# CHAPTER 17

# *IN VITRO* PROPAGATION OF *FRAXINUS* SPECIES

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## 1. INTRODUCTION

The genus *Fraxinus*, a member of the Oleaceae family, includes over 65 ash species native to the temperate regions of the northern hemisphere (Miller, 1955). Several of the ash species are important forest trees noted for their tough, highly resistant to shock, straight grained wood as well as being excellent shade trees for parks and residential areas (Dirr, 1998). Economically, the most important species include white ash (*F. americana* L.) and green or red ash (*F. pennsylvanica* Marsh.) in the United States and Europe or common ash (*F. excelsior* L.), flowering ash (*F. ornus* L.), and narrow leaf ash (*F. augustifolia* Vahl.) in Europe and Asia Minor.

Most ashes are deciduous trees that produce inconspicuous apetalous flowers in terminal or axillary clusters in the spring just before or with the leaves (Dirr, 1998). Fruits are bore in open panicles of elongated, winged, mostly single seeded samaras that mature in late summer or fall. Mature samaras can be dried to 7 to 10% moisture and stored under refrigeration in sealed containers for more than 5 years with little loss in viability (Bonner, 1974). Most species of ash exhibit some form of seed dormancy due to immature embryos, internal growth inhibitors, and/or to impermeable seed coats. The standard treatments to overcome seed dormancy involve various combinations of after-ripening at 20 to 30°C for 30 to 90 days to mature embryos and/or stratification at 1 to 5°C for up to 150 days to overcome internal factors (Bonner, 1974).

Propagation is usually by seed collected in the fall and sown immediately or artificially stratified for 90 to 120 days before sowing in the spring. Reliance on seed propagation for conventional breeding is problematic as it may take 10 to 25 years for trees to attain reproductive maturity and then abundant seed crops may only be produced every 3 to 5 years (Bonner, 1974). Although there are no reliable methods



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for rooting softwood cuttings, ash cultivars can be propagated by budding, grafting, and possibly layering (Hartmann et al., 1997). *In vitro* propagation through axillary shoot micropropagation, adventitious shoot organogenesis, or somatic embryogenesis is promising for several of the ash species. The objective of this chapter is to describe the procedures we have used and to compare them to some of the most promising *in vitro* approaches used by other researchers for the different ash species.

## 2. EXPLANT SOURCES AND DISINFESTATION

### *2.1. Stored Seed*

We have found that *in vitro* establishment of *Fraxinus* species using vegetative buds from non-stratified embryos as explants is easier than using shoot tips or apical buds from seedlings or adult trees (Preece et al., 1987). Seed is easily peeled from the samara and harbors few microorganisms so it is easily disinfested. Seed of most ash species contains a single embryo fully differentiated into hypocotyl, cotyledons, and epitcotyl. The embryo is surrounded by endosperm and may extend from half to the full length of the seed with cotyledons pointing away from the wing on the samara (Miller, 1955; Bonner, 1974). The major problem associated with in vitro germination of ash seed is overcoming dormancy due to inhibitors within the endosperm or an impermeable seed coat (Preece et al., 1995). Dormancy can be overcome by excising the embryo from the endosperm and testa (Arrillaga et al. 1992b); however, this technique is labor intensive and frequently results in damaged embryos unable to survive surface disinfested with dilute solutions of sodium hypochlorite (NaOCl) or other less commonly used disinfectants.

Excellent *in vitro* germination of non-stratified, surface-disinfested seed of ash is achieved by excising 1 to 2 mm from the end of the seed that contains the tips of the cotyledons (Preece et al., 1989, 1995). To use this approach, each seed has to be marked with indelible ink, surface disinfestations in 1% NaOCl and 0.01% Tween-20 solution for 20 to 30 minutes followed by three rinses with sterile distilled water, then cut under sterile conditions. We have also found removal of approximately 1 mm from both the apical and basal ends of the seed coat was equally effective for white and green ash (Van Sambeek et al., 2001). Germination rates of sound, surface-disinfested seed typically exceed 95% with fewer than 10% of germinants showing microbial contamination during the first month in culture. With either cutting technique, the cotyledons start emerging from seed coat within a week of placement *in vitro* followed within a week or two by an elongating epicotyl. The cut seed technique can also be used with immature seed collected at the liquid-endosperm or seed-filling stages with germination exceeding 80% and more than 60% of these germinates producing elongating epicotyls within 4 weeks (Preece et al., 1995).

