PROPOSAL TITLE FORM

Phytophthora Root Rot of Chestnut Trees: Research Projects at Clemson University in 2024-2025

Potential Funding Agency:
The American Chestnut Foundation (TACF)
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Submitted by:
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05 September 2024
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A. Project Title

Phytophthora Root Rot of Chestnut Trees: Research Projects at Clemson University in 2024-2025

B. Summary

We will continue to conduct research projects to better understand and manage Phytophthora root rot (PRR) on American and hybrid chestnut trees. Funds will be used primarily to pay a Lab Manager and purchase supplies needed for the project. We plan to:

- Assay chestnut plant and soil samples in the eastern USA for *Phytophthora* spp.;
- Work with colleagues to better understand genes involved in resistance to *Phytophthora cinnamomi*;
- Collaborate with TACF and the USDA Forest Service to conduct the annual *P. cinnamomi* resistance screening experiment; and
- Conduct field trials to evaluate selected fungicides for managing PRR on American chestnut seedlings.

C. Principal Investigator and Institutional Affiliation

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D. Duration of Project: 01 November 2024 – 31 October 2025 = 12 months

E. Total Amount Requested: $13,798
Matching funds in the amount of $7,244 will be provided by Clemson University

F. Short- and Long-term Goals of Project

Short-term Goals

1. To continue to assay plant and soil samples, provided by TACF members, for species of *Phytophthora*, so we can identify where these plant pathogens are located in chestnut-growing and potential chestnut-growing sites in the eastern U.S.; then, culture and maintain representative isolates of *Phytophthora* spp. for future studies.
2. In 2021, we began a collaborative project to identify genes involved in resistance to *P. cinnamomi* using RNA sequencing; additional experiments were conducted in 2022, 2023, and 2024; this project will continue.

3. Identify and supply isolates recovered and stored in short-term Goal 1 (above) for use in the annual TACF *P. cinnamomi* resistance screening experiment at the Resistance Screening Center (RSC) at USDA Forest Service Bent Creek Experimental Forest in Asheville, NC; then, assist with inoculating and scoring hybrid chestnut seedlings in spring and fall, respectively, during the year. This experiment was not conducted in 2024 because the RSC was closed.
4. A trial to evaluate selected fungicides for efficacy in managing PRR on American chestnut seedlings under field conditions was initiated at Warren Wilson College (WWC) in Swannanoa, NC in 2024; this trial will continue in the coming year. At least one additional field trial will be established at Clemson University in 2025 to replicate the trial at WWC.

Long-term Goals

1. Identifying the location and distribution of *Phytophthora* spp., especially *P. cinnamomi*, is necessary, so TACF knows where it can and should not plant chestnut seedlings now and in the future. In addition, isolates of *Phytophthora* spp. recovered from diseased chestnut trees and infested soils will be maintained in a permanent collection at Clemson University, so that we can use them in future research projects—e.g., see short-term Goal 3 below. This information is essential to breed chestnuts with resistance to all species of *Phytophthora* capable of causing PRR. Eventually, we will develop a GIS map showing the geographic distribution of the species of *Phytophthora* affecting chestnut.
2. We have been collaborating with colleagues at the University of Kentucky (Tatyana Zhebentyayeva and Dana Nelson) and TACF (Jared Westbrook) and with independent consultant Bert Abbott to identify genes involved in resistance to *P. cinnamomi*. We have developed a unique and effective system to inoculate the roots of chestnut seedlings with active zoospores of *P. cinnamomi* so that seedlings can be exposed to inoculum for precise time periods. Therefore, roots can be analyzed by our colleagues using RNA sequencing to identify genes involved in the resistance process in chestnut. Experiments were conducted in 2022, 2023, and again in 2024. Currently, we are working on one manuscript and seeking funding to sequence root samples from the 2024 experiment. Eventually, we hope to identify specific genes in the resistance response, so they can be manipulated to produce chestnut trees resistant to *P. cinnamomi*.
3. By staying actively involved in the annual TACF effort to screen hybrid chestnut seedlings for resistance to *P. cinnamomi*, we can maintain continuity and consistency in this effort, which our lab initiated in cooperation with Dr. Joe James in 2004. The screening effort in 2025 depends on the operation and availability of the USDA RSC in Asheville.
4. A diverse array of oomycete-targeting fungicides has been evaluated in several greenhouse experiments, and the most effective products have been identified. Now, we need to evaluate the selected products under field conditions to determine if they also are effective under chestnut-growing conditions. Fungicides that are effective in the field could then be used to treat and protect chestnut seedlings planted in germplasm conservation orchards (GCOs).

