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

University of California, Davis
October 24-27 2002

Attendance:

California:	Pam Kazmierczak, NE-140 Chair, Neal Van Alfen, Debra Wilk, Warren Roberts (UC, Davis), Deborah Golino, Carole Lamb, Dick Hoenisch, Judy Lee (FPMS), Art DeKleine (Cal-Poly Institute), Robert Wooley (Wilson Nursery), John Ireland (Fowler Nursery), Lucienne Grunder, Daniel Titzel (Owl Creek Ranch), Harvey Corriea, Suzette Canfield (Chestnut Growers)
Connecticut:	Sandra Anagnostakis (Connecticut Agricultural Exp. Station)
Kentucky:	Chuck Rhoades (University of Kentucky)
Maryland:	Donald Nuss, Lynn Geletka, Chris Root (University of Maryland Biotechnology Institute)
Michigan:	Dennis Fulbright (Michigan State University)
Missouri:	Ken Hunt (University of Missouri, Center for Agroforestry)
New Jersey:	Bradley Hillman (Rutgers University)
New York:	Alice Churchill (Boyce Thompson Institute, Cornell University)
Ontario:	John Gerrath (University of Guelph)
Pennsylvania:	John Carlson (Pennsylvania State University)
West Virginia:	William MacDonald, Mark Double (West Virginia University)

An evening reception was hosted by Neal Van Alfen and Pam Kazmierczak on October 24, 2002 in their home. The meeting was called to order by Chairman Kazmierczak at 9:00 am on October 25, 2002 in the UC, Davis viticulture and enology classroom.

<u>OBJECTIVE 1.</u> To improve chestnut trees for timber and nut production, and determine the cultural requirements of chestnut seedlings in nursery and natural settings.

Harvey Corriea, Chestnut Grower

Corriea discussed the history of chestnut in California. Chestnuts were first imported to California during the gold rush era in the mid-1800s; many Europeans brought chestnuts with them as they migrated west. The oldest trees in CA are located in the Sierra foothills, along the north coast and in the central valley. Some trees, located in Mendocino County, were planted in 1882. Initial commercial production appears to have been started in San Joaquin County in the Linden area. Corriea showed pictures of 60-70'-tall Italian marroni chestnuts from Linden. Many of these commercial orchards were removed in the mid-1900s due to high labor costs and poor returns. Most of the current orchards in CA are in blocks of 10 acres or less and production varies from 3,000-5,000

pounds nuts/acre. He is not aware of any CA grower using American chestnut. Corriea is a member of the western chestnut grower's association and their web site is: wcga.net.

The only area in CA that has chestnut blight is the DeMartini orchard in Los Altos. The biggest problem facing CA chestnut growers is not chestnut blight but *Phytophthora*.

Cultivars:

- There are limited cultivars available from nurseries.
- Majority of plantings consist of the 'Colossal' cultivar along with pollinators, 'Nevada' and 'Okei'.
- A handful of growers graft trees to European cultivars (stock of various sources).
- Some chestnut breeding was done by Luther Burbank.
- There are few *C. mollissima* grown due to climate.

Diseases and Insect Pests

- Blight is primarily centered in one producing orchard in San Joaquin County.
- *Phytophthora* is largest problem due to poor cultural practices or unsuitable sites.
- Weevils are a limited problem reported in the Sierra foothill orchards.
- Shot hole borers are reportedly a problem in the Sierra foothills.

Harvesting

- Larger commercial operations have adapted walnut and almond harvesting equipment.
- Smaller operations still harvest by hand.
- At the Tanimoto orchard in Gridley, CA, their de-burring operation uses rubber tires to remove burrs.

Marketing

- Several growers use established brokers to sell their chestnuts.
- Smaller growers sell directly to clientele and farmer's markets.
- 'Colossal' (on average of 1.25 inch-diameter) sells for $3.50/lb.

Future Considerations

- 'Colossal' is large but considered by some not to be of high quality.
- Other cultivars should be given consideration.

Lucienne Grunder, Owl Creek Ranch

She is a nut orchardist, located outside Modesto, CA, with 85 acres of chestnuts and 400 acres of walnuts. She reported that an eastern broker requested 47,000 pounds of chestnuts; she does not have the acreage to meet that request.

Summary of her operation: She uses microsprinklers for irrigation. She planted chestnut on the poorest site on her property and they have grown like weeds. Grow tubes are used to assist in collection of water. Condensation can amount to 1/4" per tube and in a dry climate, every drop of water helps. Her 'Colossal' cultivar harvest was aided by the use of an ethylene spray to help synchronize nut drop; 77% of the nuts from this cultivar were classified as "giant." She planted several thousand trees in pots to see how they would perform. Those cultivars were:

- 'Marki', a French cultivar with good performance.
- 'Marissard' has good flavor and peels well.
- 'Precoce Migoule' is harvested three weeks after 'Colossal'.

- ‘Bouche de Betizac’ produces red chestnuts.
- ‘Montesol’ molds very easily.
- ‘Belle Epine’ is a very nice nut.
- ‘Okei’ is a good pollinator.

From the pot test, she plans to keep the following cultivars: ‘Colossal’, ‘Marissard’, ‘Bouche de Betizac’, ‘Marki’ and ‘Precoce Migoule’. She is looking for trees that: (1) do not produce late pollen; (2) have good shape; and, (3) have timber potential.

She has high boron levels at the Owl Creek Ranch and some cultivars have boron sensitivity.

Deborah Golino, Director, Foundation Plant Material Services, UC Davis

The FPMS produces, tests, maintains and distributes elite disease-tested plant propagation material. They provide plant importation and quarantine services, disease testing and virus elimination. FPMS was established in 1958 to distribute virus-tested, professionally identified grape, fruit and nut tree propagation stock. Selections originated from UC and USDA variety improvement programs. Today, FPMS has programs for grapes, strawberries, fruit and nut trees, roses and sweet potatoes. There is a staff of 35 and all operating funds are provided by industry; their most important source of income is the IAB (fruit and nut tree and grapevine improvement group). FPMS maintains collections and they also do quarantine work. There are strict quarantine regulations on chestnut importation into CA. The chestnut industry hopes to increase their cultivars, so FPMS applied for a permit to import material. A $10,000 request has been made from IAB to help establish newly imported chestnuts, although at this time, they have not yet decided on what cultivars to import nor where the trees will be planted. There is some discussion on establishing an east coast and a west coast chestnut collection site to keep germplasm intact. The west coast collection has been approved to be located at UC Davis. There is, however, no current pomology faculty member working on chestnut.

Mike Cunningham, Foundation Plant Material Services, UC Davis

He is in charge of the greenhouse and orchards at FPMS. He currently works with many varieties of nuts, strawberries and grapes, but he has limited knowledge of chestnut. FPMS already has a chestnut orchard with trees from Lucienne Grunder. The idea is that when they begin to import chestnut cultivars for the germplasm collection, they will already have rootstocks available and have some knowledge of growing and maintaining chestnut.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

She is lobbying for the east coast chestnut germplasm repository to be located in Missouri as they have a large AgroForesty program.

She posed the question, “Are chestnut stump sprouts dying out in the east?” She sees no evidence of stump sprouts not regenerating.

