Comparing the Effect of Four Propagation Techniques on Hybrid Chestnut Seedling Quality

A Final Report to The American Chestnut Foundation

Principle Investigators:

Taylor Evans and Dr. Heather Griscom
Department of Biology
James Madison University
Harrisonburg, VA

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INTRODUCTION

Hybrid Chestnut Restoration Efforts

Actively restoring forests by planting seedlings is one of the primary methods for reintroducing species into ecosystems unlikely to be restored by natural regeneration. Seedling quality, planting density, and planting costs are all considered when determining the least-cost approach to achieving a desired stocking density in a forest restoration project with seedling quality often compromised, potentially resulting in reduced survival and higher effective cost overall (Dey et al. 2008; Van Sambeek et al. 2016). Planting a greater number of seedlings of lower quality increases the effective cost of restoration projects by increasing the labor and material costs with lower associated seedling survival. A more effective approach may be to plant fewer seedlings of higher quality with a greater chance of survival and allocate resources to vegetation control or browse protection (Zaczsek, et al. 1995; Ward et al. 2000). Therefore, providing tree nurseries with simple and efficacious best practices of increasing seedling quality should assist in improving restoration outcomes overall.

Hybrid chestnut seedlings have recently been tested for their susceptibility to blight, their growth habits, and their viability for reforestation efforts throughout the historical range of the American chestnut (Clark et al. 2011; Clark et al. 2012; Pinchot et al. 2015; Skousen et al. 2018). Hybrid chestnuts have been tested in a variety of settings from orchards to former mine sites, testing the limits of hybrid chestnuts to survive and reproduce in harsh site conditions (Skousen et al. 2009; Skousen et al. 2013; Skousen et al. 2018). In addition to blight, animal browse, insect damage, ink disease caused by *Phytophthora cinnamomi*, and competition for light and water provide additional challenges to chestnut restoration (Jacobs 2007; Clark et al. 2014a) . A majority of current restoration efforts are conducted via direct seeding, though failure rates up to 50% can result due to predation and seed desiccation (Selig et al. 2005). Seed predators, especially rodents, consume chestnuts with the same frequency as more common food sources such as red oak (*Quercus rubra*) leading to a disproportionately negative effect on potential chestnut seedling recruitment (Blythe et al. 2015). Seedlings have shown increased performance, in addition to added protection from predation, when compared to direct seeded trees in field trials, (McCarthy et al. 2010; Fields-Johnson et al. 2012). While there has been research testing chestnut hybrids in the field, there remains a gap in research on optimizing nursery protocols to produce high quality chestnut seedlings for restoration projects. Pinchot et al. (2017) identified that seedling quality is important for both early growth and long-term competitive ability of chestnut seedlings. Additionally, researchers from TACF have noted that although current propagation methods are well established, they have yet to be optimized for seedling production and, in a plan for reintroducing chestnut to the United States National Forest System, researchers identified that improving seedling quality would be the most effective method in overcoming the biotic and abiotic challenges to chestnut restoration (Clark et al. 2014a; Collins et al. 2017). The restoration of the American Chestnut (*Castanea dentata*) is a century long project that has finally shown promise with the introduction of hybrid chestnut seedlings that can tolerate the fungal blight that removed American chestnuts from the forest canopy. Given the resources involved in producing blight resistant chestnuts, it is important to understand how restoration ecologists can increase the likelihood of chestnut seedling success. Improving seedling quality is a low cost and efficacious means to achieve this goal and ensure resources are maximized.

Seedling Quality

Seedling quality includes a combination of phenotypic traits associated with seedling morphology and physiology that influence survival and positive field performance of tree seedlings (Grossnickle and Macdonald 2018a). During the establishment phase, seedling morphology and physiology contribute significantly to survival and future field performance (Burdett 1983; Struve et al. 2000; Jacobs et al. 2005, Davis and Jacobs 2005). Morphological characteristics such as height, root collar diameter, number of first order lateral roots, shoot:root ratio, root architecture, and their combined effect on seedling quality have all been used to match nursery stock to site conditions and to predict the eventual performance of seedlings once planted in the field (Rose et al. 1990; Dey et al. 2010 Grossnickle 2018a; Grossnickle 2018b). Using the "target seedling concept" and a framework, nursery growers can manipulate growing conditions to produce seedlings with a morphology best suited to the conditions in which it will be planted (Rose et al. 1990). Physiological characteristics such as root growth potential, freeze tolerance, and root electrolyte leakage are also indicators for future field performance but are less commonly used as indicators of seedling quality due to the specialized equipment and additional time required to perform measurements (Davis and Jacobs 2005; Grossnickle 2018a; Grossnickle 2018b).

Morphological traits such as height, root collar diameter (RCD), number of first order lateral roots (FOLR), and root volume vary in their effect on seedling quality and performance based on local environmental conditions. Each of these traits can be manipulated in response to nursery cultural practices to increase the likelihood of survival on a given planting site.

Seedling height remains one of the fastest methods of visually assessing seedling quality; tall seedlings tend to stay taller once planted than shorter seedlings of the same age (Thompson 1985; Oswalt et al. 2006; Clark et al. 2012; Clark et al. 2014a). Decreased density facilitated by larger seedling spacing or greater container size can increase seedling height, and chestnuts readily respond to increased light levels via rapid stem growth (Wang et al. 2006, Grossnickle and El-Kassaby 2016). However, other studies of *C. dentata* suggest that seedlings may be taller when grown in sites with competition for light (Anagnostakis 2007). Long term studies of oak seedlings have shown that larger seedling size is a significant predictor of survival and dominance after more than a decade (Pinchot et al. 2018), however, site conditions may ultimately determine benefits conferred by seedling height. Sites with high light competition favor taller seedlings (Grossnickle and El-Kassaby 2016, Grossnickle and MacDonald 2018b) as opposed to sites with greater water stress where shorter seedlings may establish more readily due to favorable shoot:root ratios (Grossnickle 2012, Clark et al. 2016; Grossnickle and El-Kassaby 2016). Taller seedlings with a greater shoot:root ratio can experience stem die-back after planting, especially in xeric sites (Clark et al. 2016). If site conditions favor taller seedlings, growers should utilize nursery culture that would encourage a larger root system to balance shoot:root ratios in seedlings to prevent stem die-back. Despite challenges with stem die-back, taller seedlings may avoid terminal bud browse more frequently than shorter seedlings, depending on their growth rate. Whitetail deer (*Odocoileus virginianus*) are present throughout the historical range of the American chestnut and have been shown to browse chestnut heavily in the wild and in field trials (Clark et al. 2012; Clark et al. 2014b). Therefore, planting tall seedlings that are rapidly able to escape browse height should reduce mortality in areas with heavy deer pressure (Oswalt et al. 2006).

Root collar diameter (RCD) continues to be the morphological characteristic most associated with field performance for multiple species including hybrid chestnut (Dey and

Parker 1997; Jacobs et al. 2005; Davis and Jacobs 2005; Clark et al. 2009). RCD can be increased in nursery settings by increasing the spacing between seedlings and/or root pruning seedlings to encourage lateral branching of the root system (Pinchot et al. 2015). RCD is positively correlated with early seedling survival, establishment, and morphological characteristics such as height and number of first order lateral roots (Ward et al. 2000; Clark et al. 2009; Pinto et al. 2011; Van Sambeek et al. 2016; Pinchot et al. 2018). Jacobs et al. (2005) concluded that trees with greater diameter could withstand herbivore browse and other physical harm better than smaller seedlings. The predictive power of RCD likely lies in the relationships between RCD, root system architecture, and seedling height, i.e., RCD alone may not confer morphological benefit but is predictive of both root system size and seedling height (Dey and Parker 1997; Davis and Jacobs 2005; Wilson and Jacobs 2006). Thus, RCD offers a quick measurement of overall seedling quality readily accessible for nursery practitioners that can be used to grade trees without removing them from the soil or container.

Root systems can be quantified by overall root system size and the arrangement of roots around the root collar. Root volume is a quick, non-destructive method to assess overall root system size that can be used as an alternative to destructive fresh/dry mass sampling (Burdett 1979). FOLR count is determined by counting any root over 1 mm that emerges from the main tap root of a seedling (Davis and Jacobs 2005). These two measurements together provide a picture of the overall root system by combining total size with root architecture. FOLR count is frequently used in seedling quality assessments and generally a higher FOLR count correlates with higher survival and greater initial field performance, i.e., height and diameter gains, after out planting (Schultz and Thompson 1990; Ward et al. 2000; Jacobs et al. 2005; Dey et al. 2010; Davis and Jacobs; Pinchot et al. 2015). Studies have found root volume to be as predictive as FOLR count for survival and field performance or more effective due to its ability to account for larger FOLR and second and third order roots (Jacobs et al. 2005; Davis and Jacobs 2005; Pinto et al. 2011). Jacobs et al. (2005) determined that hardwood seedlings with larger root volumes and a higher FOLR count outperformed seedlings with smaller values, potentially due to their ability to exploit carbohydrate and nutrient reserves after planting and before soil contact is established. Root volume and FOLR can be manipulated in the nursery by root pruning, adjusting planting density, and by using a porous or high organic matter planting medium (Schultz and Thompson 1990; Struve et al. 2000; Dey et al. 2004, Davis et al. 2006). Containers designed to air prune roots have openings in the walls and/or bottom of the growing container that cause root tips to desiccate upon contact with the outside air. This process results in the development of finer roots towards the inner part of the root ball via the loss of apical dominance in the root system (Arnold and Struve 1993; Amoroso et al., 2010).

Using these relatively quick measurements, nursery growers are able to grade seedlings, i.e., sort into higher or lower quality groups, for overall quality and potential field performance. Grading seedlings also allows restoration practitioners to match seedling quality to site conditions by planting the highest quality seedings on the most stressful sites or on sites where hard mast production is of particular interest while less robust seedlings could be reserved for areas with fewer limitations allowing restoration practitioners to balance cost and quality during project planning (Rose et al. 1990; Dey et al. 2008). Based on survival and performance studies conducted on northern red oak (*Quercus rubra*), there is evidence to suggest that stringent grading of nursery seedlings would result in a similar forest stocking rate after 5-10 years even if the total number of seedlings distributed were to decrease (Ward et al. 2000). In this study we will compare two standard nursery production methods, bed grown, and container grown seedlings, with two novel nursery production methods, Root Production Method (RPM®) and air-pruning raised beds, in order to compare the effect of each methodology on chestnut seedling morphology and therefore overall seedling quality as a way to further optimize nursery cultural practices for hybrid chestnut.

Propagation Methods

Bed Grown

Bed grown seedlings are propagated by direct seeding into a prepared seedbed where seedlings are grown for one to two years. Seedlings are extracted from the soil via hand tools or heavy machinery when the seedling enters dormancy. Seedlings are then placed in cold-storage as bare-root seedlings until planting. Bed grown trees are a more cost-effective means of procuring large quantities of plant material for large planting projects compared to container grown seedlings (Wilson et al. 2007).

