Identifying Host Resistance to *Phytophthora cinnamomi* in Hybrid Progeny of *Castanea dentata* and *Castanea mollissima*

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Abstract. *Phytophthora cinnamomi* Rands, the causal pathogen of phytophthora root rot (PRR) of chestnut, is one of the main obstacles to growth of american chestnut [*Castanea dentata* (Marsh.) Bork.] in the southern part of its distribution. To facilitate introgression of PRR resistance of chinese chestnut (*C. mollissima* Blume) into a *C. dentata* genetic background, we assessed the disease resistance of 10 interspecific hybrid families derived from potentially resistant *C. mollissima* cultivars. Hybrid progeny were inoculated with *P. cinnamomi* in the nursery and assessed for root lesion severity after 1 year of growth. Asymptomatic plants were transplanted to a *P. cinnamomi*-positive orchard and evaluated for survival midway through the following growing season. During the nursery experiment, 8 of 10 hybrid families were not significantly different from susceptible *C. dentata* controls for average disease resistance scores. However, multiple asymptomatic individuals were identified in each of the eight families. Two of the 10 hybrid families were not significantly different from the resistant *C. mollissima* and *C. henryi* controls. In the *P. cinnamomi*-positive orchard, the prescreened hybrid families displayed a greater proportion of survivors than backcross families that had not been prescreened for *P. cinnamomi* resistance. Hybrid plants that have survived 2 years of growth in *P. cinnamomi*-infested potting media and soils represent an important step toward the production of genetically diverse chestnut populations in the southeastern United States that combine the PRR resistance of *C. mollissima* with the morphology and local adaptation of *C. dentata*.

PRR is a catastrophic disease of the three North American *Castanea* taxa: american chestnut [*C. dentata* (Marsh.) Borkh.], allegheny chinquapin [*C. pumila* (L.) Mill. var. *pumila*], and ozark chinquapin [*C. pumila* (L.) Mill. var. *ozarkensis* (Ashe) Tucker] (Crandall et al., 1945; Jeffers et al., 2009, 2012; Sena et al., 2018). The principal causal pathogen, the oomycete *Phytophthora cinnamomi* Rands, is suspected to have been introduced to one of the coastal ports of the southeastern United States via ornamental plants from eastern Asia in the early 19th century (Anagnostakis, 2001; Crandall et al., 1945). Analyses of historical records have implicated *P. cinnamomi* in the widespread dieback of *C. dentata* and *C. pumila* var. *pumila* in the southeastern Coastal Plain and Piedmont during the 19th century (Anagnostakis, 2001; Crandall et al., 1945). *Phytophthora cinnamomi* has been isolated from dying chestnuts and chinquapins across the southeastern United States, as far north as Pennsylvania and as

far west as Arkansas (Crandall et al., 1945; Fitzsimmons, 2016; Jeffers et al., 2009). Symptoms of PRR include necrosis of roots, leaf chlorosis, wilting of foliage, and branch dieback (Crandall et al., 1945; Jeffers et al., 2009). Unless symptoms are controlled with potassium phosphites or similar chemicals (James, 2011a), PRR will cause the death of susceptible plants 3 weeks to several years after the initial infection (Crandall et al., 1945; Jeffers et al., 2009). *Phytophthora cinnamomi* is one of the most serious obstacles to growth of *C. dentata* throughout its former range because of the prevalence of the pathogen in a variety of soils across the southeast and the fact that PRR is ultimately fatal to North American *Castanea* species.

In addition to PRR, the North American *Castanea* species are severely affected by chestnut blight, caused by the ascomycete *Cryphonectria parasitica* (Murr.) Barr (Alexander et al., 2005; Anagnostakis, 2001; Paillet, 1993, 2002). Since 1989, The American Chestnut Foundation (TACF) and

collaborators have used the backcross method to introgress blight resistance from chinese chestnut (*C. mollissima* Blume) into a *C. dentata* genetic background (Hebard, 2005; Westbrook, 2017). In a recent review of TACF's backcross breeding program, Steiner et al. (2017) reported that progeny tests have validated experimentally expectations that a blight-resistant population of *C. dentata*-type trees can be constructed using introgression of extraspecific alleles for blight resistance. However, because *P. cinnamomi* was only recently recognized as an impediment to *C. dentata* restoration, TACF's breeding program has not selected for PRR resistance. As a result, PRR has caused the failure of entire experimental orchards and forest progeny tests before advanced backcross trees could be assessed for chestnut blight resistance (Clark et al., 2014; Jeffers et al., 2009, 2012; Sisco, 2009).

