MICROPROPAGATION OF ASH (*FRAXINUS*)

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Abstract. Shoot tip explants from juvenile and adult green ash (*Fraxinus pennsylvanica*) and adult white ash (*F. americana*) formed callus and single shoots elongated in vitro. Consistent axillary shoot proliferation was not obtained. However, when new shoots that developed in vitro from juvenile white ash shoot tip explants were excised and placed into stationary liquid (1 cm deep) Woody Plant Medium (WPM) (4) with 5 or 10 mg·liter^{-1} benzyladenine (BA), axillary shoot proliferation occurred. These shoots could be rooted in vitro on WPM with 0.1 or 1.0 mg·liter^{-1} indole-3-butyric acid (IBA) and 10 g·liter^{-1} activated charcoal, or in moistened vermiculite with no plant growth regulators. Plantlets were acclimatized to the greenhouse and field. Green ash internodes produced callus in response to 2,4-dichlorophenoxyacetic acid (2,4-D). White ash seeds that were transversely cut in half germinated in vitro and callus grew from the cut cotyledons. Somatic embryos formed directly from these seedlings or from the callus, especially on a medium with BA and 2,4-D, both at 5μM.

Green and white ash are important trees in forestry and ornamental horticulture. These species are typically propagated by seeds, especially for use in forestry. Horticulturally, many ash clones, e.g. 'Marshall Seedless,' are propagated by budding. Efficient in vitro techniques could be a more rapid means of reproduction than graftage.

REVIEW OF LITERATURE

In vitro studies on ash have met with limited success. Walter and Skoog (6) developed a medium for callus growth from internodes or vascular cambial slices from *F. pennsylvanica*. They did not regenerate plants. Brown and Hicks (1) placed *F. americana* buds, from adult trees, in vitro and single shoots grew. Axillary shoot proliferation was not reported. Einset and Alexander (3) placed nodal explants from juvenile *F. pennsylvanica* in vitro and obtained outgrowth of unbranched monopodial axes.

In our clonal studies, we initiated experiments to attempt to obtain axillary shoot proliferation from juvenile and adult green and white ash. We then wanted to root the shoots and establish them in the greenhouse and field. In studies ultimately aimed towards tree improvement, we conducted experiments to obtain callus and adventitiously regenerated plants of these two ash species.

MATERIALS AND METHODS

Seedling ($\leq$ four years old) green and white ash stock plants were grown in pots with peat-lite medium (Promix BX) in the greenhouse at 25 ±5°C under night interruption (2200 to 0200 h) with high

output cool white fluorescent lamps. Local adult white ash were 15 to 25 years old and green ash were 20 to 30 years old. White ash stump sprouts were from formally seed bearing trees (10 years old) that had been cut down at the soil line. White ash seed explants were from samaras collected from local trees.

Shoot tip explants were harvested as 3 to 5 cm softwood cuttings, defoliated and placed in a 0.5% NaClO solution with Tween 20 for 20 minutes (white ash) or 14 minutes (green ash) followed by three 5-minute rinses in sterile deionized water. White ash seeds were sterilized for 30 minutes in 1.05% NaClO plus Tween 20.

Shoot tip explants were cut to 2.5 cm long and were placed into 25 × 150 mm culture tubes containing Woody Plant Medium (WPM) (4) with 30g·liter^{-1} sucrose adjusted to pH 5.8 with 1N KOH or 1NHCl prior to the addition of 7 g·liter^{-1} Difco bacto agar (when used) and autoclaving. White ash seeds were cut in half transversely prior to being placed in vitro. Explants were transferred to fresh medium monthly. Cultures were incubated at 25 ±3°C with a 16 h photoperiod and a PPF of 40 μM·m^{-2}·s^{-1} provided by cool white fluorescent lamps.

During rooting studies, 2.5 cm axillary shoots were placed either onto agar solidified WPM with or without 10 g·liter^{-1} activated charcoal or into 120 ml glass jars (baby food) with 60 ml moist vermiculite. When at least 75% of the shoots rooted, the lids were removed from the culture tubes or glass jars for five days. Plantlets were then removed from the vessels, dipped into deionized water and planted into 10 cm standard plastic pots with peat-lite medium (Promix BX). They remained in the laboratory for 5 more days and were then moved to the greenhouse. At this time 10 to 15 granules of Osmocote (14-14-14, N-P_2O_5-K_2O) were added to each pot. After 5 months in the greenhouse, plants were transplanted to the field.

