Minutes of
NE-140 Technical Committee Meeting
Biological Improvement of Chestnut (Castanea sp.)
Mountain Lake, Virginia
October 7-9, 1986

Attendance

Project leaders or representatives from participating stations or agencies:

Connecticut:	Dr. J. E. Elliston*
Kentucky:	Dr. L. Shain
Michigan:	Dr. D. W. Fulbright
USDA-FS:	Dr. E. G. Kuhlman, Mr. H. C. Smith*
USDA-CSRS:	Dr. J. M. Barnes
USDA-SEA:	Dr. J. Payne*
Virginia:	Dr. G. J. Griffin**
West Virginia:	Dr. W. L. MacDonald

*Not in Attendance **Committee Chairman

Administrative Advisor: Dean Stephen J. Kleinschuster, Rutgers University.

Other participants:

American Chestnut Foundation:	Mr. P. Rutter
Chestnut Hill Nursery:	Ms. D. Gaw, Mr. R. Wallace
Connecticut:	Dr. S. Anagnostakis
Kentucky:	Dr. S. Hiremath, Mr. K. Scibilia, Mr. J. Miller
Minnesota:	Dr. C. Burnham, Dr. D. French
Ontario:	Dr. C. McKeen
Tennessee:	Dr. S. Schlarbaum
Virginia:	Dr. R. Stipes, Dr. M. Roane, Dr. A. Dietz
USDA-FS:	Dr. J. Micales
West Virginia:	Dr. D. Hindal, Dr. J. Elkins, Dr. W. Kaczmarczyk, Mr. R. Keys, Mr. M. Double, Mr. S. Jakobi, Mr. J. Newhouse, Mr. R. Bennett, Mr. M. Likins

The annual meeting of NE-140 was called to order by Chairman Griffin at 1:15 pm on October 7 at the Mountain Lake Resort. After brief introductory remarks about this area of Virginia, Dr. Laurence Moore, Chairman Plant Pathology, Physiology and Weed Sciences at VPI and SU, welcomed us to Virginia and described the Plant Pathology program at Virginia Tech.

STATION REPORTS

Kentucky:

Shiv Hiremath

Using Ep 713, a French strain, he found five different dsRNA bands that stack up in 3% gels, but are separated in 5% gels. He electrophoretically collected the large dsRNA bands and treated with RNAse T1, and layered on a 20% gel and got the same electrophoretic pattern. To

He felt that hv conidia can protect trees up to 10 weeks. He also put grease around chestnut stems, funneled rain water into milk jugs and found no correlation between the size of a tree and the amount of rain water collected, but there was a correlation for the area in the forest; more open grown stems had more water wash from them than closed area stems.

Lou Shain

Initiated cankers with a virulent strain, allowed them to grow and then inoculated with one plug of a compatible hv. He used a methionine-requiring marker, and collected cankers 3, 6, and 9 weeks after conversion and induced cirrhi and isolated conidia and mycelium from underneath the cirrhi. He found that dsRNA initially moved around the periphery and then into the center of the canker. At no time (even up to 9 weeks) did he recover any hv mycelium from beneath the cirrhi, even though he did recover hv conidia from cirrhi.

Michigan:

Dennis Fulbright

Followed canker development over time by photos at several Michigan sites. He reported on Sally Garrod's work where she made wounds (225) below artificial inoculum sources, and found evidence of Ep in wounds that did not have cankers. Cankers also developed a number of years after the wounds were made.

He incorporated dsRNA from RC1 and GH2 in a single strain, and single-spored that strain and got several morphology types: virulent; RC1-like; and GH2-like, and some debilitated isolates. These debilitated isolates would only yield RC1 dsRNA banding patterns, but upon their single sporing that isolate, he could recover isolates which had both banding patterns (GH2 and RC1). He questioned why the dsRNA expression is masked?

He has a small plasmid (4 kb) in DNA of the CL1 strain which he is starting to work with.

