Comparative in Vitro Culture of White And Green Ash From Seed to Plantlet Production©

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INTRODUCTION

In vitro procedures have already been reported for white ash (*Fraxinus americana* L.) to establish cut dormant seeds, force axillary shoot proliferation, and induce rapid rooting to produce clonal plantlets (Preece et al., 1987, Navarrete et al., 1989, Preece et al., 1989, Preece et al., 1995). Hypothetically, a production cycle from seed to greenhouse-acclimatized plantlets can be achieved in 24 to 28 weeks. Navarrete (1989) found no differences in proliferation rates among progeny for 12 open-pollinated families; however, differences were found among selected clones when rooting microshoots. We have established field plantings with white ash microplants that continue to show normal development and growth (Van Sambeek et al., 1995, Van Sambeek et al., 1999). We attribute our success with white ash to using the cut seed technique to germinate nonstratified seed (Preece et al., 1995), incorporating thidiazuron (TDZ) and benzyladenine (BA) with MS medium to induce axillary shoot proliferation (Navarrete et al., 1989; Bates et al., 1992), accelerating axillary shoot growth using liquid overlays, and pulsing with both indolebutyric acid (IBA) and naphthalene acetic acid (NAA) to induce rapid rooting (Preece, et al. 1991).

Less is known about in vitro culture of other widely distributed ash species including English or common ash (*F. excelsior* L.) and green ash (*F. pennsylvanica* Marsh.). Hammatt and Ridout (1992) report in vitro germination of common ash was better on DKW medium than MS medium using BA to induce axillary shoot proliferation. Tabrett and Hammatt (1992) reported that adding TDZ and IBA enhanced adventitious shoot regeneration. They also report on genotypic variation among clones for axillary shoot proliferation and microshoot rooting. In vitro procedures from seed establishment to microshoot rooting have been reported for three clones of green ash on MS medium for in vitro establishment with TDZ and BA for axillary shoot proliferation (Kim et al., 1997) and subsequent microshoot rooting (Kim et al., 1998). In this paper, we compare in vitro culture of green and white ash using our white ash production cycle as well as preliminary estimates of genotypic variation between and within families for both species.

IN VITRO GERMINATION PROCEDURES AND RESULTS

We collected and air-dried open-pollinated mature fruits from both white and green ash trees in Jackson County, Illinois. The assigned North Central Research Station (NCRS) stand-tree accession numbers are 6721-13, 10799-1, 10811-1, and 10816-1 for the white ash trees and 10789-2, 10813-1, 10819-1, and 10821-1 for the green ash trees. Fruits were stored dry at 4°C under darkness in either cloth bags or sealed glass jars for up to 4 years. Samaras were detached and dormant sound seed surface-disinfested with 1% sodium hypochlorite and 0.01% Tween-20 for 20 to 30 min. Seeds were rinsed three times with sterile water and both the apical and basal ends were removed to expose the embryo without damaging the apical meristem. Cut seeds were placed in 120-ml glass jars or Magenta GA7 boxes containing establishment medium composed of MS medium supplemented with 2% sucrose, 10 µM TDZ, 1 µM benzyladenine (BA), 1 µM IBA, and 0.7% agar (Difco Bacto) if solidified. The pH was adjusted to 5.7 or 5.8 before autoclaving. Culture vessels were placed on open shelves in a climate controlled laboratory at 26 ± 3°C under 16-h photoperiod with a PAR of 35 to 40 $\mu mol \cdot s^{-1} \cdot m^{-2}$ provided by 40-watt cool white fluorescent lamps. Each culture vessel initially contained five cut seeds from a single tree. Between Week 1 and 2, we inserted each germinate vertically into the agar-solidified medium. During Week 2, we added liquid culture medium to each culture vessel as a liquid overlay. After 1 month, we determined epicotyl, cotyledon, and hypocotyl plus radicle lengths, callus volume, and presence of adventitious roots or shoots. The experiment was replicated three times using five seeds of each family and subjected to ANOVA to test for differences among species and families within species.

After the first 4 weeks in culture, we found differences between species in growth of germinates but few differences among families within each species. Less than 10% of the cultures for both species developed visible fungal or bacterial contamination and were discarded before addition of the liquid overlays. At Week 4, the expanding embryo had split open the seed coat and showed rapid expansion of cotyledons. Approximately 5% of the disinfested sound seed for both species failed to germinate. Between 40% to 90% of the germinated seed within families of both species had a visible epicotyl that ranged from 4 to 10 mm in length. Cotyledon lengths ranged from 25 to 40 mm in length by family for both species. The length of the hypocotyl and radicle ranged from 11 to 18 mm among families for both species and was combined because the high TDZ concentration strongly inhibited radicle elongation.

