Final Report: "Evaluating chemical fingerprinting as a tool to rapidly screen hybrid chestnut for disease resistance"

Principal Investigators
Anna O. Conrad, Forest Health Research and Education Center, Department of Forestry, University of Kentucky
Albert G. Abbott, Forest Health Research and Education Center, Department of Forestry, University of Kentucky
C. Dana Nelson, Southern Institute of Forest Genetics, Southern Research Station, USDA Forest Service, and Visiting Scientist, Department of Forestry, University of Kentucky

Collaborating Scientists
Pierluigi Bonello, Department of Plant Pathology, The Ohio State University
Luis Rodgriguez-Saona, Department of Food Science and Technology, The Ohio State University
Jared Westbrook, The American Chestnut Foundation
Tetyana Zhebentyayeva, Department of Genetics and Biochemistry, Genomics and Computational Biology Laboratory, Clemson University
Steven N. Jeffers, Department of Plant and Environmental Sciences, Clemson University

Summary
Screening chestnut hybrids for disease resistance is intensive because trees must be inoculated with either *Cryphonectria parasitica* (causal agent of chestnut blight) or *Phytophthora cinnamomi* (causal agent of *Phytophthora* root rot). Chemotyping (i.e. chemically phenotyping or fingerprinting) trees and identifying chemical markers associated with resistance is an alternative method that could be used to screen trees rapidly. The goals of this study were to chemotype American and Chinese chestnut parents and inter-specific hybrids, to identify potential markers of disease resistance, and to develop a method that can be used to prescreen hybrids for resistance prior to planting in seed orchards.

Objectives
(1) Chemotype American and Chinese chestnut and inter-specific hybrid families;
(2) Use chemometric analysis to identify chemotypic differences between individuals that vary in susceptibility to *Cryphonectria parasitica* (chestnut blight) and *Phytophthora cinnamomi* (*Phytophthora* root rot), respectively, and
(3) Develop and validate chemical marker-based statistical models to screen hybrids for resistance to chestnut blight and *Phytophthora* root rot.

Material and methods

Plant material

Chestnut blight assay
J. Westbrook provided us with stem and leaf tissue from 211 BC_3-F_3 hybrid seedlings from 22 BC_3-F_2 mother trees, 10 American and 10 Chinese chestnut trees (Table 1) collected prior to inoculation with *C. parasitica.* J. Westbrook also provided phenotypic data for these seedlings

collected 5-weeks after inoculation, which included blight lesion length and blight ratings from 1 (less susceptible) to 3 (more susceptible).

We originally proposed a follow-up experiment in 2016 (contingent on the previous analysis), focused on evaluating chemical fingerprinting in 150 0 – 1 year old BC_3-F_2 hybrids originating from one BC_3 mother. In 2016, we coordinated with J. Westbrook to receive additional material from a BC_3-F_3 screening in summer 2016 that would include more individuals per family than the material received in 2015. However, evidence of resistance, judged by differences in symptoms (based on stem canker size) between individuals, was not pronounced (personal communication with J. Westbrook), so no additional material was received in 2016.

Phytophthora root rot assay
For the present study, foliar tissue (provided by T. Zhebentyayeva) from HB and Nanking crosses (Table 1) was evaluated. The tissue from ~20 – 30 individuals of the more severe (e.g. root rot severity class 3) and less severe or slow dying (e.g. root rot severity classes 0 – 2) rating groups were chemotyped for both HB and Nanking lines, when available. These materials were collected as part of a separate study, involving T. Zhebentyayeva, S. Jeffers and PIs Abbott and Nelson, funded by Foundation of the Carolinas to map *P. cinnamomi* root rot resistance in American/Chinese interspecific populations.

Table 1. Sources of plant material for chemotyping study.

Experiment	**Number of individuals chemotyped***	**Number of families examined**	**Material provided by**
Blight 2015	91 BC_3-F_3 hybrids and 19 American and Chinese chestnut	21 BC_3-F_2 mother trees; Wilkinson (Chinese); Sugar Loaf East (American)	J. Westbrook
Root rot 2015	102 BC_1 hybrids	Nanking (NK4) and HB (HB2) BC_1 crosses	T. Zhebentyayeva
Total seedlings screened: 212 seedlings			
*A subset of these samples had to be removed prior to statistical analysis due to abnormal spectra, which was associated with samples whose extraction volumes were modified because < 100 mg of plant tissue was available.			