Roche Institute:

Don Nuss and Jim Tartaglia

Conducted a structural analysis of GH2 and found the following:

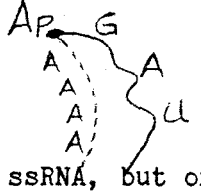
Large band	9.0 kb
Middle band	3.5 kb
Small band	0.9 kb

Using the dot blot technique the found the following homology pattern:

	L	M	S
I	•	•	
II			•

get the actual sequence, he used 2-dimensional gel electrophoresis:

25% gel, at pH 8.3
10% gel, at Ph 3.5
Yielded at poly A tail as shown:



To confirm 3' end, used S1 nuclease to get rid of ssRNA, but only got one strand labeled, and it was poly U-the other strand was not labeled and may represent structural problems. To check nucleotides at 3' and 5' ends, he subjected them to complete digestion and purified each oligonucleotide and ran them on gels, and the consensus of all five bands was as follows:

5'-...GAGCUCACUAUGAUGAUCCCAUAAAGA-3'

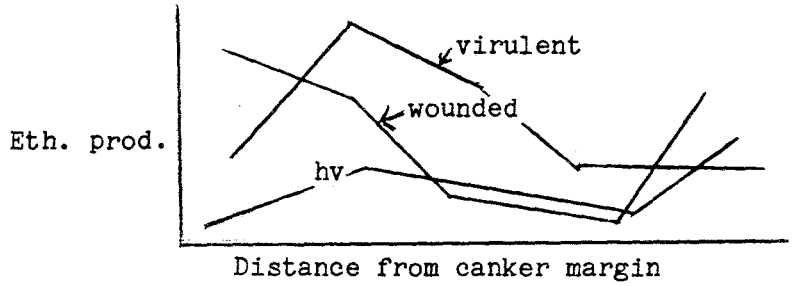
He made a small probe (190 bases), don't know where it is in the genome, but it hydridizes to all 5 Ep 713 bands; none preferentially. He thinks there is one large RNA that has internal deletions to form the smaller ones.

He reported that Ghabrial has been denaturing dsRNA with methylmercury and is trying to translate the entire dsRNA.

Fred Hebard

Measuring ethylene production-he takes bark plugs from a canker and places them in a vacuum tube, lets them sit for 24 hours, and then pulls out one ml for GC analysis. He measured ethylene production from the canker margin and on out from the canker. Ethylene production drops dramatically 3 cm from canker. He tested the production of ethylene after conversion of the canker:

-saw no effects up to 27 days after conversion
-he sampled repeatedly from the same canker



After 19 days he gets a flattening out of the all curves. He stated that he felt ethylene production occurs very early in the infection process.

Kevin Scibilia

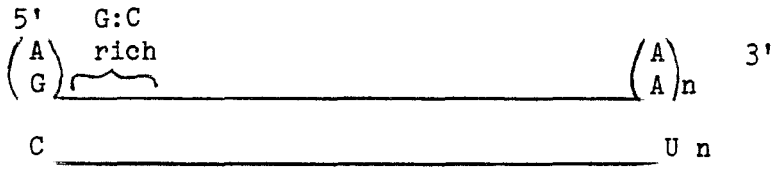
Initiated virulent cankers before and after spraying stems with hv conidia and found that conversion would occur as a result of persistant hv inoculum.

Weeks before/after spray	% virulent cankers converted
-3	83
1	58
2	63
3	50

They think that the middle and small bands are interal deletions of the larger band. They used RNAse T1, a guanine specific nuclease and found the guanine ratios at the 3' end are similar for the large and small bands They exposed the RNAse T1 material to various enzymes:

	Nondenatured	Denatured
Pancreatic RNAse	-	-
RNAse U2	-	+
Oligo(d-t) 12-18 + RNAse H	-	+

All three species (large, middle, and small) have the GC rich region at the 5' end:



Theyplan to look at various gene products,andexamine the role of replicative forms of ssRNA.

Connecticut:

Sandy Anagnostakis

Has a 2 acre plot with 200 trees that she keeps track of v-c types and sprays cankers weekly with conidial spray that contains 4 hv types. She feels recombination is occurring as evidenced by the new v-c types that are arising.

She said she is still recommending mud packs for people that call in with canker problems. She also found that compost works if left on at least 2 months, and if the canker is more than half way around, this treatment won't work.

