Application of somatic embryogenesis for development of emerald ash borer-resistant white ash and green ash varietals

Scott A. Merkle[1] · Jennifer L. Koch[2] · A. Ryan Tull[1] · Jessica E. Dassow[1] ·
David W. Carey[2] · Brittany F. Barnes[1] · Mason W. M. Richins[1] · Paul M. Montello[1] ·
Kira R. Eidle[1] · Logan T. House[1] · Daniel A. Herms[3] · Kamal J.K. Gandhi[1]

Abstract

Emerald ash borer (*Agrilus planipennis*; EAB) has devastated populations of ash (*Fraxinus* spp.) trees in dozens of U.S. states and Canada over the past few decades. The continued survival of scattered ash trees known as "lingering ash" in heavily infested natural stands, however, offers evidence of genetic resistance or tolerance to EAB. These surviving or "lingering" ash individuals may form the basis for reforestation programs in EAB-impacted areas, and clonal mass-propagation of these genotypes can help accelerate these efforts. Between 2013 and 2018, we initiated embryogenic cultures by culturing immature zygotic embryos from open-pollinated (OP) seeds collected from several surviving white ash and green ash trees in Michigan and Pennsylvania. In addition, in 2018, we initiated cultures from crosses made between lingering green ash parents from the USDA Forest Service ash breeding program in Ohio. Somatic embryos were produced by growing cultures in liquid suspension, followed by fractionation and plating on semisolid medium to produce developmentally synchronous populations of somatic embryos. Somatic embryo germination and conversion were enhanced by a combination of pre-germination cold treatment and inclusion of activated charcoal and gibberellic acid in the germination medium. Ash somatic seedlings derived from OP explants grew rapidly following transfer to potting mix and somatic seedlings representing nine ash clones were acclimatized, grown in the greenhouse and planted in a preliminary field test, along with EAB-resistant Manchurian ash (*F. mandshurica*) and EAB-susceptible control seedlings. Somatic seedlings have now been produced from cultures that originated from seeds derived from the progeny of lingering green ash parents and an ex vitro germination protocol has shown some promise for accelerating early somatic seedling growth. Results of this research could provide the basis for scaled-up production of EAB-resistant ash varieties for seed orchard production for forest restoration and cultivar development for urban tree restoration.

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Somatic embryogenesis provides a promising, scalable approach for clonal propagation of green ash and white ash genotypes derived from lingering ash parents with potential genetic resistance to emerald ash borer for reforestation.

Introduction

Ash trees, in particular white ash (*Fraxinus americana*), green ash (*F. pennsylvanica*), and black ash (*F. nigra*), are among the most abundant hardwood species in the eastern U.S., and are integral to the ecology of many ecosystems in the region. These trees are highly valued as urban tree and landscape species (Hardin et al. 2001) and ash wood is used for a variety of products, including furniture, tool handles and baseball bats (Panshin and de Zeeuw 1970). All North American ash species are under threat of extirpation from their native ranges by the emerald ash borer (EAB; *Agrilus planipennis)*, an exotic wood-boring beetle that has already destroyed millions of ash trees in North America (Herms and McCullough 2014). Mortality rates of trees greater than 10 cm DBH in some stands of ash have reached up to 99% (Knight et al. 2013, Smith et al. 2015), with cascading effects on the ecologies of the areas where ash trees once made up a substantial proportion of the forest canopy (Gandhi et al. 2014, Klooster et al. 2018).

Studies of areas long-infested with EAB have identified very low percentages of surviving, apparently healthy ash trees, referred to as "lingering ash." Lingering ash are defined as mature trees with DBH greater than or equal to 10 cm, that have survived at least two years following the death of 95% or more of ash in the stand, and have retained healthy canopies (Klooster et al. 2014, Koch et al. 2015). The survival of these trees may be due to natural genetic variation within the species. Thus, lingering ash constitute a potential source of resistance genes that could be used in selection and breeding programs to produce trees with improved resistance to EAB (Koch et al. 2015, Pike et al. 2021). EAB egg bioassays conducted at the USDA Forest Service Forestry Sciences Laboratory in Delaware, Ohio have demonstrated that some lingering green ash selections have significant differences in larval survival and development (Koch et al. 2015). Scientists have already used these lingering green ash parents to produce several full-sibling families for EAB resistance testing. Bioassay evaluation of seedling progeny from these families showed that 15 to 40% of full-sib lingering ash progeny had a more effective defensive response to EAB than either parent, indicating a polygenic or quantitative model of inheritance (Koch et al. 2020).

The impact of breeding among lingering ash parents could be greatly leveraged for accelerated ash restoration by combining this approach with clonal mass propagation programs. The combined effort would not only facilitate testing of putatively resistant material via clonal tests, but could also accelerate the establishment of EAB-resistant ash varieties for urban and landscape restoration plantings by government agencies, municipalities and private landowners. While some woody species are amenable to clonal propagation using rooted stem cuttings, cuttings of ash species have been described as difficult or very difficult to root (Kramer and Kozlowski 1979, Hartmann et al. 1997), although some exceptions have been reported. Rooting of *F. excelsior* (common ash) cuttings at rates up to 63% was reported by Jinks (1995). Douglas et al. (2017) achieved rooting rates of up to 80% for multiple ash-dieback tolerant genotypes of the same species by first rejuvenating mature trees using micropropagation (in vitro axillary shoot multiplication) to establish mother

plants, which were in turn hedged to produce crops of cuttings. While other reports of ash propagation via axillary shoot multiplication have been rare (Arrillaga et al. 1992, Preece et al. 1987), in vitro propagation via adventitious shoots has been reported for green ash (Du and Pijut 2008), white ash (Bates et al. 1992, Palla and Pijut 2011), and pumpkin ash (*F. profunda*; Stevens and Pijut 2012).

Scalable clonal propagation systems employing somatic embryogenesis have been reported for multiple hardwood forest tree species (Merkle and Cunningham 2011). Among North American ash species, somatic embryogenesis has been reported for white ash (Preece et al. 1989, Bates et al. 1992) and green ash (Li et al. 2014). Somatic embryogenesis has also been reported for several European and Asian ash species, including narrow-leafed ash (*F. angustifolia*; Tonon et al. 2001), common ash (Capuana et al. 2007), and Manchurian ash (*F. mandshurica*; Kong et al. 2012). Of these reports, only the culture systems described by Tonon et al. (2001) and Li et al. (2014) demonstrated the potential for scalable somatic embryo and somatic seedling production using suspension culture.

Here we report the application of somatic embryogenesis to clonally propagate progeny of lingering white ash and green ash parents, from both open-pollinated and control-pollinated seeds, as well as use of a scalable method for somatic embryo production employing suspension culture, and tests of treatments designed to enhance somatic embryo germination and conversion and early somatic seedling growth. We also report early survival and growth performance of somatic seedling-derived green ash and white ash trees representing clones of lingering ash progeny in a replicated field planting.

