Improving Ex Vitro Rooting and Acclimatization Techniques for Micropropagated American Chestnut[1]

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Abstract

Limited rooting and acclimatization success when micropropagating certain hardwood tree species may hinder conservation efforts of certain threatened and endangered species. Restoration efforts for such trees, such as the American chestnut [*Castanea dentata* (Marsh.) Borkh.], require a massive number of plantlets to be produced by micropropagation for testing, initial distribution, and orchard establishment. Therefore, increasing the number and quality of lab-produced plantlets is a key research focus. After previously determining that an ex vitro rooting system produced significantly more robust plantlets, we examined extending the time in elongation medium, rooting substrates, exogenous auxin applications, root-promoting substrate soaks, submerging the cut site, and light intensity. The most effective methods included seven weeks in elongation medium, using Jiffy peat pellets soaked in water as the rooting substrate, cutting off callus while submerged, then dipping in 0.31% IBA rooting gel, and placing plantlets in low light of 60 μmol·m-2·s-1 after rooting. By increasing the number of roots and improving acclimatization success, we can ensure that many more blight-tolerant American chestnuts will be available for field studies and eventual public distribution. Demonstrating the ecological safety and blight survival of these trees will help restore this foundational tree species and assist future restoration efforts for other threatened species.

Index words: Rooting, ex vitro, American chestnut, *Castanea dentata*, IBA, substrate.

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

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

Significance to the Horticulture Industry

When developing propagation protocols for woody species, it's almost imperative that each species, and perhaps each variety, will need its own regime for optimal growth. This paper will ideally give propagators new ideas of what culture factors to test or new ideas to try, and help propagators choose different products that might work well for their species of interest.

Introduction

Micropropagation is used globally to aid in the conservation of threatened and endangered woody plants and trees (Blakesley et al. 1996, Bunn 2009; Fay 1992, Sarasan et al. 2006). Tropical trees such as *Cedrela fissilis* Vell., (Da Costa Nunes et al. 2003), teak (*Tectona grandis* L.f.) (Mendoza de Gyves, Royani, and Rugini 2007), and jacaranda (*Jacaranda mimosifolia* D.Don) (Maruyama et al. 1997) are of interest as rapid production via tissue culture could potentially reduce reliance on wild stock. There is also potential in storing commercially important germplasm of fruit trees for future use, either via active micropropagation or cryopreservation (Lambardi and De Carlo 2003, Panis and Lambardi 2006, Wilkins, Newbury, and Dodds 1988). However, such stored germplasm is only of use if regeneration to rooted plantlets is possible.

In North America, many native tree species are under threat from invasive pests. Various forest health methods are being employed to protect existing trees, or to develop resistant ones, such as emerald ash borer-resistant green ash (*Fraxinus pennsylvanica* Marshall), and wooly adelgid-resistant eastern hemlock [*Tsuga canadensis* (L.) Carriere]. As such, micropropagation methods for *Fraxinus* species (Beasley and Pijut 2013, Kim, Klopfenstein, and Cregg 1998, Sambeek and Preece 2007) and eastern hemlock (Merkle et al. 2014) are in development in anticipation of genetic transformation solutions (Lee and Pijut 2018). Optimized protocols for American elm (*Ulmus americana* L.) micropropagation have been developed in order to store germplasm for future restoration projects (Shukla et al. 2012).

Having thriving plants in the greenhouse and field must be achieved in order to perform disease resistance and environmental safety testing. The use of clones enhances these experiment by allowing more direct comparisons. Recent studies on the off-target effects of transgenic American chestnut [*Castanea dentata* (Marsh.) Borkh.] on tadpoles (Goldspiel et al. 2018), honeybees (Newhouse, in preparation), mycorrhizae (D'Amico et al. 2015), native seed germination (Newhouse et al. 2018), and other organisms all required plants produced through tissue culture.

Developing reliable rooting and acclimatization methods for difficult-to-propagate woody plants and trees can be a massive hurdle for production efforts, for either experimentation, distribution, or other conservation goals. It often

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takes a swathe of experiments to determine optimal conditions for a species or cultivar, as was done in *Eucalyptus saligna* Sm. and *Eucalyptus globulus* Labill. (Fogaça and Fett-Neto 2005).

Improving rooting success and plantlet quality of American chestnut has been a goal of the American Chestnut Research and Restoration Project for many years, originally using in vitro methods (Xing et al. 1997), which was further optimized (Oakes, Powell, and Maynard 2013). The project moved to ex vitro methods once it was determined that through this method, plantlets had increased survival and vigor (Oakes et al. 2016), and was also cheaper in materials and labor.

Previous pilot studies had found that neither sand, perlite, nor rockwoll rooting pellets (Grodan, Roermond, The Netherlands), which worked well for American elm (Oakes et al. 2012), were suitable rooting substrates for American chestnut, producing large callus balls without roots. Peat pellets are cheap, self supporting, and retain moisture extremely well. We had good initial success with commercial polymerized peat pellets, and decided to test multiple brands. We used firmly packed potting mix as a control. We also tested commercial brands of IBA rooting dips and root-promoting solutions. In this study we have examined new factors and techniques to improve root initiation and elongation on micropropagated American chestnut shoots, including rooting hormone type, rooting substrate type and soaking solution, making the rooting cut while the callus is submerged, and amount of time previously in elongation medium.

