NE-140 Technical Committee Meeting
Biological Improvement of Chestnut (*Castanea* spp.)
Swift River Inn, Cummington, MA
October 23–25, 1996

Attendance:

Alabama:	James Maddox
Connecticut:	Sandra Anagnostakis, Phillip Gordon, John Anderson
Georgia:	Scott Merkle
Kentucky:	Lou Shain
Maryland:	Don Nuss, Lynn Geletka, Geoffrey Parker
Massachusetts:	Terry Tattar, Mark Mount, Robert Bernatzky, Phyllis Berman, Patricia Groome
Michigan:	Dennis Fulbright, Anita Davelos
Mississippi:	Robert Doudrick, Thomas Kubisiak
New Jersey:	Bradley Hillman
New York:	Michael Milgroom, Zizhuo Xing, William Powell, Yir-Chung Liu
Texas:	Neal Van Alfen, Pam Kazmierczak
Virginia:	Fred Hebard, Yan Shi
West Virginia:	William MacDonald, Mark Double, Clarissa Balbalian, Paul Chaloux, Stephanie Rollins
Wisconsin:	Albert Ellingboe

The meeting was called to order at 7:15 pm October 23, 1996 by Chairman Tattar. John Anderson, Administrative Advisor, suggested that the committee request a 1-year extension to complete the objectives. He will make the request of the Northeast Directors at their November meeting in San Diego. The extension is not part of the 5-year term. The project terminates September 30, 1997; he suggested that NE-140 meet prior to September 30 in 1997. This is the 4th year of the project and a paragraph is required stating specific outcomes, in layman's language, suitable for the press (see addendum, page 17).

Anderson suggested that NE-140 members should consider nominating the NE-140 project for the 1997 Secretary of Agriculture Honor Award. The Honor Awards are the highest recognition the USDA offers to publicly acknowledge contributions to agriculture, the consumers of agricultural products, and to the Department's service to America. The award must be received by CSREES by November 29, 1996. The award is limited to 25 individuals and a group photograph should accompany the application. The detailed basis for nomination, using nontechnical language, should include:

- Why does the contribution deserve special recognition?
- Is this work unique, exceptionally creative or used as a model for other research?
- What is the scope of the project?
- What specific performances exceeded expectations?
- What trials were met? What obstacles were overcome?
- Did the contribution result in increased program effectiveness?

The award is a recognition of accomplishments and the respective station directors need to participate.

Meeting minutes are limited to three pages as a project report. The NE-140 committee report must be out by March 15, 1997. 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.

Dennis Fulbright, Michigan State University

Mitochondrial hypovirulence. Their overall objective is to determine the cause of hypovirulence in dsRNA-free mitochondrial hv strains found in surviving trees in Michigan and Ontario (Kellogg Forest and Arn, respectively) They found that cyanide-resistant respiration (induction of alternative oxidase) is highly correlated with this type of hypovirulence. He showed slides of cultures with very limited pigmentation and aerial hyphae. These characteristics are maternally inherited. They found less aggressive strains are decreased in their pathogenicity. A phenomenon associated with these strains is that subcultures from the colony margin are very different; these are referred to as senescence cultures. Subcultures from the middle of the colony do not exhibit phenotypic changes, only those from the canker margin. These strains, found in Kellogg Forest, MI, appear to have an insertion in the first exon of the mitochondrial a small ribosomal subunit RNA that looks like duplication. There is also a rearrangement found 10 kbp upstream from this site. There is a lot more recovery at the Kellogg Forest than there was just a few years ago.

They have been able to induce mitochondrial hypovirulence by selecting mtDNA mutants with high levels of respiration through the alternative oxidase pathway.

A naturally occurring mitochondrial plasmid, pCRY1, is related to *Neurospora* spp. plasmids, Fiji and LaBelle (4.2 kbp). The plasmid seems to set up residence in recipient strains.

Mark Double, West Virginia University

He reported on the evaluation of a genetically engineered strain of *C. parasitica* that is currently being field tested. Don Nuss genetically engineered dsRNA from Ep 713 into Ep 155 (orange) and Ep 146 (brown). A hygromycin cassette was included to confer antibiotic resistance. The objectives of this study were to: (1) monitor the ability of the engineered strain to survive and spread from infected bark patches, used as inoculum sources, to pre-established virulent infections; (2) to determine whether ascospores and conidia carry virus particles from the engineered strain; and, (3) to compare dissemination efficiency of the engineered strain with a comparable strain that carries the same but cytoplasmically-borne virus.

Four plots were established in the Monongahela National Forest in July 1994. Within each plot, 16 healthy American chestnut trees were selected for study. Three virulent cankers were established on 8 of the 16 trees; 8 trees were left uninoculated. In October 1994, bark patches inoculated with a transfected or transformed *C. parasitica* strain were placed 10 cm above the virulent cankers. All canker/bark combinations were vegetatively incompatible but sexually compatible. Bark patches were replaced periodicaslly. Pigmentation was used as a marker for both the recipient and donor (bark patch) strains. The treatment combinations were as follows:

Plot	Virulent Canker	Donor Strain
1	Ep 146 (brown)	Ep 155 (pXH9)
2	Ep 146 (brown)	Ep 155 (cytoplasmic)
3	6-7-1 (orange)	Ep 146 (pXH9)
4	6-7-1 (orange)	Ep 146 (cytoplasmic)

Cankers were sampled by removing small bark plugs in December 1994, May 1995, November 1995 and May 1996. Cultures obtained from the plugs were evaluated for pigmentation, colony morphology, presence and absence of hypovirus and resistance to hygromycin. Findings for the mass isolates include: (1) with few exceptions, isolates recovered from the artificially established or naturally occurring cankers were representative of the strains used to establish cankers; and, (2) white, hygromycin-resistant colonies were isolated but they were not recovered over time.

