# NE-1015 Technical Committee Meeting
# Biological Improvement, Habitat Restoration, and Horticultural Development of Chestnut by Management of Populations, Pathogens and Pests

Pennsylvania State University—State College, PA
September 10-12, 2004

**Attendance:**

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|---|---|
| Connecticut: | Sandra Anagnostakis, John Anderson-Administrative Advisor (Connecticut Agricultural Experiment Station) |
| Maryland: | Donald Nuss, Lynn Geletka, Gerrit Segers, Fouyou Deng (University of Maryland Biotechnology Institute, Center for Biosystems Research) |
| Massachusetts: | Timothy McKechnie (University of Massachusetts) |
| Michigan: | Andrew Jarosz (Michigan State University) |
| New Jersey: | Bradley Hillman (Rutgers University) |
| New York: | William Powell (SUNY-ESF, Syracuse), Alice Churchill (Boyce-Thompson Institute), Steven Jakobi (Alfred State University) |
| North Carolina: | Paul Sisco (The American Chestnut Foundation) |
| Pennsylvania: | John Carlson-Chair, Haiying Liang, Tim Phelps, Kim Steiner Chien-Chih Chen (Pennsylvania State University), Sara Fitzsimmons (PA-TACF) |
| Tennessee: | Hill Craddock, Mark Alexander, Shannon Cagle, Lisa Worthen (UT Chattanooga) |
| West Virginia: | William MacDonald, Mark Double, Jenise Bauman and William Rittenour (West Virginia University) |

The meeting was called to order by Chairman Carlson at 8:30 am on September 10, 2004 at Days Inn—Penn State. Carlson welcomed the group and introduced Tim Phelps and Sara Fitzsimmons as the individuals responsible for the field trip that followed station reports.

**John Anderson, NE-1015 Administrative Advisor—Connecticut Agricultural Experiment Station**

Anderson indicated that his research interests lie outside of chestnut; he works with insects. However, over the course of many years, Sandra Anagnostakis has educated Anderson and kept him abreast of the problems with American chestnut.

The current NE-1015 project is newly revised with a timeframe of October 1, 2003 through September 30, 2008. Anderson reviewed the two objectives in the new project. The two varying objectives are a testimony to the fact that some in the project work with very practical aspects while others are doing fundamental, molecular research. The call for the group is to restore American chestnut to its former dominance in the forest, so it behooves all members to work toward the common goal.

Anderson thanked John Carlson for arranging the meeting.

**<u>OBJECTIVE 1</u>. To improve chestnut trees for reestablishment in forest ecosystems, and chestnut cultivars for nut production by selection, breeding, and marketing, and determine the cultural criteria of all chestnuts for successful production in nurseries, orchards, and/or natural settings.**

**Sandra Anagnostakis—Connecticut Agricultural Experiment Station**

Anagnostakis is involved with a breeding project to develop trees with good timber and nut qualities. She indicated that chestnut is a good orchard tree because it can be intercropped with a variety of crops. For those interested in chestnut cultivars, she suggests keeping in touch with Mike Gold and Ken Hunt of the University of Missouri Agroforestry Center in Columbia, MO. In planting hybrids into a forest, Anagnostakis likes to find a clearcut with plenty of American chestnut and use hypovirulent strains to keep the American chestnut alive so that they will survive long enough to interact with the hybrids to give plenty of diversity. To see how trees fare in the forest, she is reintroducing chestnut into a planting in Prospect, CT.

***Connecticut Water Company Land.*** This area in Prospect, CT was cleared in 2000 and 101 two-year-old hybrids were planted using 4' tree shelters. The plot represents four families of trees; they are Japanese and Chinese hybrids in different American chestnut backgrounds (Roxbury, CT and Watertown, NY). The Japanese hybrids have survived better than the Chinese hybrids. From 2000-2004, native American chestnuts that have sprouted in and around the plot have been treated with hv strains (highly debilitating French and Italian strains) to control chestnut blight. Both native and hybrid chestnuts flowered in 2004. Seed from American chestnut will be tested via a peroxidase test to see if crossing with hybrids has occurred.

***Chestnut timber planting***. In the spring of 2002, 212 siblings of the two-year-old hybrid chestnuts that were planted in Prospect were planted in an old tobacco field in Windsor, CT. The trees in this planting are not doing nearly as well (not as tall) as the trees in Prospect. This may be due to soil pH. The soil pH in Prospect is 3.5 while the pH is >5 in Windsor. Survival of Chinese hybrids was better than Japanese, 62% compared to 50%. Selections were made for timber form and those with poor apical dominance were removed. In 2002, twenty hybrids (10 Japanese and 10 Chinese) were moved to a clearcut in Session Woods in Burlington, CT.

***Sessions Woods wildlife management area***. This area in Burlington, CT was clearcut in 2001. In the spring of 2002, 20 four-year-old hybrids (from Windsor) were planted in mesh tree shelters for deer protection. Vegetation is controlled with Roundup®. Trees in this area are not as tall as the trees on the Water Company land, but there are plenty of American chestnuts, similar in size to the hybrids. Hypovirulent strains are used to control cankers; hv strains are made from the native *C. parasitica* population. Every year, cankers are sampled and analyzed for vc type and hypovirus. Both native and hybrid chestnuts flowered in 2004. Peroxidase tests will be used to determine if there is evidence for cross pollination.

***Lockwood Farms***. Seventy American chestnuts at Lockwood Farms, originally from Michigan and Wisconsin seed, were planted in 1976. Cankers on these trees were treated with a mixture of hypovirulent isolates (from 1978-1981). No treatments have

been deployed since 1981. Many of the original trees are still alive and exceed 25'. They have nearly solid, superficial cankers from the base to 20'. Many of the cankers were sampled in 2003; the samples yielded normal looking virulent and some hypovirulent isolates. Twenty-seven of the latter isolates were tested for dsRNA and 15 were positive for European-type dsRNA. Anagnostakis would like to know which of the many original hypoviruses have survived over the years.

***Native C. dentata population.*** Anagnostakis showed a map of the state of Massachusetts, based on pollen information. David Foster and Glen Motzkin of The Harvard Forest mapped the distribution of chestnut at the time of European settlement. Chestnut was not distributed evenly in the state of Massachusetts in the early 1600s.

**William Powell—SUNY-ESF, Syracuse**

***American chestnut research and restorative project***. The mission of this project is to conduct basic and applied research leading to the development of a blight-resistant American chestnut tree through genetic engineering and to reintroduce a population of these resistant trees back into forest ecosystems of New York and then the rest of the eastern US.

Powell discussed an oxalate oxidase gene (OxO), originally from wheat that has been inserted in the genome of American chestnut. OxO is a natural defense gene of grains, which can increase resistance to Septoria leaf spot in transgenic poplar and white mold in trangenic soybean by detoxifying oxalic acid. OxO also has been shown to protect lignin formation in chestnut callus grown in the presence of oxalic acid. In this construct, the OxO gene expression is controlled by a VSPB promoter (vegetative storage protein B). This promoter is from soybean; it is induced by sucrose and wounding and inhibited by auxins. In addition, there is a selectable marker, Finale (PPT). Powell is looking for hemizygous resistance in this construct. Since there is herbicide resistance, seedlings sprayed with herbicide will select for plants with the construct after outcrossing. Also, the construct has a gene for green fluorescent protein. They conducted transformation of different American chestnut somatic embryo lines to see which lines were easiest to transform. They used six clones, from northern NY and southern GA. The clones were tranformed using Agrobacterium-mediated transformation. Following a desiccation stage, fluorescence identified the transformed cells which has since grown to embryos at the cotyledon stage. Powell was not interested in the number of fluorescent cells but only those that actually developed embryos. He now has 12 stable lines. They tested germination and maturation on non-transgenic embryos from two protocols (Scott Merkle and Zizhou Xing). These two protocols differ greatly in their method steps. In their hands, the Xing method produced shoots as seen in the table below.