She examined four Rocky Hill isolates and suggests a temperature sensitive locus for vegetative compatibility that gives the following responses:

28 C gave liners
30 C gave a good barrage

She germinated scarlet oak acorns, then pulled off a leaf and inoculated leaf scar with Ep and got no infections, but when 2mm cuts were made on on the stem, cankers developed. The fungus grew up and down from the inoculation site, and large swollen areas were produced, but the seedlings were not killed.

Castanea seguini (a dwarf tree which flowers in less than one year-they are only one foot high). She grafted Rocky Hill chestnuts on the C. seguini root stock and is interested in these grafted trees for susceptibility and dwarfing.

West Virginia:

Walt Kaczmarczyk

Ep 43 found mostly linear molecules with 5% circular portions
-enzymatically digested Ep 43 and examined by HPLC and got a high peak for guanine, and the following are the base relationships:

Bases		
U	11.6%	
G	30.3%	2 to 2 1/2 times more A than U
C	30.1%	
A	27.9%	

They found at least six modified bases-the major one was methylated guanine

They treated Ep 43 dsRNA with several enzymes (separately) B-glucuronidase, pronase and ribonuclease S1 to see if there was any change in migration-they used both PAGE and agarose. They began seeing a low molecular weight band with the S1 treatment (200,000).

	Length in kb	M.W. in daltons (x100,000)
Slow species	6.7	6.7
Fast " "	4.2	6.0
New " "	?	0.2

He reported on Kathy Harper-Morris's work. She was interested in the effect of the dsRNA on protein synthesis. She looked at 4 isolates-two with dsRNA and two without. She looked at the % ribosomes that were polyribosomes and saw no differences among v or hv-they all were around 75%.

		Poly A+ mRNA extracted	
Strain	dsRNA	ug RNA/g tissue	
Ep 421	-	119.05 ab	
Ep 811	+	148.12 a	The 2 hv's produced
Ep 523	-	81.16 b	more poly A
Ep 524	+	153.22 a	

Joe Newhouse

Reported on EM observations of virus-like particles (VLPs) in hyphae and conidiaof European hypovirulent strain Ep-50, but not in its dsRNA-free counterpart Ep-67. Likewise, North American hv strain GH-2 contained many scattered vesicles in its hyphae, while dsRNA-free GH-2 E_4 strain did not. Also observed anastomosis areas between vegetatively compatible strains Ep-50 (hv) and Ep 15-7-7 (v). The Ep-50 hypha contained many aggregrates of VLPs, and some were seen in a small anastomosis bridge. Observations of anastomosis areas between vegetatively incompatible strains Ep-50 (hv) and Ep 7-5-1 (v) showed degeneration and collapse of the hypha attempting to anastomose.

Dale Hindal

Described his studies of radial growth; sporulation; orange, brown and purple pigment production; formation of concentric zones; reactions to the spot test reagents gum guaiac, naphthol and syringaldezine, and the reaction of the pH indicator bromocresol green among 125 strains of Ep. He

analyzed all data to determine if any of the cultural parameters were associated with the presence or absence of dsRNA. Cultural parameters were variable and affected by the medium used, the incubation environment and the strain of the fungus. Statistical analyses have not shown any relationship between the cultural characters and the dsRNA content of the strain.

Rick Bennett

Discussed his work with calcium oxalate in healthy American chestnut bark and bark inoculated with either dsRNA-free or dsRNA-containing E. parasitica strains. Oxalate levels in necrotic regions of cankers generally were slightly higher than in healthy bark, but not as great as at the advancing margins of the canker and were independent of the presence of dsRNA in the fungus.

Steve Jacobi

Described a dissemination experiment using v and hv strains of E. parasitica that are being introduced in an 8-year-old clearcut with abundant American chestnut. European and American dsRNA-containing strains, and a virulent brown dsRNA-free strain, were established in Oct., 1985 using a centrally located inoculum source. Each of the 216 trees in the study received 4 punch and 1 branch wound at three-month intervals for one year. Of the total 4320 wounds, only 7 were colonized by E. parasitica but four of the 72 naturally occurring cankers found to date appear to have disseminated from the inoculum source.