Materials and Methods

Plants. Four American chestnut shoot cultures were used in these experiments. The first being Ellis #1, originally a somatic embryo cell line produced from immature nuts donated by the New York chapter of the American Chestnut Foundation (Laurens, NY). The second line was Ashdale #1, a culture donated from McGill University (Montreal, Quebec) which had been established from a seedling of the Ashdale tree in Canada. The last two lines are offspring of the LM-B4SX58 Darling 58 event, the pollen from which was a controlled crossed with two American chestnut mother trees at the Lafayette Road Experiment Station (Syracuse, NY). Both offspring (D58+16001 and D58+16020) contain the gene for blight tolerance and were established in shoot culture from axillary bud tissue generated by grafting. These lines were used as all four were being propagated in high numbers for planting the following spring, and there was enough tissue in these lines to conduct experiments with appropriate levels of replication.

Culture maintenance protocol. Shoot cultures were maintained in vented MK-5 vessels (Caisson Laboratories, Inc, Logan, UT), 5 shoots per vessel, on multiplication medium consisting of full-strength Lloyd and McCown Woody Plant medium salts (WPM) (Lloyd and McCown 1980), 109 $mg \cdot L^{-1}$ Nitsch and Nitsch vitamins (Nitsch and Nitsch 1969), 1 µM benzyladenine (BA), 0.01 µM indole-3-butyric acid (IBA), 3% (w/v) sucrose, 0.7% (w/v) agar (PhytoTechnology Laboratories, Shawnee Mission, KS) , with pH adjusted to 5.5 with 1M KOH. After four weeks, healthy 1.5 cm (0.6 in) shoot tips were cut and transferred to elongation medium in vented MK-5 vessels, 5 shoots per vessel, consisting of full-strength Lloyd and McCown Woody Plant medium salts (WPM), 109 mg/L Nitsch and Nitsch vitamins, 0.5 µM benzyladenine (BA), 0.5 µM indole-3-butyric acid (IBA), 3% (w/v) sucrose, 0.7% (w/v) agar, with pH adjusted to 5.5 with 1M KOH. Each vessel contained 85 mL of medium, and all media were autoclaved for 20 min at 1.1 $kg \cdot cm^{-2}$ (15 psi) and 121 C (250 F). The vessels lids were wrapped in a layer of sealing film (PhytoTechnology Laboratories, Shawnee Mission, KS) to prevent contamination, and vessels were kept on a light bench with a 16 hr. photoperiod at 50 µmol $m^{-2}s^{-1}$.

The standard ex vitro rooting protocol. The standard ex vitro rooting procedure developed earlier (Oakes et al. 2016) began with soaking Jiffy 36 mm (1.4 in) wide peat pellets (Jiffy International AS , Kristiansand, Norway) in tap water, pH adjusted to 5.5 with pHDown (General Hydroponics, Santa Rosa, CA), and placed in clear plastic shoeboxes (Lowe's, Mooresville, NC), firmly closed to maintain high humidity during rooting. Shoots were removed from the elongation medium, excised from basal callus on a paper napkin, and cut diagonally at the basal end at approximately a 45° angle to maximize surface area. Leaves close to the basal end were removed. The shoots were dipped into Clonex Rooting Compound gel (Hydro-Dynamics International, Lansing, MI) to a depth of 1 cm (0.4 in). The shoots were then immediately inserted into the Jiffy pellets and lightly misted with distilled water, and shoeboxes firmly closed. The shoeboxes were placed on a bench with a 16 hr photoperiod at 50 µmol $m^{-2}s^{-1}$ for three weeks.

Acclimatization procotol. 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 Super Fine Germinating Mix (SFGM) (Conrad Faford Inc, Agawam, MA) and Micromax Micronutrients Granular Fertilizer (Scotts Co. LLC, Marysville OH) at 15 mL (1 Tbsp) per 20 L (0.7 cubic feet) potting mix. The potting mix was moistened with Dr. Pye's Scanmask nematode-infused water (Biologic Co Inc, Willow Hill PA) at 15 mL per 3.8L (1 Tbsp per 1 gallon) tap water, pH adjusted to 5.5, to prevent fungal gnat infestation. No NPK fertilizer was added at the time of potting. 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 (71.6 F) day temperatures and 16 C (60.8 F) night temperatures. The chambers had a 16-hour photoperiod at a light intensity of 100 to 120 µmol $m^{-2}s^{-1}$, which was raised by 100 µmol $m^{-2}s^{-1}$ each week to 600 µmol $m^{-2}s^{-1}$.