Mortality in bed grown seedlings is often highest immediately after planting (Grossnickle and El-Kassaby 2016). Compared to container grown seedlings, bed grown seedlings experience greater transplant shock and reduced survival from a reduction in the root system size at the time of extraction and a resulting increase in shoot:root ratio (Watson and Syndor 1987; Wilson et al. 2007; Struve 2009). Transplant success is also greatly decreased if bed grown seedlings are planted outside of the dormant period when the tree is no longer actively growing (Richardson-Calfee and Harris 2005; Struve 2009). Differences in field performance are most pronounced among propagation methods when seedlings are exposed to stressful site conditions, with drier soil resulting in greater mortality in bed grown seedlings compared to container grown seedlings (Landhausser et al. 2012). This may be of particular concern for hybrid chestnuts, given their superior performance on upper-slope sites with xeric conditions (Griscom and Griscom 2012). Root damage during extraction and handling of bed grown seedlings may also predispose seedlings to disease during storage (Grossnickle and El-Kassaby 2016). Mortality from disease may be of particular concern for bed grown chestnuts produced in the southern United States due to increased risk ink disease caused by *Phytophthora cinnamomi* contamination in the soil. This risk is compounded if seedlings are planted on sites with poor drainage (Rhoades et al. 2003; Clark et al. 2014a; Clark et al. 2014b; Clark et al. 2016). These negative effects can be mitigated through careful extraction to minimize root-loss (Davis and Jacobs 2005; Grossnickle and El-Kassaby 2016). When planted on sites with adequate soil moisture, open canopy conditions, and a lack of vegetative competition, bed grown seedlings can perform as well as container grown seedlings at a reduced cost (Grossnickle and El-Kassaby 2016).

Container Grown

Container grown trees are propagated in plastic pots of various sizes filled with a soilless medium - often a mixture of peat, perlite, and vermiculite. Recommended container sizes vary based on tree species and seedling age. Container-grown systems, while more expensive due to additional materials and infrastructure inputs, can offer advantages over bed grown systems. Container-grown seedlings have been shown to have a lower shoot:root ratio than bed grown seedlings as more of the root system can be retained during extraction (Grossnickle and El-Kassaby 2016). A lower shoot:root ratio has been shown to increase survival regardless of propagation method due to a reduction in transplant shock and stem dieback when planted (Thompson 1985; Grossnickle 2012; Clark et al. 2016). Container grown seedlings experience less transplant shock compared to bed grown seedlings via the transfer of the entire root system into the planting hole (Davis and Jacobs 2005). Van Sambeek et al. (2016) found that reduced transplant shock contributes to more rapid growth and lower mortality compared to bed grown seedlings. Despite increased expense, Clark et al. (2014b) has recommended the use of containerized seedlings in the southern United States where contamination from *P. cinnamomi* in the nursery can increase mortality for bed grown seedlings. Additionally, large container seedlings greater than 1.5 m tall may be above deer browse height at planting or may rapidly escape browse height (Clark et al. 2012). Despite these benefits, container grown stock types may be subject to root deformities such as circling, matted, or J-shaped roots that require pruning before planting, decreasing the advantages conferred to young trees by transferring whole root systems (Arnold and Struve 1993).

The Root Production Method®

The Root Production Method® (RPM®) is a multistep propagation procedure developed to produce containerized seedlings with high root volume, large numbers of FOLR, and large caliper diameter (Lovelace 2002). In the RPM® procedure seeds are graded based on weight with only the heaviest seeds selected for propagation. Studies on American, hybrid, and Chinese chestnuts have shown that heavier seeds produce seedlings with greater height growth, root collar diameter, and number of FOLR (Clark et al. 2012; Pinchot et al. 2015). After grading, seeds are stratified at 1℃ in a bottomless container filled with a high air-pore volume medium (4:4:2 rice hulls, pine bark, and sand by volume) amended with a slow-release fertilizer, hydrogel, and mycorrhizal spores for several months. The medium used in RPM® is composed of 35-40% air-pores by volume (Lovelace 2002). A highly aerated medium mitigates the potential for slower growth rates and increased susceptibility to environmental stressors caused by media with lower aeration, though a porous medium could increase irrigation requirements compared to a medium with higher bulk density (Mathers et al. 2007). After stratification seeds are moved into heated greenhouses to germinate. After one month, seedlings are graded by height and root collar diameter with only the largest 50% moved to the next stage of production. Root collar diameter and height have been correlated with increased survival and growth after field planting which should indicate that grading seedlings based on these characteristics increases survival probability (Jacobs et al. 2005; Clark et al. 2009; Clark et al. 2012; Pinchot et al. 2015). After grading, seedlings are transplanted into bottomless pots for an additional 60 days to facilitate additional air pruning of the roots. Finally, seedlings are transplanted into 2.5-gallon containers that are placed outdoors for the remainder of their 210 day growing cycle. Using multiple bottomless containers should initiate pruning of root tips, causing greater root initiation closer to the root collar and a more horizontally dominated root structure overall (Arnold and Struve 1993, Gilman and Paz 2014).

When comparing RPM® seedlings and bed grown seedlings, the Root Production Method® has been shown to produce oak seedlings with greater average height and basal diameter, more fibrous root systems, earlier age at first nut production, and higher survival in bottom-land site conditions (Grossman et al. 2003; Dey et al. 2004; Walter et al. 2013; Van Sambeek 2016). Grossman et. al (2003) found that RPM® seedling survival remained above 95% after two years while bed grown seedling survival dropped from 95% to 77.4% in the same location. RPM® seedlings are labor and infrastructure intensive to produce, requiring several transplants, a high cull rate, and the additional cost of transporting containerized seedlings to planting locations. The increased cost per seedling may be justified for improved seedling quality as effective cost, i.e., the cost per surviving seedling, has been shown to decrease as seedling size increases when trees are planted in the field (Spetich et. al 2002). Studies comparing the field performance and survival of RPM® and bed grown seedlings have primarily focused on *Quercus* species (Dey et al. 2004; Walter et al. 2013; Van Sambeek et al. 2016). This suggests that other members of the *Fagaceae* family, such as American chestnut, should respond similarly to the RPM® propagation method.

Air Prune Beds

An ideal propagation method would produce a large and fibrous root system with reduced labor, infrastructure, and transportation costs. Bottomless raised beds, labeled in this study as air prune beds to differentiate them from standard bed grown seedlings, have the potential to produce seedling morphology similar to RPM® seedlings with the ease of extraction and transport of bed grown seedlings by using a highly porous medium and a large, bottomless container. By increasing container size from a single pot to a raised bed, air prune beds should also mitigate root deformity issues associated with container grown seedlings produced in smaller pots. These beds can be built for any scale and can be fitted with covers to protect seeds from predation and seedlings from herbivory. This technique may be especially relevant for growers in the southern United State who would like to avoid the infrastructure associated with container grown seedlings while also avoiding mortality from ink disease where bed grown stock is subject to *P. cinnamomi* infection due to soil contamination (Clark et al. 2014a; Clark et al. 2016). While air pruning containers are common in tree propagation, there is a gap in the research on bottomless raised beds in nursery culture and their effects on both cost and seedling quality compared to other methods.

METHODOLOGY

Site Location

Container grown seedlings and seedlings in the first two of three stages in the RPM® method were placed in the greenhouse of the James Madison University BioScience building in Harrisonburg, Virginia (38.434579 N, 78.870694 W). Bed grown, air prune, and the final stage of RPM® seedlings were placed directly outside of the greenhouse with a similar aspect and orientation to the greenhouse.

Preparation

Seed Sorting

In the fall of 2019 seeds were removed from their shipping bags, placed in a large tray, and mixed by hand. Seeds were selected randomly for each of four lots corresponding to one of four propagation methods and placed into separate bags. Each time 50 seeds were removed from the tray all remaining seeds were again mixed. Seeds were cleaned, weighed, and placed into four trays for stratification.

Stratification

All seeds were stored at 1°C from December 2019 to March 2020. All seeds trays were labeled with a letter/number grid (9 rows x 17 columns) seeds were placed in a single layer with one seed per grid cell. Seeds for bed grown, container grown, and air prune propagation methods were stored in moist peat moss in molded plastic trays (10.94"x 21.44"x 2.44") covered in plastic wrap. Seeds for the RPM® seedlings were stored in a mesh flat (10.94"x 21.44"x 2.44") with 1 cm mesh covered in plastic wrap set over a molded plastic tray. The RPM® seeds were stored in a mix composed of rice hulls, pine bark, and sand (4:4:2 by volume) with slow-release fertilizer (Scotts Osmocote® Outdoor-Indoor 15-9-12) at 11.325g/gal, a wetting agent (Miracle-Gro® Water Storing Crystals, Soil Moist™ Synthetic Polymer Moisture Control) at 2.58 g/gal, and mycorrhizal spores (MycoApply® Ultrafine Endo/Ecto) at 1.12g/gal. Seeds were checked weekly for moisture and signs of mold.

Media Preparation

Seeds were placed into one of four propagation methods, each using one of three media (Figure 1). PRO-MIX Bx general purpose medium was used for peat-perlite-vermiculite (PPV) mix and soil collected from the planting area was used for the field soil mix. RPM® mix used in treatments was the same mix used to stratify seeds used in the RPM® method. All treatments were fertilized at a rate of 11.325 grams per gallon of media.

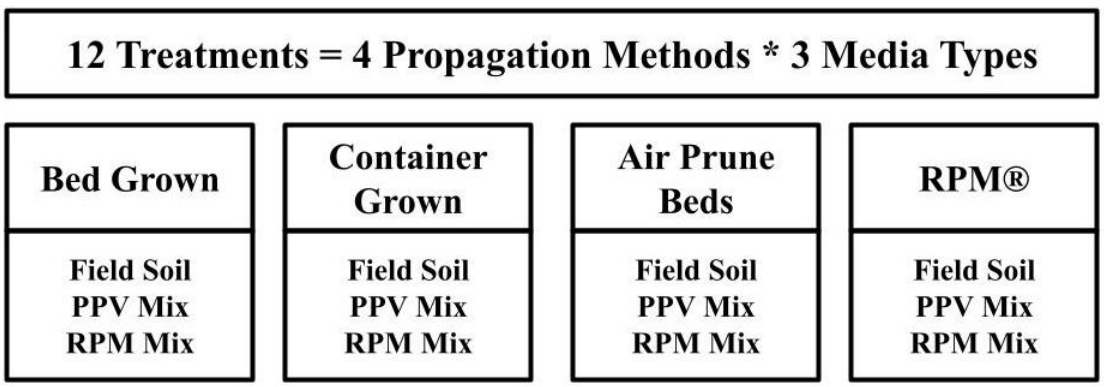

Figure 1: Overview of experimental design detailing propagation methods and media types.

Propagation Methods

Bed Grown

Three 3’x4’ beds were used to produce bed grown trees. All three beds were excavated to 10” depth, with the remaining soil loosened an additional 8” using a spading fork. The perimeter of each bed was lined with a rhizome barrier made of thick plastic to prevent tunneling rodent predation. Beds were then backfilled with one of the three pre-fertilized media and seeds were planted on March 1st, 2020 on 5” centers, 1” deep in field soil (n= 48), PPV mix (n= 45), and RPM® mix (n= 44). Each seed was given seed code tag placed 2.5” east of its planting location. Beds were irrigated daily with overhead misters.

Container Grown

One hundred and forty-five 4”x14” Treepots were placed into Treepot trays (15.75” x 15.75” x 7.5"). These trays were split into three treatments and filled with field soil (n= 48), PPV mix (n= 48), and RPM® mix (n= 48) fertilized at the recommended indoor application rate. Seeds were planted on March 1st, 2020 1” deep and the container labeled with the seed code. Trays were placed in a greenhouse under mist irrigation. Temperature in the greenhouse approximated outside temperatures year-round.