In response to PRR-induced mortality of potentially blight-resistant hybrid seedlings, a screening program was initiated in 2004 to detect PRR-resistant seedlings in TACF's backcross lines (James, 2011a, 2011b; Jeffers et al., 2009, 2012). Results of this screening program have indicated that PRR-resistant seedlings are present at low frequency in some, but not all, TACF advanced backcross lines (BC₂F_x, BC₃F_x, and BC₄F_x generations) that have been selected for blight resistance—most notably in lines derived from the blight-resistant hybrid cultivars Clapper and Graves (Jeffers et al., 2009). More recent resistance screening efforts showed that PRR resistance was also present in descendants of TACF's third major source of blight resistance, *C. mollissima* 'Nanking' (Zhebentyayeva et al., 2014).

Studies of the quantitative trait loci (QTL) associated with resistance to *P. cinnamomi* have aided breeding efforts by demonstrating that this trait is controlled by a limited number of genomic regions and may therefore be a practicable target for marker-assisted selection (Santos et al., 2017; Zhebentyayeva et al., 2014). Santos et al. (2017) identified QTLs for PRR resistance on linkage groups E and K of japanese chestnut (*C. crenata* Sieb. & Zucc.), and QTLs for PRR resistance were found on the same two linkage groups of *C. mollissima* (T.N. Zhebentyayeva, The Pennsylvania State University, personal communication). These findings suggest common resistance mechanisms to *P. cinnamomi* infection across different species within the genus *Castanea* (Santos et al., 2017). However, candidate genes within these QTLs have not been tested for their effects, and there exists the possibility that the East Asian *Castanea* species may have multiple alleles that encode PRR resistance. Until the genetic control of PRR resistance is better understood, additional sources of resistance are desirable for chestnut breeding programs in the eastern United States, because different *C. mollissima* and *C. crenata* cultivars may have different alleles encoding resistance. Efforts to identify multiple sources of PRR resistance are also important because they will facilitate the

introgression of different nut characteristics and other agronomically important traits into populations being tested for the commercial chestnut market.

Because of the high susceptibility of *C. dentata* to both PRR and chestnut blight, the growth of *C. dentata*-type trees across large portions of the southeastern United States requires planting trees that have host resistance to both pathogens. The objective of this study was to evaluate PRR resistance in 10 hybrid families derived from crosses between susceptible *C. dentata* trees and potentially resistant *C. mollissima* cultivars in a containment system, followed by outplanting the least symptomatic plants into a *P. cinnamomi*-positive orchard and assessing their survival midway through the second growing season.

Materials and Methods

Plant materials. We used three approaches to generate hybrid progeny for PRR resistance screening. With the first approach, we performed controlled pollinations between interspecific F_1 hybrids (*C. dentata* $\times$ *C. mollissima*) selected previously for high levels of resistance to chestnut blight (J.H. Craddock, unpublished data) and naturally occurring *C. dentata* trees from either Tennessee or Alabama, yielding eight first-backcross (BC_1) families (Fig. 1A; Table 1). With the second approach, an F_1 hybrid (*C. dentata* $\times$ *C. mollissima*) selected previously for blight resistance (J.H. Craddock, unpublished data) was open pollinated by its F_1 sibling planted in an adjacent position in the breeding orchard, yielding a group of F_2 progeny (Table 1). The F_1 hybrids in this experimental orchard have nonoverlapping