RESULTS AND DISCUSSION

During the first five months on agar solidified WPM juvenile shoot tip explants from both species elongated, but did not branch. Callus grew at the cut shoot bases and was greatest on medium with BA and IBA, both at 1.0 mg·liter^{-1} (Table 1). Unexpectedly, during this time, white ash shoot tips exposed to auxin in vitro produced adventitious roots. The highest rooting was 51% after three months. These rooted cuttings were easy to establish in pots in the greenhouse.

Quiescent shoots (30 cm long) from adult green and white ash trees were collected during January, February, and March and placed into containers with deionized water. The water was changed daily. New softwood shoots were excised as explants. Green ash quiescent stems forced better in the deionized water than white ash, yielding 51.6% (54 of 125) stems with shoot growth $\geq$ 2.0

cm compared to only 15.9% (20 of 150) stems for white ash. In vitro, callus grew from the cut stems exposed to high levels of BA (Table 2), but only a few shoots elongated. Actively growing stump sprouts proved to be poor explant sources because 80% became contaminated within two weeks. Thus adult ash responds more poorly in vitro than juvenile stem explants.

Table 1. The influence of BA and IBA on shoot elongation and callus formation on shoot-tip explants of green ash.[z]

Growth regulators		Time					
		Month 1		Month 2		Month 3	
BA ($mg \cdot liter^{-1}$)	IBA	Callus rating[y]	Shoot elongation (cm)	Callus rating	Shoot elongation (cm)	Callus rating	Shoot elongation (cm)
0.5	0	2.1	1.3	2.1	2.0	2.1	2.2
	1.0	2.1	1.1	2.4	2.0	2.2	2.2
1.0	0	2.1	1.1	2.2	2.5	2.0	2.0
	1.0	2.4	1.4	3.0	2.0	2.8	3.5
	SE	0.08	0.22	0.17	0.51	0.11	0.61
		NS	*	NS	NS	**	NS
		Month 4				Month 5	
0.5	0	2.1	2.6			2.1	2.6
	1.0	2.5	2.7			2.9	2.6
1.0	0	2.1	2.4			2.1	2.6
	1.0	3.6	2.7			4.1	2.7
	SE	0.19	0.15			0.17	0.11
		**	NS			**	NS

[z] Each number presents the mean of 16 to 25 replications.
[y] Callus rating 1 = no callus, 2 = 1 cm diameter callus, 3 = 2 cm diameter callus, 4 = 3–4 cm diameter callus, 5 = > 5 cm callus.
*, **, NS: Indicates a significant interaction between BA and IBA at the 5% level (*), the 1% level (**), or non-significant (NS) according to F-test with 1 and 74 d.f.

Table 2. Callus measurements from adult white and green ash shoot tip explants after nine months in vitro.

BA concentration $mg \cdot liter^{-1}$	white ash		green ash	
	Callus diameter (cm)	Total dry weight (mg)	Callus diameter	Total dry weight
5	1.28	233.6	1.12	225.1
10	1.79*	318.2*	1.77**	332.7**
15	1.87**	355.6**	1.94**	387.6**
20	1.98**	395.7**	2.10**	397.1**
5% LSD	0.40	71.96	0.42	53.20
1% LSD	0.55	99.16	0.57	57.00

F-tests for callus diameter and dry weight of inocula were significant at the 1% level.
*,**Significantly different from 5 mg 1^{-1} BA at the 5% (*) and 1% (**) level, respectively.

After juvenile shoot explants had grown 4 to 6 cm in length, the terminal 2.5 cm was excised and placed into stationary liquid or agar solidified medium. Consistent axillary shoot proliferation only occurred on white ash shoots on liquid WPM with 5 or 10 mg·liter^{-1} BA, with 10 mg·liter^{-1} being better (Table 3 and Figure 1-left). Green ash was not responsive.

Table 3. The influence of BA on axillary shoot proliferation of white ash in liquid medium.[w]

	Time after subcultering									
	1 Month		2 Months		3 Months		4 Months		5 Months	
BA mg·liter^{-1}	Mean[x]	Max.[y] Shoot	Mean	Max. Shoot	Mean	Max. Shoot	Mean	Max. Shoot	Mean	Max. Shoot
5	0.3	2	0.5	4	1.6	8	0.8	5	2.1	6
10	0.7	4	1.4	6	1.9	8	2.2	8	2.8	6
Minimum harvest[z]		4		24		36		36		

[w] New shoot growth was excised from the original explants and placed into liquid medium for up to 5 months.
[x] Each number represents the mean of 18 to 31 replications.
[y] This is the maximum number of shoots that were observed in an individual culture vessel at that time.
[z] Upon each monthly transfer, shoots were harvested for rooting studies. Only the most uniform were used, the remainder were discarded. The minimum harvest is the number of the shoots that were used. These were not counted the following month.