Table 1 Details of ash culture initiation experiments

Initiation year	Source trees or crosses	Seed collection dates	Explant type(s)	Plant growth regulator (PGR) treatments	Explants per date/ source tree or cross/explant type/ PGR treatment
2013	LA101 LA102 LA114 LA116	7/07/13 8/08/13 9/16/13	Whole seeds; Zygotic embryos	2 mg/L 2, 4-D 4 mg/L 2, 4-D	12
2015	LA102 LA111 LA112 LA113 LA115 LA116 LA132	8/11/15	Zygotic embryos	2 mg/L 2, 4-D	30
2018	#1-FA #2-FA #3-FA #4-FA #5-FP #6-FA	08/09/18	Zygotic embryos	2 mg/L 2,4-D 4 mg/L 2, 4-D 0.1 mg/L picloram	15–24
2018	DC6×DC5 DC4×DC2	7/25/18 8/14/18	Zygotic embryos	2 mg/L 2, 4-D 4 mg/L 2. 4-D 0.1 mg/L picloram	6–17

Materials and methods

Plant material and culture initiations

For culture initiations, immature samaras were collected from a variety of surviving green and white ash source trees in areas enduring EAB infestation by multiple cooperators in Michigan, Ohio, and Pennsylvania in three different years (2013, 2015 and 2018). Each year's culture initiations had different objectives and the tested induction treatments and source tree genotypes used varied among the years, so they are described separately. Table 1 summarizes all of the different seed sources, seed collection dates, explant types, plant growth regulator (PGR) treatments and explant numbers for each of the three years of culture initiations.

Fig. 1 Lingering white ash tree growing at Indian Springs Metro Park Farmland Trail, Oakland County, MI in August 2015. Trees at this site were used as seed sources for explants to initiate embryogenic cultures. Photo by Dan Herms

2013 culture initiations Samaras from four lingering female white ash trees located in Indian Springs Metro Park Farmland Trail, Oakland County, Michigan (designated LA101, LA102, LA114, and LA116; Fig. 1) were harvested by Ohio State University collaborators on July 7, August 8, and September 16, 2013, and shipped on cold packs via next day delivery to the University of Georgia, Athens, Georgia. On the final collection date, samaras were harvested from only two trees, 102 and 116, and no cultures were initiated from the harvested fruit due to weevil-infested seeds, aborted seeds, or zygotic embryos that appeared to be fully mature. The mother trees had canopy ratings of average to healthy and none of them showed signs of damage by EAB.

Samaras were surface disinfested by washing them in 70% ethanol for 20 s, 10% Roccal-D Plus (9.2% didecyl dimethyl ammonium chloride, 13.8% alkyl dimethyl benzyl ammonium chloride, 1% bis-n-tributyltin oxide; Pfizer) for 3 min, 70% ethanol for 20 additional seconds, 10% Roccal for 3 additional minutes, 50% Clorox (8.25% sodium hypochlorite) for 5 min, sterile deionized water for 3 min, and sterile 0.01 N hydrochloric acid (HCl) for 3 min, followed by three additional rinses in sterile deionized water of 3 min each. Following surface disinfestation, samaras were dissected to remove the seeds and half of the seeds were dissected to remove the zygotic embryos. Whole seeds or embryos dissected from seeds were cultured in 60 mm plastic Petri plates containing semisolid induction-maintenance medium (IMM; Andrade and Merkle 2005), which was a modified woody plant medium (WPM; Lloyd and McCown 1980), with 30 g/l sucrose, 1 g/l filter-sterilized L-glutamine, and either 2 (low auxin treatment) or 4 (high auxin treatment) mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and gelled with 3 mg/L Phytagel (Sigma). Four explants were cultured per Petri plate. For each collection date/source tree combination, three repetitions (Petri plates) were used per each auxin treatment/explant type (zygotic embryo versus embryo with seed coat) combination. Cultures were incubated in the dark at 25 °C. After 1 month, they were transferred to fresh IMM with the same concentration of 2,4-D. Explants showing evidence of embryogenesis induction after two months in culture (or in some cases, longer) were transferred individually to plates of IMM with 2 mg/L 2,4-D and maintained by transfer to fresh medium every three weeks.

2015 Culture Initiations This set of initiations was conducted to generate additional culture lines from two of the same lingering ash parents used for the 2013 initiations, plus five additional parents in the same Oakland County, Michigan population. Again, all source trees were female white ash trees (designated LA102, LA111, LA112, LA113, LA115, LA116 and LA132). Based on results from 2013, samaras were only collected on a single date (August 11). Shipping conditions, surface disinfestation, dissection and culturing were all as described above for the 2013 initiations, except all cultures were started from zygotic embryo explants cultured on IMM with 2 mg/L of 2,4-D. A total of 30 zygotic embryo explants was cultured (10 Petri plates with 3 explants per plate) for each of the seven source trees. After one month, explants were transferred to fresh medium with 2 mg/L of 2,4-D. Explants showing evidence of embryogenesis induction after two months in culture (or in some cases, longer) were transferred individually to plates of the medium with 2 mg/L 2,4-D and maintained by transfer to fresh medium every three weeks. Since the only "variable" in this initiation was source tree, no statistical analysis was performed on the embryogenesis induction data. Although different induction treatments were not tested, we are including

this information since some of the embryogenic culture lines resulting from this work were used in later germination and conversion experiments.

2018 Culture Initiations The culture initiation experiment in 2018 had two objectives: (1) to compare an alternative PGR treatment to previously used PGR treatments for embryogenesis induction, and (2) to attempt to initiate embryogenic cultures from seeds resulting from controlled pollinations between lingering ash individuals. For this experiment, open-pollinated samaras were collected by a cooperator from five lingering white ash trees (#1-FA, #2-FA, #3-FA, #4-FA, #6-FA) and one lingering green ash tree (#5-FP) in four Pennsylvania counties (Allegheny, Beaver, Butler and Mercer) on August 9, 2018. In addition, we cultured immature zygotic embryos from full-sibling seeds of two separate families. Each family resulted from controlled pollinations between two separate pairs of lingering green ash parents (DC6×DC5 and DC4×DC2) that had been confirmed to have increased EAB defenses using EAB egg bioassays, as previously described in Koch et al. (2015). Pollinations were conducted by scientists at the USDA Forest Service Forestry Sciences Laboratory in Delaware, Ohio. For controlled pollinations, pollen was collected by gently brushing or shaking from dehiscing male flowers directly into a glass beaker. To separate pollen from any debris it was passed through a 53 μm nylon sieve and used fresh or transferred into glass vials that were sealed and placed into 50 ml polypropylene tubes containing packets of silica gel and stored at −80 °C until use. Viability of all pollen was confirmed at the time of use by looking at pollen tube formation on artificial media as described in Koch and Carey (2004). Pollination bags were placed over dormant branches prior to female flowers becoming receptive. Receptivity was tested using methods described by Galen and Plowright (1987) and once receptive (in late April to early May), the bags were briefly removed, and pollen was applied directly to the stigmas of individual flowers using a paintbrush. Pollination bags were removed when stigmas were no longer receptive or when all other male ash trees in the vicinity were finished dehiscing. Seed was monitored for developing embryos and was harvested for somatic embryogenesis when the embryos were 1 to 3 mm long, which was on July 25, 2018 for DC6×DC5, and on August 14, 2018 for DC4×DC2. Harvested seed were kept at 10° C until shipped to the Merkle Lab. Shipping conditions, surface disinfestation and dissection were all as described for the 2015 culture initiations. Immature zygotic embryos were cultured on IMM supplemented with 2 mg/L 2,4-D, 4 mg/L 2,4-D or 0.1 mg/L picloram. For all the 2018 culture initiations, at least 15 zygotic embryos were cultured for each source tree by PGR treatment combination, with 3 embryos cultured per 60 mm Petri plate, except for the DC4×DC2 embryos. Because that cross had a very low filled seed percentage, only 6–8 zygotic embryos were cultured from it per PGR treatment. Culture maintenance was the same as described for the 2015 cultures, except that cultures from explants initiated on picloram were maintained by monthly transfer to fresh medium with that PGR.