Experiments

Elongation stage. We examined whether an additional transfer in elongation (3 weeks + 3 weeks) would increase

plantlet quality and survival (two treatments, 96 plantlets per treatment, n = 192), and subsequently, how long the second transfer should be (3 weeks or 4 weeks) (two treatments, 119 plantlets per treatment, n = 238). For the "Single," treatment, plantlets were rooted as per the standard procedure after three weeks in elongation medium. For the "Double," treatment, after three weeks the plantlets were moved into fresh elongation medium, leaving the callus ball intact, and inserted deeply so that the callus ball was touching the bottom of the container. For length, the second transfer was left for either 3 weeks or 4 four weeks before rooting. After the second transfer, the plantlets were rooted as per the standard rooting procedure. For the "Triple," pilot treatment (60 additional plantlets), plantlets were moved twice into fresh elongation, for a total of 9 weeks in elongation. At data collection, height, caliper, survival of plantlets, and number of emergent roots were measured.

Rooting substrate. We compared five commercial rooting substrates and plugs of potting mix (36 plantlets per treatment, n=180) . Each were soaked in enough pH 5.5 nematode-infused water to covered all the pellets prior to rooting. Pellets were removed from the soak and placed in shoeboxes, 5 of each substrate type per box, with 8 replications (5 treatments, n = 200). The five commercial substrates were Jiffy pellets, and three polymerized peat pellets, RootRiot plugs (Growth Technology Ltd., Somerset, UK), ReadyGro plugs (Botanicare, Chandler AZ); and RapidRooter plugs (General Hydroponics, Santa Rosa, CA). Faford Super Fine Germinating Mix (SFGM), moistened with nematode-infused water, was firmly packed into 2.5 cm by 2.5 cm cells (1 in by 1 in) cut from a propagation tray insert (GreenhouseMegastore.com) . The polymerized peat pellets were also inserted into cells to keep them upright. The Jiffy pellets cubes stayed oriented in the shoeboxes without support. The commercially plugs dried out quickly and were moistened with pH 5.5 tap water after two weeks, whereas the Jiffy pellets retained plenty of moisture and were not over-watered. At data collection, survival of plantlets and number of emergent roots were counted.

Rooting gel. We tested four commercial rooting gels using the standard ex vitro rooting procedure and dipping the cut end of the stem in the gel/liquid for 3 seconds: Botanicare Rhizo Gel (0.2% IBA) (Botanicare, Chandler AZ), EZ Clone Rooting Compound (0.3% IBA, plus thiamine) (EZ Clone Enterprises, Inc, Sacramento, CA), Clonex Rooting gel (0.31% IBA), and RootTech Cloning gel (0.55% IBA) (Technaflora Plant Products Ltd, Mission, BC, Canada), along with a negative control with no dip (16 plantlets per treatment, 5 treatments, n = 80). At data collection, survival of plantlets and number of emergent roots were counted.

Substrate soak. We tested four commercial root-promoting solutions, three fertilizers, and a water control to determine if soaking the pellets in low or high concentrations of NPK fertilizer before rooting would have an impact on root formation. We tested BioRoot (General Organics-USA, Santa Rosa, CA), Clonex Rooting Solution (HydroDynamics International, Lansing, MI), Bushdoctor Microbe Brew (Foxfarm, Pendleton, SC), Biobizz Root Juice (Biobizz, Los Angeles CA), MiracleGro Miracid 30-10-10 (Scotts Co. LLC, Marysville OH), Jack's 20-20-20 (J.R. Peters, Inc, Allentown PA), and Jack's 21-7-7 (J.R. Peters, Inc, Allentown PA). Each solution was adjusted to an electrical conductivity of either 0.5 or 1 S/m, pH adjusted to 5.5 with pHDown, and applied to the substrates to soak (17 treatments, 5 plantlets per treatment, n = 85). Shoots were rooted as per the standard procedure. At data collection, survival of plantlets and number of emergent roots were counted.

Submergence. We tested whether submerging the base of the stem in either water or an IBA solution (1mM or 10mM) would improve root formation. We also examined whether dipping in the preferred rooting dip (Clonex Rooting gel, 0.31% IBA) after the submerged cut would have an effect (12 plantlets per treatment, n = 96). At data collection, survival of plantlets and number of emergent roots were counted.

Statistical analysis. In each experiment, 3 or 4 of the clonal lines were used, creating experimental blocks. Results are averaged over all lines used, as there was only one instance of significant interaction, reported in the elongation stage experiment. After data collection, data was analyzed using R version 3.4.3 (R Core Team 2019). The lm() and anova() functions were used to check for statistical significant between treatments, and the package agricolae (version 1.3-1), function HSD.test() was used to determine Tukey Honest Significant Difference (Mendiburu 2019). The package ggplot2() (version 3.1.1) was used to generate figures (Wickham 2016).

Results and Discussion

We determined that the optimum rooting protocol had to be biologically sound, with high root induction and high acclimatization success, and also economically feasible and easy to perform. Therefore, our current protocol derived from these experiments includes:

- Tips taken from 4 week old shoots in multiplication medium placed onto a first round of elongation medium, there for 3 weeks
- Moving the shoots into fresh medium with no subculture, there for 4 weeks
- Submerge the basal end of the shoot in dH20, cut off callus ball, dip in Clonex Rooting Gel (IBA 0.3%)
- Insert cut end into Jiffy pellet, previously soaked in pH 5.5 tap water
- Store in clear shoe boxes on light rack for 4 weeks
- Acclimate as described in Methods

These results taken together solidify a working model for American chestnut propagation through tissue culture (Fig. 1).