Ascospores were examined from perithecia that were collected in December 1995. Ascospore progeny (n=5,000 and 12,000, respectively) were analyzed from groups of perithecia and from single perithecia to evaluate recombination with the bark patch source of inoculum. Findings from the ascospore progeny were:

• White ascospores (hygromycin resistant) were recovered from perithecia in the pXH9 plots. Recovery rates were 2% in plot 1 and 12% in plot 3, indicating sexual recombination had occurred with the bark patch inoculum source.

• In general, ascospore progeny could be categorized as follows: 12% selfing; 23% outcrossing to the bark patch; and, 65% outcrossing to wild type.

An additional collection of perithecia was made in October 1996; ascospores will be evaluated from this collection during the winter of 1996-97.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

She performed experiments with the same recombinant strains produced by Don Nuss, but with a different experimental design. Her deployment of the recombinant strains was: (1) mycelial plug deployment; and, (2) conidium-painted deployment.

Two plots (50 x 50 meters) each contained 12 healthy American chestnut stems, at least 2.5 cm dbh.. The 12 test trees in each plot were grouped as six pairs. In June 1994, three pairs in each were inoculated with virulent strain 392, one inoculation per tree. One canker of each test tree was treated with the recombinant strains while the other canker served as a control. The treatment combinations were as follows:

Plot	Virulent Canker Strain	Recombinant Strain
1	Ep 154 (v-c 39, hyg^s, orange)	Ep 146 (pXH9, hyg^r, white)
2	Ep 392 (v-c 39, hyg^s, orange)	Ep 155 (pXH9, hyg^r, white)

She evaluated the success of the recombinant strains by assessing: rainwater; insects; airborne-spore trapping; and, isolation of ascospores. From the rainwater samples, 198 *C. parasitica* isolates were recovered, but none contained the recombinant DNA. From weevils (*Acoptus suturalis*), 280 *C. parasitica* isolates were collected and 13 were resistant to hygromycin. The airborne-spore trapping yielded 5 or 35 *C. parasitica* isolates that were hyg^r. From single ascospores, 837 or 2,487 were hyg^r (34%).

Natural cankers were sampled and one isolate of 54 was hyg^r. This canker was on an uninoculated tree, one meter from a recombinant inoculum source.

Since it can be demonstrated that the recombinant strains will interact in nature, she wants to do a mass introduction of the recombinant strains to see if biological control can be accomplished through population replacement. She plans to treat a new clearcut area with a 5-gallon backpack mist blower and spray recombinant conidia two times per year. Fred Hebard suggested she look at Kevin Scibilia's data to determine the best spore concentration before she goes into a large-scale project. Scibilia's data indicated spraying should be done every two months. Lou Shain questioned what her control was and Anagnostakis replied, cytoplasmic hv. Bill MacDonald questioned the fitness of the Ep 713 hypovirus. Since it is very debilitated, if the attempt at population replacement fails, the failure may be with the hypovirus selection and not with the actual deployment strategy. Don Nuss replied that the experiment is not relying on Ep 713 conidia to survive since the spray program will occur every 2 months. Terry Tattar commented that if canker control is the goal, then maybe Euro 7 is the best choice, but if population replacement is the goal, then Ep 713 may be the better hypovirus.

Neal Van Alfen, Texas A&M University

There are characteristics of host membrane vesicles that are associated with virus replication.

• Vesicles are present in both infected and noninfected hosts.

• Contents of vesicles is similar in composition.

• Protein gels indicate similar protein profiles.
• Vesicle content does not vary with cell disruption.
• Vesicles are six-fold more abundant in infected cells.

What is the nature of vesicles in viral replication? If proteins have a system for transport, then they use the golgi system for transport to the cell surface. Vesicles could also be targeted for vacuoles. There is evidence from *Saccharomyces* that a protein, cryparin, is produced in abundance and it is transported to the cell surface. There is vesicle buildup in large numbers. On western blots there are two bands that react to cryparin antibodies, evidence it is cryparin in the vesicles.

Kex2p endoprotease cleaves the cryparin leader protein. The same endoprotease in yeast is associated with a specific type of transport system. Three randomly cloned viral down-regulated host proteins are all Kex2p processed: cryparin; laccase; and, MTSa1. The ORF of the MTSa1 gene is almost identical to that of yeast. He suggested the following:

• Virus is replicated in developmentally regulated host transport vesicles.
• Virus replication disrupts normal transport causing accumulation of vesicles.
• Cargo protein transcription adjusts to feedback signals resulting in down-regulation of cargo.
• Reduction in cargo transport results in symptoms of hypovirulence and poor sporulation.

If conidia are treated with a pheromone (peptide), it stops spore germination that is mating type specific. In *Neurospora*, conidia that land as spermatia are arrested by the ascogonium that grows toward the conidium. For a pheromone to arrest conidia germination, it must be in close proximity to the trichogyne. The main questions involving pheromones are:

• Does a pheromone influence how a conidium acts as it lands on the stroma?
• Will a conidium act as spermatia or simply as a propagule of disease?

These questions got them into the potential involvement of a protease.

Viruses may affect female fertility, but they may also affect conidia as spermatia, so male parents may also be affected. His laboratory is continuing to look at:

• As conidia germinate, do they express a pheromone?
• If Kex2p is knocked out, does cargo build up? He plans to look at transcriptional down-regulation.

William Powell, SUNY at Syracuse

Disease resistant strategy (in the tree host) includes:

• Multiple gene resistance
  -increases probable effectiveness
  -increases durability
• Genes must be typically linked to prevent segregation.
• Introduction into a multiple genetic background

He discussed three gene products:

1) ESF12 is an antimicrobial peptide (it produces pores in the membrane and causes cell lysis).