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 with resistance to both *Phytophthora cinnamomi*, which causes PRR, and to *Cryphonectria parasitica*, which causes chestnut blight. Since 2003, our lab at Clemson University has worked in collaboration with TACF to study PRR, but we have lacked the financial support to maintain a consistent research effort necessary to make significant impacts in the etiology and management of this important disease. Despite a minimum of financial support, we have made considerable progress, and our collaboration with TACF has been productive (Westbrook et al. 2019, Zhebentyayeva et al. 2019). In this proposal, I am requesting financial support to cover costs of labor and supplies to conduct ongoing research projects and initiate a new fungicide field trial that support TACF efforts to restore the American chestnut tree to our eastern forests.

Assaying plant and soil samples for species of *Phytophthora*

We began isolating *Phytophthora* spp. from chestnut trees and soils in 2003 and have continued to provide this service to TACF ever since. Isolates recovered from chestnut trees, seedlings, and soils are maintained in a permanent collection in our lab at Clemson University. Over 20+ years, we have processed hundreds of samples and have recovered *Phytophthora* spp. from 11 states—primarily in the southeastern and mid-Atlantic regions but also from a chestnut planting in southwestern Pennsylvania and from two sites in Missouri. Therefore, we now have a better understanding of the distribution of *Phytophthora* spp. in the native chestnut growing regions of the eastern U.S. We have primarily isolated *P. cinnamomi* from these samples, but occasionally we isolate other species. Based on our isolation efforts, we have confirmed PRR on chestnut seedlings or questioned the role of *P. cinnamomi* in seedling death in numerous plant samples sent to our lab for processing. We have confirmed the presence of *P. cinnamomi* in numerous chestnut planting sites or potential planting sites, which has prevented the death and loss of many valuable chestnut seedlings. To preserve American chestnut germplasm, TACF Chapters are trying to save representative seedlings and cuttings of surviving American chestnut trees. Often, these are preserved in GCOs located in states throughout the native range of the American chestnut. Before establishing a GCO, it is important to identify a site where *P. cinnamomi* is not present to prevent PRR on the planted material, which eventually will die if infected. Soil and plant samples are assayed using standard isolation protocols that have been developed in my lab over many years of studying *Phytophthora* spp. associated with fruit and ornamental crops (Ferguson and Jeffers 1999, Jeffers and Martin 1986). Recently, a graduate student in our lab improved our standard baiting bioassay to allow better detection of *Phytophthora* spp. in soil samples.

Maintaining isolates of *Phytophthora* spp. in a permanent collection

Representative isolates of *Phytophthora* spp. recovered from plant and soil samples are maintained in a permanent collection in our lab, so they are available for futures studies and projects. Over the years, we have identified five species of *Phytophthora* associated with chestnut trees, seedlings and soils: *P. cinnamomi*, *P. cambivora*, *P. cryptogea*, *P. heveae*, and *P. quercetorum* (Sharpe 2017). Before our study, only *P. cinnamomi* had been associated with American chestnut trees (Crandall et al. 1945, Westbrook et al. 2019). We currently have several hundred isolates already in our collection and will continue collecting new isolates over the coming years. Eventually, we would like to characterize the diversity of the population of *Phytophthora* spp. associated with American chestnut. At some point, the TACF breeding program will need to incorporate these other species into the annual screening procedure to be sure hybrid chestnut seedlings are resistant to all species of *Phytophthora* present in eastern forests.

RNA sequencing/root infection project

The people in my lab are collaborating with chestnut colleagues Tatyana Zhebentyayeva, Bert Abbott, Dana Nelson, and Jared Westbrook to study the initial infection process on chestnut roots. In 2021, funds were made available to fund an RNA sequencing project, which was conducted in April 2022. This involved infecting both American and Chinese chestnut seedlings with *P. cinnamomi* by immersing seedlings in a suspension of zoospores in 19-liter buckets. Seedlings then were removed of at four time points—3, 6, 12, and 24 h after initial exposure—and roots were processed immediately after removal. The root system on each seedling was divided into three parts for different assays: one part was frozen in liquid nitrogen for RNA sequencing to identify genes involved in resistance, one part was surface disinfested and used for direct isolation to determine when roots became infected, and the third part was chemically fixed for microscopic examination to visualize how zoospores enter root tissue. Root isolation and RNA sequencing data have been collected and analyzed, and we are waiting for results from the histopathology study—which has had a series of delays—so we combine the three aspects into a cohesive picture of infection of chestnut roots by *P. cinnamomi* zoospores. In June 2023, Abbott and Zhebentyayeva came to our lab at Clemson University to conduct a follow-up RNA sequencing experiment to collect additional data on gene expression. Another experiment was conducted in June 2024 using 186 F2 hybrid seedlings from a single population (one tree). Root samples from this experiment are waiting for RNA sequencing. Our team hopes to prepare a proposal for funding at the national level to continue this project.

