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Editor's Notes

Journal Winter/Spring 1994

Over the centuries writers have often likened great efforts to races - foot races, the tortoise and the hare, relay races, and biathlons and triathlons.

The work before the members, directors, and scientists of The American Chestnut Foundation has all the potential, spirit and confidence of arctic teams mushing in the Iditerod. What a challenge! To cover 1,500 arctic miles by dog sled on the strength of a vision and a team, that despite the cold and storms, always pulls together.

Restoring the American chestnut tree is the same kind of grand enterprise, requiring considerable imagination and perseverance - but stretched over decades, not just miles. As we leave behind our 10th year and enter a new phase, ACF's ability to mush on will depend largely on how well we communicate and inspire each other - daily among ourselves, to the general public and to the next generation.

For this reason we plan to infuse The Journal with new life beginning this summer. The biggest changes will be in format: expect to see more artwork and photographs, new print types and a range of page designs.

The character of the writing will likely change too as we aim to reach the greatest breadth of our membership: readers will find recollections and stories that capture the soul of our work and solid scientific reports of work in progress.

Board members have suggested new ideas for distribution as well, all with an eye to reaching curious ears that have heard little more about the American chestnut than once upon a time chestnuts were roasted by an open fire...

There will also be opportunities for members to contribute to The Journal, in the form of letters and photographs, plus some new ways as well.

As we make changes please forgive the errors and omissions that are bound to occur in any transition. Know simply we have had a strong start and will continue to gain ground for generations to come!

Meadowview Notes for 1992

F.V. Hebard
Wagner Research Farm Superintendent

editor's note: For the sake of continuity, the basic results from work conduccted in 1992 at Meadowview have been included in this issue of The Journal. The next issue of The Journal will include complete notes of work conducted in 1993 at the ACF Wagner Research Farm in Meadowview, Virginia.

In 1992, the Meadowview area was again blessed with abundant, well-spaced rains. However, it was a bit cool in late May and early June, and newly emerged seedlings did not grow as tall in 1992 as in 1991. Refer to Table 1 for the type and number of trees growing at the farm as of February 1993.

Trees continue to bear male flowers one to four years after planting. About 40 percent bear two years after planting. I attribute the precocious flowering to abundant sun, water and fertilizer as well as weed-free soil.

Our 1992 nut harvest (see Table 2) was very low, primarily due to a two-thirds reduction in nuts set per pollinated flower.

Of the 572 nuts harvested, only 286 were backcross nuts; the rest were crosses made for research rather than breeding purposes. In previous years, we harvested about 1.5 nuts per bag and 1 nut per bur; in 1992 we got .7 nuts per bag and .4 nuts per bur.

The number of bagged shoots was about the same in 1992 as it was in 1991. An additional disappointment was that the American trees we used as female parents for the production of third backcross nuts (BC3, 1516 American) succumbed to blight before setting a nut crop.

The 1992 nut set may have decreased because all chestnut trees flowered extremely late. In normal years, we finish pollinating around July 4th. In 1992 we finished on July 20th. We usually gauge flower maturity by the condition of male flowers, however, this year, I believe female flowers bloomed earlier than is normal relative to the blooming of the male flowers. Thus, we pollinated trees later than we should have, past the peak of pollen receptivity of the female flowers. We will keep this in mind the next time there is a late flowering year.

Table 1

Type and number of chestnut trees at the ACF Wagner Research Farm in 1992, with the number of sources of resistance and the number of American Chestnut lines in the breeding stock.

	Number of	
Type of Tree	Trees	Sources of Resistance
American	532	0
Chinese	377	36
Chinese x American: F1	119	7
American x (Chinese x American): B1	261	6
American x [American x (Chinese x American)]: B2	687	2
(Chinese x American) x (Chinese x American): F2	237	
[Amer x (Chin x Amer)] x [Amer x (Chin x Amer)]: B1-F2	422	
Chinese x (Chinese x American): Chinese B1	181	
Castanea sequinii	52	
Japanese	77	
American x Japanese: F1	3	1
Castanea pumila	1	
Chinese x pumila: F1	2	
Large, surviving American	91	9
Luther Burbank Cultivars	10	
Other	49	
Total	3101	

Table 2

American Chestnut Foundation 1992 nut harvest from controlled pollinations

Nut			Pollinated			Unpollinated Checks		
Type	Female Parent	Pollen Parent	nuts	bags	burs	nuts	bags	burs
B3	American	B2 from Graves B1	0	78	*	0	7	*
B2	American	Clapper B1	187	143	375	4	14	35
B2	American	Graves B1	99	263	408	1	27	42
F1	Nanking Chinese	American	31	66	91	3	8	9
F1	Meiling Chinese	American	2	11	18	0	1	1
B1	Meiling Chinese	American	2	11	18	0	1	1
B1	Mahogany Chinese	Mahogany x Amer	30	65	70	0	6	6
CxB1	Mahogany Chinese	Graves B1	9	12	14	0	1	2
B1xC	Graves B1	Mahogany Chinese	144	50	109	7	5	10
B1xF1	Graves B1	Mahogany x Amer	70	147	435	3	14	38
Totals			572	835	1520	18	83	143

* Mother trees succumbed to blight before setting a nut crop.

Chestnut Dream Recipes

Recipes are reprinted from the San Francisco Chronicle, researched and written by Sibella Kraus, and supplied courtesy of ACF member John Luigi Chiappe.

Necci: Ricotta filled Chestnut Crepes
From Italy in Small Bites,
by Carol Field.
crepes:
1 cup + 2 tbls. fresh sweet chestnut flour
1 cup minus 1 tbls. unbleached
all-purpose flour
18 tea. sea salt
14 cup sugar
112 cups + 1 tbls. cold water
2 tbls. olive oil

filling:
12 cup + 1 tbls. ricotta cheese
13 cup powdered sugar
12 tea. vanilla
Berry preserves (optional)

Directions:
Crepes: Sift both flours with salt and sugar into a bowl. Whisk in cold water and 1 tbls. of the olive oil to make a smooth liquid batter.
Warm a non-stick 6-inch skillet, and swirl remaining 1 tbls. oil oil in it over the heat. Pour off oil. Film bottom of skillet with enough batter to cover it in a thin layer. Cook until lightly browned; flip and cook briefly. Repeat, cooking 16 crepes and keeping them warm, wrapped in foil, in a low oven.
The filling: Whir ricotta in a food processor, or press through a sieve. Sift in powdered sugar, add vanilla and mix well.
Spread a small mound of filling down the center of each crepe, roll up, and serve with a spoonful of berry preserves on the side.
Serves 8. Per serving: 215 calories, 4 g protein, 35 g carbohydrate, 6 g fat (2 g saturated), 9 mg cholesterol, 55 mg sodium, 3 g fiber.

Chestnut Stuffing
Ingredients:
12 lb. pancetta, (Italian ham) cut into small pieces
2 onions, chopped
1 cup finely chopped celery
12 cup butter
2 cups sliced mushrooms
2 tbls. finely chopped fresh sage
2 tbls. finely chopped thyme
4 tbls. minced parsley
1 to 2 tea. salt
4 cups chestnuts, cooked, peeled, and quartered
4 cups small fresh bread crumbs
12 to 1 cup chicken stock

Directions:
Cook pancetta in a large skillet, drain and reserve. Using the same pan, gently saute onions and celery in butter until soft. Add mushrooms, herbs and salt; cook until tender. Mix with chestnuts, bread crumbs and pancetta in a large bowl. Moisten with chicken stock.

Yields about 10 cups. Per cup: 285 calories, 6 g protein, 30 g carbohydrate, 16 g fat (8 g saturated), 34 mg cholesterol, 548 mg sodium, 5 g fiber.