Root Study. She is examining four hybrids that have been planted in two clearcut areas in central CT (Prospect North and Prospect South) and in a nursery in Windsor, CT. Another site in a partially forested area was established in 2002 using transplants from the nursery. Trees at this site were planted in green tree shelters. Since there are native

chestnuts in the forests around these plots, she plans to collect chestnut blight samples and infect virulent strains with hypovirus and then inoculate natural infections to keep the native trees alive so they can provide pollen for the planted trees. The next generation of trees will include resistant genes from the hybrid trees. The height of all trees is being measured and all trees are doing quite well. The hybrids include: BC_3 and BC_2 (Roxbury, CT), BC_3 and BC_2 (Watertown, NY). Soil and leaf samples were taken from the forest clear-cut plots and the Windsor nursery. Results of mineral analysis after acid digestion revealed some differences. Calcium and phosphorous levels were much higher (2-fold) in leaves from the nursery trees than the forest clearcuts.

Ester Portela (Portugal) has reported that chestnut bark with higher C/N rations seem to be more resistant to chestnut blight disease. The Windsor nursery has a much higher C/N ratio because the Windsor soils have low N. Anagsnostakis is looking more closely at the C/N levels because the levels reported by Portela are much lower than the CT levels. She will contact Portela to make sure their procedures are similar.

Anagnostakis also looked at calcium levels in dormant, dried chestnut bark from American, Japanese and Chinese chestnut. Chinese chestnut has the most calcium/kg dried bark.

Ken Hunt, University of Missouri Center for Agroforestry

The Center for Agroforestry has 12 field studies with nut trees; three studies deal with chestnut. The reason behind studying chestnut is they hope to begin a chestnut industry for small farms in Missouri. The objective of their chestnut field trials are:

- To evaluate and characterize available cultivars for marketable traits (50 cultivars in this trial).
- To establish, replicated cultivar research/demonstration orchard trials in commercial areas in Missouri and Kansas (12 cultivars in this trial).
- To develop a chestnut management guide (3 cultivars in this trial).
- To work on chestnut business and marketing.

Three repositories have been planted. The first repository, planted in 1996, is intended to look at cold hardiness, quality characteristics, etc. The second trial, planted in 1999 is a cultivar trial. The third planting, begun in 2001, is intended to be a nut production orchard with a goal of 1 ton/acre. Cultivars from the first trial that look promising are 'Williamette', 'Qing', and 'Peach'.

A *Chestnut Guide for Farmers* has been developed. The guide was developed with three different clientele in mind.

- **Scenario 1**—low intensity for backyard trees
- **Scenario 2**—low input orchard (50-500 trees)—grafted trees recommended
- **Scenario 3**—high intensity (500 or more trees)—grafted trees of selected cultivars.

To maximize production of premium quality nuts in the high intensity orchard, a hedgerow system of chestnut production has been utilized. This system, developed by Dr. Hitoshi Araki from Japan (and utilized in Australia and New Zealand), suggests trees be planted on a 13' x 26' spacing. The trees are not thinned out over time but are pruned to contain tree size similar to what is done in peach orchard management. Research in Japan has shown that chestnuts require a high level of light intensity of sunlight to set and develop fruit. High light intensity stimulates nut-bearing shoot growth, which in turn

promotes large fruit size. The hedgerow pruning system strives to maximize light penetration into the entire canopy. To achieve needed tree structure, pruning should begin in the first year and continue regularly throughout the tree's life. The initial target is one leader and two main branches. The third year aims to encourage trees to spread out, thinning inward growing branches. Commercial cropping should begin by the fifth year, at which time the central leader is dehorned. This experimental hedgerow system is unproven in the Midwest US. They are looking at two pruning methods: (1) one leader and two main branches, pruned for elliptical shape; and, (2) hedgerows within the alleys. They have yet to decide the height of the first branch. The Australians recommend one meter, but the Missouri group's concern is that spray and harvest equipment cannot be used easily given the close proximity of the first branch to the ground. With a 13' x 26' spacing, trees will have to yield 15.5 pounds of nuts/tree to obtain 1 ton/acre.

Chinese chestnut trees are rather drought tolerant once established, but ample water throughout the growing season promotes good tree growth and regular nut production. Drip irrigation was installed in all three studies and preliminary evidence suggests that irrigation has increased growth 18%. There are not many problems associated with chestnut blight in the orchards.

John Gerrath, University of Guelph

Gerrath discussed the American chestnut recovery plan in Ontario.

American Chestnut in Canada:

- The species comprised ~30% of the forests in southern Ontario, pre-1900.
- Chestnut blight became established in 1930.
- Possibly 1.5-2.0 million trees in Ontario prior to chestnut blight.
- In 1990s, only 70 sites remained with about ~180 trees (>10 cm dbh).
- Its status in Canada is considered "threatened."

National Recovery Plan in Canada. John Ambrose, Greg Boland, Ken Elliott, Brian Husband, Melody Melzer and Gerry Waldron hope to restore American chestnut to self-sustaining populations. The short-term goals (5 years) in this project are: (1) identify and protect populations within its native range; (2) develop and assess management strategies to manage blight; and, (3) identify blight-free stands outside native range.

Chestnut census. This is a 2-year project to find, map and measure every American chestnut individual in Ontario. This plan also assesses ecological conditions. All trees that are found are located via GPS; a permanent ID# and tag are placed at each tree. Characteristics recorded for each tree are: height; number of stems; dbh; percent dieback; flowers; seeds; and, leaf hairs. The demographic data includes: presence/absence of chestnut blight; number of cankers; type of cankers; and, epicormic shoots. Surroundings data includes: slope; aspect; soil type; percent canopy cover; and, vegetation. Samples collected include: herbarium specimens; a sample for DNA analysis; 1 liter of soil; and, blight specimens. Gerrath reported on findings after 2 years (2001-02). They have mapped 677 trees at 116 sites; 68% of these were on public land. There is patchy distribution of chestnut in Ontario. Forty-six percent of the trees are larger than 10 cm dbh and 17% of the population is flowering. Chestnut blight is present on 21% of the trees; 25% of those show some signs of healing cankers. The project was initially intended for 2 years. It is unknown if they will continue in 2003 because there are still many more trees to map.

Chestnut hybrids. There is a project ongoing from 2002-2004 to identify interspecific chestnut hybrids of the genus *Castanea* using molecular and morphological techniques. The plan is to examine how hybrids may be affecting the native, non-hybrid trees. The non-native species includes: *C. sativa, C. mollissima* and *C. crenata*. Are there hybrids in Ontario? Gerrath will conduct a hybrid search. He will use various voucher specimens from Sandra Anagnostakis. He will collect dormant buds, leaves and branches for trees at 10 sites; these sites include 40% of the chestnut population in Ontario.

Allozyme electrophoresis. Gerrath wants to look at genetic variability in chestnuts. Hong Wen Huang has shown that allozymes are variable in chestnut, but Gerrath feels if the correct enzymes are chosen, genetic variation can be examined. If allozymes fail to produce the desired results, he can turn to DNA-based tests, RFLP, RAPD, QTL or cDNA tests. He also plans to look at morphological characters such as leaf length, leaf width, hairs and stipules. He will analyze the correlation between molecular markers and morphological traits.

Report from Adam Dale. Crosses were made successfully in 2001 on 11 American chestnut trees pollinated *in situ* with pollen from blight resistant trees from Sandra Anaganostakis. A total of 287 nuts were obtained; 162 nuts from pollen from Anagnostakis' trees, 62 nuts with R2T10 pollen and 38 nuts with R2T8 pollen. Also, 84 nuts were obtained from the trees with the Canadian *C. dentata* pollen. The nuts were germinated and 223 trees that were pollinated from resistant trees have survived along with 80 from the Canadian pollen. In 2002, 35 Canadian trees in eight southern Ontario counties were selected as female parents. These were selected to represent the geographic range in southern Ontario and be accessible for pollination. In 2002, 117 nuts were obtained from the pollination with CT pollen and over 200 with Canadian pollen.