Statistical analysis of initiation experiment data Three months following culture initiations in 2013, 2015 and 2018, each explant was scored for whether or not it showed evidence of embryogenesis induction (i.e., produced embryogenic callus or somatic embryos). For the 2013 initiation, percentages were calculated for each collection date, plant growth regulator treatment and genotype as well as the different combinations of these variables. Following arcsine transformation of the percentage data, one-way analysis of variance (ANOVA) was

performed to test for the effects of initiation date, PGR treatment, explant treatment (embryo versus whole seed) and their interactions on embryogenesis induction using R statistical software (R Core Team 2021). Means comparisons were conducted using Tukey's HSD test. Because numbers of explants per source tree×PGR treatment were unbalanced in the 2018 initiation experiment, the GLM function of R was used to analyze these data for the effects of PGR treatment and genotype on embryogenesis induction. This analysis did not employ induction percentage data, but individual explant data whereby each successfully induced explant was scored as "1" and lack of induction was scored as "0" for the GLM analysis.

Somatic embryo germination and conversion experiments

Effects of pre-germination treatments (cold, activated charcoal, gibberellic acid) on germination and conversion
Two experiments were conducted to test the effects of pre-germination treatments on germination and conversion of lingering white ash somatic embryos from cultures initiated in 2013 and 2015. While the two experiments tested some of the same treatments, the sources of the somatic embryos were different, so the two experiments are not replicates and are described separately.

In the first experiment, which used two culture lines initiated in 2013 (LA114-17B and LA114-20B), 4–6 mm long cotyledonary-stage somatic embryos were harvested individually from clusters of embryos proliferating via repetitive embryogenesis on Petri plates of semisolid IMM with 2 mg/L 2,4-D. Embryos were transferred to 60 mm plastic plates containing semisolid embryo development medium (EDM; Andrade and Merkle 2005), which was the same as IMM, but lacking PGRs, and given a pre-germination cold treatment at 8º C for 15 weeks in the dark in a refrigerator. Following cold treatment, all embryos were transferred to 125 ml Erlenmeyer flasks containing 30 ml liquid EDM and shaken overnight on a rotary shaker at 90 rpm, to remove residual 2,4-D. Before transfer to germination treatments the next day, embryos received a final wash by pipetting approximately 200 ml of liquid EDM over the embryos under mild vacuum using a Büchner funnel. Embryos were then transferred to 100 mm plastic Petri plates containing one of four treatments for germination: semisolid EDM, semisolid EDM supplemented with 10 mg/L gibberellic acid (GA_3), semisolid EDM with 0.5 g/L activated charcoal (AC), or semisolid EDM with both AC and GA_3. Ten somatic embryos were placed on each plate and 3 repetitions (plates) were used per treatment for each culture line. Table 2 summarizes the treatments and numbers of somatic embryos tested per treatment x genotype combination in this experiment. Plates with embryos were incubated in a lighted incubator under cool white fluorescent lights (100 µmol·m^{-2}·sec^{-1}) with 16 h day lengths at 25° C. Germination and conversion were scored at 12 weeks. Embryos were considered to be germinated when they exhibited taproot elongation from the radical and converted when they had produced both roots and shoots. Somatic seedlings were removed from in vitro conditions and potted in moistened peat:perlite:vermiculite (1:1:1) mix in 4-inch plastic pots, which were placed on top of water-saturated perlite in clear plastic dome-covered trays (to maintain humidity) under cool white fluorescent lights (80 µmol·m^{-2}·sec^{-1}) and 16 h day lengths. Somatic seedlings were watered and fertilized with 10 ml of Miracle-Gro fertilizer weekly. For acclimatiza-

tion, vents on the domed trays were slowly opened over the following two months until dome were removed completely and the somatic seedlings were transferred to the greenhouse to continue growth.

The second germination and conversion experiment used somatic embryos from two lingering white ash embryogenic cultures lines initiated in 2015 (LA112-10 and LA111-2). Embryos for this experiment were produced from suspension cultures. To initiate suspension cultures, approximately 0.5 g of embryogenic culture material of each line was inoculated into 30 ml of liquid IMM in 125 ml Erlenmeyer flasks and grown on a rotary shaker at 90 rpm in the dark at 25° C. Suspension cultures were fed every 3 weeks by decanting off the old medium and adding 30 ml of fresh IMM. At approximately 9 weeks, suspensions were size-fractionated by pouring them through nested stainless steel CELLECTOR® sieves (Bellco Glass) with pore sizes of 860 μm and 38 μm, such that cell clumps with diameters between the two pore sizes were collected on the 38 μm sieve. Material that collected on the 38 μm sieve was backwashed from the sieve with liquid EDM using a pipette, and collected on Nitex nylon mesh (Sefar America, Depew, NY, 30 μm pore size) using a Büchner funnel under mild vacuum. Cells were cultured along with the nylon mesh on semi-solid EDM in 100 mm plastic Petri plates. Plates were incubated in the dark at 25° C to allow somatic embryos to develop. Once populations of somatic embryos had developed on EDM, cotyledonary-stage embryos at least 2 mm long were harvested and transferred to fresh plates of semisolid EDM to enlarge for 2–3 weeks. Then, the mature embryos were given one of four treatments to test their effects on germination: (1) "Planting" (radical inserted into the medium in 100 ml of semisolid EDM in a GA-7 vessels (Magenta Corp.), with incubation in a lighted growth chamber at 22º C under cool white fluorescent light at 100 μmol·m^{-2}·sec^{-1} with 16 h of light per day, (2) Same as 1, except on using EDM supplemented with 10 mg/L GA$_3$, (3) Same as 1, except embryos were first given an 8-week pregermination cold treatment at 4º C, prior to planting in GA-7 s, and (4) Same as 3, except embryos were transferred to GA-7s of EDM with 10 mg/L GA$_3$ before placing them in the lighted incubator. Each clone by treatment combination was represented by three GA-7 vessels, with nine embryos per GA-7. Table 2 summarizes the treatments and numbers of somatic embryos tested per treatment x genotype combination in this experiment. Germination and conversion were scored after 12 weeks. Germinated somatic embryos from this experiment were removed from culture and acclimatized using the same procedure detailed for the previous experiment.

