NE-140 Technical Committee Meeting
Biological Improvement of Chestnut (*Castanea* spp.)
Rutgers University Continuing Education Center, New Brunswick, NJ
October 27-29, 1995

Attendance:

Connecticut:	Sandra Anagnostakis, Phillip Gordon, John Anderson
Georgia:	Scott Merkle
Kentucky:	Lou Shain
Maryland:	Don Nuss
Massachusetts:	Terry Tattar, Mark Mount
Michigan:	Dennis Fulbright, Andrew Jarosz, David Huber, Anita Davelos
New Jersey:	Bradley Hillman, Jim Polashock, Kevin Scibilia
New York:	Michael Milgroom, Tobin Peever, Stan and Arlene Wirsig
Tennessee:	Hill Craddock
Texas:	Neal Van Alfen
Virginia:	Fred Hebard
West Virginia:	William MacDonald, Mark Double, Clarissa Balbalian, Anne Zondlo

The meeting was called to order at 8:30 am October 28, 1995 by Chairman Hillman. John Anderson, Administrative Advisor, emphasized the minutes from the meeting must be in the mail within 30 days. The NE-140 committee report must be out by March 15, 1996. The report should be limited to 3 pages, not including the publication list.

The data presentations are organized by Regional Project objectives and presented by station.

OBJECTIVE 1. To investigate the genetic determinants of hypovirulence (H) and their effects on the establishment and dissemination of the pathogen in the forest ecosystem.

Neal Van Alfen, Texas A&M University

He made a plea for this group to coordinate information, especially with regard to a consensus on how genes are named.

His research approach has been to look at the molecular level effects of hypovirulence in *C. parasitica*, and he has concentrated on *vir*1 and *vir*2. These two genes may be very different from one another, but they have a similar ORF. He knocked out the *vir*2 phenotype and found a decrease in sexual sporulation; these isolates also are sterile (perithecia are formed but they are void of ascospores).

He has been interested in mating type pheromones. In mating types there is a mRNA that is expressed in one mating type but not the other. There is a sexual pheromone system in yeast; it is excreted as "Factor–a". *Vir*1 and *vir*2 have a similar pheromone peptide sequence, ending in –CAAX. The actual pheromone has been isolated in *Ustilago maydis*.

We do not know a lot about pheromones in filamentous fungi; they may be involved in trichogyne formation. Is there a difference between mating types? Does mating type "A" contain a pheromone that is not found in "a"? It is known that the genes for pheromones are found in both mating types, but pheromones are not expressed in both types.

Virus suppresses the presence of pheromone in mating type "A". What about mating type "a"? The sequence is a decapeptide that is repeated 7 times. The gene responsible for this has been renamed MTS for mating type specific.

The complexity of v-c types in North America have prevented viral spread in the United States. Virus is suppressing recombination in the fungus, so it is decreasing the number of v-c

genes. This is counterintuitive as to how we might think the system should work. Milgroom argued that the virus may be there because of a lack of diversity in the fungus, rather than the virus causing a decrease in diversity.

Tobin Peever, Cornell University

He discussed the evolution of dsRNA viruses in *C. parasitica*.

1. Genetic Structure of virus populations in North America, China and Japan. They are asking, what is the genetic relatedness within and among populations of *C. parasitica*? Also, what is the origin of dsRNAs in North American populations? They have screened 600 isolates of *C. parasitica* from 8 populations in North America using the immunoblot system to determine the population structure.

Percent dsRNA in *Cryphonectria parasitica* from Eight North American Populations

Population	Number	dsRNA (+)
NY	88	18 (20%)
WV	75	19 (24%)
Ontario	33	0
KY	25	2 (8%)
NH	20	0
NJ	259	100 (39%)
MD	66	5 (8%)
MI	30	23 (77%)

2. Asian dsRNAs. In order to determine the origin of dsRNAs in North America and Europe, they screened 240 isolates from China and Japan using the immunoblot system. Generally, lower rates of dsRNA infection were observed, compared to North American *C. parasitica* populations, although a definitive comparison is difficult due to the small sample sizes of many of the Asian populations that were available. When Northern blots with dsRNAs from populations in China and 6 isolates from Japan were probed with a cDNA probe from CHV1-Ep713, the probe hybridized to dsRNA from two populations in China (Beijing and Jiangxi) and 5 of 6 Japanese dsRNAs. The hybridization of CHV1 to the majority of Chinese dsRNAs confirms the results of Chinese researchers who have shown that European and Chinese dsRNAs cross-hybridize. The lack of hybridization with dsRNAs in one Japanese isolate and from one Chinese population (Xiuling), however, was unexpected and indicates that dsRNA in populations in both China and Japan may be structured to some extent.

3. Virus–Fungus Specificity. They are posing several questions to try to understand the role of variation in relation to fungal resistance and virulence of hypoviruses.

- Is there variation in resistance to viruses?
- Is there variation in virulence of viruses?
- What is the specialization in virus-fungus interactions within and between populations?

