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MICROPROPAGATION

Regeneration of plants from *Fraxinus americana* hypocotyls and cotyledons

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Abstract A plant regeneration protocol was developed for white ash (*Fraxinus americana* L.). Hypocotyls and cotyledons excised from embryos were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BA) plus thidiazuron (TDZ), and compared for organogenic potential. Sixty-six percent of hypocotyl segments and 10.4% of cotyledon segments produced adventitious shoots, with a mean number of adventitious shoots per explant of 3.5±0.9 and 2.5±1.5, respectively. The best regeneration medium (52% shoot formation; 47% shoot elongation) for hypocotyls was MS basal medium containing 22.2 μM BA plus 0.5 μM TDZ, producing a mean of 3.9±0.4 adventitious shoots. Adventitious shoots were established as proliferating shoot cultures following transfer to MS medium with Gamborg B5 vitamins supplemented with 10 μM BA plus 10 μM TDZ. For *in vitro* rooting, woody plant medium with indole-3-acetic acid (IAA) at 0, 2.9, 5.7, or 8.6 μM in combination with 4.9 μM indole-3-butyric acid (IBA) was tested for a 5- or 10-d dark culture period, followed by culture under a 16-h photoperiod. The best rooting (78% to 81%) of *in vitro* shoots was obtained with a 5 d dark culture treatment on medium containing 2.9 or 5.7 μM IAA plus 4.9 μM IBA, with an average of 2.6±0.4 roots per shoot. Rooted plants were successfully acclimatized to the greenhouse. This adventitious shoot regeneration and rooting protocol will be used as the basis for experimental studies to produce transgenic white ash with resistance to the emerald ash borer.

Keywords Adventitious shoots · *Fraxinus* rooting · Shoot organogenesis · Tissue culture · White ash

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Introduction

White ash, *Fraxinus americana* L. (family Oleaceae), is one of the most abundant and useful of the native ash species, providing both ecological and economic benefits. Endemic to North America, this species is integral to many ecosystems across the east from Nova Scotia to Texas and was recognized as a component species in 26 forest cover types (Schlesinger 1990). White ash trees provide shelter and nesting sites for a variety of wildlife. The seeds are food for several bird and mammal species, and its bark was found to be utilized by rabbits, deer, beaver, and porcupine (Griffith 1991). White ash is valued as an ornamental shade tree and as a pioneer species, making it ideal for use in urban areas as well as reclamation of disturbed sites (Nesom 2001). Ash trees composed 5% to 29% of all street trees in the Midwest, replacing many of the elm trees in urban settings since the advent of Dutch elm disease (MacFarlane and Meyer 2005). White ash has high economic value as a commercial hardwood, with timber trees valued for wood strength, straight grain, heavy weight, and elasticity. The wood is used in the production of tool handles, furniture, flooring, crates, boats, doors, and cabinets. White ash is the wood most often used in the production of baseball bats. The estimated value of the 3.8 billion urban ash trees in the USA was $2.4 trillion, with an

additional $282.3 billion in compensatory value from the 7.5 million ash trees growing on US timberlands (Sydnor *et al*. 2007).

There exists a threat to white ash in the form of the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). EAB is an aggressive, wood-boring beetle indigenous to China, Japan, Taiwan, Korea, Mongolia, and eastern Russia (Yu 1992). EAB has become a devastating invasive pest to the North American landscape since being discovered in southeastern Michigan in 2002. The adult beetles feed on the foliage of ash trees, but most of the damage is produced by the larval stage. The larvae bore through the bark and into the cambial region, feeding and producing galleries that disrupt the flow of water and nutrients, eventually killing the tree. Over one-half of a tree's canopy can be devastated within the first 2 yr, and the entire tree will usually be dead within 3- to 4-yr after first observed symptoms (McCullough and Katovich 2004). EAB is fatal to ash trees, and there is neither any known innate resistance in native trees nor any means to completely eradicate the beetle (Poland and McCullough 2006). All native North American ash species are susceptible, and there is evidence that EAB is spreading quickly throughout North America, placing ash trees in danger (Dobesberger 2002). Devastation of this tree species as a result of EAB infestation will be a huge economic and ecological loss. Development of an *in vitro* protocol for regenerating white ash plants will prove valuable in producing improved ash germplasm. Such a protocol will also be necessary to genetically modify white ash to produce resistance to the EAB, thus benefiting economic markets and ecological landscapes.

