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Regeneration of plants from *Fraxinus pennsylvanica* hypocotyls and cotyledons☆

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ABSTRACT

An adventitious shoot regeneration and rooting protocol was developed for green ash (*Fraxinus pennsylvanica*) seedling explants. The best regeneration medium for freshly isolated hypocotyls and cotyledons was Murashige and Skoog (MS) supplemented with 13.3 μM 6-benzylaminopurine (BA) plus 4.5 μM thidiazuron (TDZ), and 22.2 μM BA plus 4.5 μM TDZ, respectively. Seventy-six percent of hypocotyl segments and 24% of cotyledon segments produced adventitious shoots, with a mean number of adventitious shoots per explant of 2.7 ± 0.5 and 2.3 ± 1.3, respectively. The effect of in vitro-germinated seedling age on adventitious shoot regeneration from hypocotyl and cotyledon explants was also studied. Results showed that hypocotyl and cotyledon explants from freshly isolated embryos exhibited a higher organogenesis potential than 4–15-day-old explants. Adventitious shoots from hypocotyls and cotyledons were established as proliferating shoot cultures following transfer to MS basal medium with Gamborg B5 vitamins supplemented with 10 μM BA plus 10 μM TDZ. A high rooting percentage (73–90%) was achieved when in vitro shoots were rooted on woody plant medium (WPM) containing 4.9 μM indole-3-butyric acid (IBA) and IAA (0, 2.9, 5.7, or 8.6 μM) with a combination of 10-day dark culture period followed by a 16-h photoperiod. The highest rooting (90%) of adventitious shoots or the number of roots per shoot (3.0 ± 1.0) was obtained on WPM with 4.9 μM IBA plus 5.7 μM IAA. Rooted plants were successfully acclimatized to the greenhouse and 100% survived after overwintering in cold storage. This regeneration system using hypocotyls and cotyledons provides a foundation for *Agrobacterium*-mediated genetic transformation of *F. pennsylvanica* for resistance to the emerald ash borer.

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1. Introduction

Green ash (*Fraxinus pennsylvanica*) (family Oleaceae) is an important North American tree species. The wood is used for general solid wood products such as, crates, boxes, and for specialty products such as tool handles, furniture, oars, and canoe paddles because of the strength, hardness, shock resistance, and excellent bending qualities of the wood. Green ash woodlands are important in supporting a rich bird community by providing cover and nesting sites for various bird species (Rumble and Gobeille, 1998). Green ash is also very popular as a shade tree because of its good form, adaptability to various soil conditions, and normally relatively free from insect and diseases (Kennedy, 1990). Many of the ash trees grown as urban street, shade, and landscape trees were once planted to replace elm trees destroyed by Dutch elm disease. Ash trees now comprise 5–20% of all street trees throughout North America. However, the emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae)), an aggressive exotic phloem-feeding beetle from Asia, has been found infesting green ash, white ash (*Fraxinus americana*), black ash (*Fraxinus nigra*), as well as several horticultural varieties of ash in Ohio, Indiana, Illinois, Michigan, Maryland, and Ontario. This devastation results in significant economic loss and environmental damage in these urban areas with EAB infestation (Dobesberger, 2002; Haack et al., 2002). The pest is fatal to an infested tree, at present there is no effective means to completely eradicate the EAB, and evidence exists that the EAB is spreading throughout North America (Poland and McCullough, 2006).

Genetic engineering is a feasible tool to produce plants with resistance to pests compared to conventional breeding strategies. Successful genetic transformation of pest-resistance genes into tree species has been reported (McCown et al., 1991; Shin et al., 1994; Kleiner et al., 1995; Leple et al., 1995; Wang et al., 1996;