Air Prune Beds

Three 3’x4’x8” beds were used to grow air prune trees. Each bed was constructed out of untreated dimensional lumber with a ¼” mesh hardware cloth stapled to the bottom (Figure 2). Beds were placed on risers 6” above the ground. These beds were filled with one of the three pre-fertilized media and seeds were planted on March 1st, 2020 on 5” centers, 1” deep in field soil (n= 53), PPV mix (n= 48), and RPM® mix (n= 53). A single layer of heavily saturated newspaper was placed over the hardware cloth to prevent media from falling through the mesh as the beds were filled. Each seed was given seed code tag placed 2.5” to the east of its planting location. Beds were irrigated daily with overhead misters.

Figure 2: A 3’x4’ air prune bed using ¼” hardware cloth as the base to encourage root pruning.

Root Production Method®

Following stratification, seeds for the RPM® method were transferred into the greenhouse on March 1st, 2020. Seeds were left to germinate in the mesh tray to initiate root pruning of the young seedlings. Per the RPM® procedure, seedlings are typically graded by height and diameter at 30 days and transplanted into bottomless Band Pots after grading (Lovelace 2002). However, seedlings had not emerged in any treatment as of 30 days and as a result, were grown for an additional 30 days, 60 days total. At 60 days, all seedlings regardless of propagation method were measured for height and groundline diameter. After measurement, RPM® seedlings were transplanted from their stratification tray to into bottomless 2 7/8" x 5.5" square Anderson Band Pots and were placed on a wire mesh table. Band pots were filled with field soil (n=28), PPV mix (n=62), and RPM® mix (n=61) fertilized at the recommended application rate (11.325 g/gal). The number of containers with field soil was reduced due to a lack of medium available from excavating the bed grown propagation method. After 60 days RPM®, seedlings were moved outdoors for two days to harden off. RPM® seedlings were transplanted into 2.5-gallon pots (10 ⅛” x 9 ⅛”) filled with field soil, PPV mix, and RPM® mix. Any Band Pots that did not have a seedling that had emerged after 60 days, or had a dead seedling, were not transplanted into a 2.5-gallon pot (n~51). Seedlings remained outside for the remainder of the study. Pots were irrigated daily with overhead misters.

Data Collection

Seedling Grading

To analyze the effect of grading seedlings by height and diameter used in the RPM® method, seedling height and ground line diameter were measured at 60 days for all seedlings to use these data as covariates to determine the predictive power of these early measurements on seedling morphology at the end of one growing season. Seedling height (cm) was measured from the soil level to the most terminal visible leaf node. In the event of a forked stem, the average height of the two stems was taken. Seedling diameter, e.g., ground line diameter, was measured at the soil/medium surface using digital calipers (Traceable® Digital Calipers 8in). The RPM® mix medium in the stratification tray had lifted because of seedling germination and a consistent medium level did not exist for all seedlings. Therefore, seedling diameter was taken using the edge of the stratification tray as a consistent reference, as the tray had been filled to the top when seeds were originally stratified.

The original seed labels were lost for the bed grown and air prune treatments. A new grid system was established based on the locations of the planting tags originally placed next to the seedlings. Photographs of the original treatment labels were used to pair the original treatment codes with the new seedling codes to recover seed weight data for seedlings that could be clearly identified. For seedlings that could not be paired with the original labels, height and ground line diameter measurements were still collected.

Measures of Seedling Quality

In November 2020, seedlings were removed from their treatment for measurement. Bed grown and air prune seedlings were removed by inserting a spading fork parallel to the edge of the bed, lifting, and gently removing the seedlings to preserve as much of the root system as possible. All other seedlings were removed by overturning the container and gently removing the seedlings. Seedlings had all soil and/or growing medium removed from their root systems via immersion and spray washing. Remaining medium was removed by hand to preserve as much of the fine root mass as possible, prioritizing removing medium over preserving all extant fine root mass. Seedling root systems were kept submerged in water to avoid desiccation during measurement.

All seedlings were measured for height (cm), root collar diameter (mm), root volume (mL), and number of first order lateral roots (FOLR) greater than one millimeter. Height was measured from the root collar to the top of the apical bud. In the event of a forked stem the average height of the two stems was taken. If apical bud dieback had occurred, the seedling was treated as if forked. Root collar diameter was measured via a digital caliper placed at the root collar, i.e., the distinct line of color change found on the seedling at the soil line. Root volume was measured via immersion up to the root collar using one of three graduated cylinders, 100 mL, 500 mL, or 1000mL after removing medium from the root system (Burdette 1979). Volume was measured in the smallest graduated cylinder that would fit the root system without damage. FOLR count was measured by counting all roots greater than 1mm emerging from the main taproot. Counts were collected by the same individual to ensure consistency. If the root system was forked at the root collar the FOLR count was listed as one. After measurement, seedlings were bundled by treatment and heeled in for planting in the field in the spring of 2021.

Chlorophyll Content

Chlorosis was observed in several treatments in late summer 2020, leading to questions surrounding differences in nutrient management requirements in different propagation method and media types. In October 2020, each treatment was measured for chlorophyll content using a SPAD meter (Konica Minolta Chlorophyll Meter SPAD-502Plus). Ten trees from each treatment (n= 120) were randomly selected using a random number generator and the uppermost leaf from each tree was measured and SPAD values, proportional to chlorophyll content, were recorded. If a tree was selected by the random number generator that had no leaf color, i.e., brown, another tree was selected in its place.

Mortality

Seedling mortality was defined in this study as seedlings that had emerged at 60 days, were included in the seedling grading measurements, but were not included in final measurements due to mortality between day 60 and final measurements, i.e., no seedling was present, or the seedling was obviously dead after observing the root system and/or stem pliability.

Cost per Seedling

Cost per seedling was calculated by totaling the materials cost for each treatment, e.g., total cost of pots, amendments applied, potting mix used per treatment, and the labor cost, i.e. the number of hours dedicated to a particular treatment at a fixed hourly rate, and dividing this total cost by the total number of seedlings that survived to the end of the study. Labor time for measuring seed weight, height at 60 days, and diameter at 60 days was factored into total cost for the RPM® treatments, but not other treatments as this is only a requirement of the RPM® method.

Statistical Analysis

Analysis of the effects of propagation method and media on measures of seedling quality were analyzed using an ANOVA with simple effects to examine the effects of each media type within each propagation method, and to examine the effects of each propagation method within each media type (Table 1). Games-Howell post hoc tests used for multiple comparisons as they do not assume homogeneity of variance. Due to both non-normality and heteroscedasticity, data for root volume were analyzed using a Kruskal-Wallis test comparing propagation method, mixes, and propagation method*medium. The effects of seed weight, height at 60 days, and ground line diameter at 60 days on measures of seedling quality were analyzed using nested model comparisons. Analysis of chlorophyll levels (SPAD values) were analyzed using an ANOVA with simple effects to examine the effects of each mix type within each propagation method, and to examine the effects of each propagation method within each mix type using Tukey HSD post-hoc analyses. All statistical analyses were completed in SPSS 27 (IBM Corp., 2020).

RESULTS AND ANALYSIS

The Effect of Propagation Method and Medium Choice on Measures of Seedling Quality

Final height, final root collar diameter, and FOLR count were analyzed using a two-way ANOVA for main effects and interactions. Dead seedlings, or seedlings with root systems damaged during extraction were removed from analyses. Outlier values (n = 7) were removed from the FOLR analysis to correct for skew. An $\alpha = 0.01$ was used to account for heteroskedasticity in data for final height, final root collar diameter, and FOLR count (Tabachnick and Fidell, 2013). Final height, final root collar diameter, and FOLR count all demonstrated significant interaction effects between propagation method and medium (Table 1). Removing outliers in root volume data did not change normality test results and were kept in the final analysis.

Table 1: Statistical results of three, two-way analyses of variance (ANOVA) testing the effect of propagation method (air prune, bed grown, container grown, and RPM), medium (field soil, peat-perlite-vermiculite, RPM Mix), and their interactions on (A) final height, (B) final root collar diameter (RCD), and (C) count of first order lateral roots (FOLR) greater than 1 mm. Significance ($p < 0.001$) is denoted by an asterisk.

A

Effect	**F**	**P**	η_p^2
Prop. Method (PM)	32.409	<0.001*	0.361
Medium	103.607	<0.001*	0.210
PM*Medium	10.454	<0.001*	0.146
Model R^2		0.498	

B

Effect	**F**	**P**	η_p^2
Prop. Method (PM)	12.539	<0.001*	0.093
Medium	61.847	<0.001*	0.253
PM*Medium	6.203	<0.001*	0.092
Model R^2		0.366	

C

Effect	**F**	**P**	η_p^2
Prop. Method (PM)	20.773	<0.001*	0.148
Medium	90.533	<0.001*	0.335
PM*Medium	4.375	<0.001*	0.068
Model R^2		0.429	

There were significant main effects of propagation method ($F(3,366) = 32.409$, $p < 0.001$), medium ($F(2,366) = 103.607$), and a significant interaction effect of propagation method and medium on final height ($F(6, 366) = 10.454$, $p < 0.001$) (Table 1). Propagation method had a greater effect size ($\eta_p^2 = 0.361$) than either medium ($\eta_p^2 = 0.210$) or the interaction term ($\eta_p^2 = 0.146$) (Table 1). There was a significant difference in final height within any given propagation method when comparing seedlings grown in different media types (Figure 3A). Seedlings grown in peat-perlite-vermiculite (PPV) mix were significantly taller than seedlings grown in other media types in air prune (65.97 cm), container grown (61.78 cm), and RPM® propagation methods (37.93 cm). Bed grown seedlings grown in PPV mix (82.40 cm) were taller than bed grown seedlings grown in field soil (63.60 cm), though not significantly so ($p = 0.013$). When examining final height within different media types, there was not a significant difference between air prune (65.97 cm), bed grown (82.40 cm), or container grown (61.78 cm) trees using PPV mix (Figure 3B).