-18 amino acid sequence has a charged side and a hydrophobic side
-they change activity by changing size
-minimum inhibitory concentration (MIC) that completely inhibits fungal conidia

MIC is 10 μm for *C. parasitica*

MIC is 250 μm for *C. mollissima* pollen

-to test for potential problems in mammalian cells:
-1% Triton-X detergent produced 100% hemolysis
-Magainin 2 produced 10% hemolysis
-ESF 12 at 250 μm produced <3% hemolysis

2) Ac-AMP 1.2 is another microbial peptide that inhibits cell wall synthesis.

Powell put both ESF12 and Ac-AMP1.2 into pCCWEA1 (14,125 kbp).

3) Chitinase from poplar digests chitin in the fungal cell walls. He is looking at chitin-binding peptides via domain shuffling.

Signal sequences-*N. tabacum* ap24 gene's signal sequence my be a target peptide for cellular export for possible gall wasp control.

The gene promoters being used are:

- CaMV 355
- Win 3.12
- Win 6.39

In related research, he is looking at poplar transformation, using a gene inserted to look at *Septoria* resistance.

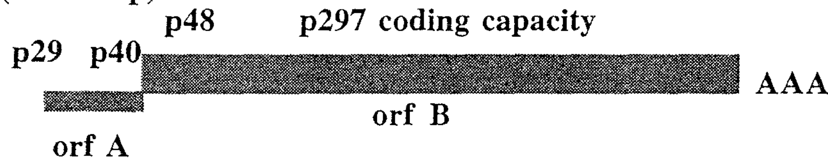
Bradley Hillman, Rutgers University

He presented an overview of members of the Hypoviridae.

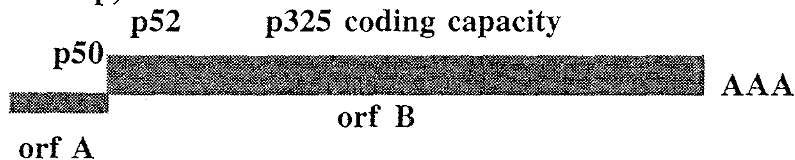
- CHV1-origin in Europe, white phenotype, infectious cDNA clone of one (Ep 713)
- CHV2-originally isolated in New Jersey, recently found in China (NB58)
- CHV3-isolated in Michigan (GH2)
- West Virginia isolates (SR2-like) result in no apparent phenotype change or hypovirulence; there is no sequence data.

1. Genomic organization of members of the Hypoviridae:

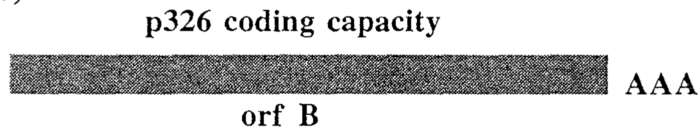
CHV1 (12.7 kbp)



CHV2 (12.5 kbp)

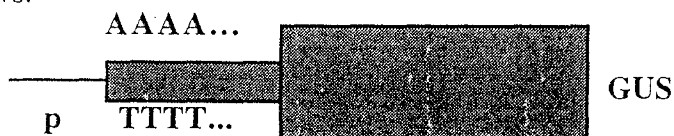


CHV3 (9.6 kbp?)



2. Differential mRNA display of NB58F. The virus-resistant mutant of NB58 has been designated, NB58F. He demonstrated that NB58F could be infected with the mitochondrial virus, NB631, but not by cytoplasmic viruses under normal circumstances. This year, he identified several up-regulated and several down-regulated genes in NB58F by mRNA differential display A complete cDNA clone of one of the up-regulated genes has been sequenced. There are no obvious homologies between any of the differentially regulated genes examined.

3. NB58F leader sequence. The leader sequence associated with CHV1 and CHV2 is as follows:



The leader sequence (the non-translated 5'-terminal 487 NT of CHV2-NB58 dsRNA) is about 500 bp and has nine start codons, short variable reading frames and a stop codon before the first gene. He posed the question: How do we overcome the leader sequence, as you never get past the stop/start codons to translate the first gene? To answer that question, four transformation constructs were analyzed for their ability to yield β-glucuronidase (GUS) activity: (1) a promotorless, leaderless GUS gene; (2) a promotorless, leader-containing GUS gene; (3) a GPD promotor-containing, leaderless GUS gene; and, (4) a GPD promotor-containing, leader-containing GUS gene. A total of six independent transformants for each of the four constructs were selected. Of the 24, only the six GPD promotor-containing, leaderless constructs and one of the promotorless, leaderless constructs led to significant GUS activity, suggesting that overcoming the translation block imposed the 487 NT non-translated leader is not as simple as gaining association host factor. To examine whether a viral factor could act *in trans* to overcome the translation block, *in vivo*, the 24 transformants were infected with CHV2-NB58 by pairing them and isolating converted transformants. No clear differences were observed in colorometric GUS assays in the uninfected and infected transformants.

4. Small dsRNAs. Isolate NB631, isolated from New Jersey, and RC1, isolated from Michigan, contain mitochondrially associated dsRNA about 3.0 kbp. Their closest relatives are yeast T and W dsRNAs from coliphage. Hillman wanted to see if these small mitochondrial agents could act cytoplasmically. To do this they mutagenized all terminators and tried to initiate infection by transcription. A full-length cDNA clone was built of NB631 dsRNA mutagenized for cytoplasmic expression from the *C. parasitica* transformation vector pCPXHY1, from a yeast transformation vector, or by transcription. The infectivity studies have been completed. Neither transformation to *Cryphonectria* or yeast, nor transfection resulted in autonomously replicating RNA, so this project has been terminated.