Annual TACF effort to screen hybrid chestnut seedlings for resistance to *P. cinnamomi*

In cooperation with Dr. Joe James, my lab at Clemson University initiated a project to screen American, Chinese, and hybrid chestnut seedlings for resistance to *P. cinnamomi* in 2004. That project has continued annually ever since, and now is conducted in cooperation with the USDA Forest Service at the RSC at Bent Creek Experimental Forest in Asheville, NC. Each year, the site where surviving seedlings from the annual trial will be planted in the field is selected. This site is based on soil samples collected from potential sites in the Mid-Atlantic and Southeastern states. Soil samples are processed in our lab to identify ones that contain *P. cinnamomi*. Isolates of this pathogen are subcultured and maintained in axenic culture in a permanent collection.

Once the field site for out-planting surviving seedlings is identified, the appropriate isolates of *P. cinnamomi* are used to produce a large quantity of inoculum. Staff from my lab participate in inoculation of seedlings in the spring and scoring seedlings for root rot severity in the fall. Our involvement helps maintain continuity and consistency in how seedlings are inoculated and rated in this annual evaluation. However, the RSC was not available in 2024 due to staffing issues, and it may not be available again in 2025. Therefore, an alternative site for growing and screening hybrid seedlings may need to be identified.

Fungicides for managing Phytophthora root rot on American chestnut seedlings

Previously, we have conducted several greenhouse experiments to evaluate fungicides that target diseases caused by species of *Phytophthora* and other Oomycetes for efficacy at managing PRR on American chestnut seedlings. Products containing the active ingredients mefenoxam and phosphonates consistently have been most effective (Jeffers et al. 2020, Schmitz et al. 2023). However, performance under controlled conditions in the greenhouse on seedlings planted in soilless container mix may not be the same as performance on seedlings growing in field soil and exposed to changing environmental conditions throughout the year in natural ecosystems. Therefore, field performance of fungicides registered for use on chestnut trees needs to be determined before they are recommended to protect chestnut seedlings in GCOs. Therefore, in Feb 2024, we established a field trial to evaluate the fungicide active ingredients, mefenoxam (Ridomil Gold) and phosphonate (Reliant) in cooperation with colleagues at Warren Wilson College in Swannanoa, NC. Seedlings were planted in a site where *P. cinnamomi* is known to occur using a randomized complete block design. The first fungicide application was made on 10 June with the second application scheduled for some time in September. We plan to establish a similar, replicate trial on the Clemson campus this fall (2024) or early next year (Jan-Feb 2025). Currently, American chestnut seedlings (approximately 100) are growing in our greenhouse at Clemson University. Seedlings will be transplanted after temperatures have cooled and plants are dormant. Fungicides will be applied at label rates three times during the growing season each year—Fall, spring, and summer. Plants will be evaluated periodically during the growing season for one to several years for symptom development and mortality. In addition, I am working with the BAYER/ENVU company to extent the label of ALIETTE fungicide for use on chestnuts in nurseries and GCOs through a FIFRA section 2(ee) recommendation. This will allow the use of ALIETTE under special circumstances not specified on the current label. ALIETTE is another phosphonate product that has performed well in our greenhouse trials.

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Zhebentyayeva, T. N., Sisco, P. H., Georgi, L. L., Jeffers, S. N., Perkins, M. T., James, J. B., Hebard, F. B., Saski, C., Nelson, C. D., and Abbott, A. G. 2019. Dissecting resistance to *Phytophthora cinnamomi* in interspecific hybrid chestnut crosses using sequence-based genotyping and QTL mapping. Phytopathology 109:1594-1604.