### *2.2. Shoot Tip and Nodal Segments*

Many of the early trials on *in vitro* propagation of ash started with the culture of defoliated shoot tips taken from seedlings or from branches cut from adult trees and forced in the laboratory (Browne & Hicks, 1983; Chalupa, 1984; Preece et al., 1987;

Arrillaga et al., 1992a; Perez-Parron et al., 1994). Excised shoot tips or apical buds are most commonly surface disinfested by immersion in 70% ethanol, then in 0.3 to 1.6% NaOCl mixed with a non-toxic surfactant like Tween 20 for 5 to 20 minutes, and, finally, in multiple rinses with sterile deionized water. Preece et al. (1987) found forcing new shoot growth on branches from adult trees was more effective with green ash than it was white ash. Browne and Hicks (1983) reported that more than 60% of white ash shoots excised from branches forced in the laboratory were still free of contamination after 2 to 4 weeks *in vitro*. Perez-Parron et al. (1994) reported less than 20% contamination after 4 weeks for narrow-leaf ash shoots excised from branches forced in the laboratory.

No reports were found that described the grafting of dormant branch tips of adult trees to seedling rootstocks to force new shoot growth as a source of explants for any of the ash species. This approach has been successfully used on black walnut, a species more recalcitrant to *in vitro* culture than ash (Van Sambeek et al., 1997). Laboratory observations indicated that tissues originating from adult ash trees may produce phytotoxic exudates *in vitro* and, like black walnut, may initially require more frequent transfers to new medium than do explants from germinating seeds (Compton & Preece, 1988).

### *2.3. Epicormic Sprouts*

We have also experimented with forcing epicormic sprouts in the laboratory or greenhouse on branch segments cut from basal branches or stems of adult trees. Dormant buds on basal branches exhibit many of the traits that the tree possessed when the buds were first formed and are a promising source of juvenile explants for *in vitro* culture. The forcing of epicormic sprouts in the laboratory or greenhouse on stem or branch segments cut from adult trees has been reported for both white and green ash trees (Van Sambeek et al., 2002; Aftab et al., 2005). Explants taken from epicormic sprouts collected in the field or forced under mist are very difficult to surface disinfest (Preece, et al., 1987). However, explants from epicormic sprouts forced in the laboratory or greenhouse with hand watering or drip irrigation are relatively easy to surface disinfest with dilute NaOCl solutions (Van Sambeek et al., 1997; Van Sambeek & Preece, 1999; Aftab et al., 2005).

No published reports were found that described greenhouse or laboratory forcing of epicormic sprouts on branch pieces as an explant source for any of the ash species; however, this technique has been successfully used with silver maple, black walnut, and eucalyptus (Ikemori 1987; Bailey et al., 1998; Van Sambeek et al., 1998a; Aftab et al., 2005). Softwood cuttings of white ash are easily rooted when excised from epicormic sprouts forced in the greenhouse on branch segments cut from basal branches of adult trees (Van Sambeek et al., 1998b; Van Sambeek & Preece, 1999). Rapid rooting of the softwood cuttings with or without auxin treatments is evidence that epicormic sprouts forced on basal branches from adult trees retain more juvenile traits than shoot tips forced on terminal branch tips that are cut from the same adult trees that traditionally are difficult to root or used as explant sources for establishing *in vitro* cultures.