5. Population biology of CHV1 and CHV2-type dsRNAs associated with Italian or Chinese isolates of *C. parasitica*. Portions of dsRNAs from three Chinese and six Italian isolates, collected by Michael Milgroom and purified by Tobin Peever, were amplified by PCR and sequenced. The Chinese dsRNAs hybridized to NB58 and the isolates looked like NB58 in culture. Over a 1500 base stretch in a conserved portion of the genome, there was no consistent differences among the three isolates or between their consensus and that of CHV2-NB58. Similarly, the six white Italian isolates did not differ from each other substantially, although they clearly differed from CHV1-Ep713. Hillman is going to a less conserved region of the genome to see if he can pick up more variation.

Don Nuss, University of Maryland

The infection process is dynamic; there are responses by the tree to fungal infection and responses by the fungus to the tree's defense. The fungus also has to respond to environmental cues. Virus-infected fungi are somehow different in their ability to change to these cues.

1. Two G protein α subunits. Hypovirus involves disruption of signal transduction; there is a plieotrophic nature of phenotypic alterations. This led him to look at G protein signal transduction that involves a large number of receptors. There is a response in the nucleus to events on the cell surface.

cpg-1 (353 amino acids)

Hypovirus infection causes a decrease in cpg-1 accumulation. This suggests an important role for G protein-linked signal transduction in fungal virulence. Hypovirus infection results in substantial reprogramming of fungal gene expression. There is observed evidence for both enhanced and reduced transcription accumulation, with the former predominating. cpg-1 could result in a change in cAMP levels, thereby blinding the fungus to certain signal events. The following table shows cAMP accumulation in a virulent strain and several disruption mutants.

Intracellular cAMP accumulation in a virulent strain (Ep 155), two *cpg-1* disruption mutants (G1-1 and G1-15), and two *cpg-2* disruption mutants (G2-37 and G2-39) of *C. parasitica.*

Strain or Mutant	cAMP ± SD, pmol/mg	Mean Canker Area ± SD, (cm2)
Ep 155	6.77 ± 0.50	22.36 ± 2.97
G1-1	15.15 ± 1.02	0 ± 0
G1-15	15.38 ± 3.91	0 ± 0
G2-37	2.67 ± 0.23	18.22 ± 2.75
G2-39	3.22 ± 0.10	16.61 ± 4.19

The results of targeted gene disruption for *cpg-1* confirm that CPG-1 is required for diverse functions of virulence, optimal hyphal growth, orange pigment production, conidiation, and sexual reproduction. In contrast, the same technique revealed that *cpg-2* is dispensable for these processes, indicating quite distinct regulatory roles for these two Gα subunits. The finding that transgenic *cpg-1* disruption and hypovirus infection both result in similar phenotypic changes also significantly strengthens the proposal that disruption of G protein-linked signal transduction constitutes one major mechanism underlying hypovirus-mediated hypovirulence.

2. Hypovirus-encoded protein, p29. Transfection-mediated hypovirus infection was compared for: *C. parasitica*; *C. radicalis*; *C. havanensis*; *C. cubensis*; and, *Endothia gyrosa*. The protein, p29 was found to have a significant impact on both sporulation and pigmentation in the fungal hosts examined. All fungal species were able to transmit virus by anastomosis within the same species. The ability to introduce hypoviruses into different fungal species holds promise for the expanded utility of virus-mediated hypovirulence for understanding and controlling fungal pathogenicity.

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

Anita Davelos, Michigan State University

1. Variation in genetic diversity of *C. parasitica*. She is looking at what factors allow dsRNA to spread in some locations and not others. The spread of dsRNAs is thought to be influenced by variation in vegetative compatibility group (vcg) diversity in the fungus. Lower vcg diversity should lead to higher rates of spread of dsRNA. She examined the relationship between the proportion of isolates that contain dsRNA. The number of vcgs in four recovering Michigan populations were examined.

Four populations in Michigan were randomly selected with 150 trees at each location. Both healing and lethal cankers were chosen at each site and on each tree, if possible.

Location	Total # Trees	Total # VCG	# VCG in Common	# Rare VCG
Grand Haven	27	8	5	3
Frankfort	20	3	2	1
Roscommon	17	2	1	1
County Line	20	1	1	0

These data indicate that the degree of recovery at these four sites is not correlated with vcg diversity. There is a positive relationship between the proportion of trees with healing cankers at a site and the proportion of isolates that contain dsRNA.

2. Effects of infection on demography of chestnut populations. A second aspect of her study involves the comparison of three types of sites (disease free, recovering and non-recovering). She will look at # burrs/tree, dbh and how many seedling survive.

Seedling density and survival for disease-free, recovering and non-recovering American chestnut populations in MI. Density is the number of seedling/9 square meters.

Type of Site	Seedling Density	Seedling Survival
Disease Free		
Missaukee	1.140	0.71
Leelanau	0.125	1.00
Recovering		
County Line	0.660	0.92
Frankfort	0.296	0.94
Non-Recovering		
Massaukee	0.580	0.83
Stivers	0.100	0.64

She looked at tree size in 1995 versus their size in 1993. Data indicate that smaller trees may have less benefit from hv than larger trees.

Dennis Fulbright, Michigan State University

Evaluation of irradiated chestnut germplasm. The objective of this study is to determine why some chestnut trees at the National Colonial Farm in Accokeek, MD and at Sugar Loaf Mountain, Dickerson, MD, have survived blight infection for an extended period of time. Seedlings from selected open pollinated mother trees were planted at Accokeek and in Michigan. At Sugar Loaf, all sources of chestnut pollen were destroyed in 1995 allowing only 5 large surviving trees to cross pollinate. This seed was collected and planted in Michigan in 1996. Finally, mother trees were cloned by rooting cuttings from the trees; these are planted in Michigan. These trees will be evaluated for increased levels of resistance. At the National Colonial Farm, susceptible seedlings planted in 1990 from open pollinated surviving trees have been culled leaving only those seedlings demonstrating positive reactions to infection. These trees are a potential source of resistance that differs from Chinese chestnut.