Biscotti Di Castagna
Chestnut Short Dough Cookies
From chef Gary Rulli, Pasticceria Rulli.

ingredients:
4 oz. unsalted butter (softened slightly)
23 cup sugar
1 tbls. honey
pinch salt
1 tbls. olive oil
1 large egg
pinch cinnamon
1 tbls. vanilla
1 tbls. rum
112 cups chestnut flour
112 cups all purpose flour
pinch baking powder

Directions:
Mix together the butter, sugar, honey and salt until blended. Add olive oil, egg, cinnamon, vanilla and rum.
Sift together the chestnut flour, all-purpose flour and baking powder.
Combine the two mixtures until they are incorporated, being careful not to overmix. Wrap in plastic wrap and refrigerate overnight.
Roll out dough on a floured surface 18 inch thick. Cut into rounds with a 1-inch diameter cookie cutter. Place on a parchment-lined baking tray and bake at 350 degrees for 15 to 18 minutes until golden brown. Let cookies cool on the pan.
These cookies may be eaten plain or sandwiched together with Chocolate-Chestnut Cream Filling (see recipe).
To fill cookies: Place the Chocolate-Chestnut Filling in a pastry bag fitted with a small round tip. Pipe a round of cream about the thickness of 1 biscotti on the flat side of half of the cookies; sandwich together with the remaining cookies.
Yields 40 cookies; 20 filled cookies. Per cookie: 75 calories, 1 g protein, 11 g carbohydrate, 3 g fat (2 g saturated), 12 mg cholesterol, 12 mg sodium, 1 g fiber. Per filled cookie: 200 calories, 2 g protein, 28 g carbohydrate, 9 g fat (5 g saturated), 29 mg cholesterol, 43 mg sodium, 2 g fiber.

Chocolate Chestnut Cream Filling

ingredients:
10 each marron glace, pureed; or use chestnut paste to equal roughly 14 to 13 cup.
4 tbls. butter softened
2 oz. dark chocolate, melted
2 tbls. brandy

directions:
Mix together the pureed marron glace, butter and melted chocolate. Stir in the brandy. Use as a filling for Biscotti di Castagna (see recipe).

Portrait of a Pioneer: Angus W. McDonald, Jr.

from interviews conducted in February 1994

Wind-swept plains, foreign tongues and food, new technology and resurrecting a presumed dead tree species are territories that would intimidate a good many people.

Then there are those who find "wide open spaces" intriguing, challenging and revealing.

For Angus McDonald, The American Chestnut Foundation's long-time Journal editor and friend, sinking his teeth into something and making a difference has been a way of life.

"He saw he could do something and he did it," said American Forests President and ACF board member Donald Willeke. "That's the beauty of the likes of Angus McDonald, and it is in the spirit of the entire American Chestnut Foundation. It's better to light a candle, than to curse the darkness. That kind of a person is always going to find something constructive to do. They're like a tall horse that's willing to lean into the harness and not hold back."

However, sometimes the challenge becomes an internal effort. Last year McDonald was diagnosed with Amyotrophic Lateral Sclerosis, also known as Lou Gehrig's disease. ALS, a virus which is as yet unknown, attacks the motor neurons that control one's voluntary muscles.

True to his way, McDonald is meeting this unknown with courage, "a belief in miracles" and the strength of family and friends-they have banded together to form Angus' Chestnuts. He said he also hopes to take part in a clinical program that will test a nerve growth factor.

After years of dedicated service to The American Chestnut Foundation McDonald has stepped down from his posts. But through his insights and example he continues to inspire and encourage us all.

Over the years McDonald has become expert in several fields, including modern Chinese and East Asian history, international business and multi-lingual computer software, and has developed interests in comparative religions and cultures. He is one of the world's experts on China's Hunan Province which is larger than the whole country of France and in 1980 he put together a delegation of most of the scholars who had written full-length studies involving Mao Zedong.

He has held numerous teaching posts both in the United States and abroad, including the University of Minnesota, Stanford University and Sophia University in Tokyo, Japan.

Then there is his interest in the American chestnut.

McDonald and Willeke both recall the day they were discussing trees on the grounds of St. Mark's Episcopal Church in Minneapolis where they are both members.

"We began to talk about trees and their importance in God's creation," said Willeke. "We talked about trees as one of, if not the most important aspect of God's creation. Certainly they are the equals of the mere mortals that plant and tend them and sometimes destroy them."

This conversation occurred just a few years after The American Chestnut Foundation had been established, and Willeke waxed enthusiastic about restoring this once great tree.

"Amazingly I felt energized," said McDonald recounting his first impressions of the work The American Chestnut Foundation planned. "It was a cause one could sink one's teeth into and make a difference."

Willeke introduced McDonald to ACF co-founders Philip Rutter and Charles Burnham. "Of course I had always known about the chestnut in theory," said McDonald. "I thought it was gone like the passenger pigeon. These two people convinced me the chestnut was not necessarily lost. Willeke tells a similar story about early conversations with McDonald. "What seemed to strike home was here was a group that was trying to do something about the problem rather than just lament the loss. He appreciated that we were trying to help God with the process of recreation. Only God can make a tree, but He's probably got a lot of other things on His mind these days."

McDonald joined up and became editor of The Journal upgrading its format and releasing issues brimming with science, news and reflections. He also joined the Finance Committee and worked together with then ACF Finance Committee Chair Mark Michaud and Treasurer William MacDonald.

For McDonald, being active in The American Chestnut Foundation also brought back to the surface his own personal memories from his childhood.

"At the Bower, my family's home near Harper's Ferry, West Virginia I helped my grandfather plant many oak trees in the acre grove," said McDonald. "My grandfather, E.P. Dandridge, was Bishop of Tennessee during the 40s and 50s, and I guess I absorbed trees and theology simultaneously."

Chestnut trees have also surfaced in McDonald's studies of Chinese history, and their story casts insight

on our own problems here in the United States. Currently the Chinese chestnut is not an important agricultural crop compared with the mainstays of wheat, rice and so forth, or apples and peaches and leechee; it is considered a secondary orchard tree, according to McDonald. However, it may have been a more substantial tree in the past, he said. Most historians understand that China's big trees were gone by the 15th century. In the North and in the Yangzi River regions all the big trees were cut, he said.

"The deforestation that occurred in China scares me. I don't want to see it happen in America."

McDonald said this mass deforestation coincides with a decline of other aspects of Chinese civilization. Just as the West began to develop new ways of approaching problems, China turned inward: architecture became constrained; navigation was limited; packaging seems to have turned from wood to cloth. The West had big trees; China did not. I suspect that the modern Chinese chestnut was developed during the same period.

Can Americans learn from this experience? McDonald says obviously technology has changed: we no longer need wooden masts and spars.

"I hope that big trees such as the chestnut will continue to live and die in America," said McDonald. "Trees are an important crop, but we need wild trees including wild American chestnuts. We need their nuts to feed the wild birds and animals. We need their height to lift our spirits. Trees are too important to be left to the forest products industry."

And as McDonald mulls over the enormous challenge before The American Chestnut Foundation, he says the greatest effort will be to actually reintroduce this tree to our forests. He says we need to take advantage of the high quality work that Fred Hebard and other scientists are doing, particularly in developing an ecologically diverse population of blight-resistant chestnuts. McDonald says some of these trees will be suited for life in the South; some will be suited for dry sites; and some will be suited for wet sites.

"We have to plant enormous numbers of trees and be prepared to suffer the loss of some that may become blighted or that aren't ecologically suited to their sites," said McDonald. "The scientists are developing important plant material. It is up to the rest of us to help nature restore the wild American chestnut. We need lots and lots of chestnut seedlings. The American Chestnut Foundation, in my opinion, has a vital role in the quest to restore this American classic."