To begin to answer these questions, they screened isolates of *C. parasitica* for the presence of dsRNA from two populations in Italy using the immunoblot system. In the Bergamo population (northern Italy) 34% of isolates (N=107) contained dsRNA, while 42% of the isolates (N=50) from the Teano population (southern Italy) contained dsRNA. Nine dsRNA positive isolates had colony morphologies that were irregular and orange, distinct from the typical European white hv isolates. All 9 isolates were found to contain a dsRNA that was approximately 2.5 kb. The remaining 12 isolates had dsRNAs of approximately 12 kb, typical of CHV1 hypoviruses. They took random samples of dsRNAs from Bergamo and Teano that hybridized to CHV1 and they are using these isolates to investigate the interactions between hypoviruses and their fungal hosts on the virulence of the pathogen within and among populations. They have randomly sampled 7 viruses and 7 isolates from Teano and Bergamo and have transferred every virus into every isolate

(49 combinations) within both of these populations. They are planning to measure the virulence phenotype of all virus/fungus combinations on excised chestnut stems.

Don Nuss, University of Maryland

1. Hypovirus Infection of Other Fungi. He reported on a transfection system using electroporation to force dsRNA into spheroplasts. Transcripts corresponding to the viral RNA coding strand were synthesized from a full-length cDNA copy of CHV1-Ep 713 dsRNA. Hypovirus infection was established in *C. parasitica* and related fungal species not previously reported to harbor virus: *C. cubensis*, the causal agent of a canker disease of *Eucalyptus; C. havanensis*, also a pathogen of *Eucalyptus*; *C. radicalis*, a nonpathogenic saprophyte; and *Endothia gyrosa*, a canker pathogen of *Quercus* spp. In addition to altering morphology, the hypovirus also greatly effects virulence. He questioned how closely related these species are. He did sequence analysis of a small ribosomal subunit ^{18}S; a 547 base intron from *C. parasitica*. He found that there was not enough difference in the ^{18}S subunit to discern any differences. He did note that between *C. parasitica* and *C. radicalis* there is a 10 bp difference in the ITS region, which is more variation than he wanted. He now has an ATCC basis for each species.

2. GTP Binding Protein. He is investigating how virus causes reduction in virulence. GTP binding proteins are instrumental in causing increases and decreases of messengers. The accumulation of heterotrimeric GTP-binding protein α subunit of G_i class was found to be reduced in hypovirus-containing *C. parasitica* strains. Cpg-1, 353 amino acids, can be detected in Ep 155 but not in Ep 713. A number of signal transduction and α subunits (cpg-1 and cpg-2) were cloned. He looked at transformants and saw no signal differences in antisense constructs, but there were differences in the sense constructs. The results of the virulence assay, done on dormant excised chestnut stems for cpg-1 sense and antisense contructs, are found in the following table.

Strain or Transformant	Mean Canker Area (cm2)
Ep 155	7.13
Ep 713	0.61
1310	0.56
1318	0.44
141	6.02
142	6.17

Transformants 1310 and 1318 contained sense constructs while 141 and 142 contained antisense constructs. These results indicate a crucial role for G protein linked signal transduction in fungal pathogenesis and suggest a molecular basis for virus-mediated attenuation of fungal virulence.
To show the difference in PCR products, he presented the following table.

Inventory of Differential Display PCR Products

	>4 Fold		2-4 Fold	
Category	Decrease	Increase	Decrease	Increase
713-Unique	8	44	38	59
713/1310-Common	23	64	58	129
1310 Unique	10	42	32	31

3. Cellulase Activity. Ping Wang has been looking at cellulase activity. He has cloned some cellulase genes to determine if they disrupt the cpg pathway; is this pathway important in the production of cellulase?

4. p29 Protein. The hypovirus in Ep 713 downregulates sporulation; isolates containing the Ep 713 hypovirus are female fertile. p29 contributes to sporulation and may also be

responsible for female infertility. He deleted p29 and the resulting isolate sporulated better and had more pigment.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

1. Recombinant Hv Strain. In June 1994, Anagnostakis, MacDonald and Nuss received official permission from USDA/APHIS/BBEP to test Nuss' recombinant hv strains of *C. parasitica* in the forest to see if they can: a) survive; b) effect a biological control of chestnut; and, c) spread more efficiently than other hv strains. A large number of *C. dentata* stems were located in Sharon, CT in the Housatonic State Forest, a typical CT woodland, dense with an open understory.

Two plots were established with 12 trees (six pairs of trees) in each. In late June 1994, three pairs in each plot were inoculated with strain 154 (MAT1-1, v-c 39) and three pairs were inoculated with strain 392 (MAT1-2, v-c 39). The treatment combinations are as follows:

Canker Strain	Treatment Strain
154 (MAT1-1, v-c 39)	146 [pXH9] (MAT1-2, v-c 9)
392 (MAT1-2, v-c 39)	155 [pXH9] (MAT1-1, v-c 40)

During the summer of 1995, she continued mapping the American chestnuts within the plot and she sampled cankers on all living stems. There are now 153 trees mapped, in addition to the 24 test trees. Forty-three cankers were sampled; 42 isolates were orange and 1 white. These isolates fell into 33 v-c groups. All 43 isolates were hygromycin sensitive. Thirty-seven isolates were inoculated into Granny Smith apples, along with Ep 155, as a virulence test; all isolates were categorized as virulent.