phenology with backcross trees in the same orchard (10–14 d difference in anthesis). With the third approach, a male-sterile F_1 hybrid (*C. dentata* $\times$ *C. mollissima*) selected previously for chestnut blight resistance (J.H. Craddock, unpublished data) was open-pollinated by neighboring advanced backcross trees (either BC_3F_1 or BC_4F_1 generations) selected previously for chestnut blight resistance and several *C. dentata* morphological traits, yielding “better backcross” (BB_1) progeny that we refer to as a family in this study (Fig. 1B; Table 1). All F_1 hybrids in this orchard were male-sterile; thus, only the selected BC_3F_1 and BC_4F_1 plants could contribute pollen. The third crossing strategy is justified because the BB_1 progeny could inherit blight resistance from two sources: TACF’s ‘Clapper’ line parent (i.e., paternal parent) and/or the *C. mollissima* cultivar used in our recent F_1 cross (i.e., maternal parent). The site conditions of the F_1 trees used for our BC_1 , F_2 , and BB_1 crosses were of particular importance, because nearly all the F_1 hybrids have exhibited several years of healthy growth in *P. cinnamomi*-positive orchards. The following *C. mollissima* cultivars were used as potential sources of resistance: ‘Amy’, ‘Byron’, ‘Gideon’, ‘Lindstrom 99’, ‘Nanking’, ‘Payne’, and ‘Petersburg’ (Table 1).

To perform controlled pollinations, we emasculated bisexual catkins and covered female flowers with corn pollinating bags about 1 week before anthesis. We collected staminate catkins from male parents, placed catkins on glass panes overnight to promote anther dehiscence, collected pollen in vials, and stored pollen at 3 °C until pollination was performed. We applied pollen to female flowers using a plastic vial lid or glass microscope slide and placed pollination bags over female flowers until nuts were harvested. To detect unintended outcrossing, for every 10 flowering shoots that were hand-pollinated, we treated one flowering shoot as a no-pollen control. Seeds were harvested in Sept. 2014 and Oct. 2014, and were stored in slightly moistened peatmoss at 3 °C until Spring 2015.

PRR-resistant and -susceptible control plants were selected from species tested previously (Crandall et al., 1945; Jeffers et al., 2009). *C. dentata* seedlings provided by the American Chestnut Foundation were used as susceptible control plants, whereas seedlings of *C. mollissima* and *C. henryi* (Skan) Rehder and E.H. Wilson obtained from a cultivar trial in Chattanooga, TN, were used as resistant control plants.

Nursery trials. In Mar. 2015, soil samples were collected from TACF breeding orchards in Greenback, TN (lat. 35.6281 N, long. –84.1040 W) and McDonald, TN (lat. 35.0982 N, long. –84.9531 W), where plant death accompanied by PRR symptoms had been observed previously. Soils were baited for *Phytophthora* spp. using the bioassay of Ferguson and Jeffers (1999). *P. cinnamomi* isolates recovered from the two breeding orchards were accessioned in the *Phytophthora* culture collection of S.N. Jeffers (Clemson University). Using *P. cinnamomi* isolates from the TACF orchard in Greenback, TN, a V8-

vermiculite inoculum was prepared following the protocol of Jeffers (2015).

In May 2015, seeds were planted outdoors in nursery containers at the University of Tennessee at Chattanooga. Nursery containers were filled with a soilless container mix consisting of pine bark, Canadian sphagnum peatmoss, and vermiculite (Sun Gro Metro-Mix 360; Agawam, MA). Seeds were planted 5 cm apart in five 229-L plastic nursery containers in a randomized complete block design.

Seedlings were inoculated with *P. cinnamomi* following the methods of Jeffers et al. (2009). Inoculations were performed in July, $\approx$ 12 weeks after planting. To inoculate plants, a thin layer of inoculum was distributed evenly in 1- to 3-cm-deep furrows between rows of seedlings. The furrows were covered and the nursery containers were watered thoroughly. Nursery containers were watered daily throughout the growing season. The nursery containers were placed in small plastic swimming pools to retain effluent and prevent the spread of *P. cinnamomi* throughout the nursery. Chlorine tablets were added to the containment pools to kill *P. cinnamomi* in the irrigation effluent.

Seedlings were assessed for PRR symptom severity using the rating system developed by Jeffers et al. (2009). In January, we removed plants from their containers and gently washed the root systems to remove potting medium. We assigned symptom severity ratings to each of the plants using the root rot phenotyping system developed by Jeffers et al. (2009): 0 = healthy, no lesions observed on roots; 1 = lesions observed on at least one lateral root; 2 = lesions observed on the tap root; and 3 = severe root rot, plant dead.

