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Regeneration of Plants from *Fraxinus nigra* Marsh. Hypocotyls

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***Abstract. Fraxinus nigra* Marsh. (black ash) is a native North American hardwood tree species that is ecologically important and has ethnobotanical significance to American Indian communities of the eastern United States. Black ash has immature embryos at seed set, combined with complex stratification requirements, making natural regeneration difficult. This, combined with the threat and devastation being caused by the emerald ash borer, makes an in vitro adventitious shoot regeneration and rooting protocol imperative for mass propagation, conservation, and genetic improvement of this species. Hypocotyls were cultured for 4 weeks on a modified Murashige and Skoog (MS) medium containing 13.3 μM 6-benzylaminopurine (BA) and 4.5 μM thidiazuron for adventitious shoot induction. Shoots continued to regenerate when explants were then cultured on MS medium supplemented with Gamborg B5 vitamins plus 0.2 g·L^{-1} glycine (B5G), 6.7 μM BA, 1 μM indole-3-butryic acid (IBA), and 0.29 μM gibberellic acid (GA_3) for 4 weeks, followed by transfer to MSB5G medium with 13.3 μM BA, 1 μM IBA, and 0.29 μM GA_3 for shoot elongation. Once elongated, the microshoots were continuously micropropagated through nodal sectioning, and cultured on MSB5G medium supplemented with 13.3 μM BA, 1 μM IBA, 0.29 μM GA_3, and 0.2 g·L^{-1} casein hydrolysate. Rooting of elongated microshoots was successful using woody plant medium supplemented with 4.9 μM IBA and 5.7 μM indole-3-acetic acid with a 10-day initial dark culture for root induction followed by culture under a 16-h photoperiod. Rooted plantlets were successfully acclimatized to the greenhouse with 100% survival.**

Fraxinus nigra Marsh. (black ash; Oleaceae) is a native hardwood species in northeastern North America occurring in Newfoundland west to Manitoba and south to Iowa, Illinois, West Virginia, and Virginia (Wright and Rauscher, 1990). Black ash has significant ecological and ethnobotanical importance. The tough, durable wood is valued commercially for paneling, flooring, doors, cabinets, interior finish, and various specialty products such as traditional snowshoe frames, baskets, barrel hoops, canoe ribs, and chair seats. The seeds of black ash are an important food source for wildlife such as quail, songbirds, and waterfowl (Leopold et al., 1998), and twigs and foliage are also used as food for white-tailed deer and moose. The strongly ring-porous wood affected by ecosystem site factors (Benedict and Frelich, 2008) is preferred by Native Americans for making splints for basketry (Benedict, 2001). Black ash populations have been declining as a result of pests, disease, loss of habitat, inadequate natural regeneration, and various interacting (climatic, edaphic, and physiographic) factors (Benedict and David, 2000; Palik et al., 2011, 2012; Ward et al., 2006). The emerald ash borer (EAB; *Agrilus planipennis*), an aggressive exotic phloem-feeding beetle from Asia, also threatens native populations of black ash. The adult beetles feed on the foliage, but the larvae bore through the bark and into the cambial region, feeding and producing galleries that disrupt the flow of water and nutrients, eventually girdling and killing the tree. The pest is fatal to an infested tree, and at present, there are no effective means of complete eradication because the EAB is spreading throughout North America (Emerald Ash Borer, 2013; Poland and McCullough, 2006).

Changes in global climate and increased global trade of wood products also have negative impacts on forest ecosystems. Forest dynamics are constantly being challenged by the invasion of exotic pests and diseases, changes in land use, and human encroachment. Changes in disturbance regimes (i.e., flooding and fire) can also affect the dynamics of a forest ecosystem. The decline of *F. nigra* populations will have negative impacts on ecosystem function, because it would open up the forest floor to invaders and disrupt the hydrology of an area, since black ash is found on moist sites, such as floodplains and along streams. Crown dieback and mortality, and radial growth, of black ash have been reported in recent years and may be related to spring droughts, wetter sites, temperature, site factors, tree age, and distribution (Palik et al., 2011, 2012; Tardif and Bergeron, 1993). Flooding appears to play an important role in the ecology of *F. nigra*, and changes in flooding regimes and timing may impact black ash population dynamics and decrease the resilience of the ecosystem (Tardif and Bergeron, 1999).