Adventitious shoot regeneration has been attempted for several ash species. Callus formation was induced using isolated petioles of *F. americana*, but no shoot regeneration from that callus was achieved (Hicks and Browne 1983). Navarrete *et al*. (1989) reported explant growth and axillary shoot proliferation from *in vitro* germinated, cut white ash seeds. Bates *et al*. (1992), using cotyledons, hypocotyls, and epicotyls from mature white ash seeds, regenerated adventitious buds and shoots, but only a few shoots elongated enough to be rooted. Adventitious shoot regeneration from hypocotyls (Tabrett and Hammatt 1992) and direct organogenesis from hypocotyls, embryonic leaves, and stems (Mockeliunaite and Kuusiene 2004) of common ash (*Fraxinus excelsior*) have also been reported. High rooting (76%) was achieved by Tonon *et al*. (2001b) for narrowleaf ash (*Fraxinus angustifolia*) by culturing shoots in the dark for 5 d on root induction medium then 15 d in the light on root expression medium. Du and Pijut (2008) developed a complete plant regeneration protocol using hypocotyls and cotyledons of green ash (*Fraxinus pennsylvanica*). Mitras *et al*. (2009) successfully used epicotyls for *in vitro* propagation of *F. excelsior*. The present study was initiated to develop a protocol for adventitious shoot regeneration and rooting of white ash for further use in genetic transformation studies.

Materials and Methods

Adventitious shoot induction. Mature white ash seeds purchased in 2004 from F.W. Schumacher Co., Inc. (Sandwich, MA) were stored in a sealed container in the dark at 5°C until used. The pericarps were cut and removed along with 2- to 3-mm of the seed opposite the radical. Any seeds with noticeable insect or fungal damage were discarded. The seeds were surface disinfected in 70% (*v*/*v*) ethanol for 30 s and then immersed in 20% bleach solution (5.25% sodium hypochlorite) for 18 min, followed by three rinses in sterile, distilled water before being stored in sterile, distilled water overnight at 24±2°C. After 24 h, the turgid embryos were extracted, and cotyledon and hypocotyl segments were cultured horizontally on Murashige and Skoog (MS) medium (1962) supplemented with 13.3 μM 6-benzylaminopurine (BA) plus 4.5 μM thidiazuron (TDZ) to induce adventitious shoot formation (Du and Pijut 2008). Four to five replications of each explant type were tested, with 12 to 17 explants per replication. Unless noted otherwise, all cultures were incubated at 24±2°C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}), and all media included 3% (*w*/*v*) sucrose, 0.7% (*w*/*v*) Difco Bacto agar, with the pH of the medium adjusted to 5.7 prior to autoclaving and dispensing at 45 ml per 100×25-mm Petri plates. After obtaining these results, adventitious shoot regeneration was optimized for hypocotyls using 0, 4.4, 8.9, 13.3, or 22.2 μM BA in combination with 0, 0.5, 2.3, or 4.5 μM TDZ (Du and Pijut 2008). Five replications with 15 hypocotyls per treatment per replication were conducted. Cultures were incubated for 4 wk to induce callus and shoot bud formation.

