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Effectiveness of a detached-leaf assay as a proxy for stem inoculations in backcrossed chestnut (*Castanea*) blight resistance breeding populations

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Summary

A recently developed detached-leaf blight resistance assay has generated interest because it could reduce the amount of time needed to evaluate backcrossed hybrid trees in the American chestnut blight resistance breeding programme. We evaluated the leaf inoculation technique on a sample of advanced progeny from the Indiana state chapter American Chestnut Foundation breeding programme, along with susceptible American chestnut (*Castanea dentata*), the recurrent parent, and resistant Chinese chestnut (*Castanea mollissima*), the donor parent for blight resistance. In experiments over 2 years using two pathogen isolates, we found no biologically meaningful relationship between leaf lesion size and the size (length and width) or severity (1–5 canker severity rating) of stem cankers on 5-year-old trees. Chinese chestnuts did develop significantly smaller leaf lesions than American or backcrossed chestnuts. We conclude that while the detached-leaf assay may have utility in some chestnut breeding applications, it is not a suitable proxy for the established practice of stem inoculations.

1 | INTRODUCTION

In cases where trees must be grown for several years in the field before inoculations and evaluations are performed, breeding resistance to pests and pathogens can be prohibitively time-consuming. Reducing the time it takes to evaluate crosses has great appeal to tree breeders. Detached-leaf assays are often used in woody plant breeding programmes as rapid alternatives to field inoculations when the leaf assay can be shown to produce estimates of resistance similar to those obtained through field inoculations; that is, known resistant and susceptible genotypes are ranked similarly by both methods (e.g. Calonnec et al., 2012; Tahi et al., 2000). Although detached-leaf assays can be effective in screens against pathogen species that naturally attack leaf tissue (Calonnec et al., 2012), a number of devastating tree pathogens (root rots caused by *Phytophthora* spp., Dutch elm disease, butternut canker disease, chestnut blight) do not, or only rarely, attack leaf tissue in nature. In spite of this, leaf assays have been successfully developed for some non-foliar pathogens (*Phytophthora* in Tedford, Miller, & Nielsen, 1990; citrus canker in Francis, Peña, & Graham, 2010). Recently, a leaf inoculation assay was developed (Newhouse, Spitzer, Maynard, & Powell, 2014) for use in one of the largest forest tree breeding programmes in North America, the backcross breeding programme of the American Chestnut Foundation (TACF).

Building on hybrid breeding work initiated during the chestnut blight epidemic of the early 20th century, TACF aims for the restoration of chestnut to the forests of eastern North America (Burnham, Rutter, & French, 1986; Gravatt, Diller, Berry, Graves, & Nienstaedt, 1953). First detected in 1905, chestnut blight, caused by the ascomycete *Cryphonectria parasitica*, spread rapidly throughout the native range of the American chestnut (*Castanea dentata* Marsh. Borkh.) and eliminated it as a canopy species (Anagnostakis, 1987). Chestnut blight causes necrotic cankers on the surface of the branches and trunk that expand to cause girdling and mortality in susceptible trees. Conversely, Chinese chestnut is the most resistant *Castanea* species to chestnut blight. Because it can readily hybridize with American chestnut, it serves as the resistance donor

for the breeding programme. Evaluations of hybrid crosses led breeders to hypothesize that a few major genes control blight resistance, so a backcrossing programme may be a reasonable way to produce trees that look like American chestnut but are highly blight-resistant (Diskin, Steiner, & Hebard, 2006). Backcross breeding is commonly used in crop breeding to transfer a desirable trait from a donor parent (here, Chinese chestnut) with mostly undesirable features into an elite genetic background (the recurrent parent; here, American chestnut) (Acquaah, 2007). Quantitative trait locus (QTL) mapping experiments (Kubisiak et al., 1997, 2013) identified three loci that together explain about 75% of the variation in blight resistance among *C. mollissima* × *dentata* hybrids and show incomplete dominance (Schlarbaum, Hebard, Spaine, & Kamalay, 1997). These three loci underpin the working inheritance model for blight resistance. TACF is in the process of backcrossing interspecific hybrids to American chestnut for three generations, and intercrossing the third backcross progeny with putative elevated resistance to produce a generation of progeny (BC_3F_2) in which a few recombinant individuals are supposed to be homozygous for all three major resistance genes. To accomplish this, large numbers of BC_3F_2 trees must be evaluated (~1,200 per family) because the desired recombinants are rare (1/64 of the F_2 progeny), and a large range of phenotypes from highly susceptible to highly resistant are present (Burnham et al., 1986; Fitzsimmons et al., 2014). The most resistant, putatively homozygous recombinants should be similar to American chestnut, but true-breeding for blight resistance; that is, they will transmit equally strong blight resistance to their offspring.

