



Clonal propagation of Oregon ash (*Fraxinus latifolia*) via somatic embryogenesis

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Abstract

Key message This first account of Oregon ash somatic embryogenesis identifies unique auxin and abscisic acid responses distinct from green and white ash, advancing clonal propagation techniques for reforestation.

Abstract Oregon ash (*Fraxinus latifolia*), the only ash species native to the Pacific Northwest, is vulnerable to the invasive beetle, emerald ash borer (EAB; *Agrilus planipennis*). EAB was first detected in North America in 2002, and subsequently spread primarily east of the Rocky Mountains, with greatest concern centered on more abundant and economically valuable ash species, such as green ash (*Fraxinus pennsylvanica*) and white ash (*Fraxinus americana*). Given the devastating effects of EAB on ash populations and limited natural regeneration capacity of Oregon ash, developing reliable propagation methods is essential for restoration efforts. Thus, our objective was to develop somatic embryogenesis (SE) protocols for Oregon ash by evaluating effects of auxin concentration, explant manipulation, and abscisic acid supplementation on embryogenesis induction, proliferation, and maturation. Embryogenesis induction was influenced by the interaction between picloram concentration and explant cutting ($p=0.03$), with optimal rates achieved using 0.2 mg/L picloram with intact embryos (16%) or 0.1 mg/L picloram with excised cotyledons (15%). During proliferation, 2,4-D significantly improved culture growth compared to picloram ($p=0.03$). Genotype also affected Oregon ash proliferation response to varied minute (0.1–10 μ M) abscisic acid (ABA) concentrations ($p<0.01$). Our findings lay the foundation for understanding SE response in Oregon ash and demonstrate the feasibility of clonal propagation for this species, providing essential tools for conservation efforts as EAB threatens Pacific Northwest ash populations.

Keywords Oregon ash · Somatic embryogenesis · Clonal propagation · Suspension tissue culture · Emerald ash borer · Pacific Northwest

Introduction

Increased global trade has accelerated the introduction and establishment of invasive, nonnative insect populations in North America, reshaping both urban and forested landscapes (Liebhold et al. 2012; Loehle et al. 2023). Emerald ash borer (EAB; *Agrilus planipennis*), a wood-boring beetle

native to East Asia and first detected in North America in 2002, ranks among the most economically destructive, as ash lumber is commonly used for furniture, baseball bats, and hand tools. (Herms and McCullough 2014; Klooster et al. 2018). As EAB populations have expanded, they have caused widespread mortality of ash (*Fraxinus*) trees (58–99% post-EAB infestation) (Nisbet et al. 2015; Kashian 2016; Klooster et al. 2018; Ward et al. 2021), driving some North American ash species toward functional extinction (Ward et al. 2021). Associated economic losses are estimated to exceed 10 billion USD (Kovacs et al. 2010; McConnell et al. 2019; Sargent et al. 2013).

Until recently, research and management efforts have primarily focused on ash species dominant in regions already affected by EAB (e.g., green ash [*Fraxinus pennsylvanica*] and white ash [*Fraxinus americana*]), as the west coast was largely unaffected until its initial detection in Oregon

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in 2022 (Zobrist et al. 2023). For example, research efforts concentrated on green ash and white ash in somatic embryogenesis (SE) have used this technique for clonal propagation and gene transformation through RNA interference (RNAi) technologies that silence EAB-specific genes within these species (Merkle et al. 2023; Tull et al. 2022). Development of these technologies within the more widespread ash species lays a strong foundation for future scaling-up of EAB-resistant ash varieties for reforestation. However, as these studies have primarily focused on ash species in the Midwest and Eastern regions of North America, it is important to expand this research to include other ash species across the diverse ecoregions where EAB continues to spread and establish populations in the presence of host species. The continued spread of EAB now threatens Oregon ash (*Fraxinus latifolia*), the sole native ash species in the Pacific Northwest (PNW). In response, research has begun to assess susceptibility of Oregon ash and model the projected ecological effects of EAB on riparian ecosystems where Oregon ash is commonly found. Additional to its prevalence in natural systems, Oregon ash has been commonly planted as an ornamental and street tree. Thus, this species' vulnerability to EAB will drastically change both urban and forest environments where it is found (Callahan et al. 2025; D'Amato et al. 2023; Shaw et al. 2023).

As Oregon ash joins other native species at risk from EAB, clonal propagation of ash by in vitro techniques such as SE could be advantageous for commercial plant production, genetic modification, and reforestation initiatives (Loyola-Vargas and Ochoa-Alejo 2016; Park 2002). SE holds promise for adaptation in the face of change and has been used with several conifer and broadleaf trees, and crop species (Bueno et al. 1992; Litz and Gray 1995; Park 2002). SE has been used for clonal propagation of ash, and when EAB-resistant genotypes are eventually identified, this technique can be applied to support large-scale production of resistant ash planting stock. However, using SE protocols for operational propagation of hardwoods, such as ash, has been hindered by associated high labor costs and inconsistent plantlet production (Andrade and Merkle 2005; Merkle et al. 2023). Updated protocols that optimize the key stages of SE are necessary to make this clonal propagation tool a realistic solution for conserving and restoring North American ash. Production of clonal plantlets by SE has been reported for the more abundant species of North American ash, such as green ash and white ash (Preece et al. 1989; Li et al. 2014), but there is currently no published literature on in vitro propagation of Oregon ash.

Oregon ash is a dominant hardwood in wetland forests throughout its native range and is also common in urban landscapes across North America. The continued spread of EAB in the PNW is likely to drastically alter forest structure,

composition, and function. These functional changes in riparian forests can lead to cascading effects in water quality, carbon sequestration, and ecosystem stability for wildlife. As Oregon ash populations decline, other trees associated with ash such as black cottonwood (*Populus trichocarpa*), red alder (*Alnus rubra*), and grand fir (*Abies grandis*) may become more prevalent. However, grand fir and red alder populations are also declining, further reshaping regional forests (Krahl and Shaw 2023). These shifts in forest function in some of the most productive forest systems will have significant ecological and economic impacts (Campbell 2002; Perez-Garcia 2003). Therefore, research on Oregon ash response in vitro is critical not only for species preservation but also for the conservation of important forest and urban landscapes in PNW and across North America.

