Ex Vitro Rooting of American Chestnut Improves Acclimatization Survival and Plantlet Quality[1]

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Abstract

Tissue culture of plants has many applications, from producing genetically identical horticultural varieties, to production of secondary metabolites, to virus indexing, and most relevantly, developing novel traits by genetic transformation. Using *Agrobacterium*-mediated transformation on somatic embryos, blight-resistant American chestnuts [*Castanea dentata* (Marsh.) Borkh.] have been developed as shoot cultures in plant tissue culture. Rooting tissue-cultured shoots and acclimatizing the rooted plantlets are key steps in tree production. In this study, *in vitro* and *ex vitro* rooting methods were compared. The *ex vitro* method resulted in a lower initial rooting percentage but an overall higher survival percentage, resulting in higher potted plant production. The higher survival was likely due to partial acclimatization taking place before the plantlets were transplanted into potting mix. After 8 weeks, plantlets rooted via the *ex vitro* method were taller, and had more, and larger, leaves than the *in vitro*-rooted plantlets. These trees are currently in high demand for inoculation studies for federal regulatory review and eventually for restoration of this keystone species to its native habitat.

Index words: *Castanea dentata*, micropropagation, rooting, tissue culture, acclimatization.

Chemicals used in this study: IBA, indole-3-butyric acid.

Species used in this study: American chestnut (*Castanea dentata* (Marsh.) Borkh.).

Significance to the Horticulture Industry

Once blight-resistant American chestnuts are available to the public, many companies may be interested in propagating these trees as we expect intense interest from landowners, orchard growers, and private citizens. Due to the relatively long time before flowering occurs, generally 5 to 7 years, propagation through tissue culture is an ideal way to produce American chestnut trees, especially since micropropagated shoot cultures may become available for purchase by the public in the future alongside limited nursery stock. Also, chestnut is an extraordinarily difficult tree to root, and therefore these techniques may also be of interest to others working with rooting and acclimatizing micropropagated trees. This paper describes an *ex vitro* method for rooting of tissue-cultured American chestnut shoots. Moving to an *ex vitro* rooting protocol may be ideal for other difficult-to-root species that have had limited success with *in vitro* protocols and are difficult to propagate via conventional methods such as cuttings, grafting or layering. The *ex vitro* method is cheaper, with significantly less labor, time, and materials needed to perform than the *in vitro* method.

Introduction

In little more than a century, the American chestnut went from a towering canopy giant of Appalachia to a stump-sprouting shrub found in scattered patches, and isolated individual trees throughout its native range. American chestnut timber had many uses, providing tough, rot-resistant lumber for log cabins, fence rails, coffins, and telephone poles, and the tannins extracted from its bark and wood were preferred for tanning leather (Davis 2005). Many forest-dwelling species such as wild turkeys, bear, deer, squirrels, and domestic pigs were fattened on its rich seeds, and chestnut leaf litter and coarse woody debris was a preferred food and habitat for stream-dwelling invertebrates, which in turn provided more food for fish (Hedman et al. 1996, Davis 2003).

The American chestnut was devastated by an exotic fungal pathogen *Cryphonectria parasitica* (Murrill) Barr, which was accidentally introduced to North America in the late 1800s via shipments of Japanese chestnut seedlings to several nurseries. In 1904, the first presence of chestnut blight was observed at the Bronx zoo (Murrill 1906, Anagnostakis 1987) and spread quickly throughout the eastern hardwood forests, which lost more than 25% of their mature, canopy trees (Hepting 1974, Griffin 2000, Freinkel 2009).

Agrobacterium-mediated transformation of American chestnut embryos with disease-resistance genes from other species is one method to help restore the species to the forests it once dominated (Polin et al. 2006). The procedure utilizes both the vast resources of molecular biology and tissue culture to produce plantlets that express a gene of interest. Because *C. parasitica* uses oxalic acid to attack the American chestnut, a transgene coding an oxalate oxidase enzyme was chosen to detoxify this acid. Without the oxalate to lower the pH and kill tissue, *C. parasitica* can only form a superficial canker and does not girdle the chestnut (Zhang et al. 2013, Newhouse et al. 2014). Although transformed plants are now growing in field test plots, getting significant numbers of trees from tissue culture to the field has been a difficult endeavor.