Awareness of chestnut blight will be promoted in Canada via radio, television and connection with local schools.

John Carlson, Penn State University

Carlson is working on an American chestnut silviculture study in conjunction with Tim Phelps and Kim Steiner. Their objective is to examine the suitability of a range of native forest sites and various silvicultural methods for reforestation of American chestnut for the eventual planting of blight resistant seedlings in a forest setting.

American chestnut silviculture study. A second round of trials for the direct seed study was begun at seven sites across central PA, as replicates of those started in 2001. The seven sites varied in soil type, elevation, aspect and competing vegetation. All sites were established in fenced areas that were cleared before planting. Fifty seeds were planted at each site. After two years, growth in the 2001 trial has been poor at all sites often due to competition from hay-scented fern and/or deer browse. Several sites suffered heavy seedling predation by rodents. These trials will be monitored for 5-6 years or until they succumb to chestnut blight.

American-Chinese chestnut B_2F_2 generation evaluation. A plantation was established in an open field near State College in 1996 with seedlings from seven seedlots from TACF's farm. The plantation is a progeny test of B_2F_2, although the trees were open-pollinated, so it is impossible to state they are BC_2F_2. There were significant differences among the seven seedlots with respect to mean height.

Molecular cytogenetics for selection in backcross generations. The objective of this study is to develop a cytological procedure for screening trees or seedlings in backcross generation individuals with the least amount of *C. mollissima* genetic material. Vegetative buds from dormant and forced shoots, roots harvested from seedlings grown in soil and root tips from seeds germinated under sterile conditions have been studied to see which provides the most suitable source of chromosomes. Root tips from newly germinated seeds proved best. The main limitations in chestnut cytogenetics are that root tips are thick and difficult to squash and seeds are usually few in number. PA chestnut cooperators harvested thousands of open-pollinated seed for this project. Seeds are obtained from American, Chinese and hybrid trees for use in the development of the genomic *in situ* hybridization (GISH) technique for chestnut. Hybridization of labeled nucleic acids probes are added to denatured chestnut chromosomes and fixed to glass slides. Probes are detected by fluorescence microscopy and the images are captured digitally. The GISH procedure will allow for visualizing Chinese chromosome fragments in chromosomes of advanced backcross populations. After a GISH protocol is developed for chestnuts, they will begin to test the effectiveness to directly select for the smallest amounts of the Chinese genome in progeny of the BC_3 generation.

Chuck Rhoades, University of Kentucky

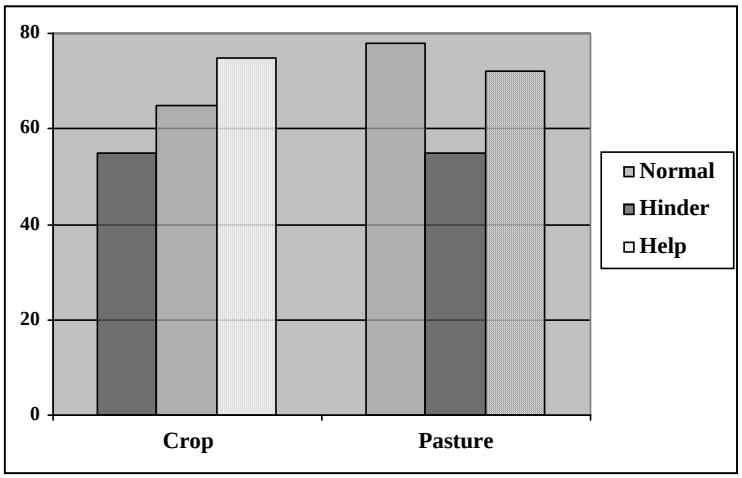
Soil factors influencing root disease. Rhoades is working in conjunction with Fred Hebard at TACF's Price farm to look at areas of the farm with high chestnut mortality. Some areas are very good for chestnut growth while other areas are poor. Previous land-use history is important. Areas that were previously pastureland give good growth while long-term cropping usage results in poor growth due to a compacted plow pan. Previous cropped areas were planted to tobacco and corn. Cropped lands tend to be more acidic, have more organic matter and have no depth trend. Phosphorous levels are not different in crop versus pastureland. The pilot study objectives were:

- Evaluate combinations of physical, chemical, and biological soil conditions that inhibit or enhance root disease in chestnut plantings.
- Assess individual treatment components in compliment greenhouse and subsequent field experimentation.

Soil treatments were: (1) normal growth; (2) hinder growth-root disease; and (3) improve growth.

Improve aeration/drainage	*Reduce aeration/drainage*
-perlite usage	-compact soil
-irrigation	-heavy irrigation
-mesh landscape fiber	-plastic mulch
-mycorrhizae spores	-Benlate to reduce mycorrhizae

Results from this study were mixed as shown by the following graph.



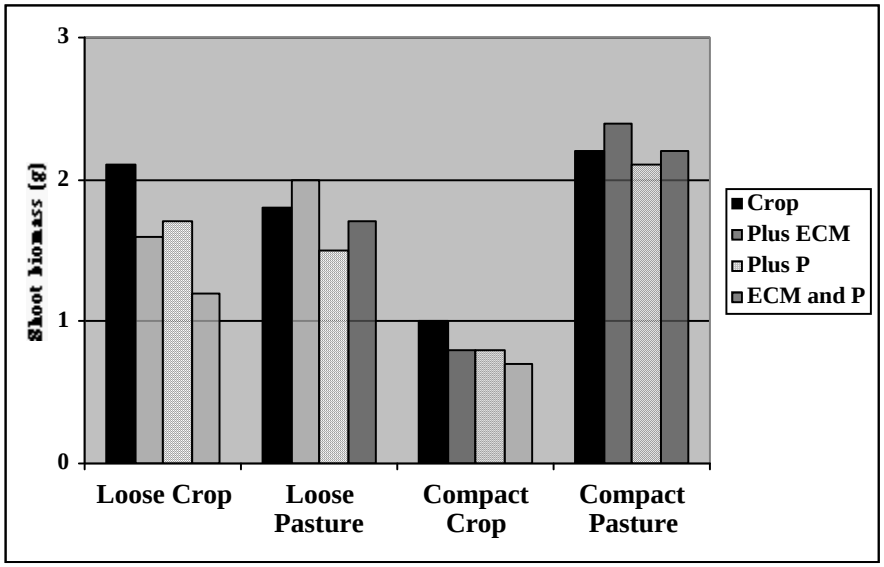
Field Seedling Survival (harvested 1200 seedlings)

Also confounding was seedling height. Seedlings in the 'hinder' treatment were significantly taller than the 'help' treatment.

Assessment of disease and mycorrhizae was done visually. Root necrosis was not found in 'normal' or 'helpful' treatments. Similar mycorrhizal infection was found between 'normal', 'unamended soil' and 'mycorrhizal-amended soil'. They only found mycorrhizal infection on 1/3 of the trees to which mycorrhizae was added. Good-looking trees may have no mycorrhizae while poor-looking trees may have plenty of mycorrhizal infections. They could not detect mycorrhizal infection based on tree morphology or growth of seedlings. Upon examining roots, there seems to be more than one species of mycorrhizal fungi. Preliminary evidence from the field plots—*Phytophthora* was found but they could not identify the species. Initial results from the mycorrhizae study are as follows:

- Mycorrhizae amendments were ineffective at altering colonization.
- Effects on initial seedling growth were less clear.
- Interaction effect on seedling survival; increased survival in cropped soils with both treatments.

