NE-140 Technical Committee Meeting
Biological Improvement of Chestnut (*Castanea* spp.)

Patuxent Research Refuge, College Park, Maryland
September 10-12, 1998

Attendance:

Alabama:	James Maddox
Connecticut:	Sandra Anagnostakis, Phillip Gordon, John Anderson
Georgia:	Scott Merkle
Kentucky:	Lou Shain, Jeffrey Lewis
Maryland:	Don Nuss, Baoshan Chen, Shin Kasahara, Nobuhiro Suzuki, Clarissa Balbalian, Lynn Geletka
Massachusetts:	Robert Bernatzky, Terry Tattar, Patricia Groome
Michigan:	Dennis Fulbright, Andy Jarosz
Mississippi:	Thomas Kubisiak
New Jersey:	Bradley Hillman, Daniela Linder-Basso, Weiming Yuan
New York:	Michael Milgroom, Yir-Chung Liu, Kiril Sotirovski, Isabel Agudelo
Tennessee:	Hill Craddock
USDA-CSREES:	Robin Huettel
Virginia	Fred Hebard, Paul Sisco
West Virginia	William MacDonald, Mark Double, William Jones, Paul Chaloux

A reception, hosted by the University of Maryland, was held at the Center for Agricultural Biotechnology on September 10, 1998 from 5:30 - 7:30 pm. The meeting was called to order on September 11, 1998 at 9:15 am by Chairman Nuss at the Patuxent Research Refuge. He introduced John Anderson, the Administrative Advisor for NE-140.

John Anderson, Director, Connecticut Agricultural Experiment Station

Anderson congratulated the group on their project approval by the NE Directors. He commentated that efforts put forth by the NE-140 group are hard to match. The publication record of the group is superb. Over 150 papers have been published by members of NE-140; not a single paper has been published by scientists at the NE Forest Experiment Station (NEFES). That is not a criticism of the NEFES, but it shows the dedication of members of NE-140 and their commitment to American chestnut.

The project has been approved for another 5 years, 1 October 1999 to 30 September 2003. As directed by the NE Directors, minutes of the meeting must be taken and a summary of the minutes must be limited to three pages. This summary should include: highlights; decisions and plans; special interest items; and, a brief comment on research by objective. This summary must be distributed within 30 days of the meeting. This will allow the minutes to be forwarded to USDA-CSREES on time.

This year is the final year of the previous project, thus a termination report must be prepared in lieu of an annual report. The termination report is due 15 March 1999; it

will summarize accomplishment of the past five years (research activities and benefits to the USDA, institutions, foresters, lumber companies and citizens).

Anderson suggested that the group consider writing a comprehensive document that covers research on American chestnut. He pledged support for funds to publish this document.

Robin Huettel, USDA-CSREES

She reported that Jack Barnes, previous USDA-CSREES representative, retired last June. She stated that a significant act was passed by Congress this year, the Ag. Reform Act of 1998, part of the Farm Bill. This bill provides $600 million for agricultural research ($120 million/year). She sees good opportunities for funding. The call for proposals will be listed on the CSREES home page. These are nonappropriated dollars; the money may not actually be available. She explained that Congress recently zeroed out the Rural American Fund to cover the cost of flooding in North Dakota. The budget process is very confusing as Congress did not pass a budget prior to their recess. As a result, Huettel sees political infighting over the next few months. By 1 October 1998, if there is not budget approved by Congress, then nonappropriated funds will be allocated and put in place.

Within the agency, there have been some key changes this year. A scientist was hired to head the biocontrol projects. Food safety is a very big issue, especially with regard to food contaminants. It is possible that all agricultural water may have to be tested for microbial contamination. This issue is being pushed by Vice President Al Gore.

<u>Objective 1.</u> To investigate the genetic determinants of hypovirulence (H) and their effects on the establishment and dissemination of the pathogen in the forest ecosystem.

Nobuhiro Suzuki, University of Maryland

He updated the work on the ORF A encoded protein, p29 in CHV1-Ep 713. ORF A encodes for a p29 protein and ORF B encodes for a p40 protein. Mark Craven had made a deletion mutant of p29 and found it was not essential for viral replication, but he did find that p29 causes a 1000-fold increase in sporulation. Suzuki wanted to investigate where the symptom determinant lies. He made p29 deletion mutant constructs that were used for *in vitro* transcription.. He checked the viability of the constructs. The isolates with the smallest deletion mutant of p29 were more like Ep 713; they also produced less conidia. These data show that p29 is not enough to confer all of the suppression accounted for by the virus. His conclusions were:

- suppressive activity in pigmentation and conidiation has been mapped to residues 25-73.
- p29 has at least two functional domains; the currently identified symptom determinant and the previously identified papain-like protease catalytic domain.

