The West Virginia Chapter
of
The American Chestnut Foundation
NEWSLETTER
In the heart of American chestnut's natural range
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Find us on Facebook: @WVTACF
National Office: 50 N. Merrimon Street, Asheville, NC 28804
Phone: 828-281-0047

Email: WVachestnut@acf.org
Newsletter Editor: Mark Double

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Darling 58 Saga

Long-time members of TACF know all about Darling 58, the genetically-modified American chestnut tree developed at the State University of New York (SUNY). For those new to TACF and/or the WV chapter, let's start at the beginning. Back in the late 1980s, a few years after TACF began its backcross breeding program, **Drs. William Powell** and **Chuck Maynard** at SUNY began searching for a gene that could break down oxalic acid, one of the key acids produced by the chestnut blight fungus. Oxalic acid kills tissues in the vascular cambium of American chestnut. Once the tissues are disrupted, the fungus then utilizes the cell contents as food. Powell and Maynard searched among both animals and plants and chose a gene from wheat, oxalate oxidase (OxO), that breaks down oxalic acid into carbon dioxide and hydrogen peroxide, thus neutralizing the ability of the fungus to kill tissues. They began investigating the effect of OxO after it was inserted into the DNA of an American chestnut tree. This research at SUNY continued for several decades, eventually leading to the development of Darling 58, named for Herb Darling, a past president of the NY chapter of TACF.

One of the shortcomings of Darling 58 was the promoter for OxO that was chosen. A promoter is a region of DNA upstream of a gene where proteins bind to initiate the transcription (formation) of that gene. In simple terms, the promoter acts to make a specific gene function within the cell. The promoter in the case of the Darling line is referred to as a 35S promoter. This particular promoter acts to produce OxO constitutively (continuously). The analogy is having a fever all day, all week, all year. It's hard to function and perform daily activities if a person has a constant fever. It's the same with the OxO in a chestnut tree--the tree doesn't have sufficient energy to grow similarly with sibling trees that do not contain the OxO gene as seen in the photo on Page 2. Trees with OXO grow 15-25% slower than trees without OxO.

Many trees were produced at SUNY with the 35S promoter. These trees all were in the 'Darling line'. Two trees in the line were Darling 54 and Darling 58. Researchers early on knew there were problems with Darling 54 and they stated so in the documents that were compiled for the Federal government. Somehow Darling 54 and Darling 58 were switched. This happened at the very beginning of the program, likely in 2016. It is a "switched at birth" scenario, where pollen which was thought to have been gathered from a D58 tree was actually gathered from a D54 tree. Pollen from those two originating trees were the basis for all research moving forward on

Darling 58 trees with the OxO gene on the left and full sibling trees without OxO trees on the right. The OxO+ trees grow 15-25% slower than the OxO- trees. Photo courtesy of TACF.

what was thought to be Darling 58. Since 2016, all trees that were assumed to be Darling 58 were actually Darling 54. So what is the difference between the two trees?

Members of the Indiana chapter established field trials of Darling progeny in 2019 under permit from the USDA. In 2022, they inoculated about 150 Darling progeny with the chestnut blight fungus. Approximately half of the progeny inherited OxO and half did not inherit OxO. The progeny that inherited OxO initially had significantly smaller chestnut blight cankers than their siblings that did not inherit OxO; however, a subset of the OxO positive progeny had large, severe cankers similar to the OxO negative trees. Our Indiana collaborators kept the OxO positive trees that had the smallest cankers and cut down the trees that had larger cankers. One year after the inoculations, the cankers on the OXO+ selections continued to expand on some of the trees (see photo in next column). A similar finding of expanding cankers was noticed on some of the oldest Darling progeny at SUNY. It is thought that the 35S promoter is somehow silenced over time. The failure (silencing) of the 35S promoter has been seen in other plants. An alternative hypothesis is that there are additional important mechanisms of blight resistance besides oxalate detoxification by OxO. TACF is following up with studies of how expression of the OxO gene over time corresponds to relative canker expansion.

Expanding canker on an OxO+ tree (photo courtesy of TACF).

The next part of the explanation is to understand homozygous versus heterozygous. While these two terms can be intimidating, the differences are rather simple.

Homozygous is a genetic condition within an organism's genetic makeup in which identical alleles for a specific gene are present on each pair of chromosomes through inheritance from both parents. The following diagram of a Punnet Square helps to explain the two conditions.