Non-transgenic shoot regeneration started 3-31-04 with 108 clumps/clone

| Line | Merkle | Xing |
|---|---|---|
| 30015 | 0 shoots | 7 shoots |
| Pond 1-1 | 0 | 7 shoots |
| WB296 | 0 | 0 |
| WB322 | 0 | 0 |

***Estimated timeline***. The first transgenic embryos were produced in the spring 2004 and the first transgenic potted plants are expected in the summer 2005. The first

field planting should be established in 2005 or 2006. Time needed for regulatory approval for release is unknown.

Powell commented that the antimicrobial peptide used in American elm, which is ready for field testing, can be outplanted until they begin to flower, at which time they have to be either rouged out or approved for release.

***Seedling inoculation.*** Powell has developed a technique where 1.5-mm-diameter seedlings can be tested for resistance with Ep 42 and Ep 155. In a recent inoculation trial, only 2/24 Chinese chestnut seedlings had tops that died following inoculation and all the American chestnut seedling had tops that died..

**Hill Craddock, University of Tennessee at Chattanooga**

***The Chattanooga chestnut tree project (CCTP)***. A broad genetic base of locally adapted American chestnut trees is required when attempting to breed for resistance. To ensure adequate genetic diversity, genetic resources have been collected from across the southeastern portion of the native range. Finding and breeding surviving American chestnuts stump sprouts is problematic due to the relatively short life span of blooming stems and their remote habitat. They have employed GIS to overlay soil, topography and satellite imagery for the generation of predictive, site-specific, chestnut range maps. Between 2002 and 2003, seven trees were located in TN. The trees were hand-pollinated using American chestnut and TACF hybrids in an attempt to capture native germplasm. Eighteen trees were added to the collection in 2004. Recently, 16 wild American chestnut trees were rescued from a highway construction site, which prompted coverage in the local newspaper. They hope to expand their target areas to western TN and MS. There may be some outliers in these states that survived as part of the ice refugium (an area of relatively unaltered climate that is inhabited by plants and animals during a period of continental climatic change (as a glaciation) and remains as a center of relict forms from which a new dispersion and speciation may take place after climatic readjustment).

A number of American chestnut trees from various states (KY, TN, GA) have been grafted onto container-grown *C. mollissima* and/or *C. dentata* rootstock.

***Backcross breeding for blight resistance***. Craddock is still conducting breeding work in TN. Pollen from the TACF farm in Meadowview is used to cross with local American chestnuts to create 3$^{rd}$ and 4$^{th}$ backcross trees. Future crosses will be made to get $BC_3$ $F_2$s. In 2004, they placed over 1000 bags for controlled pollination. The results were: 43, 271, 510 and 225 bags for $F_1$, $BC_3$, $BC_4$ and $BC_5$, respectively. Bendabout Farm is one area in which many of their crosses are made. In 2004, intercrosses from the CCTP project were made. All of the CCTP seed is grown in containers in the greenhouse and then planted out. With the TN chapter of TACF, he can provide seed to member growers; they often direct seed. Craddock noted that more than a dozen seed this year were lost to *C. parasitica* on the nuts. The TN 'mother trees' used in the CCTP ranged from large survivors in Lincoln, Rutherford and Blount counties to large escapes in Dekalb and Polk counties.

***Oral tradition***. Craddock indicated that he would like to document oral history associated with some of the large, surviving American chestnuts. People have many stories to tell regarding trees that they feel some emotional attachment to and Craddock feels these stories should be documented. Many stories from the original epidemic are lost because no one took the time to write them down.

***Cultivar evaluation in TN***. More than 50 named cultivars of *C. crenata*, *C. mollissima, C. denata*, *C. henryi* and *C. pumila* have been planted. Twenty cultivars with twenty trees each have been planted in four complete blocks. Cultivars that could not be obtained commercially were grafted; he hopes to have every named US cultivar in this trial. In the first two years of the study, cultivar differences have been noticed for vigor, survival, precocity of bloom, onset of anthesis, full anthesis, ripening and harvest date, tree form and habitat, insect attack and incidence of fungal disease. Craddock noted that many insects attack these trees. Polyphemus moths can defoliate a tree in one day. Other insect pests include: ambrosia beetles, bagworms, yellownecked caterpillars, Japanese beetles and fire ants. Ladybird beetles are beneficial insects.

**Paul Sisco—TACF-Asheville**

***TACF breeding program***. Marshal Case, TACF's President and CEO, made the push to get state chapters going and involve more people in the breeding program. It was his feeling that the more people that are involved, the more expertise is acquired. For example, Hill Craddock has more expertise in grafting than do staff members at Meadowview. At the Carolina chapter meeting, many members of the audience had advanced degrees, and through their knowledge, they greatly added to the member sharing. There are state chapters in Connecticut, Indiana, Kentucky, Maine, Maryland, Massachusetts, New York, NC/SC, Pennsylvania and Tennessee, with Alabama and Georgia in the process of becoming chapters.

In terms of the lines of resistance in the breeding program, the Carolinas Chapter is investigating one additional source of resistance, a Japanese chestnut tree at Fort Defiance, NC, the plantation of General William Lenoir (Revolutionary War patriot).

In an $F_1$ generation, trees are nearly uniform in resistance. In 1989, Fred Hebard started with two moderately resistant $BC_1$ trees as sources of resistance: the 'Clapper' tree from the USDA program and the 'Graves' tree from the CAES program. The grafts of the 'Clapper' tree maintained at CAES were crossed as males to pure American chestnut trees at CAES's Lockwood Farm to create $BC_2$ families (see report by Anagnostakis on Lockwood Farm). Most of the first $BC_2$'s were from the Lockwood Farm, but later crosses of both 'Clapper' and 'Graves' were to trees in the Mount Rogers National Recreation Area of SW Virginia. These $BC_2$ trees were then used as males onto other Virginia trees to create the $BC_3$ families at Meadowview. Each chapter is using Meadowview $BC_2$ pollen to pollinate American chestnut trees in its region to create locally-adapted $BC_3$ families. The current $BC_3$'s should be on average 94% American chestnut. The $BC_3$ intercross, begun at Meadowview in 2002, will be used to get resistance up to its full level. The final product should breed true (Chinese resistance and American form).

Paul discussed the difference between American and Chinese burs and leaves. American burs have a dense mass of long, slender, 2-3 cm-long spines. Chinese burs have a sparse mass of short, thick 1-2 cm-long spines. American leaves are long in relation to their width. Teeth are large and prominent. The leaf blade tapers sharply and the leaves are thin and papery. In contrast, Chinese leaves are oval-shaped with smaller teeth. The base of the leaf is rounded and leaves are thick and waxy.

The breeding efforts are progressing so that American chestnuts with blight resistance will be ready for forest restoration efforts over the next 3-10 years. $BC_3F_3$

chestnuts will be outplanted by TACF and PA-TACF between 2006-08. Beginning in 2008-10, the Maine, Indiana and Massachusetts chapters will outplant the $BC_3F_3$trees while Kentucky, Tennessee and the Carolinas will outplant beginning in 2011.

***Chromosome maps***. Sisco is interested in mapping resistance genes. John Carlson at Penn State is putting together maps of either pure American or Chinese chestnut, so that we can get alignment of the 12 chromosomes. The comparison of linkage groups of American chestnut (*Castanea. dentata*), Chinese chestnut (*C. mollissima*), European chestnut (*C. sativa*) and European oaks (*Quercus robur* and *Q. petraea*) is shown in the table below. This information was generated by a cooperative project between the labs of Fiorella Villani at Porano, Italy, Antoine Kremer at INRA, Bordeaux, France and Tom Kubisiak at SIFG in Saucier, Mississippi. Manuela Cassasoli, graduate student with Drs. Villani and Kremer, drew up the following chart.

