THE GENETICS OF RESISTANCE OF AMERICAN BEECH TO BEECH BARK DISEASE: KNOWLEDGE THROUGH 2004

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Introduction and Background

In stands long affected by beech bark disease (BBD), a small percentage of American beech trees (*Fagus grandifolia*) remains disease free. Trials using an artificial inoculation technique have indicated that these trees are resistant to the scale insect (*Cryptococcus fagisuga*) portion of the disease complex (Houston, 1982, 1983). In many cases, resistant trees were observed to occur in close proximity to one another. A study of the patterns of disease-free trees in two stands in Nova Scotia demonstrated that the majority of resistant trees within these stands were located in groups (Houston 1983). Based on the spatial arrangement of these resistant trees, Houston and others concluded that these groups "suggest a genetic resistance transmissible both vegetatively and sexually" (Mielke et al. 1986). However, in the 1986 study, it was not determined if the resistant clusters of trees were from root sprout or seed origin. Similar findings were reported in stands located in Massachusetts, West Virginia, Prince Edward Island, Maine, New York and New Hampshire (Houston and Houston 1994, 2001). All the stands included in these studies were selected based on the presence of at least one group of disease-free trees.

The Search for a Marker Correlating with Resistance

In northern hardwood stands that have been long affected by BBD, large numbers of severely deformed American beech trees persist. To further complicate stand management practices, root sprout initiation can be stimulated by the large number of trees in decline due to BBD, resulting in the formation of "thickets" of diseased, deformed beech sprouts. To improve stand quality, silvicultural approaches are needed to reduce the number of susceptible trees and increase the number of resistant beech trees. Such approaches rely on the ability to distinguish between resistant and susceptible trees. One difficulty forest managers encounter in carrying out recommended treatments is that even in heavily infested areas, trees that remain free of scale may be escapes and not truly resistant. In the initial stages of infestation, larger, more mature trees are attacked first while smaller juvenile trees do not show scale build-up until later phases of infestation. Currently, the only known method to test for resistance is the artificial infestation method developed by Houston (1982). Drawbacks to this method include the minimum 1-year wait for results and the reliance on live scale eggs, which could result in spread of the scale insect if this method were used in areas where the scale was not yet found. Identification of a marker correlating with resistance would be beneficial not only as an aid to management of the disease, but also in genetics research.

Houston and Houston (1994, 2001) used isozyme analysis to assess the genetic structure of several BBD affected stands. Individual trees were analyzed using nine polymorphic enzymes to generate an isozyme profile. Based on these isozyme profiles, an indication of relatedness of individuals (either clonally or by family structure) could be estimated. Although many resistant individuals located within the same stand shared an identical isozyme pattern, there was no distinct pattern that correlated to resistance. In fact, there were cases where resistant individuals were shown to have the same isozyme pattern as a susceptible individual. In searching for a marker correlating to resistance, the number of isozyme loci that can be scored is limiting and a system that generates a larger number of polymorphic loci will be required.

We used RAPDs (randomly amplified polymorphic DNA) (Williams et al. 1990) to analyze subsets of the individuals that Houston and Houston (1994, 2001) had included in their isozyme analysis. This polymerase chain reaction (PCR)-based technique relies on short (10 base) primers to detect random polymorphisms in the genomic DNA. One hundred and forty-four individuals were screened with 24 different primers to uncover 34 reliable polymorphic loci (Carey et al. 2001). Using this technique, we were able to show that some individuals with identical isozyme profiles were not truly clonally related. An estimate of the probability of misidentifying two individuals as clonal was generated by taking the inverse of the product of the frequency of the most prevalent phenotype. The phenotype in this case is referring to whether a particular band is present or absent at each of the 34 polymorphic loci. Based on this

calculation, there is only a 1 in 49,500 chance of clones being inaccurately identified using these loci. Although the numerous polymorphic loci that can be assessed quickly using RAPDs are more sensitive in determining clonal relationships than the limited number of isozyme loci, there was still no specific RAPD pattern or band that was associated with resistance. To identify a marker linked to resistance, a more informative marker system will be required.