## 3. MICROPROPAGATION

### *3.1. Laboratory Procedures*

#### *3.1.1. Basal Media*

Much of the early research on *in vitro* culture of ash consisted primarily of testing various media and plant growth regulators to identify conditions leading to successful establishment and rapid axillary shoot proliferation. High-salt basal media normally produce the best results based on screenings done with various combinations of nine different basal media and three ash species (Chalupa, 1984; Navarrete et al., 1989; Perez-Parron et al., 1994). For *in vitro* propagation of white and green ash, we use slightly modified versions of MS (Murashige & Skoog, 1962) and DKW (Driver & Kuniyuki, 1984) using 10 or 20 ml of six stock solutions (Table 1). As shown in Table 2, the basal medium is supplemented with various combinations and concentrations of the plant growth regulators thidiazuron (TDZ), benzylaminopurine (BAP), isopentenyladenine (2iP), indole-3-buytric acid (IBA), naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) for the different *in vitro* propagation stages. The pH of the medium is routinely adjusted to 5.6 to 5.8 before addition of plant growth regulators and heating to melt agar when used. No published reports were found comparing effects of different gelling agents; however, 7 to 8% Difco Bacto agar is most often used for agar-solidified medium. Approximately 20 ml of basal medium is added to 25 × 150 ml glass culture tubes capped with semi-transparent, autoclavable Magenta closures or 30 ml of basal medium is added to 120 ml glass jars or Magenta GA7 vessels capped with autoclavable Magenta lids. Basal media are routinely autoclaved at 121°C (1.2 Kg $cm^{-2}$) for 20 to 30 minutes depending on size of culture vessels.

#### *3.1.2. In Vitro Environment*

Established cultures are routinely transferred or subcultured to new medium monthly inside a laminar flow hood disinfested with 70% ethyl alcohol. Cultures are normally maintained on open shelves in climate-controlled laboratories (26 ± 3°C). Shelves are lighted with 40-watt cool white fluorescent lamps providing 35 to 40 $umol{\cdot}s^{-1}{\cdot}m^{-2}$ of photosynthetically active radiation with a 16-h photoperiod.

### *3.2. Micropropagation by Axillary Shoot Proliferation*

#### *3.2.1. In Vitro Establishment from Seed Explants*

For *in vitro* germination of white or green ash seeds, we typically place surface disinfested, cut seeds on agar-solidified medium (Figure 1). We have published on several techniques that can be used to accelerate the establishment phase when using cut seeds for both white and green ash (Navarrete et al., 1989; Preece et al., 1995; Van Sambeek et al., 2001). Changing the concentration of the cytokinin analog TDZ in EEM (Table 2) affects *in vitro* establishment and growth to a greater extent than does changing concentrations of 2iP or BAP (Figure 2). We found epicotyl elongation and axillary shoot initiation from the cotyledonary node of germinating ash embryos can be accelerated by adding a liquid overlay of the establishment medium

midway between the monthly transfers to new agar-solidified medium. Inserting the radicle end of the emerging germinate into the solidified EEM before applying the liquid overlay will strongly inhibit radicle elongation and promote axillary shoots from the cotyledonary node. Typically over half the white and green ash germinants possess visible epicotyls ranging from 4 to 10 mm in length and cotyledons ranging from 25 to 40 mm in length after the first month of culture.

***Table 1.*** *Composition of stock solutions for preparation of MS and DKW basal media.*

| *Stock solution and components* | *for MS medium g/L* | *for DKW medium g/L* |
|---|---|---|
| *Stock solution A (nitrogen):* | | |
| Ammonia nitrate | 82.5 | 98.0 |
| Potassium nitrate | 95.0 | — |
| Calcium nitrate | — | 98.0 |
| *Stock solution B (sulfates):* | | |
| Magnesium sulfate heptahydrate | 18.5 | 37.0 |
| Potassium sulfate | — | 78.0 |
| *Stock solution C:* | | |
| Calcium chloride dihydrate | 22.0 | 7.35 |
| Potassium phosphate | 8.5 | 13.0 |
| *Stock solution D (chelated iron):* | | |
| Ferric sulfate heptahydrate | 1.39 | 1.65 |
| Sodium ethylene dinitrotetraacetic acid | 1.88 | 2.25 |
| *Stock solution E (micronutrients):* | | |
| Manganese sulfate monohydrate | 1.110 | 1.700 |
| Zinc sulfate heptahydrate | 0.430 | — |
| Zinc nitrate hexahydrate | — | 0.850 |
| Boric acid | 0.310 | 0.250 |
| Potassium iodide | 0.042 | — |
| Sodium molybdate dehydrate | 0.013 | 0.020 |
| Cupric sulfate pentahydrate | 0.0013 | 0.0125 |
| *Stock solution F (organics):* | | |
| Myo-inositol | 5.00 | 5.00 |
| Glycine | 0.10 | 0.10 |
| Pyridoxine hydrochloride | 0.025 | — |
| Nicotinic acid | 0.025 | 0.05 |
| Thiamine | 0.005 | 0.10 |