To address the need for effective propagation strategies for Oregon ash, we focused our efforts on evaluating feasibility of SE for clonal propagation of this species. Our goal was to develop a reliable SE protocol that can support reforestation efforts using resilient Oregon ash genotypes, while also informing improvements to in vitro propagation techniques for other North American ash species. Accordingly, we conducted a series of experiments to: (1) test the effects of auxin concentration and explant manipulation on embryogenesis induction (Experiment 1); (2) evaluate auxin type effects on culture proliferation (Experiment 2); and (3) assess ABA concentration effects on culture proliferation and embryo maturation (Experiments 3 A-B). These efforts represent the first comprehensive attempt to optimize SE protocols for this species and aim to support future propagation of genetically diverse, potentially EAB-resistant Oregon ash in both urban and forest ecosystems.

Methods

Experiment 1: Auxin and explant effects on embryogenesis induction

The objective of Experiment 1 was to evaluate effects of auxin concentration and explant manipulation on induction of somatic embryogenesis in Oregon ash. Immature samaras from four Oregon ash source trees were collected in early-mid July 2024 by US Forest Service collaborators at the Dorena Genetic Resource Center in Cottage Grove, Oregon. Samaras were shipped on cold packs via next day delivery to the University of Georgia, Athens, Georgia and stored at 10° C until used within one week of arrival. Following de-winging, samaras were surface disinfested in 150 mL beakers on a stir plate, with transfer to new beakers at each step in the sterilization process. The surface-disinfestation sequence included two successive washes in 70% ethanol

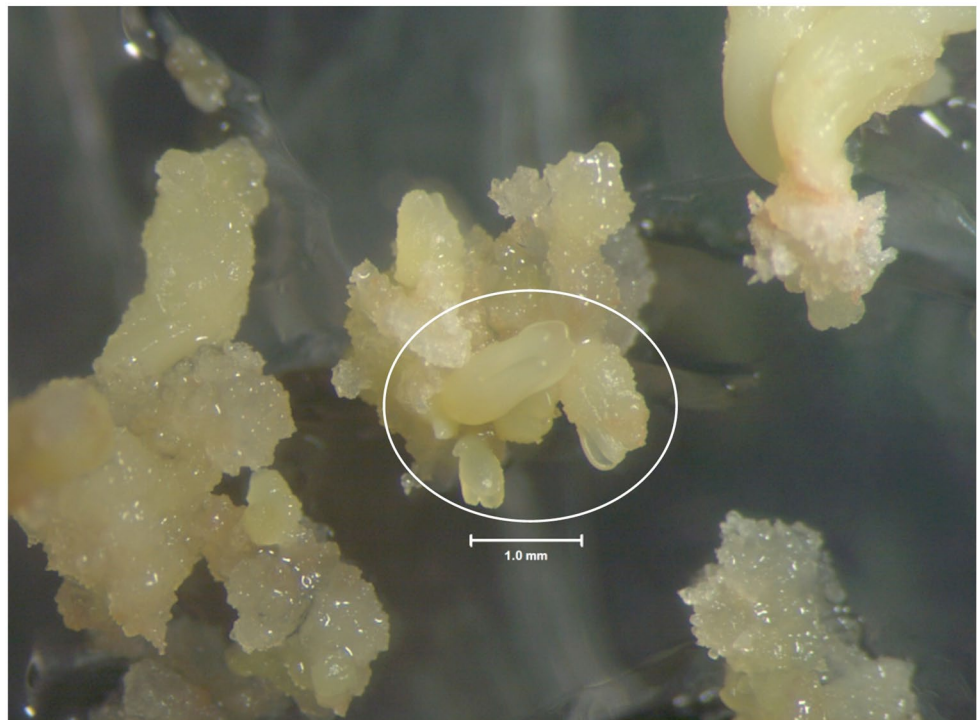
(20 s each), followed by two successive washes in 10% Roccal-D Plus (9.2% didecyl dimethyl ammonium chloride, 13.8% alkyl dimethyl benzyl ammonium chloride, 1% bis-n-tributyltin oxide; Pfizer; 3 min each). This was followed by a 5-minute wash in 50% Clorox (8.25% sodium hypochlorite), 3-minute rinse in sterile deionized water, 3-minute rinse in sterile 0.01 N HCl for 3 min, and three additional 3-minute rinses in sterile deionized water. Throughout this 10-step process, samaras were agitated with a spoonula, in addition to being spun on a stir plate with a magnetic stir bar (Merkle et al. 2023). Disinfested samaras were dissected to remove zygotic embryos for use as explants.

Immature zygotic embryo explants were cultured on semisolid induction maintenance medium (IMM; Andrade and Merkle 2005) supplemented with three different concentrations of picloram: 0.414 μM (0.1 mg/L, low auxin treatment), 0.828 μM (0.2 mg/L, reference treatment), or 0.166 μM (0.4 mg/L, high auxin treatment). IMM is a modified woody plant medium (WPM; Lloyd and McCown 1980) with 30 g/L sucrose, 0.5 g/L filter sterilized L-glutamine, and gelled with 3 g/L Phytigel (Sigma). Media was dispensed in 60 mm plastic Petri dishes. In addition to culturing whole zygotic embryos (uncut treatment), we also tested excising the cotyledons from the embryo axis with a scalpel and culturing the cotyledons and axis separately (cut treatment). In both cases, three embryos were cultured per Petri plate. For each source tree (genotype), five repetitions (Petri plates) were used per each auxin treatment and whole embryo/cotyledons-cut from axis treatment, for a total of 15 embryo explants for each genotype x auxin

treatment x embryo cutting treatment combination. Cultures were incubated in the dark at 25° C. After one month, cultures were transferred to fresh plates of IMM with the same auxin treatment. Explants remained on these plates and the numbers of explants that showed evidence of embryogenesis induction (Fig. 1) were transferred to individual plates as developed, and all remaining culture units were scored after four months. Petri plates were also monitored for potential contamination, and contaminated plates were immediately discarded, and scored as 0 for GLM analysis. Each newly identified embryogenic culture was transferred individually to its own plate of IMM with 0.828 μM picloram and maintained in the dark at 25° C with transfer to fresh medium every three weeks.

To evaluate treatment effects on induction, we used a binomial generalized linear model (GLM) with a logit link function. The binary response variable was whether an embryo produced morphogenic callus (1=yes, 0=no). Fixed effects included auxin concentration, explant condition (cut or uncut), and their interaction. The reference treatment was 0.828 μM picloram with uncut embryos. Source tree was excluded from final model because its effect was not significant. Post hoc pairwise comparisons were conducted using estimated marginal means (EMM), with an $\alpha=0.05$. Analyses were performed in R (Version 4.4.2) (R Core Team 2024) using emmeans (Lenth 2025), ggplot2 (Wickham 2016), ggthemes (Arnold 2024), and grid (R Core Team 2024).