Amplified fragment length polymorphism (AFLP) is a molecular marker generated by a combination of restriction enzyme digestion and PCR amplification (Vos et al. 1995). This technique is more powerful than RAPDs because a large number of polymorphic loci can be generated in a single amplification. To date, we have screened 80 primer pairs using subsets from the original stands described by Houston and Houston (1994, 2001). This has identified 274 polymorphic loci, or about three times as many polymorphisms per primer as compared to RAPDs. These AFLP primers were used to screen "bulks" consisting of equal quantities of DNA from 10 different individuals. For two different stands in Maine and one in Nova Scotia (Houston and Houston 2001) a bulk of resistant trees was screened along with a bulk of susceptible trees, for a total of six bulks. Three of the 80 primer pairs showed bands that were present only in the resistant bulks and not in the susceptibles. However, when these primer pairs were used to screen an expanded population of individuals (N = 144), the relationship with resistance did not hold.

Even though the 80 primer pairs screened did not produce a marker that correlates to resistance, we believe that using AFLPs is currently the most efficient way to search for such a marker. In populations where the relatedness of individuals is not absolutely clear, the search for a marker is very much like looking for a needle in a haystack. Nevertheless, due to the ease of generating large numbers of polymorphisms with AFLPs, in combination with the low sample number for screening by using bulks, it should be possible to screen through thousands of markers. With such high numbers, the odds of success increase. However, by using this semi-random population approach, any marker that is potentially linked to resistance would have to be confirmed through breeding and studies of inheritance.

Controlled Cross-Pollinations in American Beech

Without question, looking for markers correlated with resistance should ideally be done through breeding so the segregation pattern of the phenotype (resistance) can be assessed directly in comparison to the marker to determine if the marker co-segregates with the phenotype. However, generating a full-sib family using resistant American beech parents is not a simple task for many reasons. First, the estimated minimum age for seed production in American beech is 40 years, and a beech tree of that age can reach heights of 70 to 120 feet (Rudolf & Leak 1974). Second, flowers are usually most prevalent in areas of the canopy that are in direct sunlight, generally toward the top of the tree. Third, flowering in beech is variable and flowers are extremely susceptible to spring frost. In general, good beech seed crops are produced every 2 to 8 years (Rudolf and Leak 1974).

Fortunately, we were able to identify scale-free trees that were very near a paved campground roadway in Ludington State Park, MI. The site is located in a killing front, so the disease pressure was high enough to be able to select "clear" trees with reasonable confidence that they were resistant. The parent trees were tested for resistance using the artificial infestation technique. An 8 ½ x 11 ½ inch foam pad failed to enhance the formation of scale colonies underneath after being in place for a year on all parent trees. The following year, 300 eggs were placed under the foam pad. Again, little to no scale insects colonized underneath the foam. In cases where insects were found under the foam, there was no evidence of egg production. Susceptible control trees also were tested in a similar manner, and in these cases the foam alone was sufficient to detect an enhanced scale population on the tree.

Details of the methods used to perform controlled crosses are outlined in Koch and Carey (2004a). Crosses were performed between two resistant (R) parents, between a resistant and an intermediate (I) parent (initially thought to be scale free, but eventually developed low level infestation) and between a resistant and susceptible parent. The reciprocal cross between the susceptible (S) and resistant parent was also performed.

Table 1.—Controlled Cross-Pollinated Seed

Cross (♀ x ♂)	Seeds						Germinative Capacity	Number of Plants
	Full	Germinated	Rotten	Empty	Total	% Full		
1506 (S) x 1504 (R)	11	84	0	146	241	39	81 %	77
1504 (R) x 1506 (S)	49	31	10	585	675	13	12 %	11
1504 (R) x 1501 (I)	35	0	0	98	133	26	37 %	13
1505 (R) x 1504 (R)	28	33	0	170	231	26	84 %	51

Individual seeds were dissected from their seed coats to determine if they were full, germinated (radicle evident), rotten (including a small percentage that showed evidence of insect damage), or empty. The germinative capacity is the percent of full seeds + germinated seeds that once sown resulted in a seedling. *Table from Koch & Carey (2004b)*

Table 2.—Open-Pollinated Seedlings.