	R	r
R	RR	Rr
r	Rr	rr

The RR and rr represent homozygous pairs while Rr are heterozygous. The large R is dominant while the small r is recessive. In terms of the Darling line, all the trees that have been approved for outplanting at Penn State, SUNY, Meadowview and at the University of New England (UNE)

in Maine have been Rr or heterozygous. The problem with Darling 58 (nee Darling 54) is that we had to wait for the original Darling trees to mature so that female flowers could be produced. Male flowers (catkins) can be produced early under high light conditions, but not so for female flowers. **Dr. Tom Klak** at the University of New England (UNE) was able to produce OxO+ female flowers in the greenhouse that were fertilized with OxO+ male catkins to produce nuts that were homozygous for OxO+. The nuts were stratified and will be planted out. The problems with Darling 58 were not fully understood until trees were produced in which both the female and male flowers were both OxO+. Tom Klak was able to complete this task.

Our partners at the UNE began intercrossing flowering Darling trees indoors in their high light pollen production room. After fertilization, the expectation is that 25% of the progeny will inherit two copies of OxO, one from each parent, in a homozygous RR state. The UNE researchers enlisted a geneticist from the University of Maine (**Dr. Han Tan**) to genetically test the trees for homozygous inheritance. Through the process of testing for homozygosity, they learned that the OxO gene was actually located on chromosome 4 (and not on chromosome 7 as was expected). That insertion deleted over 1000 base pairs in the nearby SAL1 (saline) gene. In a review of over 40 progeny tested from the intercrosses at UNE, these results imply that either constitutive expression of OxO is lethal in a homozygous state or the disruption of a native chestnut gene (SAL1) by OxO insertion is lethal. Further research is required to disentangle these two possibilities. Thus, of the 40 trees examined, only 1 was homozygous, and it died. The other 39 trees were heterozygous (an unexpected result). It appears that homozygosity is difficult to obtain. While lethality is an issue with homozygous trees, TACF cannot distribute trees to the public where most of the trees canker and die. That would be disastrous and unethical.

Since the OxO gene was found to be on a different chromosome than expected, the question is can this problem be circumvented in the future? We can now do whole genome sequencing to verify where genes are as we move forward. The bottom line is that TACF did not expect a problem, so there was no reason to search for these issues until the recent events of shorter growth, expanding cankers and lethality came to light.

Are there other issues with the Darling line? Yes, leaf scorching has been observed with the Darling transgenic line as seen below (photo from TACF).

OxO- trees on the left and OxO+ trees on the right

Thus, the shorter stature of Darling 58, the development of large cankers after a year, the lethality of most trees and leaf scorching collectively led TACF to withdraw it support of Darling 58. This is not withdrawing of transgenic technology, or OxO, just Darling 58.

Since Darling 58 was actually Darling 54, why not use the real Darling 58? There are only a handful of putative D58 trees, and perhaps even only 1. This tree may only be in tissue culture. If work is to start over, it makes the most sense to focus on a line with better and more advanced technology. We suspect the constitutive 35S promoter in these Darling lines has caused some or even most of the performance issues we are seeing. Therefore, to most efficiently and effectively use our time and other resources, we should focus on lines which are likely to provide better competitive performance.

What is a more effective line? Recall that the 35S promoter is constitutive, making OxO all the time. An alternative is a wound-inducible promoter. A wound-inducible promoter only produces OxO when a chestnut tree is wounded or infected by the chestnut blight fungus. This saves a lot of energy for a tree. Rather than produce OXO all the time, a tree can use most of its resources to grow and produce a sound root system and top growth. The problem is that wound-inducible promoter lines of trees have only been grown, for the most part, in the laboratory. Field testing has yet to occur.

Does TACF still support transgenic research? Yes, TACF still supports transgenic research, including ongoing research

with the OxO gene. We have such trees already in the pipeline and with plans to create and review more. In addition, using genes from sexually compatible species, i.e. from within the *Castanea* genus, will significantly reduce or even the eliminate regulatory process.

Even if we start today to attempt another transgenic line, it takes a good two years from inserting a gene in a chestnut trees' DNA to having a plant that can be put into the ground. There is tissue culture, somatic embryogenesis, hardening off in a greenhouse, then to a shade house and then outplanting. The many steps each involve time.

There are other avenues to pursue beyond transgenics. First and foremost is crossing Best X Best. For those who read the December 2023 WV chapter newsletter, there was information from **Dr. Stacy Clark**, U.S. Forest Service, who tested backcross trees in 2013-2015 plantings. She used backcross trees from 2009, the best material available at that time. Stacy concluded that the 2009 trees did not have sufficient resistance to grow and reproduce in forest settings in TN, VA and KY. However, we have much better material in 2024 compared to 2009. The issue is time. It will take another 15-20 years to test the 2024 material in forest settings to determine if there is sufficient resistance in these newer lines.