One bottleneck is the rooting and acclimatization stage. This stage occurs after sufficient shoots are multiplied *in vitro*. The base of the shoot is treated with a rooting hormone and the plantlets are left to form adventitious roots. Then the rooted plantlets are moved to high-humidity growth chambers for acclimatization and hardening off. Similar difficulties with the rooting stage have been observed for other members of the *Fagaceae* family (Ramirez et al. 2007).

The main loss of plantlets occurs in the acclimatization stage. Plantlets that do not continue to grow new leaves and

[1]Received for publication February 29, 2016; in revised form May 16, 2016. This work was supported by the SUNY-ESF 10,000 Chestnut Challenge and The American Chestnut Foundation. Thanks go out to the undergraduates who worked in the growth chambers over the summer; Marina Lupu and Sarah Cruz.

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roots will eventually die, resulting in a significant loss of time and labor in producing shoots that do not make it to the field. In American chestnut, this is characterized by yellowing and curling of the leaves, shoot tip browning, and early lignification of the stem, along with colonization of the dying shoot tip by fungus. Many hardwood species form roots *in vitro* with poorly developed vascular systems, and these roots are less robust than roots formed *ex vitro* (Bonga and Aderkas 1992, Kim et al. 1998, Aygun and Dumanoglu 2015), resulting in poor growth and development. However, some studies have observed significant retention and further growth of *in vitro* roots during acclimatization (Gribaudo et al. 1995).

To help overcome some of these difficulties, two methods of rooting have been developed for American chestnut shoots, *in vitro* and an *ex vitro* protocols. In the *in vitro* method, roots develop while the shoots are in sterile semi-solid medium, while in the *ex vitro* method the shoots form roots while outside of a sterile environment. In this experiment, we evaluated peat pellets within humidity bins as an *ex vitro* substrate.

Materials and Methods

Plants. American chestnut shoot cultures were derived from the clone Ellis #1, a somatic embryo cell line developed from immature nuts donated by the New York chapter of The American Chestnut Foundation (Laurens, NY). Shoot cultures were maintained in Magenta GA-7 vessels with GA-7 vented lids (Caisson Laboratories, Inc., Logan, UT).The vessels contained pre-rooting medium consisting of full-strength Lloyd and McCown Woody Plant medium salts (WPM) (Lloyd and McCown 1980), 109 mg·L^{-1} Nitsch and Nitsch vitamins (Nitsch and Nitsch 1969), 2.3 mM 2-(N-morpholino)ethanesulfonic acid (MES), 12.5 µM polyvinylpyrrolidone 40000 MW (PVP-40), 0.22 µM benzyladenine (BA), 3% (w/v) sucrose, 0.35% (w/v) Phytagel® (PhytoTechnology Laboratories, Shawnee Mission, KS), with pH adjusted to 5.5. All medium was autoclaved for 20 min at 15 psi and 121 C (250 F). Each vessel contained 85 mL (3 fl oz) of medium. The vessels were kept on a light bench with a 16 hr. photoperiod at 31 µmol·m^{-2}·s^{-1} for at least eight weeks, or two culture transfers cycles, before the rooting treatments were applied.

Shoots were classified into two tiers. Tier 1 plantlets met all three criteria: shoots were greater than 5 cm in height, had at least 4 leaves larger than 1 cm in length, and a stem caliper of at least 1 mm. Shoots which met two of the three criteria were considered Tier 2. Any excess shoots were multiplied and placed into fresh pre-rooting media to be used for future experiments and production. For each of the four treatments (*in vitro* vs *ex vitro* against Tier 1 vs Tier 2), 36 plantlets were used for a total of 144 plantlets.

