

# The American Chestnut Foundation

## Comments on the Darling 54

In 2023, the American Chestnut Foundation decided to withdraw support of the D58 [D54] transgenic chestnut petitions after a thorough review of the facts, based primarily on these factors:

1. **D58 [D54] Performance:** Observational data on transgenic trees thought to be D58 from multiple orchard locations indicated a number of corroborating deficiencies and concerns, including inconsistent blight resistance, a negative impact on growth, and decreased survival rates. See [Darling 58 Performance](#) page. [also below]
2. **Mislabeling of the D54/D58 product:** In late 2023, TACF learned of a lab error at SUNY-ESF that resulted in mislabeling and confusion between two distinct transgenic trees, D54 and D58. The error may have occurred as early as 2016, but regrettably was not discovered or reported to TACF until 2023. The mislabeling meant the regulatory petitions were filed as D58 when they should have been identified as D54. Unfortunately, this unintentional lab error concealed changes to coding regions of the genome with unknown and untested effects on growth and survival.
3. **Potential Regulatory Issues:** Based on the advice of our regulatory counsel in Washington, DC, TACF concluded that the mislabeled product raised additional questions about the data submitted and TACF could not support SUNY-ESF's submission if they proceeded. Factual errors in any petition may adversely impact credibility, call into question the accuracy of other data supplied by the applicant, and potentially fuel otherwise unfounded accusations by critics of genetic engineering (GE) that developers are not doing enough to ensure the safety of GE products.
4. **Commercialization of the D54/D58 transgenic chestnut:** Finally, we were surprised and concerned when SUNY-ESF proposed the commercialization of the D54/D58 transgenic chestnut through an exclusive partnership with American Castanea Inc, a for-profit startup company. We believed then, and continue to believe today, that pricing seedlings to yield a commercial profit would jeopardize success and is inconsistent with our commitment to advance TACF's mission as a 501(c)(3) nonprofit.
5. Even after sharing these profound concerns about entering into a SUNY-ESF exclusive license with American Castanea Inc, SUNY-ESF offered a variety of high-cost proposals to TACF, all of which **continued to demonstrate their interests in using D54, a transgenic tree TACF considers unsuitable for restoration, as a means to fund their programs.** SUNY-ESF continued to request annual six-figure licensing fees and royalties, while requesting TACF's continued financial support of SUNY-ESF research. We believe that payment of these fees would divert critical funds from our core research and the development of an effective restoration tree.

## Rescue and Restoration of the American Chestnut

The American chestnut was an ecologically, economically, and culturally significant tree species in the Eastern United States. In the late 1800s, the introduction of the chestnut blight fungus

(*Cryphonectria parasitica*) from Asia decimated an estimated four billion American chestnuts. Today, this iconic species rarely reproduces in the wild and is therefore considered functionally extinct.

The American Chestnut Foundation (TACF) is leading an unprecedented mission to restore the American chestnut tree to its native range. By employing complementary methods of traditional breeding, biotechnology, and biocontrol, TACF is working to create a disease-resistant and genetically diverse population of American chestnuts.

One of TACF's greatest achievements is the establishment of a genetically diverse, reproducing population of thousands of American chestnut trees representing hundreds of breeding lines. These trees are planted in TACF's expansive network of germplasm conservation orchards and regional breeding and backcross orchards. We have substantially increased blight-resistance and *Phytophthora* resistance in these populations. The future inclusion of transgenic and/or gene-edited trees will further increase those gains.

Restoration is an incremental process which will require the integration of multiple technologies and institutions. TACF is privileged to work with a diverse range of partners, from private landowners to university research labs, who inform the direction of our scientific inquiry. This collaboration allows TACF to compare results from across the native range of the American chestnut. Through this extensive research network, transgenic methods have shown great promise for generating a blight-tolerant American chestnut tree and play an important role in TACF's [science strategy](#) and [restoration efforts](#).

## **History of the Transgenic Darling 58 American Chestnut Tree**

In 2015, The American Chestnut Research and Restoration Project (TACRRP) at the State University of New York College of Environmental Science and Forestry (SUNY-ESF) created the first line of transgenic American chestnut trees and named it after an early supporter of the project, Herb Darling. Deeming it a promising path toward American chestnut restoration, TACF began formally supporting TACRRP's work in 2015 through significant funding and research support.

