

Hypocotyl derived in vitro regeneration of pumpkin ash (*Fraxinus profunda*)

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Abstract Pumpkin ash (*Fraxinus profunda* (Bush) Bush) is at risk for extirpation by an exotic insect, the emerald ash borer (EAB). Pumpkin ash is limited to wetland areas of the Eastern United States, and has been listed as an endangered species because of EAB activity. Pumpkin ash provides many benefits to the ecosystem, and its wood is used in the manufacturing industry. In vitro regeneration provides an integral tool for the mass propagation and genetic transformation of pumpkin ash to combat EAB. Therefore, a plant regeneration protocol was developed for pumpkin ash. Aseptically extracted hypocotyls formed adventitious shoots following 4 weeks on Murashige and Skoog (MS) medium supplemented with 0–22.2 μM 6-benzyladenine (BA) and 0–6.8 μM thidiazuron (TDZ) then transferred for an additional 4 weeks on MS medium with Gamborg B5 vitamins plus 0.2 g L⁻¹ glycine (B5G) containing 6.7 μM BA, 1 μM indole-3-butyric acid (IBA), and 0.29 μM gibberellic acid (GA₃). As adventitious shoots developed, these were transferred to a MSB5G medium with 13.3 μM BA, 1 μM IBA, and 0.29 μM GA₃ for shoot elongation. Elongated shoots were successfully micropropagated using MSB5 medium with 10 μM BA and 10 μM TDZ. Adventitious root formation was as high as 94% using woody plant medium supplemented with 4.9 μM IBA with shoots cultured for 10 days in the dark followed by culture under a 16-h photoperiod.

Acclimatization to the greenhouse was successful and normal plant growth was observed. This protocol will provide a means for genetic transformation for EAB resistance and mass propagation for conservation.

Keywords Adventitious shoots · Emerald ash borer · *Fraxinus* · Organogenesis · Rooting

Introduction

Pumpkin ash (*Fraxinus profunda* (Bush) Bush) is an integral species of North American wetland communities. It is a large tree (up to 40 m) with a disjointed native range restricted to marshes, river bottomland, coastal floodplains, and poorly drained flatlands (Harms 1990). Despite its limited range pumpkin ash is both an ecologically and economically valuable hardwood tree species. Its mast provides sustenance for many waterfowl, while leaves and shoots are consumed by a variety of mammals. Economically the timber from pumpkin ash is used to generate high valued lumber, as well as stock for tool and implement handles. As are other indigenous members of the genus *Fraxinus*, pumpkin ash is at risk of extirpation by the emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire; Coleoptera:Buprestidae). First detected in Michigan in 2002, EAB (a non-native tree pest) causes mortality through disruption of the phloem during larval development, leading to the death of more than 50 million North American ash trees (Emerald Ash Borer 2011; Mercader et al. 2011).

The narrow range of pumpkin ash further exacerbates the threat of its removal from natural environments. As EAB spreads south through the natural range of pumpkin ash, both through anthropogenic activity and natural dispersal, it becomes increasingly imperative to address its long-term

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survival as a continual 10–15 year program will be necessary to mitigate the EAB threat (Cappaert et al. 2005). In vitro culture is a powerful tool for both plant conservation and for use in genetic improvement to combat EAB. Several studies demonstrated tissue culture to be useful in preserving endangered plant species from several genera (Irvani et al. 2010; Ma et al. 2011; Piovan et al. 2010; Sivanesan et al. 2011). An effective regeneration system allows for a rapid build-up of plant material while also providing a means for developing EAB resistance through genetic transformation. However, for transformation to be practical a suitable adventitious regeneration system must be established. Such protocols exist for a number of ash species including green (*F. pennsylvanica*), common (*F. excelsior*), flowering (*F. ornus*), and white ash (*F. americana*), but no protocol has been established for pumpkin ash (Arrillaga et al. 1992; Du and Pijut 2008; Palla and Pijut 2011; Tabrett and Hammatt 1992). Previous studies have shown regeneration of ash possible from a variety of initial organs (Bates et al. 1992; Du and Pijut 2008; Hammatt 1994; Mockeliunaite and Kusiene 2004; Tabrett and Hammatt 1992; Tonon et al. 2001). Although these studies utilized a variety of plant growth regulators and culture media, Kim et al. (1997, 1998) and Du and Pijut (2008) found that a Murashige and Skoog (MS; 1962) medium with Gamborg et al. B5 vitamins (B5; 1968) in combination with thidiazuron (TDZ), 6-benzyladenine (BA), and indole-3-butyric acid (IBA) to be most effective at producing adventitious shoots in green ash. Genetic analysis showed green ash along with white ash to be the progenitor species of pumpkin ash (Wallander 2008). Therefore, because of the close relatedness, pumpkin ash would presumably respond similarly in vitro to its related species.