Add 20 ml of each stock to 800 ml of distilled water before bringing final volume to 1 L. Add sucrose and plant growth regulators, adjust pH to 5.6 to 5.7 before autoclaving, add and melt agar, then dispense into culture vessels before autoclaving.

***Table 2.*** *Plant growth regulator concentrations within different media for in vitro ash propagation.*

| PGR and Other additives elongation | Explant establishment (EEM) | Axillary proliferation (APM) | Adventitious root induction (RIM) | Adventitious root (REM) |
|---|---|---|---|---|
| Basal medium[1] | 1X | 1X | 0.5X | 0.5X |
| Sucrose (g/L) | 30.0 | 30.0 | 15.0 | 15.0 |
| TDZ (μM) | 10.0 | 3.0 | — | — |
| BAP (μM) | 1.0 | 1.0 | — | — |
| IBA (μM) | 1.0 | 1.0 | 5.0 | — |
| NAA (μM) | — | — | 5.0 | — |

[1]Basal medium at 1X uses 20 ml of each stock and 0.5X uses 10 ml of each stock.

As part of the establishment phase, we transfer germinates of both white and green ash after 4 weeks to new agar-solidified axillary proliferation medium (APM) in which the TDZ concentration has been reduced from 10 to 3 μM (Table 2). Germinants are trimmed to remove half to two-thirds of each cotyledon and all but 1 cm of the hypocotyl before inserting into new APM to the depth of the cotyledonary node. TDZ at 3 μM represents a compromise between maximizing proliferation rates and minimizing unwanted organogenic callus production from tissues touching the medium (Navarrete et al., 1989). The retention of IBA in the proliferation medium aids in keeping the unwanted callus healthy which otherwise can decline, become necrotic, and release toxic exudates with subsequent loss of established cultures. Typically over half the white ash cultures will posses two axillary shoots from the cotyledonary node in addition to the epicotyl while most green ash cultures will consist primarily of the elongating epicotyl after 8 weeks in culture (Van Sambeek et al., 2001).