Baoshan Chen, University of Maryland

He is studying the virulence of hypovirus-containing field isolates. He showed slides of cankers produced by hv isolates, Ep 713 and Euro 7 along with virulent isolates, Ep 155 and Euro 7 ssV. Euro 7 produces a much larger canker than its hv counterpart, Ep 713 and thus it may be more conducive as a field isolate. He looked at the nature of the dsRNA in Euro 7. Euro 7 dsRNA was cloned and sequenced and compared to published sequences of Ep 713 and NB 58. He found the same general genetic organization among all three dsRNAs. Homology with Ep 713 ranged from 90-96% while homology with NB 58 ranged from 45-81%.

Since the genetic organization of Euro 7 is similar to that of Ep 713, he did domain swapping of ORF A and ORF B. He found that ORF B determines fungal morphology and virulence. He is now in the process of looking at hv determinants.

Don Nuss, University of Maryland

Dissemination studies. *Housatonic State Forest.* He reported on a field experiment with the transgenic strain; this was done in conjunction with Sandra Anagnostakis. The results were published in Phytopathology 88:598-604. While there was some evidence of dissemination with Ep 713-transgenic strains, he is hopeful for better results with a transgenic strain of Euro 7.

Meshamosic State Forest. He discussed field release studies on transgenic hv strains in the central CT forest. Three indigenous *C. parasitica* isolates were transformed with pX103=Not 1 site. By spaying every few weeks in 1999 with concentrations of 10^{12} conidia/ml of the transgenic strains, it is hoped that a population replacement will occur. Risk assessment and biocontrol efficacy will be conducted.

Dennis Fulbright, Michigan State University

He reported on the work of Dipnath Baidyaroy who is examining mitochondrial hypovirulent strains. Isolates from Michigan and Ontario that have mitochondrial dysfunction are shown to have:

- high levels of cyanide resistant respiration-alternate oxidase
- reduced virulence that is transmissible

These traits are maternally inherited and can jeopardize the immortal nature of the isolates; they die upon repeated transfer. A recent study of Kellogg Forest isolates showed a 973 base pair insertion in the mitochondrial small subunit; this insertion was found in 14 of the 15 isolates examined. This subunit appears to affect ribosome assembly; no mitochondrial ribosomes were found.

Bradley Hillman, Rutgers University

He reviewed the genetic organization of GH2. It contains 4 dsRNAs, one genomic and three accessory.

9.8 kb	genomic
3.7	defective
2.0	satellite
1.0	satellite

The satellite dsRNAs are related to each other, but not to the genomic dsRNA.

Sporulation, pigmentation and laccase production are similar for dsRNA-containing and virus-free isolates. GH2 has only one ORF, it lacks ORF A. Thus, GH2 has a different genomic organization from CHV1 and CHV2 hypoviruses. He has worked on making infectious clones of GH2. To date he has found:

CHV3-GH2 dsRNA 1 (9.8 kb)

- RNA 1 represents the viral genome
- 5' nontranslated region IRES-like, has 6 short ORF's
- single long ORF
- predicted papain-like proteinase
- 22nt identify between RNAs 1, 2, 3 and 4

CHV3-GH2 dsRNA 2 (3.7 kb)

- RNA 2 is a simple deletion mutant of RNA 1
- RNA 2 is unnecessary for virus infection
- 5' and 3' untranslated regions identical to RNA 1
- reading frames of single long ORF is intact
- predicted papain-like proteinase is intact
- 22nt identity between RNAs 1, 2, 3 and 4

CHV3-GH2 dsRNA 3 (1952 nt)

- RNA 3 is a linked dimer of satellite RNA 4
- RNA 3 is unnecessary for virus infection
- short 5' untranslated region differs from RNA 4
- first AUG initiates short ORF for potential 10 kd protein
- predicted papain-like proteinase is intact
- 22nt identity between RNAs 1, 2, 3 and 4

CHV3-GH2 dsRNA 4 (938 nt)

- RNA 4 is a monomeric satellite RNA
- RNA 4 is unnecessary for virus infection
- 5' untranslated region is not IRES-like
- first AUG initiates short ORF for potential 10 kd protein
- predicted papain-like proteinase is intact
- 22nt identity between RNAs 1, 2, 3 and 4

Weiming Yuan, Rutgers University

He is constructing infectious clones of NB 58. His strategy for NB 58 has been full-length clones via RT-PCR. He has incorporated a T7 promoter and poly (A). After he began sequencing the full-length clone, he noticed a number of differences between his clone and the published results, based on nucleotide and amino acid sequences. He made a full-length cDNA clone via traditional cloning methods. He then did *in vitro* transcription and transfection; neither were infectious. He then made a mutated RT-PCR clone and it was found to be infectious. He showed slides comparing the morphology of

his mutated RT-PCR clone with that of NB 58; they looked similar. He has now put the full-length clone into a transfection vector.