Orchard planting and survival. In Spring 2016, plants in the lowest symptom severity category (plants rated 0) were planted in TACF’s Georgia Chapter’s Lake Allatoona Orchard (Allatoona, GA; lat. 34.1871 N, long. –84.7065 W), which was documented previously as *P. cinnamomi* positive. In Aug. 2016, survival was assessed of plants that had been prescreened for PRR resistance.

Data analysis. To evaluate the strength of PRR resistance in hybrid families compared with previous studies, we calculated a survival quotient (SQ) for each family, using the formula of Jeffers et al. (2009):

$$SQ = \frac{(1 \times n_0) + (0.5 \times n_1) + (0.25 \times n_2)}{\text{Total number of seedlings}} \times 100,$$

where n_0 , n_1 , and n_2 are the number of seedlings rated 0, 1, and 2, respectively. The SQ ranges from 0% to 100%. High SQ values indicate resistance whereas low SQ values indicate susceptibility.

Using the raw symptom severity data, the effects of treatment (family) and block on symptom severity were assessed with a two-way analysis of variance, and differences among families were evaluated with Duncan’s new multiple range test using the “easynova” package (Arnhold, 2013) in R version 3.5.0 statistical software (R Core Team, 2018).

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Results and Discussion

In the nursery trial, PRR symptoms and seedling mortality were observed in every interspecific hybrid family and the susceptible *C. dentata* control group, whereas no plant mortality or PRR symptoms were observed in the resistant *C. mollissima* and *C. henryi* control groups, as expected (Fig. 2). Three of the 13 *C. dentata* control plants exhibited no PRR symptoms at the end of the nursery trial, which indicates that some seedlings “escaped” infection during the first year of screening, probably because of uneven distribution of the pathogen across portions of the planting containers.

SQ values of hybrid families ranged from 56.5% to 89.6%. The susceptible control species, *C. dentata*, had the lowest SQ value (48.1%), whereas both resistant control species, *C. mollissima* and *C. henryi*, had the highest SQ values observed in the trial (100%) (Fig. 2). A comparison of our SQ values with those of Jeffers et al. (2009) revealed that all but one of our hybrid families had greater SQ values than backcross families screened in an earlier study (Jeffers et al., 2009). Only one BC₁ family in our study, TN-TTU-A4 × ALCLEB04, had an SQ value (56.5%) similar to that of the most resistant cross reported by Jeffers et al. (2009): Hyko × JB575 (56.3%). One explanation for this difference is that all hybrid families studied by Jeffers et al. (2009) resulted from a greater number of backcrosses to the susceptible species, *C. dentata*, than any of the families evaluated in our study. Furthermore, parent trees in the crosses of Jeffers et al. (2009) had not been selected for PRR resistance. Specifically, Jeffers et al. (2009) screened BC₂F_x, BC₃F_x, and BC₄F_x progeny, whereas we screened eight BC₁ families, one F₂ family, and one BB₁ family. Because PRR resistance in chestnut is thought to be under the control of multiple genes (Santos et al., 2017), it would be expected that Asian American BC₁ hybrids would retain more alleles for resistance than later generation backcrosses to *C. dentata* if the parent trees were not selected for PRR resistance at every generation. A second possible explanation is that the strength of selection in the study of Jeffers et al. (2009)

was greater. For example, the environment in the planting containers of Jeffers et al. (2009) may have been more favorable for the proliferation of *P. cinnamomi*, or our inoculum may not have been entirely successful. The latter scenario would have resulted in an

uneven distribution of *P. cinnamomi* in the tubs. The existence of three asymptomatic *C. dentata* does indicate that *P. cinnamomi* was not present uniformly within the tubs.

Analysis of variance of the nursery screening data indicated that the effect of treatment (family)

Fig. 1. (A) Crossing strategy used to generate first-backcross (BC₁) seedlings screened for resistance to phytophthora root rot (PRR). (B) Crossing strategy used to generate better-backcross (BB₁) seedlings screened for resistance to PRR.

Table 1. Hybrid *Castanea* families screened for phytophthora root rot resistance. Hybrid family names follow standard nomenclature used in the American Chestnut Foundation’s *dentataBase* breeding records management system.