Another alternative to is stack the OxO gene with other genes to increase resistance. TACF has been working for years to understand the factors that confer resistance in Chinese chestnut. New molecular tools have come to the forefront in the last few years that may help us understand what genes aid Chinese chestnut in fighting the blight fungus.

Another avenue investigates turning down genes in Chinese chestnut to see if we can make Chinese chestnut susceptible. This will aid in identifying the genes that are responsible for blight resistance. **Bruce Levine** at the University of Maryland is working to identify genes in the chestnut blight fungus to try and make the fungus less aggressive.

TACF does have wound-inducible promoters in labs outside of SUNY that are producing pollen. If SUNY doesn't want to work with TACF directly, we can work with and test the wound-inducible promoters we have as a prototypes to determine the performance and if the wound-inducible promoter will be a solu

tion to pursue further. Wound-inducible promoters have yet to be field tested in a wide variety of genetic backgrounds, both of which will be required to really see how such a product can perform. At this point, TACF does not plan to develop or deploy any trees with a 35S constitutive promoter. Given all the shortcomings of the 35S promoter, TACF will concentrate on other avenues and other promoters.

After partnering with SUNY for decades developing what we thought was a good fit for restoration, it came as a shock to all that Darling 58 did not pan out. An analogy is a car company that spends millions of dollars developing a new car line only to find out that just before production starts, it is determined that the brake system fails. The car company cannot put a faulty car on the market. Neither can TACF continue with a tree that has many faults.

Science in many cases is trial and error. For decades TACF moved toward the belief that 2-3 genes were responsible for resistance in Chinese chestnut only to find out decades later that there may be hundreds of genes responsible for resistance. New tools help us move forward with our understanding of very complex biological systems. Since it was years before a homozygous Darling 58 tree could be produced, we went on the hypothesis that everything in the Darling line was going to produce a tree that could grow with American form and have sufficient resistance to the chestnut blight fungus. Again, technology told us that the Darling 58 tree was not what we hoped. TACF recognized the problem, and rather than shield its members from the truth, the problem was made public to both TACF's members and to the federal agencies. TACF has chosen to be fully transparent, knowing the concern and powerful response it would receive.

Have we lost time? Yes, we have probably lost a decade. If we start today, it will be another 10 years before we can get back to where we are today with transgenics. It is a process of developing new lines and verifying if new lines have the characteristics needed for forest survival and growth. As **Sara Fitzsimmons** (TACF's Chief Restoration Officer) has said many times, restoration is a process not a product.

TACF still believes transgenic lines are an important part of moving forward. Most of the scientific studies will have to be repeated. However, TACF still has its own backcross material to fall back on as we move for-

ward. While this is a major setback, TACF strives to continue its goal of restoring American chestnut back into our Eastern North American forests.

What is the relationship between TACF and SUNY since SUNY owns the rights to the Darling lines? At this point, our relationship with SUNY is unclear. SUNY expressed their disappointment that TACF pulled its support of their program. TACF did not pull its support of SUNY's program, just the Darling 58 line. SUNY plans to move forward with the Darling 54 line, despite its notable shortcomings. TACF's President and CEO, **Dr. Will Pitt** has talked several times with the point person in SUNY's administration. The conversations will continue between TACF and SUNY. Thus, the relationship between the two organizations is undetermined at this point in time.

Guiding Principles for Science and Restoration Efforts: Based on lessons learned through the process of researching and testing Darling lines of trees, TACF has created the following framework to better guide future efforts.

1. Rigorous testing for efficacy throughout the life cycle of the tree life cycle (both in the lab and greenhouse, and in the field) prior to regulatory submission;
2. Rigorous testing for plant health and environmental risks;
3. Implementation of a tree improvement cooperative structure which can facilitate shared intellectual property, provide full transparency across all members, and ensure the rigor and comprehensiveness of scientific methodology and analysis;
4. Ensuring products remain in the genetic commons as much as is possible, while also protecting the quality and integrity of a given product (for example, through material transfer agreements).

Boy Scout Pack 52

Sam Muncy, the WV chapter treasurer, informed about 100 Boy and Girl Scouts and their parents about the story of the American chestnut. On Saturday, December 9, at Camp Mountaineer in Monongalia County, the scouts hiked around the camp doing various crafts and knowledge stations. One of the many stations dealt with American chestnut. Sam talked not only about American chestnut but also the history and purpose of TACF. Sam had posters that showed the progression of chestnut blight from its start in 1904 in New York to groups of 10-20 scouts over an 8-hour period from 9:00 am until 5:00 pm. There were many questions from both young and old. A few knew of the blight, but to most it was new knowledge. Sam talked about both the backcross breeding program and the transgenic trees.