The traditional method for evaluating chestnut blight resistance is a stem inoculation made in early summer on four- or five-year-old trees with a cork borer and a small agar plug of inoculum (Hebard, 2005). Cankers develop over the summer and are evaluated in late fall and/or the following summer. Resistant trees (Chinese chestnut and some BC_3F_2) will form callus tissue around the induced cankers and show little or no loss of vigour. Highly susceptible trees (American chestnut and many BC_3F_2) show no callus development, and the resulting large, sunken canker typically girdles the main stem in one season. This method is reliable but requires large amounts of time and land to grow trees. A method for inoculating the small-diameter stems of first- or second-year chestnut trees (Powell, Morley, King, & Maynard, 2007) has not been widely adopted.

The leaf inoculation method published by Newhouse et al. (2014) generated interest for several reasons – trees can be inoculated and highly susceptible trees removed in the first year, the test is not fatal to the tree being tested, and scoring is straightforward, rapid and quantitative. Newhouse et al. used young leaves from susceptible *C. dentata*, resistant *C. mollissima*, and a third species with intermediate resistance, *C. pumila*, or Allegheny chinkapin. By introducing an agar plug from an actively growing *C. parasitica* colony to a wound on the abaxial side of the mid-vein and incubating in dark sealed containers for about 5 days, they induced necrotic lesions along the mid-vein. These lesions were the largest in *C. dentata*, intermediate in *C. pumila* and the smallest in *C. mollissima*, and Newhouse et al. were able to re-isolate *C. parasitica* from the margins of the lesions. Given these promising results, it was postulated that leaf inoculations could be used to rapidly screen BC_3F_2 progeny.

Working in conjunction with the Indiana state chapter TACF breeding programme, the objective of this study was to compare leaf and stem inoculations for rating resistance to *C. parasitica* in BC_3F_2 chestnuts. As major blight resistance genes are segregating in the BC_3F_2 generation, we expected to observe stem and leaf inoculation phenotypes spanning most of the range of variability between Chinese chestnut and American chestnut – some trees are expected to inherit two susceptible (American) alleles at all three resistance loci, some will inherit two resistant (Chinese) alleles at all three loci, and most will be intermediate. Our purpose was to extend the new detached-leaf assay to a practical breeding application: Newhouse et al. (2014) tested their method on resistant and susceptible species of *Castanea*, but did not test any of the hybrids or backcrossed individuals that the TACF programme depends on. If leaf inoculation could serve as a proxy for stem inoculation of BC_3F_2 trees, it would allow TACF breeders to rogue susceptible trees at the seedling stage.

2 | MATERIALS AND METHODS

2.1 | Test populations

In 2014, 100 five-year-old BC_3F_2 trees at the Southern Indiana Purdue Agricultural Center (BC_3F_2-SIPAC) in Dubois County were screened using the detached-leaf assay. The planting consists of trees planted in blocks of 120; we subsampled 20 trees from each of the five oldest blocks. This population is designated here as BC_3F_2-SIPAC. Trees at this site represented a full-sib family (one BC_3 mother × one BC_3 father). The BC_3 parents were derived by pollinating surviving American chestnuts in Indiana with BC_2 pollen ("Clapper" resistance source) from the national TACF programme. A majority of BC_3F_2-SIPAC were tested using stem inoculations by late June 2014 (Table 1). Also in 2014, five Chinese chestnuts and five BC_3 trees planted at the Purdue University Lugar Farm (LF), along

TABLE 1 Summary of data collected on chestnut blight resistance across years, sites, and chestnut genotypes

	Leaf inoculation		Stem length[a]		Stem rating[b]	
Species site	2014	2015	2014	2015	2014	2015
BC_3F_2-SIPAC[c]	100	135	78	72	61	91
BC_3-LF[d]	5	39	–	34	–	–
CC-Lf[e]	5	9	–	5	–	–
AC-MF[f]	5	5	–	0	–	–

[a]Length of canker from current-year inoculation.
[b]Qualitative rating of reaction to previous year inoculation.
[c]F2 progeny of third-backcross trees (B3F2) grown at Southern Indiana Purdue Ag Center (SIPAC).
[d]Third-backcross (B3) trees grown at Lugar Farm (LF).
[e]Chinese chestnut.
[f]American chestnut at Martell Forest (MF).

with five American chestnuts at Purdue's Martell Forest (MF), both located in Tippecanoe County, Indiana, were screened using leaf inoculations (Table 1).