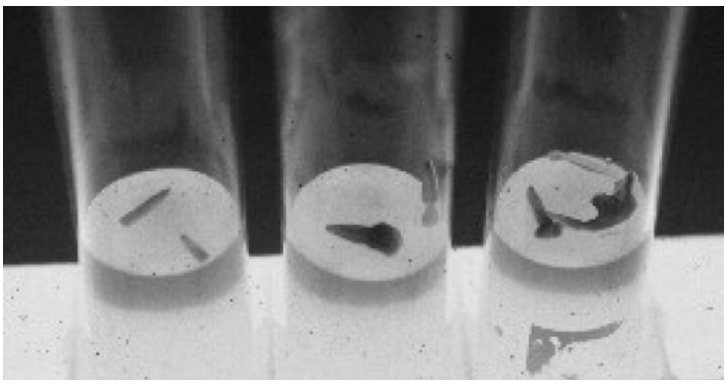

***Figure 1.*** *Disinfested cut-seed explants of white ash on EEM for 0, 1, and 2 weeks.*

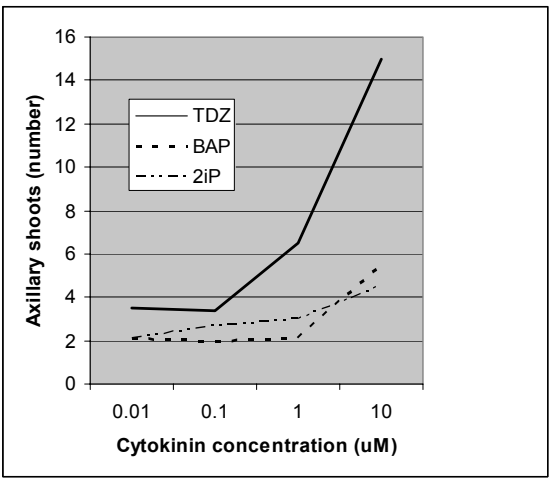


***Figure 2.*** *Influence of cytokinins on number of axillary shoots on germinants from cut, non-stratified seeds of white ash after 12 weeks in vitro.*

Kim et al. (1997) have also reported procedures for *in vitro* culture from embryo establishment to microshoot rooting for three green ash clones. They germinated their non-stratified, cut seed of green ash on MS without plant growth regulators and after 3 weeks transferred germinants to MS supplemented with 10 µM TDZ and 10 µM BAP to induce axillary shoot proliferation. When they subcultured these culture, they obtained the highest rates of axillary shoot proliferation (4 to 8 axillary shoots per culture) with a cytokinin mix of 5 µM TDZ and 5 µM BA. They also reported the production of organogenic callus on tissues touching the medium such that axillary shoots from the cotyledonary node could not be distinguished from the regenerating adventitious shoots. In contrast to our results with white and green ash, Hammatt and Ridout (1992) reported *in vitro* germination and axillary shoot proliferation of common ash was better on DKW medium than on MS medium.

*3.1.2. In Vitro Establishment from Shoot Tip Explants*

In our early research, using shoot tips taken from seedlings, we found shoot tips from white ash, but not from green ash, could be established and initiate axillary shoot prolixferation with the best proliferation occurring on liquid WPM supplemented with 44 µM BAP (Preece et al., 1987). Chalupa (1990) reported modest axillary shoot production from seedling shoot tips of European ash when cultured on MS or DKW supplemented with either 0.04 µM TDZ or 9 to 12 µM BAP and 0.5 µM IBA. With the ease that mature seed could be established as a juvenile source of ash explants, it appears few researchers have continued to pursue using explants from seedlings grown in the greenhouse to obtain juvenile explants.

We have also tried to force shoot tips on branches excised from adult white and green ash trees as a source of explants. When placed on WPM supplemented with 4.4 µM BAP and 5 µM IBA, shoots tips would elongate and form callus, but failed

to produce axillary shoots after 5 months in culture (Preece et al., 1987). Likewise, Browne and Hicks (1983) found shoots forced on branch tips from mature white trees showed little *in vitro* development on LS medium supplemented with BAP. Perez-Parron et al. (1994) successfully established shoot tips forced on branches from adult narrow-leaf ash and achieved axillary shoot proliferation when subcultured on DKW supplemented with 4.4 µM BAP and 1 µM IBA, especially if nodal segments were placed horizontally on a new culture medium when subculturing. Hammatt (1994) reported the successful establishment and axillary shoot proliferation from adult European ash when cultured on DKW supplemented with 22 µM BAP.

*3.1.3. Initiation of In Vitro Axillary Shoot Proliferation*

We achieve relatively high rates of *in vitro* axillary shoot proliferation for both white and green ash using nodal segments with monthly transfers and liquid overlays of ASP medium (Van Sambeek et al., 2001). To initiate the axillary shoot proliferation stage, 2-node explants are harvested from the elongating epicotyl and axillary shoots from 2 month old or older cultures, leaf blades excised, and then stems are placed horizontally on agar-solidified medium with the basal node slightly buried (Figure 3A). Two weeks later, a 0.5 cm deep liquid overlay of the same proliferation medium is added. Raising the TDZ concentration of the proliferation medium will increase the number of axillary shoots from the nodes; however, it will also increases the amount of unwanted organogenic callus produced on tissues in contact with the medium and can lead to abnormally thickened shoots (Figure 3B). Typically between 40 and 70% of white and green ash subcultures will produce between 0.3 and 2.5 $cm^3$ of callus between monthly transfers to new medium. The amount of callus and whether it is organogenic varies depending on the tree from which the original seed explant originated (Preece & Bates, 1995). There is a trend for organogenic callus to gradually change from producing adventitious roots initially to adventitious shoots with later subcultures.