One of the highlights for the scouts and their parents was roasted chestnuts. Sam roasted chestnuts for Monongalia County's Boy Scout Pack 52. Sam is a life-long member of the Boy Scouts of America and he spearheads the chestnut effort at Summit Bechtel Reserve (SBR), the 14,000-acre Boy Scout facility in Fayette County. There are several plantings of American chestnut at the SBR, and one scout troop expressed interest in helping with the next work day at the SBR in April 2024. Sam encourages all scout troops to become involved with the chestnut plantings at the SBR.

The WV-TACF chapter should work with scouts because scouting encourages outdoor hiking, and young people can, with some training, be able to locate native American chestnuts. The WV chapter is looking to capture and create orchards of native trees (germplasm conservation orchards, GCOs). Trees in the GCOs that flower can then be used to cross with TACF's advance backcross material to develop trees that have sufficient blight resistance, American chestnut form and adaptability to West Virginia.

Sam Muncy (middle in tan shirt) with a few of the scouts and their parents at Camp Mountaineer.

The other important aspect of the SBR is that TACF has been invited to have a station in the Antoline Family Conservation pavilion at Summit when it is constructed. A chestnut station in the pavilion can have a great impact on both scouts and their parents. What a great way to spread the news about the saga of the American

chestnut and our efforts toward restoration of a species.

A few of the scouts after Sam's chestnut presentation.

Placing chestnuts in D40 pots.

WV Chapter Spring Meeting

The 2024 spring chapter meeting will be held on Saturday, April 6 at the Westvaco Center on the campus of Glenville State University. The meeting will begin at 1:00 pm in a classroom on the second floor. Join us as new WV chapter president, **Bernie Coyle**, leads his first chapter meeting. Directions to the Westvaco Center and a link to join us via Zoom will be sent at a later date.

Potting Date in Morgantown

Even though we are in the throes of winter, it's not too early to begin preparations for spring. This year, there will be three greenhouses growing chestnut seedlings: the WVU greenhouse in Morgantown; Potomac State in Keyser; and at the home of **Dr. Lewis Cook**, WV chapter board member in Fayetteville. Potting mix and chestnuts will be delivered to Potomac State and to Dr. Cook in early March.

Backcross chestnuts will be shipped from Meadowview on February 28, so the potting date for Morgantown is Saturday, **March 9, 2024** at the Davis College greenhouse on the Evansdale campus. **Mark Double** hopes to have almost all the D40 pots pre-labeled. This makes for a quick an easy potting session. The date is weather-dependent as the March date in 2022 was met with the biggest snowstorm of the season.

The Chestnut Blight Fungus

The following article was written for TACF's Chestnut magazine, but some of the pictures are not of sufficient resolution for printing in the magazine.

Collectively, we joined The American Chestnut Foundation (TACF) for the sole purpose of helping to restore American chestnut back into our eastern North American forests. Restoration is required due to a fungus that was imported into the United States on infected Japanese chestnut. In 1904, Hermann Merkel, chief forester for the Bronx Zoo in New York City, noticed brown leaves in mid-summer on the majestic American chestnuts lining the zoo's walkways. Merkel wrote that a few scattered cases occurred [on American chestnut trees] during the summer of 1904. Early in June 1905, this disease was noticed on so many widely scattered trees of all sizes that specimen branches and an appeal for information were sent to the USDA (Merkel, 1905). Two years after the disease was first noticed, William Alphonso Murrill (Assistant curator of the New York Botanical Garden) wrote, "A new and very serious disease of our native chestnut is epidemic in many parts of New York City and threatens to destroy practically all the chestnut trees in this vicinity. An investigation of the disease was begun in the New York Botanical Garden nearly a year ago, and most

of the facts regarding it are now in our possession. The fungus in question appears to be confined to our native chestnut. A related species occurring on the European chestnut is quite different in character and totally different in habit. I have shown specimens to many mycologists, both in Europe and America, and they all pronounce it new to them and undescribed. It belongs to *Diaporthe*, a large genus whose species are as a rule confined to dead wood and are not parasitic. The name I have chosen refers to its very destructive parasitic habit. I have named the fungus *Diaporthe parasitica*" (Murrill, 1906).

After the blight fungus was discovered in New York City, plant explorer Frank Meyer found that it was present in both China and Japan, and that Asian trees were often very resistant to the disease and showed few symptoms when infected (Shear and Stevens, 1913). This was taken as proof that Asian trees imported into the United States had brought the blight with them (Anagnostakis, 1997). G. H. Powell wrote in 1900 that Japanese chestnut trees (*Castanea crenata*) were first imported in 1876 by nurseryman S. B. Parsons of Flushing, New York (in the New York City borough of Queens, at the western end of Long Island). In 1882, William Parry in New Jersey imported 1,000 grafted Japanese chestnut trees. These importations of Japanese chestnut trees could have been the source of chestnut blight. In addition, mail-order sales could have spread imported blight to all of the places where the trees were shipped (Anagnostakis, 1997).