In vitro rooting treatment. Shoots were removed from the pre-rooting medium by excision from the basal callus. The basal end of a shoot was cut at a 30° angle from vertical to increase the exposed surface area of the vascular tissue. Shoots were then dipped into a 10 mM indole-3-butyric acid (IBA) solution (dissolved in KOH, brought to volume with dH_2O) for 30 s. Shoots were placed into 'clams', clear hinged containers (Dart Solo C53PST1 ClearSeal Clear Hinged Lid Plastic Container, Webstaurant Store Food Service Equipment and Supply Company, Lancaster, PA). The clams contained rooting medium, consisting of half-strength Murashige and Skoog basal salt mixture (Murashige and Skoog 1962), 54.5 mg·L^{-1} Nitsch and Nitsch vitamins (Nitsch and Nitsch 1969), 3% (w/v) sucrose, 0.35% (w/v) Phytagel, with pH was adjusted to 5.5. All medium was autoclaved for 20 min at 15 psi and 121 C. Medium was cooled to 55 C (131 F) in a water bath before pouring into clams. Containers were placed in a dark cabinet at 28 C (82 F) for 3–4 d (Oakes et al. 2013).

Post-rooting medium consisting of full-strength WPM salts, 109 mg·L^{-1} Nitsch and Nitsch vitamins, 2.3 mM MES, 12.5 µM PVP-40, 0.22 µM BA, 3% (w/v) sucrose, 0.35% (w/v) Phytagel (Sigma-Aldrich, Milwaukee, WI), 0.5 g·L^{-1} humic acid, 2.0 g·L^{-1} activated charcoal (autoclaved separately), with pH adjusted to 5.5 with 1M KOH. All activated charcoal (Phytotechnology Laboratories, Shawnee Mission, KS) was autoclaved separately in 50 mL dH_20 and adding to cooled medium before it was poured into containers. Medium was prepared in disposable take-out containers (clams). Each clam contained three shoots. Shoots were removed from the rooting medium and placed into the post-rooting medium and on a light bench with a 16 h photoperiod at 31 µmol·m^{-2}·s^{-1} for 10 d before being potted and moved to the growth chamber.

Ex vitro rooting treatment. Jiffy 36 mm peat pellets (Jiffy International AS, Kristiansand, Norway) were thoroughly soaked in 5 mL·L^{-1} Clonex Rooting Solution (HydroDynamics International, Lansing, MI), pH adjusted to 5.5, and placed in clear plastic shoeboxes (Lowe's, Mooresville, NC) to maintain high humidity during rooting. A thin wire, similar in diameter to the shoots, was dipped in rooting gel and used to dibble a hole in each pellet. Shoots were removed from the Pre-Rooting Medium, excised from basal callus and cut diagonally at the basal end at approximately a 30° angle to maximize surface area. The shoots were dipped into Clonex Rooting Compound gel (HydroDynamics International, Lansing, MI) to a depth of 1 cm. The shoots were then immediately inserted into the dibbled holes in the Jiffy pellets and lightly misted with distilled water. The shoeboxes were placed on a bench with a 16 h photoperiod at 31 µmol·m^{-2}·s^{-1} for three weeks.

Potting. In vitro shoots that grew roots were carefully removed from the post-rooting medium and rinsed under distilled water to remove excess medium. The *ex vitro* shoots that grew roots were not removed from the pellets, instead the pellets were planted directly into containers. Plantlets from both treatments were potted into 18 cm (7 in) diam tube pots (Stuewe and Sons, Tangent, OR) containing Faford Germinating Mix (Conrad Faford Inc., Agawam, MA). All plants were placed into a Conviron CMP5090 growth chamber with a SK 300 steam humidifier (Conviron, Winnipeg, Manitoba, Canada) at 92% relative humidity with 22 C (72 F) day temperatures and 16 C (61 F) night temperatures. The chambers had a 16-hour photoperiod at a light intensity of 100 to 120 µmol·m^{-2}·s^{-1}. All pots were watered with Peter's 30-10-10 (Everris, Geldermalsen, The Netherlands), adjusted to pH 5.5 with PhDown (General Hydroponics, Stebastopol, California) as needed to keep the potting mix moist.