Fig. 1 Oregon ash embryogenic culture line DGRC04-UC2 producing morphogenic callus and repetitive embryos (circled), following initiation in 2024. This culture was initiated from an uncut zygotic seed embryo from tree DGRC04 on semisolid induction-maintenance medium with 0.828 μM picloram. Bar = 1 mm



Experiment 2: Auxin effects on culture proliferation

Previously established Oregon ash embryogenic culture lines also sourced from Cottage Grove, Oregon, were used to test the effects of different auxin treatments in semisolid medium on proliferation yields. Proliferation is a critical step in SE due to its potential to exponentially increase the number of embryos, and auxin type can strongly influence proliferation success by directing different developmental pathways within plant cells. In addition to two Oregon ash embryogenic culture lines (EVR01-1; EVR01-2) established in 2023, two embryogenic culture lines of each of green ash (AMPA-2; AMPB-1) established in 2022, and white ash (LA102-2; LA112-10), established in 2015 and 2018, respectively (Merkle et al. 2023) that had been maintained since established, were tested. Approximately 0.100 g of clumped cell starting material was plated onto semisolid IMM with the following auxin treatments: NAA (1-Naphthaleneacetic acid), picloram (reference treatment), and 2,4-D (2,4-dichlorophenoxyacetic acid), all at the same concentration (0.2 mg/L) in 60 mm plastic Petri plates. For each species and auxin treatment, four repetitions (Petri plates) were used. Culture units (inoculated Petri plates) were then incubated in the dark at 25° C for three weeks, then transferred to fresh Petri plates for three cycles, continuing in the same conditions (dark at 25° C) throughout each cycle. Following the third transfer, culture material was allowed to proliferate for an additional three weeks. In a lit, sterile fume hood, cultures were collected from the surface of the semisolid media using a spoonula and transferred to a sterile 125 mL flask with a sidearm marked with 1 mL graduations. Twenty mL of liquid embryo development medium (EDM; same as IMM, but with no PGRs) was added to the sidearm flask and the flask was swirled to suspend the culture material. Then, the settled cell volume (SCV) of culture material in each flask was measured by tipping the suspension into the graduated sidearm and allowing 5 min for the cells and cell clumps to settle before recording the delineated mL measurement. The number of embryos produced from each culture unit were counted using a hand tally counter.

Culture proliferation data were assessed using ANOVA with $\alpha=0.05$. The response variable was number of somatic embryos produced per culture unit. Fixed effects included auxin treatment and genotype. Post hoc comparisons among treatment means were performed using emmeans. Analyses and visualizations were conducted using dplyr (Wickham et al. 2023), emmeans (Lenth 2025), ggplot2 (Wickham 2016), and lmttest (Zeileis and Hothorn 2002) in R version 4.4.2 (R Core Team 2024).

Experiment 3 A: ABA effects on culture proliferation

The effects of different concentrations of abscisic acid (ABA) on proliferation of Oregon ash embryogenic material in suspension culture were assessed. Because ABA can influence both proliferation and early embryo development, mature somatic embryo production was used as the primary metric of proliferation success.

Approximately 0.05 g of embryogenic cell material from two genotypes (DGRC04-C1 and EVR01-2), maintained on semisolid IMM with 0.828 μ M picloram, were inoculated into 15 mL of liquid IMM in 50 mL Erlenmeyer flasks. Four IMM treatments were tested: a control (0.828 μ M picloram only), and three experimental treatments with added ABA concentrations of 0.1 μ M, 1.0 μ M, or 10.0 μ M. Each genotype $\times$ treatment combination was replicated three times. Cultures were incubated on a rotary shaker at 90 rpm in the dark at 25 °C, with medium replaced every two weeks. After the second feeding, cultures proliferated for an additional two weeks before assessing embryo production.

To quantify mature embryo production, cultures were size-fractionated using nested Collector™ sieves (860 μ m and 38 μ m). Cell clumps between these sizes were collected, backwashed into sidearm flasks with EDM, and incubated for maturation on semisolid EDM in the dark at room temperature for ~45 days. Number of cotyledonary embryos produced per flask was counted and used as the response variable to evaluate proliferation success under each treatment. Elongated embryos retained on the 860 μ m sieve were counted but not used for further experimentation. Remaining fine cell material was filtered using 30 μ m Nitex mesh and used for somatic seedling production as described in the Somatic Embryo Germination and Conversion section.

Levene test revealed unequal variance in the data ($p<0.05$). To account for this, Welch's ANOVA was used to evaluate the effect of ABA treatment on embryo production and assess genotypic differences in embryo production. In both cases, statistical significance was determined at $\alpha=0.05$. The response variable was the number of mature somatic embryos produced per flask. Analyses were conducted in R version 4.4.2 (R Core Team 2024) using the packages car (Fox and Weisberg 2019), dplyr (Wickham et al. 2023), ggplot2 (Wickham 2016), and WRS2 (Mair and Wilcox 2020).

Experiment 3B: ABA effects on somatic embryo maturation

Here, the effects of different concentrations of abscisic acid (ABA) on the production of mature Oregon ash somatic embryos from suspension cultures plated on semisolid medium were assessed. The tested treatments were EDM

with no ABA (control), 0.10 μM ABA and 0.20 μM ABA. Each genotype $\times$ treatment combination was replicated three times (three Petri plates). Two established Oregon ash lines (EVR01-1; EVR01-2) were used as starting material for this experiment. Approximately 0.05 g of PEMs were inoculated into 15 mL of liquid IMM supplemented with 0.828 μM picloram in 50 mL Erlenmeyer flasks and grown for 45 days. Suspensions were maintained on gyratory shakers in the dark at 22° C. Cultures were fed every 2 weeks by removing the old medium and replacing it with approximately 15 mL of fresh IMM. The same size-fractionation technique described for Proliferation Experiment 2 was also used here. The remaining fine culture material was collected and excess liquid IMM was drained using a Büchner funnel. This material was collected on nylon mesh and the nylon mesh with cell clumps was then placed atop semi-solid EDM in plastic Petri plates. Plates were incubated in the dark at 22° C. After 45 days, somatic embryos that developed were counted, picked, and transferred to new Petri plates with fresh semisolid EDM with the same ABA treatments and allowed to continue to mature directly on EDM. After two weeks, the numbers of mature somatic embryos produced from each treatment were recorded.

