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

Submitted to:

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12 August 2018

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Signing for

Tanju Karanfil, Ph.D. — Date
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Date Submitted:

15 August 2018

A. Project Title

Development of an in vitro excised twig assay to identify resistance to *Phytophthora cinnamomi* in hybrid chestnut trees

B. Summary

There is currently only one method to identify resistance to *Phytophthora cinnamomi*, which causes Phytophthora root rot, in hybrid chestnut trees: seedling inoculation trials. Development of an in vitro test for resistance would accelerate the breeding process and provide another option for identifying resistant trees in the TACF breeding program. This project will develop and evaluate the use of an in vitro excised twig assay to measure the level of resistance to *P. cinnamomi* in hybrid chestnut trees. Validation of the new method will rely on results obtained from previous seedling inoculation trials.

C. Principal Investigators and Institutional Affiliation

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D. Duration of Project: 01 November 2018 – 31 October 2019 = 12 months

E. Total Amount Requested: $9,755
Matching funds will be provided by Clemson University – see budget

F. Short- and Long-term Goals of Project

Short-term Goal

1. To develop and validate an in vitro assay using excised twigs to rapidly and consistently identify resistance to *P. cinnamomi* in backcross hybrid American chestnut trees

Long-term Goals

1. Evaluate hybrid American chestnut trees in the American Chestnut Foundation (TACF) orchards for resistance to *P. cinnamomi*; by more rapidly and efficiently identifying hybrid chestnut trees with resistance to *P. cinnamomi*, TACF breeding program will be accelerated considerably
2. To continue to optimize the excised twig assay to produce the most reliable and consistent results possible

G. Narrative

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. dentata* × *C. mollissima*] × *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).

Background and Significance

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 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 seedling trials 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 twigs to determine resistance to *P. cinnamomi* in hybrid European × Japanese chestnut clones (*C. sativa* × *C. crenata*). These authors were able to show a significant correlation between resistance determined by an in vitro twig assay and resistance determined by root inoculation. 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 hybrid chestnut trees.

Approach and Procedures: Short-term Goal

Initially, we evaluated the method used by Santos et al. (2015), but found this method produced inconsistent and unreliable results. Therefore, we experimented with different procedures, inoculation methods, and incubation times for a more reliable assay. The excised twig assay we have developed uses twigs cut from dormant current season's shoots. In preliminary experiments, dormant tissue has been the most convenient to work with and provided the most consistent results, and these findings are consistent with results from other studies (Jeffers and Aldwinckle 1986, Utkhede and Quamme 1988). Shoots then are cut into smaller pieces with a uniform length—creating replicate twigs from a single tree.

The proximal end of the twig is inoculated with *P. cinnamomi*. We are currently using a highly virulent isolate of *P. cinnamomi* isolated from the Chestnut Return Farm in Seneca, SC. The virulence of this isolate was documented in a pathogenicity experiment conducted here at Clemson University by a former graduate student (Sharpe 2017). An agar plug containing actively growing mycelium of the pathogen is placed in the bottom of a sterile plastic, disposable culture tube and covered with 1 ml of distilled water. One twig is placed in each culture tube so that the freshly cut end of the twig is resting on the agar plug. Caps are placed loosely on the tubes to allow air exchange but prevent desiccation. Five to 10 replicate twigs are used for each chestnut tree, and the culture tubes are placed in a dark incubator at 20 or 25°C for 7 to 14 days, depending on temperature. The height of the lesion created by the pathogen as it moves upward through the tissue can be measured. Non-inoculated control twigs are always included, and lesions occasionally develop on these twigs because this is not a completely sterile system. However, lesions on non-inoculated twigs usually are insignificant and rarely extend above the water line in the culture tube.

Initially, we compared lesion heights on twigs from susceptible American and resistant Chinese chestnut trees. To date, we have consistently observed a significant difference between lesion heights on twigs from American chestnut trees and those from Chinese chestnut trees when the excised twig assay is used (Figure 1). Therefore, we will use these two species as positive and negative controls, respectively, in all future experiments with twigs from hybrid chestnut trees. If the lesion heights on twigs from hybrid trees are statistically similar to lesion heights on twigs from American chestnut trees then the hybrid trees will be classified as susceptible

with low resistance to root rot. If the lesion heights on twigs from hybrid trees are statistically less than the lesion heights on twigs from American chestnut trees then the hybrids will be classified as having a higher level of resistance than American chestnut.