Genetic transformation will become increasingly necessary as no known innate resistance to EAB has been found within North American ash populations. Transformation for developing resistance to EAB in pumpkin ash will be a feasible approach because of transformation attainability in both model and non-model woody species, as well as closely related green ash (Du and Pijut 2009; Flachowsky et al. 2010; Noel et al. 2005). To date there has been no research reported on establishing pumpkin ash in tissue culture. Therefore, the objective of this study was to develop an in vitro regeneration protocol for pumpkin ash that could be used for conservation as well as future genetic transformation.

Materials and methods

Adventitious shoot formation and micropropagation

Open-pollinated mature pumpkin ash seed were obtained from the National Seed Laboratory (Dry Branch, GA;

USA) and stored at 5°C in the dark until used. The pericarp was removed and seeds were surface disinfested in 70% (v/v) ethanol for 30 s. The seeds were then washed in 20% bleach solution (5.25% sodium hypochlorite) for 15 min, followed by three rinses in sterile water, and then stored for 24 h at 24 ± 2°C in sterile water. The next day, turgid embryos were easily removed and the hypocotyls isolated by removing the cotyledons and the tip of the radicle. Hypocotyls were then cultured horizontally on MS medium supplemented with 0, 8.9, 13.3, or 22.2 μM BA in combination with 0, 2.3, 4.5, or 6.8 μM TDZ (100 mm × 25 mm Petri plates; 50 mL medium) for adventitious shoot initiation. After 4 weeks hypocotyls that turned green and began to form callus were transferred to a MS basal shoot-bud induction medium containing 6.7 μM BA, 1 μM IBA, 0.29 μM gibberellic acid (GA₃), and Gamborg B5 vitamins plus 0.2 g L⁻¹ glycine (B5G) for an additional 4 weeks. After 8 weeks, cultures were transferred to a MS shoot elongation medium containing 13.3 μM BA, 1 μM IBA, 0.29 μM GA₃, B5G, and 0.2 g L⁻¹ casein hydrolysate. After 16 weeks from initial explant culture, hypocotyls were rated for percent shoot formation and number of shoots per hypocotyl. Three replications with 12 explants per treatment per replication were conducted. All media contained 3% (w/v) sucrose, 0.7% (w/v) Difco-Bacto agar, and the pH was adjusted to 5.7 prior to autoclaving. All cultures were maintained under a 16 h photoperiod provided by cool-white fluorescent lamps (80 μmol m⁻² s⁻¹) at 24 ± 2°C. Elongated adventitious shoots were successfully micropropagated via nodal culture on MSB5G medium supplemented with 10 μM BA and 10 μM TDZ, and were sub-cultured every 4 weeks to fresh medium prior to rooting.

Rooting of adventitious shoots

Sufficient numbers of microshoots for rooting experiments were established through sub-culturing adventitious-derived shoots on MSB5G medium supplemented with 10 μM BA and 10 μM TDZ. Microshoots (2–3 cm in length) were induced to form roots on woody plant medium (WPM; Lloyd and McCown 1981) supplemented with 4.9 μM IBA in combination with 0, 2.9, 5.7, or 8.6 μM indole-3-acetic acid (IAA), 3% (w/v) sucrose, and 0.7% (w/v) Difco-Bacto agar in Magenta GA-7 vessels (Magenta Corp., Chicago, IL; 50 mL medium). Three replications with 12 explants per treatment per replication were conducted. Shoots on root induction medium were cultured in the dark for 10 days at 26 ± 2°C prior to being cultured under a 16 h photoperiod (80 μmol m⁻² s⁻¹). Six weeks after culture on root induction medium, percent root formation, number of roots per shoot, length of roots, and the number of lateral roots were evaluated for each microshoot.