H. Timeline (Nov 2024 – Oct 2025)

Research Project	Q1: Nov-Jan	Q2: Feb-Apr	Q3: May-Jul	Q4: Aug-Oct
Assaying plant and soil samples and maintaining isolates of *Phytophthora* spp.	X	X	X	X
RNA sequencing experiment& proposal	X	X	X	
Annual TACF resistance screening effort	X		X	
Fungicide field trials				

I. Measurement and Reporting of Results

Projects will be conducted based on the timeline above. Data will be collected as it becomes available—i.e., after samples are received and assayed and at the ends of trials. Likewise, these data will be summarized and analyzed when results are available. An annual report will be prepared in a timely manner based on results obtained in the 12-month period covered by the proposal. Progress and results to date will be presented at NE-2333 Meetings. Once a project has been completed and if data are based on replicated trials, results will be published in the *Chestnut* or other suitable peer-reviewed journal.

J. Budget: One Year (Nov 2024 – Oct 2025)

Expense	TACF: Requested ($)	Clemson: Matching ($)
23% of salary for Lab Manager/Research Technician	7,750	
Fringe Benefits: 43.2% for full-time employees	3,348	
Expendable supplies:	2,700	
Lab and greenhouse = $1,200		
Fungicide field trial = $1,500		
Subtotal	13,798	
Unrecovered overhead = F&A @ 52.5% = $6,456	0	7,244
TOTAL	***13,798***	***7,244***

Budget Justification

Amount Requested from TACF ($13,798)

Salary & Fringe Benefits
Funds are requested to cover 23% of the salary and fringe benefits for the Lab Manager (Celeste Giles) who will manage and work on these projects. She is paid $16.20/hour and works 40 hours/week ($33,696 annual salary). Fringe benefits for full-time employees for the coming year at Clemson University are 43.2%. Additional funds for labor to cover expenses incurred during the 2024 RNA seq experiment are included here (previously approved by Fitzsimmons and Westbrook).

Expendable supplies

- Lab and greenhouse = $1200: Lab supplies will be used for isolation from plants and soil, isolate identification, and culture storage—e.g., disposable petri dishes, disposable multi-well plates, agar media, chemicals, medium amendments, PCR primers, centrifuge tubes, glass storage vials, etc. Greenhouse supplies for growing and maintaining plants are also needed. Additional funds for supplies purchased for the 2024 RNA seq experiment are included here (previously approved by Fitzsimmons and Westbrook).
- Fungicide field trial = $1,500: Supplies are needed to establish the new field trial on the Clemson campus – including weed barrier, irrigation materials, tree protectors, and farm usage fees.

Matching funds provided by Clemson University ($7,244)
TACF has a policy to not pay overhead (F&A – Facilities and Administration) charges because their grants are relatively small—see accompanying document. Therefore, this amount ($6,456) is used as matching funds from Clemson University.

K. Brief CV for the 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-present: Professor – Dept. of ESPS; School of Agriculture, Forestry, and Environmental Sciences; Dept. of Agricultural and Environmental Sciences; Dept. Plant and Environmental Sciences (currently)
2001-2007: Associate Professor – Depts. PP&P; 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): 2010-2020
Selected Topics/Introduction to Graduate School (PES 8060): 2023-2024
Plant Disease Diagnosis (PLPA 4110/6110)—co-instructor: 2012-present

Peer-Reviewed Publications: Previous 5 years
Bily, D. S., Yang, X., Giles, C. E., and Jeffers, S. N. 2024. *Phytophthora fagacearum*, sp. nov. Fungal Planet description sheet no. 1656. Fungal Systematics and Evolution 13:312--314. https://fuse-journal.org/images/Issues/FP1614-1696.pdf

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differential response to *Fusarium oxysporum* f. sp. *vasinfectum* race 4 in three cotton cultivars. Plant Disease 106:990-995.

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Zhebentyayeva, T. N., Sisco, P. H., Georgi, L. L., Jeffers, S. N., Perkins, M. T., James, J. B., Hebard, F. B., Saski, C., Nelson, C. D., and Abbott, A. G. 2019. Dissecting resistance to *Phytophthora cinnamomi* in interspecific hybrid chestnut crosses using sequence-based genotyping and QTL mapping. Phytopathology 109:1594-1604.

Westbrook, J. W., James, J. B., Sisco, P. H., Frampton, J., Lucas, S., and Jeffers, S. N. 2019. Resistance to *Phytophthora cinnamomi* in American chestnut (*Castanea dentata*) backcross populations that descended from two Chinese chestnut (*Castanea mollissima*) sources of resistance. Plant Disease 103:1631-1641.

L. Conflict of Interest or Commitment Statement

There are no conflicts of interest with the above-listed Principal Investigator regarding this project.