Black ash is difficult to propagate because of irregular seed production intervals [1 to 8 years] (Gucker, 2005), its immature embryo at seed set (Vandstone and LaCroix, 1975), and complex stratification and germination requirements (Benedict and David, 2003). In vitro culture is a feasible tool for conserving, propagating, and genetically improving threatened and endangered tree species. Adventitious shoot regeneration and rooting is a prerequisite for use in genetic modification technology. Adventitious shoot regeneration has been reported for common or European ash (*F. excelsior*) (Hammatt, 1996; Mockeliunaite and Kuusiene, 2004; Tabrett and Hammatt, 1992), narrow-leaved ash (*F. angustifolia*) (Tonon et al., 2001), white ash (*F. americana*) (Bates et al., 1992; Palla and Pijut, 2011), green ash (*F. pennsylvanica*) (Du and Pijut, 2008), and pumpkin ash (*F. profunda*) (Stevens and Pijut, 2012). To our knowledge, there has been no in vitro propagation research published for *F. nigra*. The development of adventitious shoot regeneration and rooting protocols would provide a means for conservation, mass propagation, and genetic improvement of black ash. Thus, the aim of the present study was to develop a complete protocol for plant regeneration of black ash from hypocotyls for further use in genetic transformation studies.

Materials and Methods

Adventitious shoot induction. Black ash seeds were obtained from the National Tree Seed Center (Natural Resources Canada, Fredericton, New Brunswick, Canada) in 2011 and stored in a sealed container at 4 °C in the dark until used. The pericarps were excised and removed along with 2 to 3 mm of the seed opposite the radical. Any seeds with noticeable insect or fungal damage were discarded. Unstratified seeds were surface-disinfested in 70% (v/v) ethanol for 1 min and then immersed in 20% bleach solution (5.25% sodium hypochlorite) for 22 min

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followed by three rinses in sterile, distilled water. Surface-disinfested seeds were stored in sterile, distilled water for 4 d in the dark at 24 ± 2 °C to soften the seedcoats. After 4 d, the turgid embryos were extracted, and hypocotyl segments were excised and cultured horizontally on Murashige and Skoog (1962) basal medium (M499; PhytoTechnology Laboratories, Shawnee Mission, KS), plus organics, supplemented with 13.3 µM BA and 4.5 µM thidiazuron (TDZ) (Du and Pijut, 2008). Three replications with 20 to 35 hypocotyl explants per replication were conducted (100 mm × 25-mm petri plates; 45 mL medium). Unless noted otherwise, all cultures were maintained at 24 ± 2 °C under a 16-h photoperiod provided by cool-white fluorescent lamps (80 µmol·m^{-2}·s^{-1}), and all media contained 3% (w/v) sucrose and 0.7% (w/v) Bacto agar (No. 214030; Becton, Dickinson and Co., Sparks, MD) with the pH of the medium adjusted to 5.7 before autoclaving. After obtaining results for percent callus formation, percent shoot formation, and mean number of shoots per hypocotyl, adventitious shoot regeneration was optimized 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. Three replications with 10 fresh hypocotyls per treatment per replication were conducted. Cultures were incubated for 4 weeks to induce adventitious shoot formation.

Adventitious shoot elongation. After initial regeneration induction for 4 weeks, hypocotyls were transferred to MS medium with Gamborg B5 vitamins (Gamborg et al., 1968) plus 0.2 g·L^{-1} glycine (MSB5G) with 6.7 µM BA, 1 µM IBA, and 0.29 µM GA_3 for shoot bud enhancement for an additional 4 weeks. Explants were then transferred to MSB5G medium with 13.3 µM BA, 1 µM IBA, 0.29 µM GA_3, and 0.2 g·L^{-1} casein hydrolysate (CH) in Magenta GA-7 vessels (Magenta Corp., Chicago, IL; 50 mL medium) for shoot elongation and subsequent micropropagation. Hypocotyls with shoots were subcultured to fresh medium every 4 weeks for shoot culture establishment and continuous micropropagation through nodal sectioning.