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Meilan et al., 2000; Wu et al., 2000; Delledonne et al., 2001; Kedong et al., 2001). Adventitious shoot regeneration in tissue culture from various plant organs is a prerequisite for genetic modification via *Agrobacterium*-mediated transformation. Adventitious regeneration has been attempted for several ash species. Successful adventitious shoot regeneration from embryo hypocotyls of common ash (*Fraxinus excelsior*) has been reported (Tabrett and Hammatt, 1992). Shoot production and proliferation was better on Murashige and Skoog (1962) (MS) media containing thidiazuron (TDZ) instead of 6-benzylaminopurine (BA). Adventitious shoot regeneration from whole leaves has also been reported for common ash on MS containing TDZ (Hammatt, 1996). Most shoots were produced from the leaflet axis, but shoots also regenerated from the cut ends of petioles and wounded sites on leaves. When using petioles as explants, three out of 33 cultures produced adventitious shoots that elongated. Mockeliunaite and Kuusiene (2004) used hypocotyls, embryonic leaves and stems for direct organogenesis of *F. excelsior*. Tonon et al. (2001a) used embryo axes and cotyledons from mature seeds of *Fraxinus angustifolia* to induce adventitious shoots, and results showed that embryo axes had a higher regeneration potential than cotyledons. Isolated petioles of *F. americana* were used as explants to induce callus, but there was no report of shoot regeneration from this callus (Hicks and Browne, 1983). Bates et al. (1992) reported adventitious bud and shoot regeneration on cotyledons, hypocotyls, excised epicotyls, or through callus from mature seeds of white ash, but only a few shoots elongated that could be rooted.

To our knowledge, there are no reports of adventitious shoot regeneration for green ash. There are two reports about micropropagation of green ash in which stable axillary shoot development was achieved on MS basal medium with Gamborg B5 (MSB5) vitamins (Gamborg et al., 1968) supplemented with BA, TDZ, and indole-3-butyric acid (IBA) (Kim et al., 1997, 1998). The aim of the present study was to develop a protocol for adventitious shoot regeneration of green ash using hypocotyls or cotyledons for further use in genetic transformation studies.

2. Materials and methods

2.1. Adventitious shoot induction from hypocotyls and cotyledons

Mature seeds of green ash were purchased from F.W. Schumacher Co., Inc. (Sandwich, MA) and stored at 5 °C in the dark until the time of use. The pericarps were removed, and 2–3 mm opposite the radical was excised. Seeds were surface disinfected in 70% (v/v) ethanol for 30 s, then immersed in 20% Clorox® bleach solution (5.25% sodium hypochlorite) for 10 min, followed by several rinses in sterile, distilled water, and the seeds were stored in sterile, distilled water overnight. The following day, the embryos were easily excised, hypocotyl and cotyledon segments were excised from the freshly isolated embryos and cultured horizontally on MS supplemented with 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 (100 mm × 25 mm Petri plates; 45 ml medium) for adventitious shoot induction. Three replications with 20 explants per treatment per replication were used. All media included 3% (w/v) sucrose, 0.7% (w/v) Difco-Bacto agar, and the pH of the medium was adjusted to 5.7 prior to autoclaving. All cultures were incubated at 24 ± 2 °C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}) for 4 weeks. To further optimize the adventitious shoot regeneration potential of the hypocotyl and cotyledon explants, the effect of age of in vitro-germinated seedlings (0-, 4-, 7-, 10-, or 15-day old) were investigated. The embryos were vertically cultured on MS without plant growth regulators (100 mm × 25 mm Petri plates; 45 ml medium) for germination. Hypocotyl and cotyledon segments were excised from different age in vitro seedlings and cultured horizontally on the best medium for adventitious shoot induction. Three replications with 20 explants per treatment per replication were used. After initial regeneration induction, shoot buds and callus together with the original hypocotyl or cotyledon explant were transferred to MSB5 medium supplemented with 10 μM BA plus 10 μM TDZ (Kim et al., 1997) to induce shoot elongation. Explants were sub-cultured to fresh media every 4 weeks for shoot elongation and culture establishment.