Bill MacDonald, West Virginia University

A stand of 2500 American chestnuts in West Salem, WI, covering 50 acres, is the largest stand of American chestnuts in the U.S. Four cooperating agencies are involved in combating chestnut blight that was discovered at the site in 1987. The agencies include: Wisconsin DNR; West Virginia University; Cornell University; and, Michigan State University. A hypovirus from County Line, MI (COLI) was introduced into the resident West Salem strain and 186 cankers were treated with this virus between 1992-94. Cankers samples from multiple locations indicated that only 30% of bark plugs each year contained the hypovirus. Further, hypovirus infection was recovered from less than 10% of samples from untreated cankers, indicating limited spread of the hypovirus. These data prompted the introduction of a second hypovirus, Euro 7, into the resident Wisconsin strain. Cankers were treated with this hypovirus in 1995 and 1996. Findings as of 1996 include:

- 95 trees are now infected with 437 cankers; 128 infections were discovered in 1996
- The Euro 7 hypovirus has become more significantly established in cankers than the COLI hypovirus. It was recovered from 41% of bark samples treated in 1995 and from 23% of bark plugs from previously untreated cankers.
- A second strain of *C. parasitica* has been discovered at a site approximately 600 meters from the existing infection center. This strain is vegetatively incompatible and sexually compatible with the original strain. The Euro 7 hypovirus has been detected at this site.

Don Nuss questioned if dsRNA is the factor for the difference between COLI and Euro 7 hypoviruses. He indicated that Ep 713 acts very differently in different species of *Cryphonectria*.

Lou Shain said the cirrus has to be sampled in order to determine the actual rate of dsRNA transmission into conidia. Dennis Fulbright said he has examined cirri and some have very low numbers of conidia with dsRNA and others are quite high. The COLI hypovirus is so debilitating

to the fungus that there may not be many cirri produced. Fulbright indicated that when examining healing cankers, stromata are very difficult to find.

With regard to the new introduction site (Schomberg property), Michael Milgroom stated that the DNA fingerprint is completely different from isolates in the original epicenter, indicating it was a different introduction.

As canker age increases, there are a variety of other fungi that become associated with cankers, most notably, *Trichoderma* species. Milgroom indicated that *Trichoderma* contaminants can alter v-c tests and he believes they may also play a role in virus transmission. Terry Tattar hypothesized that *C. parasitica* may be disarmed as a pathogen by introducing hypovirus, thus lessening its ability to colonize bark. When in competition with an aggressive saprophyte such as *Trichoderma* sp., *C. parasitica* cannot compete.

Clarissa Balbalian, West Virginia University

She has field tested some of David Huber's (Michigan State University) strains to see if unidirectionality and epistasis occur 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 forest setting. To accomplish this, seven different strains comprised of five different *vic* genotypes were used to establish virulent cankers on healthy chestnut stems in May 1996. In September 1996, these cankers were exposed to a bark patch inoculum source infected with one of the seven strains of the fungus that is either genetically identical to the canker strain at all *vic* loci, or differs by one or two specific *vic* genes. The bark patch inoculum sources contain either a North American (COLI) or European hypovirus (Euro 7). The artificially established cankers will be sampled in November 1996 and the spring of 1997 to discern the extent of virus interaction.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

It has been 11 years since the last introduction of hypovirulence at the American chestnut planting at the CAES farm. She attempted to recover hv strains from "healed cankers" on those trees to discern if the spread of hypovirulence is due to surface propagules. Cankers were scrubbed with sterile cotton swabs and none of the *C. parasitica* isolates were "white". She plans to look at cirri next, because if dissemination of hypovirulent inoculum is not by surface propagules, then it must be insect vectored.

She relayed results from Tullio Turchetti (Florence, Italy) who believes virus infection of the fungus occurs in the gut of mites. He proposes that live fungal cells are damaged by gut enzymes and virus particles, surviving as resident in the gut, are transferred to virulent hyphae.

Michael Milgroom, Cornell University

1. Genetics of v-c types in Italy. He has been studying, in conjunction with Paolo Cortesi and Marco Bisiach at the University of Milan, the diversity and genetics of v-c types *of C. parasitica* to understand more about the transmission of hypovirulence in populations in Italy where hypovirulence has a significant effect on chestnut blight. He found that 4 v-c groups comprise 85% of all *C. parasitica* isolates in Italy. There are only 20 v-c groups in all of Italy; generally, 50 isolates fall into 4 v-c groups.

Why is the v-c diversity so low in Italy?

1. There are few polymorphic loci.
2. There is limited recombination.

Each v-c type is determined by a combination of 6 or more *vic* loci. The summary of the v-c genetics of Italy is as follows:

- 6 polymorphic loci
- 2 alleles/loci
- independent loci ($2^6 = 64$ v-c type possible)
- only 20 have been found

In one population at Teano, Italy, only 3 isolates out of 195 were putatively recombinants from two dominant v-c types. The following is a table of observed and expected v-c frequencies at Teano.

V-C	Observed V-C Types	Expected V-C Types
I-10	36	7.4
I-12	152	119.1
I-17	2	31.7
I-27	1	27.9

The DNA fingerprinting clustered to v-c type. The following problems were posed as to why there is limited recombination in Italy.

- Sexual reproduction does not occur.
  - No, perithecia were found in 10/11 populations
- There is a lack of outcrossing.
  - No, outcrossing rate is 70%
- There is mating incompatibility between v-c types.
  - No, segregation is found for v-c types.
- There is an unfavorable environment.
- The virus inhibits sex.

2. Mating type-specific markers. Gillian Turgeon has succeeded in cloning the conserved HMG domain found in the MAT-2 idiomorph of *C. parasitica.* He is attempting to clone conserved sequences that flank the MAT-2 idiomorph in order to locate MAT-1.

