Rooting American chestnut cuttings with plant growth promoting rhizobacteria

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04 August 2021
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A. Project title:

Rooting American chestnut cuttings with plant growth promoting rhizobacteria

B. Summary:

An efficient rooted cutting system will allow rapid and clonal propagation of American chestnut (*Castanea dentata*), accelerate the breeding process, and facilitate germplasm conservation. However, the species is notoriously recalcitrant. This project will evaluate the effects of plant growth promoting rhizobacteria (PGPR) in stimulating adventitious root formation in American chestnut cuttings. Upon infecting plants, *Rhizobium rhizogenes* (formerly *Agrobacterium rhizogenes*) induces the formation of "hairy roots". Bacillus species, such as *Bacillus subtilis*, are known to promote rooting. Our ultimate goals are to overcome the difficulty of rooting American chestnut cuttings and develop an easy-to apply and efficient rooted cutting system for the heritage tree.

C. Principal Investigators (PI) and Institutional Affiliation

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D. During of project:

12 months from the time funding is received.

E. Total amount requested

$10,000. Matching funds will be provided by Clemson University – see budget

F. Short and long-term goals of the project

Short-term Goal (12 months)

- To identify PGPR that can stimulate root formation in American chestnut cuttings.
- To reveal the synergistic effects among PGPR in rooting American chestnut cuttings.
- To reveal the synergistic effects of PGPR with exogenous hormones in rooting American chestnut cuttings.

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 will be studied.

- Unravel the physiological and molecular mechanisms of rooting initiation in American chestnut cuttings. Approaches will include hormone dynamics, histological analysis, gene function characterization, and epigenetics.

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.

The American chestnut was among the largest, tallest, and fastest-growing trees in the eastern United States, covering up to 45% of the forest canopy in some areas (Keever 1953). Rich in carbohydrates and other nutrients, chestnuts were an excellent food source for thousands of years, feeding wildlife, people and their livestock. The wood was rot-resistant, straight-grained, and suitable for furniture, fencing, and building. The species was a good source of tannins and had the ability to quickly colonize burned or clearcut areas. Because of its ecological and economic importance, the American chestnut was referred to as the "perfect tree" (Freinkel 2007).

Unfortunately, due to the accidental introduction of the exotic disease, chestnut blight, in the early 1900s, the American chestnut is now widely regarded as "functionally extinct", because only a small percentage of these trees reach sexual maturity. Known as the greatest ecological disaster to strike the world's forests in history, the chestnut blight, within 40 years, virtually eliminated the American chestnut trees that once dominated the eastern half of the U.S., and had survived all adversaries for 40 million years (Brewer 1995). Another exotic disease, Phytophthora root rot, was introduced in the 1700's, and is thought to have eradicated American chestnut from low elevation forests in the Southeastern U.S. prior to the introduction of chestnut blight (Anagnostakis 2012).

Because of the great economic and ecological value of the American chestnut tree, significant efforts have been made over the century to combat the above-mentioned diseases with an aim to restore the iconic chestnut to American forests. To date, hybridizing with a blight-tolerant chestnut species followed by repeated backcrossing (Steiner et al. 2017; Clark et al. 2019) and genetic engineering (Newhouse et al. 2014) have generated promising blight-tolerant varieties. In particular, a deregulation petition for the Darling 58 transgenic variety was submitted in 2020 to USDA APHIS. Once the disease-tolerant varieties become available, a rapid and low-cost method for clonal reproduction is needed to ensure the success of the American chestnut restoration efforts.

While tissue culture-based micropropagation is possible with the American chestnut, the micropropagation approach utilizes immature embryos as explants, and plantlets are germinated through somatic organogenesis. Due to fertilization, the genetic makeups of plantlets derived from individual embryos are not the same, and somatic organogenesis takes several months to generate a plantlet. As for grafting, it is a procedure that requires much practice and skill, and the rootstock genotype may influence the phenotype of the scion and compatibility. Therefore, vegetative reproduction by rooting cuttings is preferred.

2). Little success in rooting American chestnut cuttings with exogenous phytohormones.