Table 1. Synteny between the genetic maps of America/Chinese chestnut, European chestnut and two European oak species.

| Chinese/American Chestnut Linkage Group | European Chestnut Linkage Group | European Oak Linkage Group |
|---|---|---|
| A | 1 | 2 |
| B | 11 | 7? |
| C | 8 | 3 |
| D | 10 | 10 |
| E | 4 | 5 |
| F | 7 | 8 |
| G | 3 | 11 |
| H | 6 | 1 |
| I | 5 | 6? |
| J | 12 | 12 |
| K | 2 | 4 |
| L | 9 | 9 |

***Phytophthora cinnamomi***. Sisco concurred with Craddock that *Phytophthora cinnamomi* is a big problem in the southeastern US and it is moving into the mountain region. Chinese and Japanese cultivars have been tested and, so far, they are resistant to the disease. Sisco indicated that the NE-1015 group should consider inviting some Phytophthora experts (such as Steve Jeffers at Clemson and John Frampton at NC State) into the group, as Phytophthora will remain a problem with chestnut cultivation for many years. The *P. cinnamomi* that is covering the south is genetically uniform; only one mating type, A2, has been found. $F_1$ trees are uniformly resistant; the inoculation trials of Drs. Frampton and Jeffers are conducted with infected rice grains. There are some backcross trees that have died quickly while other have lived all summer. A preliminary model suggests that resistance is dominant but that it requires two independent loci. In a backcross to American chestnut, only 25% of the trees will be resistant as shown:

$R_1$--$R_2$-- resistant

| | |
|---|---|
| $r_1r_1R_2$-- | susceptible |
| $R_1$--$r_2r_2$ | susceptible |
| $r_1r_1r_2r_2$` | susceptible |

John Frampton and graduate student Mollie Bowles at NC State in Raleigh are studying the genetics of resistance to the pathogen. They have enough trees in their segregating population (96) that they hope to be able to map the loci for resistance as part of Bowles' MS thesis. They will be reporting on their studies at the national TACF meeting in Asheville on Oct. 30 -31.

**Sara Fitzsimmons—PA-TACF**

The first step in the restoration program is for volunteers to locate American chestnuts in the wild. She sends locator forms to individuals and the chestnut trees are then visited. She explained that a number of people incorrectly identify American chestnut; red maple, paw paw and horse chestnut are a few trees that people have identified as chestnut.

Fitzsimmons showed pictures of a tree in Clinton County, PA that is 22" dbh and 80' tall. This tree does not produce seed. Individuals have attempted to shoot catkins into the tree with a bow and arrow in an attempt to pollinate the tree. Fitzsimmons indicated that she is responsible for the logistics of getting bucket trucks into area to pollinate trees. Some individuals in Schuykill County used ingenuity to pollinate a tree; they built scaffolding around the tree.

The Ort tree, off I-83 in Cumberland County, is one of two lines of resistance that the PA chapter has inoculated. Fitzsimmons indicated that they do inoculation trials with two isolates, EP155 and SG2-3. Inoculations are made in June, read first in November; final measurements are made the following May. Following inoculation trials, trees are then bagged for pollination studies. Two of their best selections have yet to flower; she feels it may be a lack of phosphorous in the soil.

There are chestnut orchards all across the state of PA, including plantings of $BC_4$, $BC_3F_3$ and American chestnuts. Total number of trees with the 'Clapper' line of resistance are 5022, while 3983 trees have the 'Graves' line of resistance. They have almost 20,000 trees planted, of which 7,000 have died.

Fitzsimmons showed a map of the state of PA indicating 'mother trees.' There seems to be no correlation between present-day chestnut location and historic maps. Present-day locations are more correlated with individuals interested in chestnut than anything else.

**Tim Phelps—Pennsylvania State University**

Phelps discussed the planting trials of American chestnut in the central Appalachian mountains. These trials are under the supervision of Phelps, Kim Steiner, Chien-Chih Chen from Penn State and James Zaczek from Southern Illinois University.

***Silviculture studies***. In an effort to develop guidelines for reintroduction of American chestnuts into Appalachian forests, several silviculture trials are underway. A trial, begun in 1997, used a direct-seed tree shelter test to examine the effect on height growth using 5-foot-tall tree shelters (vented or unvented) and no tree shelter treatments. A containerized/nursery stock test examined the effect on height growth of two nursery stocks (1-0 and 1-1) and greenhouse-raised containerized stock in two container sizes (40

and 10 cu in) where one-half of each of the containerized stocks was given a 2.5-foot tree shelter. This study was conducted at two sites, Stone Valley (a shelterwood area) and Tuscarora State Forest (clearcut). At Stone Valley, 50% of the basal area had been removed. Trees in the shelters grew faster for the first two years, but 'no shelter' was actually better because it promoted more lower branching, giving a stronger form to the tree. After six years, there was no significant difference in height among 'no shelter' (21'), vented shelters (18') and unvented shelters (15'). In the Tuscarora State Forest, there was no significant difference between the two shelters (vented and unvented) but both were significantly taller than no shelter. However, those seedling were ravaged by deer, making this study inconclusive. The take-home message is that tree shelters are needed if deer pressure is high. If deer pressure is low, shelters are not necessary, as growth form is much better without tree shelters. Also, 1-0 stock grew just as well as 1-1 stock, so the extra year in the nursery is not needed. With regard to containerized seedlings, the container size had no significant effect on growth rate; the containerized material kept up with the nursery stock.

***Direct seed site evaluations***. They examined the suitability of a range of native forest sites for the reforestation of American chestnut. Five sites were planted with 50 seeds each (Rothrock, Stone Valley, Tuscarora State Forest, Bald Eagle and Owl Gap). All five sites had different ground cover. The study was begun in 1999 and replicated in 2000. They found that when the direct seed method is used, ground cover must be controlled. Further conclusions were: direct seeding is most efficient and economical, 1-0 nursery stock is just as good as 1-1 and containerized seedlings do just as well as nursery stock.

***PA-TACF hybrid chestnut orchards***. A third backcross ($BC_3F_1$) plantation was established in 2001. These individuals, with 'Graves' resistance contain 94% American chestnut genes. These trees will be inoculated, selected for blight resistance and then intercrossed (year to be determined). Another site, containing fifth generation material ($BC_3F_2$), is a partnership between the PA-TACF and Penn State. Ultimately, 31,500 trees will be planted. As of 2004, 5% of the orchard is complete. Open pollination of these seed trees should yield blight-resistant progeny for forest release.

**John Carlson—Pennsylvania State University**

***Variation in genome content among resistant backcross progeny*** . In the TACF backcross program, blight-resistance is bred into American chestnut by an interspecific cross with Chinese chestnut. American chestnut characteristics are then regained by a series of backcrosses to American chestnut parents. Carlson developed a dot blot protocol to screen individual trees in the backcross generations for those with greater amounts of American chestnut genome after the selection for blight resistance. The technique involves the hybridization of labeled Chinese chestnut genomic DNA probe to small amounts of genomic (total) DNA of individual backcross trees. The Chinese chestnut DNA probe is 'blocked' (pre-hybridized) with unlabeled American chestnut DNA prior to hybridization, to remove shared sequences. On average, progeny in the $BC_3$ generation should show lesser amounts of hybridization to the Chinese chestnut genomic DNA probe than $F_2$, $BC_1$ and $BC_2$ progeny. However there will be variation among individuals in each backcross generation for the amount of Chinese chestnut genome. The effectiveness and reliability of the dot blot approach were substantiated

using samples from chestnut parent trees and mockups (admixtures) representing the $BC_1$, $BC_2$, and $BC_3$ generations. Dot blot autoradiograms were imaged and quantified. ANOVA showed that the mean differences among the BC generations were significant, and interestingly that variance among each BC generation was equal. The dot blot hybridization data was compared to a morphological index for the same individuals and the correlation was found to be quite strong (Pearson's correlation coefficient = -0.662). He observed a significant amount of variation in dot blot signal intensity among individuals in all three backcross generations, indicating that the dot blot technique could be useful for selecting individuals with the greatest amount of American genome at each generation. A dot blot tool within the larger TACF breeding program is currently being tested.

