Enhancing American Chestnut Cuttings' Rooting Rate and Adventitious Root Number

Submitted to:
The American Chestnut Foundation (TACF)

50 North Merrimon Ave., Suite 115

Asheville, NC 28804

Phone: (828) 281-0047

Email: externalgrants@acf.org

Submitted by:
Clemson University
Department of Genetics and Biochemistry

Office of Sponsored Programs

230 Kappa Street, Suite 200 Clemson University Clemson, SC 29634-5702

August 10, 2023
Haiying Liang Date
Associate Professor
Dept. of Genetics and Biochemistry
108 Biosystems Research Complex
Clemson, SC 29634-0318
Phone: 864.656.2414
Email: *hliang@clemson.edu*

Diana Thrasher 08/10/23
for Tanju Karanfil, Ph.D. Date
Vice President for Research
Strom Thurmond Institute, Suite 200
230 Kappa Street, Suite 200
Clemson University
Phone: 864.656.7701

Date Submitted:
August 14, 2023

A. Project title:

Enhancing American Chestnut Cuttings’ Rooting Rate and Adventitious Root Number

B. Summary:

An efficient rooted cutting system will allow rapid and clonal propagation of American chestnut, accelerate the breeding process, and facilitate germplasm conservation. However, the species is notoriously recalcitrant. This project will build on the current achievements and aim to enhance American chestnut cuttings’ rooting rate and adventitious root number. The focus includes the synergistic effects between indole-3-butyric acid and naphthaleneacetic acid and with willow water and *Bacillus subtilis*, as well as the hormone and primary metabolite levels between the rooting and non-rooting sites. Our goal is to develop an easy-to-apply and efficient rooted cutting system for the heritage tree species.

C. Principal Investigators (PI) and Institutional Affiliation

Haiying Liang, Ph.D., Associate Professor
Department of Genetics and Biochemistry, Clemson University
Email: HLIANG@CLEMSON.EDU; Phone: 864-656-2414

D. During of project:

12 months from the time funding is received.

E. Total amount requested

$49,942. Matching funds ($39,649) will be provided by Clemson University – see budget

F. Short and long-term goals of the project

Short-term Goal (12 months)

- Improve the rooting system, with a focus on the potential synergistic effects between indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) and with willow water and *Bacillus subtilis*.
- Compare the hormone and primary metabolite levels between the rooting and non-rooting sites.

Long-term Goal:

- To establish an easy-to-apply vegetative propagation system that is efficient in rooting cuttings of American chestnut and its hybrids. Impacts of cutting age, collection season, and genotypes, as well as growth performance, will be studied,
- Unravel the physiological and molecular mechanisms of rooting induction in American chestnut cuttings.

- Train the next generation of the workforce.
- Work with chestnut breeders and restoration project staff to disseminate the techniques we develop.

G. Narrative (no more than five (5) pages)

1). There is an urgent need to overcome the barriers in rooting cuttings of American chestnut, the once "perfect" heritage tree.

With the imminent approval of the release of blight-resistant transgenic Darling 58, the American chestnut tree (*Castanea dentata*) (AC) has gained a lot of publicity lately. The "rebirth" of this heritage species is no longer a far-reaching dream after decades of dedicated hard work by many. To facilitate the restoration and diversity conservation of the species, it is critical to establish an easy-to-apply vegetative propagation system that is efficient in rooting American chestnut cuttings. However, American chestnut is notoriously recalcitrant to rooting by cutting.

2). Results from the last two years (2022 and 2023).

We have achieved rooting with moderate success (~ 50% rooting rate) with 2 hours of 100 ppm 1-Naphthaleneacetic acid (NAA) treatment (Figs. 1-3). Key findings are:

1. The current year cuttings that are semi-lignified and have new buds are suitable for rooting. Cuttings from springtime barely rooted.
2. It takes at least two months for adventitious roots to emerge. During this period, keeping leaves alive/green is critical. Open chambers work better than closed ones. Leaves tend to become molded and rotten in a closed chamber with high humidity. We use sphagnum moss to partly cover leaves on cuttings.
3. Adventitious roots (ARs) can be formed with and without callus formation. Adventitious roots formed without callus formation seem larger and more vigorous.
4. Due to the regulation associated with *Agrobacterium rhizogenes*, we did not proceed to apply this bacterium. Instead, we tested a growth-promoting bacterium, *Bacillus subtilis*. The strain was QST713. We found that QST713 did not increase the rooting rate, while it seemed to enhance root length. Further studies are needed to validate this result.
5. 100 ppm IBA seems more efficient in terms of rooting rate than 100 NAA with 2 hours of treatment. But the latter seems to generate more adventurous roots per cuttings. More data will be collected in ~two months for validation.
6. Compared to easy-to-root poplar, American chestnut cuttings had a low level of indole-3-acetic acid (IAA) and a high level of cytokinin (CK), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and oxylipin 12-oxo-phytodienoic acid (OPDA). Hormone distribution between leaves and stems also differed between American chestnut and poplar. This unfavorite endogenous hormone profile may contribute to American chestnut cuttings' recalcitrance to rooting.
7. A manuscript, entitled "Histology of Adventitious Root Formation and Phytohormone Analysis of American Chestnut Cuttings", has been accepted for publication in the *Journal of Environmental Horticulture* (Lu et al. in Press).