AXILLARY SHOOT PROLIFERATION ON VERTICAL CULTURES

Four weeks after initiating the cultures, we subcultured the germinates by removing the apical half of each cotyledon and most of the hypocotyl before placing each explant vertically in new agar-solidified medium. Our proliferation medium consisted of full-strength MS supplemented with 2% sucrose, 3 µM TDZ, 1 µM BA, 1 µM IBA, and 0.7% agar if solidified. Two weeks later, we added liquid proliferation medium as an overlay to each culture. Eight weeks after initiating the cultures, we excised the epicotyl and long axillary shoots from each culture and vertically subcultured them with the basal node partially buried in the agar-solidified medium. For cultures with epicotyls or axillary shoots exceeding 40 mm in length, the basal section was excised into 2- or 3-node explants and also subcultured. After decanting the liquid overlay, we placed the cotyledonary nodes back on the original proliferation medium for continued axillary shoot proliferation. Two weeks later, we

again added liquid proliferation medium as an overlay to both the epicotyl and cotyledonary node cultures. For each subculture, we determined epicotyl length, number of axillary shoots, length of longest axillary shoot, callus volume, and presence of adventitious roots or shoots. Mean response for each seed line was subjected to ANOVA to test for differences between species and families nested within species.

After 8 weeks in culture, we found no differences between species in epicotyl length; however, differences did exist between species for percentage of culture with axillary shoots and number of axillary shoots per culture (Table 1). Axillary shoots were produced mostly at the cotyledonary node that was partially submerged in the agar-solidified medium. Within families, axillary shoots were present on 50% to 80% of white ash cultures compared to less than 30% of green ash cultures. White ash cultures averaged three axillary shoots per culture and had nearlytwice the number of axillary shoots as green ash cultures.

When epicotyls and nodal explants were subcultured the following month, nearly all white ash cultures showed axillary shoot proliferation compared to slightly more than half the green ash cultures (Table 1). After 12 weeks in culture, 25% of the white ash and 35% of the green ash cultures were discarded due to slowly elongating epicotyls that failed to produce any axillary shoots. For the remaining 65% to 75% of the cultures, the average number of axillary shoots had nearly doubled during the 3rd month in culture (4.2) from the previous month (2.3). In addition, the white ash cultures had more than twice as many axillary shoots (5.8) as the green ash cultures with shoots (2.5) after 3 months in culture. Average length of the longest axillary shoot on cultures with axillary shoots was similar between species and among families within species. The high values for least significant differences indicate significant variation exist among progeny within the open-pollinated families for both species.

During germination and in vitro establishment, callus developed on 74% of the white ash cultures and 58% of the green ash cultures from the radicle, the base of the hypcotyl, and cotyledons that were in contact with the high TDZ establishment medium. The volume of callus after 4 weeks ranged from 0.1 to 0.2 cm^3 for both species with a trend for green ash to produce more callus because germinates of NCRS 10813-1 averaged almost 0.4 cm^3 of callus. Although the callus was discarded when germinates were subcultured, 66% of the white ash microshoots and 81% of the green ash microshoots produced new callus on the hypocotyl or cut surfaces of the cotyledons in contact with the proliferation medium. The volume again averaged between 0.1 and 0.2 cm^3 of callus after 1 month. The callus on 52% of the white ash cultures and 46% of the green ash cultures was organogenic producing either adventitious roots or shoots.

AXILLARY SHOOT PROLIFERATION ON HORIZONTAL CULTURES

During the 4th and 5th month in culture, we began subculturing 2- or 3-node and terminal segments cut from the longest axillary shoots. Leaf blades were excised from nodal explants before placing them horizontally on agar-solidified proliferation medium. We added liquid proliferation medium as an overlay to each culture 2 weeks after subculturing. For each subculture, we determined number of axillary shoots, length of longest axillary shoot, callus volume, and presence of adventitious structures. Percentage of cultures with two or more axillary shoots, with callus, and

with adventitious structures was also determined for each progeny. Meanresponse for each progeny was subjected to ANOVA to test for differences between species and families nested within species using progeny means for replication.