With this kind of tremendous task ahead staying focused on the work and goals will be a challenge. But McDonald said in his voyages into new territory there has been an approach that has worked for him.

"One of the things that I learned from my grandfather and from a career devoted to the study of history is that you have to pay attention to things, both big and small. You cannot ignore important trends and you cannot forget to wash the dishes," he said.

"Everyone has to find a balance between having your feet on the ground and having lofty goals. My own work has had room for the American chestnut because the tree can contribute to the renewal of America."

Chestnut Meeting in Italy

Reprinted from Connecticut Chestnuts, Volume 1, #2, Winter 1994, Page 3.

In mid-October ACF member Dr. Sandra Anagnostakis of the Connecticut Agricultural Experiment Station traveled to Spoleto, Italy to attend an International Chestnut Congress. This is her report on the state of chestnuts in that region.

It rained the whole week, but the intrepid Chestnuts went out for a look at an orchard anyway. Two tour buses took us north into the mountains to the Vallocchia Valley, drove as far as possible on a small dirt road, and let us out to slog through the mud to see the trees. Orchards in this area do not look like orchards in the United States.

Trees randomly spaced in a forest setting are owned by many people. We were told that about 200 people owned the "orchard" we were seeing, and several families might harvest the nuts from a single tree. Harvest was in process (in the pouring rain) and people were gathering nuts from the uneven ground into baskets and buckets. The trees were very old, many five feet in diameter. They had all been grafted at about five feet off the ground with the local 'Marrone' which has very large nuts. The grafts were made this high because cattle were usually pastured around the trees. They eat the sprouts from the base and would eat off the grafts if they could reach them easily.

Genetic data from Europe suggests that the Marrone are very uniform and that chestnut trees in eastern Turkey are much more diverse. The old dogma is that the Romans found chestnuts growing in the Caucasus mountains between the Black and Caspian seas where they survived the last ice age. Because they were clearly useful, the practical Romans took seed back to plant all through their empire. Chestnut wood makes good vine supports, so where you find an old vineyard in Europe you will probably find an old chestnut grove nearby-both planted by the Romans.

I have been confused recently by leaves from "European" trees in this country with lots of branched leaf hairs. The Castanea sativa that I thought typical had only simple hairs on the leaves on the veins, top and bottom. Walking through the old Italian orchard I kept pulling leaves off the trees and checking them with my hand microscope (there were lots of polite Italian men to hold my umbrella). To my surprise, the leaves on the sprouts from old trunks looked European, but leaves from the grafted 'Marrone' had many branched hairs. I would call them a different species, or maybe European X Chinese hybrids. The 'Marrone' are supposed to have been selected by Turkish monks in the 1100's. Did they have Chinese chestnut trees at their disposal?

Marco Polo returned from his famous trip to Asia in 1295, and though he may have brought chestnuts with him, this is probably too late to have influenced 'Marrone' development. The silk trade has recently been studied by economic historians in some detail, and if any of you have studied this, please call me (203-789-7253)! I am sure that there had been trade of silk with Constantinople by 1100, and chestnuts would have been logical trade items as well.

The resolution of this question is of more than academic interest. Reports of successful biological control of chestnut blight in Italy were considered in the light of our knowledge that C. sativa is almost as susceptible to blight as C. dentata. If the 'Marrone' have "Asian blood" are they more resistant? Is that why hypovirulence has been so successful in Italy at controlling blight?

I kept asking these questions as we walked through the orchards. The people who really knew about the trees spoke no English. Translation was not easy, even though there were several people there who spoke Italian and knew some English and vice versa. I think that the consensus was that blight showed up first on stump sprouts from the old trees and continues to be most serious on these. The 'Marrone' do get blight, but not as seriously. I probably made enough fuss about this to get some of the Italians thinking about the problem, so perhaps future work will result in some answers.

An American Chestnut Stand in Rockport, Maine

Peter Blanchard

An encounter with a mature, 50-foot-tall American chestnut in the Maine woods is both an unexpected and momentous event. The unmistakable pattern of the furrowed bark, the serrated lanceolate leaves, and in July, the plumes of cream-colored flowers are bound to arrest a progression through a wooded tract of red spruce, white pine, balsam fir, and red maple.

The Harkness Preserve chestnuts fit well with the national mission of the Nature Conservancy: to preserve biotic diversity and to protect the habitat of rare and endangered plants and animals. The presence and persistence of 43 American chestnuts on a 5-acre tract in coastal Maine, however, remains a mystery.

The earliest written record of the chestnuts dates to 1926. Nineteenth century stone walls defining a portion of the preserve boundary offer a possible clue about the stand's origin. While the trees are located at the northern limit of the ancestral range of Castanea dentata, which stretches from Georgia to southern Maine, the Harkness trees appear to have been the beneficiaries of a local farmer's efforts and may have been planted in anticipation of nut and timber crops. The oldest and largest of these trees may be 50 years old.

Geographic isolation has allowed the trees to escape the ravages of the blight until relatively recently. Since the 1980s the chestnut blight (Chryphonectria parasitica) has been making inroads into the stand killing several large trees and producing cankers on half of the chestnuts.

The earliest treatments of the blight were carried out by a local forester, Welles Thurber, and utilized hypovirulent strains of the blight fungus donated by Sandra Anagnostakis of the Connecticut Agricultural Experiment Station. With the best of intentions, Welles found himself in direct confrontation with the preserve's stewardship committee, a group of local residents who volunteer to watch over the preserve, when he cut down two of the largest trees without notifying them. The practice of cutting out and removing dead limbs and whole trees makes very good sense in traditional forestry practice.

A less destructive approach, however, is advocated by Anagnostakis, a mycologist who sees blight spores as being pervasive in a stand. A culling operation which attempts to halt advance of the blight by removing diseased limbs would be similar to an attempt to remove the dust mites riding air currents in a room. Rather, Anagnostakis' emphasis is on large scale conversion of the virulent to the hypovirulent form of the blight.

Since 1989, TNC's stewardship program at Harkness has carried out systematic inoculations of all diseased trees, drawing on the fungal cultures and guidance supplied by Anagnostakis. I continue to culture the French and Italian hypovirulent strains in my farmhouse kitchen in Somerville, Maine, a two-hour drive north of the Rockport preserve. To this is added cultures Anagnostakis has prepared from two Maine hypovirulent strains taken from bark samples of severely diseased Rockport chestnuts-thus a truly international fungal mix is introduced into the chestnut trees on our monthly visits during the growing season, from late May to early October. One wonders how, at a microscopic and biochemical level, the various strains fare in each other's company.

The Nature Conservancy's inoculation efforts have faced some technical hurdles. The slurry is injected with an oral syringe into shallow holes ringing the canker. Battery-powered drills allow for an efficient work pace and coverage of the trees, but carry the danger of drilling too deeply, thus penetrating the xylem and allowing insects and decay fungi to enter.

Anagnostakis has urged the use of cork borers instead which are rotated by hand to produce holes in the bark, however, we have found them to be very time consuming and difficult to use in the field given that we may need to treat 25 cankers in a day.

Linda Wirtz, a TNC volunteer and her husband Joe, have improvised a cork borer bit for the power drill that is less disruptive to the tree than a standard drill bit. After one or two punches with the borer, though, the tool has to be cleared with a metal rod which slows down the process.

While efforts to curb the blight at levels below 35 feet (the height to which our tallest ladders can reach) appeared effective, we realized that the disease was making its most rapid and devastating progress at the higher elevations of the tree. In the summer of 1992, we hired licensed arborists, tree climbers who use rope and harness to scale the heights of the American chestnut.

One of the largest chestnuts of the preserve seemed to be particularly stressed; it was producing a distinctly thinner crown with smaller leaves during the past growing season. When the tree surgeon

investigated the area immediately below the canopy, he found an extensive canker which had progressed 3/4 of the way around the stem. Plastic bags of slurry, a syringe and duct tape to cover inoculation holes were hoisted up to the surgeon at altitude.