Rooting of adventitious shoots. Elongated microshoots (2 to 3 cm) were cultured in woody plant medium (Lloyd and McCown, 1980) (WPM; L154, PhytoTechnology Laboratories), plus organics, and supplemented with 5.7 µM indole-3-acetic acid (IAA) and 4.9 µM IBA for root induction (Du and Pijut, 2008) in Magenta GA-7 vessels (50 mL medium). Cultures were placed in the dark at 26 °C for 10 d before transfer to a 16-h photoperiod (80 µmol·m^{-2}·s^{-1}) for 4 to 5 weeks. To obtain an adequate number of microshoots for rooting experiments, rooting was conducted using a single genotype of black ash regenerated from a single hypocotyl that was routinely micropropagated through nodal stem explants. Three replications with eight microshoots each were used for root induction. Rooting percentage, the number of roots per shoot, and root length were recorded 6 weeks after shoots were placed on root induction medium.

Acclimatization of rooted plants. After 6 weeks on root induction medium, rooted plantlets were transplanted into 10 cm × 9-cm plastic pots containing a moist, autoclaved, soilless medium with high porosity (Premier ProMix HP; Premier Horticulture Inc., Quakertown, PA). Agar was removed from roots by rinsing with distilled water before potting. Plants in pots were then 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. Plantlets in pots were acclimatized by gradually opening the bag over a 2-week period and watered as needed until the bags were fully opened. After ≈4 weeks, plants were then moved to the greenhouse and once actively growing, transplanted into Treepots™ (Stuewe and Sons, Inc., Corvallis, OR) for continued growth and development. Plants were irrigated as necessary with acidified water supplemented with a combination of two water-soluble fertilizers (3:1 mixture of 15N–2.2P–12.5K and 21N–2.2P–16.6K,

Fig. 1. Plant regeneration from hypocotyls of *Fraxinus nigra* (black ash). (**A**) Adventitious shoot regeneration on a hypocotyl after 4 weeks on shoot induction medium (MS medium with 13.3 µM BA and 4.5 µM TDZ); (**B**) black ash hypocotyl explant on shoot bud enhancement medium (MSB5G medium with 6.7 µM BA, 1 µM IBA, and 0.29 µM GA_3); (**C**) black ash shoots elongating on MSB5G medium with 13.3 µM BA, 1 µM IBA, 0.29 µM GA_3, and 0.2 g·L^{-1} casein hydrolysate; (**D**) root production on a black ash microshoot; (**E**) acclimatization of a rooted plantlet in the culture room; (**F**) black ash plant acclimatized in the greenhouse. MS = Murashige and Skoog; BA = 6-benzylaminopurine; TDZ = thidiazuron; IBA = indole-3-butryic acid; GA_3 = gibberellic acid.

respectively; The Scotts Co., Marysville, OH) to provide the following (in mg·L^{-1}): 200 nitrogen (N), 26 phosphorus, 163 potassium, 50 calcium, 20 magnesium, 1.0 iron, 0.5 manganese and zinc, 0.24 copper and boron, and 0.1 molybdenum. Nitrate form was 76% of N provided. Irrigation water was supplemented with 93% sulfuric acid (Brenntag, Reading, PA) at 0.08 mL·L^{-1} to reduce alkalinity to 100 mg·L^{-1} and pH to a range of 5.8 to 6.2. In the fall, plants were placed in cooler conditions for hardening off before overwintering in a controlled cold-storage environment (3 to 4 °C in darkness). After overwintering, the plants were returned to the greenhouse the next year, allowed to acclimatize to this environment, initiate new growth, and survival data were recorded.

Statistical analysis. Number of shoots per hypocotyl explant was recorded when explants were transferred to MSB5G medium with 6.7 μM BA, 1 μM IBA, and 0.29 μM GA_3 (total of 8 weeks from initial culture). Means and SE were calculated for number of shoots, roots, and root length using SAS 9.1 (SAS Institute, 2002). The data for shoot regeneration optimization were analyzed using a NPAR1WAY in SAS, an analysis of variance for non-normal data.

Results and Discussion

Adventitious shoot regeneration. Open-pollinated seed was used in this study to try to normalize the regeneration process across a random mix of black ash genotypes. The best adventitious shoot induction medium (MS medium supplemented with 13.3 μM BA and 4.5 μM TDZ) developed in our laboratory for adventitious shoot regeneration from green ash hypocotyls (Du and Pijut, 2008) was initially tested to determine the response of black ash hypocotyls for percent callus and shoot formation. Adventitious shoot initiation occurred on black ash hypocotyls after 4 weeks on this medium (Fig. 1A). An average of 52.4% of black ash hypocotyls produced callus, and 52.3% produced shoots with a mean of 2.2 ± 0.3 shoots per hypocotyl (Table 1).

