Effects of *Phytophthora cinnamomi* Rands on growth, nutrient uptake, and water relations of American chestnut seedlings under varying pH

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Objectives

The overall goal of this research is to gain insight into the physiological mechanisms by which *Phytophthora cinnamomi* attacks American chestnut, and the potential variation incurred at different levels of soil pH, as well as investigating the potential of inoculation with the ectomycorrhizal fungus *Pisolithus tinctorius* to enhance *Phytophthora cinnamomi* resistance upon outplanting.

Specific objectives of this experiment include:

1. Determine the interactive effects of soil pH and ectomycorrhizal inoculation in the presence and absence of *Phytophthora* infestation on morphological and physiological response of American chestnut

2. Help ensure useful guidelines prior to reintroduction, through increased knowledge of American chestnut response to substrate pH and presence of the soil borne pathogen, *Phytophthora cinnamomi* Rands, as well as the potential to mitigate these deleterious effects through the use of ectomycorrhizal inoculation

Methods of Monitoring and Evaluation

-Statistical design

This experiment was established as a completely randomized 3 x 2 x 2 factorial design, with three levels of pH (4.0, 5.5, and 7.0), two *Phytophthora cinnamomi* Rands treatments

(inoculated and not inoculated), and two *Pisolithus tinctorius* treatments (inoculated and not inoculated). There were 8 replicates of each treatment combination, using 96 seedlings. Treatments were randomly assigned across each replicate, and individual seedlings were re-randomized on the greenhouse bench monthly.

-Seedling establishment

Putatively blight resistant (BC3F1) American chestnut seedlings were used for this experiment. Seedlings were obtained through the breeding program of the Hardwood Tree Improvement and Regeneration Center (HTIRC) at Purdue University, in cooperation with the Indiana DNR Nursery near Vallonia, Indiana. Stocktype was one-year-old bareroot seedlings (1+0).

Figure 1. Established BC3F1 seedlings prior to *Phytophthora cinnamomi* inocculation.

Chestnut seedlings were obtained in early April 2011, and planted into 12 L nursery containers. Soil substrate consisted of three treatments of a 1:1:1 mixture of peat, perlite, and sand, with pH levels of 4.0, 5.5, or 7.0. Substrate pH treatments were established through additions of $CaCO_3$, and were carefully monitored throughout the growing season. Prior to container planting, seedlings were presorted to ensure damaged and/or too small and too large seedlings were eliminated from the sample, in an effort to reduce potential confounding factors. Half of all seedlings were inoculated with *Pisolithus tinctorius* basidiospores at the time of planting. All containers were then be placed in the Lilly greenhouse facilities (Purdue University, West Lafayette, Indiana, USA), and grown under ambient greenhouse conditions for one growing season. Pots were monitored daily, and watered regularly throughout the growing season. All laboratory work was carried out in the Purdue Plant and Pest Diagnostic Laboratory located in the Lilly Hall of Life Sciences (Purdue University, West Lafayette, Indiana, USA).

-*Pisolithus tinctorius* Inoculation

Basidiospores of *Pisolithus tinctorius* were applied to the rooting zone of the soil mixture at the time of planting, at a rate of approximately 2.75×10^7 spores plant^{-1}. This corresponds to 25 mg of dried spores plant^{-1} (Marx and Bryan 1975). Seedlings were then well watered to encourage mycelia growth. Basidiospores were provided by Mycorrhizal Applications Inc.

-*Phytophthora cinnamomi* Rands Inoculation

Cultures of *Phytophthora cinnamomi* Rands for use in the inoculation treatments of all three experiments were obtained from the American Type Culture Collection (e.g., ATCC # MYA-4085). These cultures were shipped on ATCC medium 343, V8 juice agar. This medium consists of V8 juice (200.0 mL), $CaCO_3$ (3.0 g), agar (15.0 g), and tap water (to 1.0 L).

Inoculation treatments were carried out following the methodology of Burns and Benson (2000) as adapted by Jeffers et al. (2009), in which rice grains were used to develop an inoculum. Two isolates of *Phytophthora cinnamomi* were used to colonize two separate sets of rice grains. A composite inoculum was developed beginning in July, using equal parts of each set of colonized rice grains.