<u>Objective 2.</u> To study the ecology and physiology of *Castanea dentata* and ecology and dissemination of the pathogen, *Cryphonectria parasitica*.

Terry Tattar, University of Massachusetts

His main area of research is microbial ecology and antagonistic microorganisms. He is now at the point of assessing which microorganisms have the most potential. There is a need to be able to release and recover these microorganisms, but it is very difficult without adequate markers.

Patricia Groome, University of Massachusetts

She has been working with *Trichoderma* spp. that have been recovered from American chestnut cankers. She worked with three isolates that exhibited a high degree of antagonism to *C. parasitica*. These isolates were "trained" to grow on high levels of fungicides in the hope that this could be used as a marker for release and recover studies. While the *Trichoderma* isolates were able to grow on the fungicides, this tolerance was not stabile.

She changed her focus to three bacterial strains that exhibited antagonism *to C. parasitica*. These isolates have tentatively been identified as *Bacillus megaterium*. Each of the strains has been screened for antibiotic resistance with streptomycin and rifampicin. She is currently using this marker for field release and recovery experiments to test for the viability of these bacterial isolates on the bark tissue of American chestnut. The field experiments are being conducted with potted seedlings, a nursery site and in a forest setting. Preliminary results indicate survival of the bacteria for four weeks, *in situ*.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Nathan Hale State Forest study. She is testing the hypothesis of Lou Shain that hypovirulent conidia can persist on American chestnut stems. Part of the forest was clear-cut in 1990-91. She chose two 1/4 acre plots in the forest; one plot serves as a control while hypovirulent isolates are released in the second plot. Ten cankers in the hv treatment area were sampled, v-c typed and converted with Italian hv strains. Water suspensions of hv conidia representing three v-c groups have been sprayed six times since 1993. There are many dead stems in the hv plot, but also many large swollen cankers. Data from both plots will be taken in the fall of 1998.

New Haven tree. A seedling from Rock Hill, CT was planted in New Haven in 1986. Cankers were first detected in 1996; these were treated with a French hypovirus. Cankers are callused; she will continue to monitor this tree.

Lockwood Farm. A plot of American chestnut trees was planted by Richard Jaynes at CAES in 1976. By 1978, the trees were cankered and subsequently treated with a mixture of hv strains from Italy, France and North America. Treatment continued for four years. Currently, survival of original trees and sprouts is 16% and 33%, respectively. Five cankers were sampled in 1997; one appeared virulent and four appeared hypovirulent. Isolates were all orange-pigmented. These isolates were sent to

C. Balbalian for confirmation of dsRNA; no virus was detected. Isolates were then sent to H. Bertrand to assay for abnormal mitochondria.

Lou Shain, University of Kentucky

Polygalacturonase (PG). He summarized the work of S. Gao who isolated a PG produced by *C. parasitica*. Gao found basic PG isoform and thought it might be a virulence factor. He "knocked out" the PG and found it did not affect virulence, as tested in excised stems. Gao found in a cup-plate assay that the virulent isolate, Ep 155 and several transformants all produced similar amounts of PG. Upon reisolation from cankers, Gao found acidic rather than basic isoforms. The question still remains if PG is a virulence factor. Shain spent the past year getting PG in large quantities to do additional disruption experiments. After several attempts, he failed. He hopes to work will continue on this project; he is retiring in June 1999. He believes PG could provide some resistance information for the host.

Putative mechanisms of host defense. He has been examining β-1,3-glucanase and chitinase in ethylene-treated bark. He has found both glucanase and chitinase to be produced in greater amounts in Chinese as compared to American chestnut. He questioned if this finding would hold up with a segregating population. He received an F_2 population from Fred Hebard. He used 12 cankers (6 small and 6 large). His findings were that the F_2 group produced chitinase in higher amounts.

Coryneum dieback. This fungus was shown to cause cankers at the ACF farm. Shain identified this slow-growing fungus as *Coryneum castaaneicola*; it takes 1-month to sporulate in excised stems. Hebard inoculated 10 trees (5 large and 5 small branches) for a total of 50 inoculations. *C. castaneicola* was reisolated from 20 inoculation sites.

Andy Jarosz, Michigan State University

He published a mathematical model on the hyperparasite (dsRNA) spread within a pathogen population (American Naturalist, 1998 issue). He speculated that when the virulence of dsRNA-containing isolates is high, the dsRNA spreads but trees still die. Conversely, when virulence of dsRNA-containing isolates is low, then hv isolates do not spread. He believes there should be some optimum level of virulence for dsRNA to work effectively.