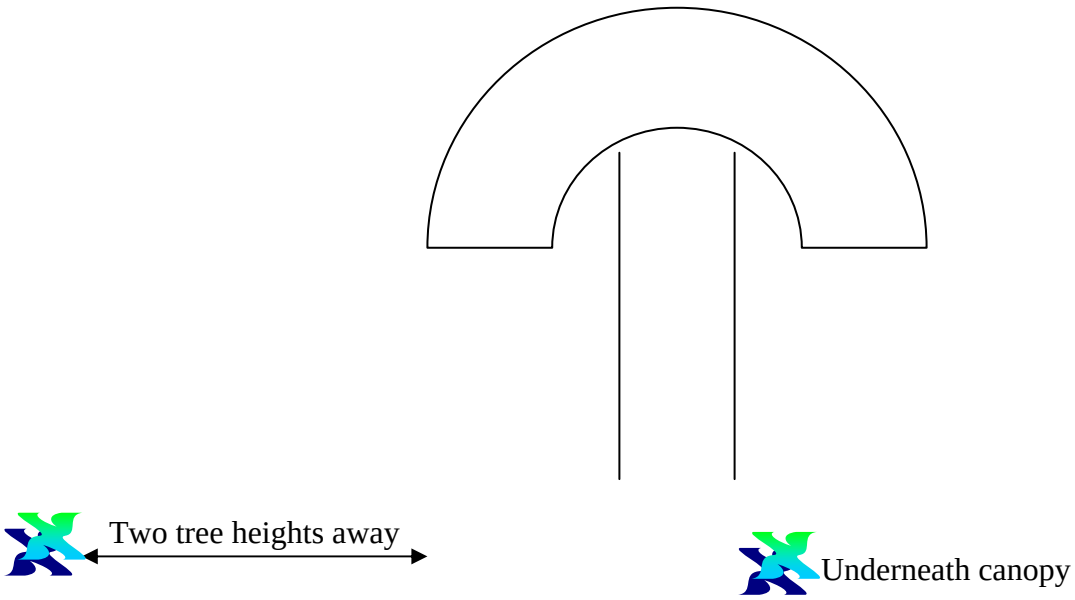
Greenhouse study. Three soils were used in growth tubes: (1) normal soil; (2) compact soil; and, (3) loosened soil. Results indicated that pasture soils performed much better than the crop soils and loosened soils resulted in poor growth. They then looked at mycorrhizae (ECM) and phosphorous amendments to see what effect they had on soil and root biomass. Four soil treatments (loose crop; loose pasture; compact crop; compact



pasture) were used along with four amendments (crop; plus ECM; plus phosphorous; plus ECM and phosphorous). The data for shoot growth is found in the graph below.

West Salem soil study. Rhoades is examining the effects of soil properties on individual American chestnut trees at West Salem. These properties may effect succession. West Salem is in the driftless area of Wisconsin. The objective of this study is to assess the effect of individual American chestnut trees on mineral soil, chemical and biological properties.

Mineral soil samples and litter samples were taken in a single-tree sampling design that is shown below.



The sites were broken into aspects (North, West and East). The north sites were much more mesic. There were eight individual plots; four on the upper slope and four on the lower slope. Five trees were sampled at each site and he looked at total carbon and nitrogen. Total C&N values were much richer on the upper slopes. Similar findings were made for magnesium and potassium. He found an interesting textural analysis; the lower slopes were much sandier and the upper sites were richer for ammonia. With regard to chestnut effects, in the upper sites, American chestnut is not affecting the total carbon while on the lower sites, American chestnut is affecting total carbon. There was a consistent increase across all sites for ammonia and nitrate, but the largest increase was on a south lower slope with the highest silt. Preliminary findings are:

- Chestnut effects vary between slope positions but not aspect.
- There is an increase in cations, phosphorous and total carbon and nitrogen and plant available nitrogen on lower slopes.
- Chestnut effects related to soil texture; the effect increases with sand and decreases with silt.

He hopes to continue to: (1) characterize litter chemistry; (2) examine chestnut litter dynamics on various soil textures; (3) characterize the soil microbial community; and, (4) relate soil changes to understory communities and forest regeneration.

Dennis Fulbright, Michigan State University

Cultivar trial. Ten years ago he put out a cultivar trial of edible chestnuts. Area farmers were planting nuts and they had poor production once the trees began to bear fruit. Fulbright suggested they plant cultivars but he did not know what specific cultivars to suggest. Thus, he began the trial to test various cultivars. The field trial was planted in 1992 in Benton Harbor, MI. A 10-year-old tree should yield about 20 pounds of nuts/tree. He found that 'Colossal' had significantly more yield than, 'Revival', 'Eaton', 'Williamette' or 'Mossbargger'. A pollination study was initiated in 2000 to determine if pollination would be a problem in a two-cultivar orchard. There was plenty of pollen but the burrs fell off unpollinated. Up until now, all Michigan orchards had either seedlings or multiple cultivars such as in the cultivar trial. He raised the question, "where is all the pollen?" A 'Colossal' orchard, established on the MSU campus in 1997, was planted on a 27' spacing with the variety, 'Nevada' as the pollinator. Fulbright showed that trees closest to the pollinator trees had the highest yield of chestnuts. Overall, trees yielded four pounds of nuts/tree, but close-by trees yielded 13 pounds of chestnuts on average. This data points to planting more pollinator trees as the sphere of pollen drops off about 50-60' from the source tree. He thinks pollen is mostly wind-driven as opposed to insects.

Restaurant trial. Chinese, 'Colossal' and other chestnut varieties were supplied to restaurant chefs in Michigan for their use. The chestnuts were scored, peeled and vacuum packed. Fresh chestnuts were supplied also for use in various recipes. Chefs then rate chestnuts that they used in various recipes.

Chestnut Peeler. Michigan State University has purchased a chestnut peeler from Italy. This 2001 purchase was funded by: (1) USDA World Development Grant; (2) State Marketing Improvement Program; and, (3) Michigan State University. Fulbright showed pictures of the peeling process. The chestnuts go into a kiln where the shell is burned. The nuts then hit the thrasher and then a steam parbioler. The last stages of the process include a scrubber to pull of the pellicle; the final stage is a brush.

Irradiated trees. In the 1950s, chestnuts were irradiated with cobalt. Everywhere the irradiated trees are planted, the trees have large healing cankers with an overproduction of callus tissue. Trees were planted at: The National Colonial Farm, Accokeek, MD; Sugarloaf Mountain, Dickerson, MD; Bob Evans Farm, Rio Grande, OH; Virgil Down, Mansfield, OH; and, Milan, OH. In the late 1980s, an experiment was begun where irradiated trees were inoculated with the standard virulent isolate, Ep 155. Seeds were collected from those trees and the nuts were planted at the National Colonial Farm and at Jackson, MI. Of the 1248 trees that were planted at Jackson, 1010 survived. These trees are large enough to inoculate with Ep 155, but the inoculations did not take. Fulbright is trying to determine if the increased callus production is a result of where the trees are planted. He does not believe there is any hypovirulence factor involved because he has collected hundreds of samples and the apple tests all indicated the isolates were normal. His Jackson planting is now fenced in to keep out deer. These trees were inoculated again in 2002 using the 'near base' technique utilized by Hebard.