ANOVA was used to determine any significant effects of the three ABA EDM treatments on embryo production with $\alpha=0.05$. Analyses were conducted in R (Version 4.4.2) (R Core Team 2024) using ggplot2 (Wickham 2016) and lmttest (Zeileis and Hothorn 2002).

Results

Experiment 1: Auxin and explant effects on embryogenesis induction

This experiment tested the effect of different concentrations of picloram, in combination with excising the cotyledons from the zygotic embryo axes, on the induction of embryogenesis in Oregon ash. An initial binomial GLM indicated that source tree did not have a significant effect on induction probability (all associated p -values > 0.05). A second binomial GLM with source tree removed as a factor, and relevelled with reference treatments of uncut embryos, and 0.828 μM picloram observed several differences amongst treatment combinations on induction. To further analyze GLM results, estimated marginal means model with 95% confidence intervals were used to compare probabilities of morphogenic callus production across each of these treatments. At 0.414 μM picloram, cut embryos had a higher probability of induction ($15\% \pm 4.6$) than uncut embryos ($5\% \pm 2.8$). At 0.828 μM picloram, uncut embryos had a higher probability of induction ($16\% \pm 4.9$) than cut embryos (8%

± 3.6). At 0.166 μM , induction rates were uniformly low for both treatments ($3\% \pm 2.3$). Collectively, these results indicate that the positive effect of removing cotyledons is concentration-dependent, enhancing induction only at the lowest picloram concentration tested of 0.414 μM by 15%. In addition, we observed Oregon ash somatic embryos frequently enlarged and germinated precociously, producing chlorophyll and taproots before completing maturation (Fig. 2).

Experiment 2: Auxin effects on culture proliferation

A two-way ANOVA detected an interaction between species and auxin type ($F=3.6$; $df=4$; $p=0.02$), indicating that auxin response varied among species. Estimated marginal means (EMMs) with pairwise contrasts showed that proliferation did not differ among auxins for green ash or white ash (all $p>0.05$). In Oregon ash, however, 2,4-D significantly improved proliferation compared to picloram ($p=0.031$; estimate=1.75; $t=2.7$). Overall, these results suggest that auxin type influences proliferation in a species-specific manner, with 2,4-D enhancing proliferation in Oregon ash relative to picloram (Fig. 3).

Effect of ABA concentration during suspension on proliferation and somatic embryo development

Experiment 3 A: ABA effects on culture proliferation

Suspension cultures of two Oregon ash genotypes (DGRC04-C1 and EVR01-2) were evaluated for proliferation under varying ABA concentrations over a 45-day period. Accordingly, Welch's ANOVA was used and showed no significant effect of ABA concentration on proliferation ($F=0.07$, $df=3.00$, $p=0.98$) but identified a strong genotype effect ($F=16.16$, $df=1.00$, $p<0.01$) with genotypes producing different amounts of cell material (Fig. 4). A bootstrapped one-way ANOVA further detected a significant genotype $\times$ ABA interaction ($p=0.01$; $\eta^2=0.46$; effect size=0.68). Post-hoc comparisons (lincon, WRS2) showed DGRC04-C1 proliferated significantly less than EVR01-2 in the control treatment ($\text{psihat}=-6.92$, $p=0.002$) but more under 0.01 μM ABA ($\text{psihat}=6.83$, $p=0.005$).

Subsequent embryo development did not differ significantly among ABA treatments ($F=2.68$, $df=3$, $p=0.11$), nor was a genotype effect detected (Welch's ANOVA: $F=3.64$, $df=1$, $p=0.11$). The genotype $\times$ treatment interaction was also nonsignificant ($p=0.10$; $\eta^2=0.22$; effect size=0.47). Average embryo counts are shown in Fig. 5, with representative morphology in Fig. 6.

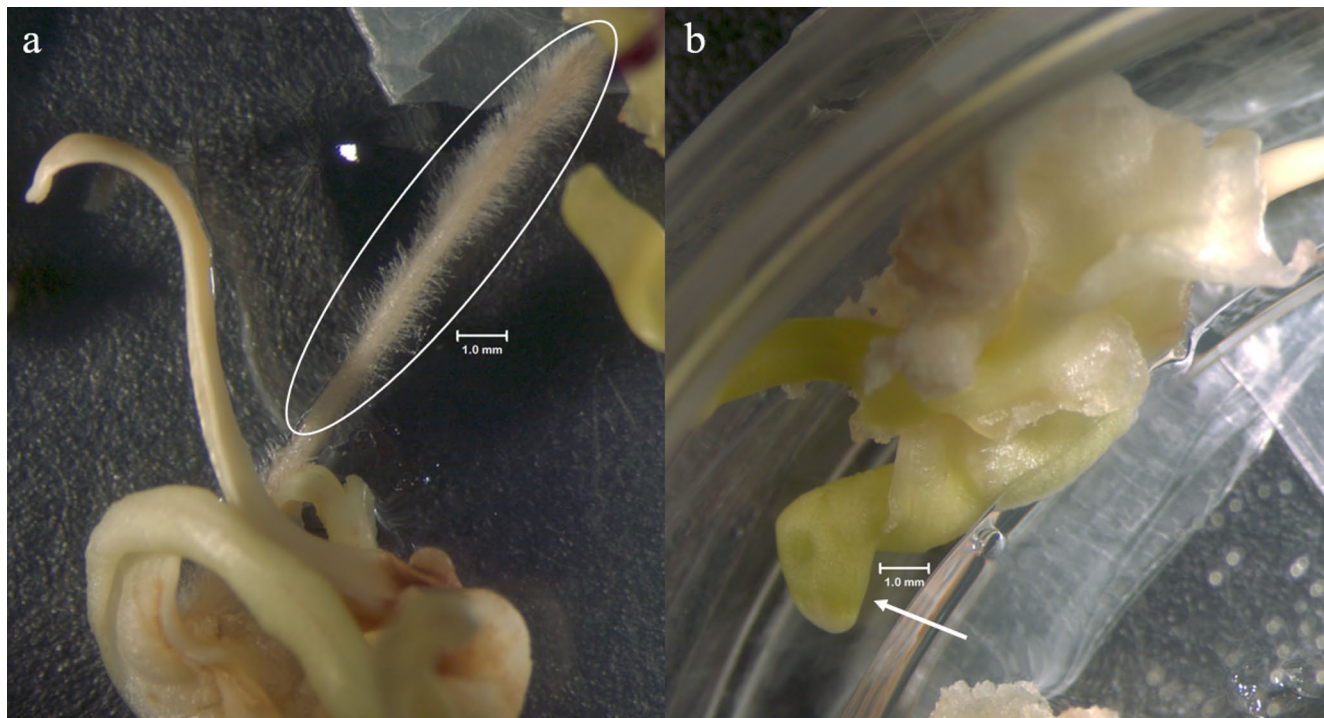


Fig. 2 Precocious germination of Oregon ash (DGRC04-UC1) somatic embryos on IMM with $0.828\mu\text{M}$ picloram, 3 months after initiation. **a.** Development of a tap root (circled) **b.** Production of chlorophyll