Elongation stage. Two, three-week transfers (6 weeks total) in elongation had very significant beneficial effect on

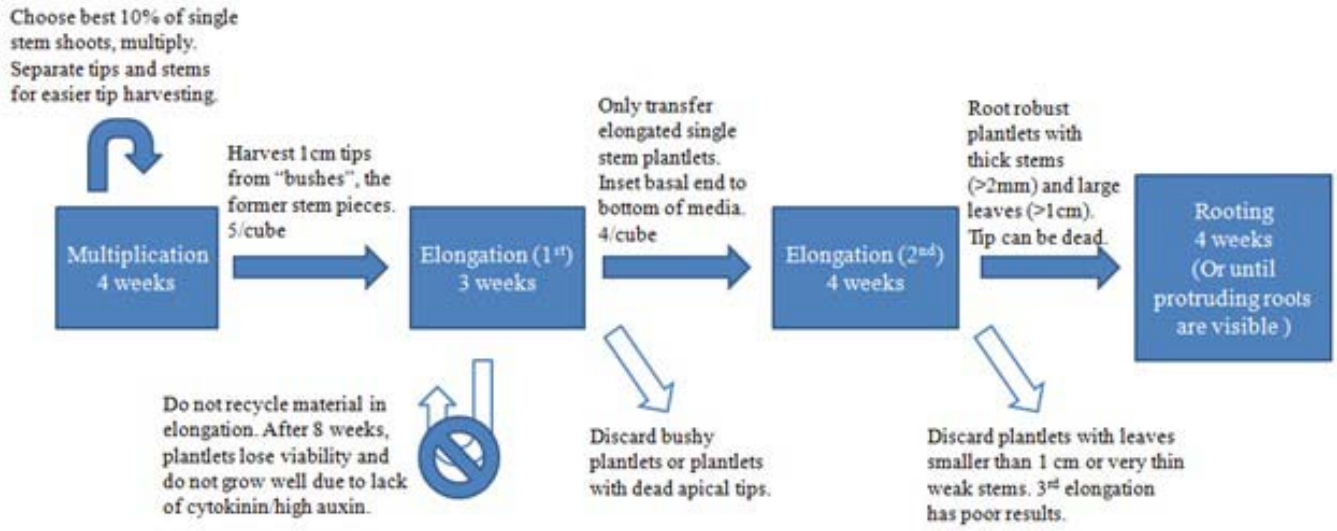

Fig. 1. Working model for American chestnut propagation.

both rooting survival ($F = 9.22$, $p = 0.003$) and acclimatization success ($F = 9.11$, $p = 0.003$) in comparison to 3 weeks (data not shown). The percentage of plantlets which survived with only one elongation transfer was 29.1 ± 4.7%, while after two transfers the rooting survival rose to 57.3 ± 4.6%. Acclimatization success increased from 27.1 ± 4.6% to 46.9 ± 5.1% with the longer period of elongation.

Adding a third transfer in elongation medium resulted in the elongated stems dying back, and new sprouts emerging from basal buds, which were not suitable for rooting. We adopted the double elongation procedure for production, and noticed a slight improvement if the cultures had been delayed by an extra week before rooting. Thus, we examined whether the second elongation treatment should be three or four weeks long.

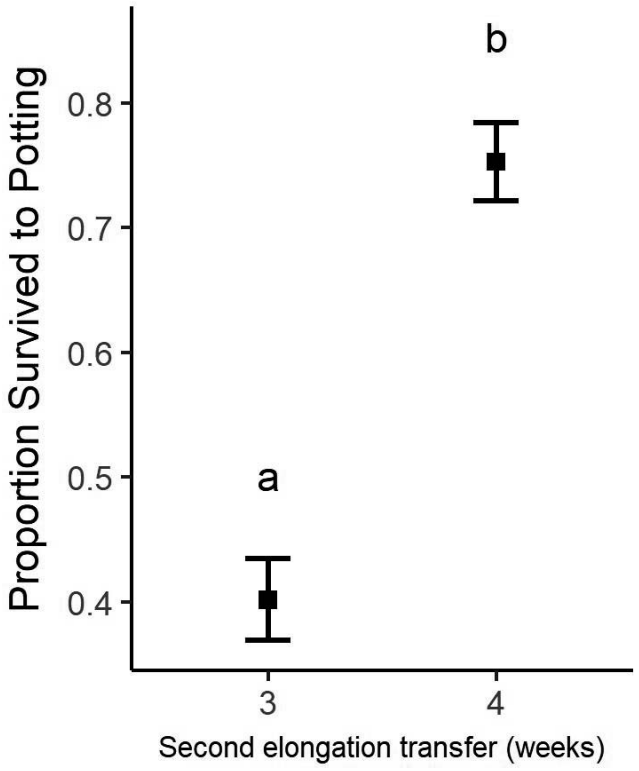

Fig. 2. Plantlet survival by length of second elongation transfer.