2. Rainwater Samples. Funnels were attached to seven trees to collect rainwater samples. the water was filtered, first through #2 filter paper and then through 1.2 micron filters. The filters were transferred to tannic acid/water agar medium and the resulting colonies were transferred to hygromycin agar. Some of the isolates have been identified as *C. parasitica*.

3. Trapped Aerial Spores. A Burkhard spore trap was used to collect spores. To date, 564 colonies have been isolated and transferred to hygromycin medium. Of these, 49 are hygromycin resistant; they are orange.

4. Insects. Very few carpenter ants were seen in the summer of 1995. She did collect 36 weevils (*Acoptus saturalis*) that appeared to be grazing on fungal stroma. These weevils were squashed and spread onto water agar. Two-hundred-eighty isolates of *C. parasitica* were collected; 17 were resistant to hygromycin.

Brad Hillman, Rutgers University

Taxonomy for Hypovirus (family Hypoviridae) was approved last year by the ICTV. This is the first and only virus family that has no protein coat. The Hypoviridae encompasses three genera: CHV1-Ep 713; CHV1-NB58; and, CHV1-GH2. The Reovirus-like isolates, C-18 and 9-B-2-1 are not included in this description.

Jim Polashock, Rutgers University

1. dsRNA of NB631. This dsRNA is 2.7 kb and based on amino acid seuqence, it is closely related to yeast dsRNA elements and bacteriophages. It is a mitochondrial dsRNA. He posed the question, "Do whole mitochondria move?" and the answer is that they probably do. He is excited about NB631 because it is so small. There are a lot of UGA stops (the code for tryptophan) so it is difficult to get a full-length clone. He altered all the UGA stops to UGG, via mutagenesis. He has located two proteins, 84 kd and 97 kd. He would like to: epxress the coding system in *E. coli*; begin nucleic acid binding studies; express full-length clone in yeast; and, epxress full-length clone in *C. parasitica*.

2. Virus Resistance in NB58F. A virus-free sector was obtained from the NJ hv isolate, NB58; this sector was referred to as NB58F. When NB58F is mated with Ep 155, he

obtained 3 F forms to every 1 normal ascospore. He looked for chromosomal abnormalities. On a CHEF gell, he obtained a 2.8 megabase chromosome, referrred to as the "B" chromosome. Normal ascospore progeny do not contain the "B" chromosome. He would like to get at NB58F by using differential display. Another avenue is to look at spheroplasts of NB58F infected with CHV1-Ep 713 infectious transcripts.

OBJECTIVE 2: To study the ecology and physiology of *Castanea dentata* and ecology and dissemination of the pathogen, *Cryphonectria parasitica*.

Dennis Fulbright, Michigan State University

He showed slides of chestnut blight from Grand Haven, Michigan cankers that were time-sequenced. He talked about recovering sites; even in these stands there are some recovering and some dying stems.

Andrew Jarosz, Michigan State University

He is comparing sites in Michigan to look at patterns across populations and the dynamics that are involved. He wants to know what is going on with the trees, fungus and dsRNA. His graduate student, Anita Davelos is focusing on trees at 6 sites, but data are currently available for only two sites, County Line and Grand Haven. She rated trees at these sites based on the following criterion:

Rating	Criterion
1	No disease
2	With disease and all cankers appear to be of killing type
3	With disease and cankers appear to be a mixture of killing and recovering types
4	With disease and all cankers appear to be of recovering type

All living stems were counted and dbh recorded.
Distribution of tree types in two recovering chestnut populations in Michigan

	Location	
Tree Rating	County Line	Grand Haven
1	30 (8%)	54 (20%)
2	63 (17%)	78 (29%)
3	190 (52%)	100 (37%)
4	79 (22%)	37 (14%)

Recovery is a general term. Despite the fact these are two recovering sites, there are still a number of stems that are not recovering and they seem to stay that way for a long period of time. A statistical analysis for County Line, rating #4 (recovering trees) occur at random; they are not clumped. The same pattern occurs at the Grand Haven site.

Fred Hebard suggested the use of "reactive" and "non-reactive" to rate cankers as these terms describe the canker and not the fungus. Reactive would be one that has callus while non-reactive contains no callus.