**Pam Kazmierczak—University of California, Davis (not present at meeting)**

***Foundation Plant Materials Service***. Deborah Golino, Director of Foundation Plant Materials Service, has acquired the necessary permits for chestnut importation. There is still a 5-year quarantine for all chestnut material—held to check for gall wasp, etc. Seeds were collected from 22 cultivars in one U.C. Davis grove as the entire section of property utilized by FPMS is being turned into housing. These seeds will be moved to the USDA germplasm repository. Luciene Grunder donated 30 trees, including 'Colossal' and various Chinese seedlings. Rootstock for scionwood is maintained at the holding location at FPMS; cultivars also will be held at that site.

**<u>OBJECTIVE 2</u>. To evaluate and integrate multiple approaches for the biological control of the chestnut blight fungus and other pathogens and pests that threaten chestnut, by investigating host/pathogen/parasite relationships from the molecular to the ecological level.**

**Sandra Anagnostakis—Connecticut Agricultural Experiment Station**

***Nathan Hale State Forest.*** This plot was clearcut in 1990-91 and sprayed with conidia of hv strains of *C. parasitica* to see if hv conidia were sufficient to keep trees alive. Trees were sprayed once in 1992, twice in 1993, and once each in 1995 and 1998. A control plot, located 0.5 miles from hv plot, had no treatment. The plots were examined in 2004 and both the hv and control plots contained a dense population of black birch; the birch overtops the American chestnut. The table below lists some of the data.

| Data taken | Control Plot | HV Treated Plot |
|---|---|---|
| Sprout Clumps ≥ 1 inch | 6% | 28% |
| % Sprout Clumps Alive ≥ 1 in | 27% | 60% |
| % Sprout Clumps Dead | 67% | 15% |
| Mean dbh | 1 inch | 1.7 inches |
| Sprouts ≥ 1 inch | 2 | 18 |
| # *C. parasitica* isolates | 9 | 21 |

| | | |
|---|---|---|
| recovered | | |
| VC types | 4 | 5 |

The plots will continue to be monitored. She would like to remove some of the birch competition to see if the some of the sprout clumps become invigorated by the influx of light and resprout. She plans to take a light meter into the stand and take readings.

***Meshomasic State Forest.*** In conjunction with Don Nuss, this was the site of release of transgenic strains in the late 1990s. The area, consisting of hv-treated and non treated control plots, was clearcut in 1990-91 and sprayed once with transgenic isolates in 1997, four times in 1998, six times in 1999 and four times in 2000. Competition in both plots was maintained annually with Roundup®, although the herbicide has not been applied since 2000. Seven years following treatment, stems in the hv-treated plots are larger than trees in the control plots and there are more stems. There were no apparent dead sprout clumps in either the hv-treated or control plots. Of the 121 *C. parasitica* isolates collected from both plots in 2004, none were hygromycin resistant. All isolates will be checked for hypovirus. An aspect effect seems to be present in both plots. In the hv-treated plot, sprouts on the west and south edge are significantly larger than sprouts on the east and north edge or in the center. Likewise, in the control plot, sprouts on the west edge and in the center are largest. She has no explanation for these results.

***Housatonic State Forest***. In 1994, two 50 x 50 m plots were established in a mixed oak forest. Each plot contained 12 American chestnut sprouts which were inoculated with a virulent isolate. One-half of the trees were treated with a transgenic isolate in the summer of 1994. Sprout clumps have been mapped continually. In 2004, 40 C. *parasitica* isolates were recovered from new cankers; none were hygromycin resistant. There is no discernable biological control evident in this study.

Anagnostakis indicated that she sends an annual report to the Biotechnology Division of CSREES to report on both areas where transgenic isolates were released. She commented that Ep 713 does not seem to persist in the forest. It works well as a treatment for individual cankers but it is not a biological control agent.

**Don Nuss—The University of Maryland Biotechnology Institute, Center for Biosystems Research**

As of May 2001, there were only 30 different *C. parasitica* genes in Genebank, and thus there wasn't much information as to what genes are being altered by hypovirulence. In order to accomplish a survey of *C. parasitica* sequence data that provides for identification of new genes, their efforts have involved sequencing using ESTs (expressed sequence tags). The procedure using ESTs is as follows:

- ESTs are derived from mRNA sequences and therefore represent only expressed genes.
- The library is stored as individual bacterial transformants.
- Once plasmids are recovered, the insert is sequenced once, from one end only (single-pass sequencing).
- Sequence data is collected and analyzed.

The disadvantages of ESTs are that there is no genomic data, sequencing is redundant and highly expressed genes are found more often.

Why use ESTs?

- It is easier to generate than genomic data (smaller DNA fragments).
- They can be used to generate spotted cDNA arrays.

Nuss' lab set out to answer the question: To what extent does the presence of prototypic hypovirus isolate CHV1-EP713 alter the transcriptional profile of virulent isolate, EP155? To answer this question, source mRNA was taken from EP155 and EP155/CHV1-EP713. They trimmed the vector, analyzed for quality and eliminated short sequences. Contigs were assembled. Over 4200 sequences were analyzed for the different gene inserts (gene products). Approximately 2200 gene products were found. Those that were highly represented were: cryparin (270) and CHV1-EP713 Orf B (277).

The sequencing took place from May 2001 to September 2002. The analysis occurred between October 2001 and February 2003. The procedure was as follows:

- mRNA was isolated and cDNA libraries were made.
- Individual bacterial colonies were generated.
- Cataloged and stored in 96-well microtiter plates.
- "Minipreps" were used to recover plasmids containing cDNA sequences.
- Sent to sequencing facility.
- BLAST (Basic Local Alignment Search Tool) was used to compare translated EST data to non-redundant protein database (BLASTX); database from National Center for Biotechnology Information.
- DNA sequences were compared to all known and predicted protein sequences.

To date, Angus Dawe has produced the following:

| | |
|---|---|
| Minipreps | 5000 |
| cDNA sequences returned | 4448 |
| Useful sequences | 4217 |
| # individual gene products represented | 2200 |
| Total base pairs sequenced | 2,703,587 |

20-25% of the coding potential of the *C. parasitica* genome is now available at: Cogeme phytopathogen database—cogeme.ex.ac.uk (Nick Talbot's lab at Exeter University) and NCBI/Genebank, accession number (CB686454-CB690670).

***Microarray Spotting.*** Todd Allen developed the microarray/spotting analysis technique. These are 2-dimensional arrays (grids) of biological molecules. Classically, these are segments of DNA that are expected to code for proteins. This analysis provides the relative abundance of mRNA and a comparison of underlying mRNA abundance between two biological samples, a reference and an experimental sample. Microarrays are glass slides with 10,080 spots of DNA within a 20 x 56 mm area. The spot spacing is 300 μm and the spot diameter is 100 μm. Allen has spotted 3,864 *C. parasitica* clones. After RNA samples are obtained, fluorescently labeled probes are prepared (i.e. Ep 155 is labeled with green fluorescence and Ep 155 (CHV1-Ep713) is labeled red). Samples are mixed, hybridized, washed and spotted. The data is then analyzed to identify differently expressed genes. It is a good system for *C. parasitica* since hypovirus infection is stable and persistent. Strains are isogenic and both virus and host can be modified with ease. Differentially expressed genes can be disrupted.

