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ATTEMPTS TO DEVELOP AN OAK WILT RESISTANT LIVE OAK

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ABSTRACT

Oak wilt, caused by *Ceratocystis fagacearum*, was confirmed in Texas in 1961 and has since been found in 60 Texas counties. Much of the epidemiology of oak wilt in Texas has been elucidated. Protocols have been developed to hinder local and long distance spread of the pathogen, to treat infected high-value live oak trees, and to protect high-value trees situated next to diseased trees. What is lacking in the arsenal to defeat this epidemic in Texas is resistant live oak (*Quercus fusiformis*) stock. One unique aspect of the Texas epidemic is the apparent, partial resistance in live oak to the disease. This suggests that either genetic or environmental components are responsible for variable survivability to the pathogen. Previous research at Texas A&M University found evidence for heritable, genetically-determined resistance and for phenotypic markers (allozymes) associated with disease tolerance. In order to expand on these findings, we used clone and seedling crops to test for genetically- determined resistance to the pathogen. In one study, resistance of clone groups and seedling groups was tested for a potential correlation with prior levels of disease tolerance exhibited by the parental post-epidemic trees. We also conducted population experiments to test prior findings of a correlation between survival and two allozyme alleles (genetic markers). Some half-sib groups and some clonal groups do perform better than other groups when grown in greenhouses and inoculated with the pathogen. This makes a strong case for the presence of genetic resistance. However, no significant correlation between prior parental tolerance under natural disease conditions and seedling tolerance was found. We attribute this finding to a strong environmental component in determining the survival of live oak trees in natural settings. In the study comparing allozyme allele frequencies between pre- and post-epidemic populations, we found no evidence of markers linked to resistance. Further research will be required for the identification of superior live oak selections with reliable oak wilt resistance.

Key words: *Ceratocystis fagacearum*, *Quercus fusiformis*, resistance screening

The oak wilt epidemic in Texas, caused by *Ceratocystis fagacearum* (Bretz) Hunt, has taken a severe toll on urban and rural oak populations (Appel and Maggio 1984, Appel 1995). This epidemic is presently attenuated by applying a variety of management tools aimed at preventing pathogen spread and protecting high risk trees (Appel et al. 2003, Billings, this proceedings). These tools include cautious treatment of firewood, the elimination of inoculum sources and infection courts, trenching to destroy root connections between diseased and healthy trees, and intravascular injection with systemic fungicides. The worth of these measures has been proven and accepted, so they are routinely applied throughout Texas where warranted. They are not, however, infallible and they can sometimes be expensive and environmentally disruptive. Therefore, additional measures are needed to control oak wilt and preserve valuable trees and woodlands.

There are 26 *Quercus* species in Texas. Some are affected by oak wilt more so than others. The most susceptible oaks are members of the deciduous red oak group (genus *Quercus*, sub genus *Erythrobalanus*), such as *Q. buckleyi* and *Q marilandica*. These red oaks do not recover from infection (Figure 1). At the other extreme, deciduous white oaks (genus *Quercus*, sub genus *Leucobalanus*) are extremely resistant to oak wilt and rarely succumb to the disease. They are not, however, the most common component of the central Texas oak savannahs. Semi-deciduous live oaks, *Q. virginiana* and *Q. fusiformis*, are the most common tree in the central Texas rangelands and show variable tolerance to the oak wilt fungus (Appel 1986). The live oaks are classified as white oaks, but exhibit several anatomical characteristics inherent to the red oaks (Muller 1961). Fifteen to twenty percent of infected Texas live oaks survive infection, with the survivors ranging from no crown death to near total mortality (Figs. 2, 3) (Appel et al. 1989). This variable disease response presents a unique opportunity to search for the sources and causes of tolerance, or resistance, to *C. fagacearum* in the native live oak population.

Due to the ability of native live oaks to withstand site disturbances, and their extreme popularity as planted shade trees, live oak comprises a majority of urban trees in Central Texas cities and communities. Given the popularity of live oak in Texas, there is a sizeable potential market for an oak wilt resistant selection. The development of such a resistant selection will not be possible until there is further information on the heritability of resistance and whether live oak survival is the result of one or many resistance genes. Previous researchers presented evidence that the resistance of live oaks was genetic and heritable (Bellamy 1992, Greene and Appel 1994, McDonald et al. 1998). Half-sib groups of live oak seedlings were shown to differ in their resistance (Greene and Appel 1994). A post-epidemic population of live oaks had different allozyme allele frequencies than surrounding pre-epidemic trees by McDonald et al. (1998). Allozymes are different forms of enzymatic proteins that can be visualized by gel electrophoresis to infer different alleles of specific genes (Soltis and Soltis 1989). Differences in allele frequencies between the pre- and post-epidemic populations are evidence for natural selection being exerted on the host by the pathogen, perhaps indicating a shift toward greater resistance in the host.