3. Hypovirus population biology. He screened 600 isolates of *C. parasitica* from 8 populations in North America using an immunoblot procedure to determine the population structure in North America. The most commonly found dsRNA is the SR2-type found in West Virginia, Kentucky, Maryland, New York, New Jersey and Michigan. The second most common type was found primarily in Michigan and hybridizes to dsRNA from GH 2. The last hybridization group was restricted to one population in New Jersey (NB58) and does not hybridize to any previously known dsRNA.

With regard to Asian dsRNAs, the majority of Chinese and Japanese dsRNAs hybridize to CHV1 (Ep 713). The exception were isolates from the Xiuling province. These isolates hybridized to CHV2-NB58. Brad Hillman sequenced part of the polymerase genes of three Xiuling isolates and found that they are very similar to the sequences found in CHV2 in New Jersey.

Paul Chaloux, West Virginia University

His project involves hypoviruses that were released in dissemination experiments 10-15 years ago. These sites were revisited and cankers were sampled. Some isolates contained dsRNA that hybridized to GH2 and SR2. The recovered isolates had no morphological changes due to the dsRNA. He has developed single conidial progeny from these isolates, with and without dsRNA, and he inoculated them into live stems in October 1996. The objective is to see if hypoviruses have evolved toward avirulence.

Michael Milgroom noted that although European and North American viruses were released in these sites, only the North American hypovirus is recovered.

Jimmy Maddox, Tennessee Valley Environmental Research Center

He works in watershed protection at the Environmental Research Center at Mussel Shoals, AL. He is looking at the nutritional problems associated with American chestnut in the seedling stage. There might be some endomycorrhizal effects in acidic soils. There are some endomycorrhizae that will mitigate potential ink disease, promoted by overwatering.

1. Effects of soil pH and mycorrhizae inoculations. Forty seedlings were planted in to pots containing limed or nonlimed Mountview soil, supplemented with P, K,

micronutrients and Pro-mix. His treatments were: control; no VAM, *Glomus etunicatum*; *Glomus diaphanum*; and, *G. etunicatum* with *Pisolithus tinctoris*. At 101 days after planting, growth was greater in the nonlimed treatment and all inoculants increased growth. His conclusions were that American chestnut can be limited by too high soil pH and the magnitude of this response can be accentuated by the presence of certain mycorrhizae.

2. Effect of VA-mycorrhizae on seedling growth, acid tolerance and disease resistance. Sixty seedlings were planted into conetainers with peat moss and fertilized with miracid. Twelve seedlings were inoculated with *G. diaphanum*, *G. etunicatum* (GES 329), GES 312, GE TVA or none. Inoculated plants grew 16% taller, basal stem area was 13% greater with 23% more leaf area, 84 days after planting. Chloride uptake from fertilizer scorched the leaves and was accentuated by the GE TVA. Seedling damping off was ameliorated by the GE TVA and the acid tolerant *G. diaphanum*. In a second experiment, thirty plants were planted into a limed silt loam , pH 6.2 amended with peat moss and miracid. Treatments were GES 329, GE TVA and no mycorrhizae. Soil-plants grew over three times larger than the conetainer-peat growth plants. Half of the seedlings produced in the soil-peat medium were planted into a reclamation site at Copper Basin, TN to evaluate their response to an acidic (pH 4.2) and highly eroded soil due to copper mining activities. The other half were planted into a field trial at the ACF farm in Meadowview, VA to evaluate their susceptibility to *C. parasitica*. After 2 years, the trees are doing well with roots 15 feet from the trees.

He is using air-pruning and this has produced good results as no cutting bar is required. He has also worked with double-cupping (32-oz). After becoming root bound in one cup, the seedlings are transferred to a larger cup; this provides a quasi-hydroponic situation. These are then outplanted.

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. This isolate was identified as *T. atroviridae* H393. In 1994, 700 American chestnut seedlings were planted in the Quabbin Reservoir Forest in Belchertown, MA. Half of these seedlings were soaked in a suspension of *T. atroviridae* prior to planting. *T. atroviridae* can still be isolated from treated trees and *Trichoderma* sp. can be found on both treated and control trees. None of the trees show any signs of *C. parasitica* infection.

An additional test has been set up (8' x8' spacing) to examine protection by *Trichoderma* and other antagonists. Tattar sees *Trichoderma* as a protectant as opposed to an eradicant.

He is continually looking for bark antagonists but *Trichoderma* is the most consistently isolated. Graduate student Patricia Groome has an additional 37 isolates of *Trichoderma*.

He presented an article from Turkey (FEMS, Microbiology Letters 176 (1995) 249-256, by Inci Arisan-Atec, Erich Heidenreich and Christian Kubicek).

Antagonism of *C. parasitica* (Euro 7) chestnut blight by some Trichoderma species.

Trichoderma	Browning Area (sq. cm)
None (Control)	3.7
T. viride (ATCC 32173)	0.5
T. parceramosum (ATCC 28019)	0.6
T. sp (ICMP 3088)	1.2
T. reesei (QM 6a)	3.4

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

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

There was poor nut production in Connecticut this year due to heavy rain in July. Her hand-controlled crosses were also poor, only 27 good nuts were produced. Nuts from 'Lockwood' open pollinated will be sent to Chestnut Hill Nursery in Alachua, FL for rootstock selection. M. Byrne from Propagation Technology in MI has successfully rooted cuttings of 'Sleeping Giant', 'Dwarfest', and 'Lockwood'.

Zizhuo Xing, SUNY at Syracuse

He is working with mature somatic embryo of American chestnut from ovule culture. His objectives are:

- embryo development
- embryo maturation
- embryo germination

The process he employes includes separating embryos using an embryo initiation medium. Within weeks he can observe somatic embryos (globular, heart and torpedo stages). The embryos are then transferred to a development medium where he has cotyledon development. The embryos then go to a maturation medium (60 g sucrose). After 4-5 weeks, there is elongation into 2 cotyledons at which time they are transferred to germination medium #1. After 2 weeks, embryos begin to germinate and after one month, he has the entire plant (root and shoot). Germination medium #2 elicits more shoots. His conclusions are:

- 2-5% of ovules produce embryogenic tissues
- 606 embryos developed from 1 gram of embryogenic tissue
- 31% of mature somatic embryos produce whole plantlets

He will use embryogenic tissue for transformation using particle bombardment and *Agrobacterium.* His constructs are <5kbp.