In vitro versus ex vitro somatic embryo conversion This experiment compared conversion percentage and early growth measurements of pre-germinated somatic embryos under vitro versus ex vitro conditions. It employed somatic embryos derived from embryogenic suspension cultures using the same procedure described above for the first germination and conversion experiment for producing synchronous populations of embryos via size fractionation and plating. Four embryogenic ash culture lines (LA112-10, LA111-7 A, LA115-5, and LA102-2) were used in the first replication of this experiment. Approximately five weeks following fractionation and plating of the suspension cultures, 3–4 mm-long embryos that developed from the plated material were individually harvested using a dissecting microscope, transferred to 60 mm plastic Petri plates containing semisolid EDM and allowed to enlarge for another two weeks in the dark at 22° C before being given a pre-germination cold treatment at 8º C for eight weeks in the dark. The plates were then removed from the

Table 2 Details of somatic embryo pre-germination treatment experiments

Genotypes	Cold treatments	Gibberellic Acid (GA$_3$) treatment	Activated charcoal (AC) treatment	Somatic embryos per genotype/ GA3 x AC treatment	Somatic embryos per genotype/ GA3 x cold treatment
Experiment 1					
LA 114-17B LA 114-20B	15 Weeks at 8° C	No PGRs; 10 mg/L GA$_3$	No AC; 0.5 g/L AC	30	-
Experiment 2					
LA 112-10 LA 111-2	No cold; 8 weeks at 4° C	No PGRs; 10 mg/L GA$_3$	No AC	-	27

cold and somatic embryos were transferred to 100 mm plastic Petri plates of semisolid EDM with 10 mg/L GA$_3$ and 0.5 g/L activated charcoal. The embryos were stored in a dark growth room at 22° C for 1 week and subsequently moved to a lighted incubator at 22° C under cool white fluorescent light at 100 µmol·m^{-2}·sec^{-1} with 16 h of light per day until radicles (taproots) elongated to at least 1 cm. The germinated embryos from each clone were divided into two groups for the two treatments. For the in vitro conversion treatment, 20 germinated embryos representing the each of the four lines were planted, five per vessel, in GA-7 vessels (Magenta Corp.) with 100 mL semisolid EDM with no GA, but with 0.5 g/L activated charcoal, and returned to the lighted incubator. For the ex vitro conversion treatment, 20 germinated embryos from each of the same lines were transferred to Fafard #3 potting mix in Hilson Rootrainer planting containers (Beaver Plastics). To plant the germinants in potting mix, a 1 cm deep hole was pushed into the potting mix with a forceps tip; then, holding the germinated embryo with forceps, its root tip was positioned into the hole so that the cotyledons remained above the surface. The planting containers with germinants were placed on top of water-saturated perlite in clear plastic dome-covered trays (to maintain humidity) under cool white fluorescent lights (80 µmol·m^{-2}·sec^{-1}) and 16 h day lengths and watered and fertilized with 10 ml of Miracle-Gro fertilizer weekly. For both treatments, data for conversion percentage, somatic seedling shoot length and numbers of leaves and first order lateral roots were recorded after 10 weeks. Table 3 summarizes the treatments and numbers of somatic embryos tested per treatment x genotype combination in this experiment. A second replication of this experiment was performed, although due to the failure of

Table 3 Details of in vitro versus ex vitro conversion experiment

Genotypes	Cold treatment	Gibberellic acid (GA$_3$) treatment	Activated charcoal (AC) treatment	Conversion treatment	Somatic embryos per genotype x conversion treatment
Replication 1					
LA 112-10 LA 111-7 A LA115-5 LA 102-2	8 weeks at 4° C	10 mg/L GA$_3$	0.5 g/L AC	In vitro Ex vitro	20
Replication 2					
LA 112-10 LA 115-5 LA 102-2	8 weeks at 4° C	10 mg/L GA$_3$	0.5 g/L AC	In vitro Ex vitro	20

one of the culture lines to produce sufficient material, only three culture lines (LA112-10, LA115-5, and LA102-2) were used.

Statistical analysis for germination and conversion experiments Data from both germination and conversion experiments were analyzed using R statistical software (version 3.12; R Foundation for Statistical Computing) using analysis of variance. Germination and conversion percentage data were subjected to arcsine transformation prior to analysis, although original percentage data are shown in the graphs. Means comparisons for treatments were conducted using Tukey's HSD test.

Common garden study

Lingering ash somatic seedlings and control trees were planted in April 2018 in a fenced nursery space (~0.9 hectare) at the Whitehall Forest, University of Georgia, Athens, Georgia. EAB was detected in neighboring counties by that time, although not in Athens-Clarke County. We used a randomized complete block design with six blocks, five rows, and four columns. Blocks were sized 12×12 m and spaced 12 m from each other. Rows and columns were spaced 3 m from each other. Species/types planted included: (1) Manchurian ash grafted saplings (EAB resistant controls); (2) conventional green ash seedlings (EAB susceptible control 1); (3) conventional white ash seedlings (susceptible control 2) and (4) white ash somatic seedlings (test trees). The white ash somatic seedlings represented nine clones derived from open-pollinated seeds collected from Michigan lingering ash source trees. Sizes of the seedlings at planting varied widely with type/species, ranging from ≤ 1 m in height for most of the somatic seedlings up to ≥ 2 m for the Manchurian ash grafts. We randomly assigned five trees of each type to rows and columns within each block. Hence, 30 trees were planted for each type, except that 26 white ash somatic seedlings were planted in the garden. Plants were fertilized annually in the spring with 10-10-10 (N:P:K), watered by an irrigation system (once a week in spring and fall, twice a week during summer; irrigation was turned off during the winter). The nursery space was mowed as needed to reduce competition from other plants.

We measured the diameter and height of the plants soon after planting (early June 2018), September 2018, April 2019, October 2019, July 2020, and November 2020. Since trees were of different sizes at planting and we took measurements at different time intervals, we calculated the relative growth rates (RGR) during 2018–2020 for the diameter (cm) and height (cm) of each plant type/species (Manchurian, green, white, and lingering white ash). The formula used for RGR was: $[\log_e(\text{Diameter}_2 \text{ or Height}_2) - \log_e(\text{Diameter}_1 \text{ or Height}_1)]/(\text{Time}_2 - \text{Time}_1)$. Time was calculated for each plant per year as 2018 (T_1 = date of planting, T_2 = fall measurement); 2019 (T_1 = spring, T_2 = fall); and 2020 (T_1 = spring, T_2 = fall). RGRs were averaged for all five plants per type within a block, and then across each tree species (N=6).

At each measurement time, the saplings were assessed for any evidence of EAB activity by checking the foliage for adult feeding and any evidence of larval presence (D-shaped exit holes, bark splitting, epicormic branching, etc.) (Smith et al. 2015). Survivorship of trees was also noted. We placed and monitored 5 purple panel traps in April/May of each year

(except 2020 because of shut-down due to COVID-19) at the nursery's perimeter for assessing EAB adult activity in the general area.