Plant material for blight and root rot assays were analyzed separately according to the following procedure:

Chemotyping chestnut
Frozen plant tissue from each experiment was finely ground in liquid nitrogen using a mortar and pestle. For each plant tissue sample, 100 mg ± 1 mg of frozen powdered plant tissue was extracted two times with 500 ml of HPLC-grade methanol, extracts were centrifuged each time and the supernatants from each of the two sample extractions were pooled and stored at –70ºC (Nagle et al., 2011).

Extracts were analyzed using Fourier-transform infrared (FT-IR) spectroscopy, which can be used to rapidly chemotype extracts. FT-IR spectroscopy measures changes in molecular absorption of infrared radiation (Diem, 1993; Guillén and Cabo, 1997; reviewed in Rodriguez-

Saona and Allendorf, 2011). This technique does not individually separate chemicals in plant extracts, but instead produces a chemical fingerprint (Figure 1) based on levels of all the chemicals (i.e. chemical groups) present within an extract that can be detected over a specific spectral range (e.g. mid-infrared spectrum, 700 to 4000 cm^{-1}). A Varian 3100 FT-IR spectrometer equipped with a triple bounce zinc selenide attenuated total reflectance (ATR) accessory was used to analyze samples based on the methods of Conrad et al. (2014). For each sample analyzed, 10 µl of extract was loaded onto the ATR crystal and allowed to sit for ~60 seconds in order for the methanol, which interferes with the spectral signal, to evaporate. Two technical replicates were analyzed for each biological replicate. Spectra were collected using Resolutions Pro version 4.1.0.101 (Varian, Inc., now Agilent Technologies Inc., Santa Clara, CA, USA).

Statistical analysis

Data collected from FT-IR spectroscopy were analyzed using the chemometrix software Pirouette version 4.5 (Infometrix, Inc., Bothell, WA, USA). With Pirouette, data is easily organized, visualized, and mined; quantitative and qualitative analyses can be performed and complex signals can be deconvoluted (Infometrix, Inc., 2014). Two approaches were used to analyze data: soft independent modeling of class analogy (SIMCA) and partial least squares regression (PLSR). SIMCA combines principal components analysis with classification analysis, creating principal components models for each training group (e.g. resistant and susceptible trees). PLSR combines data reduction methods with regression allowing for the development of quantitative predictive models (reviewed in Conrad and Bonello, 2016). SIMCA was used to detect chemotypic differences between groups (e.g. *Phytophthora* root rot rating groups), while PLSR was used to examine the association between chemotypes and quantitative measures of susceptibility (e.g. blight lesion length). From these analyses, predictive models based on regions of the chemical spectrum were also developed. Finally, data were transformed (e.g. second derivative function) and outliers were trimmed as needed, based on preliminary SIMCA and PLSR analyses.

Results

Chestnut blight assay

In a preliminary test of FT-IR, we compared methanol extracts from stems versus leaves of 18 BC_3F_3 hybrids, to determine which tissue was better for distinguishing between blight rating groups. We focused subsequent blight analyses on methanol extracts from seedling stem tissue since better group separation was achieved with these extracts in the preliminary test. There were clear chemotypic (i.e. chemical fingerprint) differences between American and Chinese chestnut (Figure 2). A 2-factor SIMCA was used to distinguish between second derivative transformed spectra (spectral range: 1402 – 1805 cm^{-1}) collected from American chestnut ('Sugar Loaf East') and Chinese chestnut ('Wilkinson') stem methanol extracts (Figure 3). 100% of American (biological replicates: n = 6) and Chinese (biological replicates: n = 8) chestnut samples were correctly classified by the model, with an interclass distance of 3.39 (the larger the interclass distance the less likely trees are to be classified as both American and Chinese chestnut by the SIMCA model).

In order to remove potential family bias from the analysis of hybrid chestnut (i.e. to avoid incorporating chemotypic differences associated with family and not with variation in susceptibility to blight), only 0 – 3 individuals per family per blight rating group (1, 2, or 3) were analyzed. There was no clear association between blight susceptibility (based on either blight rating or blight lesion length) and spectral data across all 21 hybrid families analyzed. As a result, families were split into two groups based on the original BC_1 hybrid from which they were derived, i.e. 'Clapper' versus 'Graves'.