**Controls and Validation**

- Control spots on chip (glass slide)
  - Viral coding regions

    - Genes encoding ribosomal proteins
    - Non *C. parasitica* sequences
- Multiple RNA preps, reciprocal labeling (dye swap)
- Different expressed clones must be changed in 4 of 6 or 3 of 4 independent hybridizations
- Differently expression verified for a subset of putatively changed sequences using "real time" PCR.

**Interpretation**

- Gridding (*Spotfinder*)
- Normalization (*MIDAS*)
- Identification of differentially expressed clones
- Association of chip locations to clone ID and BLAST data (*Excel*)

**What do we do with a Cryphonectria chip?**

Ask the following questions:

—How does infection of Ep 155 with CHV1-Ep 713 hypovirus alter the trascriptional profile? The answer, substantially. Of the 2200 genes expressed, 295 (13.4%) are altered at the transcript level in the presence of CHV1-EP713—132 are up-regulated and 163 down-regulated. This procedure validated the microarray platform and increased the number of CHV1-EP713 responsive host genes from 20 to an estimated 295. Some of the genes altered during persistent hypovirus infection are:

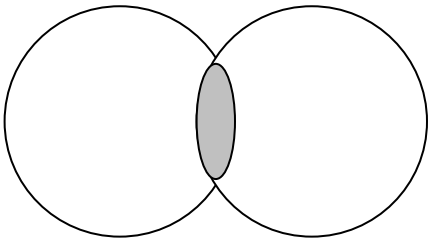
| | |
|---|---|
| S-adenosyl-L-methionine synthetase | up 7X with virus |
| S-adenosyl-L-homocysteine hydrolase | up 5X |
| Glutathione-S-transferase | up 10X |
| Heat shock protein | up 3X |
| Phosphoglutanase | down 3X |
| α-tubulin | down 3X |
| HoxX | down 11X |
| Endothiapepsin | down 4X |

(113 of the 295 differentially expressed clones do not match anything currently available at NCBI—no useful BLAST information available).

Gene Ontology was then used to develop a controlled, machine-readable vocabulary. What specifically was altered by CHV1-EP713?

| Altered | Up-Regulated | Down-Regulated |
|---|---|---|
| Amino acid metabolism | 3 | 4 |
| Carbohydrate metabolism | 1 | 13 |
| Development | 1 | 5 |
| Lipid metabolism | 1 | 3 |
| Stress response | 3 | 1 |
| Cell cycle | 1 | 3 |
| Cell wall growth | 5 | 10 |
| Electron Transport | 14 | 1 |
| Nucleic Acid metabolism | 4 | 7 |

—How do these changes differ when compared to infection with the milder CHV1-Euro 7 isolate?



EP713 vs 155
(295 genes)

Euro 7 vs 155
(166 genes)

Overlap (80 genes)

Do Euro 7 and EP713 cause similar or different changes? In the overlap, 73 of 80 genes changed in the same direction. Generally, greater magnitude of change in EP713 is greater.

Up-regulated in both cases was S-adenosyl-L-homocysteine hydrolase, a compound implicated in fungal senescence. Down-regulated in both was α-mannosidase, a compound implicated in autophagy (the feeding of the body upon itself, as in fasting; nutrition by consumption of one's own tissues). Up- and down-regulated was glutathione-S-transferase, noted in cellular detoxification.

The next step was to compare *C. parasitica* strains that are hypovirulent to those in the absence of viral infection. To accomplish this, mitochondrial mutants (*mit*2) were used. A mitochondrial mutant from Helmut Betrand (Michigan State University) was employed; this mutant has alternative oxidase activity. The microarray analyses of the *cyt*2 and *mit*2 mutants were:

Ep155 vs *mit*2: 201 of 2200 altered; 124 up and 86 down
Ep155 vs *cyt*2: 51 of 2200 altered; 11 up and 40 down

Forty-seven percent of the changes expressed between CHV1-EP713 and EP155 are also expressed between *mit*2 and EP 155, indicating there is a similarity between mitochondrial dysfunction and virus infection. Carbohydrate metabolism and stress response are both changed in similar directions. Thus, either the virus affects the mitochondria or mitochondria affect things that impinge on the virus. Mitochondria are responsible for:

Programmed Cell Death

Apoptosis Autophagy

Senescence (disintegration of mito. over time)

Does a common list of differentially expressed genes reveal itself when comparing CHV1-EP713 (viral), CHV1-Euro7 and *mit*2(mt DNA mutation)? They found that there are 19 genes that commonly changed in all three hypovirulent strains. Are any of the 19 genes functionally associated with either apoptosis, autophagy or senescence? Autophagy caught their eye since it affects vesicles and hypoviruses are packaged in lipid

vesicles. They found that the 12.7kbp dsRNA results in persistent reprogramming of a suitable portion (14%) of the *C. parasitica* transcriptome.

**Bradley Hillman—Rutgers University**

***Reoviruses.*** Ninety percent of the *C. parasitica* viruses found in nature are the hypovirus type, designated CHV. Reoviruses are very different from the CHV type. Two reoviruses of *C. parasitica,* C-18 and 9-B-2-1, were obtained from the West Virginia University group about ten years ago. While reoviruses are very important for RNA studies, Hillman is not proposing reoviruses as biological control agents. Reovirus properties are as follows:

- Found in mammals, invertebrates, plants and now fungi
- Many human diseases are associated with reoviruses, as are viruses of cattle and insects
- 10-12 dsRNA segments.
- They are found across kingdoms.
- Terminal sequences are conserved.
- Particle is important for replication.
- There is no known nuclear component.

Reoviruses are fundamentally particle-associated viruses. There are double or triple structures containing the core, which is transcriptionally active. The covered core is not transcriptionally active, but it is infectious. Reoviruses contain exactly one segment of each of the 10-12 segments of dsRNA that constitute the viral genome, encapsidated in a single complex virus particle comprised of 6-8 proteins.

All eleven segments of C-18 and 9-B-2-1 are found in one particle and are transmitted in an all-or-none fashion. Each core is transcriptionally active and all the enzymes necessary are in the particle itself.

**C-18 and 9-B-2-1**

- Were isolated in West Virginia about 20 miles apart.
- 11 segments segregate in an all-or-none fashion.
- dsRNA segments of 9-B-2-1 do not cross hybridize.
- Low cross-hybridization with homologous segments of C-18.
- Different affects on virulence and phenotype.
- 9-B-2-1 virus causes more dramatic phenotype changes (greater debilitation).
- 9-B-2-1 virus substantially reduces *C. parasitica* virulence; it doesn't grow in apples.

Hillman tried to isolate these viruses while working in Nobohiro Suzuki's laboratory in Okayama, Japan. He succeeded in producing a good band in a sucrose gradient and they looked like reovirus particles; they were 80 nanometers, had 11 segments of dsRNA and proteins. He began working with 9B21 since it had a more stable phenotype. C18 in not a well-adjusted fungus; it loses its virus consistently. It has numerous sectors that are virus-free.

C18 and 9B21 were officially named Mycoreoviruses in the 8$^{th}$ ICTV report. Reoviridae is now subdivided into 12 general. Mycoreovirus has 3 species:

MyRV-1/Cp9B21
MyRV-2/CpC18

MyRV-3/RnW370 (for *Rosellinia necatrix,* a white rotter) Mycoreovirus is most closely related to Coltivirus (a group of reoviruses that infect ticks).