The American chestnut trees at the preserve are in a dynamic balance with the blight. Welles Thurber's assessment of the situation was very apt.

"It's as if we've gotten hold of a raging bull by its horns," he said. "We have the hope of controlling it, but it may throw us."

Indicative of this tenuous hold on the blight are the cankers which show a dramatic growth of callus tissue and are healing, and the cankers which once healed that now reveal a re-invasion of the orange pycnidia, those spore-producing bodies of the virulent fungus.

The stand continues to live under assault, and a long-term prognosis for the Harkness chestnuts is difficult to determine at present, thus underscoring the need for a propagation program. Burs have been collected over the years, nuts germinated and seedlings nurtured at Harkness, but large-scale collection and propagation were initiated only recently.

The sweet chestnut, a great temptation, immediately drew competitors to the propagation program, and the services of a cherrypicker were donated to give TNC a head start over the squirrels waiting below. But the machine, donated by Bangor Hydro-Electric Co., arrived after a windy period and too late in the season; as the volunteers who arrived to help collect nuts put it, the squirrels were lined up to greet the Bangor Hydro-Electric truck. No nuts were to be seen.

Other collection efforts have been more successful and TNC now keeps a galvanized can with a squirrel-proof lid and a sign enlisting the help of passersby in gathering the burs. In the late fall of 1992, chestnuts have been sent to The American Chestnut Foundation, to the Cincinnati Zoo and Botanical Gardens for cyrogenic treatment (preservation through deep-freezing), to a landowner in South Carolina who has strong memories of the old giants at the border of his pastures, and to our own restocking efforts in Maine. Some of the nuts may be transported to remote coastal islands in Maine, where hopefully they will gain a roothold.

The cold winds are now whipping down from Canada on this remnant population of a great race of trees. Both the American chestnut and the blight fungus are quiescent as the winter descends.

But with the spring thaw the conflict will resume: a siege between the tree and its blight, between the host and a parasite that behaves more like a predator.

Peter Blanchard is an active ACF member.

Breeding Blight-Resistant American Chestnut

Albert H. Ellingboe
Professor, Plant Pathology and Genetics
University of Wisconsin at Madison
and
Chair, Science Committee
The American Chestnut Foundation

Introduction

The American Chestnut Foundation was established with the hope of restoring the American chestnut, *Castanea dentata*, to a position of importance as a timber tree and as a source of chestnuts for human and animal consumption. In order to restore this tree, however, we must find a way for the trees to resist the fungal pathogen, *Cryphonectria* (*Endothia*) *parasitica*, which causes blight.

The American Chestnut Foundation's breeding program is a test of a hypothesis developed by Dr. Charles Burnham that states resistance to blight is controlled by a relatively small number of genes and that by backcrossing for several generations to American chestnuts it will be possible to develop trees that look like American chestnut.

The concept behind the breeding program is to introduce blight-resistant genetic traits found in the Chinese chestnut to the American chestnut through backcross methods. Some Chinese chestnuts are highly resistant to blight, and they usually cross well with American chestnuts.

Several different sources of disease resistance are used, and several different American chestnut trees are used as recurrent parents. Offspring from such crosses usually survive and develop successfully, and appear to exhibit a reasonably complete range of genetic variability.

The breeding strategy assumes that the genetic inheritance of blight-resistance is less complex than the array of genetic relationships that determine the shape and growth habits of the American chestnut, characteristics we want to preserve; thus, we backcross to the American parent.

The assumption seems reasonable based on experiences in breeding field crops for disease resistance. Commonly, the inheritance of disease resistance looks very complex, particularly if the resistance is obtained from an interspecific cross (a cross between different species), but systematic analysis often shows the inheritance is rather simple.5

Clapper3,4 has also shown that the segregation for resistance among progeny from a cross of Chinese x American backcrossed to Chinese approximated a 3 to 1 resistance to susceptibility ratio. The ratio suggests that two pairs of genes control resistance to blight. The results also suggested that the resistance to blight was only partially dominant because the presumed homozygotes were more resistant than the heterozygotes.

The backcrossing program was suggested1,2,6 because the principal need is to transfer only one trait, that is, blight resistance, into the American chestnut. Theoretically, a Chinese x American hybrid crossed twice to American trees should give a population that is genetically 7/8 American, except for the chromosomal regions that contain the genes for blight resistance. A population that has 7/8 of its genes of American chestnut would, hopefully, be primarily of American type in morphology and growth characteristics, i.e. a timber type tree with resistance from the Chinese parent.

However, the population would not be expected to breed true. The American chestnut has a self-incompatibility system and cannot self-pollinate - another plant is required for pollination to take place. Intercrossing among the individuals of a backcross population may reveal considerable differences in morphology and growth characteristics. The degree of genetic difference is expected to be greatest in the F2 population, but progressively less with each additional generation of backcrossing to American chestnuts. The number of backcrosses to American needed to establish a population that is sufficiently like an American chestnut is not yet known.

This situation leaves a question that can be approached from two perspectives. How similar to American chestnut in morphology and growth characteristics must a tree be to be an acceptable American chestnut? Or, what proportion of the population fits the description of being an American type tree?

If 3/4 of the progeny from BC2 plants are acceptable American type trees, it will be necessary to prepare plans as to what should be done with the 25% of plants that do not have the desirable American type. Will these trees be self-eliminating in a dense stand or will they have to be removed? For example,

plants with a more traditional Chinese form, that is, similar to an apple tree, may be shaded by the timber type trees and be unsuccessful in competing in a dense stand. Or, we may have to physcially remove them.

A generation of intercrossing within a population created by backcrossing will also be necessary to make plants homozygous for one or more genes for blight resistance. Plants with two genes for resistance are expected to show a higher level of resistance than plants with only one such gene. Since resistance does not appear to be completely dominant, heterozygous plants are not expected to have as high a level of resistance as homozygous plants. Furthermore, the program must determine the proportion of plants that have resistance, the level of resistance among individual plants and reasonably sized populations of plants capable of yielding timber and/or nuts. It is also expected that some populations of American chestnut may need to be more or less blight-resistant in order to thrive in their respective ecological niches.

SOURCES OF RESISTANCE

Chinese chestnut is the source of resistance to be backcrossed into American chestnut, and as much as possible, the program draws from existing F1s and F2s of Chinese x American crosses.

The "Clapper", "Graves", and "Douglas" trees and/or their descendants have been used in backcrosses to American chestnuts. The chestnut trees at the Lockwood Farms and Sleeping Grant State Park in Connecticut have also been available as a source of breeding materials. The Connecticut germplasm represents an array of interspecific hybrids that have differing potentials as parents in the backcrossing programs. Not all *C. mollisima* collections are resistant to *C. parasitica*, and different collections of Chinese chestnuts may have different genes for blight resistance. The tentative conclusion is that there is an array of germplasm that has resistance to blight, and some of the necessary crosses have already been made with American chestnuts so that F1 hybrids and BC1 plants exist for use in further backcrossing.

An interesting possibility is that some of the large, surviving American chestnuts may have low levels of resistance. They may have survived because of the presence of hypovirulence in the cankers, but based on experiences in breeding other plant species for disease resistance it is expected that these individuals possess low levels of resistance.

The Amherst tree in Virginia, for example, has many cankers, but many of them are quite superficial. Other trees have large cankers, but they tend to be few in number. It may be possible to combine the trait of few cankers with the trait of superficial cankers to develop a level of resistance that is adequate to grow an American chestnut that is large in size and dependably produces nuts.