<u>Ongoing efforts:</u>

1. Compare secondary compounds in AC and easy-to-root poplar cuttings.
2. Primary compounds of AC and easy-to-root poplar and willow cuttings are being analyzed in Clemson University's Multi-user Analytical Lab (MUAL) & Metabolomic Core. Samples were sent in July 2023.
3. Histology to identify key stages of AC adventitious root formation is expected to complete in about two months. RNAseq for global gene expression and endogenous hormone content will be conducted for the key stages of AC adventitious root formation.

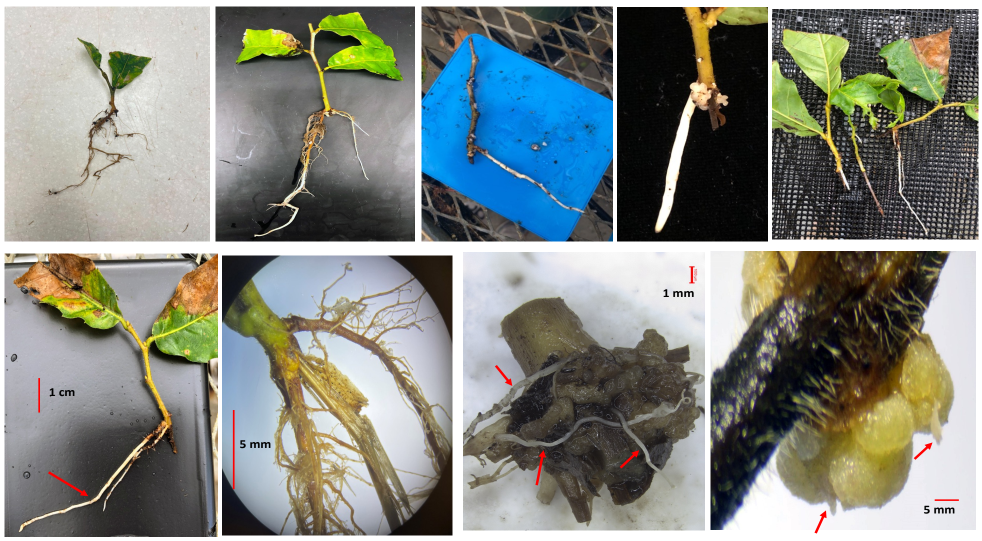

Fig. 1. Adventitious roots formed on chestnut cuttings.