Size of largest stem (DBH) by rating category at County Line and Grand Haven

Grand Haven		County Line	
Rating	Size	Rating	Size
3	9.4 cm	4	10.6 cm
4	8.7	3	8.8
2	1.5	2	2.4
1	0.9	1	1.6

They also will attempt to estimate the intrinsic rate of increase for disease-free, non-recovering and recovering chestnut populations in Michigan. At present, they have preliminary data that suggests that hv strains may have a significant negative effect on chestnut populations. Trees at County Line were measured originally in 1993 and remeasured in 1995. Data indicate that trees of 10 cm or less often experienced negative growth, while larger trees (>20 cm) never decreased in size.

David Huber, Michigan State University

He conducted a genetic analysis of vegetative incompatibility and horizontal cytoplasmic transmission. He indicated that transmission of hypovirulence sometimes occurs, sometimes it does not, and the time of transmission varies. He used small pieces of chestnut bark for his v-c typing and talked about heterokaryon color test. These two tests allowed him to test many isolates.

His genetic summary was as follows:

1. Three new *vic* loci were named: *vic*3; *vic*4; and, *vic*5.
2. Heterokaryons form under nonselective conditions between v-c strains.
3. Hyphal tips of heterokaryons contain both nuclear types in variable ratios.
4. All five *vic* loci prevent heterokaryon formation when mycelium is grown on chestnut.
5. Heteroallelism at *vic*4 and *vic*5 is associated with abnormal mycelium interactions on PDA that may represent inhibited heterokaryotic growth.

Effects of each *vic* Locus upon the Horizontal (Cytoplasmic) Transmission of Viruses in *Cryphonectria parasitica*

vic Locus	Effect upon Transmission	Exceptions (Due to Epistasis)
vic1	nonreciprocal vic1-1 ← vic1-2	None
vic2	reciprocal inhibition vic2-1 → | ← vic2-2	1. Unidirectional transmission where vic1-1is epistatic over allele vic2-1. 2. Unidirectional transmission associated with vic4-2 and 5-2 in recipient. 3. Unidirectional transmission where vic3-2 may be epistatic over vic2-1.
vic3	nonreciprocal vic3-1 → vic3-2	1. Heteroallelism at vic2 prevents unidirectional trans-mission by vic3. 2. vic1 effects are epistatic over vic3: 1-1,3-1 1-2,3-2 3. vic1-1 may be epistatic over 3-1 as long as 2-2 is not present.
vic4	no inhibition vic4-1 ↔ vic4-2	None
vic5	no inhibition vic5-1 ↔ vic5-2	None

He showed slides of dsRNA-free isolates, subcultured over time, that sometimes senesce; this is a feature of cyanide resistant respiration and may cause mitochondrial dysfunction.

Transmissible Senescence Summary:

1. Senescence agent is cytoplasmically transmissible
2. Transmission is restricted by *vic*1 but not by *vic*4.
3. Indicators of possible mitochondrial involvement.

Clarissa Balbalian, West Virginia University

She will use some of David Huber's strains in the field to see if unidirectionality and epistasis occurs in the field as it does in the laboratory. The primary objective of her study will be to evaluate the effects of specific *vic* gene differences on the transmission of hypoviruses in a field setting. Virulent cankers were established on healthy chestnut stems and will be exposed to hypovirulent inoculum. The hv inoculum will consist of a hypovirus contained within a fungal

strain that is either genetically identical to the canker-inducing strain, or that differs by one or two specific *vic* genes. Two different viruses will be used to determine whether or not transmission is affected by the particular virus; the viruses are (80-2 [a Euro 7 virus]) and a virus from County Line, MI. She will used strains that differ at:

0 genes

Single gene differences at, *vic*1 and *vic*2

She established cankers in October 1995 and will place bark patches above established cankers in March 1996. Sampling will begin in June 1996.

William MacDonald, West Virginia University

He reported on the West Salem, Wisconsin stand. This project has involved Jane Cummings-Carlson (Wisconsin DNR), Dennis Fulbright (Michigan State University) and Michael Milgroom (Cornell University). To date, approximately 60 trees are infected and a total of 250 cankers have been sampled. From 1992-94, cankers were treated with a virus from County Line that had been introduced into the resident West Salem isolate. Resamples from cankers indicated that only 30% of the bark plugs yielded hv isolates. Further, virus was recovered from less than 10% of the samples from untreated cankers, indicating very limited virus transmission from canker to canker. In addition, only 2-3% of the conidia contained the County Line virus. As a result of the 1994 findings, a second virus was used to treat cankers in 1995. The virus from Euro 7 was introduced into the resident West Salem strain and 70 cankers were treated in 1995. This virus is transmitted into >95% of conidia.