Cross name	Pedigree	Cross type	Seed type	n	Resistance source
TN-TTU-A4 × ALCLEB01	(TNCLA1 × Gideon) × ALCLEB01	F ₁ × <i>C. dentata</i>	BC ₁	27	<i>C. mollissima</i> ‘Gideon’
TN-SM2-F1 × opB ₃	(TNMON8 × Nanking) × op B ₃	F ₁ × opB ₃	BB ₁	77	<i>C. mollissima</i> ‘Nanking’
TN-TTU-A4 × ALCLEB04	(TNCLA1 × Gideon) × ALCLEB04	F ₁ × <i>C. dentata</i>	BC ₁	23	<i>C. mollissima</i> ‘Gideon’
TN-TTU-A4 × opF ₁	(TNCLA1 × Gideon) × opF ₁	F ₁ × opF ₁	F ₂	116	<i>C. mollissima</i> ‘Gideon’
TNSUM1 × TN-CN-6-193	TNSUM1 × (TNLIN1 × Payne)	<i>C. dentata</i> × F ₁	BC ₁	15	<i>C. mollissima</i> ‘Payne’
TNMAC1 × TN-CN-4-195	TNMAC1 × (TN-BF1-E10 × Amy)	<i>C. dentata</i> × F ₁	BC ₁	40	<i>C. mollissima</i> ‘Amy’
TNMAC1 × TN-CN-2-127	TNMAC1 × (TNRUT1 × Lindstrom 99)	<i>C. dentata</i> × F ₁	BC ₁	27	<i>C. mollissima</i> ‘Lindstrom 99’
TN-TTU-A4 × ALCLEB05	(TNCLA1 × Gideon) × ALCLEB05	F ₁ × <i>C. dentata</i>	BC ₁	12	<i>C. mollissima</i> ‘Gideon’
TN-CN-5-238 × ALCLEB01	(TN-BF3-L10 × Byron) × ALCLEB01	F ₁ × <i>C. dentata</i>	BC ₁	29	<i>C. mollissima</i> ‘Byron’
TN-CN-3-262 × TNCAR1	(TNLIN1 × op Chinese) × TNCAR1	F ₁ × <i>C. dentata</i>	BC ₁	49	<i>C. mollissima</i> ‘Petersburg’
<i>C. mollissima</i>			Control	11	
<i>C. dentata</i>			Control	13	
<i>C. henryi</i>			Control	17	

BC₁ = first backcross; op = open pollinated; BB₁ = better backcross; F₂ = second filial generation.

Fig. 2. Survival quotients (SQs) of hybrid *Castanea* families, *C. henryi* seedlings (resistant controls), *C. mollissima* seedlings (resistant controls), and *C. dentata* seedlings (susceptible controls) inoculated with *Phytophthora cinnamomi*. Families are arranged from high to low SQ value. $SQ = \{[(1 \times n_0) + (0.5 \times n_1) + (0.25 \times n_2)] / \text{Total number of seedlings}\} \times 100$, where n_0 , n_1 , and n_2 are equal to the number of seedlings rated 0, 1, and 2, respectively.

on symptom severity was highly significant ($P < 0.001$). Analysis of variance also indicated a highly significant effect of block ($P < 0.001$). Multiple comparisons using Duncan's new multiple range test indicated that 8 of 10 hybrid families had mean symptom severity that was not significantly different from the susceptible *C. dentata* controls (Fig. 3). Although the average symptom severity of these families was high, all eight families contained multiple seedlings that were asymptomatic at the end of the nursery experiment. These individuals were retained for outplanting in the *P. cinnamomi*-positive orchard.

Two of 10 hybrid families (TN-TTU-A4 x ALCLEB05 and TN-TTU-A4 x ALCLEB01) had average symptom severity that was not significantly different from the PRR-resistant species *C. mollissima* and *C. henryi* (Fig. 3). Both of these BC₁ families are descended from