<u>OBJECTIVE 2</u>: To better understand the interactions and ecology of the host/pathogen/parasite system at the molecular, organismal and environmental levels in order to develop effective biological controls for chestnut blight.

Sandra Anagnostakis, Connecticut Agricultural Experiment Station

Transgenic release. The first transgenic hypovirulent *C. parasitica* release was made in 1994 as a single-season experiment in the Housatonic State Forest. She has been following blight since the 1994 release; in general, the experiment has done poorly. Twenty-four experimental trees were examined in October 2002 along with 241 numbered chestnut sprouts. It was difficult to find any living trees larger than 1" dbh. She only took two samples from the plot because she limited herself to cankers on sprouts. Both isolates were orange and both contained dsRNA.

The second transgenic release was made in 1997 in the Meshomasic State Forest. Transgenic strains were sprayed in suspensions of water (10^{10}-10^{2} conidia/ml). A control plot was sprayed with water. Suspensions were sprayed for 3 years. Both the control and treated plots were examined in 2002. Most of the chestnut sprouts in the treated plot were bigger than those in the control plot. Bark samples were taken from three cankers on live sprouts in the treated plot, six cankers on live sprouts in the control plot and from one canker just outside the treated plot for isolation of *C. parasitica*. All were orange in culture and all contained dsRNA. The isolates have been plated to a hygromycin medium to determine if the isolates are transgenic.

Report from Scott Schlarbaum. Anagnostakis and Schlarbaum have been looking at Ozark chinquapin (*Castanea pumila* var. *ozarkensis*) because they may have resistance to gall wasp. She is worried about the loss of chinqupin because they are very susceptible to blight; chinquapin may be lost before they can ascertain its gall wasp resistance. When Ozark chinquapin dies, it does not put out sprouts that have new root systems; they maintain growth via the existing root system. For this reason, they are dying out. Ozark chinquapin from the Ouachita National Forest in southern Oklahoma have been used to obtain seed that will be propagated in a Georgia nursery.

William MacDonald, West Virginia University

***West Salem update*.** Assessment of disease progress, spread of two hypoviruses and canker evaluation continues at the West Salem, WI site. Because of the ever-increasing task of sampling and assessment, twelve plots were established in 2001 in three areas of the stand: Disease Center; Disease Front; and, Beyond the Disease Front. In 2002, cankers within the plots were sampled and subjectively rated. As of mid-October, approximately 90% of the bark samples removed from cankers in May 2002 have been cultured and assessed for hypovirus content and vegetative compatibility. Findings for 2002 include:

- Seven-hundred-twenty-one of the 1330 cankers that exist in the 12 plots were sampled.
- CHV1-EURO 7 continues to be the most commonly identified hypovirus but CHV3-COLI11-1 still persists at the site.

- Hypovirus was associated with 87% of the cankers that received hypovirus treatment from 1992-1997.
- Eighty percent of non-treated cankers on trees with treated cankers acquired hypovirus.
- Non-treated cankers on non-treated trees acquire hypovirus but at a greatly reduced rate.
- Subjective canker ratings show improvement in canker morphology in successive years; this is most notable for trees where hypoviruses were applied.
- A nomenclature system has been adopted, in cooperation with Andy Jarosz, Michael Milgroom and Cristina McGuire, for vegetative compatibility groups at the West Salem site. All v-c groups have been designated with a WS number. The original v-c type (Bockenhauer) was designated WS-1, the Schomberg type as WS-2 and the Rhyme 133 type as WS-3. Nine additional vegetative compatibility groups were identified from isolates collected in 2000-2001 and designated WS-4 through WS-12. Ninety-five percent of the 2002 isolates are vegetatively compatible with the Bockenhauer strain (WS-1). Two WS-2 and 20 WS-3 also were identified in 2002 as were WS-5, WS-8, WS-10 and WS-12.
- To determine if cankers are infected by multiple v-c types, 61 cankers were selected for intense v-c screening. Eight-to-ten virulent isolates from each canker were paired among themselves on Bromocresol Green Medium. Only 3 cankers yielded more than one v-c type per canker.

Mark Double, West Virginia University

Introduction and evaluation of hypoviruses for the biological control of chestnut blight. In June 1998, ninety-one American chestnut trees (infected and uninfected) were identified in the Monogahela National Forest (MNF) near Parsons, WV to evaluate the effect of treating cankers with compatible or incompatible hypovirulent inoculum. Trees were examined twice each year (June and November) and bark samples removed from cankers when initially discovered. A resulting virulent isolate from each canker was paired in the laboratory with four hypovirulent isolates with broad conversion capacities. Cankers were then placed into one of three treatment groups based upon conversion results with the hypovirulent isolates. Group 1 cankers were scratch-treated with a hypovirulent isolate that converted the inciting virulent strain, *in vitro*. Group 2 cankers were scratch-treated with a hypovirulent isolate that did not convert the inciting strain, *in vitro*. Group 3 cankers were scratch-treated with water agar to serve as a control. Cankers were treated only once with their respective treatment inoculum and then rated on a subjective scale (1-3) as long as the trees remained alive. Conclusions are as follows:

- Nearly 300 cankers have arisen on the 93 test trees.
- Water agar-treated cankers produced consistently less callus than hypovirus-treated cankers.
- Host response to canker treatment was similar for cankers treated with compatible or incompatible hypovirulent inoculum.
- Spring-treated cankers responded marginally better to treatment than fall-treated cankers.

- Tree longevity is greater after 5 years for trees inoculated with compatible hypovirulent inoculum than with incompatible hypovirulent inoculum or water agar.
- The interval between canker discovery and treatment was too lengthy to provide the tree with adequate protection.

Dennis Fulbright, Michigan State University

Mitochondrial insert. Carmen Medina-Mora, a Ph.D student working on the mitochondrial insert, has quit the program to raise a family. Medina-Mora had been working on the role of a 973 bp mitochondrial insert. The Kellogg Forest trees on which this insert was found yield isolates that have reduced sporulation and enhanced senescence. Fulbright reported that the Bockenhauer strain (WS-1) from West Salem, WI has the mitochondrial insert. Double sent Fulbright ten West Salem isolates that were compatible with WS-1; of those ten, one isolate, 29-8, yielded many morphology types that were hypovirulent in the apple test. Both 'normal' and 'lobed' isolates of WS-1 contain the insert but Fulbright does not know what proportion of the mitochondria carry the insert. Isolates WS-2 and WS-3 also were checked and they do not contain the insert.