The goal of the Darling line of trees was to confer blight tolerance by inserting a gene from wheat called oxalate oxidase (OxO). The OxO gene detoxifies oxalic acid produced by the chestnut blight fungus and could hypothetically prevent lethal cankers on the tree. Many "events" (random insertions of the gene into the American chestnut genome) were created using the 35s constitutive promoter which causes the OxO gene to always be "switched on" in the tree's tissues. These different events were given names such as Darling 4, Darling 54, and Darling 58, and were tested for gene placement and OxO expression. One, named Darling 58, was selected for further lab and field testing.

Initial laboratory and greenhouse tests on Darling 58 trees (also known as D58) showed that high disease-tolerance was being conferred by the OxO gene. Extensive safety tests showed no significant difference in the Darling chestnut's interactions with other organisms compared to

American chestnut (read about [Nutrition](#), [Wildlife](#), [Plants & Fungi](#)), and the Darling 58 product was poised to play a significant role in American chestnut restoration.

As a result, TACRRP submitted petitions for deregulation to USDA APHIS, EPA, and FDA with the goal that the Darling tree could eventually be used in the wild to restore American chestnut populations. Based on these results and its ongoing support for biotech research, TACF extensively supported TACRRP throughout the research efforts as well as the petitions for deregulation.

## **Discovery That Darling 58 Was Actually Darling 54**

In late October 2023, partners at the University of New England and University of Maine informed TACF that there appeared to be a possible mix-up of pollen early in the D58 breeding program and shared with us their results.

In November 2023, TACF independently verified that information, confirming that the O<sub>x</sub>O gene of all trees thought to be Darling 58 was on a different chromosome than expected (chromosome 4 instead of chromosome 7). We have subsequently confirmed that all the trees we and other partners have been researching since 2016 are in fact descendants of a different event in the Darling line called Darling 54 (D54).

## **Why TACF Withdrew Support for the Darling Line**

Throughout 2023, TACF and its partners observed disappointing performance results from broad-scale field and greenhouse tests of advanced generations of Darling trees across several geographic locations. As discussed in the September 15, 2023 episode of TACF's Chestnut Chat webinar series ([Darling 58 Update](#)), analysis indicated striking variability in Darling trees' blight tolerance, significant losses in growth competitiveness, reduction in overall fitness including stunted growth, leaf browning and curling, and increased mortality. View an outline of the body of evidence concerning Darling's performance issues on the [Darling 58 Performance](#) page.



These are 10% best selections from Purdue after inoculation with weaker SG2,3 strain of *C. parasitica*





In Darling 54, the OxO gene has been inserted into a coding region, causing a deletion of 1,069 base pairs in a salinity tolerance gene called SAL1. It has since been shown that there is only a single occurrence of this gene in the American chestnut genome (there are no backup copies). Research has also indicated that the homozygous state of Darling 54 (when an individual plant

inherits a copy of the OxO gene from both parents, which occurs in 25% of offspring) is largely lethal, and that a great majority of homozygous offspring die in the embryonic stage.

The fact that the 35S OxO construct in D54 interrupts a known gene, and has caused a large deletion of that gene, combined with the disappointing performance of the D54 trees in the field and the observed lethal homozygosity, have led TACF to conclude that this product should not be distributed or propagated beyond permitted sites. TACF believes these genetic issues should not be proliferated into current or future restoration or commercial chestnut populations.

In addition to those baseline scientific concerns, TACF is also concerned that further production or development of D54 would decrease public acceptance of the use of biotechnology in forest health and restoration efforts. Knowingly producing and proliferating trees with genetic issues, especially after establishing that these were at least partially the result of an early error, is bound to erode public trust in both the process and the producers of such a product.