But the intercrossing of surviving American chestnuts is not a high priority for the American Chestnut Foundation. We do, however, intend to use these trees as parents in the backcrossing program to compare with crosses with American chestnut trees for which we have no indications of any resistance. The comparison will look at the blight resistance of the resulting offspring. As the Foundation's program has progressed (see accompanying article in this issue) it has become possible to cross very young plants, younger than previously believed possible.

Some hybrid plants have produced male catkins at only 2 years of age. Hybrids that have flowered have been crossed to American chestnuts even though the individual plants have not yet been tested for resistance; the crossing program is actually progressing faster than the evaluation of progeny for resistance.

Because plants are available for crossing before we know which are resistant, several plants from each generation are backcrossed to American chestnut trees. Later, tests for resistance will show that some of the progenies used for backcrossing were susceptible, and they can be discarded.

USE OF RFLPS IN BREEDING

The process of selectively breeding for blight resistance can be greatly accelerated by a recent advance in biotechnology.

The ability to develop genetic maps of organisms took a quantum leap forward with the development of the DNA restriction fragment length polymorphism (RFLP) technology. This technology works best if the DNA in question show a high degree of difference.

Because our breeding program is based on making crosses between two distinct species, and because such crosses are particularly likely to yield variable DNA, this technology is well-suited to ACF's purposes.

There are two things that the American Chestnut Foundation wishes to determine from the use of RFLP maps. First of all we would like to find RFLP markers that bracket chromosomal regions that contain genes for blight resistance, and secondly we would like to select plants in each generation with the greatest similarity to the recurrent parent.

The first point is important because while we are able to cross plants at a very young age, the plants are too young to yield reliable evaluations of blight resistance. To counteract this problem we currently make crosses with a large number of plants in each generation in which the offspring do not appear alike under the assumption that only a few plants will have blight resistant traits. However, using RFLP markers that bracket disease resistance genes, we can focus on parents that are most likely to be resistant to blight.

A second use of the RFLP technology is to select plants in each generation with the greatest similarity to the recurrent parent for each generation of backcrossing. In a typical backcrossing program, the proportion of the genes from the recurrent parent is expected to be 1/2, 3/4, 7/8, 15/16 etc. for the F1, BC1, BC2, BC3, etc., generations respectively. Whether this proportion of genetic material from the recurrent parent is achieved has been difficult to determine. Analyses of segregating generations using RFLPs have shown that the proportion of RFLP markers with the polymorphism of the recurrent parent is often less than predicted in each population. Backcrossing does not apparently move a population toward homozygosity with the recurrent parent as rapidly as predicted from theory.

For example, of four resistant plants one may have the polymorphism of the American parent for only two RFLPs, while another has the polymorphism for seven RFLPs. Obviously, the latter is closer to the American parent than the former.

But by selecting individuals in each generation that have the highest percentage of markers with the polymorphism of the recurrent parent, and using these individuals for the next generation of backcrossing, it is possible to approach the genotype of the recurrent parents in fewer generations than predicted by theory for random selection of individuals for backcrossing in each generation. The potential use of RFLP technology for hastening achievement of goals in breeding of plants with long generation times becomes quite obvious.

ECOLOGICAL ADAPTATION

Yet another consideration in breeding the American chestnut for blight resistance is ecological adaptation. The American chestnuts were adapted to a wide geographic range from Maine to Georgia and west to the Mississippi River. It was a dominant species in the Appalachian Mountains. Whether trees adapted to one geographic area will perform well in another area is an interesting question.

Currently much of ACF's backcrossing program involves crossing to American chestnuts within the vicinity of the Wagner Research Farm in Meadowview Virginia. But the Foundation has many members in different regions who wish to be physically involved in the breeding program, and we plan to make pollen available from the backcrossing program for the purpose of crossing onto American chestnuts in different parts of the country.

Pollen from resistant plants (of some generation of the backcrossing program) could be used to pollinate surviving American chestnuts in New York, or Georgia, or Kentucky, and other areas. Crosses made with pollen from the breeding program with regionally adapted American trees should lead to the production of regionally adapted populations of blight-resistant trees.

There is also the possibility that intercrosses among surviving American chestnuts may have low levels of resistance which will yield populations with higher levels of resistance. If some of these trees do, in fact, possess low levels of resistance, they may be useful to incorporate into the backcross breeding program.

COMPLICATIONS

Any program of breeding for disease resistance must take into account that the entity to which resistance is sought is a living entity which can change. Genetically it can mutate, recombine genes, etc., and therefore the breeding program must draw from several sources of resistance.

Each source of resistance must also be backcrossed with American chestnuts to develop blight-resistant chestnuts that are similar in morphology and growth characteristics to American chestnuts. These lines will also have to be tested for their levels of resistance and growth characteristics in several geographic locations.

There must also be a constant search for new sources of resistance. Resistance obtained from different collections of Chinese chestnuts may or may not have the same genes for resistance to blight. Resistance obtained from Japanese and European chestnuts may be due to different genes that can be used separately or together with resistance obtained from Chinese chestnuts. We know that there is resistance to blight in several species of Castanea, but we don't know if the resistance is due to different genes.

The molecular mapping of resistance in interspecific crosses may be very informative in telling us whether resistance in Chinese, Japanese, European, and possibly in American chestnuts is in the same

chromosomal locations (which would suggest they contain the same genes) or in different chromosomal locations (which would suggest they have different genes for resistance).

The search for new sources of resistance is also based on the assumption that the introduction of trees from the breeding program will trigger selection within the pathogen population producing new races of the pathogen. Likewise, the breeding may unintentionally develop tree populations more susceptible to pests other than blight that will then become important problems for growing American-type chestnuts. There must be a continuing vigilance for changes in all problems in growing chestnuts.

Testing populations at all stages of the breeding program in various geographic locations should give warning signs of other parasites of chestnuts, and give opportunities to adjust the breeding program accordingly. Production of a blight-resistant plant that is essentially indistinguishable from an American chestnut is only the beginning of a program to restore American chestnuts as a major component of the United States' eastern forests. But to date, thanks to the cooperation of various breeding programs and people with similar goals, the results look very encouraging.

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The American Chestnut Foundation Breeding Plan: Beginning and Intermediate Steps

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Introduction

The American Chestnut Foundation intends to restore the American chestnut, Castanea dentata (Marsh.) Borkh., as a viable component of our eastern hardwood forests. The primary breeding approach we use is to backcross the blight resistance of Chinese chestnut, Castanea mollissima Blume, into the American species3. The basic plan is to breed hybrids of the two species, cross these back three times to the American parent, and intercross the B3s to recover trees homozygous and true-breeding for blight resistance. We will select seedlings for blight resistance from among the backcross and intercross progeny. We will not attempt any further improvements to the American chestnut until we are certain the backcross method will succeed, and even then, we would limit improvements to those characteristics which are absolutely necessary to restore the species in order to retain as much genetic diversity as possible. Improving more conventional aspects of the American chestnut, such as increased growth rate or improved wood characteristics, could be undertaken with trees which we have released to the public.

We are using conventional breeding methods and this paper details how we screen progeny for blight resistance, where and how we breed trees, and how many progeny we produce within each backcross and intercross generation. This paper also explains how many lines of American chestnut we plan to carry, and the number of sources of Chinese chestnut we will use. The final steps of the program include testing the performance of B3-F2 or B3-F3 progeny in forest situations and developing methods for introducing suitable trees back into the forest, but these steps we will discuss at a later time.

Screening Progeny for Blight Resistance

Because current evidence indicates that trees with intermediate to low levels of blight resistance, such as backcross progeny, cannot be distinguished reliably by direct inoculation until they are 2.5 cm in DBH5, we begin screening for blight resistance when they have achieved this diameter at about 4 to 5 years of age.