In 2015, based on results from 2014, BC_3F_2-PWP was excluded from further leaf inoculations. Surviving BC_3F_2-SIPAC plus a set of younger trees (5 years old in 2015) at the same site and from the same full-sib family that had received stem inoculations in early June 2015 (total n = 135) were evaluated following leaf inoculations. Additionally, an expanded set of BC_3 that had been stem-inoculated in 2014 were screened at Purdue using the detached-leaf assay (n = 39) in the summer of 2015 (Table 1).

2.2 | Stem inoculations

Stem inoculations were performed in June of 2013, 2014 and 2015. Around 600 BC_3F_2-SIPAC trees (five blocks of 120) were inoculated 2013–2015. A disc of bark was removed with a 6-mm cork borer, and an agar plug containing mycelium from either a highly aggressive *Cp* strain (Ep155) or a less-aggressive strain (Sg88) was placed next to the cambium and taped into place following standard TACF protocol (Griffin, Hebard, Wendt, & Elkins, 1983). Trees were inoculated sequentially; Sg88 was used the first year and Ep155 the second year on trees that survived Sg88. Fungal cultures were obtained from Dr. Fred Hebard of TACF in Meadowview, VA, and multiplied on acidified PDA at Purdue University; only the actively growing margins of 3- to 5-day-old colonies were used for inoculation. Stem cankers were evaluated in two ways to facilitate different types of analyses: they were rated on a standard qualitative scale ("stem rating" in Table 1) in June and July, a year after inoculation: 1 (small canker completely surrounded by callus tissue) to 5 (large, sunken canker with no callus formation) (Hebard, 2005). Length and width (mm) of developing cankers ("stem length" in Table 1) were also measured in September 2014 (measurements of developing cankers from June 2014 inoculations) and September 2015 (measurements of cankers developing cankers from June 2015 inoculations) using a digital calliper.

2.3 | Leaf sampling

In 2014, after selecting the most resistant trees (stem resistance ratings 1, 2 or 3) in each block, based on scores from 2013 stem inoculations, a stopwatch was used to randomly select trees until three or four trees were sampled from each of six rows for a total of 20 trees per block; that is, if the last digit was eight, the 8th tree in the row was selected. This method was adopted instead of a truly random sample because resistant BC_3F_2 were relatively rare, and we needed to samples as many of them as possible to test the leaf inoculation method. We sampled 5–8 leaves per tree for BC_3-LF, and BC_3F_2-SIPAC and 10 leaves per tree for resistant and susceptible species controls, taking care to select leaves that were fully expanded but still tender, generally from the ends of shoots. Leaves were collected throughout June and July. Leaves were removed from the tree by hand by cleanly breaking the petiole/branch junction. Detached leaves were placed in labelled plastic bags in a cooler and transported to the laboratory for inoculation within 2 hr of being picked.

In 2015, leaves were resampled from the same trees as 2014, in June and July, using the same sampling protocol. Trees were randomly selected (using the method described above) in a block that was stem-inoculated in June 2015. Many of the trees that were resampled had died back and resprouted from the base. On trees that exhibited basal shoots and surviving live crown branches, four leaves were taken from first-year basal shoots and four from older crown branches, to test whether the different characteristics of these leaves had any effect on leaf inoculations. If a tree had only basal shoots, or only live crown branches, six leaves were picked.