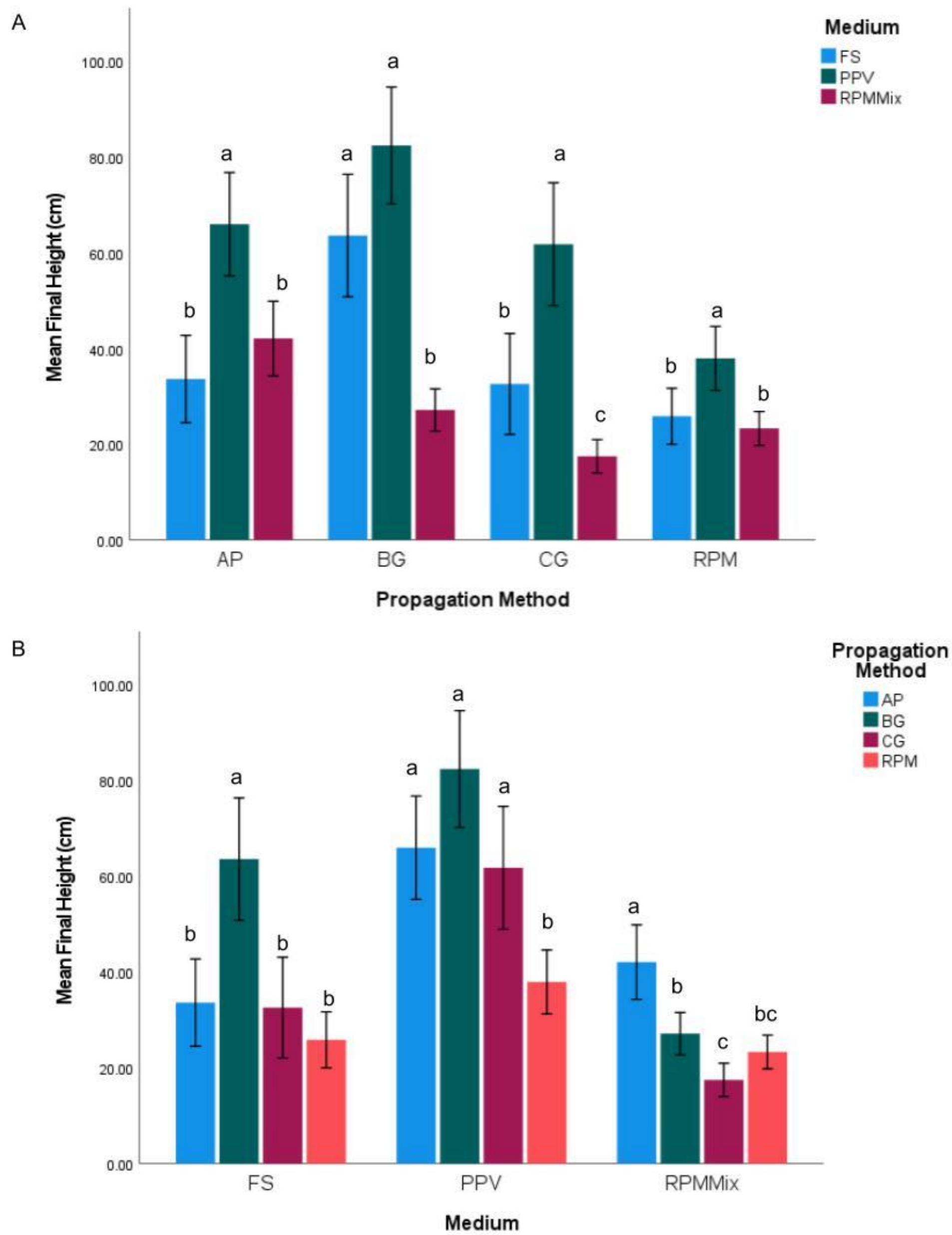

Figure 3: Bar charts showing the effect of medium type within propagation method (a) and propagation method within medium type (b) on mean final height of seedlings. Significance (P <0.01) is indicated by lowercase letters (a>b>c). Bars with the same lowercase letters are not significantly different from each other within the same propagation method (a) or medium (b). Propagation method: AP = Air prune, BG = Bed grown, CG = Container grown, RPM = RPM®. Medium: FS = Field soil, PPV = Peat-perlite-vermiculite, RPMMix = Pine bark, sand, and rice hulls.

There were significant main effects of propagation method ($F(3, 366) = 12.539$, $p < 0.001$), medium ($F(2, 366) = 61.847$, $p < 0.001$), and a significant interaction effect of propagation method and medium on final root collar diameter ($F(11, 366) = 6.203$, $p < 0.001$) (Table 1). Medium type had a greater effect size ($\eta_p^2 = 0.253$) than either propagation method ($\eta_p^2 = 0.093$) or the interaction term ($\eta_p^2 = 0.092$) (Table 1). There was a significant difference in final root collar diameter within any given propagation method when comparing seedlings grown in different media (Figure 4A). Seedlings grown in PPV mix were larger than seedlings grown in other media in container grown (9.20 mm), and RPM® propagation methods (9.19 mm). There was not a significant difference ($p = 0.271$) in final root collar diameter between bed grown seedlings grown in PPV mix (11.02 mm) and field soil (9.80 mm). There was also not a significant difference ($p = 0.031$) in final root collar diameter between air prune seedlings grown in PPV mix (8.94 mm) and RPM Mix (7.60 mm) (Figure 4A). When examining final root collar diameter within different media there was no significant difference among propagation methods using PPV mix (Figure 4B). Air prune seedlings grown in RPM mix had significantly greater average root collar diameter (7.60 mm) than any other propagation method using RPM mix (Figure 4B). Bed grown seedlings grown in field soil had significantly greater average root collar diameter (9.79 mm) than any other propagation method using field soil (Figure 4B).

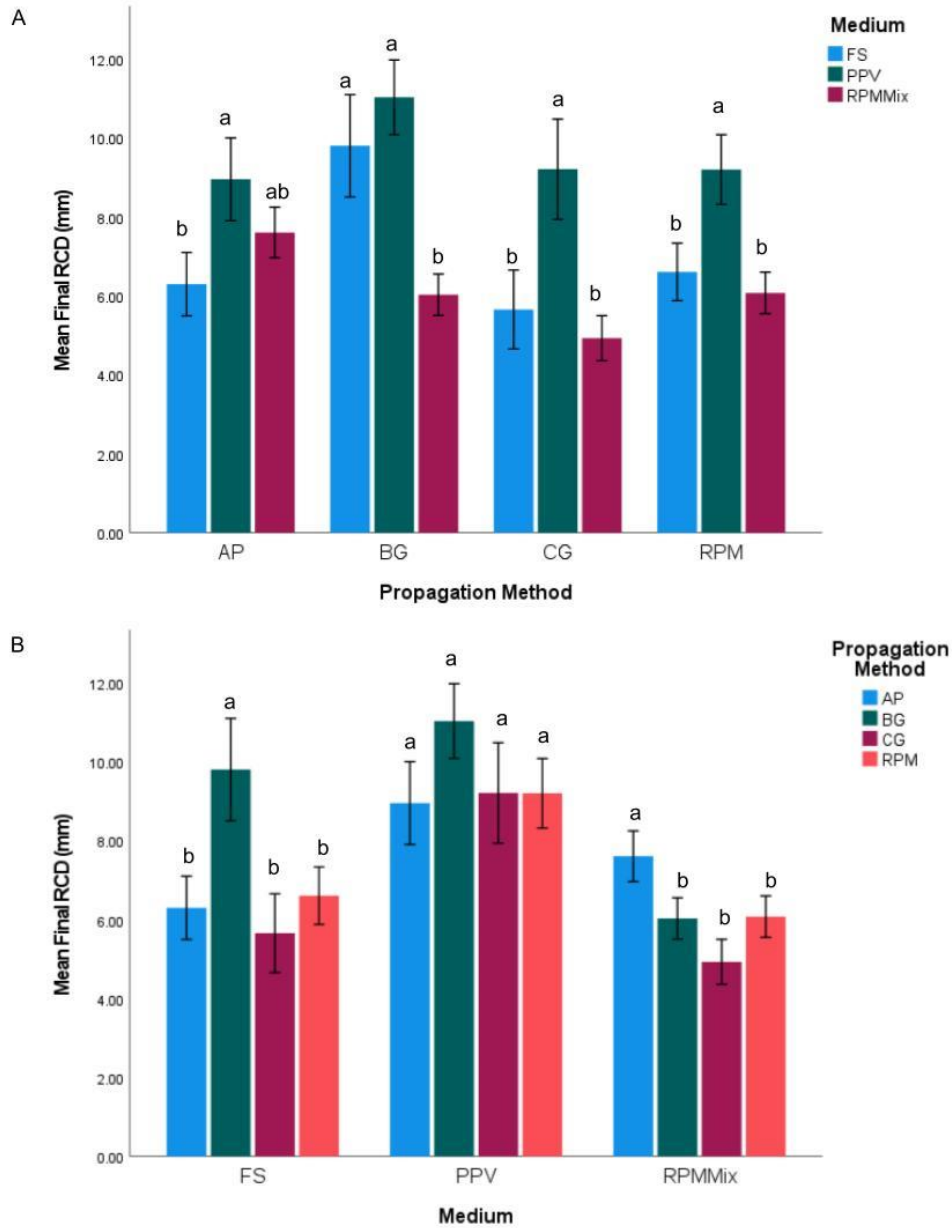

Figure 4: Bar charts showing the effect of medium type within propagation method (A) and propagation method within medium type (B) on mean final root collar diameter (RCD) of seedlings. Significance ($p<0.01$) is indicated by lowercase letters (a>b>c). Bars with the same lowercase letters are not significantly different from each other within the same propagation method (A) or medium (B). Propagation method: AP = Air prune, BG = Bed grown, CG = Container grown, RPM = RPM®. Medium: FS = Field soil, PPV = Peat-perlite-vermiculite, RPMMix = Pine bark, sand, and rice hulls.

There was no significant difference in median root volume across propagation methods (χ^2 (3) = 2.64, p = 0.451), however there was a significant difference in median root volume between media (χ^2 (2) = 2.64, p < 0.001) (Figure 5A,B). Seedlings grown in PPV mix had a significantly greater median root volume (32.5 mL, p < 0.001) than seedlings grown in field soil (14.5 mL) or RPM Mix (12.5 mL) that were not significantly different from each other. There was also a significant difference in root volume between the 12 combinations of propagation method and medium (χ^2 (11) = 96.75, p < 0.001). After adjusting for multiple comparisons there were significant differences in median root volume in different propagation method and medium combinations. Container grown seedlings in PPV mix had the largest median root volume (45 mL), followed by bed grown seedlings in field soil (35 mL) and bed grown seedlings in PPV mix (35 mL) . These three combinations were not significantly different from each other (p = 1.000). There were also not significant differences between air prune seedlings in PPV mix or RPM mix, bed grown seedlings in field soil, container grown seedlings in PPV mix, or RPM® seedlings in PPV mix.