Results

Embryogenesis induction

2013 culture initiations Proliferation of embryogenic material appeared on some explants beginning as soon a one month following explanting. The embryogenic material varied from relatively unstructured callus to cell clusters resembling proembryogenic masses (PEMs; Fig. 2a, b). Both the callus and PEMs were pale yellow and soft or friable. Clusters of repetitive somatic embryos emerged from callus while it was still on media containing 2,4-D (Fig. 2c, d). Collection date had a significant effect on embryogenesis induction (P<0.001), with explants collected on August 8th producing higher mean embryogenesis induction rates (27%) than those collected on July 10th (0%; Fig. 3a). This suggested that early to mid-August was the optimal time for harvesting lingering white ash fruit from the Michigan trees for induction of embryogenic cultures, as zygotic embryos were apparently too immature in July to produce embryogenic cultures. While embryogenesis induc-

Fig. 2 Lingering ash culture initiation. **(a)** Embryogenic callus derived from lingering white ash zygotic embryo explant. **(b)** Proembryogenic masses derived from lingering white ash zygotic embryo explant. **(c)** Somatic embryo production from lingering white ash proembryogenic masses while still on induction-maintenance medium with 2,4-D. **(d)** Clusters of proliferating somatic embryos of lingering white ash clone LA115-5. Petri plate diameter is 60 mm. **(e)** Repetitively embryogenic lingering green ash culture initiated on medium with picloram. **(f)** Development of synchronous lingering white ash somatic embryos following size-fractionation and plating of embryogenic suspension culture. Bar in all photos=1 mm
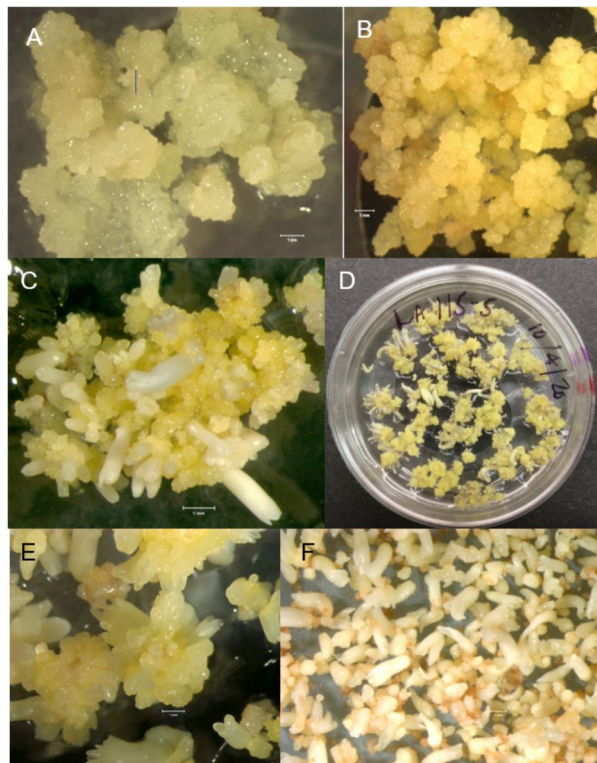
Fig. 3 Embryogenesis induction percentages from the 2013 and 2018 experiments. **(a)** Effect of seed collection date and source tree on embryogenesis induction from zygotic embryos from lingering white ash source trees (LA-101, LA-102, LA-114 and LA-116) in 2013 experiment. Means represent 10–12 Petri plates per genotype (all 2 and 4 mg/L 2,4-D and plus or minus seedcoat on explant treatments combined), with 4 explants per plate. **(b)** Effect of PGR treatment on embryogenesis induction from zygotic embryos from lingering green and white ash trees in 2018 experiment. Means represent 8 ash genotypes, each with 6–24 explants per treatment. Bars in both plots represent standard error
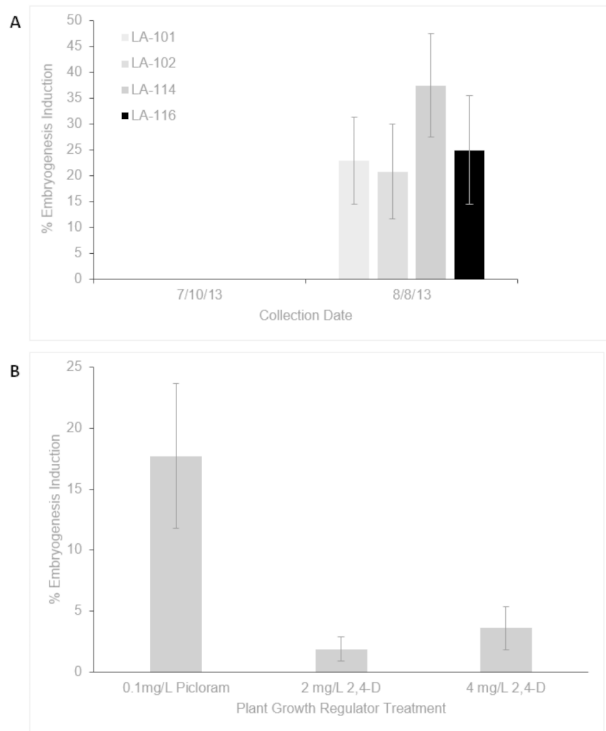
tion rates varied among the four mother trees from 15.6 to 37.5% for the seed collection (August 8th) that resulted in embryogenic cultures (Fig. 3a), the variation among source trees was not significant (P=0.467). Another variable that significantly affected embryogenesis induction (P<0.001) was whether the cultured explant included all of the seed tissues or only consisted of the zygotic embryo. Whole only 5% of whole seed explants collected on August 8th produced embryogenic cultures, 42% of embryos dissected from seeds produced embryogenic cultures (data not shown). Finally, embryogenesis induction rates for explants cultured on 4 mg/L 2,4-D (19%) were not significantly different from those cultured on 2 mg/L 2,4-D (12%; P=0.263; data not shown).

2015 culture initiations Among the seven white ash trees used for 2013 culture initiations, the induction percentages ranged from 0% (for a single source tree) to 16.7%. The overall induction percentage of 12.2% (data not shown) was lower than for the 2013 culture initiations.

2018 culture initiations The 2018 culture initiation experiment compared three PGR treatments for induction, using seed explants collected from both open-pollinated and control-pollinated green ash and white ash trees. Overall, 7.5% of cultured zygotic embryos produced embryogenic cultures. Induction rates differed significantly among PGR treatments (P<0.001). The 0.1 mg/L picloram treatment resulted in a higher average induction rate (17.7%) than either the 2 mg/L or the 4 mg/L 2,4-D treatments (1.9% and 3.6%, respectively; Fig. 3b). In fact, for explants from four of the eight source trees, the only PGR

treatment that induced embryogenesis was the picloram treatment. While the picloram treatment resulted in higher induction rates, this treatment tended to result in cultures that proliferated via repetitive embryogenesis with little or no production of PEMs or embryogenic callus (Fig. 2e). Most of the 32 cultures initiated came from open-pollinated seeds, but four embryogenic cultures were obtained from seeds derived from the controlled-pollinations between lingering green ash parents conducted by USDA Forest Service collaborators.