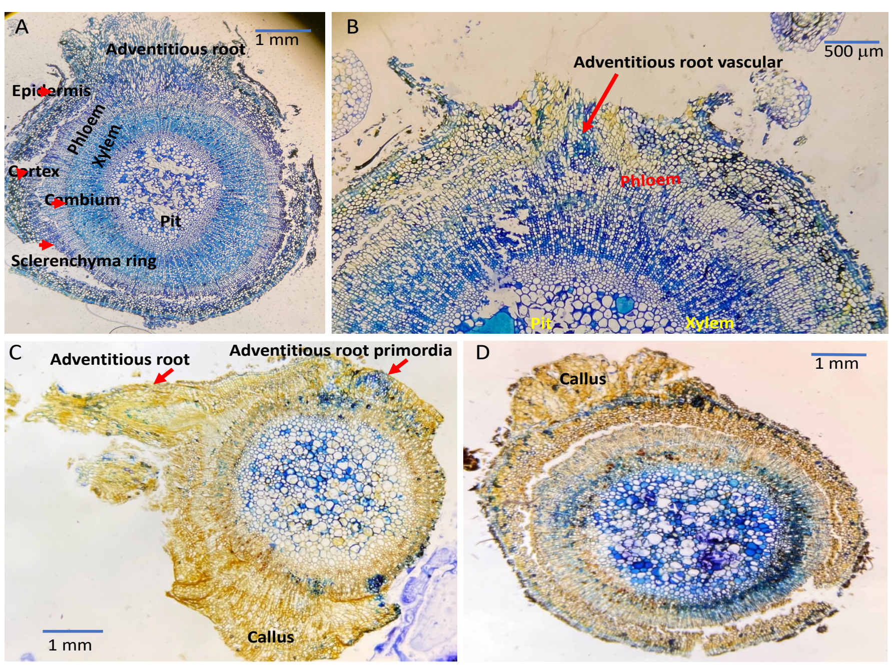

Fig. 2. Ongoing histology to identify key stages of AC adventitious root formation.

Fig. 3. Chestnut plants propagated by cutting in 2022. Photos taken in spring 2023.

3). Objectives of the current proposal.

While we have obtained induced adventitious roots from cuttings, more efforts are needed to improve the rooting rate and root number per cutting, as well as understand adventitious root formation in American chestnut cuttings at the physiological and molecular levels. In this one-year project, we aim to achieve the following five specific objectives:

- Improve rooting rate and root number with willow water.
- Compare the effects of *Bacillus subtilis* strains (QST713 and local isolates) on the length of induced AC adventitious roots.
- Investigate the effect of 2,4-Dichlorophenoxyacetic acid (2,4-D) on AC cutting rooting. Both high rooting activity and high toxicity have been reported for this synthetic auxin on cuttings, depending on the species (Tofanelli et al. 2014).
- Investigate the synergistic effect of IBA and NAA on AC cuttings' rooting rate and root number.
- Compare the hormone and primary metabolite contents between stem segments with and without nodes, because adventitious roots seem to be more likely to emerge from areas close to nodes (Fig. 4).

Fig. 4. Chestnut adventitious roots seem to emerge from areas close to nodes.

4). Approaches and methods.

Current-year's semi-lignified AC twigs are to be cut into segments that are 10-15 cm in length, containing at least two buds and 2~4 leaves. The potting substrate will be a mix of Canadian sphagnum peat moss, bark, perlite, and vermiculite (Sungro, Fafard 3b). Wet sphagnum mosses are to be used to partly cover the leaves of cuttings (Fig. 5). The cuttings are put in a transparent box covered with a screen. A temperature-controlled (22-23 °C) mist room will be employed (mist is

14 minutes off and 6 seconds on). For all hormone treatment, the basal part of the AC cuttings will be dipped into the hormone solution for two hours.

Fig. 5. Humidity is kept by using sphagnum mosses and a screen cover.

Willow water will be prepared by cutting weepy willow branches into ~ 1-inch pieces and soaking them with tap water for 24 hours (v:v 1:2). *Bacillus subtilis* cultures are to be grown with tryptone soya broth according to the description in González et al. (2018) (1×10^8 CFU mL^{-1}). Willow water or *Bacillus subtilis* cultures will be poured into AC cuttings one week after 100 ppm IBA treatment. Reports of the effect of 2,4-D on adventitious root induction are limited. Therefore, we will test a wide range of concentrations (10, 50, 100, 150, and 200 ppm). To investigate their potential synergistic effect, we will test the combinations of 100 ppm IBA+100 ppm NAA, 200 ppm IBA+200 ppm NAA, and 100 ppm IBA+200 ppm NAA. It has been reported that *Magnolia wufengensis* cuttings pre-treated with NAA:IBA (2:1) exhibited the best rooting performance (Wang et al. 2022).

There will be at least 10 cuttings per treatment. The experiments will be conducted at least three times, depending on the availability of twigs. Rooting rate, root length, and root number will be recorded. The data will be processed using SPSS v. 23.0 software (SPSS Inc., IL, USA). One-way analysis of variance is to be performed to identify statistically significant differences among treatments, followed by Duncan's multiple range test at $P < 0.05$.

Stem segments with and without nodes will be separated. Endogenous hormone analysis will be conducted in the Donald Danforth Plant Science Center, and primary metabolites will be analyzed in Clemson University's Multi-User Analytical Lab (MUAL) and Metabolomic Core. Four replicates will be included for the hormone analysis, while six replicates will be used for primary metabolites, as suggested by the facilities.

5). Potential pitfalls.

American chestnut cuttings are notoriously recalcitrant to rooting. However, we have succeeded in rooting AC cuttings (Figs. 1-4) and have enhanced the rooting rate from 33% in 2022 to ~50% in 2023. We are confident that we will be able to further improve AC's rooting efficacy through the research described in this proposal. The main potential obstacle can be getting adequate AC cuttings for the experiments. We will continue to reach out to AC breeders. There are also AC plants in our Clemson greenhouse that can provide materials for year-round use.