Similarity of MyRV-1/Cp9B21 and MyRV-2/CpC18 and 9B21 is shown below:

| Segment | % Similarity |
|---|---|
| S1 | 43% |
| S2 | 33% |
| S3 | 24% |
| S4 | 23% |
| S5 | 16% |
| S6 | 29% |
| S7 | 11% |
| S8 | 13% |
| S9 | 26% |
| S10 | 17% |
| S11 | low |

The genome size of the C18 virus is 25,050 bp while 9B21 is 23,426 bp.

The codon usage of C18 and 9B21 is dissimilar. Hypoviruses, particularly CHV1 and CHV4, are more similar to *C. parasitica* codon usage than are the reoviruses.

| | CpC18 | Cp9B21 | *C. parasitica* |
|---|---|---|---|
| G+C | 47% | 42% | 54% |

Myristoylation is a process of a short chain fatty acid addition to a protein. This is important in that this process leads to short-term association with proteins; cleavage of the fatty acid then allows for the release of a protein into the cytosol. *Rosellinia* reovirus will not be myristoylated because it has no G pattern. The mycoreovirus has a myristoylated protein in its outer capsid. Hillman is investigating this phenomenon.

**Reoviruses—current and future research**

- Complete the genome characteristics of CpC18
- Determine the particle protein—cyro EM in collaboration with José Castón
- Examine affects on fungal gene expression by northern blot and microarrays in collaboration with Don Nuss
- Examine CpC18 and Cp9B21 gene function and expression in collaboration with Nobuhiro Suzuki
- Infectivity in other fungi and other host types
- Expression of individual viral proteins in *C. parasitica*

**William Rittenour—West Virginia University**

This research project focuses on testing the forest fitness and biological control potential of strains containing a cDNA copy of the Euro-7 hypovirus. The CHV1-Euro7 hypovirus, created at the Nuss Lab (University of Maryland Biotechnology Institute), has been found to be less debilitating to the host fungus than CHV1-EP713, thereby allowing for better canker establishment and greater hypovirulent inoculum production. The study will examine: 1) artificially established cankers and subsequent inoculum production; 2)

spermatization and subsequent ascospore production of wild and artificially-initiated virulent cankers; and, 3) dissemination of *C. parasitica* strains to other trees and dissemination of cDNA copy to other vc groups. There are 12 plots in the study; the plots are split into three groups of four trees with each group receiving a main treatment of transgenic, cytoplasmic or virulent strains of EP155 and EP146. Within each plot, there are 12 trees that are assigned one of three sub-treatments. Trees with two scratch-initiated cankers were started from inoculum that corresponded to the plot's main treatment. These scratch-initiated cankers will be analyzed for conidia production by collecting bark disks, diluting stroma contents, and assessing germinating conidia. The second sub-treatment in each plot is canker treatment trees. These trees host either wild-type virulent cankers or artificially-initiated virulent cankers that were treated with spermatizing-conidia of mixed mating types corresponding to the main plot treatment. These cankers will be analyzed for hypovirulent ascospore production by diluting perithecia contents in a similar process as the conidial analysis. Any hypovirulent ascospores collected will be tested for conversion against 29 representative vc types collected from the site in December 2003. This will provide a measure of cDNA dissemination to other vc types. The third sub-treatment within each plot is on trees that did not receive a treatment and are monitored as a measure of fungal dispersal. Bark plugs will be collected from cankers on these trees to examine movement of fungal strains. Overall, results will provide information on the establishment and dissemination potential of transgenic *C. parasitica* compared to cytoplasmic hypovirulent and virus-free strains of the same background. Currently, plots are established and treatments have been administered. Bark plugs and disks will be collected in October 2004 and analyzed over the winter.

**Jenise Bauman—West Virginia University**

Transfection is an alternative laboratory method of hypovirus transmission that can be used to create hypovirulent strains. This technique involves the insertion of a synthetic hypovirus dsRNA into individual fungal spheroplasts by electroporation. The spheroplasts are regenerated on specialized media and the successfully transfected colonies can be chosen based on phenotype. A transfected strain has the same phenotypic traits as a strain infected via anastomosis. One benefit of this technique is that it overcomes the barriers imposed by vegetative incompatibility. Results from a preliminary study at West Virginia University in 1998-99 indicated that recovery of hypovirulent isolates was similar between CHV1-infected transgenic and anastomosed isolates. The objective of this research was to compare transfected with anastomosed isolates in three experiments. The first study confirmed the performance of both isolate types previously tested in a 1998-99 field study. The size of cankers and the reisolation of V and HV isolates were evaluated. The second study evaluated stroma production between anastomosed and transfected isolates. The third study involved laboratory tests to evaluate virulent and hypovirulent inoculum production. The study site, located in the Monongahela National Forest, utilized 96 American chestnut trees. Four inoculations/tree were established to assess hypovirulent isolates while two inoculations/tree were used for virulent isolates. After one year, all cankers were measured to determine growth rates of the isolates. To evaluate the recovery of V and HV isolates, three bark samples were collected from the margin of each canker using a

bone marrow biopsy instrument. Bark samples were cultured, and resulting colonies were analyzed for morphology to determine if the isolates retrieved were similar to those used to initiate the cankers. To evaluate stroma production, all cankers were observed and scored on a subjective 0-3 scale for the production of pycnidia. To evaluate the production of hypovirulent inoculum *in vitro*, conidia produced in Petri dishes were single spored on PDA. Germinating spores were observed for hypovirulent morphology to determine the influence of the isolate and the infection method on the production of hypovirulent conidia. Results evaluating size of canker were comparable to the 1998-99 study. These results showed that no differences existed in the size of cankers produced when isolates were infected either by anastomosis or transfection. When reisolated, there was no clear trend observed in either anastomosed or transfected isolates with regard to hypovirus retention. Very few stroma were produced after one year in the field from either transfected or anastomosed hypovirulent isolates. Single spore results showed HV was the prominent isolate recovered among all isolates regardless of virus acquisition.

**Mark Double—West Virginia University**

Double presented an update on the spread of two hypoviruses (COLI and Euro7) at the West Salem, WI site. Because of the ever-increasing task of sampling and assessment, twelve permanent plots were established in 2001 in three areas of the stand: 'Disease Center', 'Disease Front' and 'Beyond the Disease Front'. In 2001 and 2002, in the absence of hypovirus introduction, cankers within the plots were sampled and subjectively rated. Based on the lack of hypovirus spread, hypovirus treatment was reinstated in 2003 in the Disease Front and Beyond the Front plots. Time constraints prohibited treatment of plots in the Disease Center. Two-thirds of all trees in each plot were treated with hypovirus CHV1-Euro 7 by either the traditional bark punch method or initiation of scratch wounds to create reservoirs of hypovirulent inoculum. One-third of the trees in each plot was left untreated to serve as trap trees to assess tree-to-tree spread. In 2004, all plots, including the Disease Center, were treated and sampled.

***General findings at West Salem for 2004 include***:

- A total of 1850 cankers are present in the 12 plots; 805 were sampled in June 2004. Of the 1050 cankers not sampled, 450 were located on dead trees and 600 were beyond a reachable height.
- 213 new cankers were discovered among all twelve plots.
- Disease development (number of infected trees) has risen in all three regions of the stand. From 2000 to 2004, the percent infected trees rose from 92% to 96% in the Disease Center, from 29% to 75% in the Disease Front and from 11% to 56% in Beyond the Front.
- Vegetative compatibility testing continues. WS-1 continues to be the dominant vc type in the stand, but its rate of recovery is decreasing. Between 1994, when vc testing was initiated, and 2000, WS-1 decreased from 100% to 74%. Conversely, WS-2 and WS-3 rose to 11% and 13%, respectively.
- Tree death in the Disease Center, Front and Beyond the Front is 35%, 20% and 17%, respectively.
- Hypovirus treatment seems to play a role in tree longevity. Twenty-three percent of the trees in the Disease Center that initially were treated with hypovirus from 1992-

1997 are dead as of 2004. In contrast, 45% of the trees infected more recently (1998-2003), in the absence of treatment are dead. Since trees within the Disease Center are of similar size, the difference in mortality rate may be related to hypovirus treatment. The state record tree, located in the West Salem stand, is an 80-foot tree with a 54-in diameter. The tree was uninfected until May 2003 when four cankers were detected on the root flares. All four cankers were of the WS-2 vc type. Sixteen additional cankers were found in September 2003, representing WS-1, WS-2 and WS-3 vc types. Four additional cankers were found in 2004. Cankers were treated in 2003 and 2004 with the appropriate hypovirus-containing isolate.