Elongation of adventitious shoots. After 4 wk culture on induction medium, all explants initiating shoot buds were transferred to MS basal salt medium plus Gamborg B5 vitamins (MSB5) supplemented with 10 μM BA and 10 μM TDZ (Kim *et al*. 1997) to induce shoot elongation. Magenta GA-7 vessels (Magenta Corp., Chicago, IL) containing 50 ml of medium were used. Cultures were incubated under a 16-h photoperiod for 4- to 5-wk. Elongated shoots were continuously removed from the original explant when approximately 3 cm in length and placed directly onto root induction medium.

Establishment of in vitro germinated shoots. To supplement the number of adventitious shoots in culture for rooting experiments, proliferating shoot cultures were established

from *in vitro* germinated embryos in order to obtain a significant number of microshoots. White ash seeds were surface disinfected and the embryos extracted as described previously. Intact embryos were then cultured vertically on MSB5 medium supplemented with 10 μM BA plus 10 μM TDZ. Magenta GA-7 vessels containing 50 ml of medium were used. The *in vitro* shoots established from the embryos were micropropagated on the same medium and continuously subcultured to fresh medium every 4 wk for shoot culture establishment.

Rooting of adventitious and in vitro germinated shoots. Elongated adventitious and *in vitro* germinated shoots were pooled and randomly placed on woody plant medium (WPM; Lloyd and McCown 1980) supplemented with 4.9 μM indole-3-butyric acid (IBA) plus 0, 2.9, 5.7, or 8.6 μM indole-3-acetic acid (IAA). Magenta GA-7 vessels containing 50 ml of medium were used. Cultures were placed in the dark at 26°C for either 5- or 10-d, then incubated for 4- to 5-wk at 24±2°C under a 16-h photoperiod, after which rooting percentage, the number of roots per shoot, and root length were recorded. Nine replications with four shoots per treatment per replication were conducted.

Acclimatization of rooted plants. Rooted plantlets were removed from the Magenta vessels and transplanted into 10×9-cm plastic pots containing a moist, autoclaved, soilless medium with high porosity (Premier ProMix HP; Premier Horticulture Inc., Quakertown, PA). Any agar clinging to the roots was gently removed with distilled water prior to potting. Plants in pots were then placed in sealed 3.8-L plastic bags to provide a high relative humidity and grown at 24±2°C under a 16-h photoperiod. Watered as needed to maintain moisture, the plants were acclimatized to the culture room over a 2- to 3-wk period by progressively opening the bags until plants were able to survive without wilting. Plants were then transferred to the greenhouse, transplanted to larger pots containing a peat- and perlite-based, soilless medium with high porosity (Premier ProMix HP/Mycorise® Pro; Premier Horticulture Inc.), and watered, as needed, with fertilizer water [15N-5P-15K commercial fertilizer formulation (Miracle Gro© Excel© Cal-Mag; The Scotts Co., Marysville, OH)] that was adjusted to a pH range of 5.7 to 6.0 using 93% sulfuric acid (Ulrich Chemical, Indianapolis, IN). Three replications of 12 plants per replication were acclimatized.

Statistical analysis. SPSS (software version 17; SPSS 2009) was used to analyze data. An analysis of variance (ANOVA) was performed using the General Linear Model procedure on the individual replicate means by treatment for percent shoot formation, number of shoots per hypocotyl, percent root formation, number of roots per shoot, root length, and number of lateral roots. If the ANOVA indicated a statistical significance, a Duncan's comparison test with an alpha level of 0.05 was used to distinguish the differences between treatments.

Results and Discussion

Adventitious shoot induction. A collection of open-pollinated seed was used to normalize the regeneration process across a random mix of white ash genotypes. The tips of the cotyledons were removed during the surface sterilization process to not only make it easier to excise the embryos post-imbibition, but because cutting one-third of the cotyledons had been proven to increase germination rates (Preece *et al.* 1989, 1995). After 1 wk culture on MS medium supplemented with BA and TDZ, callus formation occurred on the cut ends and wounded surface area of hypocotyls, and shoots began to initiate. Cotyledon explants took longer to form callus, with many of these explants never regenerating shoots (Fig. 1). Both types of explants regenerated most shoots at the cut end previously nearest the shoot apical meristem.