2.2. Rooting of micropropagated shoots

In order to obtain an adequate number of microshoots for rooting experiments, shoot proliferating cultures were established from nodal explants of juvenile seedlings on MSB5 containing 10 μM BA plus 10 μM TDZ. Microshoots approximately 2–3 cm long from each of three genotypes (GAS-001 and GAS-002 were in vitro-grown seedlings; mature seeds purchased from F.W. Schumacher Co., Inc.), and GA-JP03-10 was a 2-year-old seedling grown in the greenhouse; originally field-grown state nursery stock) were used in experiments to induce root formation using woody plant medium (WPM) (Lloyd and McCown, 1980) supplemented with 4.9 μM IBA in combination with 0, 2.9, 5.7, or 8.6 μM IAA, 3% (w/v) sucrose, and 0.7% (w/v) Difco-Bacto agar. Twelve microshoots per genotype per treatment were used and three replications were conducted. Microshoots used for the rooting experiments were cultured in Magenta GA-7 vessels (Magenta Corp., Chicago, IL; 50 ml medium) and kept in the dark at 26 °C for 10 days prior to transfer under a 16-h photoperiod (80 μmol m^{-2} s^{-1}). Rooting percentage, the number of roots per shoot, root length, and the number of lateral roots were recorded 6 weeks after shoots were placed on root induction medium.

2.3. Rooting of adventitious shoots regenerated from hypocotyls and cotyledons

Elongated adventitious shoots from hypocotyls and cotyledons were pooled and placed on the best root induction medium obtained above. The procedure was the same as for rooting of micropropagated shoots. Data were collected 6 weeks after shoots were placed on root induction medium.

2.4. Acclimatization of rooted plants

Rooted plantlets (12 plantlets for each seedling genotype (36 total); 12 plantlets from hypocotyls and cotyledons) were gently rinsed in distilled water to remove any agar from the roots. Plantlets were transplanted into 10 cm × 9 cm plastic pots containing a moist, autoclaved peat moss:vermiculite:perlite mixture (1:1:1) and pots were placed in 1-gal Ziploc® plastic bags to provide a high relative humidity. Plantlets in pots were watered every 2 days with regular water and acclimatized over a period of 2 weeks in the culture room by progressively opening the bag until plants were ready for transfer to the greenhouse. After 2 weeks in the culture room, the plantlets in small pots were moved to the greenhouse for 1 week, and then plantlets were transplanted into tall Treepots™ (Stuewe and Sons, Inc., Corvallis, OR) and watered daily with fertilizer water (15N-5P-15K commercial fertilizer (Miracle Gro Excel Cal-Mag; The Scotts Co., Marysville, OH). In mid-October plantlets were placed in cooler conditions for hardening off before overwintering in late November in a controlled cold-storage environment (3–4 °C in darkness). After overwintering, the plants were returned to the greenhouse the following year (early March), allowed to acclimatize to this environment, initiate new growth, and survival data were recorded.

2.5. Statistical analysis

Data were analyzed with an analysis of variance (ANOVA) using the GLM procedure of SAS (Software Version 8) (SAS, 1999). When the ANOVA indicated statistical significance, a Tukey's comparison test was used to distinguish differences between treatments at the 5% level of probability.

3. Results and discussion

3.1. Adventitious shoot induction and elongation

After culture on MS supplemented with BA and TDZ for 1 week, adventitious shoot regeneration occurred on hypocotyl ends opposite to the radicle position and on the wounded surface area of hypocotyls. For cotyledons, most adventitious shoot regeneration occurred from the end previously near the hypocotyl and the surface of cotyledon after cultured on medium for 3 weeks (Fig. 1A and B). Most explants produced adventitious shoots through direct organogenesis and occasionally through a callus stage. The frequency of adventitious shoot regeneration from hypocotyls and cotyledons ranged from 15.5 to 75.5% and 0 to 24.4%, respectively (Table 1). There were no adventitious shoots produced from hypocotyl and cotyledon segments on plant growth regulator-free MS. The best percent shoot regeneration for hypocotyls (75.5%) and cotyledons (24.4%) were obtained with 13.3 μM BA plus 4.5 μM TDZ and 22.2 μM BA plus 4.5 μM TDZ, respectively. In the present study, hypocotyls had a greater regenerative potential than cotyledons. Similar results were reported by Tonon et al. (2001a) who used embryo axes and cotyledons of *F. angustifolia* to induce shoot regeneration. The shoots regenerated from wounded tissue in contact with the medium by direct organogenesis and no callus production was associated with regeneration. It was reported that different portions of the embryo axes had different regenerative capacities. The embryo axes with root and apical meristems removed exhibited the highest regenerative capacity. It may indicate that cotyledon presence on the explants resulted in a strong inhibition of organogenesis potential.