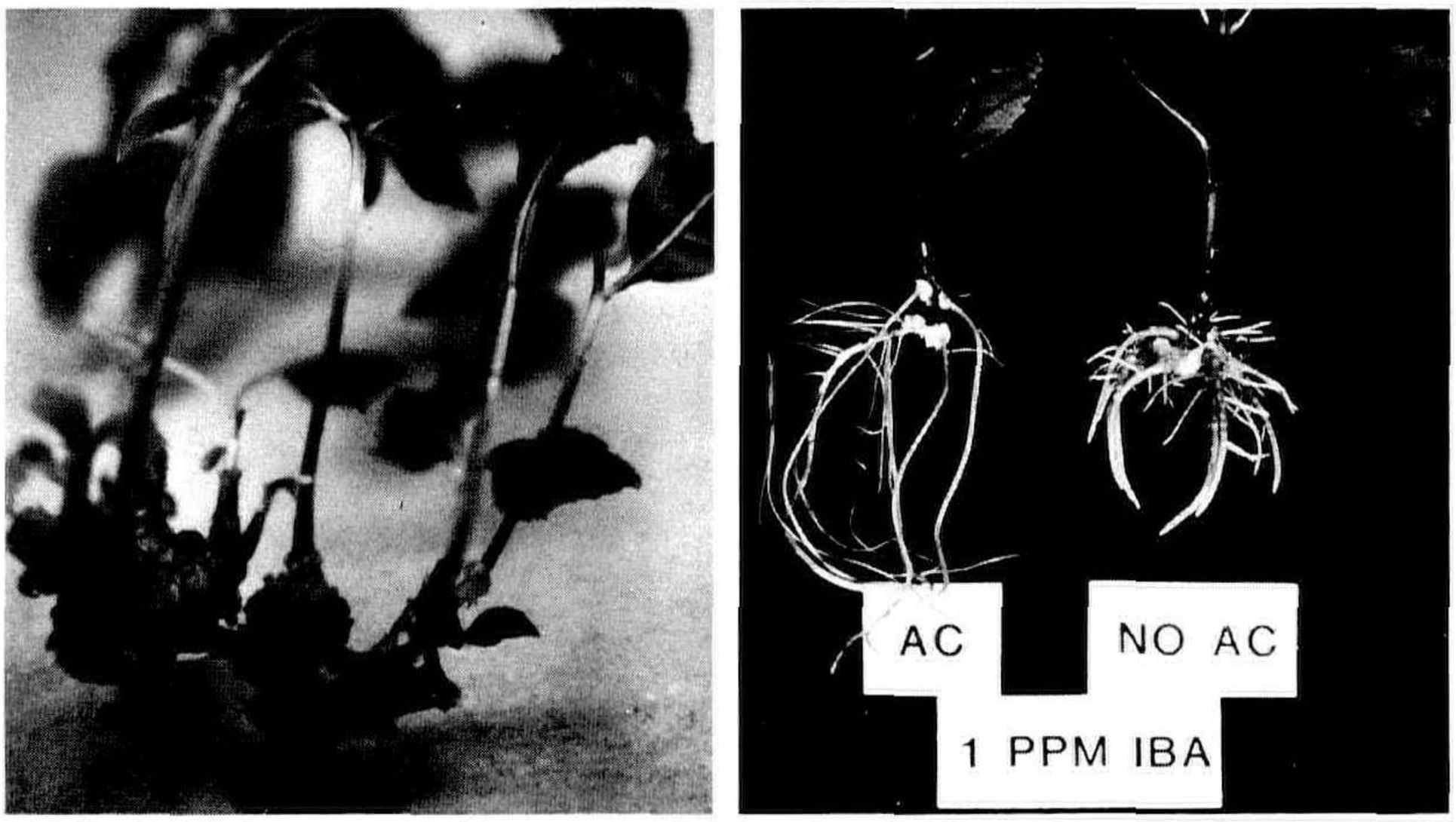

Figure 1. White ash. *Left:* Shoot multiplication from shoot tip explant on liquid medium. *Right:* Root quality was better on microshoots when activated charcoal (AC) was incorporated into the medium.