Auxin is considered the "master regulator" of rooting, and indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are commonly used, either individually or in combination (Davies et al. 2011). Currently available studies of rooting in American chestnut are mostly focused on tissue culture-regenerated plantlets. With 10 mM IBA, 0.5 g/L humic acid, and 2.0 g/L, Oakes et al. (2016) achieved an initial rooting rate of 88.5% and 44.5% for tissue-cultured shoots with *in vitro* and *ex vitro* method, respectively. However, only an average of 16.5 and 39% of plantlets survived eight weeks after acclimation. The only success on rooting cuttings was reported by Galic

et al. (2014). In this study, a rooting rate of 59% and 65% was achieved for juvenile softwood and semi-hardwood cuttings, respectively, with 1% IBA. These cuttings were collected in July 2007. However, none survived the winter or cold storage. When using cuttings collected in April and May from a grafted plant, the same authors reported that 46% of cuttings rooted, and 15% overwintered successfully (Galic et al. 2014).

For Chinese chestnut (*C. mollissima*), Zhu et al. (1998) reported a rooting rate of at least 98% for cuttings from 1-year-old seedlings and 76% and 51%, respectively, for cuttings from 3-year-old and 6-year-old mother trees. This study was conducted in China, and the commercial rooting agents employed (PRA, HL-43, IBAN, and ABT) are not available in the U.S. market.

3). Success of plant growth promoting rhizobacteria (PGPR) in stimulating formation of adventitious roots in other species.

Rhizobium rhizogenes (formerly *Agrobacterium rhizogenes*) is a natural plant genetic engineer. Upon infecting plants, the bacterium integrates a portion of its transfer DNA from the root inducing plasmid into the plant genome, resulting the formation of "hairy roots". Morphologically, *R. rhizogenes*-induced hairy roots are very similar in structure to wild-type roots, however, hairy roots have more roots and are longer, and their root systems are more branched and exhibit an agravitropic phenotype (Veena and Taylor 2007). Applications of *R. rhizogenes* include production of metabolites, study of gene function in plants, phytoremediation, drought resistance, and root development (especially for recalcitrant woody species) (Veena and Taylor 2007).

Currently, most available rooting reports with *R. rhizogene* are on micro-shoots. Examples include *Sequoia sempervirens* (Mihaljević et al. 1999), walnut (*Juglans regia*) (Caboni et al. 1996), *Papaver somniferum* (Le Flem-Bonhomme et al. 2004), and filbert (*Corylus avellane*) (Sánchez et al. 2009). In the study of bare root stock almond trees, inoculation of *R. rhizogenes* led to a larger root number and root mass, as well as significant increases in leaf number, stem diameter and shoot elongation during the first growing season after treatment (Strobel and Nachmias 1985). In jujube (*Ziziphus jujuba*) cuttings, *R. rhizogenes* was very effective in increasing rooting percentages and root number (Hatta et al. 1995). More recent reports include *Prosopis alba* (Felker et al. 2005), apple rootstocks (Azmoode et al. 2017) and *Juniperus communis* (Sarmast et al. 2019).

It is well known that PGPR, including endophytes, can increase nutrient uptake, provide various growth hormones and mitigate biotic and abiotic stresses (Deshwal et al. 2013). Generally, they form symbiotic relationship with plants and are able to produce growth hormones, organic acids, enzymes, antioxidants, etc. Recently, there are studies reporting the capability of PGPR to improve or strengthen the plant root system architecture (Sakthivel and Balachandar 2019). For instance, *Bacillus*, *Pseudomonas* sp., as well as the genera *Chryseobacterium*, *Mucilaginibacter*, and *Rhodococcus* sp. were found effective in increasing the adventitious rooting of minicuttings of *Eucalyptus nitens* × *E. globulus* (González et al. 2018). For apple rootstock, *R. rubi* (A-18) and *Bacillus subtilis* (OSU-142) work synergistically with IBA and sorbitol (Karakurt et al. 2009).

4). Current status of research on formation of adventitious roots.

Studies in other species show that there are four stages for forming adventitious roots: 1) dedifferentiation of parenchyma cells in the phloem ray area, 2) formation of root initials, 3) formation of the root primordia, and 4) elongation (Davies et al. 2011). A study with micropropagated shoots of European chestnut (*Castanea sativa*) showed very low IAA level in auxin untreated materials, which did not root (Review in Vielba et al. 2020). In Chinese chestnut microshoots, an increase of polyphenol oxidase activity and a decrease in the activities of IAA-oxidase and peroxidase-oxidase were found concomitant with the increase of auxin (Hou et al. 2010). Molecular and genetic studies on a chestnut species' adventitious root formation are

scarce. Only a few genes have been studied, and they are all from *C. sativa*: *Scarecrow-like 1* (*CsSCL-1*), an ERF transcription factor gene (*CsRap2.12 like-1*), and a MYB-like 61 gene (*Cs714F2G*) (Review in Vielba et al. 2020).