He reported on the work of Anita Davelos who is doing an ecological study on chestnut in Michigan. The main question of her work is, to what extent do dsRNAs promote the ecological recovery of chestnut populations? Her findings indicate that small juvenile trees do not survive in recovering populations. She has looked at various populations of American chestnut: healthy, non-recovering and recovering. At each site she has measured survivorship, size of stems, number of seeds produces and the status of disease. From these data she produced transition matrices and looked at stands over time. She found a distinct pattern; there is some negative growth, even in healthy populations and reproduction is occurring, but only in larger trees. She looked at growth rate of all stands and determined if rates were expanding, contracting or remaining stable. While the data indicate that all of her stands are contracting, there does appear to be enough recruitment that the American chestnut population will survive.

Daniela Linder-Basso, Rutgers University

She is a native of Switzerland, working on a Ph.D. in Hillman's lab. She is working on a partial characterization of hv isolates of *C. parasitica* associated with viruses in Switzerland. She has examined populations of the fungus where virus had been released for biological control. She is attempting to characterize the virus in this population to see if there is any diversity. She has identified potential diagnostic restriction enzyme sites. Her sample population includes:

15 white, dsRNA-containing isolates
1 released isolate

Four v-c groups are represented in these 16 isolates.

Her criteria for diversity will include:

- morphological characteristics of the fungal hosts
- sequence analysis of the released virus and the background viral population
- comparison with the Italian hypovirus, CHV1-Ep 747

Her results, to date, are:

Fungal hosts:

• There are significant differences in radial growth rate and virulence on apple test.

• Small differences are found in phenol oxidase activity.

dsRNA virus:

• ~4000 nucleotide sequences from the released virus.
• Sequence of the background population is in progress.
• There are similarities with CHV1-Ep 747.

Michael Milgroom, Cornell University

His overall research interest is the population biology of *C. parasitica* and hypoviruses.

Mating type genetics in *C. parasitica*. *C. parasitica* preferentially outcrosses, but it can self in nature. There is mating type switching that occurs.

Virus population biology. A collection of *C. parasitica* isolates from Japan was acquired recently. They screened for hypoviruses and found 30 isolates that contain dsRNA. He visited Japan and saw some very straight, tall forest-type trees of *C. crenata*.

Vegetative incompatibility. They are investigating the role of incompatibility in preventing virus acquisition. They are attempting to answer this problem by:

• mapping of *vic* genes
• vegetative compatibility in Asian isolates
• virus transmission studies

Vegetative compatibility diversity. He has examine populations of *C. parasitica* from Italy, USA (Maryland), Japan and China. There appears to be much diversity in Asian populations as indicated by the following table:

Population	**N**	**V-C Types**
Italy	716	20
USA (Finzel, Maryland)	57	25
Japan	79	71
China	30	29

The 71 Japanese isolates were compared to the Italian isolates; only 3 v-c types were found in common.

He showed a table on the effect of each *vic* gene on virus transmission.

***vic* locus**	**Probability of Transmission**
vic 1	0.02/0.98
vic 2	0.07/0.20
vic 3	0.68
vic 4	1.0
vic 5	1.0
vic 6	0.11/0.38
vic 7	1.0/0.32

The proportion of replicate pairing for *vic* 4 and *vic* 5; there is not effect on virus transmission. For numbers in the above table that have a slash (i.e. 0.02/0.98); there is a statistical difference in the proportion of virus transmission. For *vic* 1, the chance of having allele 1 in the donor and allele 2 in the recipient is 2%. Two strains are compatible if they have identical alleles at all *vic* loci. He is looking at 1 locus differences.

He has found similar transmission for all viruses, CHV1, CHV2 and CHV3.

Yir-Chung Liu, Cornell University

She is looking at the question, is there virus-fungus interaction? A population of *C. parasitica* from Bergamo, Italy was sampled. She did virus screening, immunoblotting, column purification and Northern hybridizations on the population. She chose six dsRNA negative and 6 dsRNA positive isolates. For those 12 isolates, she measured virulence, sporulation and conidial germination. These isolates were inoculated into stems in Italy and after six months, the dsRNA infected isolates produced smaller cankers. There was also significant virus-fungus interaction ($P<0.001$). With regard to conidia production, the dsRNA infected isolates exhibited reduced sporulation.

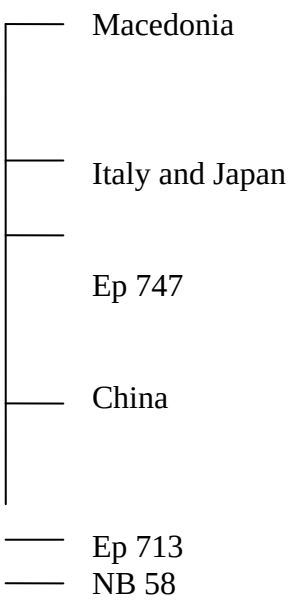
She then examined the dsRNA transmission into conidia by looking at 100 conidia from each population. The results were: >95% germination for both dsRNA infected and dsRNA free conidia; virus was transmitted into >95% of the conidia; there is no significant difference of fungus/virus on conidial germination and dsRNA transmission.