Why not then return to the “real” Darling 58? The first reason is that the Darling 58 also uses the 35S constitutive promoter which appears to result in a variety of metabolic costs to the tree and likely causes the majority of performance problems observed in the Darling line. Secondly, there are only a handful, and perhaps even only one, D58 tree(s) in existence. These trees are at either the T0 or T1 stage (the original event and first-generation offspring). If work is to start over at those early diversification stages, it makes sense to focus on new OxO lines that express the gene only in tissues infected with blight. Confining OxO expression to blight infected tissues should reduce the metabolic cost of expressing this gene, thus these new lines are more likely to have enhanced forest competitiveness.

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## Darling Performance Results

This page outlines results and conclusions from lab and field trials of the Darling 58 transgenic American chestnut tree (now known to be Darling 54 progeny) conducted by The American Chestnut Foundation (TACF) and various research partners as of December 2023. For more information about the Darling 58 (D58) tree, please visit the [Darling 58 page](#).

In these results, trees that have inherited the oxalate oxidase (OxO) gene are referred to OxO positive and denoted as OxO+. Those that did not inherit the gene are referred to as OxO negative (OxO-).

### Evidence of poor performance of Darling progeny

#### 1. Variability of blight resistance among Darling progeny that inherited oxalate oxidase

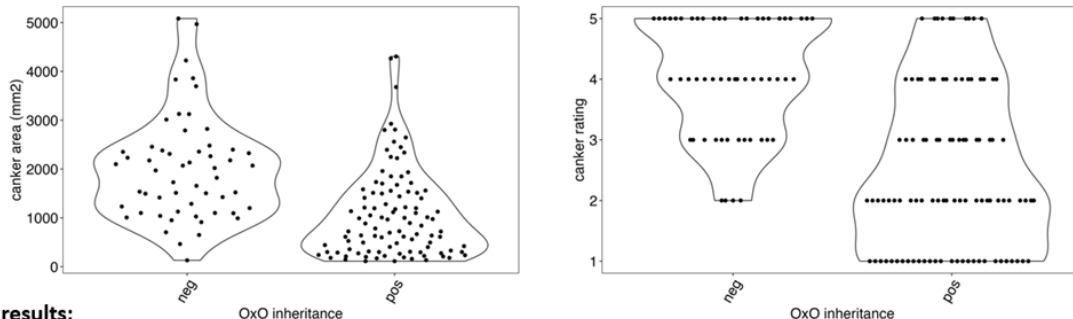
Our partners from the Indiana chapter established field trials of Darling progeny in 2019 under permit from the USDA. In 2022, they inoculated about 150 Darling progeny with the chestnut blight fungus. Approximately half of the progeny inherited OxO and half did not inherit OxO. The progeny that inherited 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 selections continued to expand on some of the trees. We observed similar phenomenon of expanding cankers on some of the oldest Darling progeny in existence. We hypothesize that delayed canker expansion is due to the expression of the OxO is being suppressed over time in some trees leading to delayed canker expansion. An alternative hypothesis is that there are additional important mechanisms of blight resistance besides oxalate detoxification by OxO. We are following up with studies of how expression of the OxO gene over time corresponds to relative canker expansion.



Note: These are 10% best selections from Purdue after inoculation with weaker SG2,3 strain of *C. parasitica*

Variable resistance in Darling 58 progeny at Purdue University.



**Key results:**  
 OxO + D58 progeny tend to have less severe cankering than non-transgenic siblings, but there is a lot of variation in canker severity among OxO + progeny

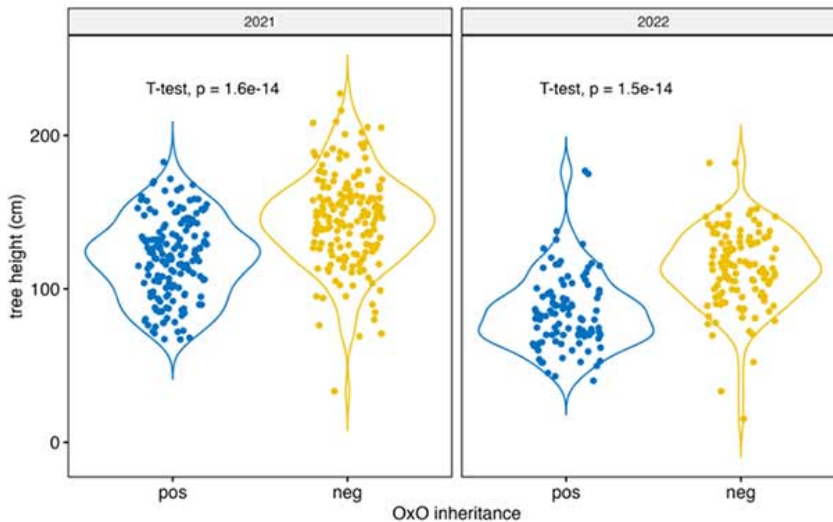
Variability in canker sizes observed in Darling 58 progeny at Purdue University, July 2023.