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. He completed his Ph.D.).

Final results from 1995 culture initiation:

-collected burrs from four north Georgia trees
-all explants have been cultured on woody plant medium
-generated 12 new embryogenic cultures representing all four trees.

1996 culture initiation progress:

-burrs were collected from four trees at the American Chestnut Foundation farm
-basal medium: WPM and Gamborg's BS medium
-plant growth regulators: 2,4-D & IBA (primary) and 2,4-D, NAA & BA (second.)
-over 280 cultures have been initiated

1. Mature Tree Tissue Experiment

Rationale: Seed embryo-based system currently in use does not clone source tree genotype, so resistant trees produced by the American Chestnut Foundation (ACF) breeding program could not be cloned via this route.

Approach: Culture inflorescence tissues of mature trees.

Background: He has produced embryogenic cultures of *Quercus* and sweetgum.

Variables: Source trees (4 ACF selections), using plant growth regulators, thidiazuron (TDZ) and 2,4-D. He is using pulse and continuous exposure to the pgr's.

Results: 24 explants produced bud-like structures. Most buds observed on elongating inflorscences and most buds became overgrown with callus. No somatic embryos have been produced, to date.

2. Storage Produce Accumulation Experiment

Rationale: Treatments that result in somatic embryos accumulate storage products that should result in improved somatic embryo formation. He is using sucrose or fructose, PEG and ABA.

Results: The ABA, PEG and carbohydrates had an impact. The average zygotic embryo gave mean starch content of 426. The best treatment was ~260, or less than 63% of the average zygotic embryo. Sucrose was better overall than fructose. From the SDS page, a 21kD protein was found in zygotic embryos but in none of the explants.

3. Current Maturation and Germination Experiment

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. He felt that embryo exposure to 2,4-D has been for too long a time period, so he did an inventory of existing cultures that had been treated with 2,4-D. They have higher concentrations of sucrose and activated charcoal. He is using cold and desiccation pregermination as treatments, along with glutamine, PEG and ABA.

4. RITA Temporary Immersion Culture Vessels

The rationale is that many plant tissue cultures do not perform will in conventional media. He is using a temporary immersion system (RITA, manufactured in France) for embryos; embryos are immersed in a liquid rinse for various time periods. He has immersed embryos in two treatments: 4 times in 24 hours for 15 minutes each; and, 4 times in 24 hours for 5 minutes each. To date, he has some somatic embryos turn green, so he has had some success.

Lou Shain, University of Kentucky

He presented data to indicate that polygalacturonase (PG) produced by the chestnut blight fungus may be a virulence factor. The evidence is as follows:

• A basic PG purified from culture filtrates caused browning of chestnut inner bark and hyphal cell wall components.

• Greater PG activity was detected in cankers on American chestnut as compared to Chinese chestnut and in cankers induced by virulent strains as compared to isogenic hv strains.

• Purified PG was inhibited significantly more by protein extracts such as PG inhibitor (PGIP) from Chinese chestnut bark than from American chestnut bark.

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

When the gene encoding PG (enpg-1) was disrupted, the disrupted mutant caused cankers similar in size to the nondisrupted virulent parental strain. This result demonstrated that the basic PG produced in culture is not required for virulence. But, two additional PGs, acidic rather than basic, were produced in these cankers. The presence of these acidic PGs in cankers raises two questions:

1. What role do these acidic PGs play in pathogen virulence? The could be resolved by additional gene disruption studies.
2. How are the acidic PGs affected by PGIPs that may be produced by American and Chinese chestnut?

The second question was addressed by extracting and concentrating protein from the margin of a naturally induced canker. The PI's of the PGs extracted from this canker were largely acidic, as determined by the IEF followed by overlay activity gel staining. PGIP from bark of five Chinese chestnuts inhibited the Pgs from culture and from bark similarly. While PGIP from bark of five American chestnuts inhibited the Pgs from culture, both sources of PG were inhibited more by Chinese than by American chestnut PGIP.

Fred Hebard of the ACF supplied stem/branch samples from resistant and susceptible chestnuts. Chitinase and β-1,3 glucanase activities were determined. A correlation between hydrolase activity and disease rating was not evident. These results could be due to differences in the vitality of the samples, so Hebard has supplied 18 additional samples (3 American, 3 Chinese and 12 hybrids) for further testing of hydrolase and PGIP activities.

Robert Bernatzky, University of Massachusetts

He is trying to identify regions of the genome of American chestnut that confer host resistance. He looked at the material from the following crosses:

American Chestnut	X	Chinese Chestnut	x	American Chestnut
Roxbury East #1		Mahogany		Roxbury #1

He looked for low copy sequences from the chestnut genome to use as a simple genetic marker.

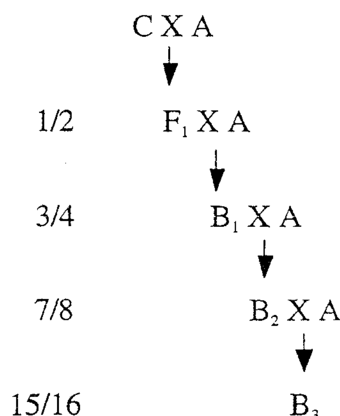
- took DNA inserts from bacteria
- hybridized DNA to chestnut
- looked for clones that do not hybridize

He found that there is a good deal of repeated DNA. He did the same thing for Chinese chestnut and got similar result. He has left this work because it is not a good source of markers. He has since isolated mRNA from leaf and made a simple library. He also has generated linkage maps from NA clones. He is working with RbcS gene that relates to carbon dioxide fixation in leaves.