In contrast, trees with high to intermediate levels of blight resistance, such as what we expect from intercross progeny, cannot be distinguished by direct inoculation if they exceed 1 cm in DBH (Hebard & Shain, unpublished); thus, intercross progeny are screened when they are 2 years old using the micro-direct inoculation technique appropriate for smaller trees.

The direct inoculation technique entails removing a plug of bark from a tree and inserting a disk of agar of mycelium of the chestnut blight fungus into the hole. In the micro-direct method the hole is smaller, about 1 to 2 millimeters in diameter versus 4 - 6 millimeters in diameter for the regular method.)

Trees with intermediate levels of blight resistance are being screened for resistance at 4 to 5 years of age; they are planted at 20-foot by 7-foot spacing. Trees with high levels of resistance are screend at 2 years of age, planted at 10-foot by 2-foot spacing. We are using these orchard spacings to eliminate crowding of trees at the time they are screened for blight resistance. These spacings were determined based on experiments done by Uchida14 with Japanese chestnut, Castanea crenata Sieb. & Zucc. He found that crowded trees were more susceptible to blight than uncrowded trees; thus, crowding might hamper our ability to distinguish resistance classes.

Because progenies are not screened for blight resistance until they are several years old, seeds are planted directly.7 Compared to transplanting seedlings from the greenhouse or nursery, direct seeding at orchard spacing results in faster plant growth and requires much less labor.

In the last three years more than 80 percent of nuts have sprouted and developed into viable seedlings. Many seedlings, including pure American chestnut, are bearing male flowers at 1 to 3 years of age, and female flowers at 3 to 4 years.

Trees planted within orchards are arranged in a statistically random fashion. American and Chinese chestnut, their first hybrid and Chinese chestnut cultivars- 'Nanking', 'Meiling', or 'Kuling'-are also planted in the orchards to serve as standards for evaluating the blight resistance of progeny from crosses. Six to ten

control plants of each type are planted for every 500 trees.

After trees have been screened for blight resistance, most will be cut down to reduce the amount of blight fungus at the farm. Undesirable trees will be removed as appropriate for space and experimental needs while selected trees will be allowed to resprout. However, those select trees which appear ready to flower will not be pruned, although cankers with blight stromata will be excised.

Pollen is collected from selected trees as soon as they flower. To speed up production of the next generation, selected trees will be used primarily as pollen parents until the intercross generation. This speeds up production because numerous female flowers can be pollinated by one catkin, whereas female flowers yield only one to three nuts because seedling chestnut trees generally bear male flowers prior to female flowers, and because chestnut trees generally bear many more male flowers than female flowers.

How and Where Trees Are Bred

To maintain adaptations to local conditions and enable us to start breeding trees immediately, as many crosses as possible have been being made on flowering American chestnut sprouts growing at their original locations. Hand pollinations are employed using methods described by Rutter11. Currently in Pennsylvania, Virginia, North Carolina, South Carolina, Kentucky, Tennessee and Georgia, numerous flowering trees occur in clearcuts, and other disturbed areas, in the national forests. The flowering period of most chestnut trees occurs between 5 and 10 years after the overstory of other tree species has been removed.

Flowering is terminated by blight acting together with suppression of new sprouts by competing vegetation. Thus, removing competing vegetation in cutover areas could prolong the flowering period and greatly increase the yield of nuts. In addition to pollinating these trees, we also are planting American chestnut seed, and transplanting naturally occurring seedlings and sprouts at easily accessible locations. Additionally, the ACF distributes American chestnut seed to members so they can raise mother trees for breeding.

Controlled hand-pollination of chestnut is very labor intensive, generally about one nut is produced per pollination bag, and one person does well to place 200 bags in a 12-hour period. There are several alternative methods of producing controlled-pollination progeny which would be much less labor intensive.

First, desirable plants could be grafted onto rootstock in clearcut areas where there is an abundance of American chestnut sprouts (it is necessary to graft at ground level and cover the graft union with soil to exclude blight). Seed from these plants would be pollinated by nearby American chestnut trees.

Second, outside the natural range of chestnut, isolated pairs of trees could be planted; the two trees in the pair making a desirable cross. The composition of the cross could be altered by grafting scions of another tree into one member of the pair.

Third, in the Midwest there are large, isolated American chestnut trees already growing; scions from other trees could be grafted into the crowns, and ungrafted portions of the crown would supply pollen to the grafts and vice versa.

Fourth, in the East some American chestnut trees survive blight due to hypovirulence, and many of these flower. If the American chestnut tree is reproductively isolated, nearby Chinese chestnut trees could be grafted with American chestnut above ground level. In the East one can graft above ground level into Chinese chestnut trees , but not into American chestnut trees because blight will rapidly invade the graft union.

One disadvantage of these hands-free methods of pollination is that they take several years to begin producing progeny, but then produce abundantly. Since we do not want a single American chestnut tree to dominate our backcross pedigrees, many of the methods probably would be of limited use in the backcrossing stage of our program, although they could very useful at the intercrossing stage. Additionally, every year we need F1 seed to plant as controls, and it can be difficult to avoid pollen contamination with bagged progeny. The hands-free methods could fill this immediate need very well.

How Many Progeny from Each Cross

The number of progeny needed for each cross is calculated based on the number of genes believed to confer blight resistance. Current evidence indicates two genes control blight resistance in Chinese chestnut, and several elements point to this conclusion.

Evidence from Clapper's4 report shows that about 14 of progeny from a backcross of an F1 Chinese American hybrid back to Chinese had high levels of blight resistance.

Also, the 'Clapper' first backcross8 and undescribed 'Graves' first backcross1 show levels of resistance

comparable to F1 hybrids-these hybrids were selected from no more than 50 siblings each.

Finally, large populations of F2 and B1-F2 progeny at the ACF's Meadowview farm are being screened for blight resistance (the trees were inoculated in June 1993), and results show that blight resistance is controlled by one to three major genes, probably two. We expect additional evidence will come from the results of screening our backcross progeny for blight resistance, which is due to begin this year.

Until the number of genes controlling blight resistance is verified, however, our plans assume that resistance is controlled by three genes, an approach that provides a safe margin of error for the program.

In each backcross generation we want to obtain four or five nuts which carry all major genes for blight resistance. From these nuts we should be able to grow two to three trees and select the most American-type trees.

Growing 73 backcross progeny will give us a 99 percent chance of obtaining at least four plants with the three genes, according to the following binomial formula:

$$p = 0.99 = 1 - \sum_{m=0}^{3} (74/m) * 0.125^{m} * 08.75^{74-m}$$

For intercross (F2) progeny, the same formula indicates (after substituting in the appropriate numbers) that 149 intercross progeny must be grown to be 99 percent certain of obtaining four plants that will be homzygous for two resistance genes.

However, it would be desirable to produce even more progeny of each cross because then we could select among blight-resistant progeny for American traits, thus by-passing several generations of crosses needed to recover American chestnut traits.2 But the space and time requirements of the current method of screening trees for blight resistance make it impractical to grow many more progeny than we presently do.

A genetic molecular marker approach is attractive in that we might be able to screen offspring using samples of nut meat or true leaves from freshly germinated nuts. Currently we can generate numerous offspring once trees start flowering, but the rate at which we can advance the breeding program is limited by the time it takes trees to flower.

On the other hand, the molecular marker approach may be too expensive for routine use. Also, molecular markers provide indirect evidence of blight resistance, rather than the direct evidence provided by inoculation. If we use them, we will have to be very careful to ensure that major genes for blight resistance are mapped accurately and that linkages to minor genes are not missed altogether.

However, if there are more than three genes for blight resistance, molecular markers linked to each gene could be used to help backcross them separately into American chestnut, in parallel. The genes from the parallel backcrosses would be combined after backcrossing was complete in order to recover highly blight-resistant trees. It would be preferable if each of these blight resistance genes were detectable by direct-inoculation tests to ensure precise mapping.