For the 'Clapper' data set (Duncan farm descendants, N = 55), a 5-factor PLSR model with leave-one-out cross validation explained > 99% of the variation (SEV = 4.78) of second derivative transformed (35-points) and trimmed (~33% of technical replicates were removed based on preliminary PLSR analysis) spectral data (spectral range of 901 – 1622 cm^{-1}) (Figure 4). Furthermore, measured blight lesion lengths and predicted lesion lengths (based on spectral data) were highly correlated (r_{val} = 0.79). We were unable to find a similar association for the 'Graves' data set (Wagner farm descendants), although our approach of including many families with a small number of individuals per family may have introduced too much variability into this model.

Phytophthora root rot assay

SIMCA was used to examine the relationship between BC_1 chestnut hybrid susceptibility to *P. cinnamomi* and constitutive chemical fingerprint data collected from leaf methanol extracts from two families, NK4 and HB2. Similar to the blight assay, there was no clear association between *P. cinnamomi* susceptibility and chemical fingerprint data across both families, which differ in their sources of resistance. As a result, families were analyzed independently.

Using a 4-factor SIMCA (with ~22% of technical replicates removed), second derivative transformed spectra (35-points) from the HB2 family could be used to distinguish, although with a relatively low discriminating power and small interclass distance, between the most susceptible root rot rating groups, 2 (lesions on the tap root) and 3 (dead) (Figure 5). Of note, no HB2 individuals with ratings of 0 or 1 were available for the present FT-IR analysis. For the NK4 family, we focused on examining differences in chemical fingerprint data between rating groups 1 and 3 using SIMCA analysis. While there was some clustering of samples within groups, the relationship between spectral data and root rot rating was very weak. Furthermore, due to the number of factors included in the *Phytophthora* root rot models, it is possible that the models are over fit and will not yield accurate predictions of naïve cases (i.e. the models will be unable to accurately predict the level of susceptibility of individuals that were not included in the data set used to generate the model). Additional individuals are needed to refine and validate models, since we did not have enough individuals to separate out our data into training (model development) and testing (model validation) sets.

Conclusions

Results suggest that chemical fingerprinting may be a useful tool for screening seedlings for disease resistance; however, further evaluation is needed due to the relatively small scale of this experiment. Clear differences in the constitutive (pre-infection) chemical spectra (i.e. chemical fingerprint) of American and Chinese chestnut were observed, suggesting that the technique is sufficiently sensitive to pick up on chemical differences between species. The strong positive

correlation between predicted (based on constitutive chemical spectra) and measured stem lesion lengths from the 'Clapper' blight data set, suggest that the method is also capable of detecting chemotypic differences within hybrid groups that vary in susceptibility to blight. Further tests are required to refine and validate existing models for both chestnut blight and *Phytophthora* root rot, and additional phenotypic data from more hybrid chestnut trees is needed. Additional funding has been requested to continue this project.

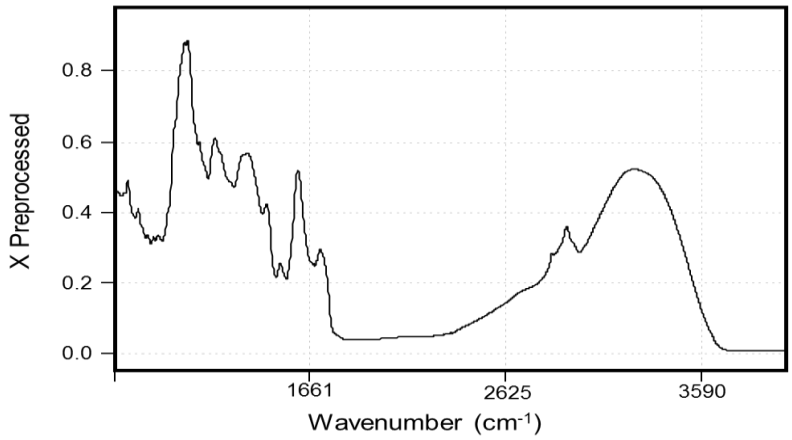

Figure 1. A representative chemical fingerprint of a methanol extract from hybrid chestnut stem tissue. Figure from 2015 – 2016 analysis.