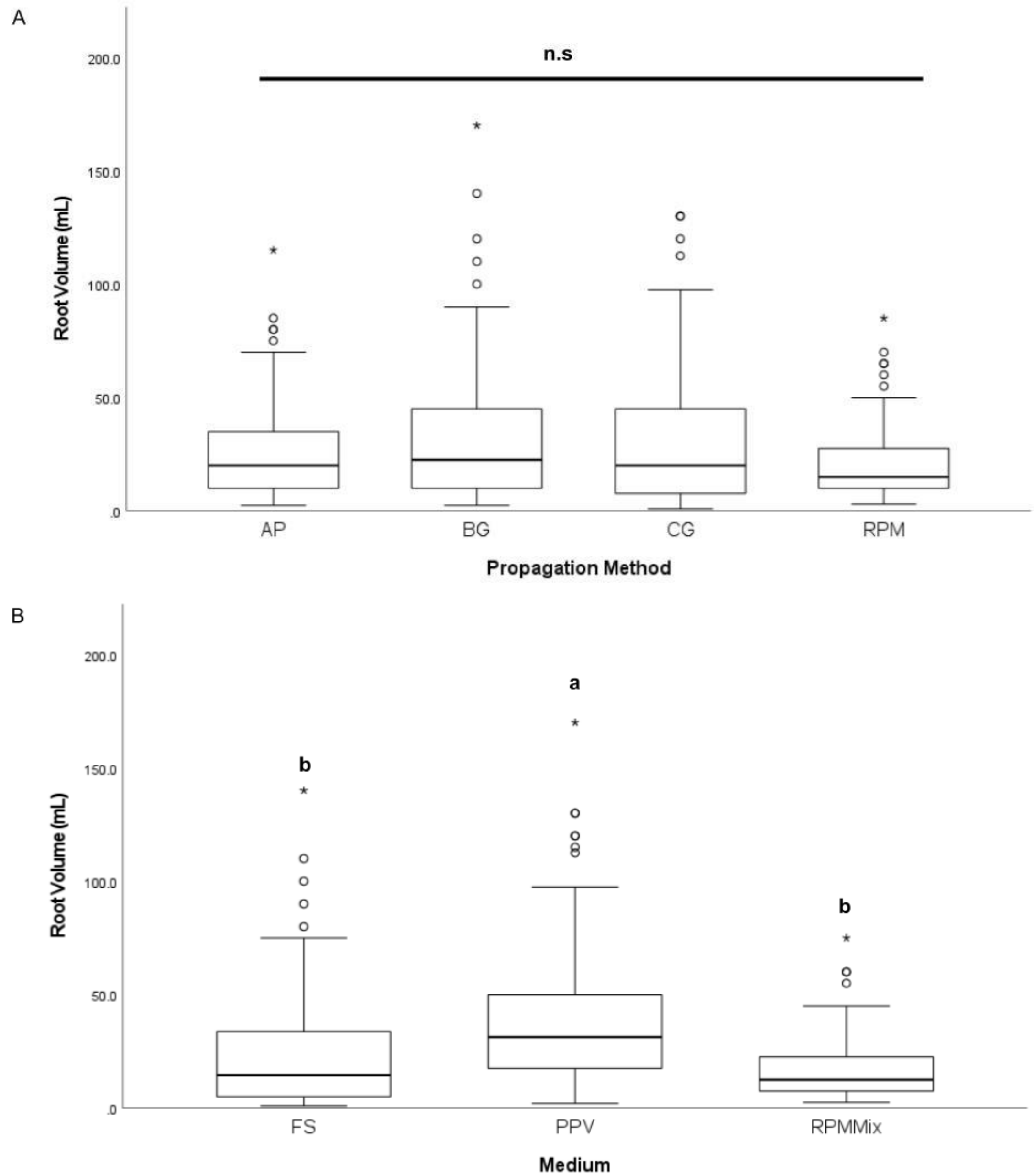

Figure 5: Box plots showing the effect of propagation method (A) and medium type (B) on root volume of seedlings. Significance ($p<0.05$) is indicated by lowercase letters(a>b>c). Boxplots with the same lowercase letters are not significantly different from each other. Propagation method: AP = Air prune, BG = Bed grown, CG = Container grown, RPM = RPM®. Medium: FS = Field soil, PPV = Peat-perlite-vermiculite, RPMMix = Pine bark, sand, and rice hulls.

There were significant main effects of propagation method ($F(3, 359) = 20.773$, $p < 0.001$), medium ($F(2, 359) = 90.533$, $p < 0.001$), and a significant interaction effect of propagation method and medium on FOLR count ($F(6, 359) = 4.375$, $p < 0.001$) (Table 1). Medium type had a greater effect size ($\eta_p^2 = 0.335$) than either propagation method ($\eta_p^2 = 0.148$) or the interaction term ($\eta_p^2 = 0.068$) (Table 1). There was a significant difference in FOLR count within any given propagation method when comparing seedlings grown in different media. Seedlings grown in PPV mix had significantly higher average FOLR counts than seedlings grown in other media in air prune (17) bed grown (21), container grown (11), and RPM® propagation methods (13) (Figure 6A). When examining FOLR count within a given medium, there were significant differences between propagation methods. Among propagation methods using field soil, bed grown trees had the highest FOLR count (13) (Figure 6B). There was not a significant difference between bed grown (21) and air prune seedlings (17) using PPV mix, though the bed grown seedlings using PPV mix had significantly greater FOLR counts than RPM seedlings (13, $p < 0.001$) and container grown seedlings (11, $p < 0.001$) (Figure 6B). Among propagation methods using RPM Mix, air prune seedlings had a significantly higher FOLR count (9, $p < 0.001$) than any other propagation method (Figure 6B).

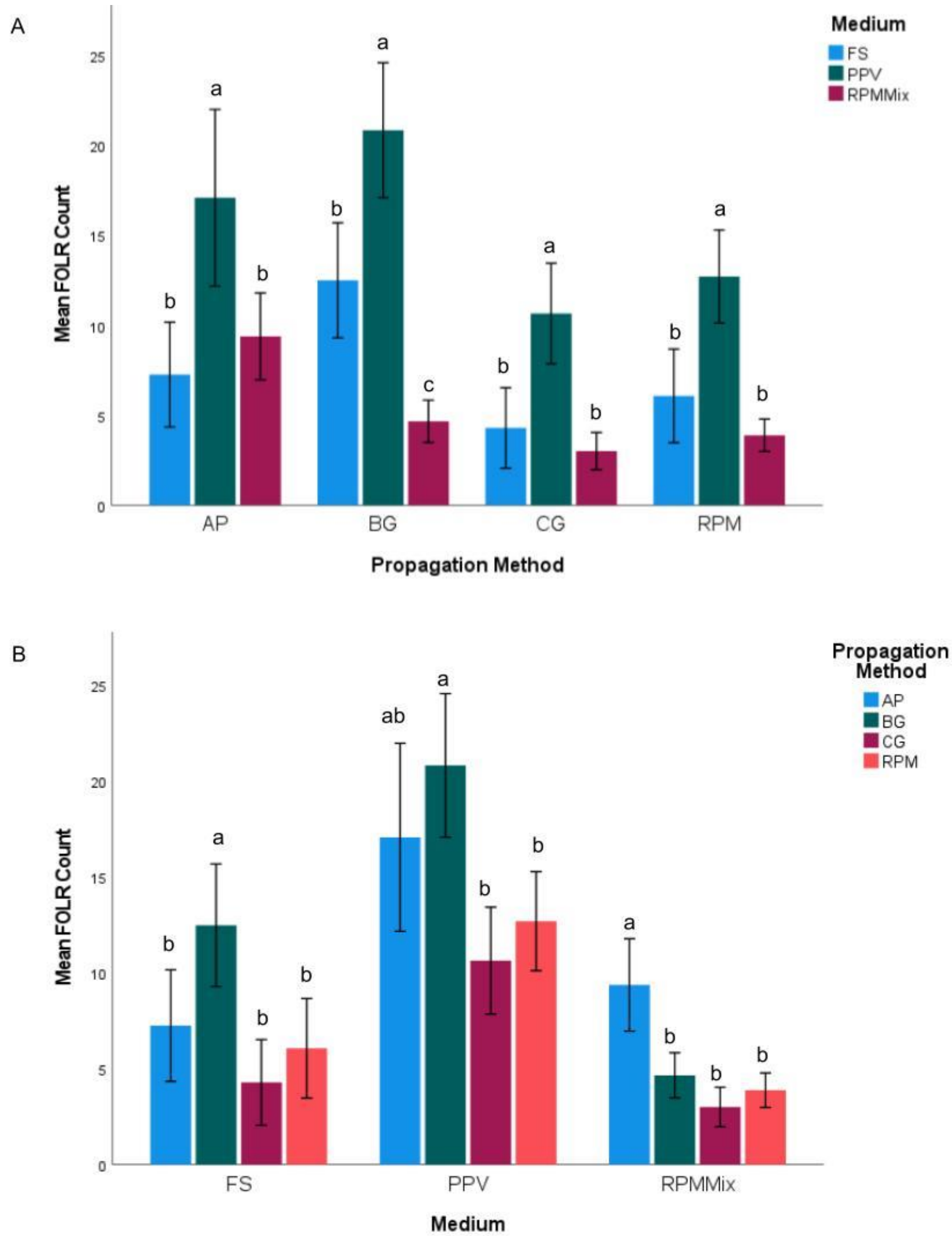

Figure 6: Bar charts showing the effect of medium type within propagation method (A) and propagation method within medium type (B) on mean count of first order lateral roots greater than 1 mm of seedlings. Significance (p<0.01) is indicated by lowercase letters(a>b>c). Bars with the same lowercase letters are not significantly different from each other within the same propagation method (A) or medium (B). Propagation method: AP = Air prune, BG = Bed grown, CG = Container grown, RPM = RPM®. Medium: FS = Field soil, PPV = Peat-perlite-vermiculite, RPMMix = Pine bark, sand, and rice hulls.

Qualitative Observations

Root architecture was varied among treatments. Fine root mass was greater in seedlings grown in PPV mix and RPM® mix when compared to seedlings grown in field soil. Seedlings grown in field soil tended to have larger lateral roots and less fine root mass that could be extracted at lifting. Taller container grown seedlings often showed evidence of root constriction in the form of J-shaped and circling roots, while RPM®, air prune, and bed grown seedlings were free of such root system deformities (Figure 7). Air prune and RPM® seedlings had extensive root branching a clearly defined point at the bottom of the root system where lateral root branching increased where it could be observed that air pruning of the root tip had occurred (Figure 8). This point was absent in both bed grown and container grown seedlings.

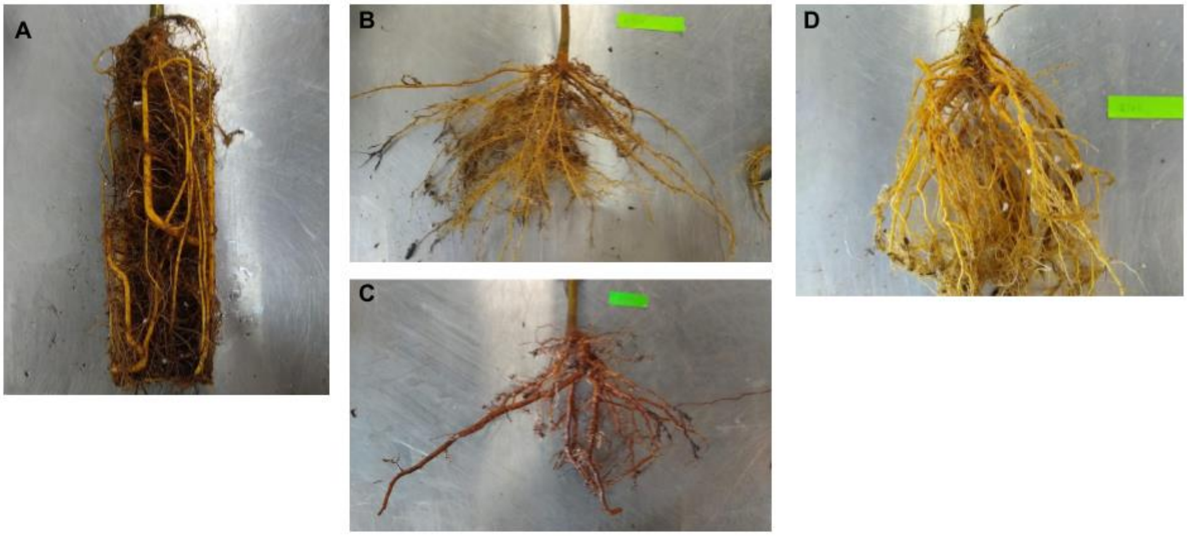

Figure 7: Differences in root system architecture between propagation methods. Container grown seedlings (A) show significant root deformities in larger seedlings. Air prune (B) and RPM® (D) seedlings show evidence of root binding and show a highly branched root system characteristic of seedlings that have had their tap root pruned. Bed grown seedlings (C) show thick lateral roots with less fine root mass than other seedlings.