Because axillary shoot proliferation only occurred on juvenile white ash, these were the only shoots used in rooting studies. Best rooting was achieved on agar solidified medium with 0.1 or 1.0 mg·liter^{-1} IBA, however these roots were shorter and more brittle than those that formed on microcuttings with no auxin. When activated charcoal was incorporated into the agar solidified medium with IBA, rooting percentage remained high (up to 89%), and the roots were not stunted (Figure 1-right). Rooting of white ash microshoots was 100% using sterilized vermiculite and no plant growth regulators. Rooting occurred within one month.

Following a slow acclimatization process, 100% of the rooted shoots survived in the greenhouse after one month. Initially the plantlets produced simple leaves, but after approximately one month, they produced compound leaves. Plants lined out into the field had 100% survival.

In an attempt to produce callus, 1 cm long softwood internodes from juvenile green ash were placed on various media with either 1 or 10μM 2,4-D for 8 weeks (primary media). They were then transferred to secondary media for four weeks with either no plant growth regulators or with BA plus naphthaleneacetic acid (NAA), both at 0.5 μM. Callus production was greater with 1 μM than 10 μM 2,4-D, regardless of the nutrient salt formulation, and on MS salts, regardless of the 2,4-D concentration (Table 4). No plantlets regenerated from the green ash callus.

Table 4. The influence of 2,4-D and nutrient media in the primary media on the growth of green ash callus on the secondary media after 4 weeks.[x]

2,4-D (μM)	Fresh weight (mg)	Dry weight (mg)
1	1519.2	194.0
10	882.4	108.4
	**	**
Nutrient medium	**Fresh weight (mg)**	**Dry weight (mg)**
MS (5)	1750.7 a[y]	181.9 a
WPM (4)	1118.3 b	150.0 ab
DKW (2)	1019.5 b	142.9 b
WS (6)	915.5 b	132.0 b
	**	*

[x] Each number represents the mean of 79 to 80 (2,4-D) and 39 to 40 (nutrient medium) replications, respectively.
[y] Means followed by the same letter are not significantly different using Duncan's new multiple range test for alpha = 0.05 level.
*,**: indicate significance at the 5% (*) and 1% (**) levels according to F-test with 1 and 142 (2,4-D) and 3 and 142 (nutrient medium) d.f., respectively.

Cut white ash seeds that had been placed in vitro germinated and callus grew where the cotyledons touched the agar solidified MS or DKW medium. Directly from the explant itself and from the

callus, somatic embryos formed (Figure 2-left). The highest percent embryogenesis occurred when BA and 2,4-D, both at 5 μM, were incorporated into the primary medium for 4 to 6 weeks.

The somatic embryos were variable and had from one to several cotyleons. They elongated (Figure 2-left-below) then some germinated into plants (Figure 2-right).

Techniques, such as somatic embryogenesis from callus and clonal micropropagation from juvenile explants, as described herein, might be utilized to improve important tree species, such as ash.