Parent Tree	Number of seeds	% full	Germinative capacity	Number of plants
1506 (S)	802	35	60 %	168
1504 (R)	2081	28	8.5 %	49
1511 (S)	478	24	2.6 %	3
ME (R)	283	75	53 %	149

The germinative capacity is defined as the percent of full seed + germinated seeds that once sown resulted in a seedling. *Table from Koch & Carey (2004b)*

The results of the controlled cross-pollinations are listed in Table 1. The amount of full seed ranged from 13 to 39 percent. The germinative capacity (the percent of sound seed that germinated) varied from 12 to 84 percent, providing some evidence of mating incompatibilities . The susceptible tree 1506 was used as both a pollen donor and a maternal parent along with the resistant tree 1504. Interestingly, when 1504 was used as a pollen donor with both 1506 and the resistant tree 1505, seeds with a high germinative capacity (81 and 84 percent) were produced. But when 1504 was used as a maternal parent with either 1506 pollen or 1501 pollen, the seeds produced had germinative capacities of 12 and 37 percent, respectively. For tree 1506, the opposite pattern was observed; this tree was more successful as a maternal parent than a pollen donor (Koch and Carey 2004a).

As a control, open-pollinated seed was collected from two of the parents used in the crosses, the resistant tree 1506 and the susceptible tree 1504. Open-pollinated seed also was collected from 1511, a susceptible tree in the same part of the Ludington State Park (LSP) campground, and from a tree in Maine that was part of a 150 acre stand where all susceptible trees have been removed (Table 2). The open-pollinated seed collected from LSP was similar to the cross-pollinated seed in the range of the percentage of barren seed observed. This similarity between the germinative capacities of cross-pollinated seed compared to open-pollinated seed from the same parent (1504, 1506), indicates that the pollination bagging process did not negatively effect seed development. Overall, 24 to 35 percent of the seeds collected from trees at Ludington State Park (1506, 1504, and 1511) were full. This figure is only slightly higher than the reported 13 to 29 percent of sound nuts collected from 20 trees in East Lansing, MI (Gysel 1971). Interestingly, the percentage of sound seeds collected from the ME tree was much higher (75 percent) than those collected from Ludington State Park. This value was comparable to those reported by Leak and Graber (1993) for seed collected from beech in the White Mountain National Forest. Over a 6-year period of time seeds from White Mountain were consistently between 75 and 88 percent sound.

Although some steps were taken to minimize self-fertilization during the controlled cross-pollination experiments, including forcing pollen production for use

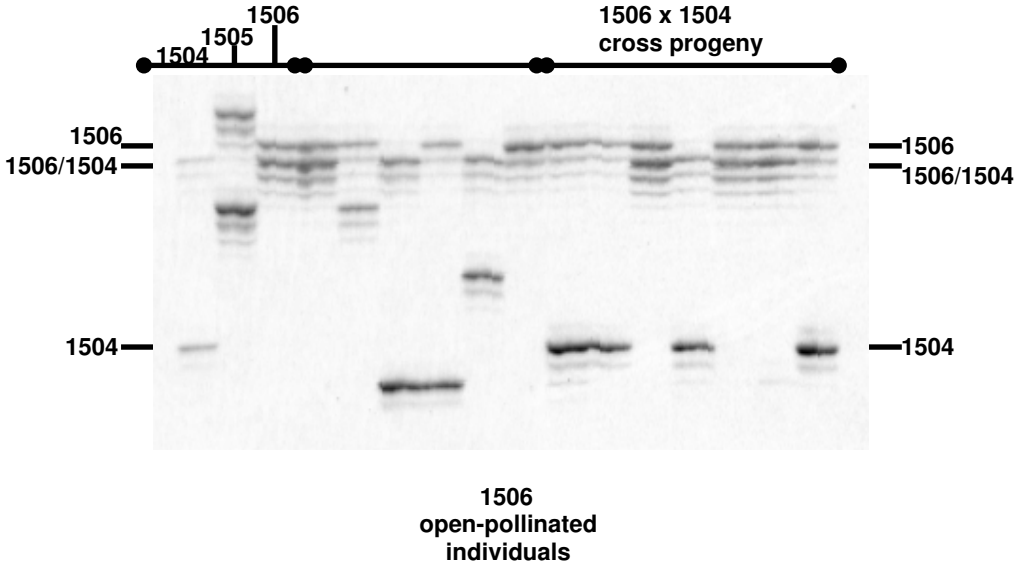

Figure 1.—Confirmation of parentage using SSRs. Only the alleles observed in the 1504 and 1506 parents on the left are also seen in the cross-progeny, indicating that each of the individuals was pollinated by 1506. Two of the open-pollinated individuals have alleles present that are also from either 1506 or 1504, indicating that some of these individuals may be the result of a cross between 1506 and 1504. Other individuals, as expected, have bands not seen in 1506 or 1504. To confirm parentage, each individual must be screened with several different SSR markers. The data shown here is a preliminary indication of parentage. To confirm parentage, a larger number of markers will be used.