Marker-directed selection would also be useful in combining, (known as pyramiding), the genes conferring low levels of blight resistance in large, surviving American chestnut trees with the hope of obtaining trees with high levels of blight resistance. Using markers to follow the process, relatively few plants might be needed compared to the number of plants needed under conventional breeding methods, such as recurrent selection for phenotype. Thor has outlined a conventional breeding program for large, surviving American chestnut trees.12

Micropropagation is another tool of biotechnology which could be useful to our program. Micropropagation, cloning chestnuts at the bud stage in tissue culture using plant growth hormones, of highly blight-resistant B3-F2 (or earlier F2 stages) trees would facilitate evaluating their performance in the field. The technology for micropropagation is immediately available for the small-scale use envisioned here. Micropropagation of selected B3-F2 nuts would also accelerate and increase the production of highly blight-resistant B3-F3 nuts which we intend to use as our primary vehicle for distributing blight-resistant progeny.

How Many Lines of American Chestnut

The key question is how many lines of trees to advance. Namkoong10 estimated that "A few thousand samples are needed to save most alleles in most populations..." In alfalfa, which is a cross-pollinated plant like chestnut, 125 lines were used by Stanford and Houston13 in backcrossing resistance to bacterial wilt, mildew and leaf spot into 'California Common' to produce the Caliverde variety. We can handle 60 breeding lines at the Meadowview facility. Five additional breeding locales advancing 20 lines each would

give us 160 lines.

The contrast between 160 lines and Namkoong's estimate of a few thousand samples makes it clear that the genetic diversity of our products will be less than that which existed prior to blight. It is also clear that there cannot be too many locales where American chestnut trees are bred for blight resistance!

Our current breeding program is concentrated in the vicinity of Meadowview, Virginia, but our goal is to restore the American chestnut throughout its native range. Thus, to preserve adaptations to local conditions, we hope to replicate at least part of the Meadowview breeding effort every few hundred miles from Maine to Georgia. Alternatively, we could breed trees adapted to local conditions by backcrossing highly blight-resistant B3-F2 trees from Meadowview into local populations followed by a large intercross generation. However, this might require long-term testing to select trees adapted to the local conditions. A few additional backcrosses to locally adapted American chestnut trees prior to intercrossing is a more rapid, but more labor-intensive means of achieving this goal.

A breeding line of American chestnut is defined here as the product of one intercross of a Chinese chestnut tree and an American chestnut tree and three backcrosses to American chestnut. For each backcross within a line, the American chestnut parents would be separate individuals, in order to avoid inbreeding. Thus, one Chinese and four American chestnut trees would be the parents of one line. After three backcrosses, the progeny will have to be intercrossed. We will probably intercross between lines, but within sources of Chinese chestnut resistance.

The 1 to 4 ratio of Chinese to American parentage is the basic concept behind our breeding lines, however, in the field the makeup is somewhat complicated. In actuality, more than four American parents may be involved in the makeup of each line because we need 73 progeny per line at each backcross step, but we cannot generally obtain this many offspring from any single flowering American chestnut tree in a typical clearcut. Thus, we have decided to equate each managed area, referred to as a clearcut, to a single American parent. We will try to have the clearcuts which are the "parents" of a single line be no more than 10 km apart, and at similar elevations. Due to blight, generally it is not possible to use American chestnut trees in a clearcut for more than one backcross generation.

Each clearcut may include multiple Chinese parents, and thus multiple sources of resistance. We are keeping pedigrees and noting when progeny from different Chinese parents have the same American parent tree from a clearcut; use of the same American tree with different Chinese sources occurs infrequently because most American chestnut trees in clearcuts bear only one crop of nuts before succumbing to blight.

How Many Sources of Chinese Chestnut Resistance

The purpose of backcrossing is to recover all characteristics of the recurrent parent except for the trait being transferred from the donor parent. Thus a high level of blight resistance is the only characteristic we use in evaluating Chinese, and other chestnut trees, as sources of blight resistance. For backcrossing, the best sources of resistance are those which confer the most resistance with the fewest genes. Sources are evaluated using the direct inoculation technique to compare their resistance, and that of F1, F2 and backcross progeny. Where possible, F2 and backcross progeny from various sources will be interplanted so their performance can be compared.

If two Chinese chestnut cultivars have identical genes for resistance, creating separate sets of lines for each cultivar would be redundant. We will try to determine whether Chinese chestnut cultivars have identical genes for resistance, hopefully by examining progeny with molecular markers. Molecular markers should at least tell us whether major blight resistance genes are mapped closely together if they cannot tell us whether they are multiple alleles for resistance at the same location.

We have room to carry three sources of resistance in 20 lines each at the Meadowview facility. Twenty lines are chosen as the absolute minimum number necessary to preserve genetic diversity on the American side for a single source of resistance.

Three sources of resistance currently have the highest priority at the Meadowview facility The first source is the triplet of Chinese chestnut cultivars, 'Meiling', 'Nanking' and 'Kuling.' These three cultivars are considered a single source of resistance because they came from the same, or very similar seed lots.9 They have high priority because in contrast to many Chinese chestnut trees6 they have demonstrated high levels of blight resistance: there are few, if any, blight cankers on most trees of these cultivars. We have advanced one line of 'Nanking' to B1.

The other two sources of resistance are the 'Graves' and 'Clapper' first backcrosses. The 'Graves' and 'Clapper' sources have high priority because they are our most advanced breeding lines and we wish to prove the utility of the backcross method. We are beginning to advance these to B3 in blind crosses.

Unfortunately, we have only one line of American chestnut in the 'Graves' and 'Clapper' trees. It will be necessary to broaden their genetic base into 20 American lines. This probably will require one or two additional backcross generations. The Chinese grandparent of the 'Graves' tree is still living, as well as some F1 hybrids between it and American chestnut. These trees can provide additional lines for the 'Graves' source.

Ideally, we would like to have perhaps 100 individual Chinese chestnut trees comprising our source(s) of blight resistance, in order to maintain genetic diversity at the Chinese genes which remain in our final breeding products. A more realistic figure will probably be ten or twenty because of the pressing need to use numerous American parents.

Conclusion

Editors Note: This paper explains in detail how we are breeding Chinese and American chestnut trees to produce a blight resistant chestnut that also looks like our American native. As you can see from this article it is a process requiring careful attention to detail from a whole host of perspectives. One way you can get a better sense of the progress we have made at Meadowview is to come and visit! The Wagner Research Farm is open to the public year-round and those who plan longer visits can get a hands-on sense of chestnut breeding by helping to pollinate trees in the summer months. For more information on visits and volunteer efforts, please contact Fred Hebard at the farm.

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Somatic Embryogenesis and Gene Transfer in American Chestnut

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Introduction

American chestnut [Castanea dentata (Marshall) Borkhausen] could potentially be restored as a component of eastern hardwood forests if a solution to the chestnut blight fungus [Cryphonectria parasitica (Murrill) Barr.] could be found.

Now new tools are available which may have considerable impact on efforts to control chestnut blight. These are the tools of molecular biology. These techniques have already been applied to transform the chestnut blight fungus with a DNA sequence that produces hypovirulence-associated viral dsRNA (Choi and Nuss, 1992).

This transformation permits the transmission of hypovirulence by sexual as well as asexual means, thus reducing the effect of vegetative incompatibility which may have limited the spread of hypovirulence in the United States to date (Anagnostakis, 1977). The release of transformed strains may lead to a greater incidence of hypovirulence in C. parasitica populations, however, hypovirulence alone is unlikely to bring about the restoration of the American chestnut as a dominant forest tree species.