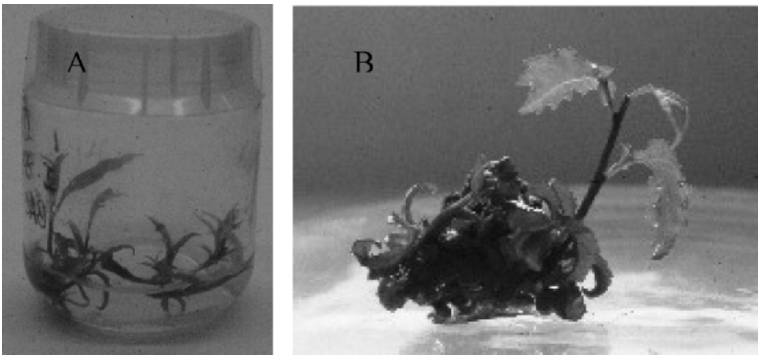


***Figure 3.*** *A) White ash subculture with proliferating axillary shoots at the stage when liquid APM overlays are added to improve axillary shoot growth. B) White ash culture from a germinating embryo on EEM with 10 µM TDZ showing extensive callus formation and indistinguishable axillary and adventitious microshoots.*

After 1 month on proliferation medium, white ash 2-node segments typically produce an average of 5.8 new axillary shoots which is more than double the 2.3 shoots from green ash nodal segments (Van Sambeek et al., 2001). In addition, the longest axillary shoot after 1 month in subculture tend to be slightly longer on white ash than on green ash nodal explants although there can be substantial variation among clones within an open-pollinated family or species. Genotypic differences in axillary shoot proliferation rates among cultures arising from open-pollinated seed of different trees also have been reported by others for green and European ash (Tabrett & Hammatt, 1992; Kim et al., 1997).

### *3.2. Micropropagation by Regeneration of Adventitious Buds and Shoots*

For adventitious shoot regeneration, cut seeds (either immature or mature) are prepared and best placed on agar-solidified MS medium supplemented with 10 μM TDZ and 0.1 or 1 μM 2,4-D. Organogenesis occurs in the callus formed where the cut ends of the cotyledons and hypocotyls touch the medium (Bates et al., 1992; Preece & Bates, 1995). If cotyledons are detached from the embryonic axis, organogenesis is reduced and shoot development will be slower than if cotyledons remain attached to the embryonic axis. If organogenic cultures are transferred to the ASP medium, buds are more likely to elongate into shoots that can be excised, rooted under mist, and acclimatized to a normal greenhouse environment. We have also achieved adventitious shoot regeneration on the unwanted callus that forms on tissues touching the medium during the *in vitro* establishment and axillary shoot proliferation stages of both white and green ash (Navarrete et al., 1989; Van Sambeek et al., 2001). Adventitious shoots typically have a thinner more transparent stem and narrower more succulent unifoliate leaves than the stem and leaves on the developing axillary shoots in these cultures. Kim et al. (1997) also reported formation of organogenic callus around the nodes on their green ash subcultures in contact with the culture medium.

Tabrett and Hammatt (1992) reported high rates of adventitious shoot regeneration on excised hypocotyls from immature and mature seed of European ash when cultured on MS supplemented with 20 μM TDZ and 0.5 μM IBA. Hypocotyls from immature seed tended to have higher rates of regeneration with fewer necrotic cultures. When using seed that had been dried and stored, the best regeneration rates from excised hypocotyls occurred on MS supplemented with 0.5 μM TDZ and 0.5 μM IBA. Higher levels of TDZ tended to result in more cultures that produced vitrified (hyperhydrous) adventitious shoots. Most excised hypocotyls exposed to a primary medium with TDZ for 2 to 5 weeks and then transferred to DKW supplemented with 20 μM BAP produced adventitious shoots that could be rooted.