Adventitious shoot induction was then optimized using a combination of BA and TDZ at various concentrations. The response of black ash hypocotyls was extremely variable, but all combinations of BA and TDZ in the induction medium produced some level of adventitious shoot formation, except treatments 0 μM BA and 0.5 μM TDZ, 4.4 μM BA and 0 μM TDZ, and 13.3 μM BA and 0.5 μM TDZ (Table 2). No significant differences were seen across treatments that produced adventitious shoots when the data were analyzed for non-normal data. The plant growth regulator combination of 13.3 μM BA and 4.5 μM TDZ resulted in 50% of the black ash hypocotyls forming shoots with a mean of 4.6 ± 1.6 shoots per hypocotyl. Therefore, this treatment was determined to be the best for black ash adventitious shoot induction from hypocotyl explants. For North American *Fraxinus* species, Du and Pijut (2008) reported that MS medium with 13.3 μM BA and 4.5 μM TDZ was also best for green ash regeneration from hypocotyls (76% shoot formation; 2.7 ± 0.5 shoots per hypocotyl), whereas Palla and Pijut (2011) reported that MS medium containing 22.2 μM BA and a low concentration of TDZ (0.5 μM) was best for white ash regeneration from hypocotyls (52% shoot formation; 3.9 ± 0.4 shoots per hypocotyl). In contrast, Stevens and Pijut (2012) found that 22.2 μM BA and 4.5 μM TDZ were optimum for regeneration from pumpkin ash hypocotyls (43% shoot formation; 1.1 ± 0.2 shoots per hypocotyl).

Table 1. Percent callus and shoot formation from hypocotyls of *Fraxinus nigra*.

Replicate	Callus Formation (%)	Shoot Formation (%)	No. of shoots per hypocotyl[z]
1	42.3	53.8	3.0 ± 0.7
2	58.8	47.1	1.7 ± 0.3
3	56.0	56.0	1.8 ± 0.2
MEAN	52.4	52.3	2.2 ± 0.3

[z]Values represent means ± SE for responsive explants (20 to 35 hypocotyls per replicate) on Murashige and Skoog medium supplemented with 13.3 μM 6-benzylaminopurine and 4.5 μM thidiazuron.

Continuous shoot bud enhancement and elongation experiments with *F. nigra* was attempted using the best medium for green ash (MSB5G medium supplemented with 10 μM BA and 10 μM TDZ; Du and Pijut, 2008), but results were unsuccessful. Regeneration using this medium produced bushy explants with minimal shoot elongation. Thidiazuron has been shown to have varying effects on explants and shoots in tissue culture, including preventing shoot elongation and excessive callus production (Chalupa, 1988; Huetteman and Preece, 1993). When TDZ was continuously included in our black ash induction and elongation medium, explants produced excessive callus, bushy shoots, and shoots failed to elongate. Elevated levels of other cytokinins such as BA can also inhibit the elongation of shoots if explants are exposed for a prolonged period of time. Vengadesan and Pijut (2009) found that a lower concentration of BA was required for shoot elongation and prevention of shoot-tip necrosis of northern red oak (*Quercus rubra*) shoots regenerated from cotyledonary node explants. Mitras et al. (2009), using epicotyl segments of in vitro seedlings of *F. excelsior*, found that increasing BA to 17.8 μM promoted multiple shoot formation, but shoots were short, often vitrified, and in some cases fasciated. When the concentration of BA was lowered to 13.3 μM, no vitrification occurred and normal shoot development was achieved. The ratio of cytokinin to auxin in the medium also has an important effect on the direction of cellular development.