in crosses prior to natural pollen release, we did not emasculate flowers. Experiments looking at self-pollination in American beech indicated a high degree of self-sterility (Koch and Carey 2004a). In addition to controlled crosses being contaminated by self-pollination, there is also always the small possibility of other sources of pollen contaminants, such as entering through a small tear in the pollination bag. To confirm the parentage of the cross-progeny and rule out contaminating pollen donors, we have begun working with SSRs (simple sequence repeats). These markers consist of tandem repeats of sequence units usually less than 5 bp in length (Bruford and Wayne, 1993). They differ from both RAPDs and AFLPs in that they are codominant and therefore can identify heterozygotes, which greatly simplifies parentage analyses. One disadvantage is in the extensive process involved in screening for microsatellites. However, once they have been identified and primers developed, the PCR-based protocol is relatively simple. Several microsatellite loci have been identified in several *Fagus* species, including *F. crenata, F. japonica, F. sylvatica* and *F. orientalis* (Tanaka et al. 1999; Pastorelli et al. 2003), so we started by screening these for informativeness in *F. grandifolia*. To date, the nine primers developed in *F. crenata* have been screened and five have been found to amplify polymorphic bands in *F. grandifolia*. We have begun screening all of the cross-progeny and parents with these primers, and the preliminary data (Figure 1) has revealed no evidence of contaminating pollen.

Methods

Screening for Resistant Seedlings

The artificial infestation method developed by Houston (1982) was used to test both the full- and half-sib families for resistance to the beech scale insect. To collect insect eggs, polyurethane foam traps measuring 21 ½ cm x 28 cm and backed by masonite were affixed to susceptible trees at the Holden Arboretum (Kirtland, OH) in the summer of 2002. One year later, the pads were peeled back to reveal an enhanced scale population underneath. Using a paintbrush, the eggs were brushed off the tree and into a one gallon plastic ziplock bag. The eggs were kept on ice and stored at 4º C. Prior to use, the eggs were sieved through 200 µm nylon mesh to separate them from debris and adult insects. Using a dissecting microscope, individual eggs were counted out and 150 eggs were placed on pieces of moistened polyurethane

Table 3.—Results of Scale Challenge Tests

Seedling source	Number of resistant	Number of susceptible	Total	Hypothesized Ratio Tested	P
1505(R) x 1504(R)	21	26	47	(9:7)[a] 4:5[b]	(.110) .974
ME(R), Open-pollinated	31	40	71	(9:7)[a] 4:5[b]	(.033) .893
1506(S) x 1504(R)	12	41	53	(9:7) 1:3[c]	(<.0001) .692
1506(S) Open-pollinated	22	67	89	(4:5) 1:3[c]	(.0014) .950
1504(R) Open-pollinated	7	26	33	1:3[c]	.616
1510(S) Open-pollinated	3	22	25	1:3[c]	.133

[a]Ratio is derived from a dihybrid cross between two heterozygous individuals where a single dominant allele for each gene is required for resistance, yielding a 9:7 ratio.
[b] Ratio derived from a dihybrid cross resulting in a 9:7 ratio, but if the homozygous, dominant condition for either of the genes is assumed to be lethal, the resulting ratio is 4:5.
[c]Ratio is derived from assumption that R tree must again be heterozygous for both genes and S tree is homozygous for both genes.
R = resistant, S = susceptible

foam measuring 3 cm x 7 cm. The foam was affixed to the stem of the seedlings using plastic coated wire, with the eggs facing directly against the bark. The potted seedlings were kept in a lathe house and fertilized weekly with a 12-12-12 fertilizer throughout the growing season and brought into a cold storage facility (4º C) from November to April. In July of 2004, the foam pads were removed and the number of insects that had established on the bark under the pad were counted using a 10 X hand lens. Individual trees with five or fewer insects were deemed resistant. Two size classes of scale were observed and in all cases where five or fewer insects were observed, they were of a smaller size class compared to those observed on susceptible trees. Many times they also appeared brown and shriveled, not round and yellow as observed on susceptible trees. Chi-square analysis determined the goodness of fit with the hypothesized ratios. The probability (P) was determined using the chi-square to P calculator at http://faculty.vassar.edu/lowry/tabs.html.