We have also achieved embryogenesis on white ash by placing cut seeds on MS medium containing a high auxin to low cytokinin ratio (Preece et al., 1987; Bates et al., 1992; Preece & Bates, 1995). The highest rates of embryogenesis have been observed when cut white ash seed where cultured either on MS containing 10 μM 2,4-D with 0.1 or 1 μM TDZ or on DKW medium supplemented with 5 μM BAP and either 1 or 5 μM 2,4-D. The number of germinants showing embryogenesis on the DKW medium could be increased to 20% by transferring germinants with callus

to DKW medium without plant growth regulators. When embryogenic callus was subcultured, new embryos were produced from either the callus or on the surface of the first-formed embryos. Only callus still attached to the original germinant remained embryogenic. A high percentage of the embryos showed abnormal development and only a few could be germinated and developed into normal plantlets when the epicotyls were excised and rooted *ex vitro* (Bates et al., 1993).

## 4. ROOTING

### *4.1. In Vitro Rooting*

We developed a two step procedure under which both white and green ash microshoots can be synchronously rooted *in vitro* (Navarrete et al., 1989; Van Sambeek et al., 2001). For step one, 2 to 6 cm long microshoots are pulsed in the dark for 4 to 8 days with the basal end set 1 cm deep into agar-solidified RIM (Table 2). We have observed that minor changes in the NAA concentration can markedly alter the number of adventitious roots produced both within and among clones. For step two, pulsed microshoots are transferred to individual culture tubes with 7 to 10 cm deep REM (Table 2). Adventitious roots typically emerge in 10 to 14 days after initiating auxin pulse for green ash and 12 to 15 days for white ash (Figure 4A). Green ash microshoots generally have slightly higher rooting percentages and produce more adventitious roots (greater than 80% with 3 or more adventitious roots) than white ash microshoots. There is some evidence that the number of adventitious roots initiated during the auxin pulse does not increase during greenhouse acclimization and following planting into field studies (Van Sambeek et al., 1999). Preece et al. (1991) did find that reducing the MS macrosalt and sucrose concentrations in REM would increase the number and length of secondary roots but did not substantially alter the number or length of the primary adventitious roots. Perez-Parron et al. (1994) reported rooting percentages in excess of 90% for microshoots originating from both juvenile and adult narrow-leaf ash when rooted on WPM supplemented with 1 μM IBA as the auxin.

Several studies have shown that microshoots of some ashes can be rooted *in vitro* without an auxin treatment (Preece et al., 1995, Kim et al., 1998); however, more synchronous rooting is achieved using auxins. Kim et al. (1998) showed that both the culture medium and auxin concentrations could dramatically alter the number and elongation rate of adventitious roots on green ash microshoots. Microshoots continuously exposed to 5 μM IBA in liquid MS averaged between 4 and 6 adventitious roots while microshoots in agar-solidified MS averaged fewer than 2 adventitious roots after 5 weeks. The addition of NAA to the culture medium doubled or tripled the number of adventitious roots although most roots were thick and did not elongate normally when they remain on the root induction medium. Preece et al. (1987) also reported that roots of white ash microshoots when left in agar-solidified WPM supplemented with either 0.5 or 5 μM IBA for 1 month were abnormally thickened and brittle. Stunting could be minimized by the addition of 10 g $l^{-1}$ activated charcoal to the rooting medium.


***Figure 4.*** *A) White ash microshoots a few weeks after being transferred to REM after having been in RIM for 1 week. B) One-month-old rooted microshoots of white ash ready to be transferred to potting medium and set under mist in a greenhouse.*

### *4.2. Ex Vitro Rooting*

We have also successfully rooted white ash microshoots under mist in the greenhouse both with and without the use of auxins. For *ex vitro* rooting microshoots are normally placed in peat plugs or a soil-less peat medium. Bates et al. (1992) reported excellent rooting on microshoots excised from adventitious shoots using a 15 second quick dip in 1,000 μM IBA dissolved in 10% ethanol before setting in trays of sterile vermiculite kept under mist in a greenhouse for 3 to 4 weeks.