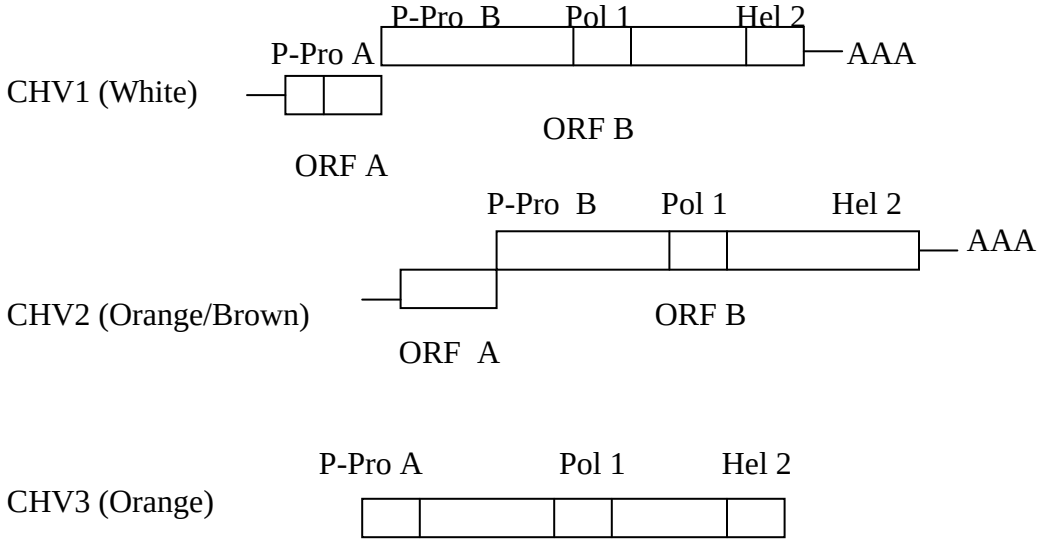
Bradley Hillman, Rutgers University

Hillman is working on viruses and transposons of *C. parasitica* with Nobuhiro Susuki at Okayama University in Kurashiki, Japan.

Properties of *C. parasitica* viruses are as follows:

- Cytoplasmic, transmissible by anastomosis.
- Most have no coat protein; dsRNA is in host-derived lipid vesicles.
- No extracellular component of the life cycle.
- Have widely varying effects on fungal host.
- Are phylogenetically diverse.
- Many viruses found in the fungus are asymptomatic.
- Genetically related to *Bymovirus* genus of plant-infecting *Potyviridae* family.

Hillman explained the genetic elements of *C. parasitica* as follows:



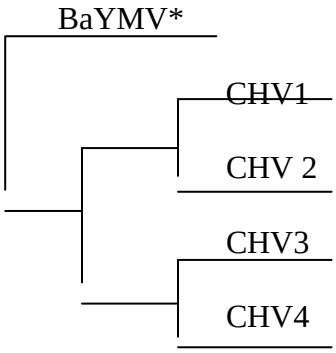
— — AAA

CHV4 (Orange) one open reading frame

The great prevalence of viruses in *C. parasitica* are hypovirulent. Only the top dsRNA band is genomic; others are accessory.

CHV4 was officially accepted at the Virology meetings in 2002. Hillman has finished the CHV4-SR2 sequence, in comparison with other hypoviruses. CHV-4 is most closely related to CHV3; only one open reading frame.

CHV1 (12.7 kb)—2 open reading frames
CHV2 (12.5 kb)—2 open reading frames
CHV3 (9.8 kb)—1 open reading frame
CHV4 (9.1 kb)—1 open reading frame



*BaYMV (barley yellow mosaic virus) is the most closely related virus to the Hypoviridae.

The sequences of CHV2 in NJ and China are nearly identical.

Findings with regard to CHV4:

- Proteases are not yet known.
- No morphological distinction with or without virus.
- Many are carried in 100% of conidia; the dsRNA titer in CHV4 is very low.
- They are the smallest of the hypoviruses.

Hillman is interested in:

- Proteases in GH2, it is very clear. However, in CHV4, the protease site is not clear; he cannot find good catalytic and cleavage sites.
- Glucosyltransferase—there is no such homologue in CHV1 or CHV2. It is found in CHV3 and CHV4. The essential signature of the sequence of glucosyltransferase is a pretty big enzyme. Fortunately, activity assays can be used with this system.

Glucosyltransferase homologue:

- Cellular enzyme is normally active inside the endoplasmic reticulum.
- Absent in RNA viruses, present in some DNA viruses of insects.

CHV4 population variability:

- He has examined eight isolates from Maryland, Michigan, Kentucky, New Jersey and Ep 102. Among the eight isolates at the 5' end, all isolates fall into the same clade

except the NJ isolate. Thus, there are two distinct groups—the NJ isolate and the majority of the population.
- CHV4 probably spread when the *C. parasitica* variability was low.
- It spread very slowly but now there are too many compatibility groups.
- The NJ population, an independent founder population of virus, resulted in a unique local population spread.
- No CHV4 has been found in Asia, so where did it come from?

While in Japan, Hillman is working on C-18 and 9-B-2-1. They have 11 dsRNA species. He first characterized C-18 but 9-B-2-1 is easier to work with. They do lose dsRNA through ascospores and 9-B-2-1 is found in only 5% of conidia.

Similarity of 9-B-2-1 to Reovirus:
- 5 of 10 clones show similarity to *Coltivirus* genus of *Reoviridae*.
- 2 *Coltivirus* species were sequenced: Colorado Tick Fever virus and Eyach virus.
- Sequence similarity with *Rosellinia necatris* W370 virus.

Chrysoviridae:
- This virus was isolated in Japan form *Cryphonectria*, but not from *C. parasitica*.
- It was approved as a new virus family in 2002.
- There are 4 segments of dsRNA contained within rigid particles.

Alice Churchill, Boyce Thompson Institute

She is dissecting the pathway for the orange pigment biosynthesis in the chestnut blight fungus. She is looking at the structure and biological activity of anthraquinones (polyketide pigments).

What are the roles of anthraquinone pigments in the biology of *C. parasitica*?
- Chemical defense
- Antimicrobial
- Antiviral
- Cytotoxic
- Apoptosis induction (DNA fragmentation)
- Antioxidant
- Active O_2 production

In fungal development, how are anthraquinone pigments sythesized?

Emodin (yellow)	**Skyrin (orange)**	**Rugulosin (yellow)**
♦ chrysrophanol	♦ oxyskyrin	
♦ aloe-emodin		
♦ hydroxyemodin		

Predicted synthesis is:
Acetyl-CoA + 7-Malonyl-CoA➔ Emodinanthrone ➔ Emodin ➔ Chrysophanol ➔ Hydroxyemodin

Proposed biosynthesis:
Hydroxyemodin + Emodin ➔ Oxyskyrin

Emodin + Emodin ➔ Skyrin

Emodin is found everywhere:
- Medicinal plants (aloe)
- *Aspergillus terreus*
- *Heterodermia* (lichen)
- Rhubarb
- St. Johnswort
- *Senna* (ringworm bush)

Emodin has many biological activities:
- Antibacterial
- Antiseptic
- Antiviral
- Inhibitor of nitric oxide production

Biological activity of skyrin:
- Cytotoxic
- Antiviral
- Induce apoptosis
- Viral RNA transcription inhibitor

It is tempting to say these compounds have something to do with virulence. They are mostly produced in fruiting structures; thus, they are not produced when the fungus is actively growing. What happens when the virulence of the fungus is reduced—are the pigments altered?

The approach to answering these questions:
- Clone genes involved in pigment pathway.
- Knock out first genes in the biosynthetic pathway.
- Compare knockout strains with isogenic strains.

The approach to cloning the anthraquinone pigment gene:
- Gene tagging by instertional mutagenesis.
- Targeted PCR amplification.
- Functional complimentation of pigment mutants.

The polyketide pathway is the pathway that produces anthraquinones in fungi. Polyketide synthase gene (7-10kb) ➔ mRNA ➔ Polyketide Synthase ➔ Lovastatin.

Insertional mutagenesis was Churchill's choice for generating mutants. Non-related DNA was inserted into the fungus and screened for mutants that had changes in pigments. Fifteen-hundred transformants were screened and she found 15 mutants. The hope was that she could take some of the mutants and look at the DNA that flanks the

vector. She did sexual crosses with the mutants. Many of the vectors had multiple insertions. Most of the mutants did not cross well. They were used as females because they did not sporulate well enough to act as males. The bottom line was that she found no crosses in which the mutations were tagged.