Somatic embryo germination and conversion

Effects of cold treatment, activated charcoal and gibberellic acid on germination and conversion The majority of the somatic embryos produced on IMM to be used in the first germination/conversion experiment were well-formed, with good hypocotyl development and two cotyledons (Fig. 2 c). Some morphological abnormalities were observed, such as fewer or more than two cotyledons, but we had sufficient numbers of well-formed embryos that we did not need to use those with abnormal morphologies for the experiment. All embryos in this experiment were given a 14-week cold pre-germination treatment. The four germination medium treatments (control, AC, GA_3 and $AC+GA_3$) differed significantly ($P<0.001$) with regard to germination (radicle elongation) and conversion of embryos to somatic seedlings. GA treatment alone improved germination percentage over the control, but AC alone

Fig. 4 Effects of activated charcoal and gibberellic acid on (**a**) germination and (**b**) conversion of embryos harvested from two culture lines derived from seeds of the same source tree (LA114-17B and LA114-20B) maintained on semi-solid induction-maintenance medium
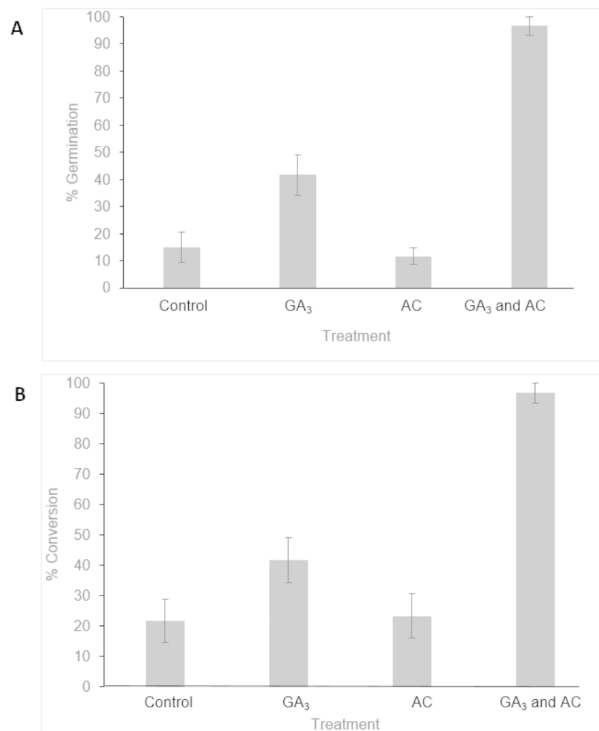
did not. The combination of GA$_3$ and AC resulted in higher germination frequency (97%) than any of the other treatments (Fig. 4a). Similarly, both GA$_3$ alone and in combination with AC improved conversion over the control and AC alone, with the GA$_3$ plus AC treatment giving a significantly higher conversion percentage (97%) than GA$_3$ alone (Fig. 4b). Thus, it appears from this experiment that using a combination of GA$_3$ and AC in the germination medium was the best treatment for ash somatic seedling production.

The use of size-fractionated and plated suspension cultures to produce somatic embryos for the second germination/conversion experiment resulted in fairly synchronous populations of somatic embryos, from which embryos of similar maturity and size could be picked for the use in the experiment (Fig. 2f). As was the case for the previous experiment, we had sufficient numbers of embryos with normal morphology that we did not need to use those with abnormal morphology for the experiment. Both cold treatment and GA treatment affected germination (P<0.001), but the interaction between the two variables was also significant (P<0.01). GA treatment greatly increased germination percentage when no cold per-germination treatment was applied, but did not appear to have much effect on germination when the somatic embryos were given either 8 or 12 weeks of cold (Fig. 5a). GA but not cold treatment significantly affected conversion percentage (P<0.05). Plantlets of both clones from this experiment (Fig. 7a) were successfully acclimatized and continued growth in the greenhouse.

Fig. 5 Effects of 8-week cold pre-germination treatment and gibberellic acid on (**a**) germination and (**b**) conversion of synchronized embryos of two culture lines derived from seeds of two different source trees (LA111-2 and LA-112-10). Embryos were produced from suspension cultures that had been size-fractionated and plated to synchronize embryo develpment. Bars in both plots represent standard error
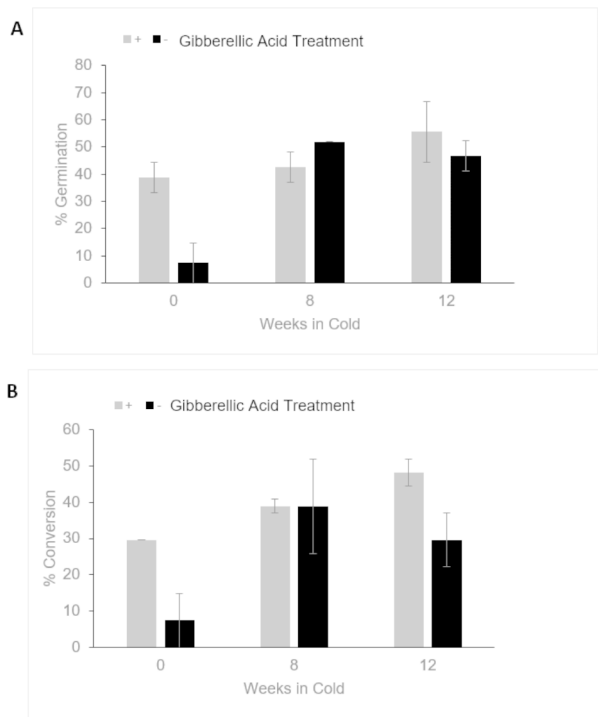
In vitro versus ex vitro somatic embryo conversion The first replication of the experiment comparing in vitro versus ex vitro conversion employed somatic embryos of four lingering white ash lines produced from size-fractionated suspension cultures, as in the cold treatment/GA experiment described above. Both genotype and conversion treatment significantly affected conversion percentage, with lower somatic seedling production from all genotypes for the ex vitro treatment than for the in vitro treatment (Fig. 6a). One genotype failed to produce any somatic seedlings using the ex vitro treatment. Conversely, measurements taken at 10 weeks on those somatic seedlings that were produced appear to show that the plantlets resulting from ex vitro conversion were taller and had more leaves and more first order lateral roots than those derived from in vitro conversion (Figs. 6b–d and 7b, c). However, analysis of variance results indicated that of these three traits, only number of leaves was greater for ex vitro-converted plantlets (P<0.01), probably due to the large within-genotype variation in these three traits for the ex vitro-converted plantlets (Fig. 6d). The second replication of the experiment only included three of the four culture lines used in the first replication. Results were similar to those obtained from the first replication, with the exception of the performance of the culture line for which no somatic seedlings were produced in the first replication (LA115-5). In the second replication, not only were somatic seedlings produced by this line, but the ex vitro conversion percentage for it was higher than the in vitro conversion percentage, which is the reverse of what was observed for the other lines (data not shown).
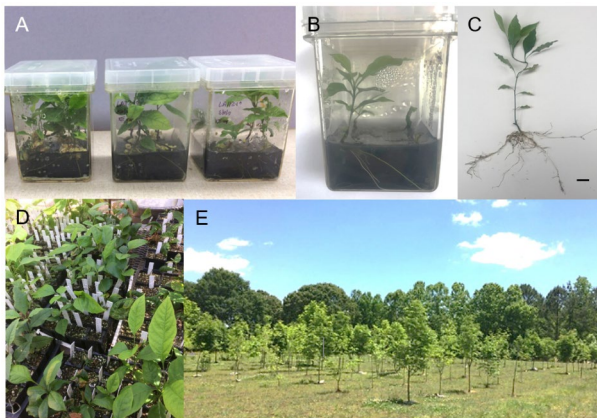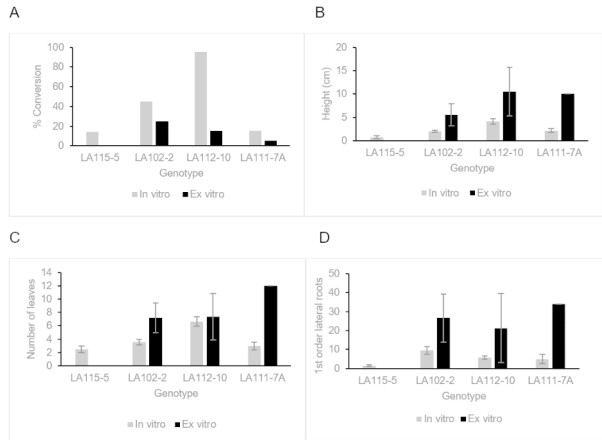