We found the additional week markedly improved rooting survival, increasing from 40% to 75% as shown in Fig. 2 ($F = 58$, $p < 0.001$). At rooting, the 3 week old shoots (6 weeks in elongation total) were more soft and green, and sometime bent when being inserted into the peat pellet. The 4 week shoots (7 weeks in elongation total) were sometimes slightly chlorotic, or had dead apical tips with sprouting axillary buds, but had firmer, thicker stems that were slightly lignified. The number of roots significantly ($F = 8.21$, $p = 0.004$) increased from 3.2 ± 0.1 to 4.1 ± 0.2 when plantlets spent an extra week in elongation medium (data not shown).

The younger, greener stems were more susceptible to infection by surface fungus than the older ones, and death due to fungal infection counted for most of the losses of the younger stems (data not shown). We also saw a significant interaction effect ($F = 6.94$, $p=0.008$) in that one T1 line, the D58+16001, produced significantly more roots when shoots were rooted at three weeks old compared to the other lines. However, the increased root production (~1.5 roots per plantlet) did not appear to contribute to survival (data not shown).

Commercial or large-scale production projects may be loath to add an extra in vitro step to their production timeline, but for such a recalcitrant woody species the extra elongation step is helpful in reducing wasted time and materials from unsuccessful rooting. A pre-rooting high auxin treatment on apple (*Malus domestica* Borkh.) rootstock cuttings increased future rooting success (Howard 1968). A pre-rooting step using vented vessels improved root length in micropropagated artichoke (*Cynara cardunculus* var. *scolymus* L.) (Pacifici et al. 2007). In pistachio (*Pistacia vera* L.), transferring to a plant growth regulator-deprived medium containing activated charcoal helps the shoots harden and increases subsequent rooting (Benmahioul et al. 2012).

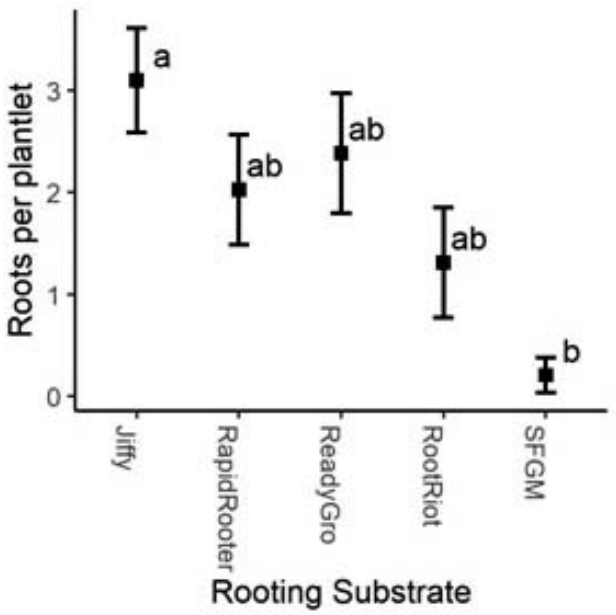

Fig. 3. Number of roots produced per plantlet by rooting substrate. Error bars indicate standard error of the mean. Different letters indicate significant difference between treatment means via Tukey's range test.

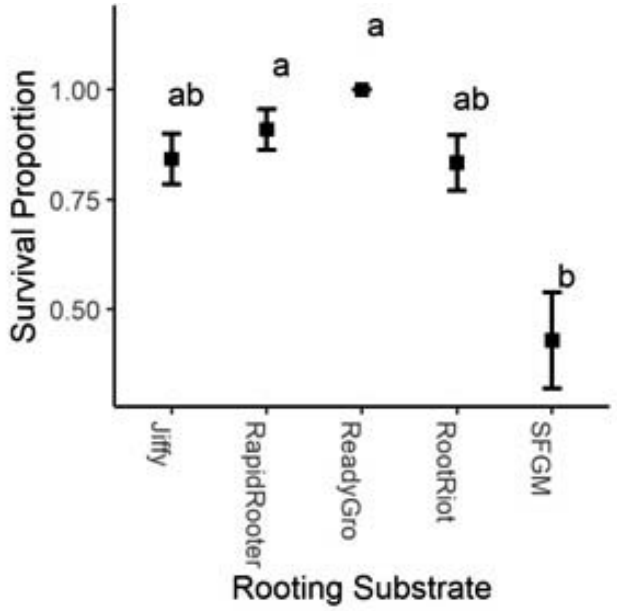

Fig. 4. Proportion of overall plantlets which were alive after 6 weeks in the growth chamber. Error bars indicate standard error of the mean. Different letters indicate significant difference between treatment means via Tukey's range test.

Rooting substrate. All the commercial substrates and potting mix were inclined to dry out much faster than the peat pellets and required additional water application at three weeks, while the Jiffy pellets never fully dried over the course of six weeks. However, it appeared that opening the boxes to apply water increased the overall chance of fungal contamination in this experiment relative to other runs, although specific fungal contamination rates were not collected. Differences between rooting substrates were significant ($F=3.46$, $p=0.009$) with Jiffy pellets producing the most roots overall with 3.1 roots per plantlet (Fig. 3). Acclimatization success was also significantly affected, with the Superfine germinating mix underperforming all the commercial pellets (Fig. 4). Size and health of surviving plants (height, longest leaf, caliper) was not significantly different 6 weeks after moving to the growth chambers (data not shown).