(arrow). These changes would normally be expected during later SE stages of germination and conversion, rather than so early in the SE process. Bar = 1 mm

After the 45-day liquid culture period, size-fractionated cultures were transferred to semisolid EDM to evaluate maturation. EVR01-2 failed to produce mature somatic embryos under any ABA treatment, so comparisons were limited to DGRC04-C1. For this genotype, there was a significant overall effect of ABA treatment on mature embryo production ($H=9.31$, $df=1$, $p=0.025$). However, Bonferroni-adjusted Wilcoxon tests did not detect significant differences among individual treatments (all $p>0.05$).

Experiment 3B: ABA effects on somatic embryo maturation

ANOVA indicated no statistically significant differences between genotypes ($p=0.50$), treatments ($p=0.96$), or the interaction between the two variables ($p=0.46$), failing to reject the null hypothesis that the tested ABA treatments would not affect production of Oregon ash embryos.

Discussion

This study establishes feasible propagation methods for Oregon ash via SE and explores the different species response in-vitro to important plant compounds (hormones, auxins) of interest. Our Oregon ash initiation experiment

found the varied effect of cutting at different picloram concentrations. We found that cutting embryos only increases induction success at lower picloram concentrations, suggesting a dynamic relationship between wounding and stimulating plant response in-vitro. We compared the effect of auxin type on proliferation of Oregon ash to white and green ash species culture lines to understand differential tissue culturing response between ash species. Our results determined that culture response to plant auxins commonly used in tissue culture such as picloram, NAA, and 2,4-D; varied depending on ash species. Oregon ash responded significantly better to 2,4-D, with higher cell proliferation compared to the other auxins, while green ash and white ash proliferation did not vary across auxin type. We found the benefit of ABA supplementation during SE proliferation of Oregon ash varied based on genotype.

Oregon ash SE induction has not been previously published in literature, though preliminary cultures in our lab showed induction rates below 2% (Merkle Lab, unpublished data). In our first experiment, we demonstrate that the effectiveness of embryo dissection depends on picloram concentration within the gelled medium. Our results indicate that the positive effect of separating cotyledons from the embryo axis during induction depends on picloram concentration. Cutting embryos improved induction at the lower concentration $0.414\mu\text{M}$ picloram, whereas higher concentrations

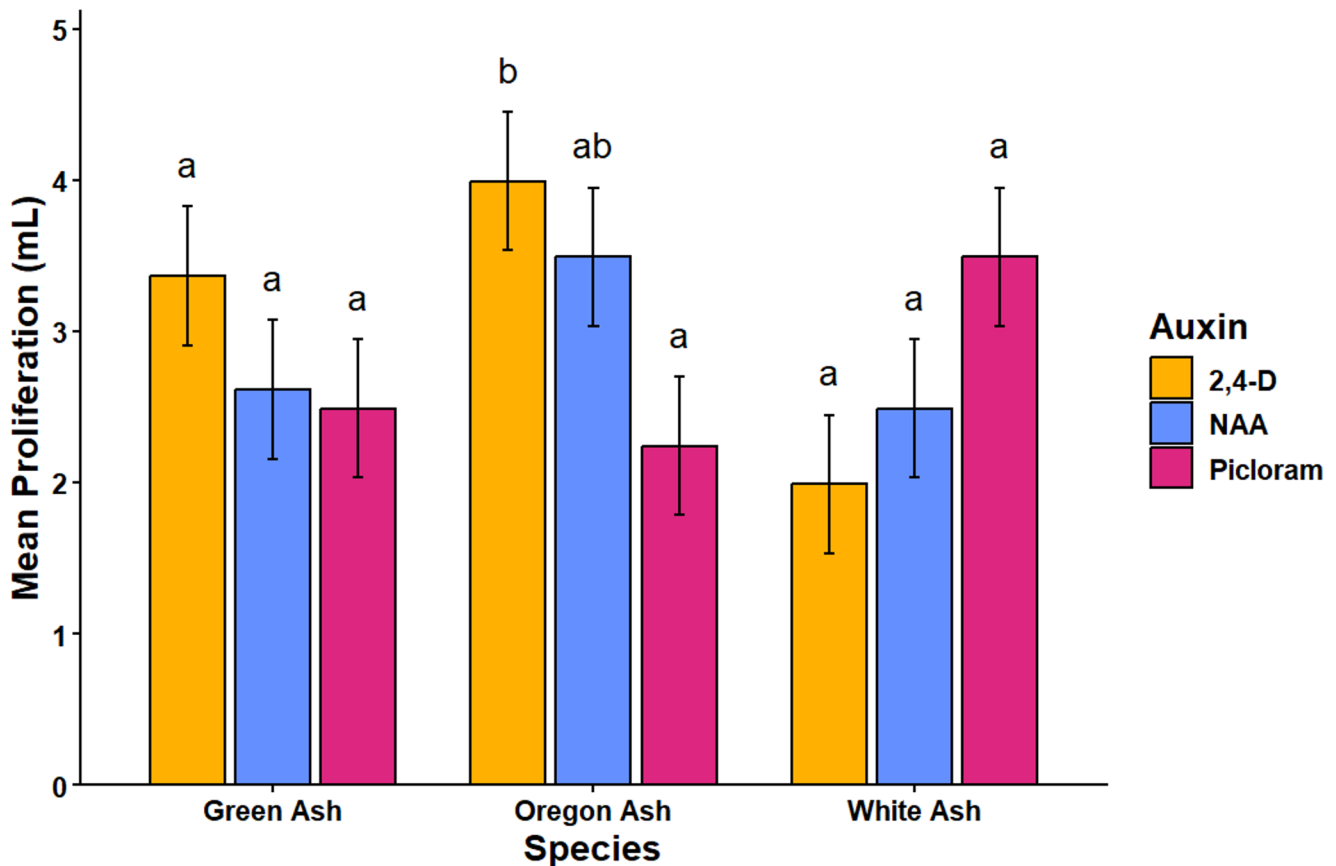


Fig. 3 Mean proliferation of embryogenic suspension cultures from green ash, Oregon ash, and white ash in response to 2.0 mg/L of three different auxin treatments (2,4-D, NAA, picloram) added to semisolid IMM. Different letters indicate significant relationships between treatments, with Sidak-correction applied for multiple comparisons, with $\alpha=0.05$. Error bars represent 95% confidence around sample mean.