The percentage of subcultures that had two or more elongating shoots 2 weeks after subculturing was higher for white ash subcultures (76%) than for green ash subcultures (32%) with no differences among progeny for either species (Table 2). The majority of the green ash subcultures failed to undergo axillary shoot proliferation and usually showed elongation of a single axillary shoot on nodal cultures or the apical meristem on terminal explants. Four weeks after initiating new subcultures, the percentages of cultures with axillary shoot proliferation increased to 46% for green ash and 88% for white ash subcultures. On a few white ash subcultures, the explants callused over and were discarded because we could not reliability distinguish between axillary and adventitious shoots.

Table 1. Variation in axillary shoot proliferation from Week 4 to 8 for explants with cotyledons and from Week 8 to 12 for explants without cotyledons[1].

Species and NCRS Accession number	Explants with cotyledons			Explants without cotyledons		
	Epicotyl length (mm)	Cultures w/axillary shoots (%)	Number of axillary shoots (#)	Cultures w/axillary shoots (%)	Number of axillary shoots (#)	Longest axillary shoot (mm)
White Ash:						
6721-13	34	78	3.6	96	7.3	22
10799-1	36	60	3.2	100	4.7	10
10811-1	23	50	3.6	100	7.0	14
10816-1	34	58	2.7	82	3.6	11
5% LSD:	13	64	1.4	28	4.1	12
Green Ash:						
10789-2	34	22	1.5	80	2.5	16
10813-1	31	28	1.0	52	2.0	16
10819-1	36	8	2.0	40	2.4	11
10821-1	28	8	1.0	37	3.2	5
5% LSD:	11	29	1.7	64	2.1	21
Species[2/]:	ns	**	*	**	**	ns
Within Species	ns	ns	ns	ns	ns	ns

[1] Values are means from 4 to 10 seeds or progeny for each open-pollinated family. ANOVA computed using individual progeny means for replication.

[2] Nonsignificant differences and differences at the $P<0.05$ and $P<0.01$ between species and among families within species are indicated by ns, *, and **, respectively.

Two weeks after subculturing, white ash cultures averaged 3.3 elongating axillary shoots compared to only 1.7 shoots on green ash cultures with axillary shoots (Table 2). The length of the longest axillary shoot averaged 20 mm for white ash and 21 mm for green ash subcultures.

Two weeks after application of a liquid overlay, green ash subcultures showed a slight increase in number of elongating shoots (2.2) while white ash subcultures nearly doubled the number of axillary buds and shoots (6.0). The differences found in number of axillary shoots among the white ash families after subculturing for 2 weeks, no longer existed after subculturing for 4 weeks primarily due to the wide variation found among the progeny within each family. Four weeks after subculturing, the longest axillary shoot on white ash subcultures averaged 33 mm in length compared to only 24 mm for green ash subcultures.

After 1 month in culture, small amounts of callus were produced where tissues were in contact with the proliferation medium for both white and green ash. Forty

Table 2. Variation in axillary shoot proliferation on horizontally-oriented 2-node microshoots 2 and 4 weeks after subculturing for 12 and 16 week old cultures[1/].

Species and NCRS family Number	2 weeks after subculturing			4 weeks after subculturing		
	Cultures with ≥2 shoots (%)	Number of axillary shoots (#)	Longest axillary shoot (mm)	Cultures with ≥2 shoots (%)	Number of axillary shoots (#)	Longest axillary shoot (mm)
White Ash:						
6721-13	81	4.2	25.2	98	7.9	39.7
10799-1	86	3.8	19.9	95	6.0	29.5
10811-1	56	2.4	19.6	82	5.8	28.9
10816-1	72	2.9	20.3	77	4.2	32.1
5% LSD:						
Green Ash:						
10789-2	36	1.7	20.9	53	2.5	22.5
10813-1	36	1.8	24.4	66	2.7	25.2
10819-1	27	1.8	19.1	30	1.8	28.2
10821-1	30	1.4	18.3	36	1.8	19.5
5% LSD:						
Species[2/]:	**	**	ns	**	**	**
Within species:	ns	**	ns	*	ns	ns

[1] Family values are averages from 2 to 7 progeny which were the means from 1 to 9 subcultures per progeny. ANOVA was computed using individual progeny means for replication.