The objectives of the present research project were designed to expand on the results of both Greene and Appel (1994) and McDonald et al. (1998). This was done by: 1) challenging large numbers of greenhouse-grown half-sib live oak seedling groups with *C. fagacearum* to look for differences in resistance, 2) creating and comparing clonal offspring from post-epidemic trees by screening them for response to challenge by *C. fagacearum*, and 3) evaluating the allozyme profiles of live oaks growing in additional disease centers to test for allele frequency changes in post-epidemic populations. One purpose of the project was to determine if allozymes can be used as markers to recognize trees containing resistance genes. If those efforts are reliable, then they may be selected for breeding and propagating future populations of resistant trees.

MATERIALS AND METHODS

Experiment 1

Seedlings grown for inoculation with *C. fagacearum* were collected from plateau live oaks (*Q. fusiformis*) in 1997 and 1998. One group of live oaks (post-epidemic trees) consisted of survivors in a disease center near Round Rock, TX. The post-epidemic trees exhibited a complete range of crown death (see Fig. 1), so that diseases responses of the artificially infected seedlings could be compared to that of their naturally infected parents. Progeny from these trees were compared to progeny derived from live oaks growing outside of, but adjacent to, the

expanding disease center (pre-epidemic trees). Seedlings were grown for one year in pots with a 4:1 sand/bark mixture and slow release fertilizer and then inoculated with a suspension of *C. fagacearum* conidia (1 x 10^6 spores/ml). The seedlings from each tree were place in random blocks in a shade house and treated uniformly to minimize effects due to environmental variation. Typical disease symptoms were monitored regularly for a year, until disease progress ceased. Responses of seedling groups were compared for each crop using two measures: 1) percentage of group survivors after one year, and 2) percentage of group with less than 25 % crown stem death.

Clonal trees were grown from root spouts collected from post-epidemic tree in 1997. As with the seedlings, the parent trees for clonal sprouts were rated for disease response so that the responses of the clones under artificial inoculation with *C. fagacearum* could be compared to the response of the surviving parent in the infection center. Ramets were cut from live oak root systems, treated with the root stimulating hormone indole-3-butyric acid (Sigma, St. Louis, MO), and planted in "d" pots contain a 4:1 sand bark mixture (Wang and Rouse 1989). The plants were placed in a mist chamber until they grew substantial root systems (approximately 4 months), and then transferred into one gallon pots with the same mix supplemented with a slow release fertilizer. The clones were inoculated after two years growth using identical techniques as those for the seedlings and disease progress followed as previously stated. Clonal group disease responses were measured and compared using average stem death.

Experiment 2

Selected allozymes were analyzed in the leaves of live oaks growing in oak wilt centers at two separate locations in central Texas. One was approximately 10 ha., located in the Balcones Canyonlands Reserve (BCR) in western Travis County near Austin, TX. The other was a rural site north of Lampasas, TX, and was approximately 15 ha. The allozyme profiles of two distinct live oak populations were compared in each of the locations. The first population consisted of post-epidemic, surviving trees located on the interior of the disease center. The second consisted of healthy, pre-epidemic trees on the perimeters of the disease center. Trees from each site were chosen and marked with the limitation that no trees less than 10 m distant from another selected tree was included to avoid clonal individuals. Several leaves (10 - 20 per tree) were collected, transferred to the lab on ice, and processed to provide enzymatic proteins. The leaves were kept refrigerated in the laboratory at 4^o C and processed with 3 days of collection. Standard enzyme extraction, electrophoresis, and gel evaluation procedures were used for allozymes from four polymorphic loci (Stuber et al. 1988). The allozyme frequencies from pre-and post-epidemic populations were compared to determine such population history dynamics as selection, migration, and genetic drift (Ayala 1982, Nei 1978).

