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Via regulations.gov

Dr. Alan Pearson
Assistant Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
U.S. Department of Agriculture
4700 River Rd., Unit 97
Riverdale, MD 20738

RE: Comments of the American Chestnut Foundation on Draft Environmental Impact Statement and Draft Plant Pest Risk Assessment for Determination of Nonregulated Status for Blight-Tolerant Darling 54 American Chestnut; Docket No. APHIS-2020-0030

Thank you for the opportunity to comment on the revised Plant Risk Assessment (PPRA) and Environmental Impact Statement (EIS) for the Darling 54 American chestnut (SUNY-ESF, 2025). The American Chestnut Foundation (TACF) assessed the performance of Darling 54 progeny, beginning in 2018. The original APHIS petition for nonregulated status was first submitted in January 2019 ("Petition"). Some of our performance observations differ from those subsequently reported by the petitioner, the State University of New York College of Environmental Science and Forestry (SUNY-ESF), as reflected in the draft PPRA and EIS. We observed lower than expected inheritance of the oxalate oxidase (OxO) construct, growth and survival penalties associated with transgene inheritance, and a lack of long-term robust blight resistance among the Darling 54 progeny that inherited OxO. These comments address these performance limitations and the uncertain significance of certain physiological characteristics of the Darling 54. This information is critical to understanding the prospects for using Darling 54 as the basis for restoring the American chestnut to its natural range.

I. The Transformation of Darling Appears to Disrupt a Single Copy Gene

Unlike the Darling 58, in the Darling 54, the existing *SAL1* gene (Caden.04G062600) is disrupted by the transgene insertion. The draft PPRA minimizes the significance of this disruption of the underlying American chestnut genome based on an understanding that the American chestnut genome contains multiple copies of *SAL1*. In our own research, TACF has not been able to duplicate this finding. When TACF performed a BLAST search of the *C. dentata SAL1* gene against the *C. dentata* genome, the most similar gene (Caden.05G115700) was only 42% similar in amino acid sequence. To further assesses the extent to which multiple copies of Caden.04G062600 may exist in the American chestnut genome, TACF used the program GENESPACE, which uses OrthoFinder to assign genes to gene families (orthogroups) (Lovell et al. 2022). When we performed orthogroup analysis on all genes in the 'Ellis-1' American chestnut genome and four Chinese chestnut genome assemblies, the *SAL1* orthologs

grouped into a single orthogroup in which there was only one copy of this gene in each genome (Westbrook et al. 2025).

In the model plant species *Arabidopsis thaliana*, *SAL1* is a negative regulator of drought and high light responses. Knockout of this gene in Arabidopsis results in reduced growth and abnormal leaf and flower development when the knockout is made homozygous through self-fertilization (Phua et al. 2018; Estavillo et al. 2011; Ashykhmina et al. 2021). Based on such previous studies, and as a matter of good stewardship and practice in the industry, it would be prudent to examine whether the disruption of the single copy *SAL1* gene in Darling 54 represents a deleterious mutation that could be spread to the remaining wild population of American chestnuts if released in the wild. TACF's observations from field trials indicate an early growth and survival penalty for OxO hemizygous offspring, though it is not clear whether this is related to *SAL1* or the transgene construct itself.

II. Low Recovery of OxO/SAL1 Deletion Homozygotes Suggests Deleterious Effects on Reproduction of Darling 54 Progeny

Researchers at the University of New England successfully intercrossed Darling 54 progeny in a contained greenhouse setting and recovered homozygous OxO progeny at a much lower rate than expected; only 1 of 37 progeny were homozygous compared to the expectation of approximately 9 (25%) (Klak et al. 2025). To our knowledge, the homozygous offspring produced by the Petitioners and cited in the draft PPRA and EIS did not result from natural seed production, but rather from embryo rescue, where immature embryos are treated with hormones to generate clonally propagated plants through tissue culture (Klak et al. 2025). While useful for some research purposes, this approach by itself does not provide competent or reliable evidence that homozygous offspring can be obtained through natural sexual reproduction.