Mark Double, West Virginia University

He reported on the characterization of two Reovirus-like strains of *C. parasitica*; this was a portion of Scott Enebak's Ph.D. study, undertaken after Enebak received his degree. Two Reovirus-like isolates were used, C-18 and 9-B-2-1. Field inoculations were established in July 1994 using virulent strains that are known to be vegetatively compatible or incompatible with isolates C-18 and 9-B-2-1. These cankers were challenged with either C-18 or 9-B-2-1 in September 1994. During the summer of 1995, bark samples from the cankers were obtained to determine how effective the viruses were in spreading throughout the canker thallus. Results indicate that neither virus was effective in limiting the rates of canker expansion. Although the viruses have spread within the thallus of the canker-inciting strain, they are not consistently reisolated from the canker.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Lázló Radóçz, a visiting scientist from Hungary studied v-c among the 33 isolates he brought from Hungary. Radóçz divided Hungary into 4 regions as he thought each region had a single v-c type. Typing of these isolates yielded 14 v-c groups; the most common were v-c 36 and v-c 19. Only one strain was as virulent as the CT virulent standard, Ep 155. There were a number of isolates that were as debilitated as Ep 713; they look like typical European dsRNA-containing strains. Tests in Brad Hillman's lab confirmed the presence of dsRNA in some of the strains. Radóçz plans to use the hv strains to improve biological control in Hungary.

Phil Gordon, New York Botanical Garden

He started out in the field 8 years ago. His background is in tissue culture, working with soybeans, tomatoes and corn. His maize work gave him a good background for chestnut. While he works for the New York Botanical Garden, he does his scouting in Connecticut. He began a population study with lots of help; when he got up to 2 million chestnuts and a fruiting population of 50,000, he quit counting. Now, he concentrates on large trees (>10 inches).

He questions why there is still so much chestnut germplasm. He went to the Connecticut telephone company and requested streets in CT that had chestnut in the name. He visited many of

these streets and interviewed residents to confirm the presence of chestnut. His conclusion is that CT was uniformly populated by chestnut. He has a similar belief about chestnut in the 17 other states that are in the range of American chestnut.

He has an 80-acre tract of land near his home in Old Lyme, CT; he has visited this site religiously over the past 5 years and made observations. Some of his observations pertain to survival:

1. Shoots are produced by somatic embyogenesis. There are only 3 tissues that will work in tissue culture: apical meritstem; tissue from root crown collar; and, embryogenic tissue.
2. Leaf Types. There are sun leaves for photosynthesis and shade leaves for transpiration. Chestnut transpires like few other trees. Chestnut puts down a deep root system and during drought it will still have water.

Michael Milgroom, Cornell University

Population biology of *C. parasitica* and its hypovirulence.

1. Population Structure.

- Geographic subdivision

  Continental ⟶ Among Trees

- Clonal ⟶ Random Mating

(clonal populations seem to be areas where hv is most successful).

- Selfing in Nature (~25%)

Eleven different populations of *C. parasitica* were sampled in Italy, from the Alps to Sicily; approximately 50 samples were collected from each population. Twenty v-c groups were found among 716 isolates. Eighty-five percent of the isolates were in 4 v-c groups. Three of the most common isolates were found mostly in northern Italy, while two other v-c groups were found mainly in the south. The diversity of v-c groups was greater in the north than south.

Paolo Cortesi (post-doc from Milan) made numerous crosses of isolates to determine inheritance. To date, four polymorphic loci have been found, each with two alleles. The genetic information will be used to study population structure of *C. parastica* and hv transmission in Italy.

2. Reproductive Biology. Although selfing occurs at a rate of approximately 25% in nature (~10% in Switzerland), it is observed infrequently in the lab. Most selfing in the laboratory appears to be from contamination. They have only one isolate that was a confirmed "self".

With regard to the segregation of mating types in selfing, they have examined ascospores from perithecia formed in the field and laboratory. In addition, PCR primers from the highly conserved HMG domain of the *Neurospora crassa* mating type "a" gene amplify a band of similar size (~300 bp) from only one *C. parasitica* mating type (Mat1-2) and not the other (Mat 1-1). This gene amplifies mating type signal in other fungi.

Terry Tattar, University of Massachusetts

Microbial antagonists may play a role in the survival of American chestnut in Massachusetts. A *Trichoderma* sp. was isolated from a 35 cm tree in Sunderland, MA; this particular isolate is antagonistic to *C. parasitica*, as shown on PDA plates co-inoculated with the two fungi. The effectiveness of *Trichoderma* was tested in the laboratory, using bark and xylem tissue from freshly peeled bark of American chestnut. Hyphal growth of *C. parasitica* was stopped within 2 days after application of the *Trichoderma* spores and when pretreated with *Trichoderma*, no *C. parasitica* growth was observed.

The *Trichoderma* isolate was used in the field on 5-10 cm trees in the Cadwell Experimental Forest in Pelham, MA. A 10^9 *Trichoderma* spore suspension was applied to actively growing blight cankers and the cankers were then wrapped with Saran wrap. This was done in the fall of 1993 and samples were taken in November 1993, May and August, 1994. *Trichoderma* could still be recovered in 1995. He believes there is potential for use with seedlings; hence a seedling study was established. *Trichoderma* was used as a prophylactic treatment and a certain degree of success

was obtained. This year, the experiment was moved into the greenhouse, however, mites and excessive heat were detrimental to the trees. Seven treatments were made, as follows:

1. *Trichoderma* followed in 2 days by *C. parasitica*
2. *C. parasitica* followed in 2 days by *Trichoderma*
3. *C. parasitica* followed in 2 days by water
4. Water followed in 2 days by *C. parasitica*
5. *Trichoderma* followed in 2 days by water
6. Water followed in 2 days by *Trichoderma*
7. Water followed in 2 days by water

Results indicate there was a suppression of cankers in treatment 1, compared to treatments 2, 3, and 4. Disappointingly, there was not suppression of canker growth with treatment 2. The explanation may be the excessive greenhouse temperatures (>40 C); Trichoderma's optimal temperature is 27 C. This experiment is being repeated outdoors.