We will use shoots cut from hybrid chestnut trees with known levels of resistance to validate this new excised twig assay. To do this, previous results from Phytophthora root rot seedling trials must be used to establish a positive correlation between lesion height in vitro and disease severity on seedlings in the field for specific hybrid families. Therefore, results from seedling trials for hybrid families screened previously will be used to validate and confirm resistance ratings obtained with the excised twig assay.

To accomplish the short-term goal of this project, we must have a reliable supply of suitable chestnut shoots during the winter dormancy period. Obtaining chestnut shoots for this project is the single greatest challenge for this project. Consequently, we will rely on collaborators at TACF to supply cut shoots, which will need to be shipped by an overnight courier service (e.g., Federal Express or UPS). Once shoots are obtained, it is a simple matter of processing twigs and running repeated trials with each batch of shoots as quickly as possible to reduce the amount of time cut shoots are stored, which could compromise results.

Approach and Procedures: Long-term Goals

Once an excised twig assay has been developed and validated, we will begin to assay backcross hybrid chestnut trees growing in TACF orchards to expedite the identification of resistant parents that can be used in the breeding effort to produce seedlings with high levels of resistance to *P. cinnamomi*. At least two trials with the excised twig assay will be used for each hybrid family to insure consistent results. We will continue to improve the excised twig assay as time goes on, optimizing incubation times and temperatures and evaluating additional isolates of *P. cinnamomi*. We also will continue to validate results obtained with the excised twig assay with results from seedling assays using selected chestnut families.

The observations obtained from the assay must be tested further with the use of multiple independent trials and replications using twig tissue from multiple trees. We have also conducted the excised twig assay on hybrid chestnuts. The results of three independent trials can be seen in (Figure 1). Eight out of ten hybrid trees exhibited lesion heights smaller than the mean lesion height of the American twig tissue tested.

Preliminary Results

To date, we have conducted several experiments to develop an excised twig assay using various combinations of incubation times and temperatures. Figure 1 shows the results from one such experiment—in which lesion heights on excised shoots from 10 hybrid chestnut trees were compared to those on twigs from American and Chinese chestnut trees. Three independent trials were conducted to generate treatment means. These trials were conducted at 20°C and lesion heights were measured at 14 days after inoculation using twigs cut from dormant,

previous-season's shoots that were ≤ 15 mm in diameter and collected in February at Chestnut Return Farms in Seneca, SC. In each trial, ten twigs were used for each chestnut tree, so means are based on 30 replicate twigs. Data were analyzed using one-way ANOVA, and means were compared using Tukey's HSD test. Eight of the hybrid trees were more resistant than American chestnut.