The choice of rooting substrate modifies the water, nutrients, and air available to the developing adventitious roots. Peat pellets worked best for our purposes with their high water retention for chestnut's slow pace of root regeneration, low pH, low cost, and ease of use. Other species will display similar pickiness about substrate choices, either due to their ability to retain water, or availability of nutrients, or pH. Softwood elm (*Ulmus* spp.) cuttings rooted very well in Grodan root cubes, not minding a wetter substrate, whereas this has never worked well for chestnuts (Oakes et al. 2012).

For commercial production, pelleted or individual substrates may be disadvantageous, and large bins or planters would be ideal. Loose substrates were used to root *Cordia alliodora* (Ruiz & Pav.) Oken, a flowering tree in the borage family, and mineral substrates of gravel and sand were more effective than sawdust (Mesén, Newton, and Leakey 1997). However, sawdust was the most effective substrate for rooting *Prunus africana* (Hook.f.) Kalkman (African cherry) (Tchoundjeu et al. 2002). Researchers tested fine sand, coarse sand, sawdust, and a 1:1 mix of coarse sand: sawdust on rooting of leafy stem cuttings of *Milicia excels* (Welw.) C.C. Berg (African teak), and found that the sawdust was most efficient, and that rooting percentage correlated directly to higher water content in the substrate (Ofori et al. 1996).

Rooting gel. Between commercial gel dip treatments, plantlet survival was not significant (data not shown). However, number of roots was significantly improved in the 0.3% and 0.31% IBA treatments compared to the 0%, 0.2% and 0.55% IBA treatments ($F=4.84$, $p=0.001$) (Fig. 5). Higher IBA concentration does not preclude increased root regeneration, so finding an optimal concentration for the target species is imperative.

In *Cordia alliodora* (Ruiz & Pavon) Oken, leafy stem cuttings rooted best at 1.6% IBA, and higher concentrations inhibited root development (Mesén, Newton, and Leakey 1997) In micropropagated *Malus zumi* (Matsumura) Rehd, a Japanese apple rootstock, a moderate application of IBA (120mg/L) had a significantly higher rooting rate (86%), compared to either 60mg/L (69%) or 240mg/L (55%) (Xu et al. 2008). For leafy stem cuttings of *Milicia excelsa*, higher IBA concentration (>0.2%) increased root formation but also increased cutting mortality (Ofori et al. 1996). In Tali (*Palaquium polyanthum* Engl.), a native Bangeldashi tree, different IBA concentrations had significantly different outcomes on cuttings: the most roots occurred at 0.8% IBA, the longest roots occurred at 1.2% IBA, and the highest plantlet survival occurred at 0.4% IBA (Jannat et al. 2017).

Many research groups are actively determining both substrate choice and IBA concentration in combined

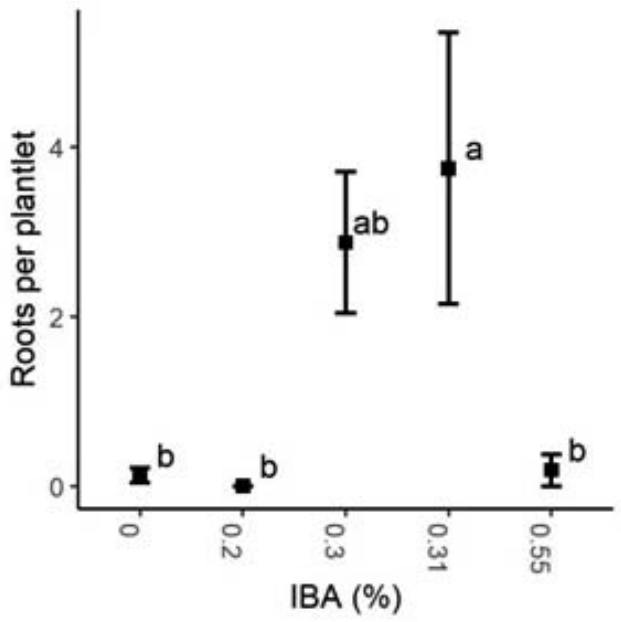

Fig. 5. Number of roots (>1cm) observed per plantlet after rooting dip treatment and 3 weeks in Jiffy pellets. Treatments were a water control (0%), Botanicare Rhizo Gel (0.2% IBA); EZ Clone Rooting Compound (0.3% IBA, plus thiamine); Clonex Rooting gel (0.31% IBA); and RootTech Cloning gel (0.55% IBA). Error bars indicate standard error of the mean. Different letters indicate significant difference between treatment means via Tukey's range test.

experiments. For stem cuttings of *Warburgia ugandensis* Sprague, an evergreen tree also known as Ugandan greenheart, both IBA concentration (0, 0.3, 0.6, 0.8%) and substrate choice (pine bark, soil, sand) were compared in a 4 by 3 factorial. Milled pine bark and 0.8% IBA gave the highest incidence of root and shoot development (Akwatulira et al. 2011). A similar study on the woody perennial honeybush (*Cyclopia subternata* Vogel) developed specific recommendations for commercial substrates and rooting gels, and determined the best time of year to take and establish rooted cuttings even in a mild climate (Mabizela, Slabbert, and Bester 2017). In addition to cutting survival times being influenced by the season, auxin requirements may change seasonally. In plum cultivars (*Prunus* sp.), a 50% higher auxin concentration was required to reach the rooting level obtained in summer when attempting to root in autumn or dormant seasons (Sharma and Aier 1989). The auxin concentration may also differ from *in vitro* methods or a thicker or stickier consistency such as a gel or powder could be used to ensure contact with the plant tissue rather than diffusing through the rooting substrate.