Allozymes were analyzed as alleles at individual loci. Therefore, the collection of allozymes at each tree represents its genotype. Allozyme data for all the trees at each site were entered into the software population genetics program "POPGENE-VERSION 1.31" (Yeh and Boyle 1997). The POPGENE program can be used to evaluate allele frequencies, genotype frequencies, genetic diversity, Hardy-Weinberg equilibrium, and a variety of other parameters reflecting the genetic structure and evolutionary background of a population. Details of these analyses will be discussed only in general terms during this presentation.

RESULTS

Experiment 1

Some groups of half-sib seedlings following inoculation had significantly greater average survival after one year than other groups from both the 1998 and 1999 seedling crops. Comparisons among 21 first year (1998) half-sib groups (seedlings surviving for one year after inoculation) resulted in five groups (numbers 1, 2, 3, 12 and 13) that had significantly higher percentages of survival than the five poorest groups (numbers 6, 7, 8, 10 and 15) ($p = 0.05$) (Fig. 4). The best performing seedling group (number 12) had a significantly higher percentage of successful seedlings than the poorest 15 groups. In the second year's crop, 1999, one group (number 13) had a significantly higher percentage of surviving seedlings than 11 out of the other 31 groups with the fewest survivors ($p = 0.05$) (Fig. 5).

When analyzing the proportions of the half sib seedlings with less than 25% stem death, group no. 20 from the 1999 crop was significantly more tolerant than 8 of the 30 other groups. Three of the groups were significantly more tolerant than nine of the least tolerant groups (Fig. 6).

In the comparisons of nine clonal groups with at least three members, one clonal group (number 6) was more tolerant than the three least tolerant groups (Fig. 7). The variances of tolerance within the clonal groups were surprisingly uniform.

The tolerances of half-sib seedling and clonal groups from post-epidemic trees were compared to their parent trees' performances in the field under natural infection by *C. fagacearum*. In general, the seedlings and clones that exhibited increased tolerance tended to have more tolerant parents as estimated by crown survival (Fig. 8). But, the correlation coefficients were all low.

Experiment 2

No specific data comparing the tree allozyme frequencies between pre- and post-epidemic areas of two disease site will be presented in this talk. There were no specific allozymes that had significant pre- to post-epidemic differences in both sites. These results will be discussed in general terms below.

DISCUSSION

These experiments were conducted to find potential sources of resistance to the oak wilt pathogen in native live oaks. Two general approaches were used. Both of these approaches were used in previous, preliminary studies to test for resistance in surviving live oaks growing in oak wilt centers in central Texas (Bellamy 1992, McDonald et al. 1998). The first was to test for unique enzyme profiles (allozymes) in surviving live oak populations to determine whether the pathogen is exerting natural selection for resistant host genotypes. If this was the case, then those survivors are potential sources of selection and breeding efforts to develop superior trees. The second was to collect acorns and root sprouts from those survivors as sources of seedlings and clones, respectively, for inoculation screenings (Green and Appel 1994). Results from the preliminary projects were sufficiently promising to extend them to a broader sample of trees growing over a wider geographic range in the present study.

In the screenings of seedlings, two responses were measured to evaluate potential resistance. The first was average group survival, for which there were differences among groups of seedlings derived from both the 1998 and 1999 acorn crops. Presumably, the best performing seedling groups would reflect some degree of resistance in their parents and point to those trees

as candidates for further analyses. The second response measured in the seedlings was the proportions with less than 25% stem loss. Again, differences among the seedling groups for this criterion indicated there may be variability in resistance to *C. fagacearum* among the parents.

Since live oaks are open pollinated, out-crossing trees, variability in a population of seedlings grown from acorns from a maternal parent for any phenotype such as disease resistance may exist. For this reason, clones from the parental trees were developed to undergo similar screening. There was one clonal group with a significantly greater proportion of stems with less than 25% dieback. This tolerance in one of the groups lends further evidence for a genetic basis for resistance to oak wilt in the surviving native live oaks.

The methods used in the present study also have been used for identifying sources of resistance to a variety of diseases in other tree species. For example, clones have proven effective in testing for disease resistance in other species, such as elms for Dutch elm disease (Solla et al. 2005). In addition to the genetic basis for resistance, Solla et al. (2005) mentioned a wide number of other factors as being influential in the disease response. These included time of inoculation, environmental conditions, and even height of the inoculated elm saplings. These factors were probably influential in the present study on oak seedlings and saplings, adding to the variability in disease response and perhaps confounding the discovery of a clearly resistant selection. Nonetheless, the results are sufficiently encouraging and some useful materials have been found for continued propagation and testing.