HPLC method. A HPLC method was developed to quantify pigments. The wild type strain, Ep 155, had a high skyrin peak with no chrysophanol or emodin. Mutant Fp8 overproduced skyrin and had slight emodin. Cr1a (cream) has no skyrin or emodin. Ep 155 produces phloroglucinol-like compounds. Phloroglucinol compounds can act as floral-UV pigments. They can also act as insect toxins and feeding deterrents, antioxidants and they have biocontrol activity in *Pseudomonas fluorescens*. The cream isolate produced a lot of polar compounds; some had the same HPLC peaks as phloroglucinol.

Complimentation studies. She employed complementation of pigment mutant phenotype with cosmid library genomic DNA. The steps were:

- Introduced a mixed cosmid library.
- Protoplast transformation into Ep6W.
- Regenerated protoplasts and hygromycin selection.
- Cosmid rescue via lambda packaging.
- Transformation of cosmid p1.2cr9 into Ep6W.

There was a low frequency of pigmented transformants.

She repeated the above work at Boyce Thompson Institute and rescued PKS-like gene fragments cloned from *C. parasitica*. The best hits from the databank is to melanin. She ordered a cosmid library and probed the polyketide synthase with PKS genes. Complementation of Ep6W with groups of cosmids; she pulled out about 50 cosmids that hybridized to the PKS fragments. She then groups the cosmids:

- Sm1 (5 cosmids)
- Sm2 (5 cosmids)
- Sm3 (5 cosmids)
- B5 cosmid

The B5 cosmid complementation gives a brown phenotype. There was better complementation with Sm1, Sm2 and Sm3.

Don Nuss, University of Maryland Biotechnology Institute

Genomic organization and expression strategy of CHV1-Ep 713.

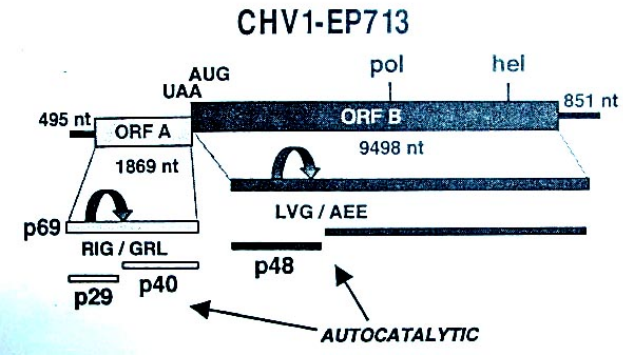
- dsRNA virus of 12 kb with two open reading frames.
- Does not encode any capsid and has no extracellular form.
- Significantly alters host phenotype.

He explained the use of cDNA clones.

Virus-free C. parasitica spheroplasts

Transfection *OR* *Transformation*
vector
Regeneration-take viral cDNA; with full-length
CHV1
make coding strand transcription and use electroporation Ep 713 DNA

The emerging functional map of the genome is shown below:

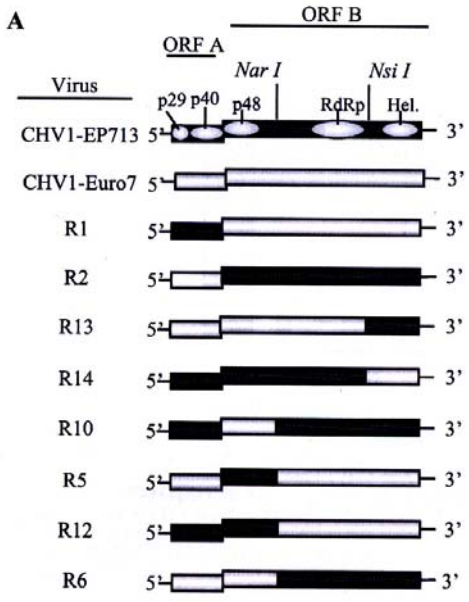


The following areas have been identified:

- p40—RNA accumulation
- p48—canker morphology
- ORF B—colony morphology

The first 24 codons of p29 are responsible for virus replication and p29 suppresses pigmentation and conidiation. It is not responsible for virulence attenuation.

Ep 713 and Euro 7 are similar enough that chimeras can be made as shown:



Hypovirus CHV1-Ep 713 shows extensive sequence similarity with CHV1-Euro 7 at both the amino acid level (90%-98% identity) and at the nucleotide level in the 5' and 3' noncoding sequences (93% identity). However, CHV1-Euro 7 and CHV1-Ep 713 confer distinct phenotypes on infected *C. parasitica* strains. CHV1-Ep 713 can be classified as a severe hypovirus strain, causing a greater reduction in fungal growth rate on synthetic

media and more severely attenuating both the development of spore-containing stromata and canker expansion on chestnut tissue than the milder CHV1-Euro 7 strain. Characterization of chimeric viruses indicates that the primary determinants responsible for the differences in symptom expression observed between CHV1-Ep 713 and CHV1-Euro 7 are located in ORF B.

Using deletion mutants, it was found that deletion of the p40 coding domain had a significant effect on virus-mediated alteration of host colony morphology. Progressive extensions of the p40 coding domain were used to address alteration of colony morphology. The domain responsible for the p40-mediated contribution to alteration of colony morphology can be defined within the region extending from Thr(288) to Arg(312). The effect of progressive p40 repair on fungal asexual sporulation paralleled that observed for colony morphology. Thus, the determinant for p40-mediated alteration of colony morphology colocalizes with the determinant that contributes to the suppression of fungal asexual sporulation. While △p40 mutants produce small cankers in excised stems, they do sporulate more than CHV1-Ep 713 as shown in the table below.

Fungal Strain or transfecting virus	No. of conidia/ml	Canker area (cm^2) at 4 wk
Ep 155	1.5×10^9	31.09 ± 5.92
CHV1-Ep 713	7.8×10^3	1.36 ± 0.11
△p29	5.3×10^3	1.34 ± 0.70
△p40a	2.0×10^7	3.27 ± 1.72

There is a direct correlation between restoration of p40-related suppressive activity and an increased level of viral RNA accumulation. There is strong evidence that the peptide sequence extending from Thr(288) to Arg(312) is responsible for the p40-mediated amplification in RNA accumulation. Also, p40 may have some role in the expression of ORF B.

A summary of p40 is as follows:

- Is dispensible for CHV1-Ep 713 replication.
- Is not required for hypovirulence.
- Does result in increased pigmentation, sporulation and viral RNA accumulation.
- Functional domain mapped to Thr(288) to Arg(312).
- Indirectly contributes to virus-mediated suppression of pigmentation and conidiation by providing an accessory function in hypovirus RNA amplification.

Field study at Meshomasic State Forest:

- Three indigenous *C. parasitica* strains representing two mating types and three different vegetative compatibility groups were transformed with a plasmid.
- Transgenic release was conducted over a 3-year period using a condial spray, 10^{12} spores/ml, delivered in a backpack mistblower.
- Control plot was sprayed with water.
- Transgenic CHV1-Ep 713 strains are ecologically unfit.

- No evidence of spread of transgenic strains or derived viral RNA to control plot or buffer zone.
- After examining 3,000 insects, they do not appear to be major vectors.
- Some transgenic *C. parasitica* isolates with atypical morphology were recovered.