Substrate soak. There was no significant difference in number of roots produced by the shoots in different soaks relative to pH 5.5 tap water (data not shown). There was a significant drop in survival (F=5.91, p=0.017) for the plantlets exposed to higher doses of fertilizer, specifically the highest dose of RootJuice (Fig. 6). Micropropagated

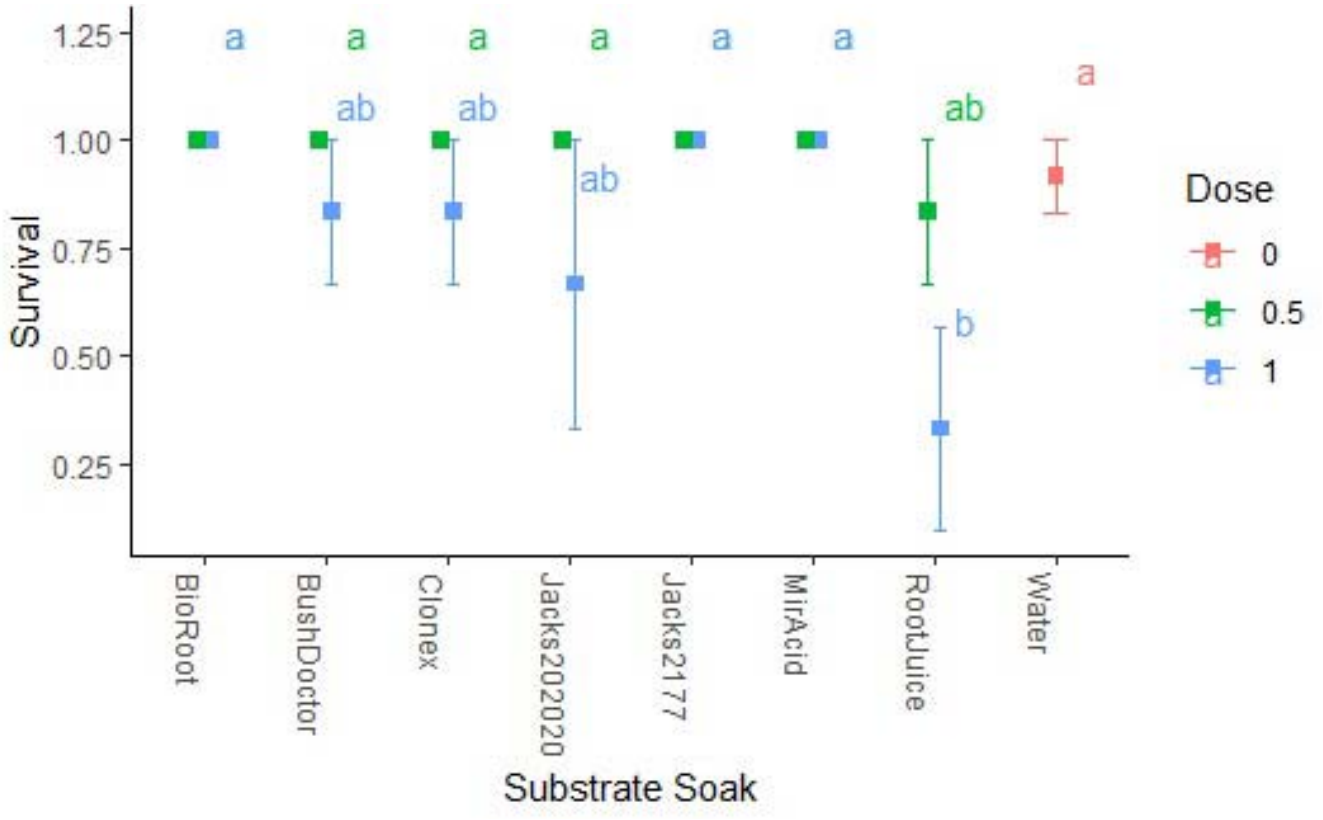

Fig. 6. Acclimatization survival by soak type and dose after 6 weeks in the growth chamber. Error bars indicate standard error of the mean. Different letters indicate significant difference between treatment means via Tukey's range test.