6). Literature Cited.

González P, Sossa K, Rodríguez F, Sanfuentes E (2018) Rhizobacteria strains as promoters of rooting in hybrids of *Eucalyptus nitens* × *Eucalyptus globulus*. Chil J Agric Res 78:3–12.

Tofanelli MB, Freitas PL, Pereira GE (2014) 2, 4-dichlorophenoxyacetic acid as an alternative auxin for rooting of vine rootstock cuttings. Revista Brasileira de Fruticultura 36:664-672.

Wang Y, Khan MA, Zhu Z, Hai T, Sang Z, Jia Z, Ma L (2022) Histological, morpho-physiological, and biochemical changes during adventitious rooting induced by exogenous auxin in *Magnolia wufengensis* cuttings. Forests 13(6):925.

X Lu, M Cuarto, H Liang (In Press) Histology of adventitious root formation and phytohormone analysis of American chestnut cuttings. Journal of Environmental Horticulture.

H. Timeline (December 2023 – November 2024)

Research Work	**1st quarter**	**2nd quarter**	**3rd quarter**	**4th quarter**
Rooting with willow water				
Rooting with *Bacillus subtilis* strains				
Rooting with 2,4-D				
Synergistic effect of IBA and NAA				
Hormone analysis				
Primary metabolite analysis				
Data analysis & interpretation				
Present results and manuscript drafting				

I. How results will be measured and reported

Rooting rate, root length, and root number will be recorded and compared. Results will be presented at two meetings. A manuscript will be drafted.

J. Breakdown of how and when funds will be spent

Expense	**Amount Requested**
One graduate student's stipend	$27,000
One graduate student's fringe (8.6%)	$2,322
Attending two meetings (PI and one student)	$2,300
Greenhouse rental	$3,600
Hormone analysis: $260 per samples, two types of stem (with and without internodes, 4 timepoints, 4 replicates)	$260*2*4*4=$8,320
Primary metabolite analysis: $100 per samples, two types of stem (with and without nodes, 4 timepoints, 6 replicates)	$100*2*4*6=$4,800
Expendable supplies—rooting reagents, medium, Petri dishes, trimmers, gloves, postage, etc.	$1,600
Unrecovered overhead = F&A @ 52.5%, $26,220	0
Graduate student's tuition $13,429	0
Total	**$49,942**

Budget Justification

- Amount Requested from TACF

The fund requested from TACF includes one graduate student's stipend and associated fringe, greenhouse rental, fees for hormone and primary metabolite analyses, and consumables (rooting reagents, culture medium, Petri dishes, gloves, paper towels, and shipping materials such as cuttings), as well as meeting expenditures for the PI and one graduate student (registration fee, hotel, transportation, etc.). The greenhouse rental fee covers pots, potting mix, water, labels, fertilizers, and electricity).

- Matching funds provided by Clemson University ($39,649)

TACF has a policy not to pay overhead (F&A – Facilities and Administration) charges since its grants are relatively small. Therefore, this amount ($26,220) is regarded as part of matching funds from Clemson University. In addition, College of Science in Clemson University has waived the graduate student's tuition fee, which is $ 13,429. In total, the match fund from Clemson University is $39,649.

K. Brief Curriculum Vitae (CV) of Haiying Liang- Clemson University

a) Professional Preparation:

Beijing Forestry University, China	Forestry	B.Sc.	1990
Beijing Forestry University, China	Plant Biology	M.S.	1993
College of Environmental Science &Forestry State University of New York	Plant Science & Biotechnology	Ph.D.	2000
College of Environmental Science & Forestry State University of New York	Plant Science & Biotechnology	Postdoc	2000~2004
The Pennsylvania State University	Plant Functional Genomics	Postdoc	2004~2006

b) Appointments:

07/2012—present: Associate professor, Clemson University, Clemson, SC
09/2006—06/2012: Assistant Professor, Clemson University, Clemson, SC.
04/2004—08/2006: Postdoctoral Fellow, The Pennsylvania State University, State College, PA.
08/2000—03/2004: Postdoctoral Fellow, College of Environmental Science and Forestry, State University of New York, Syracuse, NY.
07/1993—07/1996: Instructor, Beijing Forestry University, Beijing, China.

c) Publications in the last four years:

1. (In Press) X Lu, M Cuarto, **H Liang**. Histology of Adventitious Root Formation and Phytohormone Analysis of American Chestnut Cuttings. Journal of Environmental Horticulture.
2. (2022) L Guo, J Shen, C Zhang, Q Guo, **H Liang**, X Hou. Characterization and bioinformatics analysis of ptc-miR396g-5p in response to drought stress of Paeonia ostii. Non-coding RNA Research. June 20.
3. (2022) C Shen, Q Li, Y An, Y Zhou, Y Zhang, F He, L Chen, C Liu, W Mao, X Wang, **H Liang**. The transcription factor GNC optimizes nitrogen use efficiency and growth by driving the expression of nitrate uptake and assimilation genes in poplar. Journal of Experimental Botany, May 8.
4. (2022) O Gailing, M Staton, SE Schlarbaum, MV Coggeshall, J Romero-Severson, **H Liang** & J E Carlson Progress and Prospects of Population Genomics of North American Hardwoods. In: Population Genomics. Springer, Cham
5. (2021) B Lei, CJ Frost, T Xu, JR Herr, JE Carlson, **H Liang**. Poplar allene oxide synthase 1 gene promoter drives rapid and localized expression by wounding. Biotechnology Journal International, 25(5): 16-28.

6. (2021) W Lu, E Wang, W Zhou, Y Li, X Song, J Wang, M Ren, D Yang, S Huo, Y Zhao, **H Liang**. Morpho-histology, endogenous hormone dynamics, and transcriptome profiling in *Dacrydium Pectinatum* during male cone development. Forests. Nov;12(11):1598.
7. (2021) X Wei, S Wu, X Liang, K Wang, Y Li, B Li, J Ma, **H Liang**. Paclobutrazol modulates endogenous level of phytohormones in inducing early flowering in *C. tamdaoensis*, a golden Camellia species. HortScience 56(10):1258-1262.
8. (2021) KX Li, K Liu, Y Chen, X Huang, W Liang, B Li, Y Shen, **H Liang**. Comprehensive transcriptome and metabolome analysis of *Lithocarpus polystachyus* leaf revealed key genes in flavonoid biosynthesis pathways. Journal of the American Society for Horticultural Science. 1(aop):1-11.
9. (2020) S Li, **H Liang**, L Tao, L Xiong; W Liang, Z Shi, Z Zhao. Transcriptome sequencing and differential expression analysis reveal molecular mechanisms for starch accumulation in chestnut. Forests 11:388.
10. (2020) Y-Y Xia, D-X Wang, B-Q Hao, Z-P Jiang, G-C Chen, **H Liang**. Nitrogen fertilizer mitigates water loss and restores pigment composition in camellia oleifera, an oilseed crop. Journal of Soil and Plant Biology 2020:106-112.
11. (2019) X Wang, **H Liang**, D Guo, L Guo, X Duan, Q Jia X Hou. Integrated analysis of transcriptomic and proteomic data of tree peony (*P. ostii*) seed reveals key developmental stages and candidate genes related to oil biosynthesis and fatty acid metabolism. Horticulture Research. 6, Article number: 111
12. (2019) HY Zhao, **H Liang**, YB Chu, N Wei, MN Yang, CX Zheng. Effects of salt stress on chlorophyll fluorescence and the antioxidant system in *Ginkgo biloba* L. seedlings. HortScience 54(12):2125-2133

d) Courses taught:

1. GEN3000 (Fundamental Genetics)
2. GEN3020 (Molecular & General Genetics)
3. GEN 4930 (Undergraduate Senior Seminar)

e) Synergistic Activities:

1. Academic editor for Horticulturae (2021 to present).
2. Served in the University's Graduate and Undergraduate Integrity Hearing Boards (2015-2021).
3. Chaired (2019-2022) or serve (2017-present) in the Clemson University Genetics and Biochemistry Department's Graduate Committee; Chaired the Clemson University Genetics and Biochemistry Department's curriculum committee for three years.
4. Program Committee member for the 2013 Southern Forest Tree Improvement Conference and recipient of a USDA conference award for a career development workshop and supporting students and postdoctoral fellows to attend the conference.
5. Held Visiting Professorships at Henan University of Science & Technology and Guangxi Forestry Research Institute in China.

6. A science fair judge for local and regional schools for more than 10 years. A frequent manuscript reviewer for various journals, including Plant Physiology, Tree Physiology, BMC Genomics, Plant Cell, Tissue and Organ Culture, PLoS ONE, and Horticulture Research.

L. A Conflict of Interest or Commitment (COI or COC) statement.

There is no known COI or COC regarding this project.