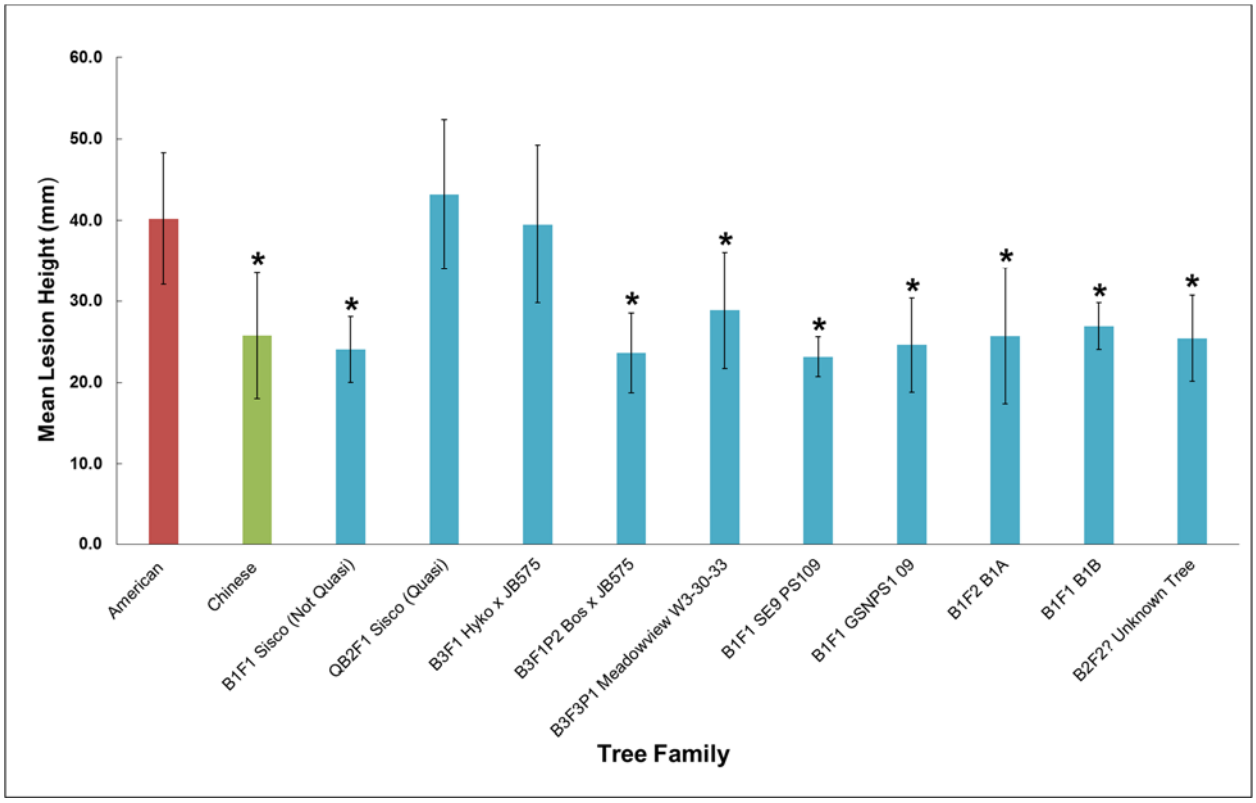

Figure 1. Mean lesion heights on twigs from American (red bar), Chinese (green bar), and ten hybrid chestnut trees (blue bars) were calculated from three independent trials using an excised twig assay. Means with asterisks are significantly different ($P < 0.0001$) from the American chestnut tree; error bars are standard deviations.

Literature Cited

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Sharpe, S. R. 2017. *Phytophthora* species Associated with American, Chinese, and Backcross Hybrid Chestnut Seedlings in Field Sites in the Southeastern United States. MS Thesis. Clemson University, Clemson, SC.

Utkhede, R. S., and Quamme, H. A. 1988. Use of the excised shoot assay to evaluate resistance to *Phytophthora cactorum* of apple rootstock cultivars. Canadian Journal of Plant Science 68:851-857.

H. Timeline (November 2018 – October 2019)

Research Work	3 months	6 months	9 months	12 months
Continue to develop assay	X			
Validate assay	X	X	X	
Begin testing trees in TACF orchards		X	X	X
Analyze results	X	X	X	X
Summarize and present results			X	X

I. Measurement and Reporting of Results

All Phytophthora lesion heights measured will be compared to the control lesion heights of American and Chinese controls. To validate the use of the excised twig assay, all hybrid tree family lesion height results will be cross checked with the level of disease severity observed during Phytophthora seedling trials using the same tree families. A final report of the results from this project will be prepared for TACF and all collaborators. Preliminary results will be presented at the NE-1333 Meeting at State College, PA in September 2018.

J. Budget: One Year (November 2018 – Oct 2019)

Expense	**TACF: Amount Requested**
20% of salary for Mr. Andrew Gitto	$6,240
Fringe Benefits: 40.3% for full-time employees	2,515
Expendable supplies—including overnight shipping of shoots	1,000
Subtotal	9,755
Unrecovered overhead = F&A @ 52.5% = $5,121	0
TOTAL	*$9,755*

Budget Justification

Amount Requested from TACF

Most of the funds requested in this proposal will be used to cover 20% of the salary and fringe benefits for Mr. Andrew Gitto, the Research Assistant who is working on this project. Mr. Gitto will be making $15.00/hr by the time this proposal is funded. The remainder of the funds will be used to pay for expendable lab supplies and overnight shipping of chestnut shoots from various locations.

Matching funds provided by Clemson University

TACF has a policy to not pay overhead (F&A – Facilities and Administration) charges since their grants are relatively small—see accompanying document. Therefore, this amount ($5,121) is used as matching funds from Clemson University.