They wanted to see if nursery seedlings can be protected with *Trichoderma*, so a number of seedlings were inoculated at a forest in Belchertown. The data is in found in the following table.

Reisolation of *Trichoderma* sp. from American Chestnut Seedlings Planted at Quabbin Reservoir Forest in Belchertown, MA

	Sample Location			
	High*	Low*	Total	Percent
Control	23/46	11/46	25/46	54%
Pretreated	38/41	31/41	39/41	95%

* High=80 cm above ground and Low=40 cm about ground

Kevin Scibilia, Tree-Tech, Bridgewater, NJ

He believes that spraying conidia for control should not be abandoned, as conidia persist. Test strains should be tested prior to use. He tested isogenic lines, initiating virulent cankers and then sealing the wound with latex. Resulting cankers were then sprayed with a conidia suspension. His data is found in the following table.

Conversion Efficiency of Conidia from Aggressive and Debilitated Hv Stains Painted on Chestnut Stems Inoculated with Corresponding Virulent Strains

Hv Strain	Relative Aggressiveness	% Converted
758	+++	87%
63	++	96%
779	++	100%
901	+	17%
1105	+	70%
717	-	100%

He isn't sure how many conidia are necessary for control, but his data indicate it may be as few as 50/ml. The advantage of spraying conidia is the entire tree can be treated, not simply individual cankers.

OBJECTIVE 3. Continue efforts toward developing blight-resistant chestnuts utilizing both tissue culture and traditional breeding methods.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

1. Gall Wasp Progress. Jerry Payne reported that her test planting in Georgia still has no infestation. Her planting consists of trees that are susceptible or resistant to gall wasp. Payne indicates that trees are usually 5 years-old before they become attractive to the wasps. A site on TVA land in Tennessee will be prepared by Scott Schlarbaum and she will be planting seedling trees from Schlarbaum's crosses there next spring. She will be using chinquapin as gall wasp resistant material. She also has a total of 93 trees planted in the Pisgah National Forest at Bent Creek, NC. She stated that gall wasp was introduced into the U.S. to a Georgia nurseryman who brought scion wood from China into the U.S., bypassing the plant quarantine process. Gall wasp has spread from its introduction site in Georgia; at this time there is no chemical control.

2. Nut Harvest. With regard to nut harvest, she reported that in 1993, her crosses resulted in 9.5% of potential (based on counting filled and unfilled nuts); she assumed drought was the cause. In 1994, her success rate was 36%. In 1995, there was drought as severe as the one in 1993, but the success rate was 30%. Clearly, the lack of water during the growing season in 1993 was not solely responsible for the poor yield.

James Hill Craddock, Tennessee State University

1. Chestnut Variety Trials. He is conducting chestnut variety trials in conjunction with Scott Schlarbaum at the University of Tennessee. This is a cooperative study between the TVA and the University of Tennessee. This planting has the oldest *C. mollissima* in TN. Nuts are evaluated for the following attributes: size; peelability; flavor; and, multiple embryos.

2. Asiatic and Hybrid Chestnut Test. Evaluations continue to be made of chestnut plantations that were established over the last six decades in North American by J.D. Diller and R.B. Clapper. The plots range from Connecticut to Alabama and Missouri.

3. Ambrosia Beetle. This particular insect (shot hole borer, *Xyleborus dispar*) has been a very big problem for west coast chestnut growers. Males are flightless and spend their entire lives within the galleries or near tunnel entrances where mating occurs. Female emergence is timed with the beginning months of spring. Once the female has made contact with a suitable host, she crawls around on the trunk searching for a site to begin boring an entrance hole. This entrance hole is about the diameter of a pencil lead. Hill showed slides of small trees infested with Ambrosia beetles.

Scott Merkle, University of Georgia

The goal of his lab is to develop embryogenic cultures of American chestnut for mass propagation and gene transfer applications. His approach to resistance is from the side of the host plant. He believes that a combination of hv and resistant hosts will be the best way to develop a timber-type tree. He is trying to develop a gene transfer system via somatic embryogenesis (*in vitro* regeneration system). His laboratory has been working with various tree species, such as Yellow poplar, magnolia and oak. He has explants from immature embryos from Wisconsin, New York, Connecticut, Pennsylvania, North Carolina and Georgia. (Most of the work reported is from Daniel Carraway's dissertation work; Daniel is now working for International Paper Company).