Figure 8: The base of the root system of an air prune seedling showing extensive lateral branching at the point where the taproot tip was desiccated via air-pruning.

The Effect of Grading on Final Seedling Quality

Only seedlings with complete data, i.e., all covariates and all outcome variables, were used to examine the ability of covariates to predict measures of seedling quality (n = 309). Seed weights were compared using a one-way ANOVA and Tukey HSD post-hoc tests for all treatments. There was no significant difference found in seed weight among the twelve treatment combinations ($F(11,298) = 1.628$, $p = 0.09$). Covariates were analyzed using simple linear regression to determine the predictive relationship between seed weight on height at 60 days and ground line diameter at 60 days. Seed weight did not significantly predict height at 60 days ($R^2 = 0.010$, $p = 0.078$), but there was a significant, though weak, relationship between seed weight and ground line diameter at 60 days ($R^2 = 0.035$, $p = 0.001$). Height at 60 days and ground line diameter at 60 days were then analyzed using simple linear regression. Height at 60 days and ground line diameter at 60 days had a significant relationship ($R^2 = 0.399$, $p < 0.001$).

In building the nested model comparison, bed grown propagation method and field soil were used as the reference category. Propagation method and medium type were placed in blocks and added to the model first. Subsequently, seed weight was added to the model, followed by height at 60 days, and ground line diameter at 60 days and R^2 change, and significance was examined for each added co-variate. Significant interaction terms were then added last to build a complete predictive model. An $\alpha = 0.01$ was used to account for heteroskedasticity in data for final height, final root collar diameter, and FOLR count (Tabachnick and Fidell, 2013). After adding propagation method and medium to the regression model, the addition of seed weight and height at 60 days significantly increased the R^2 value for all measures of seedling quality. Seed weight significantly increased R^2 for final height (Table 2A), final root collar diameter (Table 2B), root volume (Table 2C), and FOLR count (Table 2D). After accounting for seed weight, height at 60 days significantly increased R^2 for final height (Table 2A), final root collar diameter (Table 2B), root volume (Table 2C), and FOLR count (Table 2D). The addition of ground line diameter at 60 days did not significantly increase the R^2 value for final height (Table 2A), final root collar diameter (Table 2B), root volume (Table 2C), or FOLR count (Table 2D). In addition to these results, it is worth noting that, after accounting for propagation method, medium, and all covariates, the interaction term did not significantly increase R^2 for either root volume (Table 2C) or FOLR count (Table 2D).

Table 2: Summary statistics for a nested model comparison of (A) final height, (B) final root collar diameter, (C) root volume, and (D) FOLR count using stepwise addition. R^2 change and F change refer to change from the previous model. Bed grown propagation method and field soil are used as the reference category for all models. Significance ($p < 0.01$) is denoted by an asterisk.

A

Model	**R**	**R^2**	**σ_{est}**	**ΔR^2**	**ΔF**	**df1**	**df2**
M1: Prop. Method (PM)	0.405	0.164	25.791	0.164	19.966*	3	306
M2: M1 + Medium	0.679	0.461	20.765	0.298	84.026*	2	304
M3: M2 + Seed weight	0.703	0.494	20.159	0.033	19.562*	1	303
M4: M3 + Height (60 days)	0.785	0.617	17.572	0.123	96.764*	1	302
M5: M4 + Diameter (60 days)	0.785	0.617	17.600	0.000	0.055	1	301
M6: M5 + PM * Medium	0.806	0.650	17.000	0.033	4.605*	6	295

B

Model	**R**	**R^2**	**σ_{est}**	**ΔR^2**	**ΔF**	**df1**	**df2**
M1: Prop. Method (PM)	0.246	0.061	3.094	0.061	6.571*	3	306
M2: M1 + Medium	0.563	0.318	2.646	0.257	57.241*	2	304
M3: M2 + Seed weight	0.610	0.372	2.543	0.054	26.143*	1	303
M4: M3 + Height (60 days)	0.701	0.492	2.291	0.120	71.234*	1	302
M5: M4 + Diameter (60 days)	0.702	0.493	2.292	0.001	0.712	1	301
M6: M5 + PM * Medium	0.703	0.522	2.247	0.029	3.025*	6	295

C

Model	**R**	**R^2**	**σ_{est}**	**ΔR^2**	**ΔF**	**df1**	**df2**
M1: Prop. Method (PM)	0.145	0.021	26.380	0.021	2.202	3	306
M2: M1 + Medium	0.469	0.220	23.621	0.199	38.826*	2	304
M3: M2 + Seed weight	0.510	0.260	23.042	0.040	16.468*	1	303
M4: M3 + Height (60 days)	0.594	0.353	21.584	0.093	43.314*	1	302
M5: M4 + Diameter (60 days)	0.594	0.353	21.620	0.000	0.003	1	301
M6: M5 + PM * Medium	0.615	0.378	21.423	0.024	0.024	6	295

D

Model	**R**	**R^2**	**σ_{est}**	**ΔR^2**	**ΔF**	**df1**	**df2**
M1: Prop. Method (PM)	0.351	0.123	8.235	0.123	14.351*	3	306
M2: M1 + Medium	0.629	0.395	6.862	0.272	68.359*	2	304
M3: M2 + Seed weight	0.659	0.434	6.649	0.039	20.841*	1	303
M4: M3 + Height (60 days)	0.692	0.479	6.390	0.045	26.059*	1	302
M5: M4 + Diameter (60 days)	0.695	0.483	6.379	0.004	2.063	1	301
M6: M5 + PM * Medium	0.711	0.506	6.298	0.023	2.300	6	295

The Relationship Between Measures of Seedling Quality

All measures of seedling quality were analyzed using simple linear regression to determine the relationship between measurements. All regression analyses were significant ($p < 0.001$), though the coefficient of determination varied between comparisons (Table 3). Final height and final root collar diameter showed the strongest predictive relationship, ($R^2 = 0.739$, $p < 0.001$), followed by final root collar diameter and root volume ($R^2 = 0.733$, $p < 0.001$), and final root collar diameter and FOLR count ($R^2 = 0.677$, $p < 0.001$). Final root collar diameter showed the greatest predictive ability for all other outcome variables (Table 3).

Table 3: Coefficient of determination (R^2) values of simple linear regression models of final measures of seedling quality for hybrid chestnut seedlings. All regression slopes were positive. Significance ($p < 0.01$) is denoted by an asterisk.

	Final RCD (mm)	Root Volume (mL)	FOLR Count
Final height (cm)	0.739*	0.586*	0.643*
Final RCD (mm)	--	0.733*	0.677*
Root Volume (mL)	--	--	0.562*

Mortality

Likelihood ratio tests determined that propagation method contributed significantly to mortality outcomes ($\chi^2(3) = 45.47$, $p < 0.001$) while medium type did not ($\chi^2(2) = 3.77$, $p = 0.152$). Bed grown seedlings were more likely to survive (OR = 19.190) than seedlings grown by any other propagation method while RPM seedlings were least likely to survive when compared to other propagation methods (Table 4). Though not significant, seedlings grown in field soil were less likely to survive (OR = 0.508) than seedlings grown in any other medium (Table 4).

Table 4: Parameter estimates for a logistic regression to determine the relationship between propagation method, medium, and survival. "Did not survive" is the reference category for mortality. RPM® seedlings grown in RPM® mix (pine bark, sand, and rice hulls) are used as the reference category for propagation method and medium type. PPV Mix = Peat-perlite-vermiculite. Significance ($p < 0.05$) is denoted by an asterisk.

Parameter	B	σ	Wald	df	Odds Ratio
Intercept	1.136	0.302	14.170*	1	--
Air prune	2.220	0.507	19.156*	1	9.207
Bed grown	2.991	0.751	15.871*	1	19.910
Container grown	0.738	0.344	4.601*	1	2.092
Medium: Field Soil	-0.678	0.393	2.976	1	0.508
Medium: PPV mix	-0.054	0.371	0.021	1	0.947

Relative Chlorophyll Levels

Relative chlorophyll content (SPAD values) among seedlings ($n = 120$) showed a significant interaction effect between propagation method and medium type ($F(6, 108) = 8.31$, $p < 0.001$). Regardless of propagation method SPAD values were highest for seedlings grown in field soil, though only the bed grown propagation method showed significantly higher SPAD values when compared to seedlings grown in either PPV mix or RPM mix (38.61, $p < 0.001$, Figure 7a). SPAD values were consistently lowest for seedlings grown in RPM mix, regardless of propagation method (Figure 9a). Container grown seedlings in the PPV soil had significantly higher SPAD values compared to seedlings grown in PPV soil in the other propagation methods (33.75, $p < 0.05$, Figure 9b). SPAD values were lowest for propagation methods using RPM® mix, though not significantly so in air prune seedlings (Figure 9a).