Green and white ash are in the Melioides clade and therefore have high nuclear and morphological similarities (Wallander 2008). The adventitious shoot regeneration protocol developed in our lab for green ash (*F. pennsylvanica*; Du and Pijut 2008) was therefore used initially to compare the response of white ash hypocotyls and cotyledons and to determine the best explant for further development of the regeneration protocol. Hypocotyls proved to have a significantly higher organogenic potential than cotyledons in white ash (Fig. 2*A*; Table 1). Sixty-six percent of hypocotyl segments produced adventitious shoots, but only 10.4% of cotyledon segments regenerated in this way, with a mean number of adventitious shoots per explant of 3.5±0.9 and 2.5±1.5, respectively. This was consistent with results

Figure 1. Callus formation and shoot initiation on a cotyledon explant of *Fraxinus americana*. Cotyledon cultured for 4 wk on Murashige and Skoog medium with 13.3 μM 6-benzylaminopurine plus 4.5 μM thidiazuron.

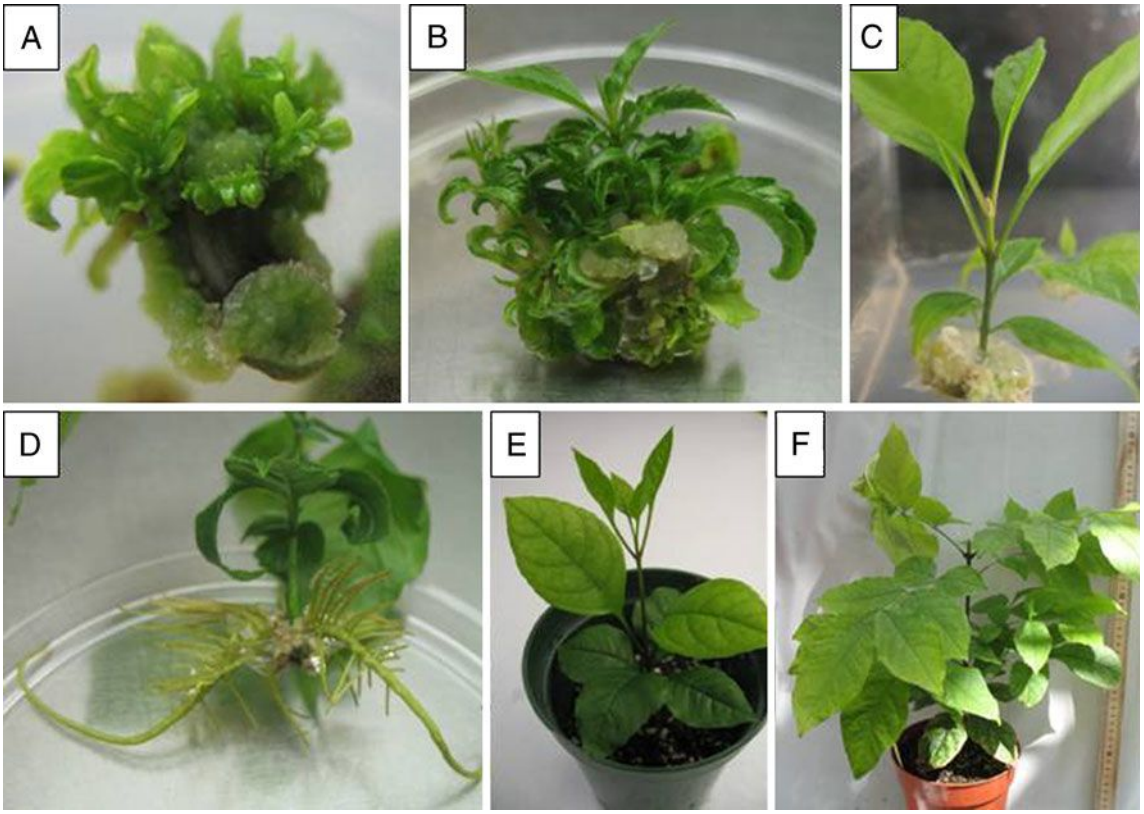

Figure 2. Plant regeneration from hypocotyls of *Fraxinus americana*. *A*: Callus formation and adventitious shoot induction on a hypocotyl explant after 4 wk on Murashige and Skoog (MS) medium with 13.3 μM 6-benzylaminopurine (BA) plus 4.5 μM thidiazuron (TDZ). *B*, *C*: Elongation of adventitious shoots from hypocotyls on MS with 10 μM BA plus 10 μM TDZ. *D*: Shoot with roots on woody plant medium with 2.9 μM indole-3-acetic acid plus 4.9 μM indole-3-butyric acid after 5 d in the dark and then 4 wk under a 16-h photoperiod. *E*: Acclimatized white ash plantlet in the culture room producing simple leaves. *F*: Acclimatized white ash plant in the greenhouse exhibiting compound leaf growth.