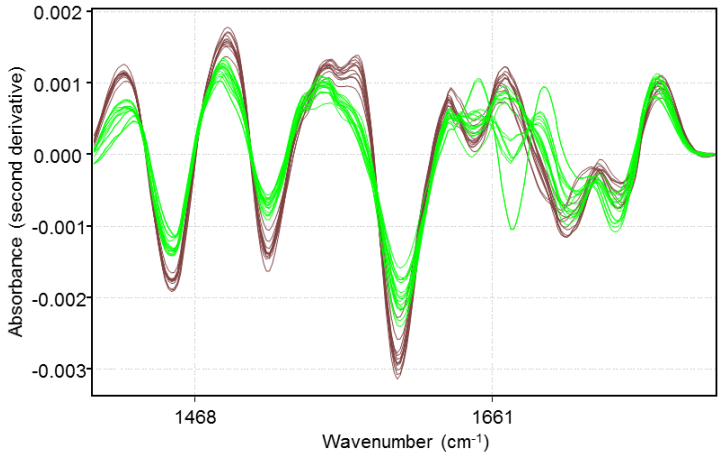

Figure 2. American (brown) and Chinese (green) second derivative transformed spectra, ranging from 1402 – 1805 cm^{-1}. Each line represents one spectrum (2 spectra were collected per individual). Chemotypic differences (areas of spectrum where American and Chinese chestnut differ, i.e., where green and brown lines do not overlap) are evident, in particular around 1422 and 1599 cm^{-1}.

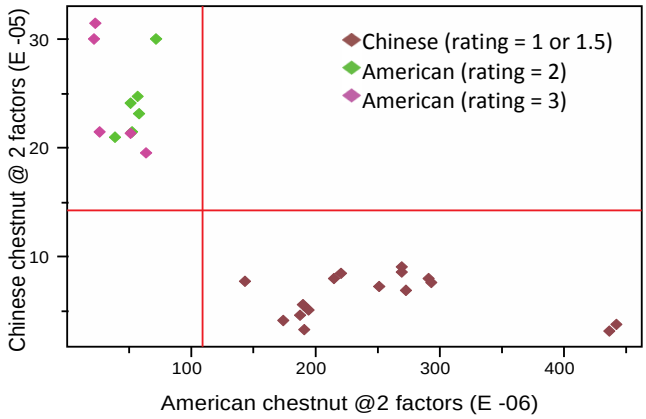

Figure 3. Class distances plot from 2-factor SIMCA analysis showing the relative dimension-free distance between American and Chinese chestnut seedlings based on second derivative (21 points) transformed chemical fingerprint data collected from a spectral range of 1402 – 1805 cm^{-1} (N = 28, with 2 technical replicates per biological replicate). Red lines indicate critical sample residual thresholds.

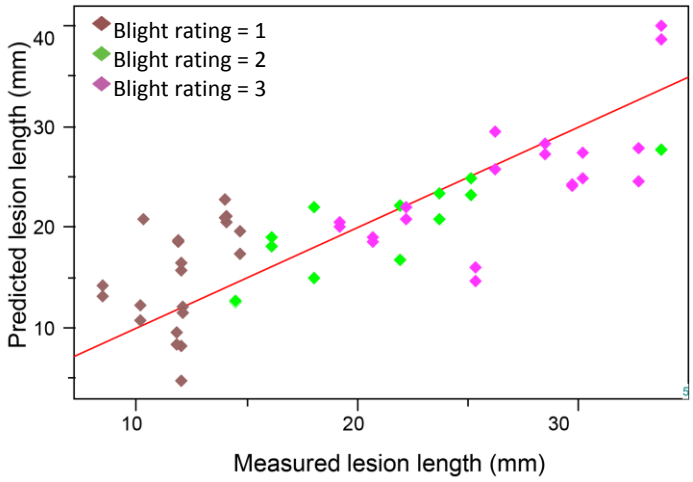

Figure 4. Correlation plot from 5-factor PLSR analysis of the 'Clapper' blight data set showing the relationship between measured and predicted lesion lengths, based on second derivative transformed spectral data (spectral range of 901 – 1622 cm^{-1}). Two technical replicates were analyzed independently for each biological replicate and replicates were trimmed as needed based on preliminary SIMCA analysis (N = 55).