**Andrew Jarosz—Michigan State University**

***Census of American chestnut populations in Michigan.*** The State of Michigan has a number of varied populations of American chestnut. Some areas have chestnut blight, others are blight-free and some sites are recovering, due to hypovirulence. Jarosz looked at the demographics of six American chestnut populations, 2 uninfected, 2 with the blight-epidemic only and 2 recovering (chestnut blight and hypovirulence). Jarosz stated that large trees are not evidence of recovery. He believes that juvenile stems need to get into a fast-growing state to show recovery. He has worked with six stands in Michigan and followed the change in size class over time. A net replacement rate ($\lambda$) is a measure of population growth (>1 indicates a growing population; <1 indicates a declining population).

| Stand | Stage | $\lambda$ |
|---|---|---|
| Leelanau | Uninfected | 1.012 |
| Missaukee | Uninfected | 1.005 |
| Missaukee | Non-recovering | 0.995 |
| Stivers | Non-recovering | 0.999 |
| County Line | Recovering | 1.008 |
| Frankfort | Recovering | 0.990 |

Jarosz felt the above was a poor measure or evaluation. He continued to ask the question, "what can you predict about chestnut stands?" He subsequently divided chestnut populations in a series of size classes.

| Size-Class | Size |
|---|---|
| 1 | Seedlings (seed still attached) |
| 2 | Seedlings <50 cm off ground |
| 3 | Seedlings >50 cm off ground |
| 4 | Seedlings up to 100 cm |
| 5 | Stems 100 cm to 1 m |
| 6 | Stems 1 m to 10 m |
| 7 | Stems above 10 m |

In a disease-free stand, he projects domination of size-class 2. Thus, there would be few large trees. In an epidemic area, there are probably few small stems (1-10 cm); most trees are in size-class 6. Larger trees in an epidemic area do not reach size-class 7 but are killed back to size-class 2 or 3. Uninfected sites have different predicted class structures than diseased sites. Recovering stands are unlike uninfected stands in that recovering

sites have more size-class 2 individuals and more size-class 6 and 7. From his data, Jarosz summarizes that it appears there is more ecological recovery in Michigan in stands with hypovirus.

***Scion wood experiment***. Hypoviruses have been introduced into the West Salem stand for many years. In 2001, Dennis Fulbright questioned why some trees recovered and others died, given the same amount of hypovirus treatment. Are these differences due to genetics of the tree host? To answer that question, scion wood was collected from trees in West Salem for grafting in 2003-04. Scion was grafted onto trees at Michigan State University. The grafted trees will be allowed to grow to a reasonable size prior to inoculation with virus-free and hypovirulent (Euro 7) strains of *C. parasitica*. Trees will then be scored for recovery.

***Disease spread at West Salem, WI***. Jarosz is monitoring spread of disease, hypovirus and stand dynamics at the West Salem chestnut stand. He monitored newly infected trees in 1998 and 1999 by using data on vc and hypovirus recovery. He identified the nearest tree with a matching vc type and dsRNA when appropriate and measured the distance from the source to the new infection. The following table indicates that the virulent form of the fungus moves faster than hypovirus infections.

Rates of spread measured as newly infected trees per standing infected tree

| ***C. parasitica*** | **# Trees in 1997** | **Rate in 1998** | **Rate in 1999** |
|---|---|---|---|
| WS-1 | 109 | .14 | .56 |
| WS-2 | 11 | .46 | 1.0 |
| WS-3 | 3 | .67 | 2.2 |
| Euro | 94 | .06 | 0.33 |
| COLI | 38 | .00 | 0.08 |

The average distance moved by virulent and hypovirulent isolates was 29.9 m and 27.6 m, respectively. The nearest tree is, on average, 8 m from its neighbor. The rate of movement between virulent and hypovirulent isolates is different but the distance moved is the same. Most virulent isolates moved south, particularly SW, while hypovirulent isolates moved NE and SE. He has no explanation for these data, as neither follow the prevailing wind—from the south the summer and from the north in the winter.

**Alice Churchill—Boyce-Thompson Institute**

***Pigments of the chestnut blight fungus***. The chestnut blight fungus produces a family of orange and yellow pigments that exhibit diverse biological activities. These pigments have antimicrobial, antiviral and antioxidant activities in vitro, as well as perturbing normal cell death and signaling pathways. Some of the known anthraquinones are the dimeric compounds, skyrin and oxyskyrin (orange pigment) and rugulosin (yellow pigment). Some of the monomer precursors to the three mentioned pigments are: emodin, chrysophanol and aloe-emodin. The roles that the pigments or their precursors play in the biology of *C. parasitica* as it interacts with its tree host are unknown. By using genetic approaches that prevent or interfere with the expression of genes predicted to contribute to pigment production, we can determine the role of pigments in the biology of this devastating tree pathogen. Specifically, our approach is to compare the development and disease-causing ability of a wild type, pigment-producing strain of the fungus with genetically identical strains that differ only by their inability to make

pigments due to a site-specific mutation in the pigment biosynthetic machinery. In this way, we will determine if the pigments produced by *C. parasitica* contribute to its pathogenicity or development in chestnut trees. The major pigments produced by *C. parasitica* are classified as a family of chemicals called aromatic polyketides. The first step in the biosynthesis of polyketides in fungi requires the activity of a large multifunctional, multidomain enzyme called a polyketide synthase (PKS). Previously, we used a gene amplification process called PCR to clone seven unique PKS fragments from *C. parasitica*. Each of these PKS gene fragments was hybridized to a genomic cosmid library of *C. parasitica*; 60 unique cosmids that contain identical or similar PKS sequences were identified. One cosmid was chosen for further analyses since it encoded a PKS gene whose expression was clearly correlated with orange pigment production by *C. parasitica* cultures. These and other results suggested that this gene could be involved in polyketide pigment biosynthesis. A 37 kb genomic DNA insert was sequenced from this cosmid and 14 putative genes were identified in a presumptive secondary metabolite gene cluster. Most of the genes are highly similar to other fungal genes involved in secondary metabolite biosynthesis and transport. In addition to a PKS gene, the gene cluster contains genes similar to those that encode a protein folding enzyme, two transcription factors, a transcription enhancer, two monooxygenases, an oxidoreductase, a hydrolase, and part of a toxin transporter. These are the types of genes we predict might be present in a pigment biosynthesis pathway. Targeted disruption of the Cp-*PKS1* gene is in progress to determine if altering the function of this gene by mutation affects pigment production and/or the ability of *C. parasitica* to cause disease in American chestnut.