Fred Hebard, The American Chestnut Foundation, Meadowview Research Farm

He is following the traditional backcross method to get to 15/16th American by the third backcross. Whether this will work depends on the number of genes involved. He will intercross B_3 to get B_3F_2 and then work toward B_3F_3. He plans to make F_2 and F_3 generations and screen for resistance and cull out nonsurvivors. Screening will be done by direct inoculation using Ep 155 and a virulent isolate with low pathogenicity, SG 2-3.

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



He wants to avoid inbreeding so he will use different American parents; he has separate lines of American parents. Also, he does not want to rely on only one source of resistance. He expects to move onto third backcrosses and produce B_3F_3 in about 10 years.

The Wagner farm was planted full in 1995. Thus, in order to save space, he began challenging at a younger age. As trees get older, cankers get larger, on average. He found that resistance is incompletely dominant in 1-year old trees and he cannot distinguish between Chinese and Chinese X American, the opposite of what he expected; he thought the data from 1-year old trees would be what he expected from 5-year old trees. Resistance can be screened for on 1-year old trees, but the tendency is to throw the baby out with the bath water, as a lot a potentially good material is discarded. He is now collecting data on 2-year old trees.

The genetic map from one Chinese-American F_2 population was discussed. This work is in conjunction with Tom Kubisiak and Robert Doudrick (USDA-Forest Service, Saucier, MS) and R. Bernatsky (University of Massachusetts). The map incorporates RFLP, RAPD, isozyme, morphological and resistance loci.

Al Ellingboe, University of Wisconsin

Charles Burnham asked Ellingboe to be the science chair of the ACF; only in the last few years has he worked with chestnut. He discussed the history of conceptual framework of host-pathogen interactions. Scientists felt that a resistant host produced a toxic compound. In 1946-47, Harold Flore pointed out that there are corresponding genes on both the host and the pathogen. It took 10 years for the one gene (virulence) for one gene (resistance) theory to catch on. Vanderplank then developed the theory of horizontal resistance (resistance in the host is independent of the pathogen). This was accepted in two ways–either as fact or as a hypothesis that needs to be tested. With recombinant DNA, genes can be cloned to see how they function. There

have been about 35 avirulence genes cloned and sequenced; this has not given any real significant insight.

With regard to rice blast disease at Wisconsin, a strain of the fungus (*Magnaporthe griseae*) was developed with high fertility to look at host/parasite relations. The following cross was made:

Avirulence		**Virulent**
P12	X	p12, yields 1:1

The question was raised, "Is the above actually correct"? The following crosses were made to answer that question.

				A:V
A	X	V	yielded	1:3
A	X	A	yielded	3:1
V	X	V	yielded	1:3

Two genes are segregating and there is a second locus involved; it is very specific to the first. There are suppressers that suppress avirulence; this is a deviation from 1:1 ratio. The second locus is very mportant in suppressing expression.

The reason cloning has not given any insight is that the genes that control specificity are controlled by other genes. We need to go back and see if the genes we are looking at (cloned) actually control specificity.

What genes are present in field isolates of *M. griseae*?

- 4 different gene in *M. griseae* in a Texas isolate
- 8 genes in rice blast

Will these questions help us with American chestnut? Ellingboe wants to intercross different strains of *C. parasitica*.

Van Alfen asked if we should expect a gene for gene system in chestnut. Ellingboe said that disease is dependent on genes in both organisms but it is more complicated if enough test crosses are done in both the host and the pathogen. Ellingboe stated that in the past five years, no matter what system has been looked at, they are never controlled by a single gene. Van Alfen argued that the above is valid for long evolved systems but may not be applicable for introduced pathogens.

Yan Shi, The American Chestnut Foundation, Meadowview Research Farm

He joined the ACF in April 1996. He will examine:

- sources of blight resistance (will inoculate with Ep 155 to see if there is variability among Chinese chestnut)
- male sterility (made crosses to look at male sterility-some have much higher % of sterile males)
- precocity
- combining age (to find best Chinese to act as parents)
- tree form (there are variations in ACF B_2; he is looking at branching pattern)

Phillip Gordon, New York Botanical Garden

The American chestnut is not an endangered species because he has looked at American chestnut growing in its natural range. It has changed its niche from an overstory tree to a shrub. When a field or forest is cleared, American chestnut can come up. Even with our new technology, he predicts American chestnut will not be quite the same as it was in its original form.

Tom Kubisiak, USFS, Saucier, MS

He is working with Fred Hebard's F_2 population. He has identified 200 markers and identified markers that explain about 30% of the phenotype. He will confirm these regions by looking at backcrosses using Nanking and Mahogany.

Business Meeting

Don Nuss was elected secretary for 1997-98. Chairman for 1996-97 is Neal Van Alfen.

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 thanked Terry Tattar for the local arrangements. The annual report, by Tattar, must be in the mail by March 15, 1997. The meeting minutes, by Van Alfen, must be out by the end of November, 1996. The project extension needs a rewrite committee; he needs a one page justification. Sandy Anagnostakis will spearhead the committee for the project rewrite. Van Alfen suggested that Anagnostakis chose members of the committee as she sees fit.

The award application should be in the mail by mid-November. Tattar will chair the award committee with the help of Bill MacDonald.

Next year's meeting will be held at the Delaware Water Gap at a site to be chosen by Brad Hillman.

The meeting was adjourned at 12:00 noon on Friday, October 25, 1996.

Addendum

The project expires in 1998, not in 1997 as reported in the minutes. Therefore, the paragraph of outcomes, suitable for the press and written the the fourth year of the project, is not necessary until 1997. Also, the project extension need not be written until 1997.