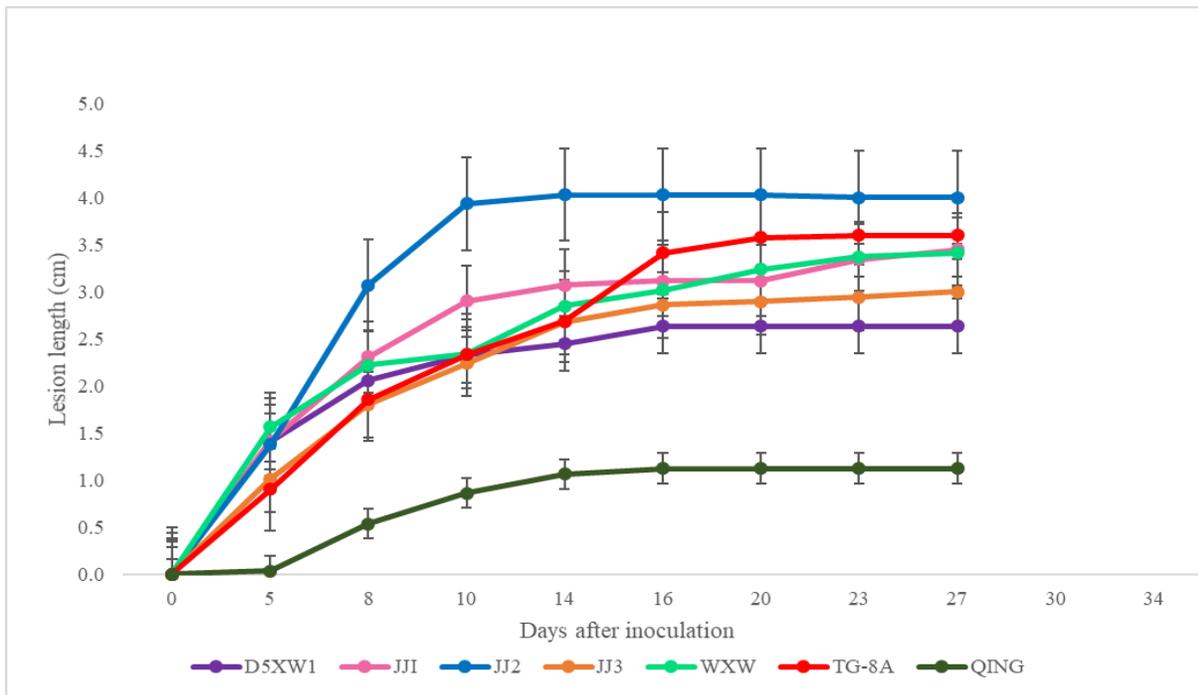


Fig. 8. Experiment 3 results. Stem lesion lengths on American chestnut (TG-8A), Chinese chestnut (Qing), BC3 (JJ1, JJ2, JJ) and BC3F3s (WxW, D5xW1) *in vitro* shoots at different lengths of time following inoculation with *P. cinnamomi*. Bars indicate standard error. See text for full names of genotypes.

Proposed for Year 2 work

Short term goal 4: Validate results of in vitro screen with ex vitro tests in soil.

It is possible that apparent PRR resistance demonstrated by chestnut plantlets under *in vitro* conditions may not accurately reflect their resistance in soil. The *in vitro* test we developed relies on direct infection of shoots by *P. cinnamomi* mycelia growing in gelled medium, rather than infection of roots by swimming oospores, which is the infection mechanism that occurs in soil. Therefore, we will test the resistance or susceptibility of the genotypes used in the *in vitro* tests in soil to see how closely the responses correspond to those obtained with *in vitro* shoots, by inoculating whole plantlets with *P. cinnamomi* in potting mix in the greenhouse.

Plantlet production. The first step in setting up the greenhouse experiment is to generate sets of complete plantlets from the same cultures that produced the shoots used in the *in vitro* tests. Since we have proliferating shoot cultures of these clones (media and protocol described above in the Progress Report), we will harvest 2-3 cm-long shoots from the cultures and root them *in vitro*. Briefly, after the shoots are excised, cut basal ends will be dipped in a solution of 1 mg/ml K-IBA and stuck into gelled “rooting” medium, which is the same as pre-rooting medium, but lacking BA or meta-Topolin, in GA7 vessels. Shoots undergoing rooting will be incubated in the dark at 22° C for 10 days, before being transferred to a lighted incubator with a 16-hour photoperiod at 80 $\mu\text{moles}/\text{m}^2/\text{sec}^1$ at 22° C. Once rooted, plantlets will be removed from *in vitro* conditions and their root systems will be washed clean of any medium before potting in Hillson-type Rootainers (Rootainers International) containing a 1:1:1 mixture of peat:perlite:vermiculite. Plantlets will be acclimatized to greenhouse conditions in trays with clear plastic domes with adjustable vents, allowing the RH inside to be gradually lowered to ambient levels. Plantlets will be grown in the greenhouse for 2-3 months before being repotted in 0.5-gal pots for resistance screening.