**Hill Craddock—University of Tennessee at Chattanooga**

***Host-pathogen interactions.*** Many pictures of forest American chestnut trees are shown at meetings; these trees are in various states of disease. What we have not controlled for is the variation in host resistance. Response to biological control is different on different trees. In 2003, a population of 56 $BC_2F_2$ Chinese/American hybrids were inoculated at Bendabout Farm with Ep155 and isogenic Ep155/CHV1-EP713 and EP155/CHV1-Euro7. As measured by 95-day-old canker length and by survival at one year, *C. mollissima* was highly blight resistant while *C. dentata*, *C. sativa* and *C. pumila* were highly blight susceptible. Segregation, in terms of blight resistance/susceptibility was noticed in the $BC_2F_2$ population. There was no significant difference in canker lengths between EP155 and EP155/CHV1-Euro 7. As a result, he may conduct future inoculation trials with EP155/CHV1-Euro 7, as he had to convert the EP155 cankers in this field test to keep it from killing valuable germplasm. Craddock would like to measure the expression of hypovirulence on this set of trees. He in contemplating coring cankers and measuring the depth of necrotic tissue.

A clonal orchard, on a 13-acre site, was used for graft-propagation on some of the best trees Bendabout Farm trees. This will add to the clonal orchard for future host-pathogen interaction studies. Trees were whip and tongue grafted on container-grown *C. mollissima* ‘Sweethart; and/or *C. dentata* seedling rootstock. He has noticed some graft incompatibility at this site and he questions what control graft compatibility. He grafts at the ground level as this gives a more vigorous shoot. Some trees in this study were lost

suddenly, probably to due Phytophthora. This may be a large problem in the southeastern US in years to come.

**Pam Kazmierczak—University of California, Davis (not present at meeting)**

***Intracellular processing and secretion of the fungal hydrophobin, cryparin***.

Their lab is looking at the biochemical and cellular-level to determine how the virus affects the fungus. They have found three genes that are down-regulated by the virus. Many of these down-regulated products are enclosed in vesicles. These products are: cryparin, a cell wall hydrophobin, laccase and MF1-1 pheromone. Hydrophobins are ubiquitous, important in aerial mycelium production and very important for spore dispersal. They found that cryparin accumulates outside the cell wall in virulent isolates but in hypvovirulent isolates, cryparin accumulation is inside the cell; it cannot get out. Kazmierczak found cryparin on aerial hyphae and on the outside of fungal pustules. When cryparin was deleted (strain Δ119) pustules were unable to break the surface of the bark of American chestnut; there was no eruption of stromatal pustules. Secretion of cryparin is abundant (20-30% of mRNA is comprised of cryparin) and it is conducted via a Kex2 pathway. (Note: The Kex2 protein of the yeast *Saccharomyces cerevisiae* is a membrane- bound, Ca2(+)-dependent serine protease that cleaves the precursors of the mating pheromone alpha-factor and the M1 killer toxin at pairs of basic residues during their transport through the secretory pathway). The hypothesis is that CHV1 disrupts cryparin production by interfering with a Kex2 protein secretory pathway. To test this hypothesis, they created mutants of cryparin that have incorrect signals for signal peptidase and Kex2 recognition. They created GFP (green fluorescent protein) constructs of wild type strains to follow cellular disruption. A cryparin/GFP fusion vector was created to see where GFP is accumulating in the cell. The GFP is losing expression as *C. parasitica* does not hold GFP well. She has gone to liquid shake cultures, which are better than plates when viewing GFP. In her control, the expression of GFP was throughout the mycelium while the mutant expressed GFP only in the vesicles. They hypothesized that the virus affects the system such that accumulation should be in the hyphae. However, the buildup was only seen in the septa and cell wall; this was an unexpected result. The current hypotheses are:

- As CHV1 uses vesicles for regulation, the proteins are not excreted but build up inside the hyphae.
- Proteins build up and do not fold correctly, as with Trichoderma sp.

*Conclusions*:

- Kex2 processing is not necessary for secretion of cryparin.
- Mutating Kex2 processing site does not affect the wettability of the fungus or its ability to break through the bark.
- Cryparin/GFP fusion mutants appear to contain the fusion protein in discrete vesicles within the hyphae.

**Robert Bernatzky—University of Massachusetts (not present at meeting)**

RFLP markers for *Castanea* species have been developed but the technique is laborious and time consuming. The purpose of this project was to consider the feasibility of converting several of these to PCR- based markers. Eight sets of primers were

developed from six different cDNA sequences (randomly cloned from *C. dentata* leaves): *cd141, cd146, cd147, cd155, cd175*, and *rbcS* (known from sequence data to represent the small subunit of ribulose-1,5-bisphosphate carboxylase). Two types of primers were developed. The first type, which was made for all the cDNA sequences, were developed using a computer program (Primer3) that identifies primers that would produce specific fragments between 150 and 250 base pairs long. A second type of primer combination was also developed for *cd175* and *rbcS* that was derived from the most terminal sequences available from the clones. These eight sets of primers were used in PCR on five different chestnut samples: 'Hopa' and 'Bursa' (*C. sativa*), 'Clapper' ((*C. dentata* x *C. mollissima)* x *C. dentata)*, and 'Nanking' and 'Mahogany' (*C. mollissima*).

All of the primers amplified DNA except for the *cd175* terminal primers. Most of the products were monomorphic among the samples. Two combinations showed reproducible polymorphism, *cd146* and *rbcS*. These need to be further verified and applied to additional samples to determine their usefulness as markers.

**Business Meeting**

John Anderson, Administrative Advisor, indicated that our Federal representative is Robert Noweirski, National Program Leader-Biobased Pest Management, USDA-CSREES-PAS. Noweirski had intentions of attending the meeting, but he did not have sufficient funds. Noweirski sent along information with regard to national research initiative programs relevant to pest management, opportunities in: 1) agricultural research enhancement awards for postdoctoral fellows and new investigators, 2) integrative biology of arthropods and nematodes, 3) biology of weedy and invasive plants, 4) biology of plant-microbe associations, and 5) functional genomics of agriculturally important organisms. Further information can be obtained at the CSREES website: http://reeusda.gov. Robert Noweirski's email address is: rnowierski@csrees.usda.gov.

Current chair is John Carlson and the Chair-elect is Alice Churchill. Nominations for the 2005-06 secretary were discussed. Hill Craddock nominated Bill MacDonald for Secretary; the motion was seconded by Donald Nuss. Election was unanimous. Chairs of the committee are: John Carlson (2004), Alice Churchill (2005), Bill MacDonald (2006).

Anderson indicated that he represents the Directors of the Northeast Agricultural Experiment Stations. The report of this group must be delivered to him within 60 days from the close of the meeting. The minutes should include publications from 2003-04.

Anderson informed the group that he will be stepping down as the Director of the Connecticut Agricultural Experiment Station in November 2004 and this meeting will be his last as administrative advisor. Anderson indicated that he has enjoyed his tenure with the group; he particularly thanked Sandra Anagnostakis for her friendship. He does not know who will fill in as his replacement. Bill MacDonald spoke for the entire group when he voiced his appreciation for John's astute nature. Several years ago, Anderson 'went to bat' for NE-140 and the project may not have survived without Anderson's support. MacDonald went on to say that Anderson has become a colleague and a champion for the chestnut cause. Anderson remarked that the NE-1015 project has firm 'legs' on which to stand and he envisions that the project will continue to move forward. Anderson indicated that, as a group, this project has wonderful minutes.

Churchill will host the 2005 meeting in Ithaca, NY. Paul Sisco stated that the chestnut harvest is mid-September through the first of October. The consensus of the

group was to hold the meeting following chestnut harvest but prior to the October 23, 2005 annual TACF meeting. The meeting was adjourned at 12:00 pm on September 12, 2004. Immediately following the scientific exchange, a field trip was conducted; research sites included The Arboretum at Penn State Chestnut Seed Orchard, the PA-TACF $B_3$ Graves orchard and the Stone Valley Chestnut Demonstration Orchard. Dinner, held at Whipple Dam State Park following the field trip, concluded at 7:00 pm.

Respectfully submitted,

Mark Double
*September 2004*

**NE-1015 Publication List 2002-2004**

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