An additional, useful measure of heritability for resistance is the relationship between the responses of progeny to artificial inoculation compared to the performance of the parents under natural infection in the field. Similar considerations are being made for other tree diseases, such as efforts to find disease resistance in native butternut (*Juglans cinerea*) to the exotic canker causing pathogen *Sirococcus clavigignenti-juglandacearum* (Michler et al. 2005). No significant trends were detected when the response of seedlings were compared to those of their parents, indicating a parent's prior performance cannot be used to confidently predict the tolerance or resistance of the offspring toward the pathogen.

The two allozyme alleles that McDonald et al. (1998) found associated with survival were tested in this study and were not associated with survival in either disease center. We found no decrease in genetic diversity as was reported in that previous study. We did find that all live oak populations were in Hardy-Weinberg equilibrium which shows that these populations are maintaining genetic diversity through sexual reproduction instead of being only large populations of a few clones. Allozyme profiles of individual trees and GPS mapping did allow us to find several clonally propagated motts within the larger populations. The allozyme data also was used to show that the two disease sites, although separated by approximately 80 km, had nearly identical allele frequencies. This finding was evidence that gene flow is widespread and that natural populations of live oaks through out the Edward's Plateau in central Texas should be expected to share similar genetic profiles.

CONCLUSIONS

A genetic basis for tolerance to oak wilt caused by *C. fagacearum* does exist in live oaks, and this tolerance is heritable. However, the level of crown loss in post-epidemic trees is a poor predictor of how offspring from those trees will perform when challenged with the fungus. Environment or chance plays a substantial role in the outcome of this disease in live oaks. Individual clonal groups show a more normal variation of response to the fungus. Two clonal groups have been found that showed consistent tolerance and may indicate a source of tolerant

trees. The allozyme markers that were studied in this project are not useful marker to identify resistant live oaks. Future research should take advantage of artificially-created live oak clones and revisit environmental effects upon the disease process.

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Figure 1. Red oaks in central Texas killed by *Ceratocystis fagacearum* exhibiting no survival.

Figure 2. Live oak within an oak wilt center in central Texas exhibiting partial survival following infection by *Ceratocystis fagacearum*.

Figure 3. Variable survival rates of live oaks within a typical central Texas oak wilt center.

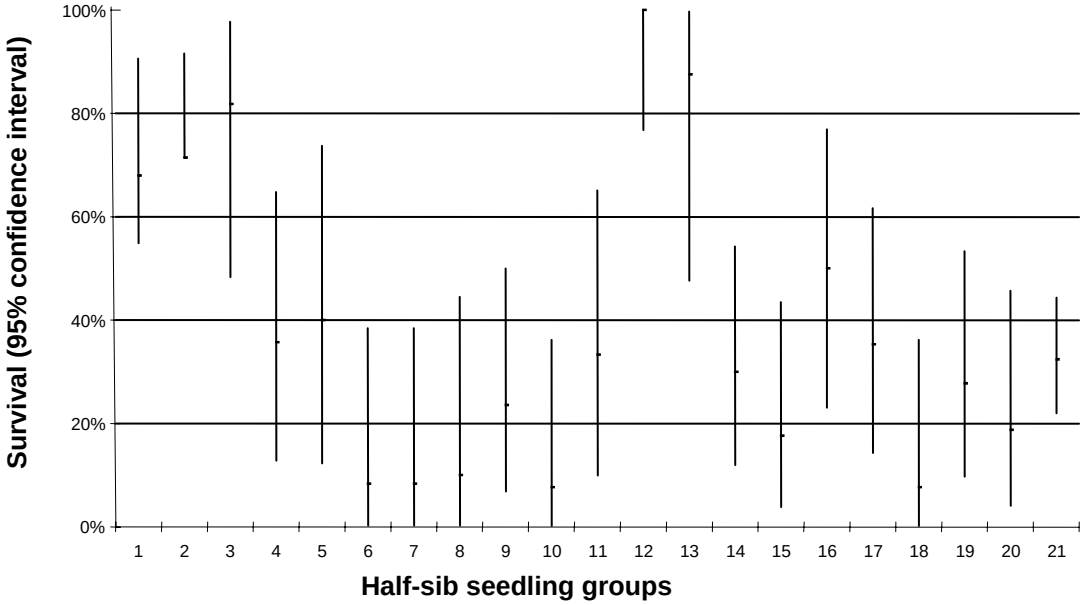

Figure 4. Comparison of survival (% alive) of 21 seedling groups grown from the 1998 acorn crop from live oaks in central Texas one year after inoculation with *Ceratocystis fagacearum*.