5). Objectives.

As seen above, studies to understand the adventitious root formation in American chestnut lag that of other species. The goal of this one-year project is to stimulate rooting in American chestnut cuttings with *Rhizobium rhizogenes* and *Bacillus* species. There will be three specific objectives:

1) Assess rooting promoting efficacy of PGPR.
2) Assess synergistic effects among PGPR.
3) Assess synergistic effects of PGPR with exogenous hormones.

This proposal aims to overcome the difficulty to root problem with PGPR. Our initial efforts with PGPR have generated some promising results: Figure 1A shows a chestnut cutting that has been surviving for two months, while Figure 1B shows a shoot being rooted in a living chestnut.

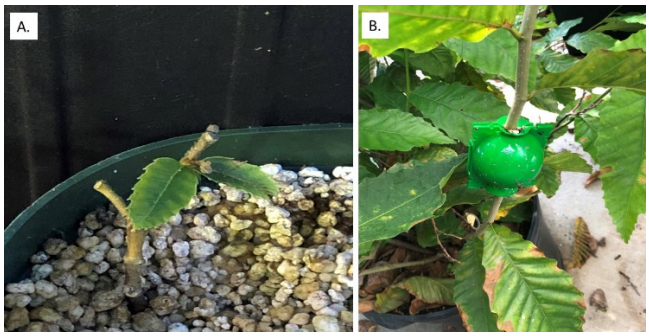

Figure 1. Preliminary rooting experiments. A cutting chestnut plant with expanded leaves (A). A chestnut shoot is being rooted in a living plant (B).

6). Approaches and methods.

We will reach out to the American Chestnut Foundation and the Chestnut Return Farms in Seneca, SC. for 1~2-year-old twigs. We also have a few chestnut plants growing in the greenhouse. The twigs are to be cut into segments that are 10-15 cm in length, containing at least two buds or leaves. Field materials will be from summer, fall, and winter when buds have formed. Our preliminary results showed that spring materials before bud break were not suitable. Potting substrates are peat and vermiculite (1:1), as referenced from Galic et al. (2014). A temperature-controlled (22-23 °C) mist room will be employed (mist is 14 minutes off and 6 seconds on). Sorbitol (55 mM) will be sprayed as food energy supplement. Carbohydrate solutions were found effective on rooting of fruit rootstock, such as apple (Karakurt et al. 2009).

<u>6-1. Obj#1. Assess rooting promoting efficacy of PGPR.</u>

We have *R. rhizogenes* strain K599, ATCC15834, and R1000 in our collection. USDA APHIS guidelines will be followed. Dr. H. Wang at Clemson University will provide *Bacillus subtilis* QST 713 and other *Bacillus* strains. These biological agents will be tested individually for their rooting promoting effect. Cultivation of *R. rhizogenes* will follow the protocol described in Samaan et al. (2017) with Luria Bertani medium. *Bacillus* cultures will be grown with Reasoner's 2A medium as described in González et al. (2018). The basal part (~2 cm) of freshly wounded cuttings is dipped in bacterial suspension for 24 hrs for *R. rhizogenes* (an optical density 0.6 at 600 nm wavelength) in complete darkness (Samaan et al. 2017) for 1 min for *Bacillus* (1×10^8 CFU mL^{-1}) (González et al. 2018), before being transferred into potting mix.

6-2. Obj#2. Assess synergistic effects among PGPR.

Synergistic effects between *R. rhizogenes* and *Bacillus* are documented (e.g. Karakurt et al. 2009). This objective aims to determine if inoculation of both *R. rhizogenes* and a *Bacillus* strain will improve rooting in the American chestnut cuttings. *R. rhizogenes* and *Bacillus* will be cultured separately as described in Samaan et al. (2017) and González et al. (2018), then are mixed right being used for cutting treatments. Bacterial concentration will be maintained the same as single treatments in Obj#1.

6-3. Obj#3. Assess synergistic effects of PGPR with exogenous hormones.

