a. Project Title

Variation in blight severity and inhibitory properties of bark against *Cryphonectria parasitica* in 20-year-old full-sib progeny of *Castanea mollissima* ('Mahogany' x 'Nanking')

b. Summary (100 words)

The project has two goals. First, we will phenotype a 20-year-old full-sib population of 150 Chinese chestnut crosses (Mahogany x Nanking) for variation in blight severity from natural infection. Second, we will test the potential of *in vitro* bark extract plate assays as a phenotyping method to screen the bark from a subset of the phenotyped trees for variation in inhibitory properties against *Cryphonectria parasitica*. This project will aid in identifying the genetic variation underlying bark chemistry and blight resistance in Chinese chestnut, and in developing phenotyping tools to identify trees with high blight resistance.

c. Principal Investigator and Institutional Affiliation

- **PI - Dr. Susanna Keriö,** Assistant Agricultural Scientist, Department of Forestry and Horticulture, Connecticut Agricultural Experiment Station (CAES), susanna.kerio@ct.gov, 203-974-8491
- **Collaborator: Bruce Levine.** PhD Student, University of Maryland, Department of Plant Science and Landscape Architecture, LevineBJ@umd.edu, 202-549-3187

d. Duration of project

Project is expected to last 6 months, January-June 2022.

e. Total amount of funding requested

- **Total requested in TACF grant: $10,000**
    - **Part-time research assistant salary: $10,000**
        - Salary costs for a part-time research assistant in the Keriö lab. Covers salary costs for 2 days/week of work over 6 months ($5712 salary, $4288 fringe).
- **Matching funding: $5139**
    - **$4139 for lab supplies.** Covered through Keriö's hatch/lab startup funding.
    - **$1000** for indirect costs: As TACF does not cover overhead fees, the CAES will cover the indirect costs (10% of requested grant amount).

f. Short and long-term goals of the project

- **Short term:** Study variation in chestnut blight resistance in the 20-year-old full-sib 'Mahogany' x 'Nanking' Chinese chestnut progeny in CAES research farm:
    1. Phenotype the progeny (150 trees) to detect variation in blight resistance
    2. Test variation in inhibitory properties of bark extracts prepared from the phenotyped trees
    3. Identify candidate 'Mahogany' x 'Nanking' trees for in-depth chemical and genetic analysis to identify metabolites and genomic regions associated with variation in resistance to natural blight infection.
- **Long-term:**
    - By end of project, report results of the phenotyping and plate assays.

g. Project narrative (5 pages)

Variation in chestnut blight resistance in 20-year-old full-sib progeny of *Castanea mollissima* ('Mahogany' x 'Nanking') based on field phenotyping and *in vitro* bark extract plate assays.

Chinese chestnuts (*Castanea mollissima*) are highly blight resistant, and several hybrids of American and Chinese chestnuts have been created as part of the breeding program coordinated by TACF. Among the parent trees are two Chinese chestnut trees, 'Mahogany' and 'Nanking', which both have high blight resistance (Steiner et al. 2017). The Chinese-American hybrids including the progeny of 'Mahogany' and 'Nanking' have been used in backcross breeding with the goal of creating hybrids with high American chestnut heritage and high blight resistance associated with the Chinese chestnut genes. Recent research on the genomics of chestnut blight resistance indicates that the resistance to chestnut blight is a polygenic trait, involving multiple loci on several chromosomes (Westbrook et al. 2020). The current breeding program scores the trees for breeding value based on a combination of small stem assays and performance in field plantings. Even though promising results have been acquired through this approach, getting the phenotypic values takes time. Therefore, alternative approaches that would help in identifying factors contributing to resistance in Chinese chestnuts could prove valuable to the breeding efforts.

A central location for host-pathogen interaction in the chestnut blight pathosystem are the active defenses in the inner bark. Bark of trees forms an important first line of chemical and physical defense against several fungal pathogens. The chestnut blight pathogen *Crypnonectria parasitica* establishes infection through wounds and natural openings such as lenticels on host epiderm or periderm (Lovat and Donnelly 2019). An important aspect of host resistance is the ability of the tree to compartmentalize the bark lesions and the pathogen. This is done through the formation of a lignified zone around the lesion. In

resistant trees and typically in Chinese chestnuts, the pathogen is not able to breach this cell layer (Lovat and Donnelly 2019 and references therein). However, it is not fully clear which compounds and molecules are responsible for the inhibitory properties of Chinese chestnut phloem and bark.