(0.828 μ M and 0.166 μ M) cutting were less effective. At 0.414 μ M picloram, cutting embryos significantly improved morphogenic callus production, whereas at 0.828 μ M, uncut embryos had a higher likelihood of induction. Both treatment combinations outperformed other tested conditions, while the highest picloram concentration (0.166 μ M) was not beneficial. Picloram was selected based on previous *Fraxinus* studies showing higher induction rates than 2,4-D (Merkle et al. 2023), and lower picloram concentrations have been associated with improved induction and proliferation (Dai et al. 2015; Karami et al. 2007).

Wounding explants through cutting increases stress, leading plant cells to change their molecular programming, initiating cell proliferation, the second stage of SE (Méndez-Hernández et al. 2019). Although explant wounding has not been reported for SE in North American ash, dissection of seed explants has improved induction outcomes in various plant species, such as Paraguay peanut (*Arachis Paraguariensis*) Aina et al. 2012), Chinese tallow (*Sapium sebiferum*) Hou et al. 2015) and *Arabidopsis thaliana*

Letters indicate significant differences ($p < 0.05$) from across all treatment and genotype combinations using compact letter display (CLD); so that bars sharing a letter are not significantly different from one another. White ash and green ash proliferation did not vary across the auxin types tested (denoted “a”). Oregon ash exhibited a unique species response to 2,4-D (denoted “b”)

(Horstman et al. 2017). Not only have studies found the benefit of embryo cutting to induction, separating the cotyledons from the embryo axis may also lead to induction happening sooner, with cut embryos producing globular embryo structures as early as one week post culture initiation (Aina et al. 2012). Thus, by wounding embryo explants during SE initiation, we can improve the productivity of SE as a useful clonal propagation technique, as several studies have now evidenced. Due to natural variation of plant response to stressors such as desiccation or physical injury, further exploration of these wounding effects could yield important insights for improving SE protocols in both ash and other woody or crop species.

Given that this is the first reported account of SE in Oregon ash, we observed differences in culture response between Oregon, green, and white ash. SE in green ash and white ash has been successfully established, with particular focus on these species because green ash is the most abundant, and white ash is the most economically valued, of the *Fraxinus* genera (Li et al. 2014). We tested three plant auxins that

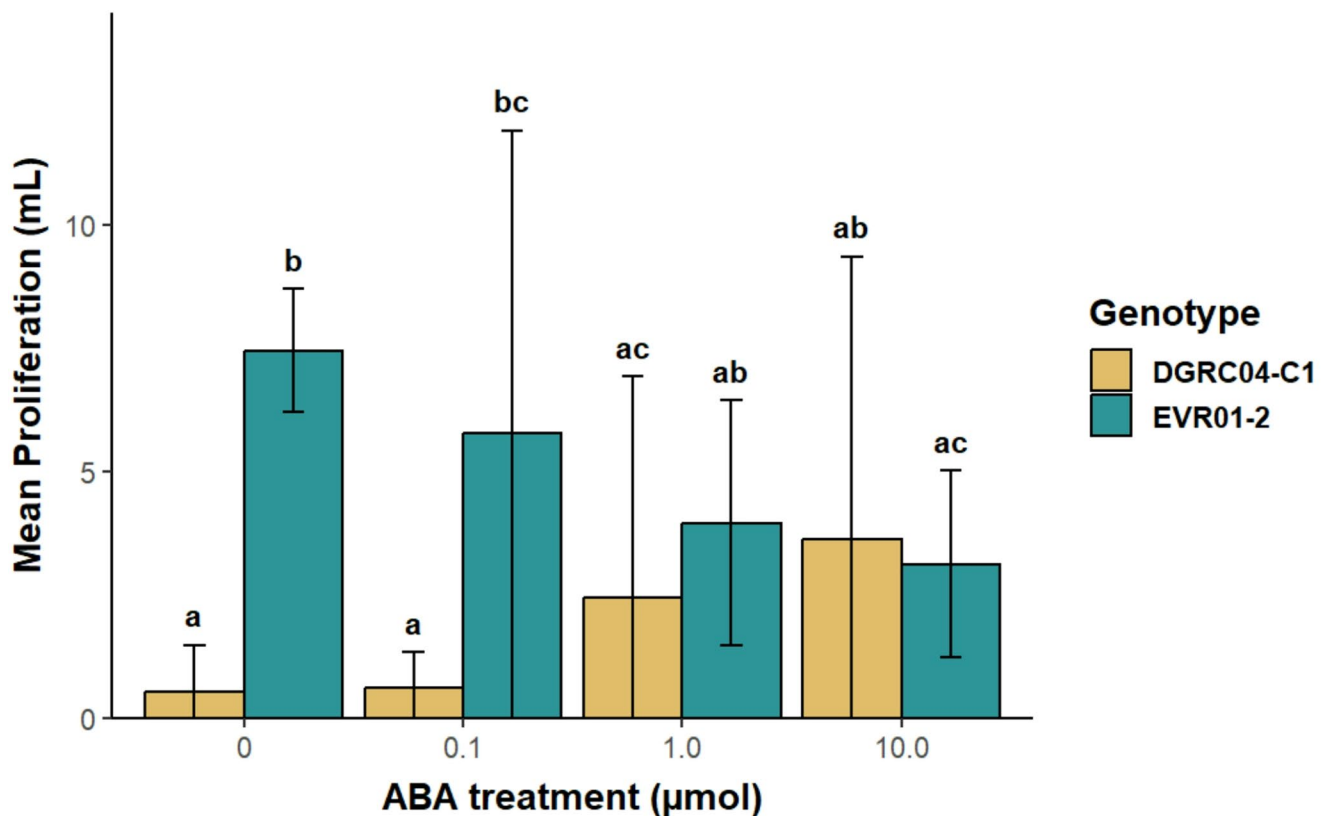


Fig. 4 Mean proliferated cell material for two Oregon ash genotypes (DGRC04-C1; EVR01-2) across the tested concentrations of ABA during suspension culture. Error bars represent the 95% confidence around sample mean. Letters indicate significant differences ($p < 0.05$) across all treatment and genotype combinations using compact letter