reported by Preece and Bates (1995) and Tonon *et al*. (2001a) which noted a lower organogenic potential of *Fraxinus angustifolia* cotyledons compared to embryo axes. The lower shoot formation rate observed for white ash cotyledons (10.4%) compared to green ash cotyledons (24%; Du and Pijut 2008) could be a result of white ash having a stronger and more complicated hormonally controlled dormancy than green ash (Ashley and Preece 2009).

All combinations of BA and TDZ in the induction medium produced some level of adventitious shoot formation on hypocotyls. Seventy-three percent of hypocotyls produced shoots on medium containing only 0.5 μM TDZ, but this percentage did not accurately represent the best regeneration medium, as only 19% of the shoots elongated (Table 2). To better elucidate which medium would produce a high percentage of shoot formation along with shoot elongation, we present the percentage of hypocotyls producing shoots, percent shoot elongation, and mean number of shoots elongating per explant (Fig. 2*B*, *C*; Table 2). This was a more accurate representation of what medium was the most successful to use for white ash regeneration. The medium supplemented with 22.2 μM BA plus 0.5 μM TDZ proved to be the best adventitious shoot induction medium overall. Even though the percent shoot formation (52%) appears low, this medium produced the highest percentage (47%) of shoots able to elongate and the

Table 1. Effect of explant type on *Fraxinus americana* adventitious shoot regeneration

Explant type	Callus formation (%)	Shoot formation (%)	Mean no. of shoots per explant
Cotyledon	28.9±25.2	10.4±8.0	2.5±1.5
Hypocotyl	91.5±6.4	66.1±19.5	3.5±0.9

Values are means±standard deviation of responsive explants (49 cotyledons and 72 hypocotyls) on Murashige and Skoog medium supplemented with 13.3 μM 6-benzylaminopurine and 4.5 μM thidiazuron

Table 2. Effect of cytokinins on adventitious shoot regeneration from hypocotyls of white ash

