IMPROVED MICROPROPAGATION OF WHITE ASH (FRAXINUS AMERICANA L.)[1]

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Abstract.--Cut, nonstratified seeds of white ash (Fraxinus americana L.) were surface-sterilized and germinated on four cytokinin-containing media. The best germination, explant growth, and axillary shoot proliferation occurred on Murashige and Skoog medium (MS) and on broad-leaved tree medium (BTM). Cultures on woody plant medium (WPM) and Driver - Kuniyuki walnut medium (DKW) grew slowly. Because less callus formed from explants on MS medium, it was chosen over BTM for subsequent experiments. The best in vitro germination, explant stem growth, and axillary shoot proliferation were on MS medium supplemented with 3 µM thidiazuron (TDZ), 1 µM benzyladenine (BA), and 1 µM indole-3-butyric acid (IBA). Isopentenyladenine (2iP) was ineffective for initiating axillary shoot proliferation. Best rooting of axillary microshoots occurred using an 8 day dark pulse on MS supplemented with naphthalene acetic acid (NAA) and IBA both at 5 µM. Rooted microshoots were successfully acclimatized under mist in the greenhouse.

White ash (Fraxinus american L.) occurs widely throughout the central hardwood forests and is an important species for timber, landscaping, and wildlife (Fowells 1965). It is generally propagated from seeds (Bonner 1974); however, seedling variabililty is problem within this species because of natural heterozygosity and multiple ploidy levels (Clausen et al. 1981). White ash can be propagated vegetatively by means of cuttings, chip or T-budding, and whip and tongue grafting (Dirr 1977; MacDonald 1986). Compared to budding and grafting, micropropagation techniques could reduce costs and greatly increase the number of plants produced from selected clones.

Initial attempts to micropropagate white ash by Browne and Hicks (1983) resulted in some growth on dormant buds; however, long term growth and axillary shoot proliferation were not achieved. Recently, Preece et al. (1987) were successful in micropropagating white ash from seedling shoot tips. After 5 months on woody plant medium (WPM) supplemented with 5 or 10 mg liter^{-1} benzyladenine (BA), axillary shoot proliferation averaged 3.5 shoot per culture. Axillary shoots could be rooted in WPM supplemented with indole-3-butyric acid (IBA) or in sterilized vermiculite without plant growth regulators. Subsequently, white ash was also micropropagated via somatic embryogenesis (Preece et al. 1989).

The choice of media and ratio of cytokinin to auxin can markedly affect axillary shoot proliferation and rooting. For black walnut, axillary shoot proliferation and elongation are much greater on Driver-Kuniyuki walnut (DKW) medium than on woody plant medium (WPM) (Hiele- Sudholt et al. 1986). Preece et al. (1989) found no differences in growth of white ash callus on four media supplemented with 2,4-D; however, similar studies have not been done to determine effects of media and cytokinins on axillary shoot proliferation. Although initial results by Preece et al. (1987) indicated white ash microshoots are easily rooted, the roots were often thickened and brittle suggesting additional refinements were needed.

The objectives of our experiments were to develop improved in vitro procedures for rapid production and rooting of white ash microshoots.

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MATERIALS AND METHODS

Mature fruits were collected in 1987 from 10 widely-spaced trees throughout Jackson County, Illinois and stored dry in cloth bags at 4±2 °C in darkness until used as explants. Samaras were detached and the apical one-fourth of each seed was removed. Cut seeds were sterilized in 1.05% sodium hypochlorite (NaClO or 20% Clorox) with 0.01% Tween-20 (polyoxyethylene sorbitan monolaurate) for 30 minutes. Seeds were rinsed three times in sterilized water and the apical end recut to reduce seeds to two-thirds the original size before placing on culture medium.

Stock solutions for woody plant medium (WPM) (Lloyd and McCown 1981), broad-leaved tree medium (BTM) (Chalupa 1984), Driver-Kuniyuki walnut medium (DKW) (Driver and Kuniyuki 1984), and Murashige and Skoog medium (MS) (Murashige and Skoog 1962) were prepared and stored at 4±1 °C. All nutrient media was made up with 20 g $liter^{-1}$ sucrose and 1 µM indole-3-butyric acid (IBA). The cytokinins, thidiazuron (TDZ), benzyladenine (BA), and isopentenyladenine (2iP), were dissolved in a small amount of 1 N KOH. The auxins, IBA and naphthalene acetic acid (NAA), were dissolved in a small amount of ethanol before adding to nutrient media. The pH of each medium was adjusted to 5.8 with 1 N KOH or 1 N HCL before addition of 7 g $liter^{-1}$ Difco Bacto agar (when used). Media were distributed in 20 ml aliquots into 25 x 150 mm borosilicate glass culture tubes or in 30 ml aliquots to 120 ml glass jars (baby food) capped with clear autoclavable lids. Vessels and media were autoclaved for 20 minutes at 121 °C.