Regeneration of adventitious shoots from hypocotyls has also been reported by Tabrett and Hammatt (1992) using *F. excelsior* dried seeds. MS supplemented with TDZ instead of BA increased the proportion of ash embryo hypocotyl explants that produced adventitious shoots, and adventitious shoots were transferred to a Driver and Kuniyuki medium (DKW) (1984) with 22.2 μM BA for proliferation. Regeneration of adventitious shoots from cotyledons

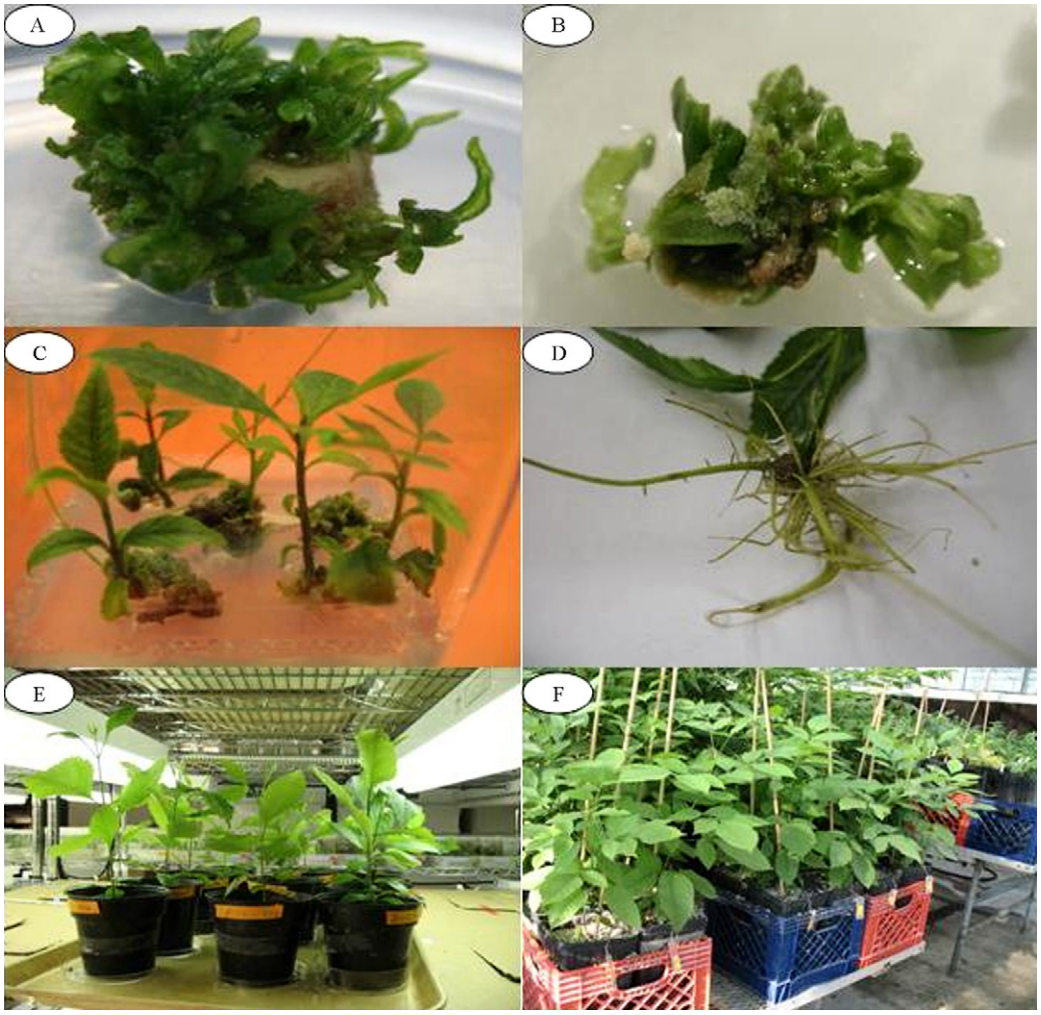

Fig. 1. Plant regeneration from hypocotyls and cotyledons of *Fraxinus pennsylvanica*. (A) Adventitious shoot induction from hypocotyls on MS with 13.3 μM BA plus 4.5 μM TDZ after 4 weeks. (B) Adventitious shoot induction from cotyledons on MS with 22.2 μM BA plus 4.5 μM TDZ after 4 weeks. (C) Adventitious shoot elongation on MSB5 with 10 μM BA plus 10 μM TDZ. (D) Rooting of shoots on WPM with 4.9 μM IBA plus 5.7 μM IAA. (E) Normal growth of potted green ash in culture room after 2 weeks. (F) Acclimatization of plantlets to the greenhouse after 4 weeks.

Table 1
Effect of cytokinin combinations on adventitious shoot regeneration from freshly isolated hypocotyls and cotyledons of green ash

Plant growth regulator (μM)		Hypocotyl		Cotyledon	
BA	TDZ	Shoot formation (%)	Mean no. shoots	Shoot formation (%)	Mean no. shoots
0	0	0e	0b	0c	0a
4.4	0	33.3 ± 0bcde	3.1 ± 1.5a	0c	0a
8.9	0	46.7 ± 26.7abcd	2.7 ± 0.5a	0c	0a
13.3	0	55.5 ± 20.4abcd	2.1 ± 0.1ab	0c	0a
22.2	0	66.7 ± 6.7ab	2.7 ± 0.6a	8.9 ± 7.7bc	1.5 ± 1.3a