[2] Nonsignificant differences and differences at the $P<0.05$ and $P<0.01$ between species and among families within species are indicated by ns, *, and **, respectively.

to 60% of the white ash subcultures produced between 0.3 to 1.4 cm^3 of callus compared to 50% to 70% of the green ash cultures that produced between 0.8 to 2.5 cm^3 of callus with no statistical differences between species or families within species. Of subcultures with callus, 28% of white ash and 10% of green ash subcultures had organogenic callus with adventitious shoots mostly.

IN VITRO ROOTING PROCEDURES AND RESULTS

Microshoots were rooted using the procedure previously developed for white ash (Navarrete, 1989; Preece et al., 1991). Twenty to 28 weeks after initiating the culture, we harvested 2- to 6-cm long microshoots approximately 2 mm below a node. Microshoots were individually placed in culture tubes approximately 1 cm deep into agar-solidified root-induction medium composed of quarter-strength MS macrosalts, full-strength MS microsalts and organics, 1% sucrose, 5 μM IBA, 5 μM NAA, and 0.7% agar. Microshoots were set in the dark for 1 week before transferring pulsed microshoots to a root elongation medium that was composed of the same components as the root induction medium minus the plant growth regulators. Pulsed microshoots were maintained under low light and evaluated for number and length of roots 1 and 2 weeks later. Microplants were transplanted to a commercial potting medium and placed under intermittent mist for several weeks until terminal buds initiated new growth. Plantlets were then moved to a greenhouse bench for continued growth for several months before transferring to refrigerated coolers.

Green ash microshoots have a higher rooting percentage and produce more adventitious roots than white ash microshoots with no statistical differences among families within species (Table 3). Approximately 90% of the green ash microshoots rooted with an average of 3 roots per microplant. In contrast, approximately 70% of the white ash microshoots rooted with an average of only 2.2 roots per microplant. Three weeks after initiating the auxin pulse, green ash microshoots also had longer adventitious roots (41 mm) than white ash microplants (27 mm). Emerging root tips were usually visible 10 to 15 days after initiating the auxin pulse for both species. Root emergence occurred earlier on green ash than white ash because no differences were found in daily growth rate of the adventitious roots between the second and third week. We observed that once a microshoot had produced visible adventitious roots, the number of adventitious roots seldom increased even when transplanting to potting medium.

DISCUSSION

More than half the seeds from all four families of both white and green ash were successfully cloned to produce plantlets using our white ash production cycle. Of the 15 sound seed for each open-pollinated family, we produced cloned plantlets from 3 to 7 seeds from each white ash family and from 2 to 4 seeds with each green ash family. Of the 90 white and green ash seeds that were not taken through the production cycle, 8% showed fungal contamination within the first 2 weeks, 7% were apparently nonviable, 35% grew very slowly on our proliferationmedium, and 50% had low proliferation rates or seldom initiated more than one axillary shoot on nodal explants.

The cut seed technique overcame dormancy in mature nonstratified seeds for all four families of white ash and green ash. Our results are consistent with previously reports that nonstratified embryos must be dissected from the seed or the testa of the seed must be cut to permit germination for white, green, and common ash

(Navarrete et al., 1989; Hammett and Ridout 1992; Preece et al., 1995; Kim et al., 1997). The addition of 10 uM TDZ to MS medium effectively induced rapid germination with minimal development of basal callus for both white and green ash. Other studies report the germination of both green and common ash on MS without plant growth regulators (Kim et al., 1997; Hammit and Ridout, 1992).

As previously reported for white ash culture, liquid medium overlays enhanced axillary shoot elongation and promotion of additional axillary shoots from submerged nodes. Liquid medium overlays were less effective on green ash subcultures, especially for subcultures from horizontally oriented nodal explants. Two weeks after application of liquid overlays, there were only small increases in number or length of the longest axillary shoots. Medium aeration maybe important as in vitro rooting of green ash is improved using rooting plugs and liquid MS medium as

Table 3. Variation in rooting and microplant characteristics 3 weeks after initiating a 1-week auxin pulse on microshoots harvested from 20- and 24-week old cultures[1].