2.4 | Leaf inoculations

Cryphonectria parasitica was cultured on acidified potato dextrose agar; cultures were stored in the dark at room temperature until they had reached sufficient size (4–5 days). In 2014, the strain Sg88 alone was used; in 2015, Ep155 and Sg88 were inoculated pairwise on leaves. An agarose plug containing mycelium was taken from the actively growing edge of the colony using a 4-mm cork borer. Agarose plugs were balanced on a cut (about 5 mm in length) in the mid-vein of the abaxial side of the leaf, made with a razor blade (sterilized with 70% ethanol). Control inoculations using agarose plugs without mycelium were performed in each set of inoculations. In order to avoid confounding of tree effects on leaf lesion size with effects due to different boxes in which inoculated leaves were stored, a random number generator was used to assign 6–12 individual leaves to each box.

Leaves were rinsed in one bath of 0.1% Tween-20 and two baths of distilled water, patted dry, labelled with permanent marker, inoculated and stored for 5–6 days in Sterilite 16-cup gasket-sealed plastic Ultra-Seal food storage boxes that were lined with damp paper towels (Newhouse et al., 2014). Sealed plastic boxes were held in the dark at room temperature until symptoms were measured. Lesion length and width were measured with a digital calliper 5 or 6 days after inoculation (Newhouse et al., 2014).

2.5 | Statistical methods

We used ANOVA with Tukey's HSD multiple comparison test in R (R Foundation for Statistical Computing 2015) to test for differences in the mean leaf lesion length of different chestnut genotypes (Chinese chestnut, American chestnut, BC_3, BC_3F_2) and BC_3F_2 from different sites (PWP, SIPAC). ANOVA (R function: aov) was also used to analyse the variability of leaf lesion dimensions among BC_3F_2 chestnuts grouped in different blight resistance classes. We used linear regression (R function: lm) to identify whether there was a relationship between stem canker length and leaf lesion length, between stem canker width and leaf lesion width, and to identify correlations between canker length and width, and assess correlations between years for BC_3 and BC_3F_2 leaf lesion dimensions. Because the stem and leaf canker measurements were quantitative, homoscedastic and normally

distributed (not shown), we were confident that the basic assumptions of parametric regression and analysis of variance were met.

3 | RESULTS

3.1 | Leaf inoculation protocol

Control inoculations with blank agar plugs failed to produce lesions, and in cases where the agar plug rolled away from the inoculation site, no necrotic lesions were observed along the cut; these leaves were noted as failures and not scored. Secondary infections away from the inoculation point were only observed on a few leaves, and inoculations rarely failed to produce visible lesions, although failures were more frequent on Chinese chestnut than on American or hybrid leaves. In 2014, 35 of 50 Chinese chestnut leaves developed lesions. This was compared to 49 and 50 of 50 leaves that developed lesions on American chestnut and BC_3, respectively. Failed inoculations were excluded from all statistical estimates of leaf lesion dimensions.

Inoculations with Sg88 resulted in leaf lesion sizes that were not significantly different than inoculations with Ep155 based on 95% confidence interval estimates of the means for lesion length and width. The mean lesion width (pooled across 2015 genotypes) for Ep155 was 13.25 ± 0.75 mm (mean, standard error [*SE*]) compared to 12.76 ± 0.82 mm for Sg88; mean lesion length for Ep155 was 28.69 ± 1.43 mm, and for Sg88, 28.48 ± 1.61 mm. In BC_3F_2 -SIPAC, the difference between the two strains was similarly small (Ep155 mean length = 28.51 mm; Sg88 = 28.01 mm). For this reason, lesions from the different strains were pooled for subsequent analysis. Leaf lesion length and width were significantly correlated (Pearson's correlation coefficient: .79).

3.2 | Variability in leaf lesion size by site, year and species

ANOVA tests conducted with genotype/site as a grouping variable (Table 2) for 2014 ($F_{3,136}$ = 25.48, p < 0.001) and 2015 ($F_{3,193}$ = 31.61, p < 0.001) were both significant, indicating that at least one genotype/site sample had a significantly different mean from the others. When Tukey's HSD test was used to test differences of means in the 2014 data, American chestnut (27.47 ± 0.83 mm), and BC_3 (19.94 ± 2.28 mm) were not significantly different from each other according to the Tukey test; neither were American chestnut and BC_3F_2-SIPAC (28.76 ± 0.51 mm). The mean length of Chinese chestnut (8.19 ± 0.27 mm) was significantly smaller than all others. When the same test was performed on the 2015 samples, Chinese chestnut was significantly smaller than other samples (10.71 ± 1.31 mm), but American chestnut (32.9 ± 0.63 mm) and BC_3 (32.17 ± 1.12 mm), and American chestnut and BC_3F_2-SIPAC (29.31 ± 0.44 mm) were not significantly different from each other.