Explants were placed on agar-solidified medium and transferred monthly to new agar-solidified medium. Two weeks following each transfer, 10 ml of the same medium without agar was added aseptically to each culture. After one month in culture, the radicles were removed from germinated seed explants. The cotyledonary nodes were removed after two months in vitro. Cultures were incubated at 26±2 °C with a 16 hour photoperiod and a PPF of 40 µM m^{-2} s^{-1} provided by cool white fluorescent lamps.

Explant epicotyl length, axillary shoot number and length, and callus volume ($2/3\ \pi r^2 h$) were determined at 2 week intervals. Number and length of roots were recorded at four day intervals in rooting studies. All experiments were arranged in a completely random design with a factorial combination of treatments. Data were subjected to analysis of variance.

RESULTS AND DISCUSSION

Axillary shoot proliferation studies

Explant germination percentage, stem length, number of axillary shoots after 12 weeks in culture were greater on BTM and MS medium than on WPM or DKW medium (Table 1). In addition, callus volume was greater on explants when grown on WPM and BTM than when grown on MS or DKW medium. Older parts of the callus mass frequently became brown which stained the culture medium and appeared to reduce shoot growth in these cultures. MS medium was used in all further experimemts because explants on MS showed good epicotyl elongation and shoot proliferation with minimal production of callus. Although not quantified, axillary shoots on MS medium usually had larger, greener leaves than on the other media. To reduce callus browning, 1 µM IBA was added to the medium.

Table 1.--Effects of media and BA after 12 weeks in vitro on explant germination, epicotyl length, axillary shoot number, and callus volume.

Treatment	Germination	Epicotyl length	Axillary shoots	Callus volume
	(%)	(mm)	(no.)	(mm^3)
MEDIA				
MS	100 [a]	33.3 [a]	1.0 [a]	78 [a]
BTM	94	30.0	1.9	161
WPM	65	26.6	0.4	215
DKW	83	17.2	0.4	27
5% LSD=		6.1	0.7	80
BA (µM)				
0.0	79	25.0	0.2	0
1.0	78	25.2	0.7	35
5.0	94	26.1	0.8	127
10.0	94	31.3	1.4	261
5% LSD=		6.1	0.7	81

[a] Each value represents the mean of 30 - 40 cultures.

Explant epicotyl length and axillary shoot production after 12 weeks in vitro were greater on medium supplemented with 10 µM BA than on medium supplemented with either 0.0, 1.0, or 5.0 µM BA (Table 1). Earlier work by Preece et al. (1987) using seedling shoot tips showed that consistent axillary shoot production on WPM was induced by 22 and 45 µM BA (5 and 10 mg l^{-1}, respectively). These BA levels, however, resulted in extensive callus formation on explants, especially if 4.9 µM IBA was added to the medium (Christ 1984). Preece et al (1989) showed that nonstratified seeds required transverse cutting to remove the apical end of each cotyledon before seeds would germinate. In our studies there was a trend for increasing amounts of BA to improve germination from cut unstratified seeds.

In experiments to test the effects of three cytokinins on axillary shoot proliferation, we found an interaction between TDZ, BA, and 2iP and their concentrations on axillary shoot production after 12 weeks in vitro (Figure 1). At 0.01 and 0.1 µM, TDZ, BA, or 2iP did not induce high axillary shoot proliferation rates. At 1 and 10 µM, TDZ was more effective than either BA or 2iP.

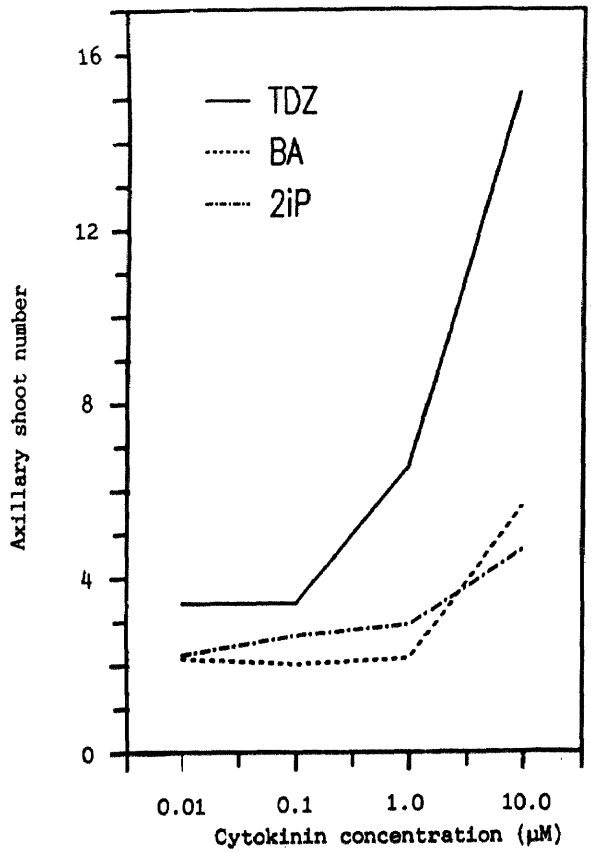

Figure 1.--Effect of cytokinins after 12 weeks on axillary shoot proliferation from cut seeds germinated in vitro.