## 2. Growth and survival penalties associated with inheriting OxO among Darling progeny

At multiple stages of trees' lifetimes and in multiple contexts, we have observed reduced growth and survival of Darling progeny that inherited oxalate oxidase calling into question the competitive ability of these trees in the forest. In Indiana field trials, OxO positive trees were 15% to 25% shorter on average than OxO negative siblings among at age 2 to 3 years. At a testing site in New York, we observed stark visual differences in the heights of OxO positive trees as compared to OxO negative siblings among trees that were at least six years old. At Meadowview Research Farms in 2021, the OxO positive trees exhibited reduced growth and increased leaf injury relative to OxO negative siblings. In a field trial at Virginia Tech, the survival of OxO positive trees at age 5 was much lower (5 OxO positive survivors of 24 planted) than OxO negative full siblings (19 survivors of 24 planted).

### *Evidence 1*

Growth penalties associated with OxO inheritance, Purdue 2023.



### *Evidence 2*

Cross-lab greenhouse trial (VA, PA, NY)2022. Significant growth penalties observed in OxO positive trees. Pooling across 3 replicate sites OxO inheritance predicts seedling height, with OxO positive siblings being significantly shorter than OxO negative siblings.

### *Evidence 3*

Central NY. March 2022. Growth differential shown between OxO- trees and OxO+ trees.



Central NY. Growth differential between OxO- trees (left) and OxO+ trees (right)

*Evidence 4*

Virginia Tech Kentland BRAG plot, September 2022. Higher mortality rate in OxO positive seedlings. 19 of 24 OxO negative survived vs 5 of 24 OxO positive survived.



TACF staff observed blight cankering on some surviving OxO positive trees at the VT Kentland BRAG plot, September 2022

#### *Evidence 5*

Meadowview Research Farms nursery , Meadowview, VA 2023. D58 vs DarWin trial. Darling 58 positive seedlings had considerably lower first season survival than D58 negatives, DarWin positives, and DarWin negatives. All seedlings shared the same mother tree. Survived: 95% D58 neg, 65% D58 pos, 75% DW neg, 93% DW pos. This was a preliminary trial with 160 plants total.

#### 3. Lethality in homozygous state and insertion of OxO into a native chestnut gene

Our partners at the University of New England began intercrossing flowering Darling trees indoors in their high light pollen production room. The expectation is that 25% of the progeny will inherit two copies of OxO, one from each parent, in a homozygous state. The UNE researchers enlisted a geneticist from U. Maine 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. That insertion deleted over 1000 base pairs in the SAL1 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.

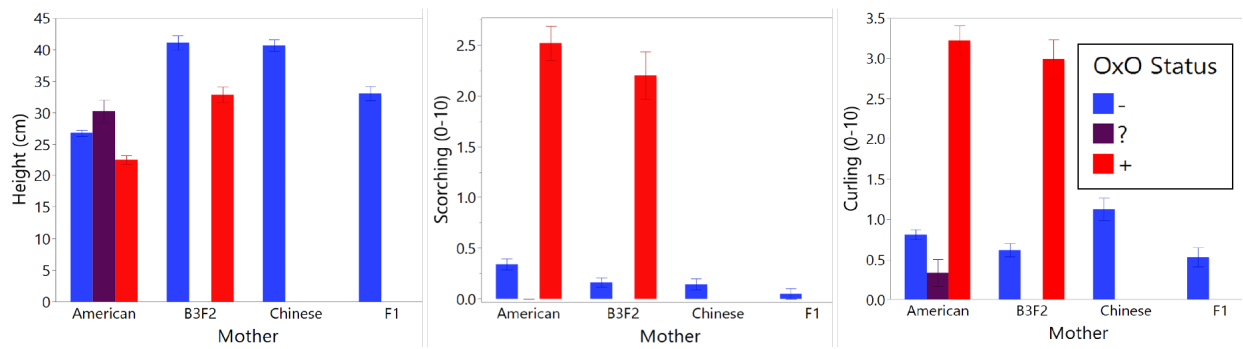
#### 4. Leaf curling, browning, stunting