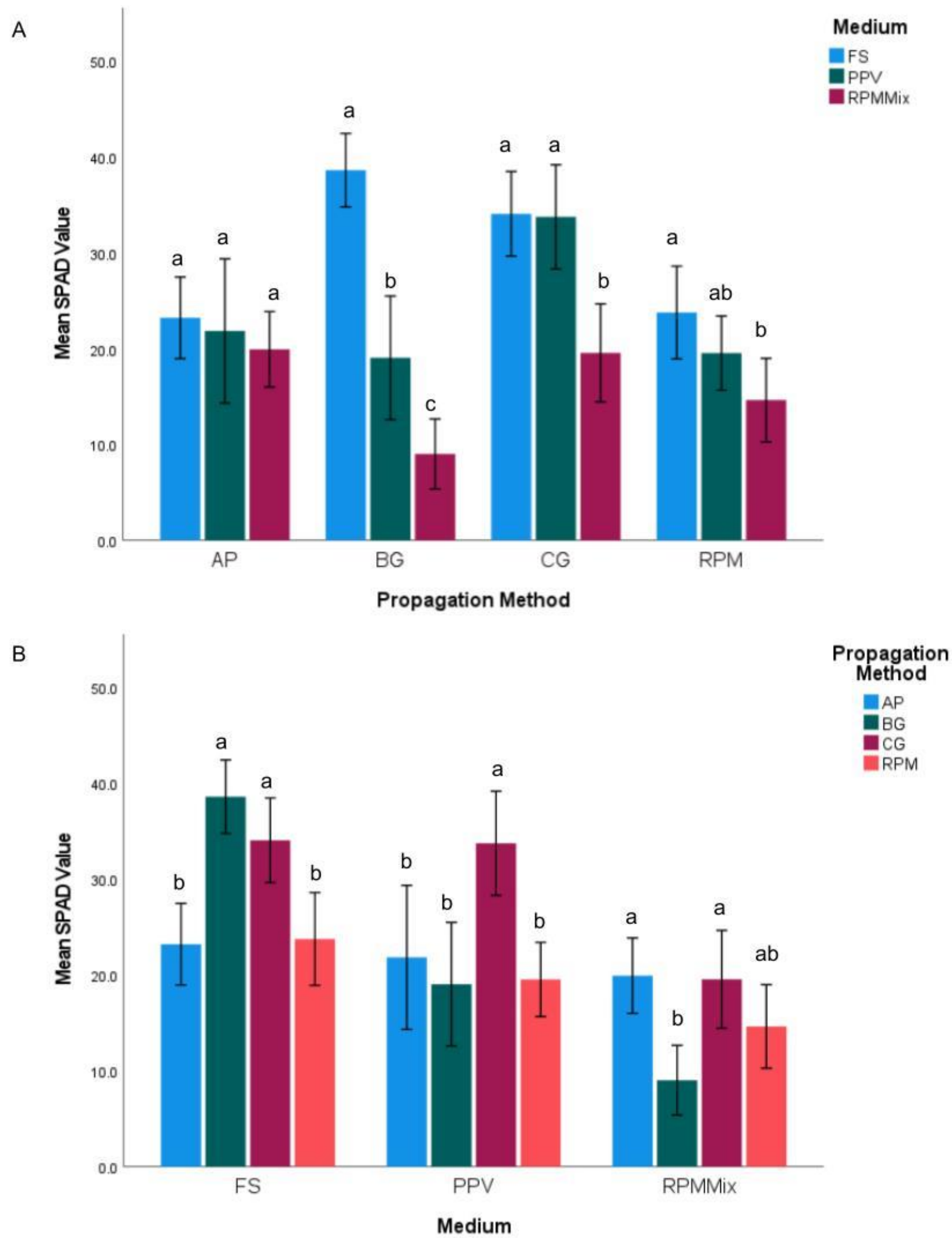

Figure 9: Bar charts showing the effect of medium type within propagation method (A) and propagation method within medium type (B) on mean relative chlorophyll content (SPAD values) of seedlings. Significance ($p<0.05$) is indicated by lowercase letters (a>b>c). Bars with the same lowercase letters are not significantly different from each other within the same propagation method (A) or medium (B). Propagation method: AP = Air prune, BG = Bed grown, CG = Container grown, RPM = RPM®. Medium: FS = Field soil, PPV = Peat-perlite-vermiculite, RPMMix = Pine bark, sand, and rice hulls.

Cost per Seedling

After accounting for both materials and labor costs and dividing this total by the number of seedlings that survived to the end of the study, bed grown seedlings grown in field soil were the least expensive seedlings to produce ($1.72 per seedling) while RPM® seedlings grown in field soil were the most expensive seedlings ($6.06 per seedling), likely due to the high labor costs and low survival (Table 6). Soil present on-site was used for the field soil medium, nurseries who purchase topsoil may see an increased cost for this treatment compared to costs shown in this study. Materials costs and labor costs were highest for the RPM® method due to the number of specific containers required and the time required to grade seedlings and perform multiple transplants. Of the treatments most likely to be used in a nursery setting, bed grown trees in field soil were the cheapest ($1.72 per seedling) followed by air prune seedlings in PPV mix ($2.46 per seedling), container grown seedlings in PPV mix ($3.73 per seedling), and finally RPM® seedlings in RPM® mix ($5.75 per seedling) (Table 6).

Table 5: Cost per seedling by treatment. Cost was calculated based on cost for materials and labor in treatment preparation divided by the number of seedlings that survived at the end of the study. Costs marked with an asterisk are "reference categories", i.e., treatments that may be used in a nursery setting rather than experimental controls. PPV mix = Peat-perlite-vermiculite, RPM mix = Pine bark, sand, and rice hulls.

Propagation Method	Medium	Materials Cost	Labor Cost	Total Cost	Surviving Seedlings	Cost per Seedling
Air prune	Field soil	$35.63	$38.79	$74.42	29	$2.57
Air prune	PPV mix	$67.08	$38.79	$105.87	43	$2.46*
Air prune	RPM mix	$69.92	$38.79	$108.72	40	$2.72
Bed grown	Field soil	$30.99	$22.33	$53.32	31	$1.72*
Bed grown	PPV mix	$86.03	$22.33	$108.37	34	$3.19
Bed grown	RPM mix	$88.94	$22.33	$111.27	27	$4.12
Container grown	Field soil	$87.57	$25.46	$113.03	28	$4.04
Container grown	PPV mix	$112.38	$25.46	$137.84	37	$3.73*
Container grown	RPM mix	$113.67	$25.46	$139.13	23	$6.05
RPM®	Field soil	$29.91	$67.06	$96.97	16	$6.06
RPM®	PPV mix	$125.38	$67.06	$192.44	36	$5.35
RPM®	RPM mix	$128.60	$67.06	$195.66	34	$5.75*

DISCUSSION

Effective cost remains one of the largest challenges facing any restoration effort and increased seedling survival offers one of the simplest means of reducing effective cost and improving restoration outcomes. Seedling survival is especially important when attempting to reestablish compromised species such as the hybrid chestnuts investigated here. Each of the measures of seedling quality investigated in this study are directly linked to seedling survival and performance as seedlings establish themselves in the field (Dey and Parker 1997; Davis and Jacobs 2005; Wilson and Jacobs 2006; Grossnickle and El-Kassaby 2016, Grossnickle and

MacDonald 2018b). Research focused on reestablishing the American chestnut has identified improving seedling quality as the most efficacious means of increasing the success of restoration programs (Clark et al. 2014a; Collins et al. 2017). Our study aimed to determine effective means of increasing seedling quality by testing several propagation methods, media types, and grading methodology. We found that specific treatments produced higher quality seedlings than others, including promising results from the novel air prune bed propagation method that produced seedlings of comparable quality and cost to time-tested treatments across all measures of seedling quality. In addition to these findings, we discovered that grading seeds by weight and seedlings by height at 60 days offers nurseries a viable means of selecting seedlings for improved seedling quality overall. These findings fill a gap in the research dealing with methods of improving hybrid chestnut seedling quality without substantially increasing costs.

Effects of Propagation Method and Medium on Measures of Seedling Quality

Propagation Method

Propagation method showed the greatest effect size for explaining final height (Table 1). Bed grown propagation, the least expensive method, resulted in the tallest seedlings when grown in field soil and PPV mix. Across several studies, bed grown seedlings are generally taller than container grown seedlings of the same age. This is potentially due to bed grown seedlings having a larger "container" volume to exploit than seedlings grown in pots, densities being equal (Grossnickle and El-Kassaby 2016). RPM®, the most expensive and time-consuming method, produced smaller seedlings than other propagation methods despite being grown at decreased densities, i.e., in ten-inch pots rather than at five-inch spacing found in other treatments. *Castanea dentata* readily responds to increased light levels via rapid shoot growth and the lack of response to increased light availability due to decreased density indicates that growth may have been stunted by other factors (Wang et al. 2006). While propagation method had the greatest effect on variability in height, medium had the greatest effect on variables related to root system size and architecture.

Soil Medium

Soil medium had the greatest effect size for final root collar diameter and first order lateral root (FOLR) count (Table 1) and, unlike propagation method, different media showed significant differences in root volumes (Figure 5b). Across all treatments peat-perlite-vermiculite (PPV) mix resulted in the greatest root collar diameter and the highest FOLR count (Figures 5b, 6a). Seedlings grown in PPV mix also consistently had the highest seedling quality measurements of any medium, regardless of propagation method. This is likely due to PPV mix's excellent water holding capacity and low bulk density when compared to RPM® mix and field soil, respectively (Grossnickle and El-Kassaby, 2016). Root system size and architecture data suggest that PPV mix remains an excellent choice for containers or air prune beds. These results also suggest that nurseries may improve bed grown seedling quality overall by decreasing soil bulk density of beds by increasing soil organic matter (Davis et al. 2006). Seedlings grown in PPV mix and RPM® mix were predicted to show greater root volumes than seedlings grown in field soil because of the lower bulk density of these two media. Seedlings grown in PPV mix produced significantly greater median root volumes than those grown in RPM® mix but there was no significant difference in root volume between seedlings grown in field soil and seedlings grown in RPM® mix (Figure 5b).

The lack of significant difference between field soil and RPM® mix could be due to several factors. Field soil placed in containers had higher compaction than RPM® mix – potentially preventing more extensive root systems and lead to greater loss of fine root mass at extraction (David and Jacobs, 2005, Cambi et al. 2018). The high porosity and lower water holding capacity of the RPM® mix could have contributed to water stress and allowed nutrients to wash out of the medium at a faster rate than could be absorbed by seedlings leading to stunted growth. Seedlings grown in RPM® mix showed lower seedling quality outcomes than other mix types within any given treatment (Figures 3a, 4a, 5b, 6a). Furthermore, seedlings grown in RPM® mix had lower average SPAD values and showed greater signs of chlorosis, and therefore greater potential nutrient deficiencies, across treatments than other media (Figure 9a). Although the effect size for the interaction between propagation method and medium was consistently smaller than that of propagation method and medium alone, these results provide context for refining potential treatments in the nursery.

Interactions between propagation method and soil medium

Combinations of medium and propagation method must be considered when predicting final seedling quality due to a significant interaction effect (Table 1). The combination of the bare root propagation method and PPV mix resulted in the most robust seedlings (Figures 3b, 4b, 6b). Bed grown seedlings may perform exceptionally well if grown in beds with high soil organic matter which may also ease extraction and reduce root system loss as a supplementary benefit. Studies examining the effects of increased soil organic matter in *Fraxinus pennsylvanica* and *Quercus rubra* found that increasing the organic matter content of soils increased both seedling height and root collar diameter (Davis et al. 2006). Our results indicate the need for further research exploring changes in seedling quality across various levels of soil organic matter.

Our prediction that the two novel propagation methods, air prune beds and the Root Production Method® would both produce taller seedlings with greater diameter, root volume, and higher FOLR count than the two standard propagation methods, bed grown and container grown, was not entirely supported. Outcomes between these two novel methods varied depending on the medium used. Air prune seedlings in PPV mix, a practical combination of medium and propagation method, significantly outperformed RPM® seedlings in the same medium in final height and were not significantly different from RPM® seedlings across other measures of seedling quality, supporting our initial prediction about the equivalency of these two methods. Air prune seedlings grown in PPV mix also performed comparably across height and root collar diameter and showed higher FOLR counts to two standard treatments - bed grown seedlings grown in field soil and container grown seedlings in PPV mix (Figures 3b, 4b, 6b). While RPM® mix may be an unlikely medium choice, air prune seedlings were significantly greater than RPM® seedlings across height, root collar diameter, and FOLR count when using this medium (Figures 3b, 4b, 6b).

Seedlings performed worst in the most unlikely combination of propagation method and medium, containers filled with excavated field soil, (Figures 3b, 4b 6b). Field soil most likely became highly compacted when placed in containers, as was observed in air prune, container grown, and RPM® treatments. Experiments on the effect of soil compaction on seedling growth have shown that increased compaction led to poor growth outcomes when compared with trees grown in less compacted soil (Cambi et al. 2018). Examining the interaction effect between propagation method and medium allows for greater refinement in seedling production while direct comparison between industry standard and novel techniques can allow nurseries greater flexibility in choosing the propagation method most aligned with their desired seedling quality outcomes.