## 5. ACCLIMATIZATION AND FIELD PERFORMANCE

### *5.1. Acclimatization*

White and green ash microplants rooted *in vitro* can be easily acclimatized to a greenhouse environment if left on the REM until adventitious roots start to curl at the bottom of the culture tube or until shoots start to produce new leaves (Figure 4B). Microplants are then removed from the REM, rinsed free of agar, and transferred to an autoclaved soil-less medium. Plantlets are placed in a high humidity environment until they develop secondary roots along the adventitious roots and additional new leaves with normal functioning stomata (Preece & Sutter, 1991). Initially, plantlets produce simple leaves with a gradual transition to compound leaves with increasing numbers of leaflets (Figure 5). After 2 to 4 weeks under decreasing humidity, plantlets can be moved to a greenhouse bench for additional shoot and root growth and then forced to set a terminal bud.

Dormant plantlets can be transferred to refrigerated coolers for three or more months to meet normal chilling requirements before field planting. Occasionally, we have had difficulty in acclimatizing microplants from somatic embryos because the roots would not elongate or develop secondary roots. These plantlets can be successfully acclimatized by excising the shoot and treating it as a softwood cutting. Following the gradual exposure to reduced relative humidity over a 2 to 4 week period, survival of over 65% has been reported for microplants of white, European, flowering, and narrowleaf ash (Preece et al., 1987; Chalupa, 1990; Arrillaga et al., 1992a; Perez-Parron et al., 1994).

***Figure 5.*** *White ash microshoots acclimatized to a greenhouse environment beginning to transition from simple to compound leaves.*

### *5.2. Field Performance*

We have successfully spring-planted, in-leaf white ash plantlets in several field studies with little transplant mortality. In one study we are following the growth of white ash microplants from 12 clones (Van Sambeek et al., 1999). Six years after planting, survival averaged between 70 and 100% except for a single clone where all the microplants had died. No relationship has been found between numbers of adventitious roots and fifth-year stem height and diameter for the eleven surviving clones. Few clones changed their relative ranking when ranked by average height from the second through the sixth year. Initially more variation in stem height and diameter existed within clones than among clones; however, after 6 years variation among clones was twice that within clones. All clones except the one that did not survive exhibited normal growth and morphology including the clone originating from organogenic callus. Chalupa (1984) reported successful establishment after one winter of European ash microplants in the field. To date, Bates et al. (1992) are the only researchers to report the occurrence of abnormal development and that was on one white ash microplant from a somatic embryo that exhibit atypical phyllotaxy on the main stem.

## 6. CONCLUSIONS

Successful *in vitro* propagation from explant establishment through field planting of microplants has been reported for white, green, European, flowering, and narrowleaf ash. In most cases, explants from adult trees have not been established *in vitro*. Embryo dissection or the cut seed technique works well to overcome embryo dormancy in mature, non-stratified seed for all five species. Axillary shoot proliferation is commonly reported on shoots harvested from *in vitro* germinants when subcultured monthly on a high-salt, agar-solidified medium supplemented with thidiazuron (TDZ). The use of liquid overlays of the same medium has been shown to increase

proliferation and axillary elongation rates. Organogenic callus producing adventitious shoots is frequently produced on cut surfaces of germinants and axillary shoots when exposed to TDZ with or without 2,4-D. Pulsing microshoots for a week on a low salt medium supplemented with both IBA and NAA and transfer to medium without plant growth regulator leads to synchronous adventitious rooting. With gradually declining humidity, ash microplants are easily acclimatized to a greenhouse environment where they can put on additional height and growth. Field plantings show most microplants develop normally and the amount of phenotypic variation among clones is much greater than the variation within a clone after a few years.

## 7. REFERENCES


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