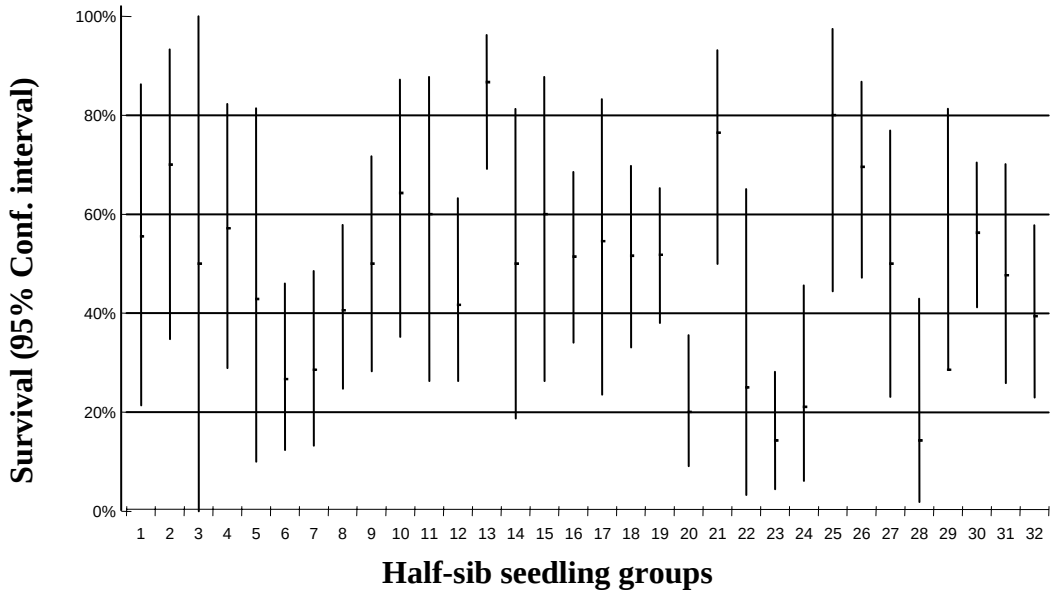

Figure 5. Comparison of survival of 32 seedling groups grown from the 1999 acorn crop from live oaks in central Texas one year after inoculation with the *Ceratocystis fagacearum*.

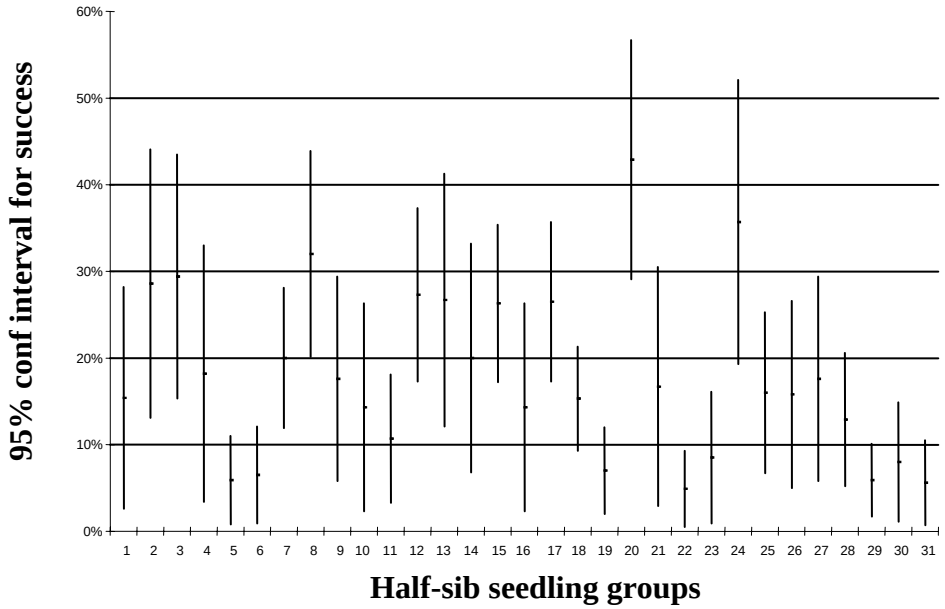

Figure 6. Tolerance, as defined by less than 25% stem loss, in groups of half sib seedlings one year following inoculation with *Ceratocystis fagacearum*.