TABLE 2 Mean leaf lesion length (mm) for chestnut genotypes and sites tested with results of Tukey's HSD test performed following ANOVA

Species site	2014*	2015
BC_3F_2-SIPAC[a]	28.76 a	29.31 a
BC_3-LF[b]	19.94 b	32.17 a
AC-MF[C]	27.47 ab	32.9 a
CC-Lf[d]	8.19 c	10.71 b

[a]B3F2 trees grown at Southern Indiana Purdue Ag Center (SIPAC).
[b]Third-backcross (B3) trees grown at Lugar Farm (LF).
[c]*Castanea dentata*.
[d]*Castanea mollissima*.
*Means followed by the same letters were not significantly different from others within the same year according to a Tukey's HSD test.

For all sampled genotypes, leaf lesion length was significantly, but not strongly, correlated between 2014 and 2015 samples (r^2 = .30; p < .001), indicating moderate reproducibility in length of leaf lesions across years. There were no apparent effects of year on leaf lesion length or width, when all genotypes were pooled (overall mean length 2014 = 30.8 mm; 2015 = 29.4 mm; mean width 2014 = 12.37 mm; 2015 = 12.57 mm). When we compared the mean lesion lengths of leaves from stump sprouts versus those from established branches, the lesions that developed on stump sprout leaves were, on average, slightly larger (29.1 mm vs 28.5 mm).

3.3 | Relationship of leaf lesion length to canker rating

ANOVA tests and Tukey's multiple comparison of means tests were used to determine whether leaf lesion size differed for BC_3F_2-SIPAC genotypes from different resistance categories based on stem inoculations (1–5; Fig. 1). Of the four ANOVA tests performed, only one (2015 leaf lesion length by 2015 stem canker rating) indicated that there was any difference in leaf lesion dimensions among trees in the different stem canker rating categories ($F_{(1,87)}$ = 10.67, p = .001; Fig. 1). In this case, the Tukey's HSD test supported a difference in mean leaf lesion length for trees in category 1 (most resistant based on stem lesion phenotype, mean leaf lesion length = 20.58 mm) versus genotypes with stem cankers rated 3, 4 or 5 (mean leaf lesion length 26.93 mm, 27.06 mm and 27.42 mm, respectively). All the trees with stem canker ratings of 2 in 2014 had stem canker ratings of 3, 4 or 5 by 2015. A majority of B3F2 trees at SIPAC were rated highly susceptible (rated either 4 or 5) in both 2014 and 2015. Some BC_3F_2 trees that rated highly resistant (1 or 2) for stem cankers and had small (2 standard deviations less than the mean) leaf lesions in 2014 deteriorated in both categories by the summer of 2015. The very small number of highly resistant B3F2 trees rated 1 or 2 (n = 9 in 2014 and n = 3 in 2015) had significantly smaller leaf lesion dimensions in 2015 (Fig. 1, Fig. 2), but there was no significant difference in leaf lesion size between moderately resistant (stem canker rating = 3) and highly susceptible trees (Fig. 1). All the BC_3F_2-SIPAC trees rated "1" in 2015 were stem-inoculated in 2014, and all BC_3F_2-SIPAC trees stem-inoculated in 2013 showed symptoms rated > 2 by 2015. The few trees that maintained a low-to-moderate (1, 2, 3) canker severity rating in both 2014 and 2015 (n = 4) had relatively small leaf lesions: 2014 mean lesion length for these four trees was 23.53; in 2015, it was 26.18 (compared to overall means of 30.8 and 29.4 for 2014 and 2015, respectively).