Mike Likins/Dale Hindal (reporting for Adam Michna)

Reported on studies that have been initiated to compare the function of the dsRNA in the E. parasitica population at three sites in Michigan and West Virginia. The E. parasitica population in two sites in Michigan contains dsRNA; these sites are recovering. The third site is dsRNA-free. The population of the fungus in all three sites in West Virginia contain a mixture of dsRNA-free and dsRNA-containing strains, and there is no evidence of recovery. Sexual and asexual reproduction is being compared among these sites, and work to date indicated asexual sporulation among cankers collected from an apparently dsRNA-free site in Michigan (Kellogg) is quite variable (ranging from 0 to 25 pycnidial stroma per cm^2). Early results suggest asexual sporulation of West Virginia cankers is higher. The distribution of dsRNA in cankers, the capacity of the dsRNA to be transmitted to asexual and sexual progeny as well as the vegetative compatibility of single conidial isolates also will be compared between the Michigan and West Virginia populations.

Mark Double

The spread of European and American v and hv isolates, after their introduction into an American chestnut sprout stand, has been followed over a 4-year period. This study includes 20 v, or 100 hv inoculated and 120 noninoculated stems that were evaluated twice each year (May and November) by sampling new infections to detect the dissemination of inoculum. The rate of canker development was similar on inoculated and noninoculated stems, although hv cankers were seldom found on noninoculated stems. Most significant is that v inoculation sites were converted to hv by the introduced (vegetatively compatible) hv strain.

Bill MacDonald

Attempted to generate discussion by hypothesizing how hypovirulence may become established in the Endothia population. No discussion resulted. Either the hour was too late or the stimulus was lacking!

Roy Keys

Described shoot multiplication of juvenile American chestnut using a medium consisting of Heller's macronutrients and Murashige and Skoog micronutrients and organic constituents. The addition of 132 mg/l $(NH_4)_2SO_4$ and 350 mg/l $Ca(NO_3)_x$ resulted in significantly better elongation of the shoots. The latter medium also was the best for American chestnut shoots from mature trees. Shoot cultures of hybrid chestnuts, however, grew best on a Chevre medium. Shoot multiplication of mature American and hybrid cultures was greatest using 1×10^{-6} M BAP. Woody plant medium was inferior for all of the tissue types.

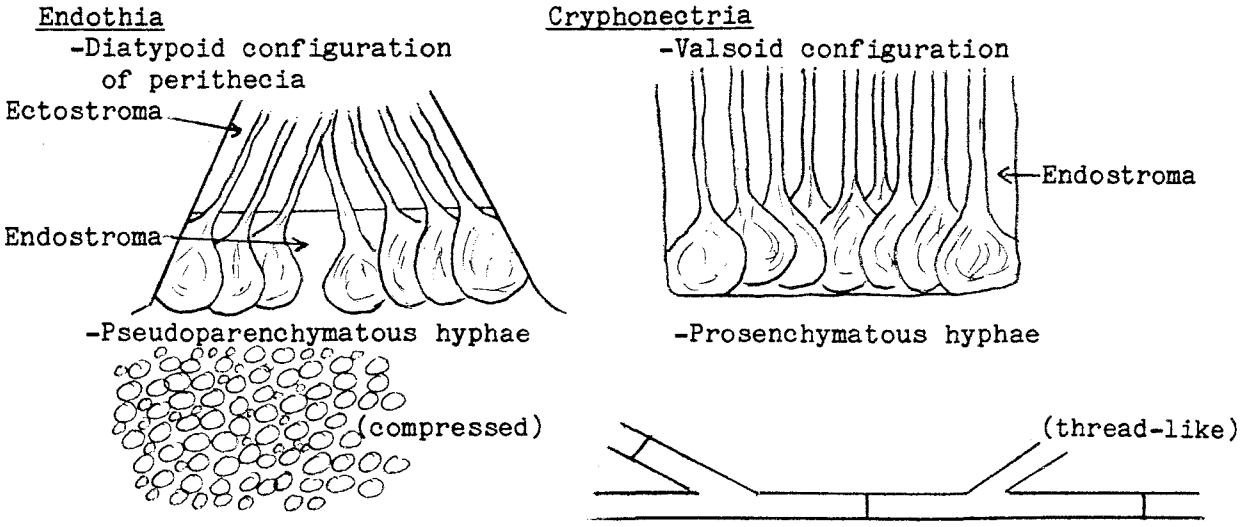
Rooting was accomplished by dipping the shoot base in an IBA solution, 3 g/l being the best concentration. Rooting of hybrid shoots was affected by the nutrient medium on which the shoots were grown, and by prior culture conditions. Rooting was affected by clone and age of the parent material. Shoot tip necrosis continues to be the major obstacle in the successful transfer of plantlets to soil.