Bark extractions using various solvents have been tested to identify chemicals and molecules associated with resistance. Based on previous findings, the inhibitory properties of chestnut bark are the result of several molecules and compounds rather than of any single compound. Anagnostakis (1992) studied the inhibitory potential of acetone tannin extracts prepared with green bark from two Chinese, two Japanese, and two American chestnut trees each prepared from branch samples collected over the course of one year. The study found that tannins from different species of chestnut did not inhibit the growth of the EP155 isolate of *C. parasitica*. In fact, tannins seemed to stimulate fungal growth compared to control medium (Anagnostakis 1992). In another study (Gao and Shain 1995), acetone-extracted tannins from American chestnut were found to be more inhibitory to *C. parasitica* polygalactouronases (PGs) than tannins from Chinese chestnut. PGs are enzymes involved in plant cell wall modification, and are considered to be a potential *C. parasitica* virulence factor (Lovat and Donnelly 2019). McCarroll and Thor (1985) found that aqueous phloem extracts of Chinese chestnuts were more inhibitory to *C. parasitica* PGs and cellulases than American chestnut extracts. Samman, Schell and Thor (1978) found two probably unsaturated fatty acid compounds in petroleum solvent extracts from Chinese chestnut bark that were 100 and 75 percent inhibitory against *C. parasitica* in bioassays (Samman et al. 1978).

Phloem proteins have also been of interest as inhibitors of *C. parasitica*. Gao and Shain (1995) found that a phosphate buffer extract of Chinese chestnut phloem contained proteins that inhibited *C. parasitica* PG activity. The highest inhibitory activity was found in extractives prepared from samples collected in June and October in Kentucky (Gao and Shain 1995). Another study tested the impact of extracts prepared from freeze-dried young bark of Chinese and American chestnuts with hot water, various solvents, and phosphate buffer (Miller et al. 1992). The samples were collected either during the growing or dormant season. Solvent extracts were not inhibitory, but protein extracts from Chinese chestnuts were able to inhibit *C. parasitica* growth and lyse hyphae. Especially bark extracts prepared from ethylene-treated bark were inhibitory. The protein extracts of Chinese chestnut contained chitin isoforms and beta glucanases not found in American chestnut extracts, and these proteins were present independent of time of year (Miller et al. 1992). McCarroll and Thor (1985) found Chinese chestnut protein extracts from phloem twice as inhibitory as similar extracts from American chestnut. However, these and other results above were obtained on a limited number of trees. It would be of interest to screen a larger set of Chinese chestnuts to detect variation in the inhibitory properties of bark.

Research at the University of Maryland (2018) found that *C. parasitica* mycelium grown on a medium made with water-extracts of Chinese and American chestnut bark grew slower and accumulated less biomass on the medium made from Chinese chestnut, and failed to produce fruiting bodies, which were abundant in the American chestnut-derived medium . (Levine and Yu 2018). Further work is under way this summer at William and Mary comparing more replicates of bark from more Chestnut species and including ethanol-extract media as well. Based on these preliminary results, we will be able to expand the selection of plant material and extraction methods used in the suggested experiments.

In this project, we propose to analyze the segregation of the inhibitory properties of Chinese chestnut bark against *C. parasitica* in a 20-year-old full-sib progeny of highly blight resistant Chinese chestnuts 'Mahogany' x 'Nanking' established in CAES experimental farm. We will apply a combination of field phenotyping and bark extract plate assays to study the association of natural blight severity with *in vitro* inhibitory properties against *C. parasitica*. This unique set of plant material consisting of approximately 150 trees will offer an opportunity to associate natural blight infection and blight severity with the antifungal properties of bark extracts. These experiments will help in identifying the chemical, molecular, and genetic components associated with variation in chestnut blight resistance in Chinese chestnuts.

Planned experiment 1: Field phenotyping of natural blight infection. Per the phenotyping guidelines used by TACF and collaborators, phenotypic variables of interest include presence of cankers, containment of bark cankers, sporulation, exposure of wood, main stem viability, stem branching, DBH, height (Westbrook et al. 2020). This data will be collected by the PI and the hired research assistant. The phenotypic data will be used to select a subset of trees to prepare the bark extracts for plate assays.