Species and NCRS family Number	Progeny tested (#)	Micro shoot length (mm)	Micro-shoot rooting (%)	Advent-itious roots (#)	Average root length (mm)	Root elongation rate (mm day^{-1})
White Ash:						
6721-13	4	29	80	2.2	25	2.0
10799-1	8	19	58	2.1	24	2.2
10811-1	3	24	75	2.2	42	3.4
10816-1	6	28	57	2.4	17	1.6
5% LSD:	---	5	32	0.8	17	2.1
Green Ash:						
10789-2	2	22	100	2.5	55	5.1
10813-1	4	27	100	3.5	40	2.9
10819-1	4	25	83	3.2	35	2.8
10821-1	2	25	82	2.7	34	2.5
5% LSD:	---	6	39	2.2	29	2.8
Species[2]:	---	ns	*	**	**	ns
Within species:	---	**	ns	ns	*	ns

[1] Family values are averages from indicated number of progeny. ANOVA was computed with replication from individual progeny means computed as average from five to ten microshoots.

[2] Nonsignificant differences and differences at the $P<0.05$ and $P<0.01$ between species and among families within species are indicated by ns, *, and **, respectively.

compared to agar-solidified MS medium (Kim, et al., 1998). We hypothesis that the liquid overlays may have decreased aeration to our horizontally-oriented subcultures and slowed axillary shoot development on green ash but not white ash subcultures.

Navarrete (1989) reported optimum axillary shoot proliferation with minimal development of callus on MS medium supplemented with 3 μM TDZ, 1 μM BA, and 1 μM IBA. Epicotyls and axillary shoots of green ash subcultures had elongation rates similar to white ash subcultures; however, the concentrations of plant growth regulators were not sufficient to overcome apical dominance and promote axillary shoot proliferation. Kim, Schumann, and Klopfenstein (1997) used 10 μM TDZ or 40 μM BA to induce axillary shoot proliferation on three clones of green ash. Apparently, green ash subcultures will require higher cytokinin concentrations than white ash to achieve cloned plantlets within the similar time frame as for white ash.

White ash subcultures producing 4- to 9-axillary shoots in 4 weeks with less than 1.4 cm^3 of callus that did not adversely affect axillary shoot elongation, which confirmed earlier observations on the effectiveness of TDZ for white ash micropropagation (Navarrete, 1989;Navarrete et al., 1989). Thidiazuron is a highly effective plant growth regulator for enhancing axillary shoot proliferation and promoting callus on many hardwood cultures (Huetteman and Preece, 1993). Higher concentrations of TDZ cannot be recommended because we had some white ash subcultures spontaneously initiated rapid callus formation that eventually covered the nodal explants and had to be discarded. As previously shown by Navarrete et al (1989), the addition of IBA to the culture medium keep the callus from becoming necrotic and releasing potentially toxic exudates. Our green ash subcultures usually produced less than 3 axillary shoots after 4 weeks with as much as 2.5 cm^3 of callus with 3 μM TDZ in the proliferation medium. Kim, Schumann, and Klopfenstein (1997) obtained higher rates of axillary shoot proliferation (4 to 8 axillary shoots per culture) with a cytokinin mix of 5 μM TDZ and 5 μM BA.

In our study approximately half of the subcultures of both white and green ash produced some callus that was organogenic on 10% to 30% of the cultures. Kim, Schumann, and Klopfenstein (1997) also reported formation of organogenic callus around the nodes on their green ash subcultures in contract with the culture medium. Although not quantified, we observed a trend for organogenic callus to initially produce adventitious roots and gradually switch to adventitious shoots after several subcultures. Adventitious shoots typically had a thinner more transparent stem and narrower unifoliate leaves than the stem and leaves on axillary shoots. Adventitious shoots were not used as source of nodal explants for subculturing or for rooting because adventitious shoots may have an increased frequency of somaclonal variation than meristem-derived tissues (Huetteman and Preece, 1993).

Several studies have shown that microshoots of both white and green ash can be rooted without an auxin treatment (Preece et al., 1995; Kim et al., 1998); however, auxin treatments result in more synchronous rooting and more adventitious roots (Navarrete 1989). Kim et al. (1998) showed that both the culture medium and auxin could dramatically alter the number and elongation of adventitious roots on green ash microshoots. Microshoots 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. They also found that addition of NAA to the culture medium could double or triple the number of adventitious roots although

most roots did not elongate normally. We achieved excellent rooting on white and green ash by pulsing microshoots on agar-solidified MS medium with 5 µM IBA and 5 µM NAA and then transferring to MS medium without auxins. Our results suggest that pulsing microshoots in a high auxin medium for 1 week initiates synchronous rooting and subsequent transfer to auxin-free medium allows the adventitious roots to elongate normally.

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