Acclimatization of rooted plantlets

Rooted plantlets were gently removed from the agar medium and potted in 12.5 cm × 8 cm plastic pots containing a moist autoclaved potting soil (1:1:1 (v/v/v), peat moss:perlite:vermiculite) and grown under a 16 h photoperiod ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$). All agar was removed from the roots prior to transplanting. Pots were placed in 3.8 L plastic zip-lock bags to provide a high relative humidity, and had four holes punched below the zip-lock to allow for air exchange. The bags were gradually opened over a period of 2–3 weeks in the culture room to allow plantlets to acclimatize to ambient conditions. Plants were watered every 4–5 days until the bags were fully opened, and then watered as needed. After approximately 4 weeks plants were transferred to the greenhouse, and eventually transplanted into Tall Treepots™ (Stuewe and Sons, Inc., Corvallis, OR) for continued growth and development. Plants were given a 5–6 month slow-release fertilizer 15–9–12 Osmocote® (The Scotts Company LLC).

Statistical analysis

Data were analyzed using an analysis of variance (ANOVA) using R statistical software (R development Core Team 2006). When the ANOVA indicated a statistical difference, Tukey's comparison test was used to identify differences between treatments at the 5% level of probability.

Results and discussion

Adventitious shoot formation and micropropagation

Pumpkin ash hypocotyls, similar to other species, retained enough cellular plasticity to achieve plantlet regeneration (Annapurna and Rathore 2010; Shaik et al. 2011; Slater et al. 2011; Sridevi et al. 2010). Adventitious shoot formation was observed for all BA and TDZ concentrations tested. A positive correlation was noted between TDZ concentration and percent shoot formation with the optimum regeneration medium being supplemented with $22.2 \mu\text{M}$ BA and $4.5 \mu\text{M}$ TDZ (Table 1). TDZ has been well documented to be a powerful inducer of shoot organogenesis in woody species (Huetteman and Preece 1993), and research with white and green ash showed TDZ concentrations as high as $10 \mu\text{M}$ to be effective for adventitious and axillary shoot formation (Bates et al. 1992; Kim et al. 1997; Navarrete et al. 1989). However, we found that TDZ concentrations higher than $4.5 \mu\text{M}$ in combination with BA had a deleterious effect on pumpkin ash adventitious shoot formation as percent shoot

formation decreased (Table 1). An over-abundance of TDZ has been shown to have negative effects in vitro, such as inhibition of shoot elongation and hyperhydricity, and could be a factor limiting pumpkin ash adventitious shoot formation.

Responsive hypocotyls began to form callus and shoots after 4 weeks on shoot initiation medium (Fig. 1). After transfer to a shoot-bud induction medium with lower BA and no TDZ fully formed shoots were clearly evident after an additional 4 weeks (Fig. 2a). There was no effect on the number of adventitious shoots formed regardless of treatment (Table 1). Although white and green ash were capable of producing 4.6 and 3.5 shoots per hypocotyl, respectively, pumpkin ash produced no more than 1.5 shoots per hypocotyl (Du and Pijut 2008; Palla and Pijut 2011). However, all shoots were quick to elongate normally. Adventitious shoots originating from green callus (Fig. 2a) and the duration of time before complete formation (8 weeks) both suggest an indirect pattern of organogenesis.

Elongation and micropropagation were successful using the MSB5G medium supplemented with $13.3 \mu\text{M}$ BA, $1 \mu\text{M}$ IBA, $0.29 \mu\text{M}$ GA₃, and 0.2 g L^{-1} casein hydrolysate, however, this medium was inconsistent and some microshoots failed to elongate. Therefore, the elongation medium was changed to MSB5G medium with $10 \mu\text{M}$ BA and $10 \mu\text{M}$ TDZ, which had been found to be effective in stimulating green ash (*F. pennsylvanica*) and white ash (*F. americana*) shoot elongation (Du and Pijut 2008; Palla and Pijut 2011), and proved to be effective with pumpkin ash as well (Fig. 2b). Shoots grew rapidly on this medium and required sub-culturing monthly. Nodal sub-culturing which proved successful for pumpkin ash has also been effective with other North American hardwoods, such as many species of oak (Vieitez et al. 2009).