The 15 axillary shoots per explant produced after 12 weeks in vitro with 10 µM TDZ is nearly 5 times the best rate previously reported for white ash (Preece et al 1987). Enhanced axillary shoot proliferation by TDZ has also been reported for apple and silver maple (Nieuwkerk et al. 1985; Ashby et al. 1987).

In other experiments to examine the type of explants to use for subculturing, we tested 2-node stem segments with and without leaves on factorial combination of 1, 3, and 10 µM of TDZ and BA. We found no differences in the number or length of axillary shoots between 3 and 10 µM TDZ; however, axillary shoots produced with 10 µM TDZ were abnormally thickened. The best axillary shoot proliferation with good elongation rates occurred when 1 µM BA and 3 µM TDZ were incorporated into MS medium. This experiment also showed that when leaves were retained on the 2-node stem segments, it increased the length of new axillary shoots.

Results from the above experiments suggest that our best axillary shoot proliferation and elongation with minimal callus formation was achieved by placing cut nonstratified seeds on agar-solidified MS medium supplemented with 3 µM TDZ, 1 µM BA, and 1 µM IBA. To maintain vigorous cultures, explants and subdivided axillary shoots need to be transferred monthly to new medium with the addition of liquid medium two weeks later.

Rooting studies

Pulsing white ash microshoots for 8 days with 5 µM IBA in half-strength MS medium increased both the percentage of shoots rooting and the number of roots compared to pulsing without IBA (Table 2). The first adventitious roots were visible 10 to 14 days after initiating the auxin pulse. Shoots pulsed with 10 µM IBA showed more synchronous rooting than shoots pulsed without IBA. The later microshoots showed gradually increasing rooting percentages throughout a 42 day period. Whether the shoots were pulsed in the light or dark for 8 days with IBA had no effect on rooting; however, subsequent experiments showed that darkened pulses were slightly more effective than lighted pulses. The initiation of one or more roots inhibited the subsequent initiation of additional roots from the main stem. Overall rooting percentages in our experiments were lower than those reported by Preece et al. (1987); however, it was not necessary to add activated charcoal to the medium to prevent development of thickened, brittle roots observed with longer IBA pulses by Preece et al (1987).

Factorial combinations of NAA and IBA at 0, 1, and 5 µM as an 8 day darkened pulse were then tested. This was to determine what combination of auxins would stimulate rapid synchronous root initation on a high percentage of the shoots without producing thickened, brittle roots. There was an interaction between IBA and NAA for both the number of roots per microshoot and average root length 32 days after initiating treatments (Table 3). More microshoots were rooted when exposed to high auxin concentrations compared to low auxins concentrations after both 16 and 32 days. The combination of 5 µM NAA and 5 µM IBA

Table 2.--Rooting response of axillary microshoots in half-strength MS supplemented with IBA.

IBA	Rooted shoots Day 20	Rooted shoots Day 32	Roots per shoot on day 32	Average root length
(µM)	(%)	(%)	(no.)	(mm)
0	3 [a]	21 [a]	0.8 [a]	24 [b]
1	27	41	1.0	27
5	27	64	1.1	28
10	17	31	0.9	24
5% LSD=			0.2	11

[a] Each value represents the mean of 27 to 33 microshoots.

[b] For rooted microshoots on day 32 only.

Table 3.--Effects of NAA and IBA on rooting of microshoots in full-strength MS medium.

Auxin level		Rooted shoots		Roots per shoot on day 32	Average root length
NAA	IBA	Day 16	Day 32		
(µM)	(µM)	(%)	(%)	(no.)	(mm)
0	0	22 [a]	78 [a]	1.2 [a]	23 [b]
0	1	30	54	0.9	16
0	5	10	50	0.8	12
1	0	10	30	0.4	10
1	1	20	40	1.0	11
1	5	38	100	2.1	37
5	0	60	80	1.7	28
5	1	50	90	2.1	39
5	5	80	90	3.5	36
5% LSD=				0.9	15

[a] Each value is the mean of 9 to 12 microshoots.

[b] For rooted microshoots on day 32 only.

resulted in the greatest number of roots per shoot without formation of thickened, brittle roots. Rooting occurred more quickly on full-strength MS medium than on half-strength MS medium (data not presented) although the response to the auxin pulse was similar.

Rooted shoots were easily acclimatized to a greenhouse environment by transplanting to a sterilized commercial potting mixture and setting on a mist bench for approximately 4 weeks before moving to benches without mist. Initial growth consisted of simple leaves with a gradual transition to compound leaves by the third to fifth pair of new leaves.

CONCLUSIONS

Best in vitro clonal propagation of white ash occurred on MS medium supplemented with 3 µM TDZ, 1 µM BA, and 1 µM IBA. To keep producing healthy axillary shoots, it is necessary to transfer them to new medium monthly. Our rooting studies indicate that rapid rooting of axillary shoots can be achieved in full-strength MS medium with 5 µM NAA and 5 µM IBA. This auxin combination induced rapid, synchronous rooting of normal appearing roots after 10 to 14 days. Rooted plantlets were easily acclimatized in the greenhouse by placing on mist benches for 4 weeks before transferring to benches without mist.

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