Plant growth regulator (μM)		Hypocotyl		
BA	TDZ	Shoot formation[a] (%)	Shoot elongation[b] (%)	Mean no. of shoots[c]
0.0	0.0	0e	0c	0c
4.4	0.0	68.0±6.8ab	32.7±8.8ab	2.9±1.0ab
8.9	0.0	61.3±3.3abc	28.8±11.0ab	2.1±0.6abc
13.3	0.0	54.7±2.5abcd	25.0±8.6abc	2.2±0.6abc
22.2	0.0	41.3±7.1d	35.4±9.6ab	4.6±2.4a
0.0	0.5	73.3±6.0a	19.4±5.3bc	2.4±0.7abc
4.4	0.5	54.7±3.3abcd	19.7±7.1bc	1.7±0.6bc
8.9	0.5	62.7±4.5abc	27.5±10.6ab	3.2±0.9ab
13.3	0.5	61.3±5.7abc	32.7±5.8ab	2.3±0.5abc
22.2	0.5	52.0±7.4bcd	46.9±7.7a	3.9±0.4ab
0.0	2.3	62.7±5.4abc	18.6±5.8bc	2.3±0.8abc
4.4	2.3	54.7±7.1abcd	16.3±4.8bc	1.6±0.9bc
8.9	2.3	62.7±3.4abc	21.5±5.6abc	2.5±0.5abc
13.3	2.3	54.7±4.4abcd	20.1±11.9bc	2.1±0.9abc
22.2	2.3	50.7±10.7bcd	31.2±6.2ab	1.4±0.2bc
0.0	4.5	68.9±4.6ab	23.8±4.9abc	2.3±0.7abc
4.4	4.5	52.0±6.4bcd	42.6±7.9ab	3.0±0.6ab
8.9	4.5	52.0±5.0bcd	22.1±7.0abc	1.3±0.3bc
13.3	4.5	68.0±3.9ab	27.0±5.3ab	1.5±0.2bc
22.2	4.5	48.0±5.7cd	41.6±12.1ab	2.9±0.7ab

Means in each *column* followed by the same *letter* are not significantly different according to Duncan's multiple comparison test (α=0.05)

[a] Mean±standard error for 75 explants per treatment

[b] Mean±standard error for hypocotyls that produced leaf primordia and were able to elongate shoots

[c] Mean number of shoots based on the number of hypocotyls transferred to Murashige and Skoog medium supplemented with 10 μM 6-benzylaminopurine (BA) and 10 μM thidiazuron (TDZ)

highest mean number of shoots elongating per hypocotyl (3.9±0.4). The BA concentration was found to be a significant factor in determining the number of shoots initiated that would eventually elongate from the hypocotyl explants. This same BA concentration (22.2 μM) also proved successful for proliferation of adventitious shoots from *Fraxinus excelsior* hypocotyls (Tabrett and Hammatt 1992). The concentration of TDZ at 0.5, 2.3, or 4.5 μM was

Table 3. Effect of auxin concentration on *in vitro* root formation of white ash microshoots

Treatment		Rooting (%)	Mean no. of roots	Mean root length (cm)	Mean no. of lateral roots
Dark period (days)	IAA+IBA (μM)				
5	0.0+4.9	52.8±10.6abc	1.8±0.2b	4.0±0.6ab	7.7±2.0a
5	2.9+4.9	80.6±8.1a	2.5±0.3ab	3.6±0.7ab	9.1±2.0a
5	5.7+4.9	77.8±6.5a	2.6±0.4ab	4.1±0.8a	7.6±1.5a
5	8.6+4.9	69.4±10.0ab	3.6±0.6a	2.7±0.6abc	4.1±1.1a
10	0.0+4.9	36.1±12.6c	1.8±0.5b	1.6±0.7c	5.7±3.2a
10	2.9+4.9	66.7±9.3abc	2.3±0.4ab	2.5±0.6abc	5.8±2.3a
10	5.7+4.9	63.9±11.1abc	2.3±0.4ab	2.1±0.6bc	3.7±1.6a
10	8.6+4.9	41.7±11.8bc	1.4±0.4b	1.2±0.3c	4.1±1.2a

Values represent the means±standard errors for 36 explants per treatment. Means in each *column* followed by the same *letter* are not significantly different according to Duncan's multiple comparison test (α=0.05)

IAA indole-3-acetic acid, *IBA* indole-3-butyric acid

a significant factor in percent shoot formation on hypocotyls in combination with zero BA, inducing a high percentage of shoot formation of 73%, 63%, and 69%, respectively. This was consistent with the findings of Navarrete *et al*. (1989), who noted that 3 μM TDZ was the maximum concentration allowing for the most shoot proliferation of white ash while having minimal callus formation. With our protocol, only a 3- to 4-wk culture on one medium was necessary to induce shoots on hypocotyl explants. No additional plant growth regulator overlays or different medium transfers were necessary, as had previously been reported (Navarrete *et al*. 1989; Preece *et al*. 1991; Van Sambeek *et al*. 2001).