Preparation of *P. cinnamomi* inoculum and plantlet inoculation. We will collaborate with Steve Jeffers (Clemson University) to set up a pot-based screen in one of our greenhouses, since he routinely conducts these screens in his greenhouse. Dr. Jeffers has already provided us with *P. cinnamomi* isolate SC-3061 (UGA-Pc01), which was isolated from soil at Sandy Creek Nature near Athens, GA. The isolate will be grown up in V8A-10% medium, as described in the Progress Report, and 10 ml (2 teaspoons) of inoculum will be applied to the surface of each pot, mixed gently into the top layer of potting mix and covered with fine propagation mix. Pots will be carefully watered to incorporate the inoculum and to prevent its desiccation.

Scoring of infection symptoms. Plants will be scored weekly for foliage symptom severity and/or mortality using a 6-point rating scale (Table 1). Area under the disease progress curve (AUDPC) values will be calculated from these scores for each replicate in the experiment using as described by Shaner and Finney (1977). The dates of the first symptoms and mortality will be recorded for each plant showing infection symptoms. From these data, the times to first symptoms and survival will be calculated. If a stem lesion is visible, the presence of the lesion will be documented as well as the height (cm) from the soil surface to the lesion.

Harvesting of infected plants. When a plantlet dies, the soil will be carefully removed from around the root system by gentle washing under tap water. Root rot severity will be recorded using a scale from 0 to 100% in increments of 10%, with 0% being healthy and 100% being dead. The top part of the plant will be excised from the root system and fresh weights (g)

recorded for both the shoots and roots. The root systems will be wrapped in wet paper towels, sealed in Ziplock bags, and transferred to Dr. Jeffers' lab at Clemson to isolate *P. cinnamomi* from the root systems, to confirm infection by the *P. cinnamomi* isolate used to inoculate the plants. If a stem lesion is present, samples of the stem tissue will be harvested from the base of the stem up to the top of the lesion. The stem tissue will be stored at -20° C until PCR verification can be performed for confirmation of *P. cinnamomi* infection.

Experimental design and statistical analysis. At least 4 plantlets per clone will be used to evaluate resistance/susceptibility, using the scoring system detailed above. As with the *in vitro* screen, the pot-based screen will employ American chestnut plantlets as susceptible controls and Chinese chestnut plantlets as resistant controls. The entire experiment will be conducted (replicated) twice. Disease rating and AUDPC values will be subjected to analysis of variance and means separation will be performed by Tukey's test using the R statistical package (R Core Team 2013)

Table 1. Rating scale for disease symptoms.

Score	Rating	% foliage showing chlorosis, wilt, or necrosis
0	Healthy	none
1	First	1-10%
2	Moderate	11-15%
3	Extensive	51-90%
4	Severe	91-99%
5	Dead	100%

Literature Cited

- Andrade, G.M., and S.A. Merkle. 2005. Enhancement of American chestnut somatic seedling production. *Plant Cell Reports* 24:326-334.
- Oakes, A.D., W.A. Powell and C.A. Maynard. 2013. Doubling acclimatization survival of micropropagated American chestnuts with darkness and shortened rooting induction time. *J. Environ. Hort.* 31(2):77-83.
- R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.
- Shaner, G, and R.E. Finney. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. *Phytopathology* 67:1051-1056.

Project schedule (Year 2)

Timeline (Month 1 = January 2020)

Month 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Plantlet production <i>in vitro</i>	xxxxxxx
Plantlet acclimatization	xxxxxx
Plantlet growth in greenhouse	xxxxxxx
Screening for resistance	xxxxxxxx

Measurement and reporting of results

Measurements, experimental design and statistical analyses are described above in the Experimental Plan. Results from our experiment will be reported at annual meetings of TACF, meetings of the Georgia Chapter-TACF and/or Southeastern Regional TACF meetings and tree improvement meetings such as SFTIC. Results will be included in manuscript to be submitted to the Journal of The American Chestnut Foundation or a plant biotechnology journal such as Trees Structure and Function.

Budget justification and funding timing

Funds are requested for 260 hours of undergraduate student lab assistant. Funds are also requested for tissue culture supplies (chemicals, plasticware, glassware) and greenhouse supplies (pots, potting mix, insect control). Year 2 funds will be needed by January 1, 2020.

Budget (Year 2 only)

Personnel

Student Lab Assistant (260 hr @ \$12 per hr)	3,120
--	-------

Operating expense

Tissue culture & greenhouse supplies	4,774
--------------------------------------	-------

Total direct costs	7,894
---------------------------	--------------

Indirect costs (F&A)	0
----------------------	---

Total costs	\$7,894
--------------------	----------------