With softwood cuttings from juvenile American chestnut plants, Galic et al. (2012) achieved a rooting rate of 28% with a 5-second treatment of 1-2% IBA. Therefore, we will test the synergistic effects of PGPR with 2% IBA. Cuttings are to be inoculated with bacterial cultures first, and then dipped in 2% IBA for 5 seconds.

6-4. Data analyses

There will be at least 10 cuttings per treatment. The experiment will be conducted at least three times, depending on the availability of twigs. Cuttings are randomly arranged inside the mist room once a week. Rooting rate, root length, root number, and stem growth 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 $P < 0.05$.

7). Potential pitfalls.

American chestnut cuttings are notoriously recalcitrant to rooting. We are starting to see some promising results with our initial experiments. A backup plan is to use twigs from one or two- year-old seedlings, which are growing in our greenhouse facility. This will greatly enhance the rooting rate, since materials from juvenile plants are earlier to root. The efficacy of PGPR can be genotype dependent. We will continue to contact researchers in the U.S. and obtain more PGPR strains. Meanwhile, we will work with plant bacteriologists to isolate PGPR from roots of American chestnut trees if necessary. We are confident that we will be able to achieve the much-needed breakthrough in rooting American chestnut cuttings.

8). Future plan.

The main purpose of this one-year project is to utilize PGPR to stimulate adventitious root formation in American chestnut cuttings. Upon success, it will open up the opportunities to study this critical stage for the success of the species' cutting propagation. In the near future, we will focus on optimizing treatment methods and environmental conditions to achieve high rooting and survival rates and understanding the factors contributing to the species' recalcitrance to rooting. Approaches will include histological analysis, endogenous hormone dynamics, gene function characterization, and epigenetics. We will work with chestnut breeders and restoration projects staff to disseminate the techniques we developed.

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H. Timeline (December 2021 – November 2022)

Research Work	1st quarter	2nd quarter	3rd quarter	4th quarter
Rooting with winter twigs				
Rooting with summer twigs				
Rooting with fall twigs				
Rooting with twigs from a greenhouse				
Data analysis & interpretation				
Present results and manuscript drafting				

I. How results will be measured and reported

Rooting rate, root length, root number, and stem growth will be recorded and compared. Results will be presented at the NE-1333 Meeting in the fall 2022. A manuscript will be drafted.

J. Breakdown of how and when funds will be spent

Expense	TACF: Amount Requested
Part-time assistant salary	$3,600
Part-time assistant fringe (25.1%)	$904
Travel to attend TACF annual meeting	$800
Greenhouse rental	$1,500
Expendable supplies—rooting reagents, culture medium, Petri dishes, trimmers, gloves, postage, etc.	$3,196
Unrecovered overhead = F&A @ 52.5%, $5,250	0
Total	**$10,000**

Budget Justification

- Amount Requested from TACF

The fund requested from TACF is used to cover the salary and fringe for a part-time assistant, greenhouse rental, and consumables (rooting reagents, culture medium, Petri dishes, trimmers, gloves, postage, etc.). The greenhouse rental fee covers pots, potting mix, water, labels, fertilizers, and electricity.

- Matching funds provided by Clemson University

TACF has a policy to not pay overhead (F&A – Facilities and Administration) charges since its grants are relatively small. Therefore, this amount ($5,250) is used as matching funds from Clemson University.

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. (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 (in press).
2. (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.
3. (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.
4. (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.
5. (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

6. (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
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10. (2017) M Harmon, T Lane, M Staton, MV Coggeshall, T Best, C-Chih Chen, **H Liang**, N Zembower, DI Drautz-Moses, YZ Hwee, SC Schuster, SE Schlarbaum, JE Carlson, O Gailing. Development of novel genic microsatellite markers from transcriptome sequencing in sugar maple (*Acer saccharum* Marsh.). BMC Res Notes 10:369.
11. (2017) **H Liang**, B-Q Hao, G-C Chen, H Ye, J Ma. Camellia as an oilseed crop. HortScience 52(4):488–497.

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. Serve in the University's Graduate and Undergraduate Integrity Hearing Boards since 2015.
3. Chair or serve in the Clemson University Genetics and Biochemistry Department's Graduate committee since 2017; 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. Have held Visiting Professorships at Henan University of Science & Technology and Guangxi Forestry Research Institute in China.
6. A science fair judge for local and region 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.