Rooting of adventitious and in vitro grown shoots. A collection of *in vitro* shoots (adventitious and *in vitro* micropropagated) were randomly divided among eight root induction treatments. Roots were visible 10 d after transferring cultures to the light, with slight formation of callus at the basal end of the shoot. Rarely did shoots form roots without a small amount of light green callus. Significantly higher rooting (53% to 81%), number of roots (1.8 to 3.6), and root lengths (2.7 to 4.1 cm) were obtained with the 5 d dark culture treatment. The most effective root induction medium was WPM supplemented with either 2.9 or 5.7 μM IAA plus 4.9 μM IBA for 5 d in the dark (Fig. 2*D*), with 81% and 78% of shoots rooted with an average of 2.5±0.3 and 2.6±0.4 roots produced per shoot, respectively. The number of lateral roots formed was not statistically significant across all treatments (Table 3).

Preece *et al*. (1995), Kim *et al*. (1998), and Tonon *et al*. (2001a, b) reported rooting of *Fraxinus* spp. on auxin-free medium, but it was noted that the addition of auxin provided more synchronous root formation. Half- or full-strength WPM supplemented with 4.9 μM IBA had previously been proven successful for inducing root formation in *Fraxinus* spp. (Hammatt and Ridout 1992; Tabrett and Hammatt 1992; Perez-Parron *et al*. 1994; Tonon *et al*. 2001a; Du and Pijut 2008) as well as MS and MSB5 medium (Preece *et al*. 1987; Navarrete *et al*. 1989; Kim *et al*. 1998). The addition of 2.9 to 8.6 μM IAA to WPM supplemented with 4.9 μM IBA produced significant rooting (89–90%) of *F. pennsylvanica* shoots (Du and Pijut 2008), along with a 10 d dark pretreatment. Tonon *et al*. (2001b) achieved high rooting (76%) for *F. angustifolia* by exposing shoots to a root induction medium for 5 d in the dark before transferring shoots to a root expression medium, and then placing cultures in the light. A similar two-step process was used for *F. americana* and *F. pennsylvanica* (Navarrete *et al*. 1989; Van Sambeek *et al*. 2001) with a 4- to 8-d dark pulse on root induction medium followed by culture in the light on root elongation medium. Our rooting protocol eliminates the need to use two different media for root induction and elongation and required only 5 d in the dark. We achieved 81% rooting of white ash shoots with an average of 2.5 roots per shoot, a mean root length of 3.6 cm, and a mean number of lateral roots of 9.1 using one medium. Previously, approximately 70% of shoots rooted with an average of 2.2 roots per shoot after 7 d in the dark on root induction medium and culture in the light on root elongation medium (Van Sambeek *et al*. 2001).

Acclimatization of rooted plants. Thirty-eight rooted plantlets were chosen randomly for acclimatization. Plantlets with two to three internodes, four to six fully formed leaves, and well-developed roots were used (Fig. 2*D*). Simple leaves were observed on plantlets regenerated *in vitro* and acclimatized to the culture room (Fig. 2*E*), with compound leaf development observed after continued growth and acclimatization to the greenhouse (Fig. 2*F*). This was consistent with previous observations on growth of white ash started *in vitro* (Van Sambeek *et al*. 2001). No morphological abnormalities were observed with our white ash plants, and 100% of the plants survived acclimatization to the greenhouse.

Conclusions

A successful *in vitro* adventitious shoot regeneration, rooting, and plantlet acclimatization protocol for *F. americana* was developed using hypocotyls obtained from a collection of open-pollinated seeds. This protocol will be used for experimental studies to produce transgenic white ash with resistance to the EAB or transfer of other value-added traits for genetic improvement and conservation.

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