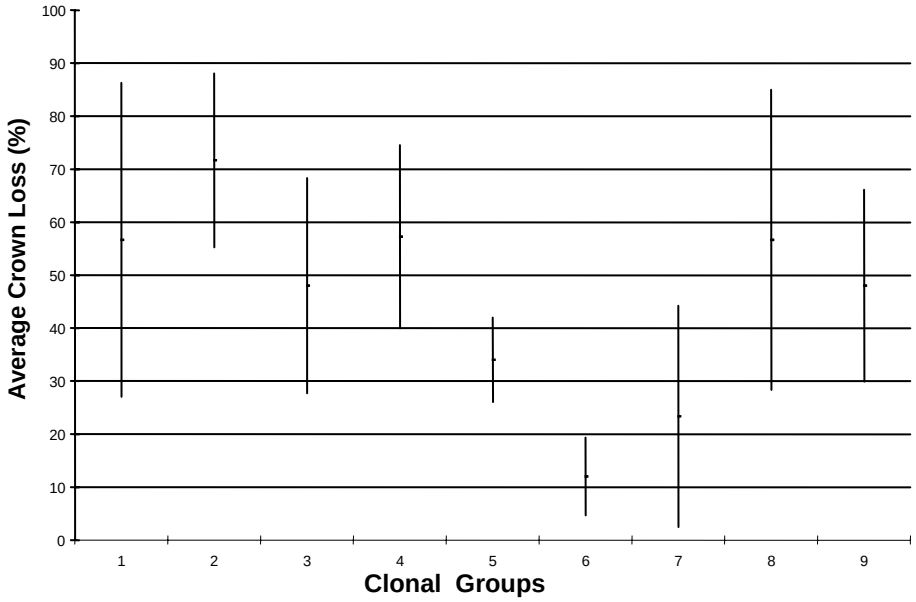

Figure 7. Average crown loss for groups of live oak clones inoculated with *Ceratocystis fagacearum.*

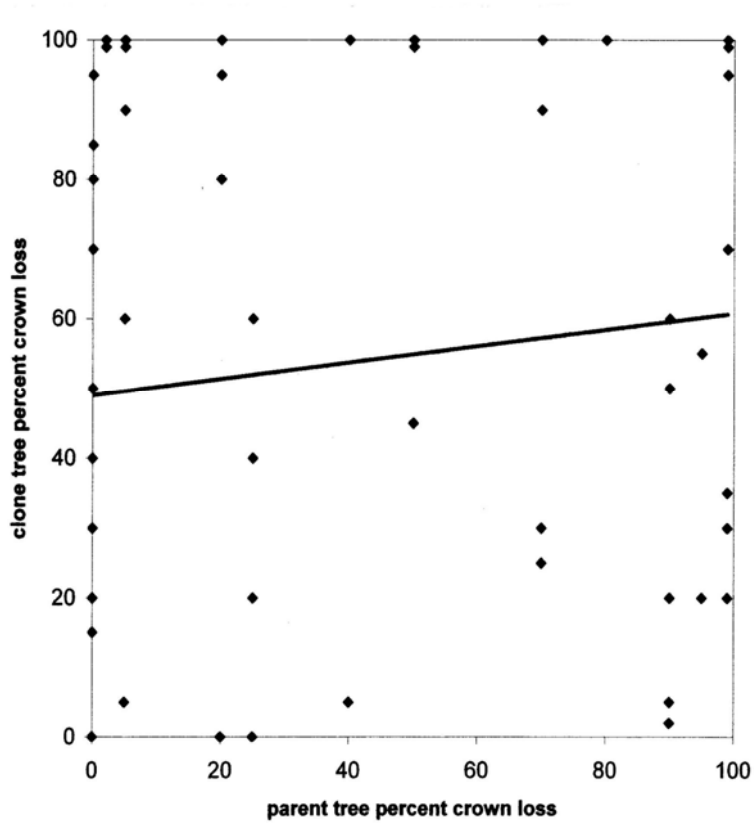

Figure 8. Correlation between the proportions (percent) of crown loss in clonal saplings artificially inoculated with*C. fagacearum* and the crown loss of their naturally-infected parents.