##### *Evidence 1*

MRF nursery 2021 leaf injury data. Leaf scorch injury was observed on average at 8.4X greater intensity on OxO positive than on OxO negative seedlings. Leaf curl injury was observed on average at 4.1X greater intensity on OxO positive than on OxO negative seedlings. OxO positive seedlings grew 17% shorter in a season than their OxO negative seedlings.



Characteristic suite of symptoms on an OxO+ seedling



Bar graphs of seedling symptoms, data collected July 2021. N = 628 OxO-, 314 OxO+.

*Evidence 2*

Purdue nursery 2021. OxO positive seedlings showed considerably more leaf margin scorching compared to OxO negative seedlings. Photos from July 2021.



Purdue 2021 OxO+ (OxO positive) seedlings. Photo credit: Caleb Kell.



Purdue 2021 OxO- (OxO negative) seedlings. Photo credit: Caleb Kell.

*Evidence 3*

Cross-lab greenhouse trial (VA, IN, NY) 2022 leaf injury data. Pooling across 3 replicate sites, OxO inheritance predicts leaf scorch and leaf curl injuries, with greater leaf scorch and leaf curl injuries in trees that inherit OxO.

## VISUAL ASSESSMENT OF BIOLOGICAL SIGNIFICANCE

Images below correspond to the mean height, scorch, curl, and chlorosis in each site x OxO status pairing

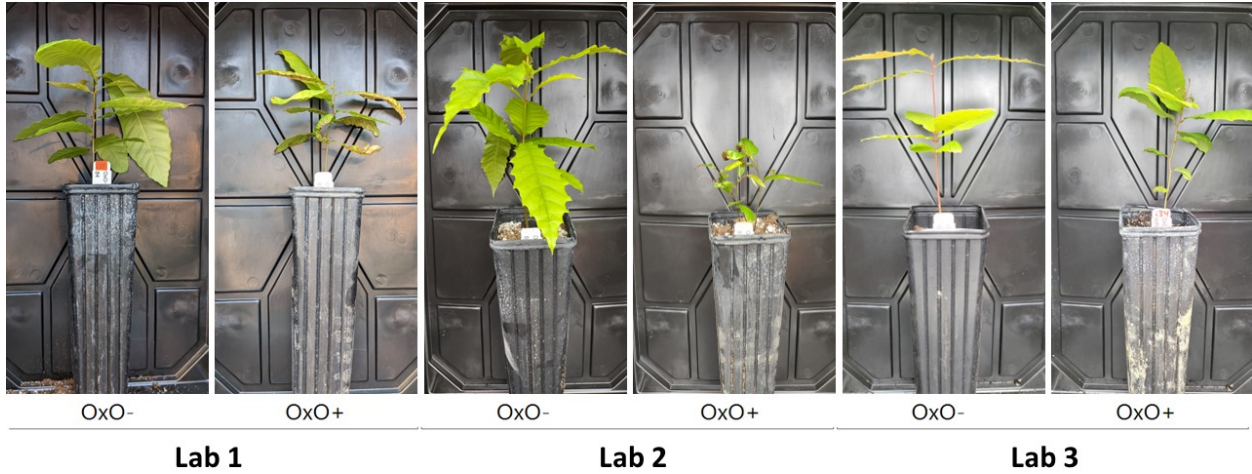


Photo of seedlings that match the mean height and leaf injury values at each replicated location, showing decreased height and increased leaf scorch and curl in OxO positive (OxO+) seedlings.

*Evidence 4*

Greenhouse NY Chapter seedlings, May 2022.



TACF NY Chapter OxO+ seedlings beginning to manifest characteristic leaf injury symptoms, May 2022.