Fig. 6 White and green ash somatic seedlings derived from lingering ash sources. **(a)** Lingering white ash somatic seedlings of genotype LA112-10 following cold treatment and germination on medium with activated charcoal and GA_3. **(b)** Somatic seedling derived via in vitro conversion after 10 weeks. **(c)** Somatic seeding derived via ex vitro conversion after 10 weeks. Bar=1 cm. **(d)** Somatic seedlings from embryogenic cultures derived from control-pollinated seeds from crosses between two lingering green ash parents (DC4×DC2) in the greenhouse. **(e)** Field test of lingering ash somatic seedlings and controls in Athens, GA in the second growing season (May 2021)

Fig. 7 Effects of in vitro versus ex vitro conversion on conversion percentage and early somatic seedling growth. **(a)** Effect of in vitro versus ex vitro conversion on conversion percentage. **(b)** Effect of in vitro versus ex vitro conversion on somatic seedling shoot length at 10 weeks. **(c)** Effect of in vitro versus ex vitro conversion on number of leaves on somatic seedlings at 10 weeks. **(d)** Effect of in vitro versus ex vitro conversion on number of first order lateral roots at 10 weeks

Common garden study

Survival was 100% for Manchurian, green, and white ash, and ~92% for lingering ash trees planted in the common garden. Three lingering ash somatic seedlings died during the first year following planting. During spring 2018 - fall 2020, Manchurian ash grew by a mean of 155% in diameter and 106% in height; green ash conventional seedlings 391% and 304%, white ash conventional seedlings 303% and 181%, and lingering white ash somatic seedlings 261% and 337%, respectively. There were little differences in relative growth rate (RGR) among the North American ash species tested, with the highest diameter growth

Fig. 8 Mean (±SE) relative growth rate for **(a)** diameter and **(b)** height of lingering ash-derived somatic seedlings, conventional green ash and white ash seedlings (EAB-susceptible controls) and Manchurian ash grafts (resistant controls) in the common garden planting in 2018–2020
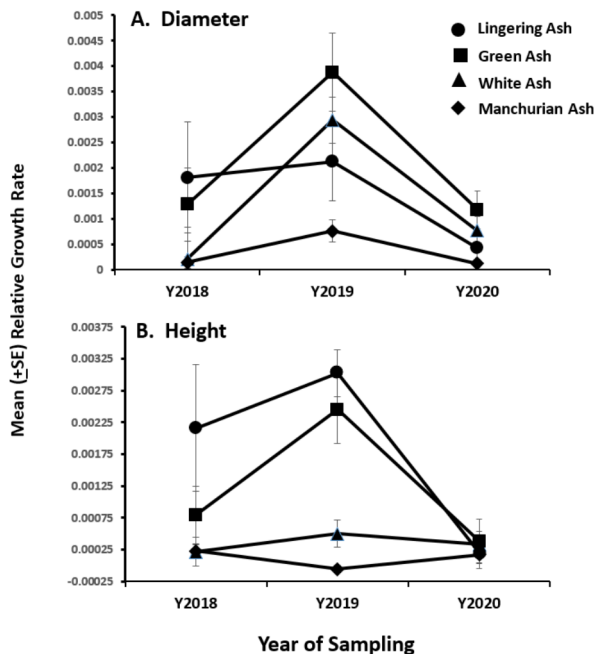
in 2019, and the highest height growth in 2018 and 2019 observed for the lingering ash somatic seedlings (Fig. 8a, b). Generally, Manchurian ash, which were the largest trees and were grafted, had the lowest relative growth rate in almost all the years. In 2020, RGR was very similar and lowest across all the tree types, likely due to acclimatizing to the local soil and climatic conditions. We did not find any evidence of EAB colonization on these trees or on the purple panel traps during three years of monitoring.

Discussion

The results reported here are the product of a critical collaboration between entomologists and other forest scientists selecting and breeding putatively EAB-resistant lingering ash trees and those working to optimize clonal propagation of the products of the selection and breeding work. Our ability to successfully generate ash clonal material and grow the trees in a common garden is evidence that we will be able to use these novel techniques to produce EAB-resistant stock for ash reforestation efforts. However, developing an operational system to address EAB in North America combining genetic resistance with mass clonal propagation via somatic embryogenesis requires overcoming a number of bottlenecks, some of which were addressed in this study.

First, we needed to demonstrate the ability to initiate highly productive embryogenic cultures from lingering ash material. Over the course of several years of culture initiations (of which only three are described in this report), we showed that standard protocols we have employed to initiate embryogenic cultures from other hardwood species, i.e., determining an optimal zygotic embryo developmental window to use as explants, employing auxin treatment (2,4-D, picloram), resulted in embryogenesis induction from most of the lingering ash genotypes we cultured, including control-pollinated material. However, embryogenesis induction levels (percentage of explants that produced embryogenic cultures) varied widely with source tree genotype and induction treatment. Most of our induction rates were in line with those previously reported for North American ash species. Li et al. (2014) reported an average of 8.3% induction with immature green ash seed explants for the best induction treatment. Preece et al. (1989) and Bates et al. (1992) reported induction rates up to 20% and 10%, respectively, for white ash, although their culture initiation protocols mainly employed mature ash seeds as explants, thidiazuron in combination with 2,4-D and DKW medium (Driver and Kuniyuki 1984) and/or MS medium (Murashige and Skoog 1962), rather than WPM. As in the current study, Preece et al. (1989) also noted the strong genotypic effect on embryogenesis induction in white ash. Although we did not attempt to initiate cultures from mature zygotic embryos due to the very low number of filled seeds in the samaras collected in September, mature zygotic embryo explants of green ash failed to produce any embryogenic cultures using a similar protocol to that used here (Li et al. 2014).

One unusual observation with regard to the embryogenic cultures, once they were established was the fact that somatic embryos emerged from the callus and completed development while still on media containing 2,4-D. This plant growth regulator usually inhibits the development of somatic embryos from embryogenic callus or PEMs of other hardwood species, including those of green ash, yellow-poplar (*Liriodendron tulipifera*) and sweetgum (*Liquidambar styraciflua*), until they are transferred to 2,4-D-free medium (Li et al. 2014, Merkle et al. 1990, Dai et al. 2004).