She looked at gene flow of dsRNA among v-c groups from two Italian populations, one from Teano and one from Bergamo. She found some restricted gene flow of viruses among v-c groups.

She looked at sequence of viruses from Teano and Bergamo, using a 1200 bp region. She found all the isolates from Teano are closely related; similar results from the Bergamo population. When she examined both populations, she found they are closely related.

She did a comparative study of CHV1 viruses from Asia and Europe. She compared the virulence of Asian and European hypoviruses and conducted phylogenetic analyses. She included Ep 713 and Ep 747 as standards and NB 58 as an outlier; an isolate from Macedonia was also included. Based on parsimony, the hypoviruses from

Italy and Japan are closely related. A crude example of her phylogenetic tree is as follows:



The only other CHV2 (NB 58) is found in China.

Kiril Sotirovski, Cornell University

He is visiting Cornell from Macedonia where he is studying European chestnut. Chestnut comprises approximately 5,800 ha located in 66 stands across the country. Some areas have been forested and chestnut is always found near villages.

Chestnut blight was first discovered in Macedonia in 1974. At present, he has a collection of 500 isolates from 11 stands. Populations of chestnut vary with regard to disease incidence, from heavily infected stands to blight-free stands.

He has studied v-c groups from his collection and has found only 5 v-c types. The v-c type that is most common is I12; it is the most common isolate in southern Italy and Greece and it comprises 97% of his Macedonia collection.

Both mating types have been found in Macedonia and he has found some isolates that "self".

James Maddox, Tennessee Valley Authority Agricultural Research Center

He is studying the effect of endomycorrhizae on seedling growth, acid tolerance and disease resistance in *C. denata*. A test plot was established in 1995 to look at the infectivity of endomycorrhizae. There appear to be benefits in the first 6-8 weeks; he has no data on long-term effects.

He also has examined the effect of *Pisolithus tinctoris* (Pt), an ectomycorrhizal fungus. When Pt is present without endomycorrhizae, the response is not as pronounced. There seems to be an additive effect with both endos and ectos.

He found *C. dentata* to be an acidophile. A pH below 5.0 is not good, especially with high amounts of aluminum. He found American chestnut to be very tolerant of high levels of aluminum.

A study was conducted to test nitrogen levels. He used 0, 100, 150 and 300 ppm nitrogen. There were no differences in tree size between 100-300 ppm, but there were differences between 0 and 100 ppm.

He has also looked at restoration of American chestnut on poor soils. Trees require more magnesium than normal on poor sites. He has not yet examined phosphorous.

In the four years he has been working on chestnut, he has developed 600 feet of artificial beds. Chestnut trees have been given away on Earth Day to a junior college as well as to individuals.

He has had an outbreak of Ambrosia beetles; about 30% of his trees have been attacked and 15% have been killed.

Mark Double, West Virginia University

Hypovirus deployment study. He reported on a study that compared methods and timing of application of transgenic conidia to virulent cankers. Virulent cankers were initiated in May 1997 on 36 trees with the orange isolate, 6-7-1. Groups of trees were spermatized in August, September and October with sexually compatible transgenic brown strain, Ep 146/pXH9 or the virulent strain, Ep 146. Hypovirulent conidia were administered by bark patches, or painting cankers with an agar slurry or liquid peptone. Virulent conidia were applied only via liquid peptone. In the fall of 1997, bark disks containing perithecia were collected. Ten perithecia were examined from each canker for a maximum of 90 perithecia/treatment/date. Twenty-five ascospores were examined from each perithecium for color. When successful spermatization with Ep 146/pXH9 occurred, ascospore progeny were orange, brown and white. Of the treatments, the hv agar slurry was most effective; 50% of the perithecia outcrossed to Ep 146/pXH9. The bark patch the least effective; 0% outcrossing. Other treatments were intermediate. This study has been modified and repeated in 1998 utilizing spermatization dates of June, July and August.

Pathogenicity study. In August 1998, 30 isolate were inoculated (5 replicates) onto American chestnut stems in West Virginia. This study will compare transfected versus anastomosed virus for six *C. parasitica* isolates and two viruses, Euro 7 and Ep 713. Virulent isolates include: Ep 155, Euro 7 ssV, two Virginia isolates (JR 10 and WR 2) and two Wisconsin isolates (Bockenauer and Schomberg). Canker size and stromata will be evaluated in 1999.