Planned experiment 2: Bark extract plate assays. A subset of the most resistant and most susceptible trees will be selected to prepare the phloem extracts. First batch of plant material will be collected in early February. If differences in inhibition are found, plant material collection can be repeated later in the spring. These experiments are planned by the PI and the collaborator.

Branches will be harvested with a pole pruner and bark will be peeled off. The bark is allowed to dry at room temperature, followed by homogenization. Alternatively, the bark samples can be freeze-dried, or extraction can be started right after sample harvest without drying the samples. Extracts are prepared by soaking the bark samples in deionized water in a refrigerator for 48 h. Based on preliminary experiments conducted by collaborator Levine, we plan to use 10 g of bark and extract it with 100 ml of deionized water for 1 hour. The extracts will be centrifuged to filter out solid particles, and freeze dried to get total dry mass. The lyophilized extracts will be adjusted to a certain concentration by dissolving in

deionized water, filter-sterilized, and stored at -20°C until used. For the plate assays, the extracts will be mixed into water agar, PDA, or EP Minimal Medium (Anagnostakis) at a set concentration. Plates will be inoculated with *C. parasitica* strain EP155. Growth of the fungus will be monitored by measuring the colony radius and fungal biomass (grown on cellophane and peeled off to measure weight). Media without the extract will be used as a control.

Planned experiment 3: Inhibitory protein extracts and plate assays. A subset of the most resistant and most susceptible trees will be selected to prepare the phloem extracts. First batch of plant material will be collected in early February. If differences in inhibition are found, plant material collection can be repeated later in the spring.

Branches will be harvested with a pole pruner and bark will be peeled off. The bark will be freeze-fried, and small amounts of tissue (0.5-1 g) will be used to extract the potential inhibitory native proteins at +4°C following the protocols described elsewhere (Miller et al. 1992; Gao and Shain 1995; McCarroll and Thor 1985; Plomion and Lalanne 2007). The protein extracts are cleaned by dialysis or with a kit, and protein concentrations are measured with Qubit protein assay. The protein extracts are stored at +4°C until used (1-3 weeks). Inhibitory assays are performed by either adding the protein extracts into 5 mm wells cut into the growth media in advance of mycelium, or by adding them onto sterile filter paper discs. The inhibitory effect will be estimated based on hyphal morphology and growth rate around the protein extract.

h. Timeline, showing start and completion dates for each goal

- **Short term:** Study variation in chestnut blight resistance in a 20-year-old full-sib ‘Mahogany’ x ‘Nanking’ Chinese chestnut progeny in CAES research farm:
    1. Phenotype the progeny (150 trees) to detect variation in blight resistance (Jan-Feb 2023).
    2. Test variation in inhibitory properties of bark extracts and protein extracts prepared from the phenotyped trees. (February 2023 – May 2023).
    3. Identify candidate ‘Mahogany’ x ‘Nanking’ trees for in-depth chemical, genetic and proteomic analysis to identify metabolites, proteins and genomic regions associated with variation in resistance to natural blight infection (June 2023).
- **Long-term:**
    - By end of project, report results of the plate assays to TACF (July 2023).

i. How results will be measured and reported

Tree phenotyping: Per the phenotyping guidelines used by TACF and collaborators, phenotypic variables of interest include presence of cankers, containment of bark cankers, sporulation, exposure of wood, main stem viability, stem branching, DBH, height (Westbrook et al. 2020). Plate assays: The inhibitory properties of the bark extracts and protein extracts on *C. parasitica* growth will be evaluated compared to control plates (base media without extracts or proteins). The PI and collaborator will disseminate the results both through presentations and peer-reviewed publications. Results will be targeted for publication in Plant Disease, Canadian Journal of Forestry, or Forest Pathology.

j. Breakdown of how and when funds will be spent

Funds are needed for research assistant salary starting January 2023 and working until June 2023. Planned work schedule 2 days/week, monthly hiring cost $1666.7. Potential candidates to conduct the work (former intern in Dr. Keriö's lab; two biology students at Southern Connecticut State University) have already been identified.

k. Brief Curriculum Vitae (CV) for each Principal Investigator.

PI CV attached.

l. Conflict of Interest or Commitment (COI or COC) statement.

No conflict of interest to declare.

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