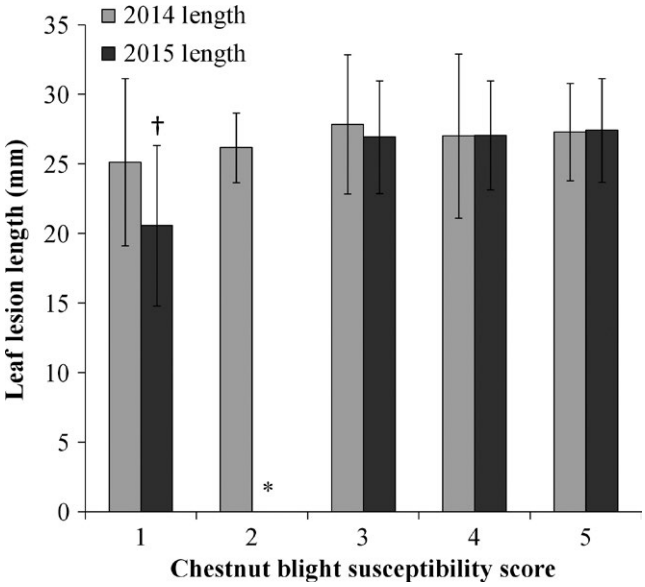

FIGURE 1 Plot of mean leaf lesion length among BC3F2 chestnuts in different resistance categories growing at Southern Indiana Purdue Agricultural Center. 1 is most resistant; 5 is least resistant based on stem inoculations. * indicates no trees were observed in that category in a given year. † indicates a mean significantly different from the others, as determined by Tukey's HSD test. Bars show standard deviation

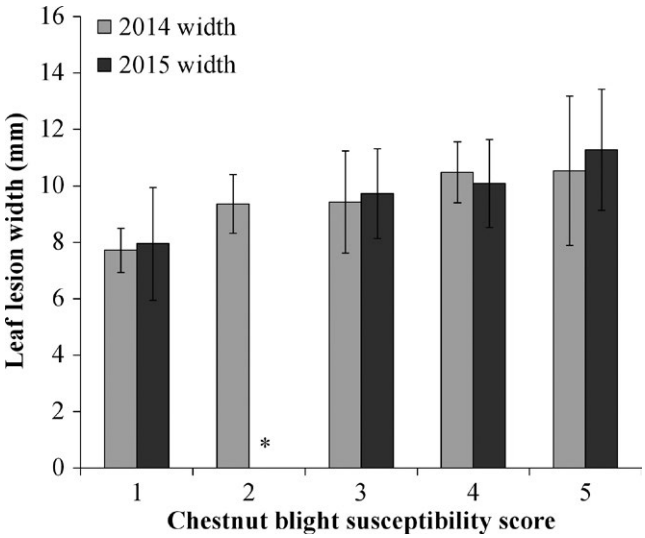

FIGURE 2 Plot of mean leaf lesion width among BC3F2 chestnuts in different resistance categories growing at Southern Indiana Purdue Agricultural Center. 1 is most resistant; 5 is least resistant based on stem inoculations. * indicates no trees were observed in that category in a given year. Bars show standard deviation

3.4 | Relationship of leaf lesion length to stem canker length

Simple linear regressions of stem lesion length on leaf lesion length and width and of stem lesion width on leaf lesion length and width were performed separately on 2014 and 2015 data from BC_3F_2–SIPAC, and only weak associations were observed, based on estimated regression coefficients and r^2 values (r^2 range: .00–.07), even if some regression coefficients were significantly different from zero (Table 3, Fig.3). When the same analysis was performed on the BC_3 and Chinese chestnuts that had received stem inoculations, no significant correlations between leaf lesion length and stem canker length were found (results not shown).

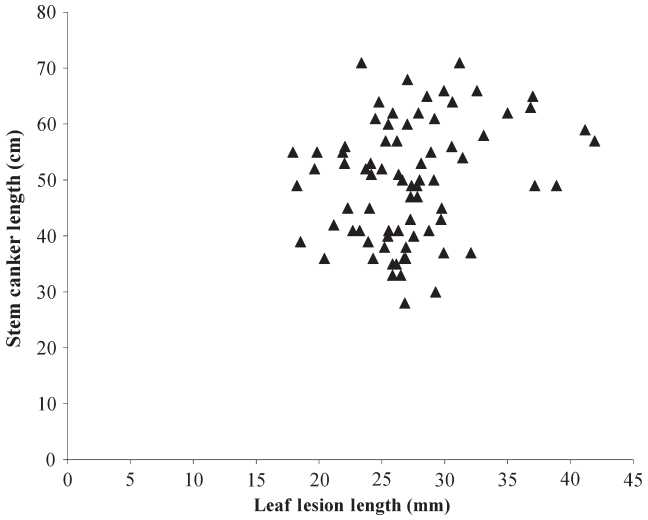

FIGURE 3 Scatterplot of leaf lesion length by stem canker length among BC3F2 chestnuts at Southern Indiana Purdue Agricultural Center. Data shown are from 2014