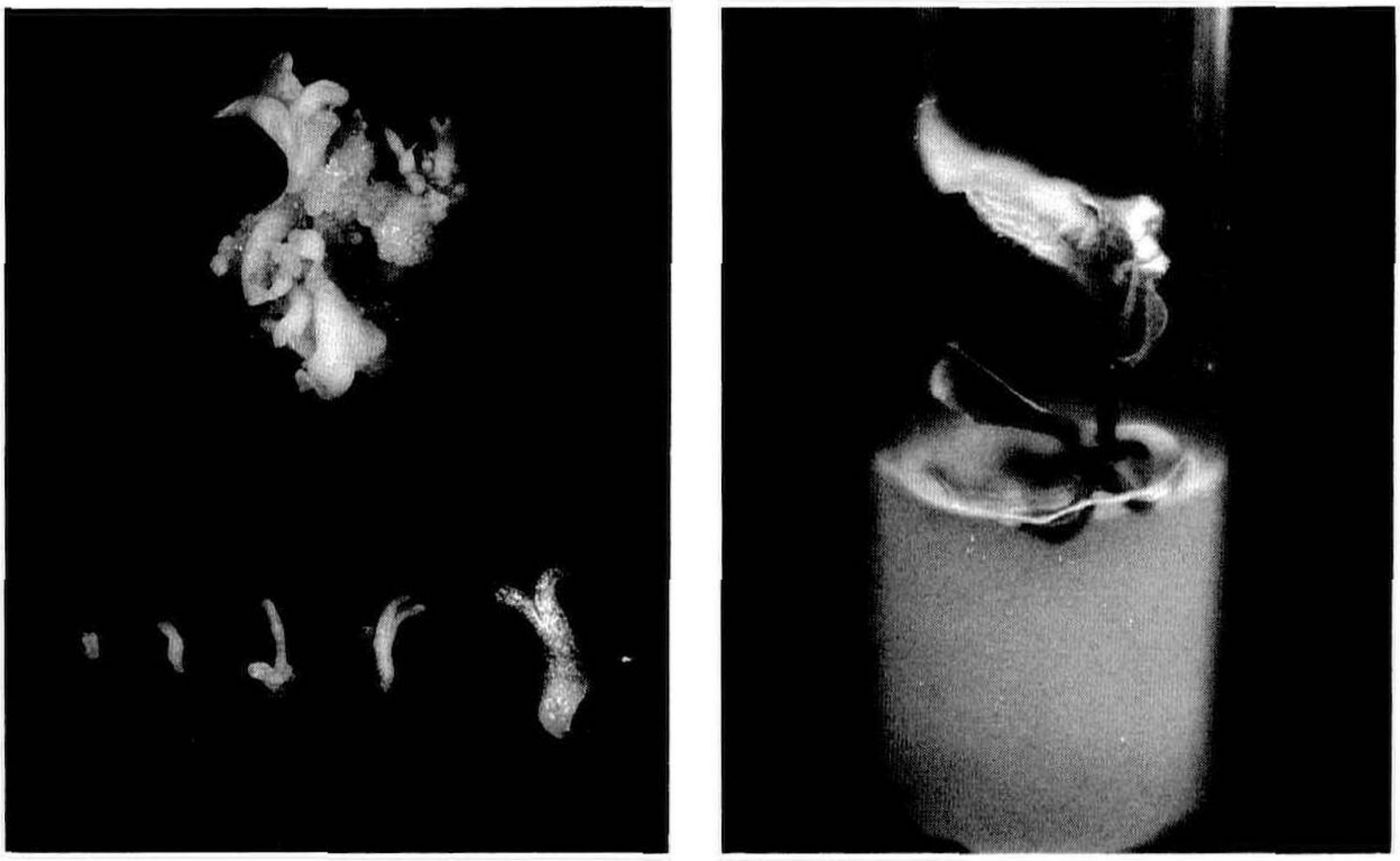

Figure 2. White ash. *Left above*: Somatic embryogenesis from cotyledon derived callus. *Left below*: Somatic embryos were variable and grew at different rates. *Right*: Some somatic embryos germinated and grew into plants.

Table 5. The influence of culture period on the primary and secondary media and plant growth regulators on embryogenesis from cut white ash seeds after 10 weeks in vitro.

Culture period		Primary medium[y]		
		Plant growth regulator		
Primary medium (week)	Secondary medium (week)	BA (μM)	2,4-D (μM)	Percent embryogenesis[x]
4	6	0	0.5	2.6
		5	5	16.7
6	4	0	0.5	2.6
		5	5	13.0

[x] Each number is based on 76 to 80 replications.

[y] The primary medium consisted of DKW salts and the secondary medium is averaged across MS and DKW salts.

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OLIVINE: A POTENTIAL SLOW-RELEASE MAGNESIUM SOURCE FOR NURSERIES

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Magnesium is essential for plant growth. About 10% of the magnesium in green plants is in the pigment chlorophyll which gives plants their green color as well as their ability to change light energy to chemical energy through the process of photosynthesis. The remainder of magnesium in a plant has many uses. High concentrations of magnesium are usually found in parts of plants where lots of energy is required such as the growing tips of roots and shoots or areas where seeds are being formed.

MAGNESIUM DEFICIENCY

The need for magnesium in higher amounts in these areas of rapid growth helps to explain why magnesium deficiency symptoms appear as they do. Magnesium moves readily from one area inside a plant to another. If there is not enough magnesium for all parts of the plant when conditions are right for rapid shoot growth or a heavy seed set is occurring, the magnesium will be preferentially transported to areas where it is needed most. When this happens, the older leaves on plants turn yellow because the magnesium needed for the rapid shoot growth or a heavy seed set is