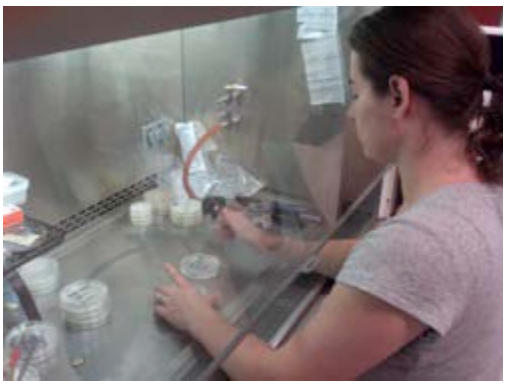


Figure 2. Culturing *Phytophthora cinnamomi* in V8 agar media in the lab.

In mid-August 2011, 5 mL of the composite inoculum was spread in a thin layer approximately 5 cm deep in those pots receiving the inoculation treatment, in a circular trench approximately 5 cm from and encompassing the seedling stem (Meadows et al. 2011). The inoculum was then covered with soil substrate, and pots were then watered to avoid desiccation of the inoculum. The growing season was then artificially extended in the greenhouse to allow sufficient time to capture results of the inoculation.

-Data collection

A sub-sample of seedlings will be destructively sampled to establish baseline morphological data (height, root collar diameter, biomass allocation, and proportion of necrotic roots as outlined below) prior to treatments.

-Morphological measurements

During destructive sampling, roots are being examined for ectomycorrhizal associations through the use of both WinRhizo 5.0 (Regent Instruments, Quebec, Canada), as well as with a visual rating system. Tree height and root collar diameter was measured on all seedlings immediately following planting to establish baseline data prior to inoculation treatment, and then monthly throughout the growing season. The final measurement occurred at the end of the growing season, just prior to the initiation of destructive sampling.

At this time, destructive sampling is ongoing. Seedlings are being excised from containers, separated into shoots and roots. Root damage due to

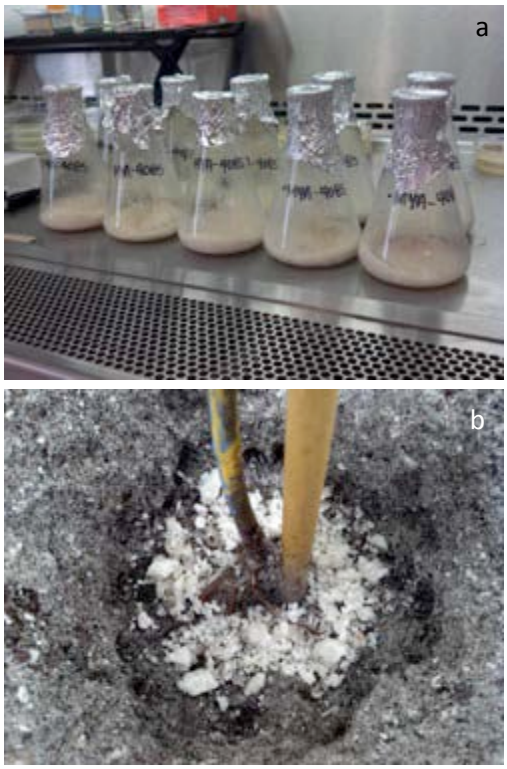


Figure 3. *Phytophthora cinnamomi* inoculum (a) in culture in flasks, and (b) applied to seedlings in the greenhouse.

Phytophthora cinnamomi Rands is being assessed using the software WinRhizo 5.0 (Regent Instruments, Quebec, Canada) to determine root lengths, surface area, and volume. The proportions of live and necrotic root tissue will be calculated for each of the above parameters, as these parameters are highly correlated with the ability to extract nutrients and moisture from the soil (Atkinson 2000). Roots are also being rated on a scale of 0 to 3 as an indicator of symptom severity in accordance with Jeffers et al. (2009). Ratings are as follows:

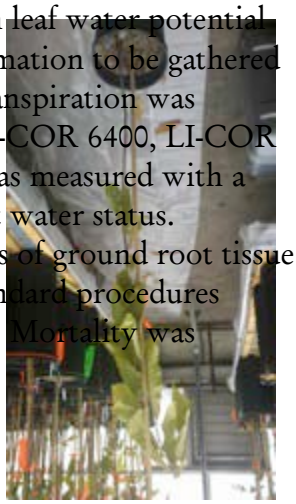
- 0 = healthy, no visible lesions on roots
- 1 = lesions on at least one lateral root
- 2 = lesions on the tap root
- 3 = severe root rot, plant dead

Post scanning, roots and shoots are being oven dried at 70°C for 72 hours to determine biomass allocation.