4 | DISCUSSION

We observed differences in leaf lesion size between American (susceptible to *C. parasitica*) and Chinese (resistant) chestnut trees: like

TABLE 3 Summary of simple linear regressions of leaf lesion and stem canker dimensions performed among BC3F2 chestnut growing at SIPAC

	2014			2015		
Model	b_1	*p*	r^2	b_1	*p*	r^2
SL[a]=LL[b]	0.12	.023*	.07	−0.02	.003*	.06
SW1[C]=LL	0.11	.064	.04	−0.01	.41	.01
SW2[d]=LL	1.15	.518	.01	−0.25	.67	.00
SL=LW[e]	0.26	.202	.02	0.29	.026*	.06
SW1 = LW	0.05	.032*	.06	−0.01	.428	.01
SW2 = LW	2.59	.044*	.06	0.24	.457	.01

[a]Length of stem lesion parallel to trunk.
[b]Length of leaf lesion parallel to mid-vein.
[c]Width of stem lesion perpendicular to trunk.
[d]Width of stem lesion perpendicular to trunk, adjusted for diameter.
[e]Width of leaf lesion perpendicular to mid-vein.
*p-value less than .05.

Newhouse et al. (2014), the resistant species developed smaller leaf lesions and the susceptible species developed larger lesions. Also in agreement with Newhouse et al., we did not observe a significant difference in leaf lesion size between *Cryphonectria* strains that show strong variation in virulence when applied to the stem. We observed variation in leaf lesion size among individual BC_3F_2 that was somewhat consistent between the 2 years of the study. The most resistant and the most susceptible B3F2 trees, based on stem inoculations, had leaf lesions that were mostly indistinguishable in size. Further, the size of leaf lesions of BC_3F_2 in general was similar to the mean size of leaf lesions of American chestnut. BC_3F_2 would be expected to have a wide range of leaf lesion phenotypes if leaf lesions reflected overall resistance to *C. parasitica* and if the model of inheritance of resistance that is assumed by the TACF breeding programme is correct. It was also expected that if leaf lesions reflected overall resistance to *Cryphonectria parastica*, and resistance is inherited as expected, the mean lesion length of inoculated BC_3F_2 leaves should be intermediate between lesion length of American and Chinese chestnuts. In both 2014 and 2015, clear differences between American and Chinese chestnut leaf lesion dimensions were observed, and B3F2s displayed a range of leaf lesion sizes, but leaf lesion sizes of hybrid trees were not intermediate but closely matched values for American chestnut, even among those hybrids that showed low susceptibility based on stem inoculations.

One reason our results do not conform to expectations of the current chestnut blight resistance breeding model may be the generally low blight resistance (based on stem inoculations) in the populations tested; most trees were rated highly susceptible (score 4 or 5). It is possible that the leaf lesion size of the most resistant trees was influenced by the greater vigour of those trees relative to moderately susceptible individuals, or more likely, leaf lesion size was influenced by the morphology of the inoculated leaves. The observed increase in both stem canker rating and leaf lesion size from 2014 to 2015 among trees rated 1 and 2 for stem cankers may have been a reflection of moderate susceptibility to *C. parasitica*, a reflection of morbidity incited by *Cp* leading to declining tree health, or both, and the reasons for the increase may not have been the same for stem and leaf tissues. There were a few ($N = 4$) BC_3F_2–SIPAC trees that maintained stem canker severity ratings of 3 or less and relatively small leaf lesions throughout both years. These four individuals represent 0.6% of the total number of trees (~600) that were screened for resistance using stem inoculations in the SIPAC planting as of 2015. The expected number of homozygous-resistant recombinants, for three segregating resistance genes, is 1/64 or 1.6%, so BC_3F_2–SIPAC either has lower overall resistance than expected or the screening methods failed to identify all the resistant trees. Based on these results, it might be practical to use leaf inoculations to eliminate the most susceptible trees at a young age (e.g. 25% of trees with largest leaf lesions could be rogued). Eliminating the most susceptible trees based on leaf lesion size would be unlikely to lead to accidentally discarding the most resistant members of the B3F2 population. However, given that leaf inoculations could not consistently distinguish moderately susceptible (possible desirable) trees from the most susceptible trees, the utility of this method to breeders is inferior to that of traditional stem inoculations.