His laboratory is working on two projects simultaneously and hoping to bring them both together:

- a regeneration protocol
- a gene transfer protocol

1. Regeneration protocol. Growth regulators are used to produce and proliferate early globular stages and then are removed to allow the developmental stages to occur. He is trying to find the optimum zygotic stage to use as the embryo and he has found that the early stages work best. Embryos go though various stages, such as early globular stage to heart stage to torpedo

stage. There are up to 20 ovules in a chestnut fruit; usually one becomes dominant. They tested plant media, plant growth regulators, IAA, NAA, cytokinins and didiazuron. He got 6065 explants from 30 parent trees. The only auxin that worked was 3 ppm 2,4-D. On 2,4-D medium, an ovule will clone itself and form a mass. Shake culture is used to separate proembryogenic masses. Then, they size fractionate the masses onto filter paper to separate the embryos. Their best "seedling" came from the following treatment: a 10 week cold treatment followed by a 6-hour desiccation. They are still looking at various potting mixes. Daniel Carraway's newest cultures are now 2 years old, so Merkle set up new cultures from new nuts.

2. Embryogenic Culture Proliferation. Cultures grown on medium gelled with 0.8% Phytagar proliferated slowly, so suspension cultures were initiated by inoculating proembryongenic masses into liquid medium containing 2,4-D. These cultures were maintained by inoculating 0.5 g of material into fresh medium monthly, but often the proembyogenic masses turned black soon after transfer. Since it was likely that the cells had become accustomed to lower levels of 2,4-D as it was degraded by the end of each culture cycle, the frequency of transfer was changed to 2 weeks. Under this regime, fresh weight of the cultures doubles every 7 days.

3. Somatic Embryo Production. The standard protocol for embryo production is to transfer clusters of proembryos from medium with 2,4-D to basal medium, thus releasing the embryos to develop to later stages. This approach led to fused clusters which were difficult to separate without damaging the embryos. To overcome this problem, they size-fractionated the clusters on a stainless steel sieve and the fraction was passed through a 380 micron screen. The embryos were grown on a basal medium with activated charcoal. Fractionated suspension-cultured proembryogenic masses gave the most total somatic embryos as well as the most single embryos for the 3 cultures tested. Activated charcoal increased the number of embryos produced per gram and increased growth rates by as much as 3-fold. In addition different carbon sources were tested at 3, 6 and 9%. Of the sources tested (sucrose, fructose and maltose), fructose performed the best with single embryos.

4. Gene transfer protocol. No new gene transfer work was conducted in 1995, although 16 cell lines under kanamycin selection are being maintained. The transgenic lines, obtained by biolistic transformation of cells from one embryongenic suspension culture with pBI121, continues to express the inserted genes (kanamycin and the GUS reporter gene).

Lou Shain, University of Kentucky

1. Chitinase and Glucanase Activities. He reported previously that chitinase and β-1,3 glucanase activities appeared to be high in ethylene-treated Chinese chestnut bark as compared to similar amounts of ethylene-treated American chestnut bark. He is now reporting the hydrolase activities of both.

Chitinase Activity (pkat/mg bark)

Material	American Chestnut	Chinese Chestnut
Fresh Frozen	9.8	8.1
Ethylene	183.8	479.3

β-1,3 Glucanase Activity (fkat/mg bark)

Material	American Chestnut	Chinese Chestnut
Fresh Frozen	22.1	22.4
Ethylene	37.7	229.5

Shain questioned why American chestnut has much less protein than Chinese. He speculates that it may involve fertilization as his samples were from Chinese chestnut trees that were field grown while the American chestnut trees were forest trees. Fred Hebard added that light may also be a factor.

2. Polygalacturonase Inhibitory Protein. PGIP might be a possible source of host resistance. To test this, a Chinese x American chestnut F_1 and 12 F_2s, provided by F. Hebard were assayed for PGIP activity against purified *C. parasitica* PG. The F_1 was intermediate in PGIP activity between American and Chinese chestnut (cv Nanking) as shown in the following table.

Polygalacturonase Inhibitory Protein (PGIP) in Bark of American and Chinese Chestnut.

Sample	Species	% PGIP Inhibition
Nanking	Chinese	100
12/1/89	Chinese	100
3/9/90	Chinese	92.6
6/22/90	American	100
Nanking x American	American	75.3
CCR	American	42.4
12/1/89	American	13.0
3/9/90	American	31.5
6/22/90	American	19.8

This very limited sample suggests that PGIP may be inherited quantitatively and therefore may be controlled by multiple genes. All genotypes designated as resistant to blight by an inoculation test were high in PGIP activity as seen in the following table.

Polygalacturonase Inhibitory Protein (PGIP) in Bark of some F_2 American x Chinese Chestnut.