Data collection. The presence of roots (Rooted) and the survival rate at potting (Potted) were both documented. Not all plants with roots retained living shoots, so at 4 and 8 weeks plantlet survival was tallied, and at 8 weeks the

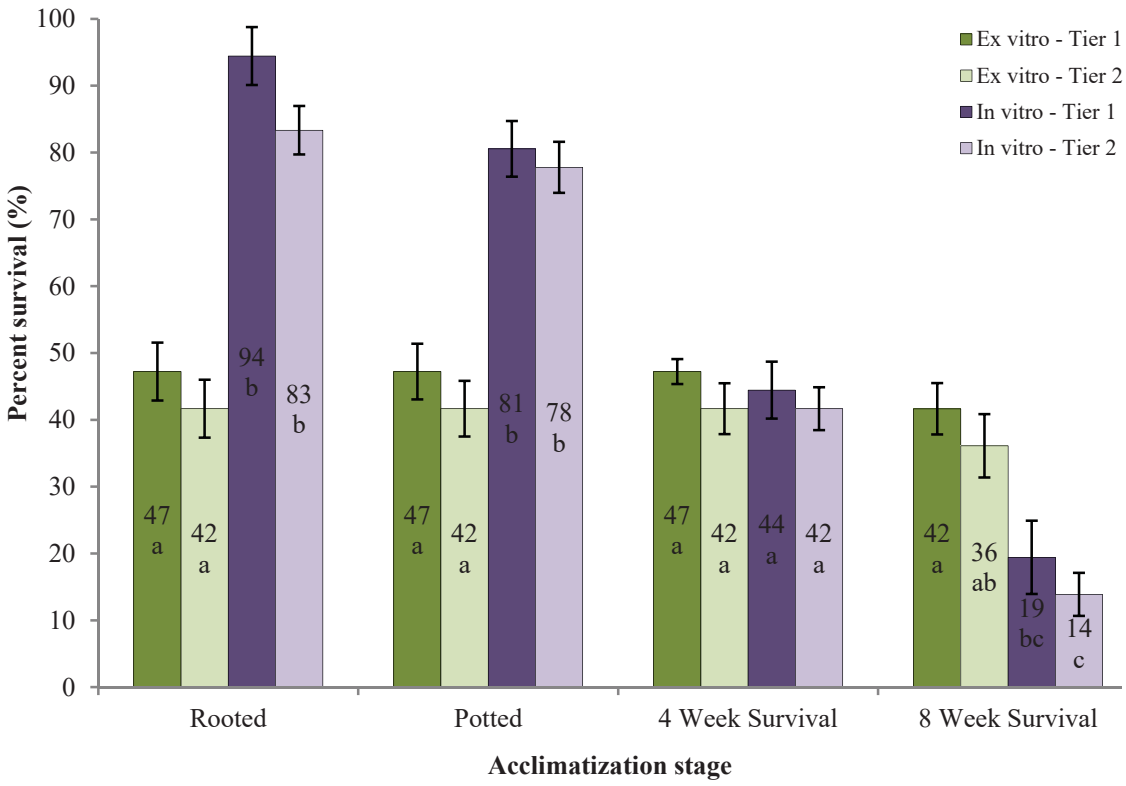

Fig. 1. Mean plantlet survival of American chestnut shoots from tissue culture for two rooting methods by acclimatization stage. 'Rooted' indicates percentage of shoots which had formed roots at the end of the rooting procedure. 'Potted' indicates what percentage of plantlets had both living shoots and roots and were therefore moved into acclimatization. '4 weeks' and '8 weeks' indicated survival in the growth chamber at those time points. Error bars indicate standard error of the mean. Different letters indicate significant differences in the mean at $\alpha = 0.05$.

heights, number of leaves, and length of longest leaf was recorded for each surviving shoot.

Data analysis. The data for each experiment were analyzed using ANOVA using SAS™ 9.0 statistical software (SAS Institute Inc., Cary, NC); means were tested with Waller-Duncan K ratio t-tests. Differences among means with a p-value of less than 0.05 were accepted as being statistically significant, and relevant F and p-values were stated.