In this study, TDZ was removed from the shoot induction medium after the initial 4-week culture period. To continue adventitious shoot bud enhancement, black ash hypocotyl explants were cultured on a medium with a lower concentration of BA (6.7 μM) plus 1 μM IBA and 0.29 μM GA_3. Shoots were continuously initiated on hypocotyls on this medium (Fig. 1B); however, not all shoots would routinely elongate enough for continued micropropagation. For routine shoot elongation, after 8 weeks from first initiation of hypocotyls into culture, we found that BA needed to be increased back to 13.3 μM along with 1 μM IBA, 0.29 μM GA_3, and the addition of 0.2 g·L^{-1} CH to prevent chlorosis (Fig. 1C). Continuous micropropagation of regenerated shoots was achieved using this medium through nodal stem sectioning.

Table 2. Effect of cytokinins on adventitious shoot regeneration from hypocotyls of *Fraxinus nigra*.

Plant growth regulator (μM)		Hypocotyl	
		Shoot	Mean
BA	TDZ	formation (%)	no. of shoots[z]
0	0	50[y]	1.0 ± 0
0	0.5	0	0
0	2.3	33.3	1.3 ± 0.3
0	4.5	16.7	3.0 ± 0
4.4	0	0	0
4.4	0.5	20	4.0 ± 0
4.4	2.3	50	1.3 ± 0.3
4.4	4.5	42.9	3.2 ± 1.4
8.9	0	50	2.5 ± 1.5
8.9	0.5	42.9	2.0 ± 1.0
8.9	2.3	30	3.0 ± 1.2
8.9	4.5	16.7	1.5 ± 0.5
13.3	0	50	1.0 ± 0
13.3	0.5	0	0
13.3	2.3	22.2	1.5 ± 0.5
13.3	4.5	50	4.6 ± 1.6
22.2	0	33.3	1.0 ± 0
22.2	0.5	50	3.0 ± 1.2
22.2	2.3	62.5	1.4 ± 0.4
22.2	4.5	37.5	1.7 ± 0.6

[z]Values represent means ± SE for 30 hypocotyls per treatment on Murashige and Skoog medium supplemented with 6-benzylaminopurine (BA) and thidiazuron (TDZ). *P* value = 0.5897.
[y]Showed root formation.

Rooting and acclimatization of black ash. Following the rooting protocol developed in our laboratory by Du and Pijut (2008) for *F. pennsylvanica*, an average of 93% of *F. nigra* microshoots produced roots with a mean of 4.1 ± 0.6 roots per shoot and a mean root length of 1.8 ± 0.2 cm (Fig. 1D; Table 3). No additional lateral root formation was observed in vitro on rooted black ash shoots. Acclimatized *F. nigra* plantlets in the culture room (Fig. 1E) showed 100% survival and normal growth and development after transfer to the greenhouse (Fig. 1F). Twenty-two rooted plantlets were acclimatized to the greenhouse for further growth, overwintered in a controlled cold-storage environment, and returned to the greenhouse the next year with a survival rate of 95.5%.

In conclusion, successful in vitro adventitious shoot regeneration, rooting, and plantlet acclimatization protocol for *Fraxinus nigra* (black ash) has been developed. The following stepwise protocol can be used for routine adventitious shoot regeneration, rooting, and continuous

Table 3. In vitro root formation of *Fraxinus nigra* microshoots after 6 weeks.[z]

Replicate	No. of roots per shoot	Mean root length (cm)	Rooting (%)
1	4.2 ± 0.7	1.8 ± 0.2	100
2	3.0 ± 0.9	3.3 ± 0.1	80
3	4.6 ± 1.2	1.1 ± 0.2	100
MEAN	4.1 ± 0.6	1.8 ± 0.2	93

[z]Values represent means ± SE for eight microshoots per replicate on woody plant medium containing 5.7 µM indole-3-acetic acid and 4.9 µM indole-3-butyric acid for 10 d in the dark followed by culture in the light.

micropropagation of black ash from hypocotyls. MS medium plus 13.3 µM BA and 4.5 µM TDZ for 4 weeks (induction) → MSB5G medium plus 6.7 µM BA, 1 µM IBA, and 0.29 µM GA_3 for 4 weeks (shoot bud enhancement) → MSB5G medium plus 13.3 µM BA, 1 µM IBA, 0.29 µM GA_3, and 0.2 g·L^{-1} CH (elongation and micropropagation) → WPM plus 5.7 µM IAA and 4.9 µM IBA for 10 d in the dark (root induction). This protocol will be used in future studies to develop a genetic transformation protocol for black ash to produce trees with resistance to the EAB.

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