C. mollissima, 'Gideon' (Table 1). Interestingly, a closely related family that shares the 'Gideon' source of PRR-resistance, TN-TTU-A4 x ALCLEB04, was not significantly different from the *C. dentata* susceptible control. There are a few possible explanations for these seemingly incongruous results. The first is that some variation in PRR resistance may exist among the pollen parents of these backcross families (i.e., the naturally occurring *C. dentata* trees ALCLEB01, ALCLEB04, and ALCLEB05). This seems highly unlikely, because previous studies did not find evidence of PRR resistance in *C. dentata* (Crandall et al., 1945; Jeffers et al., 2009). A more likely explanation can be attributed to the small number of trees in TN-TTU-A4 x ALCLEB01 and TN-TTU-A4 x ALCLEB05 (with 27 and 12 seedlings, respectively), and that some plants escaped infection, depressing the

average symptom severity for these two families. The trial in a *P. cinnamomi*-infected orchard was designed to test plants that had missed pathogen exposure in the nursery trial, as well as provide results informative of field resistance.

In the assessment of orchard survival, 101 of 144 prescreened BC₁ and BB₁ plants were alive after 8 months of growth in the *P. cinnamomi*-positive orchard in Aug. 2016. These 101 plants represent 70% of the 144 seedlings identified as least symptomatic in the 2015 nursery trials that were transplanted to the *P. cinnamomi*-positive orchard in Jan. 2016. In previous plantings at the Allatoona orchard, hybrid seedlings that had not been prescreened for PRR resistance experienced very low survival compared with PRR-resistant *C. mollissima* controls. As of Aug. 2016, for example, only 6 of 46 (13%) *C. dentata* seedlings, 5 of 40 (13%) nonscreened BC₃F₂ seedlings, 71 of 338 (21%) nonscreened BC₃F₃ seedlings, 10 of 71 (14%) nonscreened BC₁ seedlings, and 36 of 47 (77%) *Castanea mollissima* seedlings were alive after at least 1 year of growth in the *P. cinnamomi*-positive orchard. Because hybrid families that had not been prescreened for PRR resistance were planted in earlier years than our prescreened hybrid seedlings, a statistical analysis of these data would not have been appropriate. However, the fact that 70% of our nursery-screened hybrids were alive after nearly an entire growing season in a *P. cinnamomi*-positive orchard does suggest that our nursery trials were effective in reducing the number of susceptible seedlings planted in the field.

One of the major goals of the American chestnut restoration effort is to produce a population of advanced backcross trees with the chestnut blight resistance of *C. mollissima*, but with all other phenotypic traits of *C. dentata*. Researchers have pursued this goal in earnest since the early 1980s (Burnham, 1988); however, the need for host resistance to PRR in these populations only became apparent in 2003 (Jeffers et al., 2009). A population of trees with resistance to both chestnut blight and PRR would facilitate successful plantings of *C. dentata*-type hybrids in the southern portion of the American chestnut's distribution, where PRR-induced planting failures have been most common (Jeffers et al., 2009).

As a first step toward producing populations of PRR-resistant plants for the American Chestnut Foundation's breeding program in the southeastern United States, we have generated PRR-resistant seedlings in hybrid families derived from multiple sources of resistance. Although hybrid derivatives of *C. mollissima* 'Nanking' have been assessed previously for PRR resistance (Zhebentyayeva et al., 2014), our study represents the first attempt to identify PRR resistance in descendants of the following *C. mollissima* cultivars: Amy, Byron, Gideon, Lindstrom 99, Payne, and Petersburg. Of the progeny challenged with *P. cinnamomi* in our study, seedlings derived from the *C. mollissima* cultivars Amy, Byron, Gideon, Nanking, and Petersburg are still alive and growing in a *P. cinnamomi*-positive orchard as of Nov. 2018. In addition to the host resistance to PRR identified here, the cultivar grandparents of

Fig. 3. Mean symptom severity of phytophthora root rot (PRR) in hybrid *Castanea* families, *C. henryi* seedlings (resistant controls), *C. mollissima* seedlings (resistant controls), and *C. dentata* seedlings (susceptible controls). Error bars represent 1 SEM. Columns with the same letter are not significantly different according to Duncan's new multiple range test ($\alpha = 0.05$). Column colors represent seed type of the different groups screened for disease resistance.

the hybrid progeny also have several desirable agronomic and culinary traits, including high average nut mass, high yield, and superior taste (Metaxas, 2013). Consequently, the hybrid progeny screened here may also be of interest to plant breeders with a focus on chestnut as a nut crop for the southern United States.

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