Pumpkin ash proved to be amenable to in vitro culture and adventitious shoots were recovered from hypocotyls with an overall 28% efficiency and 1.2 shoots per hypocotyl (Table 2). Large standard deviations across all treatments can be explained from genetic variation among the mixed bag of open-pollinated seeds utilized in this study. A varied response among differing genotypes has been well documented for many woody species in tissue culture systems. Although similar to other *Fraxinus* species, pumpkin ash maintains a system for adventitious shoot formation, and this protocol will provide a means for future study of genetic transformation for EAB resistance, and to prevent its extirpation from natural ecosystems.

Rooting of adventitious shoots

Pumpkin ash was extremely responsive to adventitious root formation as all treatments including the control ($0 \mu\text{M}$ IBA plus $0 \mu\text{M}$ IAA) formed roots (1.6 ± 0.6) at a high

Table 1 Effect of cytokinins on adventitious shoot regeneration from pumpkin ash hypocotyls after 16 weeks

Plant growth regulator (μM)		Hypocotyl explants	
BA	TDZ	Frequency of shoot formation (%)	Mean No. shoots per explant
0	0	0a	0b
8.9	0	16.9 \pm 8.0a	1.0 \pm 0ab
13.3	0	19.4 \pm 9.6a	1.2 \pm 0.4a
22.2	0	25.5 \pm 7.6a	1.2 \pm 0.3a
0	2.3	21.7 \pm 16.4a	1.3 \pm 0.4a
8.9	2.3	19.4 \pm 12.7a	1.0 \pm 0ab
13.3	2.3	29.3 \pm 27.5a	0.9 \pm 0.7ab
22.2	2.3	35.1 \pm 7.3a	1.5 \pm 0.3a
0	4.5	38.9 \pm 31.5a	1.1 \pm 0.1a
8.9	4.5	34.3 \pm 8.5a	1.2 \pm 0.4a
13.3	4.5	38.4 \pm 14.3a	1.4 \pm 0.4a
22.2	4.5	42.9 \pm 25.1a	1.1 \pm 0.2a
0	6.8	27.8 \pm 19.2a	1.1 \pm 0.1a
8.9	6.8	28.8 \pm 17.9a	1.2 \pm 0.3a
13.3	6.8	20.4 \pm 11.6a	1.0 \pm 0ab
22.2	6.8	18.1 \pm 6.4a	1.3 \pm 0.6a

Values represent means \pm SE for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$). Shoots regenerated on Murashige and Skoog medium supplemented with 6-benzyladenine (BA) and thidiazuron (TDZ)

**Fig. 1** Responsive hypocotyl after 4 weeks on MS medium supplemented with 22.2 μM BA and 4.5 μM TDZ

percentage (83%) (Table 3). Root formation without exogenously applied auxin had been reported for the green ash clone KA 2018 (Kim et al. 1998), but had not been demonstrated in pumpkin ash until this study. Roots

formed under the control treatment were significantly longer than those which incorporated exogenous auxin, but microshoots had significantly fewer roots (Table 3; Fig. 2c, d). Although a negative correlation was observed between auxin concentration and root length, the number of roots increased with additional auxin.

Roots became evident on the microshoots 10–14 days after culture on root initiation medium, and developed directly from the stems with minimal callus formation (Fig. 2e). The rapid expression of adventitious roots and the origin would suggest pumpkin ash cells were immediately competent to changes in cell fate, and followed a direct pattern of organogenesis. This pattern of adventitious root formation could explain the ease at which pumpkin microshoots form roots across all treatments. Emergence of roots was very uniform and occurred shortly after exposure to the 10 days dark culture period. Research with white ash and oak showed a short, initial dark period worked well to provide synchronous rooting as well as higher rooting percentages (Navarrete et al. 1989; Palla and Pijut 2011; van Sambeek et al. 2001; Vieitez et al. 2009).