Detached-leaf assays developed as proxies for inoculation of tissues other than the pathogen's natural target tissue have not always been effective in cases where fruits (Liebhard et al., 2003) or roots (Irwin, Musial, Mackie, & Basford, 2003) were the pathogen's target tissue, and their utility to assay stem pathogens in forest trees has been questionable in some other cases (Parke, Roth, & Choquette, 2005). The trees we inoculated and evaluated were selected because they presented an opportunity to test the published leaf inoculation method against the current standard for measuring susceptibility to *C. parasitica*, that is stem inoculations on hybrid chestnut genotypes. Since our tests took place on 5-year-old field-grown trees, there were some potential confounding factors that would probably not be present if screening was performed using glasshouse-grown seedlings, which were the main plant material used by Newhouse et al. (2014). First, as mentioned above, there is the potential that the severity of a tree's reaction to stem inoculation affected the results of the leaf inoculation. In particular, we were concerned that very susceptible trees, which were killed above the inoculation point in the first year, might have biased results because the leaves being tested inevitably came from shoots below the inoculation point. Leaves from rapidly growing shoots tend to be larger and less suberized than leaves on twigs in the normal live crown. The sample for the comparison included shoot leaves from both highly susceptible and somewhat resistant trees, and lesions were slightly larger on shoot leaves. Therefore, any bias from the use of shoot leaves would have been in the direction of susceptible trees developing larger lesions. Tahi et al. (2000) described a case in rubber tree where a detached-leaf assay that was deemed ineffective when tested with field-grown leaves later proved to be useful when glasshouse specimens were tested. It is likely that glasshouse-grown BC_3F2 seedlings would provide better material for leaf inoculations and a test using this approach (with seedlings tested in the glasshouse and evaluated using stem inoculations in the field 5 years later) could validate the method for hybrid breeding. On the other hand, using glasshouse-grown trees would not account for environmental realities that affect disease resistance reactions in the field.

We hypothesize that the differences in leaf lesion size between susceptible American chestnut and resistant Chinese chestnut observed by Newhouse et al. and replicated in this study were caused not only by the defensive mechanisms that confer blight resistance to Chinese chestnut, but also by morphological and histological differences in the leaves of the species. Chinese chestnut has heavy, waxy leaves with a densely hairy underside, while American chestnut leaves are not hairy and are generally thinner and less heavily suberized. As gross phenotypic characters of Chinese chestnut are selected against in the TACF breeding programme, after three generations of backcrossing most Chinese-like leaf characteristics have been eliminated (Diskin et al., 2006). This could explain why leaf lesion size of BC_3F2 trees was similar to American chestnut, even among BC_3F2 trees that were moderately resistant based on stem inoculation data.

Furthermore, the intermediate species used by Newhouse et al. when first describing the assay, Allegheny chinkapin, has some leaf

characteristics in common with Chinese chestnut, namely, pubescence on the abaxial side of the leaf. Finally, two of the BC_3 trees we inoculated at Lugar Farm are now thought to be misclassified F_1 hybrids or BC_1. These trees, which had hairy, waxy leaves intermediate between Chinese and American chestnut, had smaller leaf lesions than the B3 trees assayed at the same site. Unfortunately, these were the only early-generation hybrids we had access to for the study: a test of F2 hybrid trees with varying levels of Chinese-like leaf trait expression would be a good test of the hypothesis that leaf traits control the leaf phenotype in addition to inherent blight resistance.

Our study, inspired by the excitement generated by the potential of the detached-leaf assay among chestnut breeders, sought to extend the results of Newhouse et al. (2014) from comparisons of resistant and susceptible chestnut species to the backcrossed hybrid trees that TACF hopes to use for the restoration of chestnuts to the North American landscape. We conclude that the leaf inoculation assay does not discriminate between resistant and susceptible trees under field-based breeding conditions, such as that conducted by IN-TACF. Research to improve the utility of this assay should compare glasshouse-grown versus field-grown leaves and examine the effects of leaf morphological differences in greater depth.

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