Although hypovirulent fungal infections are normally non-lethal, American chestnut stems infected with hypovirulent strains are often badly damaged by multiple infections. Thus, there remains a need to develop American chestnut trees which are resistant to infection by the chestnut blight fungus. We believe that American chestnut trees which have been genetically engineered to resist chestnut blight would, in combination with hypovirulent fungal strains, provide an integrated system of disease control that would be superior to either one alone.

Similar to the progress noted above with the chestnut blight fungus, recent advances in the fields of plant tissue culture and plant genetic engineering have provided new techniques that can be applied to develop an effective control for the chestnut blight fungus from the host side of the interaction.

The plant tissue culture regeneration system known as somatic embryogenesis involves de novo production of embryo-like structures from somatic cells (Merkle et al., 1990).

In other words, embryo-like structures are engineered in the laboratory through a process that begins with a developing embryo. Soon after fertilization the embryo is exposed to plant growth regulators.

Many people believe that in the early stages of embryo development the developmental programing in each cell is still very flexible, and we know that plant growth regulators can control the formation of roots, shoots or other cells.

In this experiment we are encouraging cells to replicate themselves to form clusters of cells. The result is a population of genetically identical chestnut embryos. If the growth regulators are removed the embryos can often be germinated to produce seedling like plants. This process is called somatic embryogenesis.

Recently, there has been increasing interest in somatic embryogenesis due to its potential to improve plants through large-scale clonal propagation selected individuals with desired genetic traits.

In addition, embryogenic cultures have been shown to be highly adaptable for gene transfer applications; embryogenic cultures of some forest trees have already been used to produce genetically engineered trees (McGranahan et al., 1988; Wilde et al., 1992).

We have already reported preliminary results on the initiation of embryogenic American chestnut cultures (Merkle et al., 1991), although optimal culture conditions for embryogenesis were not established and no plantlets were regenerated.

If we can improve our methods for producing American chestnut embryos and plantlets and combine that with an efficient procedure for transfering genes, we could develop a system for genetically engineering the American chestnut. This gene transfer system may ultimately be used to introduce fungal resistance genes into American chestnut trees. Single gene traits that confer resistance to fungal diseases have gained considerable academic and commercial interest.

Although genes known to inhibit growth of the chestnut blight fungus are not yet available, our overall goal is to develop a reliable system for producing genetically engineered American chestnut trees, so that

when such genes are developed, there will be a mechanism readily available to engineer American chestnut trees with them.

Thus, our approach involves achievement of two objectives: (1) To develop a system for in vitro propagation of America chestnut via somatic embryogenesis, and (2) To define a proceedure for incorporation and expression of foreign DNA in embryogenic American chestnut cells. Methods developed to achieve each separate goal will then be combined in order to produce trees from the transformed cell cultures via somatic embryogenesis.

Methods Used in Somatic Embryogenesis

American chestnut ovules and zygotic embryos from developing burs were used to initiate cultures. Material was collected from as many locations and from as many individual trees at each location as possible throughout the original American chestnut range. Because fertile American chestnut trees are not common, sources were dictated by the occurrence of fertile trees. During the two years (1991 and 1992) that cultures were initiated, twenty-five trees from locations in New York, Wisconsin, Connecticut, Pennsylvania, North Carolina and Georgia were sampled.

Seed tissues were cultured on two types of basal medium, woody plant medium [WPM] (Lloyd and McCown, 1980) and Driver and Kuniyuki medium [DKM] (Driver and Kuniyuki, 1984), using various combinations and concentrations of plant growth regulators. During the two years that cultures have been initiated, four auxins and two cytokinins have been tested. Auxins tested were 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), and picloram. Cytokinins tested were benzyladenine (BA) and thidiazuron.

Treatments of plant growth regulators were applied as follows: (A) no growth regulators; (B) auxin only; (C) cytokinin only; (D) auxin + cytokinin (E) 1, 2, or 3 week pulse on B followed by transfer to A or C; (F) 1, 2, or 3 week pulse on C followed by transfer to A or B; (G) 1, 2, or 3 week pulse on D followed by transfer to A.

Seed tissues from 16 of the 25 trees sampled during 1991 and 1992 produced somatic embryos. Five of these trees produced cell lines that exhibited repetitive somatic embryo production over several months (Figure 1). Those lines that consistently produced somatic embryos had been continuously exposed to a combination of 2,4-D and BA (Treatment D).

Cultures developed on the two different basal media did not perform differently.

And although plantlets have not yet been recovered, somatic embryos produced from our system are now receiving various treatments in order to promote maturation and germination. Treatments include various combinations of cold stratification (some seeds must be exposed to temperatures of about 4* C for a period of two to three months before they will germinate), drying and exposure to absicisic acid.

We have also started suspension cultures from some of our embryogenic American chestnut cultures by inoculating clusters of developing embryos into a liquid medium. The suspended cultures permit more rapid growth and gene transfer than do the solid cultures.

Gene Transfer

Suspension cultured American chestnut cells were used for gene transfer experiments in which a Bio-Rad PDS1000/He Biolistics apparatus was used to bombard the cells with microscopic gold particles. These tiny "bullets" were coated with DNA and then accelerated toward the embryogenic cells with a burst of helium gas. Some of the gold particles penetrate the walls of the cultured cells and release DNA into the cytoplasm.penetrated the walls of the cultured cells and released DNA. Once in the cell the introduced DNA may enter the nucleus and become part of the plant cell's genetic material.

The DNA used in this project encoded a reporter gene, ss-glucuronidase [GUS] (6) and a selectable marker, neomycin phosphotransferase (NPT II), which permits us to identify and separate those cells that have been successfully enetically engineered from those that have not. The process is aided by another condition; when a reporter gene has been inserted into an organism, it produces an easily identifiable trait. Cells that have been genetically engineered with the GUS reporter gene turn blue in the presence of X-glucuronide [X-gluc] (Jefferson et al., 1987).

In plants, selection is usually based on resistance to an antibiotic or herbicide that has been incorporated into the tissue culture medium. American chestnut cells expressing NPT II are resistant to the drug kanamycin, while non-transformed cells die. Thus colonies of American chestnut cells observed growing after several weeks of exposure to kanamycin are likely to have grown from cells which have integrated the NPT II gene into their chromosomes. Colonies of cells that survive on kanamycin can be checked to

confirm that they carry the transferred DNA.

Following bombardment, cells were allowed to stabilize on a medium without antibiotics for 12 days and were then transferred to a selection medium containing kanamycin. After approximately 8 weeks, resistant colonies of cells were visible against a background of dying (non-resistant) cells on some of the plates. Each resistant colony was transferred to its own plate with a fresh selection medium to continue growth. Approximately 10 weeks later, those lines that we believed had been transformed were tested for expression of the GUS reporter gene, using the method described above.

We then extracted DNA from each of the lines and examined it for the the presence of the indroduced DNA in order to confirm that the foreign DNA had been integrated into the American chestnut cells' own DNA.

Based on the results of assays for GUS expression and the DNA analysis, microprojectile bombardment produced 16 independent lines of transformed cells.

To date, no mature somatic embryos or plantlets have been produced from these transformed cultures. However, all 16 of these transformed lines were derived from a single embryogenic American chestnut suspension, which only infrequently produced well-formed somatic embryos prior to the gene transfer experiments. Other highly productive lines are available for bombardment, and we intend to apply what we have learned to obtain genetically engineered somatic embryos and plantlets from those lines using the microprojectile bombardment method.

Conclusion

We have demonstrated that somatic embryos can be induced from immature zygotic embryos of American chestnut, and that microprojectile bombardment is an effective method for genetic engineering of American chestnut cells. We believe that the integration of somatic embryogenesis and microprojectile-mediated gene transfer will allow development of a procedure that will permit us to introduce blight resistant genes into American chestnut.

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