K. Brief CV for each Principal Investigator

Steven N. Jeffers, Ph.D. – Abbreviated CV

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Education
1985: Cornell University, Ithaca, NY: Ph.D. Plant Pathology (Soil Science minor)
1980: Cornell University, Ithaca, NY: M.S. Plant Pathology (Soil Science minor)
1976: University of California, Davis: B.S. (Highest Honors) Plant Science/Pomology

Employment
Clemson University, Clemson, SC
2007-2018: Professor, Dept. of ESPS/School of AFES/Dept. of AES/Dept. PES
2001-2007: Associate Professor, Depts. PP&P and Entomology, Soils, and Plant Sciences (ESPS)
1995-2001: Assistant Professor, Dept. of Plant Pathology and Physiology (PP&P)
EcoScience Corporation, Worcester, MA: 1992-1995; Senior Scientist
University of Wisconsin-Madison: 1985-1992: Assistant Professor, Dept. of Plant Pathology

Research Experience
1995-present: Development of integrated management strategies for diseases of ornamental crops and trees in South Carolina; biology and ecology of *Phytophthora* spp. in nurseries, greenhouses, landscapes, and natural ecosystems; management of rust diseases
1992-1995: Development of biological control products for postharvest diseases of fruit crops
1985-1992: Integrated management strategies for diseases of fruit crops grown in Wisconsin

Extension Experience
1995-present: Clemson University Extension Specialist, Diseases of Ornamental Crops & Trees
1985-1992: University of Wisconsin Extension Specialist, Diseases of Fruit Crops

Teaching Experience
Principles of Plant Pathology (PLPA 3100): 2013-present
Plant Diseases and People (PL PA 310): 2010-2012
Selected Topics/Introductory Plant Pathology for Graduate Students (PLPA 8020): 2010-present
Plant Disease Diagnosis (PLPA 4110/6110)—co-instructor: 2012-present

Peer-Reviewed Publications: 2011-Present
Westbrook, J. W., James, J. B., Sisco, P. H., Frampton, J., Lucas, S., and Jeffers, S. N. 201x. Resistance to *Phytophthora cinnamomi* in American chestnut (*Castanea dentata*) backcross populations that descended from two Chinese chestnut (*Castanea mollissima*) sources of resistance. Plant Disease 102: *submitted and in review.*

Gitto, A. J., Jeffers, S. N., Graney, L. S., Loyd, A. L., and Bechtel, C. N. 2018. First report of *Phytophthora occultans* causing root rot on American boxwood planted in residential landscapes in the eastern United States. Plant Disease 102: *in press.*

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Dong, W., Jeffers, S. N., and Buck, J. W. 2013. Management of daylily rust with different fungicides and application methods. Plant Dis. 97: *accepted & in press.*

Scocco, E. A., Walcott, R. R., Jeffers, S. N., and Buck, J.W. 2013. Detection of *Puccinia pelargonii-zonalis*-infected geranium tissues and urediniospores. J. Phytopathology 161: *accepted & in press.*

Olson, H. A., Jeffers, S. N., Ivors, K. L., Steddom, K. C., Williams-Woodward, J. L., Mmbaga, M. T., Benson, D. M., and Hong, C. X. 2013. Diversity and mefenoxam sensitivity of *Phytophthora* spp. associated with the ornamental horticulture industry in the southeastern US. Plant Disease 97:86-92.

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Andrew J. Gitto, B.S. – Abbreviated CV

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Education
B.S. in Microbiology, Minor in Plant Pathology; Clemson University, 2017

Research Experience
Research Assistant and Lab Manager, Department of Plant & Environmental Sciences, Clemson University, conducting and assisting in plant pathology research and lab operations in the Dr. Steven Jeffers Lab. 2017- Present

Undergraduate Research Assistant, Department of Plant & Environmental Sciences, Clemson University: Assisted with plant pathology research in the laboratory of Dr. Steven Jeffers; 2015-2017

Undergraduate Research Assistant, Department of Biological Sciences, Clemson University: Conducted independent research in the laboratory of Dr. Min Cao focusing on interactions between the nematode *Caenorhabditis elegans* and bacteria; 2015-2017

Awards
American Society of Microbiology Undergraduate Research Fellowship, 2016

Professional Organization Memberships
American Society of Microbiology, 2014- present
The American Chestnut Foundation, 2017- present

Peer-Reviewed Publications/Presentations
Gitto, A. J., Jeffers, S. N., Graney, L. S., Loyd, A. L., and Bechtel C. N. 2018. First report of *Phytophthora occultans* causing root rot on American boxwood planted in residential landscapes in the eastern United States. Plant Disease. First Look. In press.

Gitto, A., Klees, M., Cao. Influence of probiotics on fat storage in Caenorhabditis elegans. Poster presentation at the American Society of Microbiology, General Meeting (Microbe), New Orleans, LA. June, 2017.

L. Conflict of Interest or Commitment Statement
There are no conflicts of interest for the above listed Principal Investigators regarding this project: Steven N. Jeffers and Andrew J. Gitto.