The use of the lower inorganic salt concentration found in WPM for adventitious root formation had been shown effective for many ash species. Rooting trials conducted with common ash (*F. excelsior*), narrow-leaf ash (*F. angustifolia*), and green ash (*F. pennsylvanica*) all showed root formation in excess of 79%, and successful acclimatization, using WPM and various concentrations of IBA (Du and Pijut 2008; Hammatt and Ridout 1992; Kim et al. 1998; Perez-Parron et al. 1994). High levels of root formation have also been achieved for white (*F. americana*)

Fig. 2 Hypocotyl derived regeneration of pumpkin ash (*Fraxinus profunda*). **a** Fully formed adventitious shoot after 4 weeks on MS medium with 22.2 μM BA and 4.5 μM TDZ and 4 weeks on MSB5G medium with 6.7 μM BA, 1 μM TDZ, 0.29 μM GA₃, **b** Microshoot elongation on MSB5G medium with 10 μM BA and 10 μM TDZ, **c** Successfully rooted microshoots on WPM with 0 μM IBA and 0 μM IAA, **d** Successfully rooted microshoots on WPM with 4.9 μM IBA and 0 μM IAA, **e** Adventitious root protrusion from microshoot on WPM with 4.9 μM IBA and 2.9 μM IAA after 2 weeks, and **f** Successfully acclimatized plants growing in the greenhouse after 8 weeks



Table 2 Mean number of adventitious shoot formation for all treatments

Replicate	Frequency of shoot formation (%)	Mean No. shoots per explant
1	33.5 \pm 0.6a	1.3 \pm 0.3a
2	23.7 \pm 15.8a	1.1 \pm 0.4a
3	26.1 \pm 9.4a	1.1 \pm 0.2a

Values represent means \pm SE for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)

Table 3 Effect of auxin concentration on root formation of *Fraxinus profunda* in vitro microshoots after 6 weeks

Treatment IBA* + IAA** (μM)	Root formation (%)	Mean No. roots per shoot	Mean root length per shoot (cm)	Mean No. lateral roots per shoot
4.9 + 0	94.4a	3.8 \pm 1.9abc	2.3 \pm 1.0b	12.1 \pm 4.8ab
4.9 + 2.9	80.6a	3.2 \pm 1.3c	1.4 \pm 0.5c	18.2 \pm 12.4a
4.9 + 5.7	88.9a	4.6 \pm 1.7a	1.3 \pm 0.3c	11.8 \pm 8.4ab
4.9 + 8.6	83.3a	3.3 \pm 1.2bc	1.7 \pm 1.1bc	11.0 \pm 9.4ab
Control	83.3a	1.6 \pm 0.6d	4.3 \pm 2.0a	8.3 \pm 8.0b

Values represent means \pm SE for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$). * Indole-3-butyric acid (IBA). ** Indole-3-acetic acid (IAA)

and green ash, as well as other woody species using half-strength MS basal medium (Dai et al. 2011; van Sambeek et al. 2001; Zhu and Wei 2010).

Exogenous auxin had no significant effect on the number of lateral roots formed compared to the control, except for the treatment of 4.9 μM IBA plus 2.9 μM IAA. It was found in pumpkin ash that exogenous auxin acted to decrease root length, but did increase the total number of adventitious roots formed for all treatments compared to the control (Table 3). Therefore, an optimal system for adventitious root formation might include a two-step process where the microshoots were pulsed on an auxin medium in the dark prior to being transferred to plant growth regulator-free medium. This two-step process has been shown successful for both white (*F. americana*) and green ash (*F. pennsylvanica*), and common ash (*F. excelsior*) (Mitras et al. 2009; van Sambeek et al. 2001), as well as many other woody species (Espinosa et al. 2006; Leslie et al. 2010; Sanchez et al. 1996). We have shown that adventitious rooting was possible in pumpkin ash at relatively high percentages (81–94%) (Table 3). Woody plant medium supplemented with 4.9 μM IBA and 0 μM IAA for 10 days culture in the dark, followed by culture in the light produced roots 94% of the time and were well formed and appeared healthy (Fig. 2d).

Acclimatization of rooted plantlets

Continued growth and normal development of rooted plantlets was observed 8 weeks after transfer to the greenhouse (Fig. 2f). Twenty plants were randomly transplanted to larger TreepotsTM for further growth, overwintering, and future survival data. One hundred percent of in vitro rooted plants were successfully acclimatized to ambient greenhouse conditions.

Conclusions

A successful in vitro adventitious shoot regeneration, rooting, and plantlet acclimatization protocol was developed for pumpkin ash using hypocotyls from open-pollinated seed. This protocol will be used for pumpkin ash conservation, as well as genetic improvement for developing resistance to the EAB.

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approval to the exclusion of other products or vendors that also may be suitable.

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