0	0.5	15.5 ± 3.9de	1.6 ± 0.5ab	0c	0a
4.4	0.5	55.6 ± 7.7abcd	2.1 ± 0.3ab	0c	0a
8.9	0.5	51.1 ± 13.9abcd	2.7 ± 0.4a	2.2 ± 3.9c	0.3 ± 0.6a
13.3	0.5	55.6 ± 13.9abcd	2.7 ± 0.1a	2.2 ± 3.9c	0.3 ± 0.6a
22.2	0.5	64.5 ± 3.9ab	2.9 ± 0.4a	8.9 ± 10.2bc	1.7 ± 1.5a
0	2.3	22.2 ± 7.7cde	2.1 ± 0.4ab	0c	0a
4.4	2.3	53.3 ± 20abcd	3.5 ± 2.1a	2.2 ± 3.9c	2.0 ± 3.5a
8.9	2.3	53.3 ± 6.7abcd	2.8 ± 0.3a	2.2 ± 3.9c	1.0 ± 1.7a
13.3	2.3	57.8 ± 21abc	2.6 ± 0.4a	2.2 ± 3.9c	0.7 ± 1.2a
22.2	2.3	51.1 ± 7.7abcd	2.1 ± 0.1ab	6.7 ± 6.7bc	1.5 ± 1.3a
0	4.5	44.5 ± 23.4abcd	2.4 ± 0.7a	0c	0a
4.4	4.5	57.8 ± 3.9abc	2.1 ± 0.1ab	8.9 ± 10.2bc	1.2 ± 1.3a
8.9	4.5	57.7 ± 3.9abc	2.7 ± 0.7a	4.5 ± 3.9c	2.0 ± 2.6a
13.3	4.5	75.5 ± 3.9a	2.7 ± 0.5a	15.6 ± 13.9ab	1.7 ± 1.5a
22.2	4.5	71.1 ± 3.8ab	2.2 ± 0.6a	24.4 ± 10.2a	2.3 ± 1.3a

Values represent means ± S.E. for 60 explants per treatment. Means in each column followed by the same letter are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$).

of *F. excelsior* was reported by Hammatt (1996). Shoot formation was obtained at all TDZ concentrations tested, and 2.2 μM TDZ was the best concentration for regeneration from cotyledons than 4.4 or 22 μM TDZ. Bates et al. (1992) found that adventitious shoots formed when explants (embryo with cotyledons and hypocotyl intact) of *F. americana* were exposed to TDZ at 10 μM or lower, but not on explants exposed to BA or 2iP at the concentrations tested. Our results indicate that the best adventitious shoot induction was obtained by a combination of BA and TDZ.