Future plans for field studies:

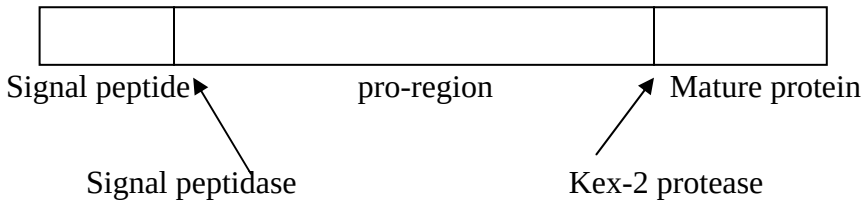
- Test performance of more ecologically fit transgenic hypovirus *C. parasitica* strains, transformed with CHV1-Euro 7 cDNA in a forest site in West Virginia.

Neal Van Alfen, University of California at Davis

The Van Alfen lab is looking at biochemical and cellular-level problems to determine how the virus affects the fungus. They know the virus inhibits protein secretion, sexual reproduction and pigment production. They have found three genes that are down-regulated by the virus. Many of these down-regulated products are enclosed in vesicles. The genes that are Kex-2 processed are:

- Cryparin (cell wall hydrophobin)—when it is deleted, virulence is not reduced, but stromatal pustules do not break the bark surface.
- Laccase
- MF1-1 pheromone

The three genes are similar in that their encoded proteins are processed and secreted by the same pathway.



The signal peptide directs information to the endoplasmic reticulum to the golgi body, etc. Van Alfen's lab used Pulse-Chase Analysis to follow cryparin. With regard to the cell wall, cryparin accumulates outside the cell wall in virulent isolates but not in hypovirulent isolates. In hypovirulent isolates, the cryparin accumulates inside the cell wall but it cannot get out of the cell.

The hypovirulent phenotype can be mimicked by a Kex-2 inhibitor. They are in the process of deleting Kex-2 to see what it does to phenotype. Is there any feedback going on? They have found that the cargo (proteins) builds up and signals the genes to stop transcribing.

Van Alfen's group continues to characterize vesicles. They used two isolates: Ep 67 (virulent) and Ep 802 (hypovirulent) and coated highly ordered vesicles (30-40 ηm). They have found that vesicles are contained in both virulent and hypovirulent isolates but they proliferate in hypovirulent strains. Clatherin is a structural protein that is highly conserved in eukaryotic organisms; it forms a triskeletal lattice around vesicles. Clatherin is enriched in virus-infected strains. It appears that the virus keeps the fungus in a juvenile stage longer, thereby interfering with the developmental process.

Proposed mechanism of viral interference:

- Proteins are carried to the cell wall.
- As the cargo in the vesicles builds up, somehow signals are sent back to reduce the cargo.
- Coded vesicles that contain virus interfere and affect normal cycling.

GFP-Green fluorescent protein. If fungus development is affected but not growth, at least one transport system must be affected. Van Alfen's group fused cryparin to GFP to look at secretion. They found secretion is not in young hyphae but in pycnidial initials. Most of the viral particles, however, are located in the hyphal tips. They will continue this work.

Business Meeting

Neither John Anderson, NE-140 Administrative Advisor nor Robert Noweirski, USDA-CSREES representative, were able to attend the meeting. Michael Gold, NE140 chair for 2003 also was not in attendance.

Bradley Hillman suggested Chuck Rhoades for the chair-elect position. Sandra Anagnostakis nominated Rhoades and Pam Kazmierczak seconded the motion. Rhoades was unanimously elected as 2003 chair-elect.

Anagnostakis read John Anderson's report; Anderson was unable to attend as Connecticut Governor Rowland restricted travel of all CT state employees. Anderson informed members that the current project terminates September 30, 2003. Anagnostakis reported that Michael Gold has already sent in the request to write a proposal entitled, "Biological improvement of chestnut by management of populations, pathogens pests and habitat restoration and its development as a horticultural crop." The request was approved at the Northeastern Agricultural Experiment Station Director's meeting held in March, 2002. The draft of the new proposal is due for review now.

- The proposal should not exceed 15 pages, excluding the bibliography.
- The committee members rewriting the proposal are: Michael Gold, Bradley Hillman, William MacDonald, Fred Hebard and Don Nuss.
- The new proposal needs to address all the following:
    - The need as indicated by stakeholders.
    - The importance of the work and what the consequences are if it is not done.
    - The technical feasibility of the research.
    - The advantages for doing the work as a multistate effort.
    - What the likely impacts will be from successfully completing the work.
- A "critical review" that summarizes the accomplishments of the current activities should be inserted in "related, current and previous work."
- The proposal must be finished by January 1, 2003 so that John Anderson can send it off for review. The NE-140 members also must provide a list of 5 potential reviewers. Anderson will telephone the reviewers personally.
- Reviews must be completed by February 2003.
- The termination report of the current project is due March 15, 2004; this report will summarize the accomplishments of the current 5-year project.

Dates suggested for the 2003 meeting, to be held in Missouri, were September 18-20 and September 12-14. These two dates will be given to Michael Gold from which to choose.

Milestones that were listed for 2001 in the previous proposal are as follows:

- Additional forest site test begun.
- Characterization of putative genetically transformed American chestnut embryogenic cultures completed.

- Field evaluation of confirmed transgenic chestnut trees containing single-transgenic constructs.

  The two goals for 2002 are:
- Sampling of 60 chestnut trees from each of 25 sites within 135-mile grid completed, to assess the level of diversity in wild American chestnut populations and to determine the geographic component of such diversity.
- Evaluation of transgenic hypovirulent strains of *C. parasitica* for biological control of chestnut blight.

Comments from the meeting:

- Bill MacDonald asked if the material being sent to Michael Gold is inclusive or generic.
- Sandra Anagnostakis stated that she sent in projects that she hopes to accomplish in the next five years but she did not include a bibliography.
- Bill MacDonald suggested that Ken Hunt ask Michael Gold to provide each cooperator with a deadline for the upcoming proposal. Bradley Hillman suggested November 10 as a deadline for everyone.
- Neal Van Alfen felt the proposed title is too long. Bradley Hillman asked if John Anderson thought the title could still be changed.
- Pam Kazmierczak read the 2001 milestones from the current proposal and then read Bill Powell's proposed outcomes from SUNY. Kazmierczak will contact Powell to address his current outcomes and how they fit into the final report.
- Bill MacDonald asked for a show of hands as to who will join the next project. Those indicating interest were: Bill MacDonald, Ken Hunt, Alice Churchill, Dennis Fulbright, John Carlson, Neal Van Alfen, Chuck Rhoades, Sandra Anagnostakis and Bradley Hillman. Don Nuss indicated he will try to join the project. The question was raised as to the intent of Fred Hebard, Scott Schlarbaum and Scott Merkle. Sandra Anaganostakis assumes all will join. Bill MacDonald suggested Purdue University and Clemson University might be interested in joining the project as both institutions were funded by TACF for 2003. MacDonald agreed to contact individuals at both institutions.

The meeting was adjourned at 4:00 pm on October 26, 2002. Following the meeting, George and Suzette Canfield of Winters, CA hosted a BBQ for the NE-140 group. Many members attended a day-long tour of the Napa Valley on October 27, a trip arranged by Pam Kazmierczak and Deborah Golino.

The URL for the NE-140 Home Page is:
http://www.agnr.umd.edu/userforms/nera/projects

Minutes respectfully submitted,

Mark Double

West Virginia University
November, 2002