Comparison of Novel and Standard Propagation Methods

In addition to comparable seedling quality outcomes, air prune seedlings offer several advantages when compared to bed grown and container grown seedlings. Seedlings with long tap roots can be easily damaged during extraction from bed grown beds, the primary cause of transplant shock (Watson and Syndor 1987; Wilson et al. 2007; Struve 2009). While this can be mitigated by using container systems, this propagation method can cause significant root deformities as seedling roots circle once they reach the edge of the container (Figure 7). Air prune beds mitigate these issues by stopping root elongation via desiccation of the root tip, resulting in root system depths that are equivalent with the depth of the bed and preventing root deformities by desiccating the root tip of any roots that would otherwise curve upon contacting the bottom of another container (Figure 8). By initiating greater numbers of FOLR, root pruning may also reduce transplant shock in air prune seedlings as they establish soil contact in the first year after planting (Jacobs et al. 2005). This result is especially relevant to nurseries in the southern USA seeking alternative propagation methods for chestnut and other tree seedlings where *Phytophthora* contamination in nursery beds can contribute to high mortality rates (Rhoades et al. 2003; Clark et al. 2014a; Clark et al. 2014b; Clark et al. 2016). These studies have traditionally recommended container grown seedlings as an alternative where *Phytophthora* is an issue. Our results show that air prune beds can produce high quality seedlings at a lower cost than traditional container grown methods (Table 6) while avoiding the long-term survival issues associated with circling roots (Arnold and Struve 1993). This research can inform future studies optimizing air prune bed construction to increase structural integrity while maintaining low cost and high seedling quality outcomes. We conclude that air prune beds will produce high quality, low-cost seedlings equivalent to those grown in bed grown beds or containers and should be studied in field trials to compare survival and performance.

Seedlings grown using the RPM® method were more expensive on average than other treatments and did not outperform bed grown and container grown seedlings as expected, making their increased cost difficult to justify (Table 6). RPM® seedlings also had the highest mortality rate of all propagation methods (Table 4). This result was surprising given prior research highlighting the high quality and vigor of RPM® seedlings across several growth measures (Dey et al. 2004). The RPM® propagation method selects the heaviest seeds and only the tallest seedlings with the largest root collar diameters are transplanted after 30 days, thereby selecting for the most vigorous seedlings to market as an RPM® seedling. Our data show that seed weight and height at 60 days, explain a significant amount of the variation in all measures of seedling quality examined in this study (Table 2), indicating that grading per the standards laid out in the RPM® method may have increased measures of seedling quality on average, though at an even greater cost per seedling (Table 6). Therefore, by retaining our entire sample we may have shown a more complete picture of seedling quality produced by the RPM® method than what would normally progress to the final stage in the RPM® methodology.

We also predicted that container grown seedlings would show greater root collar diameter, root volume, and FOLR than bare root seedlings due to increased root system size overall and an increased ability to preserve the entire root system during extraction. However, seedlings in these two treatments performed similarly across all measures of seedling quality. Our data show that media characteristics were more important than propagation method in determining root system size and architecture (Table 1, Figure 5). Previous research has primarily focused on cost, and site factors are the primary reasons for selecting between bed grown and container grown seedlings. Container grown seedlings were more expensive to produce than bed grown seedlings for comparable seedling quality and were less likely to survive the growing season (Table 4, Table 6). However, previous research has highlighted the importance of preserving a seedling's root system in reducing transplant shock and improving overall survival, especially in areas where water stress is likely (Thompson 1985; Grossnickle 2012; Clark et al. 2016). Therefore, site conditions will play a large role when selecting between bed grown and container grown seedlings where using bed grown seedlings, though less expensive individually, may result in greater effective cost due to reduced survival rates in stressful site conditions.

Predicting Measures of Seedling Quality

Seedling grading is an essential component of the RPM® methodology and could be used with other propagation methods to improve seedling quality outcomes. Our prediction that seed weight would be predictive of all measures of seedling quality was supported, as the addition of seed weight to models predicting each measure of seedling quality added significant explanatory power to each model (Table 2). These results support previous research that also found significant relationships between seed weight and final height, root collar diameter, and FOLR count (Clark et al. 2012; Pinchot et al. 2015). There was a positive relationship between seed weight and final height ($R = 0.12$), final root collar diameter ($R = 0.20$), root volume ($R = 0.16$), and FOLR count ($R = 0.15$), the additional explanatory power of seed weight is between 3.3% and 5.4%.In alignment with previous studies, our data show that seed weight contributes to marginal overall gains in predictive power for measures of seedling quality (Clark et al. 2012). This offers nurseries the option to separate seed by weight class, placing seeds with lower weights in separate areas to grow for additional time before harvest. For large projects, the time required to weigh individual seeds may be prohibitive and alternative methods, such as the aspiration tables employed by the RPM® propagation method, may be required to reduce labor costs (Lovelace 2002; Clark et al. 2012). While grading based on seed weight can increase seedling quality outcomes, using seed weight as a pre-emptive screening tool may be disadvantageous for hybrid chestnut given that selecting for larger seeds may inadvertently select for phenotypic characteristics more closely associated with Chinese chestnuts rather than American chestnuts (Clark et al. 2012; Pinchot et al. 2015). In addition to our findings on seed weight, seedling height at 60 days may be more powerful in predicting final seedling quality outcomes that previously expected.

We found height at 60 days significantly explained variation in all measures of seedlings quality, including belowground morphology (Table 2). Of the three covariates, height data is easiest to collect and is often used by nurseries to visually grade seedlings into different quality categories. This study further validates height in predicting seedling quality. Height at 60 days explains an additional 12.3% of the variation in final height, an additional 12.0% of the variation in final root collar diameter, an additional 9.4% of the variation in root volume, and 4.5% of the variation in FOLR count (Table 2). The predictive power across all measures of seedling quality and the ease of data collection makes this measurement a simple and useful means of grading seedlings. Diameter at 60 days was not as informative as height or seed weight in predicting final seedling quality. In this study, diameter at 60 days did not add significantly explanatory power to the model after accounting for seed weight and height. This variable is the least convenient and most time-consuming measurement to collect of the three grading criteria analyzed and does not significantly explain any additional variation in the measures of final seedling quality (Table 2). We conclude that if nurseries are interested in grading seedlings, height at 60 days is the most effective measurement to select for more robust seedlings and that if additional time is available, grading based on seed weight can provide additional improvements to overall seedling quality, with caveats for use with hybrid chestnuts. Selecting the appropriate propagation methods, media, and implementing grading in the nursery offer practical means of improving seedling quality. These optimization practices must be integrated with production costs and decision-making criteria to in order to select the appropriate methodology for a given nursery and its financial and site constraints.

Least Cost Approaches to Producing High Quality Seedlings

Air prune seedlings grown in PPV mix provide a promising alternative to bed grown and container grown seedlings when considering seedling quality and overall cost per seedling ($2.46 per seedling). Importantly, this method can be used on sub-par growing spaces such as old building pads or sites with high soil compaction and can be moved to new locations as needed if the beds are reasonably sized and filled with a lightweight medium, (Figure 10). Given the lack of materials required, it is unsurprising that bed grown seedlings grown in field soil remain the lowest cost approach to propagating seedlings. With this in mind, site conditions should be considered when selecting seedlings, as abiotic stressors may favor methods that preserve more of the seedling’s fine root mass, such as container grown, air prune, or RPM ® seedlings (Figure 10).

Container grown seedlings in PPV mix provide comparable quality seedlings, but at a higher cost per seedling ($3.73 per seedling) than bed grown seedlings grown in field soil ($1.72 per seedling). This is primarily due to higher materials cost, though higher mortality for container grown seedlings may have factored in as well (Table 4). Brick and mortar infrastructure, such as greenhouses, are not included in the calculation of cost per seedling and should be factored into any interpretation of these results as this would add significant cost to any nursery operation, though the ability to extend the growing season in colder climates may outweigh these costs (Figure 10). RPM® seedlings had the highest cost of any propagation method due to the high materials cost and labor required to perform multiple transplants and grading measurements (Table 6). Despite this, the RPM® method remains of interest for forest restoration and produced promising results in several other studies (Grossman et al. 2003; Dey et al. 2004; Walter et al. 2013; Van Sambeek 2016). Ultimately, survival and field performance data will provide further information on the efficacy of these treatments to improve restoration outcomes as these measures of seedling quality are most important in the first year or two after out planting as seedlings become established.

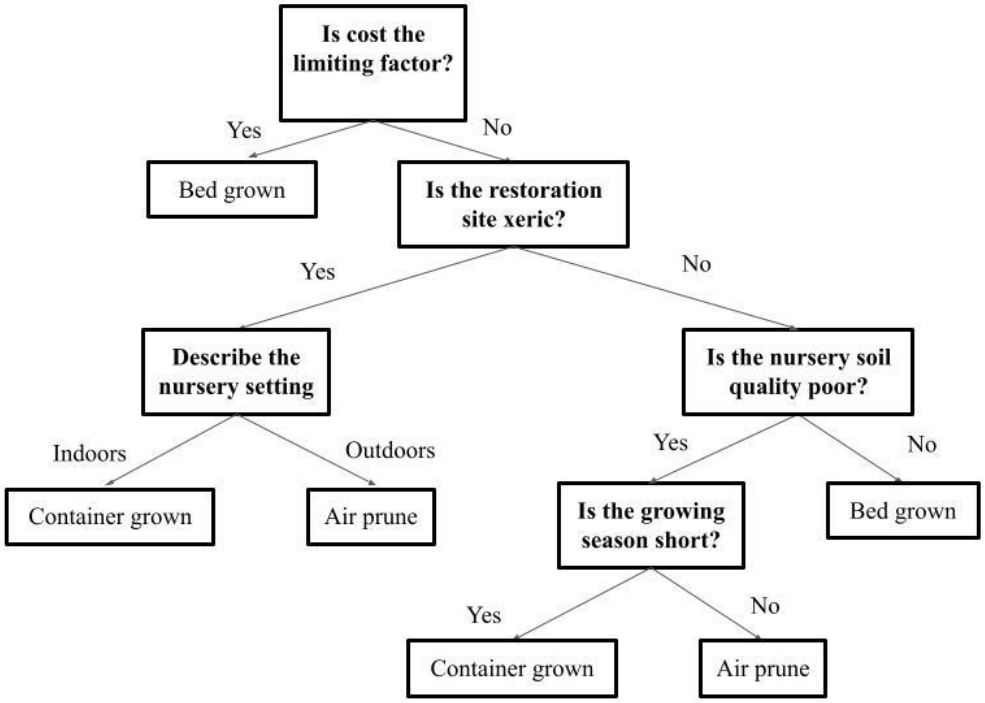

Figure 10: A decision-tree diagram for considerations in selecting the more appropriate propagation method based on cost, restoration site factors, and nursery conditions. Bed grown seedlings are assumed to be grown in field soil, while container grown and air prune seedlings are assumed to use a standard peat-perlite-vermiculite mix. RPM® were not included in this decision tree due to underperformance.

CONCLUSION

Improving seedling quality remains an efficacious means of improving restoration outcomes by increasing survival and field performance of seedlings resulting in a more effective use of resources. Propagation method, medium choice, grading seeds by weight, and grading seedlings by height can result in increased overall seedling quality. These accessible means of increasing seedling quality can be of use to any tree nursery given their low cost and ease of implementation. The promising results from air prune beds should inform future studies to optimize this propagation method and provide seedlings for long-term survival and field performance research. No one method is perfect for every situation, however, and considerations for final site conditions, existing infrastructure, and cost are important factors in selecting a propagation method and medium to produce high quality seedlings at a reasonable cost. Results and recommendations from this study will inform agencies and individuals concerned with forest restoration to effectively select propagation methods for their context to conserve time, resources, and in the case of the American chestnut, contribute to the restoration of an extirpated species to its historic range.

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