Hypocotyl and cotyledon explants from freshly isolated embryos exhibited a higher organogenesis potential than explants from in vitro-grown seedling explants (4-, 7-, 10-, or 15-day old) (Table 2). A maximum of 83.3% of hypocotyls derived from freshly isolated embryos developed shoots. With an increasing age of explants, organogenesis potential of explants was decreased. There was no adventitious shoot regeneration when 15-day-old hypocotyls and 7-, 10- or 15-day-old cotyledons were used as explants. Similar results in the morphogenic potential of explants of different ages derived from seedlings were also reported in other species. Chang et al. (1996) found that more shoots were produced on explants cut from the most basal region of cotyledons from 2- to 4-day-old seedlings than older seedlings of hybrid geranium. Ajay et al. (2002) found that adventitious shoot formation from 12-day-old hypocotyl explants had a higher regeneration rate than 14- or 18-day-old hypocotyl explants of *Sesbania restrata*. Zhu et al. (2005) found that hypocotyl explants of 3–5-day-old (>80% regeneration rate) Chinese cabbage had more potential to regenerate than 7-day-old (51.1% regeneration rate) explants.

After hypocotyl and cotyledon explants were initially induced to regenerate shoots, adventitious shoots together with the original explants were sub-cultured to MSB5 supplemented with 10 μM BA plus 10 μM TDZ to induce shoot elongation and proliferation. It has been reported that MSB5 supplemented with 10 μM BA or 40 μM TDZ provided best shoot proliferation (Kim et al., 1997). In our study, the modified MSB5 supplemented with 10 μM BA plus 10 μM TDZ worked well for adventitious shoot elongation and shoot proliferation (Fig. 1C).

3.2. Effect of plant growth regulators on root formation

Shoots from three different genotypes were transferred to WPM supplemented with 4.9 μM IBA together with different concentrations of IAA for rooting. Root induction and formation was a two-step process in our study. Microshoots were first kept in the dark for 10 days on rooting medium, and then transferred to a 16-h photoperiod. One week after transferring cultures to the light, root formation was noticed at the basal end of the shoots. All three genotypes of green ash produced high rooting percentages (Table 3, Fig. 1D).

No roots developed on plant growth regulator-free WPM. The addition of IAA to the culture medium along with IBA improved rooting percentages, but did not significantly affect the number or

Table 2
Effect of different ages of hypocotyls and cotyledons of green ash on adventitious shoot regeneration

Age (days)	Hypocotyl		Cotyledon	
	Shoot formation (%)	Mean no. shoots	Shoot formation (%)	Mean no. shoots
0	83.3 ± 2.9a	1.9 ± 0.2a	18.6 ± 3.2a	1.7 ± 0.1a
4	51.7 ± 7.6b	1.3 ± 0.04ab	9.4 ± 5.1b	1.1 ± 0.2b
7	8.3 ± 2.9c	0.9 ± 0.2b	0c	0c
10	6.7 ± 7.6c	0.7 ± 0.6bc	0c	0c
15	0c	0c	0c	0c

Values represent means ± S.E. for 60 explants per treatment. Means in each column followed by the same letter are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$).

Table 3
Effect of genotype of *Fraxinus pennsylvanica* and auxin level on root formation in vitro of microshoots

	Rooting (%)	Mean no. roots	Mean root length (cm)	Mean no. lateral roots
Treatment IBA + IAA (μM)				
Control[a]	0	0	0	0
4.9 + 0	73.2 ± 0.1b	2.9 ± 1.4a	3.0 ± 1.5a	19.1 ± 7.7a
4.9 + 2.9	88.9 ± 0.1a	2.6 ± 1.1a	2.9 ± 1.8a	18.1 ± 7.2a
4.9 + 5.7	89.8 ± 0.1a	3.0 ± 1.0a	3.0 ± 1.9a	17.8 ± 6.5a
4.9 + 8.6	88.9 ± 0.1a	2.8 ± 1.1a	3.4 ± 2.1a	17.6 ± 7.1a
Effect of clone				
GAS-001	95.8 ± 0.1a	3.3 ± 1.5a	2.9 ± 2.0b	16.7 ± 7.0b
GAS-002	81.9 ± 0.1b	2.9 ± 1.2ab	2.4 ± 1.7b	16.7 ± 6.7b
GA-JP03-10	77.8 ± 0.2b	2.3 ± 0.7b	3.9 ± 1.8a	21.0 ± 7.7a