Clarissa Balbalian, University of Maryland

She conducted a field study to evaluate the effects of *vic* 1 and *vic* 2 on the transmission of hypoviruses in the field. She used color markers in her study and recovered 249 orange-pigmented isolates that were recovered from cankers that had been initiated by brown or cream-pigmented isolates. In order to ascertain if these isolates were heterokayons, she made single conidial isolations from 117 of these isolates. Thirty-nine of these were heterokayons; most (32) were the result of fusion between a wild type and one of the brown or cream isolates used to initiate cankers. Two-thirds of the heterokayons were recovered from samples taken from the center of the canker. The majority (85%) of the wild-type orange strains came from the canker margin.

Paul Chaloux, West Virginia University

He is evaluating the effects of dsRNAs that were recovered from hv dissemination plots in which hypoviruses had been released between 1978-82. Some of the recovered isolates are associated with hv-appearing cankers. In most instances, little or no morphological abnormalities were evident among the recovered isolates that contained dsRNA. The dsRNA that was recovered was CHV3-like (GH2) and SR2-like. Isolate families were developed from single conidia progeny (scp): dsRNA-free, CHV3-only, SR2-only and both CHV3 and SR2. Field experiments were initiated with 7 families of isolates. CHV3-scp produced smaller cankers than SR2-type or dsRNA-free scp. The double infected scp produced cankers that were smaller than SR2-type scp and dsRNA-free scp, but larger than CHV3-infected scp.

William Jones, West Virginia University

He is assessing the influence of mycelium age on hypovirus transmission within cankers. He is using color markers; an orange-pigmented isolate will be used to initiate cankers and brown-pigmented dsRNA-containing strains will be used to introduce hypovirus. The dsRNA-containing strains are: CHV1 (80-2) and CHV3 (COLI). Cankers will be established, allowed to grow, and then challenged with a plug of either CHV1 or CHV3. The rate of hypovirus colonization will be examined monthly by removing small bark plugs. To establish canker growth rates and mycelium age, canker margins will be traced with a permanent marker every three weeks. Cankers will also be dissected to culture from different layers (subsurface, mid-bark and cambial level). Histochemical evaluations for suberin and lignin also will be performed.

William MacDonald, West Virginia University

He provided an update on the West Salem, WI stand of American chestnut in which hypoviruses were introduced between 1992-97. The Michigan hypovirus, COLI was intoduced from 1992-94; its performance was not ideal. The Euro 7 virus was deployed from 1995-97. Canker treatment with hypoviruses was suspended after 1997. New findings for 1998 include:

- 151 trees are now infected with 813 cankers; 142 new infections were discovered in 1998.
- Euro 7 is the most commonly identified hypovirus.
- Other organisms are commonly recovered; older cankers yield the greatest number of contaminants. *Trichoderma* spp. is the most common contaminant.
- Spread of Euro 7 hypovirus to previously untreated cankers was greater in 1998 than in any other year.

<u>Objective 3.</u> Continue efforts toward developing blight-resistant chestnuts utilizing both tissue culture and traditional breeding methods.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

She made available mailing lists of chestnut researchers, both domestic and foreign. She is continuing her backcross breeding program. She has made crosses of *C.*

pumila X J3 with a J4 X *C. ozarkensis* in both directions. These seedlings will be outplanted at the Bent Creek Experimental Forest in NC to see if the plants differ in their resistance to gall wasp.

She reported on her lateral root study that is done in conjunction with Scott Schlarbaum (University of Tennessee). She stated that the work of Paul Kormanik on oaks should be applicable to chestnut; are first-order-lateral root characteristics determined by the female parent, as in oak? The lateral roots will be measured on 550 seedlings; these will be outplanted and evaluated for survival.

Phillip Gordon, New York Botanical Garden

He discussed chestnut marketing. The megalopolis that extends from Boston to Washington, DC has approximately 1/3 of the U.S. population; this includes many former Asian and Europeans who appreciate chestnuts. While many of foreign descent are familiar with chestnuts, most Americans are not. He attributed this to: the loss of American chestnut to chestnut blight and lack of imported chestnuts after World War II.

Connecticut is a state with a large pomology industry. He feels this will lend itself to a good chestnut industry where individuals can market chestnut. Several old Japanese chestnuts are being used in a marketing test; these trees are very hardy and produce good quality nuts. Gordon is initiating orchard and marketing research at a 70 acre farm in Madison, CT. He has been given a 20-year lease; trees will be planted on the farm. The trees will be maintained by volunteers who will also help with nut harvest.

He is also developing an educational program. This is a 2-year program where chestnut trees are outplanted by school students.

Scott Merkle, University of Georgia

His new focus is to improve the quality of somatic embryos and increase the frequency of clonal plantlet production. He reported on two projects: (1) embryogenic culture initiation; and (2) pregermination and embryo maturation.