The apparent low rate of homozygous inheritance from seed-propagated plants (vs. from embryo rescue) suggests that there may be deleterious effects for Darling progeny inheriting two copies of OxO and/or the SAL1 deletion. This could have implications for both restoration and the reproductive fitness of these trees in the wild over the long term.

III. Inheritance of OxO Results in Reduced Growth and Survival of Darling 54 Progeny

In an APHIS-permitted field trial near Purdue University, 3-year height growth of hemizygous OxO+ trees ($n = 259$) was 22% less than paired non-transgenic siblings ($n = 217$; $F_{1,472} = 113$, $P < 0.0001$), indicating significant growth penalties associated with OxO inheritance.

Reduced survival of the OxO+ progeny relative to OxO- full siblings now has been observed in several field test populations. For example, at a small planting of Darling 54 at the Virginia Tech Kentland Farm, only 5 of 24 OxO+ progeny survived to age 4 years, whereas 19 of 24 OxO- full siblings survived. At a larger trial of Darling 54 progeny at TACF Meadowview Research Farms, we observed significantly reduced survival of OxO+ (164/292 = 56% survival) relative to OxO- full siblings (211/301 = 70% survival, $\chi 2$ $P < 0.05$) starting at age 2 years. These growth and survival penalties associated with the Darling 54 OxO construct are currently unpublished but will be reported in upcoming peer-reviewed papers (*e.g.*, Westbrook et al. 2025).

Growth and survival penalties may be related to the pleiotropic upregulation of defense-related genes, as seen in a previous study where OxO was constitutively expressed with the 35S promoter in sunflowers (Hu et al. 2003), or concurrent downregulation of growth-related genes due to the production of hydrogen peroxide in OxO+ trees. Regardless of the cause, the growth and survival penalties are likely to negatively impact the long-term forest competitiveness of Darling 54 progeny.

IV. Blight Resistance Conferred by OxO May Not Be Sufficient for Long-Term Survival of Darling Progeny

At Purdue University Darling 54 field trial that was planted in 2019 and 2020, we observed that the cankers of OxO+ progeny were 37% shorter than OxO- negative siblings one year after inoculation, confirming an improvement in blight resistance, at least over the short term However, canker severity ratings of the OxO+ progeny varied widely; from fully susceptible to highly resistant. Among the 15% OxO+ progeny that were rated as highly resistant at 1 year, cankers continued to expand and girdle stems 2+ years after inoculation (Figure 1). Given that American chestnut trees are long-lived perennials that will need to survive with blight over multiple decades, longer-term field trials are necessary to determine whether or not the resistance conferred by OxO is sufficiently durable for long-term survival.

V. Conclusion

The evidence to date suggests deleterious fitness effects of OxO production and possibly of the *SAL1* deletion, possible homozygous lethality, as well as doubtfully robust blight resistance. Relevant to the draft PPRA, these characteristics reduce the likelihood that the transgenic Darling 54 progeny will become invasive and weedy. It is for these same reasons that TACF discontinued its support in 2023 for using Darling 54 progeny for American chestnut restoration (https://tacf.org/darling-58/). TACF does not oppose further research on Darling 54. Indeed, useful information may be developed on the points raised in these comments through continued research and long-term field trials. While APHIS's regulations require a 60-day public comment period and a decision from APHIS on the petition within 180 days of filing, we believe it would befit the public interest purpose of this project for APHIS to maintain an open docket for Darling 54 to facilitate the sharing of information from current or future Darling 54 research and field trials.

Sincerely,

Jared Westbrook
TACF Director of Science

Bruce Levine
Interim President and CEO, TACF

Figure 1. Chestnut blight cankers on the most blight resistant trees selected from a field trial near Purdue University. The cankers pictured are on 5-year old trees, 2 years after inoculation with the chestnut blight fungus.

Literature cited

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