Results

The results of the scale counts are summarized in Table 3. Seedlings challenged included those resulting from the R x R cross and seedlings from the S x R cross. The resistant pollen donor in each of these crosses was the same individual, tree 1504. Seedlings were called susceptible if they had five or more scale insects present. In most cases, when five or fewer scale insects were observed, they were a smaller size class than what was observed in susceptible individuals with five or more scale insects present. Forty-five percent of the seedlings resulting from the R x R cross were resistant based on these criteria. In comparison, 44 percent of the open-pollinated seedlings from Maine were resistant. In this case, the stand of the maternal tree had been managed for BBD by removing all of the susceptible trees 10 years earlier (Trial, personal communication). It is probable that the pollen donors were all resistant. Consequently, the ME open-pollinated seedlings are the result of several different R x R crosses and it is not unexpected that the proportion of resistant progeny is similar to the proportion observed in the R x R controlled cross (Figure 2B).

Twenty-three percent of the individuals from the S x R cross were resistant according to the scale challenge assay.

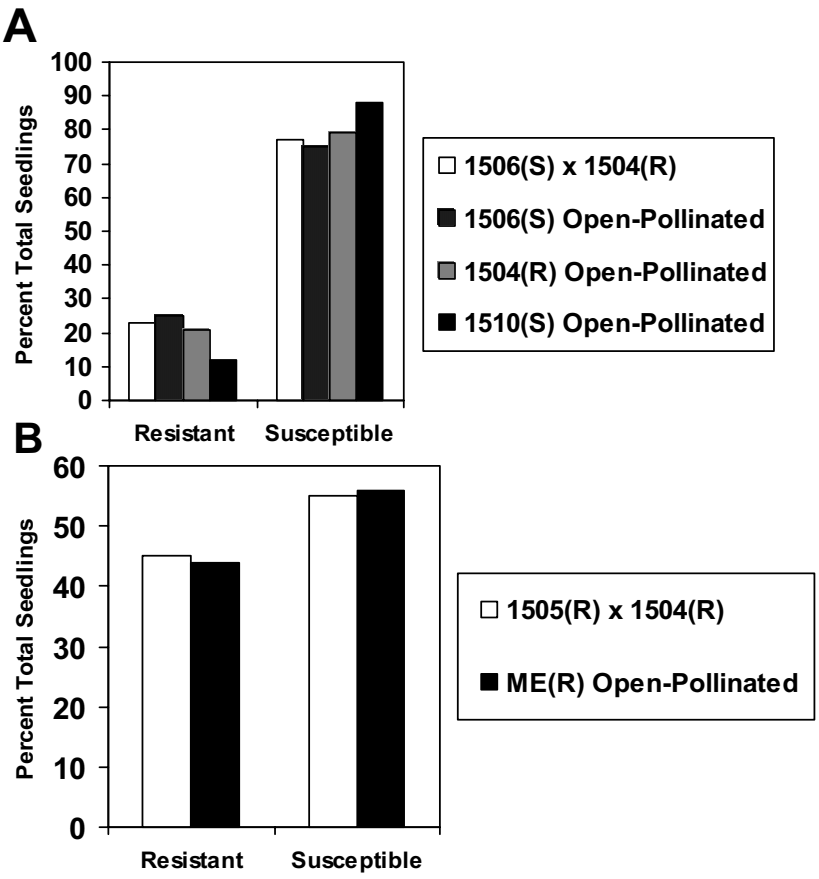

Figure 2.—Proportion of Resistant Seedlings. A. Comparison of the proportion of resistant to susceptible seedlings obtained from an S x R cross, an open-pollinated resistant tree and two open-pollinated susceptible trees. **B.** Comparison of the proportion of resistant to susceptible seedlings obtained from an R x R cross and an open-pollinated resistant tree.