have been used with SE in white ash and green ash prior; NAA, 2,4-D, and picloram on cell proliferation (Li et al. 2014; Merkle et al. 2023; Preece et al. 1989; Tull et al. 2022). We found that cell proliferation of white ash and green ash remained the same across the auxin types at the same concentration (0.2 mg/L), while Oregon ash proliferated more with auxin 2,4-D (Exp. 2). Successful SE initiation in green ash has been reported on media supplemented with 2 mg/L 2,4-D, when tested across a variety of basal medium x PGR treatment combinations (Li et al. 2014). While ash transformation experiments compared picloram and 2,4-D at varied concentrations (0.2, 2.0, 4.0 mg/L) reported successful green ash induction only occurred at the lower concentration of 0.2 mg/L picloram tested ($=0.828\mu\text{M}$; Tull et al. 2022). These contrasting results emphasize the complexity of plant-auxin relationships. Not only does auxin type provoke differential responses across species in tissue culture, but this effect also varies depending on the developmental stage of SE (Li et al. 2014; Sofiari et al. 1997). Sofiari et al. reported out of seven cassava (*Manihot esculenta*) cultivars that successful SE initiation occurred only on 2,4-D supplemented induction media, with similar findings also reported in giant

timber bamboo (*Bambusa oldhamii*) Chang and Ho 1997). Although NAA was not found beneficial during SE induction, embryos matured at a faster rate on NAA during the second SE stage of proliferation, compared to 2,4-D (Sofiari et al. 1997). These different observed effects between auxin types throughout the key stages of SE (induction, proliferation, maturation, germination and conversion) emphasize the need for continued, expanded research, identifying these discrepancies to further optimize clonal propagation across ash, and other woody species of interest.

While specific treatments were not experimentally compared for their effects on Oregon ash somatic embryo germination and conversion, mature embryos from multiple Oregon ash culture lines were successfully germinated and converted to somatic seedlings within 90 days. These embryos were transplanted into Germination Medium (GM) in GA-7 vessels following protocols described in Merkle et al. 2023. To demonstrate the regenerative capacity of this species, pictures of Oregon ash somatic embryos and plantlets during this stage are provided in Supplementary Figures (Figs S1, S2) were derived from Oregon ash culture line (EVR08-2) which, while not included in the experiments

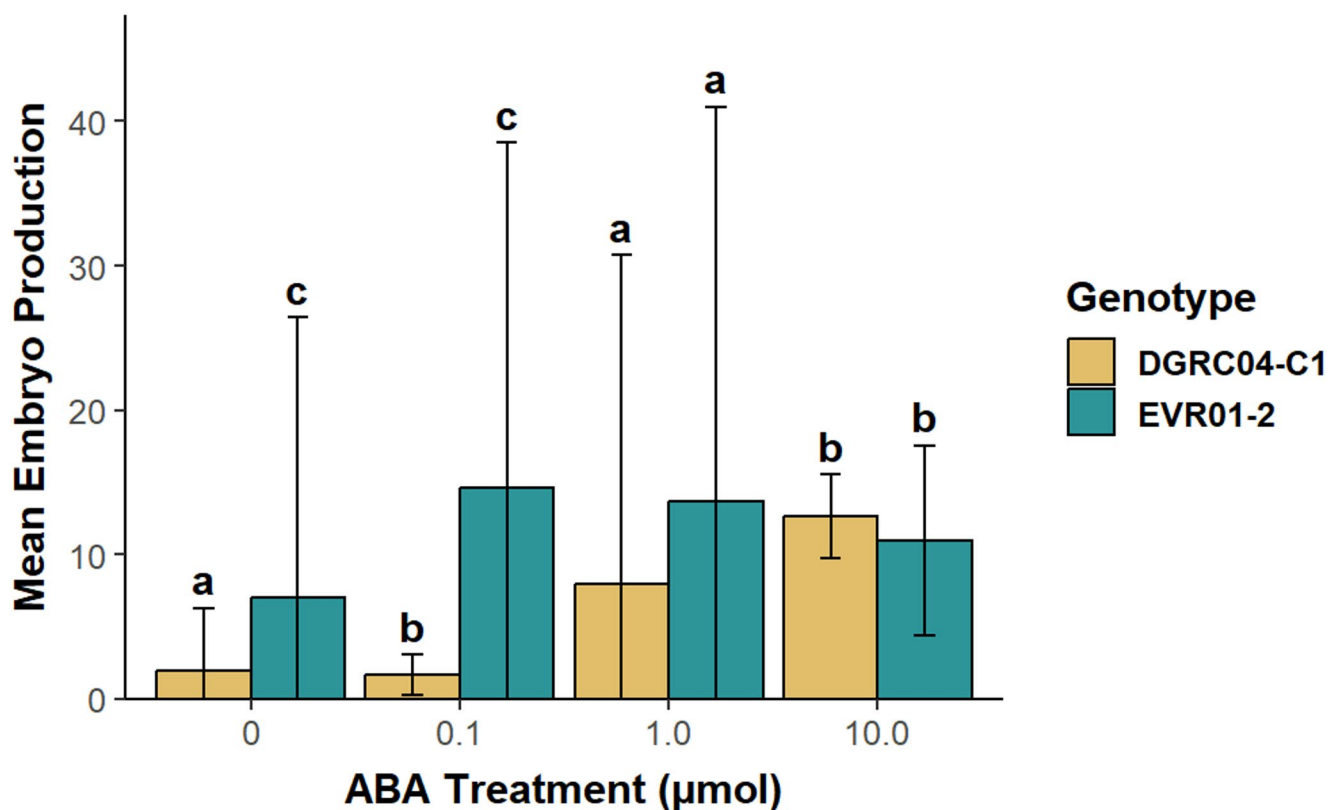


Fig. 5 Mean number of cotyledonary embryos produced per flask for two Oregon ash genotypes (DGRC04-C1; EVR01-2) across four ABA treatments in liquid suspension. Error bars represent the 95% confidence around sample mean. Letters indicate significant differences ($p < 0.05$) across all treatment and genotype combinations using

compact letter display (CLD); so that bars sharing a letter are not significantly different from one another. DGRC04-C1 exhibited its lowest embryo production at 0.1 μM and control treatments (denoted by “a” and “b”), while EVR01-2 showed significantly higher production at those same levels (denoted by “c”)

of this study, underwent identical germination and conversion protocols. Throughout maintenance of Oregon ash tissue culture, we observed that Oregon ash somatic embryos frequently exhibited precocious germination, germinating before completing full development; a phenomenon not as commonly observed in the other ash species mentioned in vitro. Precocious germination reduces process efficiency and produces lower-quality plantlets (Hugo Cota-Sánchez 2018).