As per Murrill's taxonomic description in 1906, the causal agent of chestnut blight was known as *Diaporthe parasitica*. In 1912, the name was changed to *Endothia parasitica* (Shear and Stevens, 1917). The name was changed again in 1978 by mycologist, Margaret Barr, who placed the fungus in the genus *Cryphonectria*. If no further changes are necessary, the fungus continues as *Cryphonectria parasitica*.

It is reported that 4 billion trees were lost to the fungus over the course of 50 years. The fungus co-evolved with Asian chestnut species over millennia, but found two chestnut relatives, European (*Castanea sativa*) and American chestnut (*Castanea dentata*), that have no resistance to the fungus.

The spread of the chestnut blight fungus over the range of American chestnut from Maine to Alabama was aided by two spore types, asexual and sexual. Both spore types can initiate cankers on chestnut trees. The fungus is distinctly orange-pigmented, whether as a canker on a tree (Fig. 1) or as a culture on a Petri dish. (Fig. 2). Both spore types are formed in the orange bumps on cankers, known scientifically as stroma (Fig. 3). The asexual spores (conidia) are thought to be the spore type of localized spread. These spores are produced in the stroma and exuded in a sticky matrix (cirrhi) that often resemble pig tails. Cirrhi are produced in conditions of high moisture and the tens of thousands of spores are washed over the tree during rain events (Fig. 4). The asexual conidia often find cracks as they are washed over the bark and initiate new cankers.

Fig. 1. Canker on an American chestnut tree

Fig. 2. Chestnut blight fungus on an agar medium

The sexual spores (ascospores) also are produced in the stroma in pear-shaped structures referred to as perithecia (Fig. 5). The chestnut blight fungus is classified as an Ascomycete. What makes Ascomycetes unique is that the sexual spores are formed in fragile translucent sacs called asci. Each ascus has 8 two-celled ascopores. Thousands of these packets of spores are "shot out" of the necks of the perithecia when weather conditions permit. The spores are non-motile, but when shot out of the necks, the spore packets are picked up by the wind and disseminated over long distances. A microscopic view of both spore types is shown in Fig. 6. The conidia are smaller than the two-celled ascospores. An ascus with 8 two-celled ascopores (red arrows) is shown in Fig. 7 (courtesy of D. Rigling, Switzerland). Whether a canker is initiated by an asexual or sexual spore, both sit in a crack in the bark until conditions allow for germination. Once a spore germinates, the threads of the fungus begin forming a fan (Fig. 8). The fungus grows in the vascular cambium exuding oxalic acid in advance of the threads. The acid kills the tissues in this region, and the contents of the cells are utilized by the fungus. As the fungus grows around the circumference of a branch or main stem, everything distal to the infection is killed.

Fig. 3. Orange stroma where both spore types are produced.

Fig. 4. Cirrhi in which sticky asexual spores are exuded.

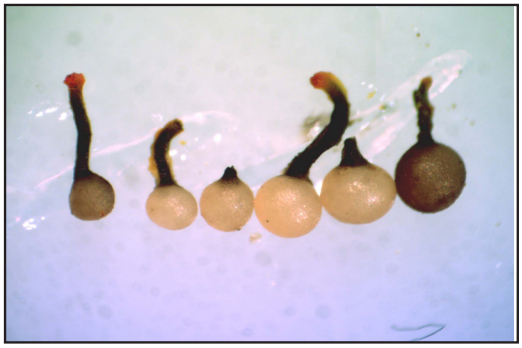

Fig. 5. Pear-shaped perithecia that produce sexual spores.

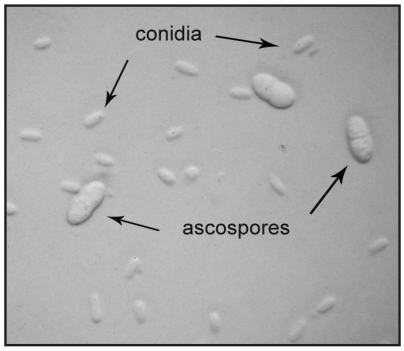

Fig. 6. Microscopic view of two spore types.

Fig. 7. Microscopic view of asci containing 8 two-celled ascospores

Fig. 8. Fans of the fungus in the vascular cambium

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