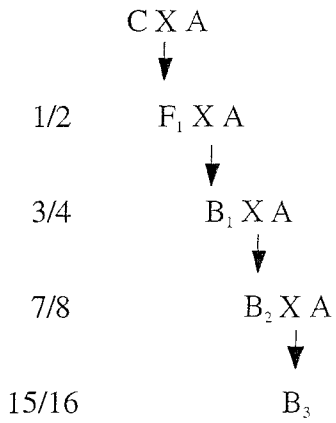
Sample	Disease Rating	% PGIP Inhibition
12	Resistant	99.6
70	Resistant	98.4
907	Resistant	73.1
395	Susceptible	38.8
600	Susceptible	50.0
21	Susceptible	14.4
389	Susceptible	35.4
901	Susceptible	31.9
387	Susceptible	97.7
292	Susceptible	97.4
459	Susceptible	95.9
879	Susceptible	89.7

The resistant F_2s from Fred Hebard had high amounts of inhibitory protein. This may suggest that PGIP may be necessary but not required for host resistance. Hebard has provided second backcrosses and the data indicates a lesser amount of the protein. Therefore, PGIP may not be as much a factor as once thought.

Fred Hebard, The American Chestnut Foundation, Meadowview Research Farm

He reported on his techniques used at the Meadowview Research Farm. He plows and fertilizes and then lays strips of plastic into which a hole is punched for the trees. He places an aluminum cylinder around the trees, piles soil around the cylinders to prevent them from blowing away and places Styrofoam cups over the cylinders to keep out rain. He has found that the cups positively effect plant emergence; he can get 85% emergence using the cups.

His breeding rationale to progressively dilute out Chinese characteristics is as follows:



The B_3s have not been tested yet, but current evidence suggests that 2, possibly 3 major genes control resistance.

He currently has 35 to 40 American chestnut lines composed of 30 to 100 progeny each that are second backcrosses from two sources of resistance, "Graves" and "Clapper". In addition, he has 10 lines of first backcrosses from "Nanking" Chinese chestnut; these are either in the ground or were harvested as nuts this year.

This year, he began producing third backcrosses (15/16th American, on average) from second backcrosses that had been screened for blight resistance in 1994. Around 800 B_3 nuts in seven American chestnut lines were harvested.

The genetic map from one Chinese-American F_2 population was discussed. This work is in conjunction with T.L. Kubisiak (USDA-Forest Service, Saucier, MS) and R. Bernatsky (University of Massachusetts). The map incorporates RFLP, RAPD, isozyme, morphological and resistance loci. Approximetely 180 markers have been identified. The current estimate of the genome size is between 780 cM and 900 cM. Based on these results, the map spans at least 73% of the Chinese X American hybrid genome. There are two regions that might be closely linked to genes conditioning resistance in the chestnut hybrids. The first was on linkage group B and the second was located on linkage group G. These two marker-intervals were responsible for explaining as much as 56% of the phenotypic variation observed. A third region is possible on yet another linkage group.

Business Meeting

Neal Van Alfen was elected secretary for 1996-97. Chairman-elect for 1995-96 is Terry Tattar; the 1996 meeting will be hosted by the Massachusetts group. The tentative dates for next year's meeting are October 23-25, 1996.

John Anderson, Administrative Advisor. He believes that the NE-140 research is focused, it is noble and it is an important scientific problem. He believes that we aim high and the research borders on the exceptional. He senses the patience of some working in classical plant pathology. He believes that side issues (i.e. entomology) are kept in proper prespective. He thanked Brad Hillman for the local arrangements.

Terry Tattar is to have the minutes in the mail within the next 30 days. The minutes will include a listing of all participants along with their addresses, phone/fax/e-mail information. Brad Hillman will put out a report (limited to 3 pages) by March 15, 1996.

We have completed the third year of the 5-year project; it is not too early to be thinking about the renewal that expires on September 30, 1997. We have three options: revise the project; develop an entirely new project; or, close NE-140 out. Next year we will have an additional paragraph to include in the report; it will have to include a statement of accomplishments. This statement of accomplishments accompanies the report in the 4th year of the project. This paragraph must be in layman's terms as it will be used to communicate with Congress.

There was discussion that next year's meeting should allow for a discussion session of "controversial" issues. Milgroom suggested that next year's meeting should include discussion of the project review.

Neal Van Alfen suggested that NE-140 adopt the gene designation system described by Yoder, et. al. in Phytopathology (vol. 76, page 383). This system follows the Neuospora practice in that Mat 1 is used, rather than Mat 1-1. Van Alfen made the following motion:

MOTION: NE-140 should adopt the Phytopathology system (Yoder, et. al.) on gene designation with one exception–we should use "A,a" for mating type.

Michael Milgroom suggested using mat 1 and mat 2, but Sandy Anagnostakis said that switching to mat 1 and mat 2 infers 2 loci. Milgroom then suggested mat–1 (using a dash).

The recommendation was seconded by Sandy Anagnostakis. The vote was unaminous to accept the recommendation.

The meeting was adjourned at 11:40 am on Sunday, October 29, 1995.