We examined ABA's influence on two critical SE stages in Oregon ash: proliferation and maturation. Liquid suspension culture is an efficient method for clonal propagation of embryogenic cells during the second SE stage of proliferation (von Arnold et al. 2002). Proliferation allows exponential multiplication of embryogenic material, underscoring SE's potential as a clonal propagation tool for effective reforestation initiatives (Merkle et al. 2023). We found that the efficacy of ABA treatment on cell proliferation depended on genotype, as Oregon ash cell line EVR01-2 had higher proliferation compared to DGRC04-C1 in the control treatment (no ABA), but DGRC04-C1 proliferated much more in 0.1 μM ABA-supplemented media (Exp. 3 A).

Equally important in SE is proper embryo development and maturation to ensure successful germination and establishment of high-quality plantlets (von Arnold et al. 2002). Our results again emphasize genotype's determinant role in plant response. For Oregon ash culture line DGRC04-C1, we found the significant overall effect ABA has on the production of mature somatic embryos but did not identify differences between the ABA treatments at the concentrations we tested (Exp. 3B). Our results suggest the fluctuating effects of ABA on plant growth and development is ultimately influenced by genotype, agreeing with several studies that have investigated ABA's effect during plant SE (Chen et al. 2020; García-Martín et al. 2005; Yang et al. 2024). Exogenous application of ABA improved induction and proliferation success rates of SE in galcham oak ([*Quercus aliena*] Yang et al. 2024). Similarly, supplementing IMM with 1 μM ABA improved somatic embryo maturation of cork oak ([*Quercus suber*] García-Martín et al. 2005), as well as holm oak (*Quercus ilex*) maturation and subsequent germination (Mauri and Manzanera 2004). Researchers found the exogenous application of ABA during maturation not only increased embryo production, but germination and

Fig. 6 Cotyledon-stage Oregon ash somatic embryos collected on 860 μm pore size sieve during fractionation step of the 45-day liquid suspension process



plantlet establishment of Persian walnut (*Juglans regia*) Vahdati et al. 2008) as well. The wide observed effect of ABA on determining successful plantlet production using SE emphasizes the need for continued research studying this hormone's potential to further improve the feasibility of SE clonal propagation of plants (Yang et al. 2024).

Future studies should explore a broader range of genotypes and ABA concentrations with higher replication to draw conclusive results on the effect of ABA concentration on both proliferation and embryo maturation. Not only has ABA been identified as a key promoter of plant dormancy and germination, ABA also regulates stomatal conductance and osmotic potential, preventing precocious germination (Chen et al. 2020; Xu et al. 1990). Therefore, ABA supplementation during both proliferation and maturation may enhance Oregon ash SE by mitigating premature germination and improving embryo quality. Targeted studies examining ABA's specific effect on precocious germination would be particularly valuable for continuing to optimize this technique for clonal propagation of Oregon ash.

The unique effect 2,4-D suggests having on Oregon ash should be further examined in studies with consistent units and subsequent concentration conversions, to consider varied auxin strengths. In this study, treatments were compared using a mass concentration of 2.0 mg/L. However, because these plant compounds have distinct molecular structures and weights, this approach does not account for differences in molarity. With molar concentration considered, the appropriate treatment doses would be 0.00186 μM NAA, 0.905 μM 2,4-D, and 0.828 μM picloram. To more accurately evaluate the physiological effects of various auxins on SE in ash species, future research should utilize molar concentrations μM to ensure an equal comparison of tested treatments. Standardizing concentration reporting in this manner will help clarify whether the observed differences in SE stages are a result of auxin potency or stoichiometric variance. Furthermore, given the highly variable tissue culture responses observed across the genus, expanding these studies to include a broader range of ash species is necessary to observe differential species responses in tissue culture.

To expand the broader applicability of these findings, future work should include individuals from across the native range of Oregon ash. The seed explants used in this study were collected near Cottage Grove, Oregon, by US Forest Service collaborators. Melton et al. (2025) report genotypic variation linked to locality, emphasizing the importance of preserving germplasm from diverse populations to maintain a broad conservation pool of potential emerald ash borer (EAB)-resistant candidates for reforestation. The need for a wide genotype pool is further supported by our results and previous studies, which found genotype to significantly influence in vitro culture response (Glock and Gregorius 1986; Hanke 1994; Merkle et al. 2023). Our results evidence that SE is a feasible propagation technique for Oregon ash, and implementation at a large scale will require continued refinement of our understanding of these relationships. Thereby, the more feasible SE becomes for clonal propagation for reforestation initiatives for foundational tree species like Oregon ash (Melton et al. 2025).

Conclusion

EAB populations continue to spread across the western coast of North America, initiating a stark decline to forest and urban landscapes where Oregon ash is present. By exploring how this species responds to SE protocols now, we can optimize them for propagating and preserving a large pool of genotypes from across the native range. When EAB-resistant Oregon ash genotypes are eventually identified, SE can be employed to multiply these genotypes for reforestation. It is important to continue this exploration of propagation of Oregon ash via SE to propel reforestation efforts for not only Oregon ash, but other North American ash species as well. This research demonstrates the potential of SE as a clonal propagation technique to reforest *Fraxinus* in both forest and urban landscapes across North America. This work is the first published report of somatic embryogenesis in this species, and we hope it will serve as the basis for future research to optimize this technique with other ash species, as EAB continues to destroy ash populations throughout North America.

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Declarations

Competing Interests The authors have no financial or proprietary interest in any material discussed in this article.

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