Results and Discussion

Surprisingly, the *in vitro*-rooted plantlets had excellent root production with Tier 1 shoots rooting 94% of the time (Fig. 1). While they started to grow, they did not fare well in the growth chambers, with a massive drop in survival between potting and 8 wk with Tier 1 shoots having only 23.4% survival and Tier 2 shoots having 17.9% acclimatization survival. The *ex vitro* plantlets that rooted were also healthy enough to be potted, with a small loss between 4 and 8 weeks, resulting in 89% and 85% acclimatization rates for the Tier 1 and Tier 2 ex vitro plantlets, respectively.

The extremely high rooting percentage in the *in vitro* shoots may be due to recent improvements in American chestnut rooting protocols, namely the addition of a dark treatment (Oakes et al. 2013) and the addition of humic acid to the post-rooting medium (Oakes et al. 2015). However, the marked decline *in vitro* shoot survival implies that rooting does not guarantee survival, and here our findings agree with other studies on Fagaceae species. In *C. sativa* × *crenata* hybrids, *in vitro* treatments initially produced more roots but had lower acclimatization survival than shoots rooted via an *ex vitro* protocol (Gonçalves et al. 1998). Gonçlaves et al. also observed that roots formed *ex vitro* had well-organized cell structure with orderly vascular cells, and there was a greater proportion of vascular tissue to total cross section area in the *ex vitro* roots. They also noted that root hairs and lateral roots only developed in *ex vitro* conditions, which we have also observed in general production of American chestnut.

The quality of the *ex vitro*-rooted plantlets was also significantly better that the *in vitro* ones, as measured by plantlet height (Fig. 2), number of leaves (Fig. 3), and length of longest leaf (Fig. 4). Tier 1 plantlets were taller and had more leaves than Tier 2 plantlets, as expected due to their placement into the tiers. However, there were not significant differences between the Tier 1 and Tier 2 plantlets in survival. Therefore, it seems from our results that our delineation between Tier 1 and Tier 2 is too conservative. In the *ex vitro* treatments, both Tier 1 and Tier 2 plantlets had excellent survival in the growth chamber, with five Tier 1 plantlets and six Tier 2 plantlets lost after 9 wks in the growth chamber, representing an 89% and 85% acclimatization rate respectively. Tier 2 plantlets were shorter and had fewer leaves than Tier 1 (Figs. 1 and 2), as expected since they were smaller plantlets to begin with, but once leaf size is compared in Fig. 4, there is no difference between plantlet tiers. Enlarged leaf size was previously identified as an indicator of successful acclimati-

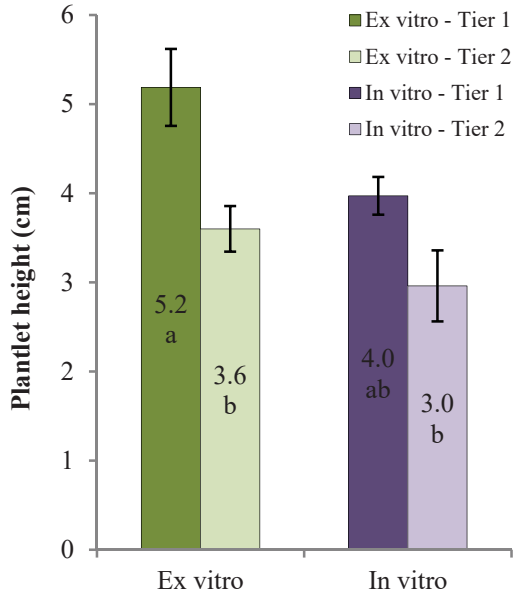

Fig. 2. Mean plantlet height at 8 weeks post-potting by rooting method and initial quality for American chestnut shoots from tissue culture. Error bars indicate standard error of the mean.