Embryogenic maturation. The objectives of this study were to: (1) generate additional embryogenic cultures for maturation and conversion experiments; and, (2) test different induction treatments to produce well-formed embryos with higher potential to produce plantlets. To test the second objective, 2000 immature open-pollinate seeds from the American Chestnut Foundation were cultured. Induction treatments were continuous exposure to 2,4-D or NAA and 2-week "pulse" treatments on high levels of 2,4-D followed by transfer to NAA or lower levels of 2,4-D. A total of 14 embryogenic cultures were initiated, for an overall rate of 0.69%. Induction frequency did not vary among culture treatments but did vary significantly among source trees.

Pregermination experiment. Desiccation and cold storage were tested as pregermination treatments. Somatic embryos were desiccated for 48 hours at 72%, 87% or 100% relative humidity (RH) using standard salt solutions. Desiccation significantly affected germination due to the fact that 72% RH killed all embryos. Germination frequency was improved by 6 weeks of cold treatment. In a second experiment, potassium carbonate was used control moisture content. Embryos were stored for 0, 4 or 8 weeks at 4 C prior to germination. While neither desiccation nor cold storage affected germination frequency, desiccation to 50% moisture content resulted in thicker roots and cold storage resulted in longer roots.

Embryo maturation experiment. Using the same cultures as above, four sets of treatments were tested for improving embryo quality and germination frequency. The following treatments were compared: 25mM glutamine, 25mM asparagine, 25 mM glutamine, no supplemented amino acids, polyethylene glycol, abscisic acid and sucrose. Fresh weight and dry weight of somatic embryos were significantly affected by culture genotype, PEG treatment and sucrose concentration. Somatic embryo fresh weight and dry weight decreased with increasing PEG concentration and generally increased with sucrose concentration; 60 mg/l sucrose gave the best results. The overall germination frequency was 8.8%.

Hill Craddock, University of Tennessee at Chattanooga

He has had three breeding seasons at the Bendabout Farm. He harvested 20 nuts the first year, 50 nuts the second year and hopes for a larger number in 1998. He is using 'Graves' and 'Clapper' material in his breeding program.

Many of his trees are dying; this may be a Phytophthora problem. He also had an infestation of Ambrosia beetles in his plantings.

He has been surveying chestnut in Tennessee. He is currently conducting forest plantings of chestnut in mixed forest areas. He has planted 300 trees, to date. Included in the planting are pure American chestnut from Lucille Griffin and F_1 and F_2 from Fred Hebard. He is protecting the trees with hardware cloth against deer browse. To propagate, he is taking cuttings from the trees and grafting, so he is not removing chestnut from the ecosystem. He prefers to use Chinese rootstock, so when shoots come up, he can distinguish from graft material from rootstock.

In a cultivar trial, he is using 20 families at 3 sites. He is treating cankers with hypovirulent strains. He has Italian and French virus transmitted into three fungal backgrounds and is treating cankers by excising cankers with a knife and spreading on a slurry of hv strains. He is also collecting mushrooms from his forest plots and is attempting to separate wood rotters from mycorrhizae.

Thomas Kubisiak, USDA Forest Service, Southern Institute of Forest Genetics

Evaluation of resistance in BC_1 populations. Genomic regions conditioning resistance to *C. parasitica* in two different backcross lines are being mapped. The backcross lines are from 'Mahogany' (BC_M) and 'Nanking' (BC_N). A total of 57 BC_M progeny are being examined. A total of 151 markers were used for mapping; two genomic regions have a significant effect on host response. In the BC_N population, Paul Sisco of the ACF scored and logged 84 RAPD markers. The data suggest that some genomic regions from Chinese chestnut could be associated with lower levels of resistance or susceptibility.

Levels and structuring of genetic variation in American chestnut. He has extracted DNA from American chestnut at 12 sites, from Connecticut to Georgia. The objective of this study is to determine if there is any geographical pattern to the observed variation; this will aid in breeding strategies. He has tested 29 simple sequence repeat primers; 15 appear to amplify polymorphic loci. To date, most variability is within populations as opposed to among populations.

Genetic markers closely linked to *vic* loci in *C. parasitica*. The main goal of this research is to identify genetic markers tightly linked to *vic* loci. Once identified,

these markers could be used for studying vc group and *vic* allele frequencies in natural populations. He has been employing bulk segregate analysis in a cross between a Japanese and an Italian isolate of *C. parasitica* known to be segregating for five of at least seven *vic* loci. None of the polymorphisms identified with this method appear to be linked to *vic* loci. His is planning to investigate PCR-based AFLPs as RAPD markers to obtain tighter linkage to *vic* loci.

Fred Hebard, The American Chestnut Foundation

He reported there are 10,000 trees planted at the Meadowview Farms. Two lines of resistance ('Graves' and 'Clapper') are being advanced into 3rd backcross. The 3rd backcross progeny will be screened for blight resistance beginning in the year 2000. It is hoped that 3rd backcross-F_3 nuts from 'Graves' and 'Clapper' will be released by the year 2005. Data indicate that theses backcross-F_3 nuts will possess adequate levels of blight resistance.