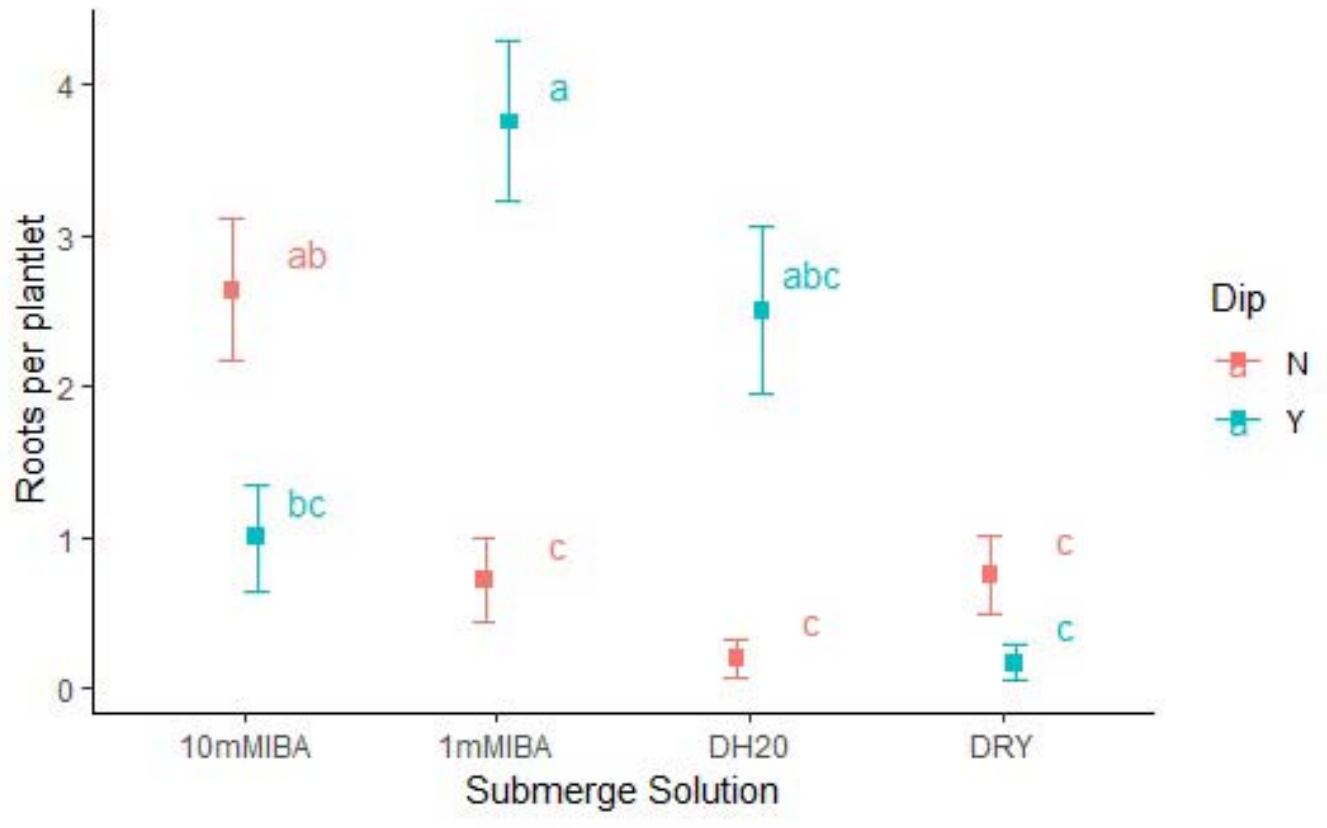

Fig. 7. Roots per plantlet by solution the stem was submerged in when the callus ball was removed, and whether the cut end was subsequently dipped in Clonex rooting gel (IBA 0.3%) – Yes (Y) and No (N). Error bars indicate standard error of the mean. Different letters indicate significant difference between treatment means via Tukey's range test.

American chestnut appears to require little to no additional NPK fertilizer to root well.

Submergence. While rooting survival was not significantly affected by submergence or rooting hormone (data not shown), the number of roots was highly variable between treatments (Fig. 7). Submerging the cut site in either water or IBA significantly increased the number of roots from dry cuts from 0.5 roots per plantlet to 1.73 roots per plantlet ($F=6.062$, $p=0.017$). When the stems were cut while submerged in the 10mM IBA solution, and also subsequently dipped in the IBA gel, there was a reduction in root formation, perhaps as a result of too much auxin (Fig. 7). However, when the stems were cut submerged in either 1mM IBA or in water, applying the extra dip in IBA gel improved root formation.

During micropropagation, plants are usually cut into multiple explants while exposed to air, and briskly returned to a very high-humidity state. One reason that cutting the plants while submerged in water might improve root formation is a lack of air embolisms in the stem tissue due to air exposure when the callus is removed. It is possible for plants to repair xylem damage cause by embolisms (Tyree and Sperry 1989), but preventing air embolisms by submerging the cut site likely prevents or reduces the frequency of occurrence.

Future research projects include determining whether an ebb and flow bioreactor system would be appropriate for rooting, as progress has been made in rooting woody plants in such systems, like *Acacia* sp. (Rathore et al. 2014). Also, optimizing the procedure for even more recalcitrant genotypes is underway, as even with a species the genotypic variation may be significant, as seen in many plants including hazelnuts (*Corylus* sp.) (Cristofori, Rouphael, and Rugini 2010), apple (Jones, Pontikis, and Hopgood 1979), peach (*Prunus* sp.) (Hammerschlag, Bauchan, and Scorza 1987), and walnut (*Juglans* sp.) (Vahdati et al. 2004).

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