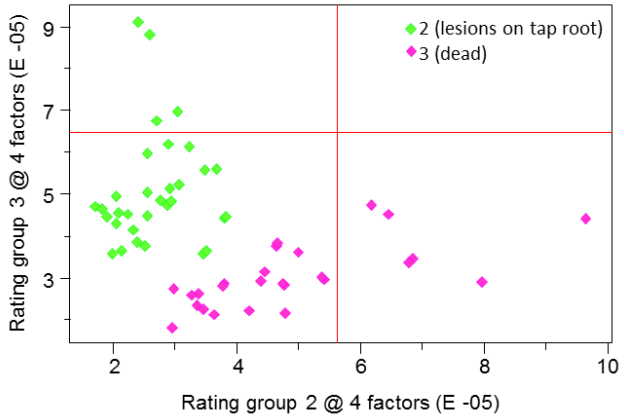

Figure 5. Class distance plot for 4-factor SIMCA analysis showing the relative dimension-free distance between individuals that differed in susceptibility to *Phytophthora* root rot in the HB2 BC_1 family based on second derivative transformed spectra. Red lines indicate critical sample residual thresholds. Two technical replicates per biological replicate were independently analyzed and replicates were trimmed as needed based on preliminary SIMCA analysis (N = 58).

Literature cited

Conrad, A.O., and Bonello, P. 2016. Application of infrared and Raman spectroscopy for the identification of disease resistant trees. Front. Plant Sci. 6:1152. doi: 10.3389/fpls.2015.01152.

Conrad, A.O., Rodriguez-Saona, L.E., McPherson, B.A., Wood, D.L., Bonello, P., 2014. Identification of Quercus agrifolia (coast live oak) resistant to the invasive pathogen Phytophthora ramorum in native stands using Fourier-transform infrared (FT-IR) spectroscopy. Front. Plant Sci. 5, 521.

Diem, M., 1993. Introduction to Modern Vibrational Spectroscopy. Wiley, New York, NY.

Guillén, M.D., Cabo, N., 1997. Infrared spectroscopy in the study of edible oils and fats. J. Sci. Food Agric. 75, 1–11.

InfoMetrix, Inc. 2014. Pirouette: Comprehensive chemometrixs modeling software. http://infometrix.com/software/products/pirouette/.

Nagle, A.M., McPherson, B.A., Wood, D.L., Garbelotto, M., Bonello, P., 2011. Relationship between field resistance to Phytophthora ramorum and constitutive phenolic chemistry of coast live oak. For. Pathol. 41, 464–469.

Rodriguez-Saona, L.E., Allendorf, M.E., 2011. Use of FTIR for rapid authentication and detection of adulteration of food. Annu. Rev. Food Sci. Technol. 2, 467–83.

Presentations

Conrad, A.O. Early screening of chestnut hybrid seedlings for resistance to chestnut blight and Phytophthora root rot. Invited talk at a meeting of the Virginia Chapter of the American Chestnut Foundation, Genomics and American Chestnut Restoration: New Tools to Identity and Increase Disease Resistance. Blacksburg, VA, October 28, 2016.

Conrad, A.O., Abbott, A., Nelson, C.D., Westbrook, J., Zhebentyayeva, T., Jeffers, S., Bonello, P., Rodriguez-Saona, L., Sisco, P., and James, J. Chemical fingerprinting: An alternative approach for screening hybrid chestnut for disease resistance. Oral presentation at NE-1333 Annual Meeting, Syracuse, NY, September 30-October 1, 2016.

Conrad, A.O., Westbrook, J. Zhebentyayeva, T., Rodriguez-Saona, L., Bonello, P., Nelson, C.D., and Abbott, A. Evaluating chemical fingerprinting as a tool to rapidly screen hybrid chestnut for resistance to pathogens. Poster presentation at American Phytopathological Society Annual Meeting, Tampa, FL, July 30-August 3, 2016.

Conrad, A.O., Nelson, C.D., Abbott, A., and Bonello, P. Chemical fingerprinting: An alternative approach for identifying disease resistant trees. Poster presentation at Society of Postdoctoral Scholars Research Symposium, University of Kentucky, Lexington, KY, June 3, 2016.

Final financial report

Expenses totaling $2,422.40 were used to cover costs associated with the project. For example, costs associated with the preparation of samples for FT-IR analysis and analysis of FT-IR data. Other expenses included travel costs, such as those associated with driving to Ohio State University, where the FT-IR analysis was performed.