The open-pollinated seedlings from the same resistant tree used as the pollen donor in the controlled crosses were 21 percent resistant. The similarity between these open-pollinated seedlings and the seedlings derived from the S x R cross is not surprising because theoretically the open-pollinated seeds are the result of many different R x S crosses (Figure 2A). The maternal parent, 1504, is resistant and it is probable that most of the potential pollen donors were susceptible. In contrast, the susceptible maternal parent, 1506, also produced 25 percent resistant open-pollinated seedlings. It was expected to produce fewer resistant offspring than the half-sib family derived from the resistant parent 1504. However, upon closer inspection, it was determined that a putatively resistant tree was located within 10 feet of 1506 and in fact, the crown of this tree actually touched the crown of 1506. This tree had no visible scale colonies and has since been challenged using the artificial infestation technique. The majority of the open-pollinated seedlings from 1506 were possibly the result of an S x R cross with this nearby resistant tree.

Open-pollinated seedlings from tree 1510, a susceptible tree, yielded 12 percent resistant individuals. The closest known resistant tree is 700 feet away from 1510, yet several other susceptible trees are located nearby. It is likely that the majority of pollen donors in this case are susceptible, but a percentage of seeds resulting from a resistant pollen donor cannot be ruled out. In future experiments, true S x S controlled crosses will be performed, using tree 1506 if possible.

Discussion and Conclusions

The percentage of resistant seedlings resulting from the R x R cross (45 %) was about a 4-fold increase over the percentage of resistant progeny from the open-pollinated susceptible tree 1510 (12 %). The higher proportion of resistant progeny observed in the R x R cross compared to the S x R cross provides the first genetic proof that resistance to the beech scale insect is a heritable trait. Although the data presented here is from a preliminary trial, the result of only a single year of challenge testing, the fact that the same proportion of resistant progeny was observed in both the full-sib family (R x R) and in the ME half-sib family provides support for the validity of

the data and the challenge test. Further support is seen in the similarity of the proportion of resistant progeny observed in both the S x R controlled cross and in the half-sib family derived from the R parent. Using the chi-square goodness of fit test, the ratio of resistant to susceptible individuals resulting from both the R x R and S x R crosses does not fit either the 3:1 or 1:1 ratios that would be expected from these crosses, respectively, if resistance were a single gene trait. The chi-square goodness of fit test was also used to test the 9:7 ratio that would be expected to result from a classic dihybrid cross. The R x R cross was found to fit this ratio, but with a very low P value of .110 (Table 3). The ME open-pollinated seedlings were found not to fit this ratio with P = .033.

Previous work by Wargo (1988) concluded that there is a correlation between high levels of scale infestation with higher levels of bark amino nitrogen. If the genes involved in resistance are somehow related to nitrogen metabolism, it is possible to imagine that if the homozygous dominant state of one of the genes results in a complete block of a metabolic pathway, not just a reduction, that lethality could be observed. If the assumption is made that the homozygous dominant condition for either of the two genes is lethal, the expected ratio for the dihybrid cross is 4:5. The chi-square goodness of fit test for this ratio gave a very high P value of .974 for the R x R cross and .893 for the ME open-pollinated family (Table 3). The resistant-to-susceptible ratio generated by the S x R cross did not fit the 9:7 or 4:5 ratios, providing support for the observation that the ratios produced by crossing two resistant individuals are different from the ratio produced by a S x R cross. However, the S x R family ratio was found to fit a 1:3 ratio (P=.692), which is the ratio that would be expected in a dihybrid cross between a resistant individual who is dominant heterozygous for both genes and a susceptible individual that is homozygous recessive for both genes.

The data presented are from a preliminary trial with a relatively small sample size. The two-gene model suggested may be an oversimplification and is presented here as a starting point, a model to be tested in future work. Furthermore, use of a biologically based assay introduces a degree of variability that could potentially result in false positive or false negative results, skewing the ratio of resistant to susceptible individuals. To determine to what extent variability within the insect challenge assay may influence the data presented here, we have repeated the insect challenge test on all of the full- and half-sib families. Data will be collected in August of 2005. In fall of 2005, we hope to establish a field planting of the cross-progeny to confirm the results of the artificial infestation experiment and to assess traits such as growth rate, form, and long-term durability of resistance. Other efforts under way include grafting of all cross-progeny as a means of preserving the germplasm and to produce replicates for further experimentation. Finally, efforts are ongoing to expand the pool of resistant parents used in genetic studies as a way to survey mechanisms involved in resistance and their mode of inheritance.

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