Two *C. parasitica* isolates, Ep 155 and SG 2-3, are used to screen trees for blight resistance. Using these two isolates, canker length and width measurements give a good separation between American and Chinese chestnut.

The selection criteria in the breeding program are to select 25% of the F_1 population and then look at those trees for the ones with the best "American" traits. The final selection is about 5% of the population.

Molecular mapping of blight resistance continues. Three crosses are being evaluated, one with 'Mahogany' and two with 'Nanking'. Data from 'Mahogany' suggest that blight resistance is mapped to loci on linkage groups BE and F. A new locus on L was also indicated.

A number of trees in the orchards have died; their death is not related to *C. parasitica*. Samples of cankers were sent to Lou Shain at the University of Kentucky. *Coryneum castaneicola* was isolated.

Paul Sisco, The American Chestnut Foundation

As a new scientist with the ACF, he discussed his breeding philosophy. He is working with markers; it is ideal to work with markers that are only present in the most resistant trees. These markers should be tightly linked to a single locus. He wants to systematically look at resistance. The backcross method is purely by phenotype; he wants to use molecular markers to choose trees that are best suited for other parts of the country.

He discussed problems associated with certain areas of the Meadowview Farm; several areas on the farm have high chestnut mortality. These areas are flat and well-suited for planting chestnut; they were originally planted with Burley tobacco. In these heavy clay soils, there is strong correlation to areas previously planted to tobacco and chestnut seedling death. A number of seedlings were pulled up and sent off for analysis; it was expected that *P. cinnamomi* would be isolated. As of yet, the fungus is still unidentified. The fungus, however, does respond to the fungicide, Ridomil. A lot of work needs to be done as to where chestnut is outplanted, i.e. avoid areas of Phytophthora.

As per the information provided by Robin Huettel on new funding, Sisco suggested a cooperative group project be submitted. This multi-institutional grant could

be a wonderful opportunity for members of NE-140. The grant could focus on tree species and/or pathogens. A germplasm collection was suggested for focus of the grant.

Robert Bernatzky, University of Massachusetts

Mapping efforts. A few years ago, he contributed RFLP markers to the project, as there were not markers for all linkage groups. There are two regions that he had markers for that looked like they contributed to resistance. He is using an F_2 population and has added new markers to linkage group F. He was asked to look at linkage group G by Tom Kubisiak. They looked at all the markers and compared results. They hope to have all markers for F_2 and BC_1 populations. The trees that went into the BC include 10 American chestnut parents and 2 hybrids ('Mahogany'). They have found only 2 markers that are unlinked; data is not complete.

Genetic diversity study. Tom Kubisiak sent 6 populations of American chestnut twigs to look at genetic diversity. Bernatzky focused on regions that showed resistance in Chinese chestnut and found a lot of diversity within a population. He used populations from Massachusetts and Virginia as examples. There is a tremendous amount of overlap at the alleles. One sidelight: can we distinguish species using these markers? Bernatzky would like to have species-specific probes. Some markers might be good for American species type against Chinese.

Business Meeting

Hill Craddock was elected secretary for 1999-2000. Chairman for 1998-99 is Dennis Fulbright. Next year's meeting site will be determined by Fulbright; first choice was Kalamazoo, MI. Potential dates are: 14-16 October and 21-23 October 1999. Don Nuss and Dennis Fulbright agreed to work on the termination report.

Germplasm discussion. Paul Sisco agreed to coordinate a grant submission project. Huettel stated that the ACF could coordinate the project but it could not act as the primary agency as funds must be appropriated to a land-grant institution. She also commented that the grant cannot be simply a repository; there have to be other avenues associated with the germplasm. Fulbright felt the ACF was a good organization to spearhead this effort as NE-140 has only 5-year approvals; hopefully the ACF is long-term. Thus, even if NE-140 would not be approved for another 5-year renewal, the project could continue if ACF-based. MacDonald questioned what would be collected. Sisco responded that storage and identification of material could be done at the ACF facility. He questioned if NE-140 wanted a long-term commitment for only 5-years of funding. It was noted that Scott Schlarbaum at the University of Tennessee would be able to provide some land. Fulbright noted that the organization that takes on this project may well be handed a "shoebox" of unknown trees. A planting can be expanded to whatever level of funding is available. Anagnostakis stated that collections could be at several locations as long as someone agrees to keep adequate records. Sisco agreed to send out a questionnaire and get interested individuals together.

The meeting was adjorned at 12:45 pm on September 12, 1998. A field trip to the chestnut plantation at the USDA-Glen Dale Experiment Station followed the meeting.