USDA-FS Forest Products Lab:

Jesse Micales

Compared criteria for Endothia/Cryphonectria systematics:

Traditional Classification	Barr, 1978
-Size & color of stroma	-Arrang. of perithecia
-Size of perithecia	-Stromatic tissue type
-Size and shape of ascospores	-Shape and septation of ascospores



Sensitivity of certain (antibiotics) fungitoxicants. She found C. parasitica is more resistant to cyclohexamide than E. gyrosa.

E. gyrosa is sensitive to <0.1 ug/ml
C. parasitica is resistant to >0.1 ug/ml

Some of the fungitoxicants which she tested:

Chlortetracycline	PCNB	Pimaricin
Nystatin	Dicloran	Iprodione
Captan	Mancozeb	MBC phosphate

Also she tested 19 enzyme systems and found the isozyme pattern to be distinctly different for Endothia and Cryphonectria.

Virginia Tech:

Jay Stipes

When pin oak becomes water stressed, E. gyrosa can grow faster in oak than it can in a petri dish.

He never failed to get E. parasitica out of swollen butts of scarlet oaks. E. parasitica is now a problem on live oak-he plans to sample a number of live oaks, type them for v-c groups and spray with a hv conidial spray.

Gary Griffin/John Elkins

Conducted a field trip to examine various chestnut plantings at Mountain Lake as well as on the Virginia Tech campus. They described the crosses and purposes of various settings where they have established trees.

Ontario:

Colin McKeen

One to two million chestnut trees in Ontario before the blight, and only a few "bush" areas are left due to deforestation. The largest chestnut tree in Ontario is 27" dbh, 50-60' tall, growing about 2/3" per year. It has a number of large cankers. He has found dsRNA in some of the cankers (dsRNA done at MSU). He is trying to get a seed orchard started for germplasm. He uses Granny Smith apples for pathogenicity tests.

USDA-FS:

George Kuhlman

Reported on his hv-spray study to control dieback with compatible hv isolates. He identified hv isolates with broad conversion capacities, and introduced these hv isolates into sprout clumps by straying the entire tree (as far as was practical with a hand-held sprayer) with a mixture of 11 hv isolates. The test for success was less dieback than the controls. Stems were sprayed once a year to run-off.

		% clumps with killed sprouts	
Year	Spores/ml	Hv-treated	Water check
82	3,700,000	24	29
83	6,000,000	15	14
84	22,275,000	31	34
85	33,460,000	39	47

Cumulative number of clumps with blight

Year	Hv-clumps	Water-clumps
82	47	36
83	53	49
84	67	62
85	76	76
86	80	88

Suggested that failure may be due to any of the following:

1. the kind of spray-only water, no sticker
2. the time of the spray
3. the number of v-c types
4. weather
5. disease incidence too great
6. inoculum density
7. virulent more successful than hypovirulent
8. hope not fact

Minnesota:

Charles Burnham

Discussed the inheritance of blight resistance. He reviewed the data from the historical breeding program and felt that those workers had taken the wrong approach. He believes that the Chinese chestnut X American crosses should be backcrossed to American chestnut, and with subsequent backcrosses to American, blight resistance can be incorporated with the form of a true American tree.

Hypothesized backcrossing:

	Recurrent parent in % (American)
F1	50%
First backcross progeny	75%
Second " "	87.5%
Third " "	93.75%

In order to restore the American chestnut we need trees that can grow in the natural range and therefore the germplasm of some of the natural trees must be preserved.

American Chestnut Foundation:

Phil Rutter

Described briefly the objectives and goals of the American Chestnut Foundation. He encouraged support from NE-140 members.

BUSINESS MEETING

1. Timetable for NE-140 revision was discussed.
2. Deadline for annual reports to Dr. Griffin set for November 7, 1986.
3. In the future each station should bring a copy of their station report to the annual NE-140 meeting for distribution.