A subsample of necrotic root pieces will be utilized in an effort to re-isolate *P. cinnamomi* using common baiting techniques.

-Physiological measurements

Stomatal conductance and transpiration were measured monthly throughout the growing season, corresponding with morphological measurements, once leaves had expanded to a size that such measurements were possible. This sampling scheme was chosen in an effort to track physiological changes resulting from *P. cinnamomi* infection. Pre-dawn leaf water potential was measured twice throughout the growing season, allowing for information to be gathered both pre- and post-pathogen inoculation. Stomatal conductance and transpiration was measured using the portable gas exchange and fluorescence analyzer (LI-COR 6400, LI-COR Biosciences, Lincoln, NE, USA), while pre-dawn leaf water potential was measured with a pressure chamber (PMS Instruments, Corvallis, Oregon) to assess plant water status. Following oven drying, root samples are finely ground, and sub-samples of ground root tissue will be analyzed for macro- and micro-nutrient concentration using standard procedures conducted by A & L Great Lake Laboratories (Fort Wayne, IN, USA). Mortality was documented throughout the experiment.



-Assessment of ectomycorrhizal associations

During destructive sampling, roots are being examined for ectomycorrhizal associations through the use of both WinRhizo 5.0 (Regent Instruments, Quebec, Canada), as well as with a visual rating system. Visual cues used to differentiate between ectomycorrhizal and non-mycorrhizal root tips included color, size, form, and texture (Quoreshi and Khasa 2007). The visual rating system is expressed as an approximate percentage of the root system that has

Figure 4. (a) Ecophysiological assessment of seedlings, and (b) American chestnut hybrid seedling exhibiting symptoms of *Phytophthora cinnamomi* infection.

formed mycorrhizal associations. Rating is as follows:

- 0 = 0%
- 1 = 1 to 25%
- 2 = 26 to 50%
- 3 = 51 to 75%
- 4 = 76 to 100%

Results

This project is part of an ongoing PhD dissertation project, and the final results are pending. Results will be made available to the American Chestnut Foundation upon project completion, in the form of manuscripts submitted to journals, and a completed PhD dissertation.

-Data analysis

Multiple analysis of variance (MANOVA) will be used to examine significant ($\alpha = 0.05$) differences in height growth, root collar diameter, water and nutrient parameters, as well as proportion of *Phytophthora* induced root necrosis (by root length, volume, and surface area) as a function of the mycorrhizal and soil pH treatments. Interaction effects between treatments will also be analyzed. Regression analysis will be employed to determine correlation between seedling responses to varying pH levels, with *Phytophthora* and *Pisolithus* inoculation as covariates. All data will be tested to ensure assumptions associated with the error term have been met, and transformed if necessary. All analyses will be carried out using SAS (Cary, NC, USA) and/or the R statistical software package (R Development Core Team 2008).

-Expected Outcomes

We expect to see decreased physiological function in response to *P. cinnamomi* inoculation, with regard to all physiological parameters measured. Foliar nutrient concentrations are expected to be lower in those seedlings experiencing the inoculation treatment. Seedlings inoculated with *P. cinnamomi* should exhibit retarded height and diameter growth, as well as decreased root volumes. Ectomycorrhizal inoculation should mitigate some of the negative effects of *P. cinnamomi* infection. We expect that this effect will vary with different levels of soil pH.

Published works and presentations

The findings of this research will be summarized and presented through various outlets. The PhD dissertation will include a complete analysis for all parameters from this experiment. The findings of this experiment will be submitted to appropriate peer refereed scientific journals. Additionally, preliminary and conclusive results will be presented at both regional and national conferences relevant to the subject material.

Press coverage

N/A

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