A second challenge was to generate synchronous populations of somatic embryos from the embryogenic cultures to use for somatic seedling production experiments. Although we did not test any variables in applying our suspension culture-based system for somatic embryo production, we found that size-fractionation of the suspension cultures and plating of cell clusters in a defined size range resulted in the production of relatively synchronous populations of singularized somatic embryos. The advantage of this approach is that the embryos can be harvested rapidly without damaging them, which often occurs when trying to separate individual embryos from fused clusters of asynchronous repetitive embryos. We have used this approach to generate large numbers of synchronous somatic embryos from embryogenic cultures of other hardwood trees including yellow-poplar, sweetgum and American chestnut (*Castanea dentata*; Merkle et al. 1990, Dai et al. 2004, Andrade and Merkle 2005). Tonon et al. (2001) also used suspension culture to scale-up somatic embryo production of *F. angustifolia*, and employed density gradient centrifugation in a Ficoll gradient to enrich for subpopulations of cell clusters with the highest potential to produce synchronous somatic embryos. The fact that the suspension cultures can be manipulated in these ways for ash somatic embryo production bodes well for scaling up production of clonal lingering ash planting stock using embryogenic cultures.

Often, the most challenging bottleneck with using embryogenic cultures for mass clonal propagation involves the somatic embryo germination and conversion (plantlet production) steps. Using somatic embryos derived from the fractionated and plated suspension cultures, we tested three variables we thought would enhance germination and conversion: cold pre-germination treatment and supplementing the germination medium with activated charcoal and gibberellic acid. All three treatments positively affected germination and conversion percentages, although there appeared to be no additive effect when both cold and GA were applied. Cold and/or activated charcoal have been shown to improve conversion of other hardwood tree somatic embryos, such as American chestnut (Andrade and Merkle 2005), hybrid *Liquidambar* (Merkle et al. 2010) and *Juglans regia* (Tang et al. 2000; Sadat-Hosseini et al. 2019). While gibberellic acid has often been included in germination media for hardwood tree somatic embryos, tests of its effectiveness on improving germination and conversion are rare and results have been inconsistent—even in the same species. GA treatment improved germination of *J. regia* somatic embryos in one study (Sadat-Hosseini et al. 2019), but failed to improve germination of the same species in another study (Tang et al. 2000). Germination of *Quercus robur* somatic embryos was not improved by GA treatment (Sanchez et al. 2003).

Based on our previous experiences with ex vitro conversion of somatic embryos of other hardwood forest species (e.g., Merkle et al. 1994), we thought pre-germinated ash somatic embryos might make good candidates for ex vitro conversion. While it appeared that, in general, early growth of somatic seedlings was accelerated by ex vitro conversion compared to in vitro conversion, as indicated by somatic seedling height growth, numbers of leaves and numbers of first order lateral roots, this advantage was off-set by the lower numbers of germinated embryos that survived potting to complete conversion. Before this propagation system can be applied for production of planting stock for ash restoration, more work on improving plantlet production remains to be done.

From our limited previous experiences growing hardwood somatic seedlings in the field, we were surprised that, overall, growth rates of the ash somatic seedlings in the common garden study appeared to be on par with those of the conventional white ash and green ash

seedlings, and better than those of the Manchurian ash grafts. However, a few of the somatic seedlings did not survive the first growing season. Our experience with American chestnut has been that somatic seedlings usually lag significantly behind conventional seedlings in early growth (Merkle, unpublished data). There may be several reasons for the unexpectedly strong performance of the somatic seedlings, including large variation in the tree sizes among the different classes of trees when planted, with the smallest trees being the somatic seedlings and the largest being the Manchurian ash grafts. Thus, the smallest trees may have had the most potential to add growth compared to the larger (and perhaps older trees). There has been no evidence of natural infestation of the planting by EAB or infestation of mature native ash trees in the general area to date, so even though many of the trees in the common garden study have reached stem diameters suitable for EAB colonization, evaluation of the trees for resistance may require artificial inoculation of the trees with EAB. We should also note here that somatic seedlings representing the genotypes resulting from controlled pollinations between lingering green ash clones were not planted in this study. The parents of the somatic seedlings planted in the common garden study were selected by field assessment alone and the presence or level of EAB defenses the parents may or may not possess have not been confirmed through further testing (EAB egg bioassays). There are plans to supply the green ash clones derived from the controlled pollinations to USDA Forest Service collaborators at the Northern Research Station for EAB resistance testing.

The results we report here are preliminary, but offer evidence of the feasibility of using in vitro propagation to leverage selection and breeding programs aimed at generating EAB-resistant ash trees in two ways. First, clones initiated from progeny of putatively EAB-resistant parents are a very powerful tool for testing if the apparent resistance observed in the parents is genetically-based or not. Data from the large numbers of ramets per clone provided via SE can provide unambiguous clonal repeatability results for the EAB-resistance trait. Second, if any of the tested clones appear to show high levels of resistance as well as other desirable traits (i.e. growth rate, form), SE provides a route for scaling up these clones to be established as varietals. While not part of this study, the same cultures used in this study have been used in experiments to optimize long-term storage of ash germplasm in liquid nitrogen. Preliminary results from these experiments indicate that the cultures should be amenable to cryostorage and subsequent recovery (Richins et al., in preparation). Thus, embryogenic cultures derived from lingering ash parents can be held for years in cryostorage while trees derived from them are tested in the field, and the best-performing clones can be recovered from cryostorage years later for scaled-up production of somatic seedlings for restoration purposes.

One further consideration in the potential application of lingering ash-derived clones for restoration is the large geographic ranges of some of the eastern North American ash species. The natural range of green ash, for example, covers over half of the continental U.S. and reaches into multiple Canadian provinces (Hardin et al. 2001). With such large ranges, much of which have already been impacted by EAB, planning to clone and preserve lingering ash material from different parts of the ranges would be necessary to ensure the availability of genotypes that are regionally adapted. These cloned genotypes could then be employed as regionally-adapted cultivars for urban tree restoration. They could also be used for testing and selection of the best performing parents for breeding, to further increase resistance and for the development of seed orchards to produce trees with appropriate levels

of resistance and genetic diversity required for forest restoration across the ranges of the species.

Authors' contributions SAM designed culture initiation and somatic embryo germination and conversion experiments and wrote all manuscript drafts; JLK supervised breeding between lingering green ash parents; ART conducted culture initiations, somatic embryo production and ex vitro conversion experiments; JEM conducted culture initiations and somatic seedling production experiments; DWC conducted breeding between lingering green ash parents; BFB installed and measured field test; MWMR analyzed culture initiation and somatic seedling germination and conversion data; PMM maintained field test trees and plotted culture initiation and somatic embryo germination and conversion data; KRE conducted ex vitro conversion experiments; LTH conducted culture initiations; DAH identified lingering white ash trees and supplied culture material from them; KJKG designed and helped install field test and plotted relative growth rate data for field test trees.

Availability of data and material (data transparency): Available upon request from corresponding author.

Code availability (software application or custom code) Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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Authors and Affiliations

Scott A. Merkle[1] · Jennifer L. Koch[2] · A. Ryan Tull[1] · Jessica E. Dassow[1] · David W. Carey[2] · Brittany F. Barnes[1] · Mason W. M. Richins[1] · Paul M. Montello[1] · Kira R. Eidle[1] · Logan T. House[1] · Daniel A. Herms[3] · Kamal J.K. Gandhi[1]

✉ Scott A. Merkle
smerkle@uga.edu

[1]   Warnell School of Forestry and Natural Resources, University of Georgia, 30602 Athens, GA, United States

[2]   USDA Forest Service, Northern Research Station, 43015 Delaware, OH, United States

[3]   Davey Tree Expert Company, 44240 Kent, OH, United States