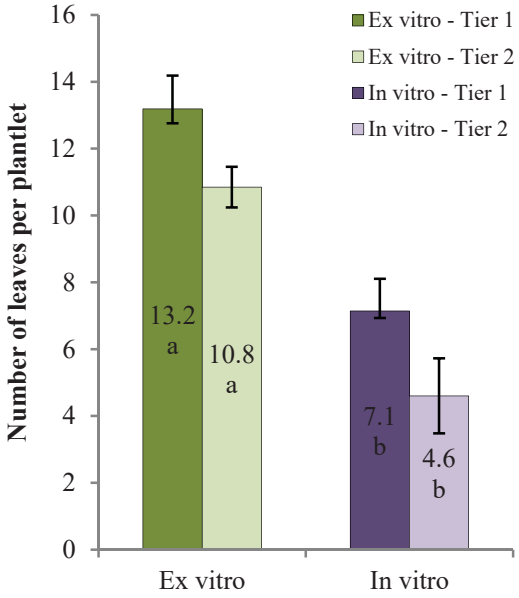

Fig 3. Mean number of leaves per plantlet at 8 weeks post-potting by rooting method and initial quality for American chestnut shoots from tissue culture. Error bars indicate standard error of the mean.

zation (Oakes et al. 2013). In order to identify a cut-off point for what shoots to root, we must lower our qualifications until we find a Tier that performs significantly worse than *in vitro* acclimatization.

Missing from this experiment are direct observations of the roots themselves. We determined that opening the peat pellets would disturb the root system and therefore nullify one of the major benefits of the *ex vitro* procedure. Therefore no roots were harmed in the production of these results. With increased shoot production, we will be able to provide enough replicates to destructively sample at multiple time points and therefore gain a better understanding of root development between treatments.

Only one clone line of American chestnut was used in this experiment due to limited availability of shoots derived from other clonal lines. This will be an important factor to examine, as other studies have noted significant differences in rooting capability between clone lines of other species. In pear, some important rootstock varieties could not be rooted with an *ex vitro* protocol (Aygun and Dumanoglu 2015), and in green ash, the clone lines had significantly different responses in shoot height, root lengths and root number to the same 1 mM IBA dip and *ex vitro* rooting treatment (Kim et al. 1998).

One factor not replicated in the *ex vitro* treatments was the 4 day dark period which the in *vitro* shoots had after the initial cutting from callus and dip in rooting hormone. Adding an initial dark period after rooting may be beneficial to the *ex vitro* shoots, as photoinhibition has been reported to lower the success of acclimatization to an *ex vitro* environment, especially to plants kept in closed vessels, such as our high humidity shoeboxes (Semorádová et al. 2002). However, experiments on pear rooting found that dark treatments had a significant positive effect between levels of exogenous auxin, but not between *in vitro* and *ex vitro* protocols (Aygun and Dumanoglu 2015) Further studies will explore irradiance levels and duration of a dark period in *ex vitro* rooting of American chestnut.

The ongoing development of a low cost, low labor, high survival *ex vitro* rooting protocol will be vital for ongoing experimentation with American chestnut plantlets derived from tissue culture. In order to pursue a non-regulated

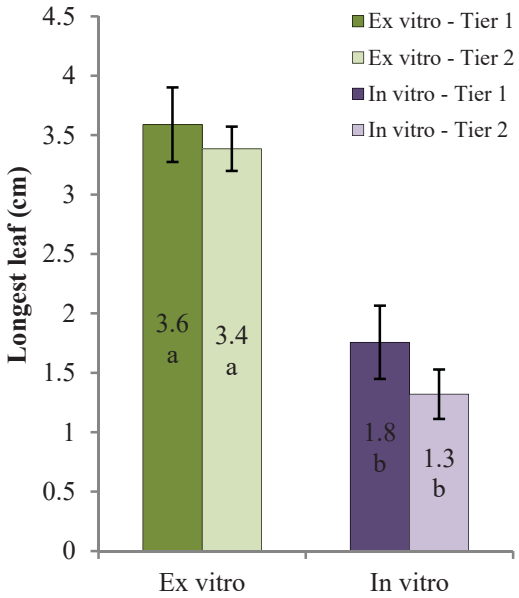

Fig 4. Mean longest leaf per plantlet at 8 weeks post-potting by rooting method and initial quality for American chestnut shoots. Error bars indicate standard error of the mean.

status, hundreds of blight resistant, transgenic American chestnuts will be needed for environmental assays of non-target impacts. Until mother tree orchards are established for controlled-pollination seed production, tissue culture will continue to be the main production method for each new line of transgenic American chestnut. Once blight resistant chestnuts are available for the public, the great task of restoring a heritage tree to its native habitat can begin.

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