Values represent the pooled means ± S.E. for three independent experiments. Means in each column followed by the same letter are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$).
[a] Plant growth regulator-free woody plant medium.

length of roots, or the number of lateral roots that developed. There was no significant effect on rooting percentage with the three concentrations of IAA tested. Each genotype showed a different response on rooting percentage, mean number of roots, mean length of roots, and mean number of lateral roots produced (Table 3). Rooting of GAS-001 (95.8%) was significant as compared to GAS-002 (81.9%) and GA-JP03-10 (77.8%). GA-JP03-10 showed a significant effect on the mean length of roots, and mean number of lateral roots produced compared to GAS-001 and GAS-002. For adventitious shoots, the greatest rooting (93%) and number of roots per shoot (2.8 ± 0.2) were obtained on medium with 4.9 μM IBA plus 5.7 μM IAA.

WPM or half-strength WPM plus the auxin IBA have been reported useful in inducing root formation in *Fraxinus* spp. For *F. excelsior*, Hammatt and Ridout (1992) found half-strength WPM containing 2.45, 4.9, or 9.8 μM IBA resulted in 67–79% rooting when proliferating axillary shoots from both shoot-tips and nodal explants were used. Tabrett and Hammatt (1992) used adventitious shoots regenerated from hypocotyls of *F. excelsior* and obtained 0–80% rooting on half-strength WPM containing 4.9 μM IBA. For *F. angustifolia*, Perez-Parron et al. (1994) reported that rooting was obtained on WPM supplemented with 0.98–4.9 μM IBA, and rooted plants were acclimatized with 85% survival. Rooting was also achieved by Tonon et al. (2001b) on auxin-free WPM and 80% rooted plantlets survived to the greenhouse. MS and MSB5 were also used for root formation in *F. americana* and *F. pennsylvanica* (Navarrete et al., 1989; Preece et al., 1989; Kim et al., 1998).

In our study, high rooting percentages (73–95%) were achieved using WPM supplemented with IBA plus IAA with a 10-day dark treatment followed by culture in the light. Plant growth regulator-free WPM did not induce root formation. We also found no roots formed if the shoots were cultured directly under a 16-h photoperiod without the dark induction time at all concentrations tested. Dark induction pre-treatment was a key factor for rooting. Tonon et al. (2001b) showed that 76% rooting was achieved after shoots of *F. angustifolia* were cultured on root induction medium for 5 days in the dark followed by culture on root expression medium for 15 days in the light. Navarrete et al. (1989) also found that pulsing white ash microshoots for 8 days in darkness was slightly more effective than lighted culture for rooting.

3.3. Acclimatization of rooted plants

Forty-eight rooted plantlets with four to six fully expanded leaves and well-developed roots were transferred to pots for acclimatization testing and hardening. Normal growth of the potted plants was observed 2 weeks after transfer (Fig. 1E), and plantlets were moved to the greenhouse for further acclimatization. After an additional 2 weeks, plants were transferred to larger pots for further root and shoot elongation (Fig. 1F). The acclimatization procedure used resulted in a survival rate of 100%. The regenerated plants did not show any morphological abnormalities during an observation period of 4 months in the greenhouse, and 100% of the plants survived after overwintering in cold storage and re-introduction to the greenhouse.

4. Conclusion

We have developed an adventitious shoot regeneration, rooting, and acclimatization system from hypocotyls and cotyledons of *F. pennsylvanica*. This protocol will be useful for experimental studies to produce transgenic *F. pennsylvanica* with resistance to the EAB, or transfer of other valued-added traits for tree improvement and conservation.

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