New Forests **16:** 43–57, 1998.
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In vitro and *ex vitro* rooting of micropropagated shoots using three green ash (*Fraxinus pennsylvanica*) clones

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Accepted 25 January 1998

Application. This *in vitro* and *ex vitro* rooting system can provide useful developmental techniques for micropropagation of green ash genotypes. In addition to supplying material for genetic mapping studies, this system can be applied to produce clonal material for basic ecological and biological studies such as drought tolerance, pest resistance, and riparian buffer function in diverse areas of forestry.

Abstract. Shoot multiplication using seedling materials was achieved by subculture on Murashige and Skoog salts with Gamborg's B5 vitamins (MSB5) medium containing a combination of 5 μM 6-benzyladenine (BA), 5 μM thidiazuron (TDZ), and 1 μM indole-3-butyric acid (IBA) with three green ash (*Fraxinus pennsylvanica*) clones, SD1009 (South Dakota origin), SD2002 (South Dakota origin), and KA2018 (Kansas origin). Shoots were rooted using *in vitro* and *ex vitro* methods. For *in vitro* rooting studies, elongated shoots were transferred to rooting plugs supplied with liquid MSB5 medium containing a 3×3 factorial arrangement of two different auxins, α-naphthaleneacetic acid (NAA) and IBA, at three concentrations (0, 5, and 10 μM). The most effective treatment for *in vitro* root number, root length, and shoot height was 5 μM IBA. The three clones also were tested for *ex vitro* rooting using a quick dip in 1 mM NAA, 1 mM IBA, or control (no auxin). The maximal *ex vitro* rooting response occurred when shoot explants of the three clones were dipped in 1 mM IBA. Significant clonal differences were noted in response to *in vitro* and *ex vitro* rooting treatments. Rooted plantlets were acclimated to the greenhouse.

Key words: adventitious root formation, auxin, hardwood, rooting sponge, shoot multiplication, woody plant tissue culture

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Introduction

Green ash (*Fraxinus pennsylvanica* Marsh.) is one of the largest and most prevalent ash species in the United States. It is widely planted for environmental protection in the form of individual shade trees, wind breaks, and watershed buffers in urban and agricultural ecosystems of the Great Plains (Solomon et al. 1993).

Provenance testing and other traditional tree improvement techniques have identified adaptable seed sources of green ash for the Great Plains (Steiner et al. 1988). Clonal propagation can help further define genetic contribution to phenotypic traits (Newcombe and Bradshaw 1996; Newcombe et al. 1996). Micropropagation could provide a potential means to clone superior selections rapidly from conventional breeding programs, or provide the basis for *in vitro* genetic manipulation or selection (Libby and Ahuja 1993). In genetic mapping programs, clonal material can be used to evaluate the utility of genetic markers across diverse environments (Bradshaw and Stettler 1995). Once genetic markers are identified for useful traits, Marker Assisted Selection can be employed on seedling material to select genotypes for clonal propagation. Thus, *in vitro* micropropagation of superior genotypes and pest/stress-tolerant selections could provide more rapid genetic gains with this species.

Green ash can be propagated vegetatively by means of cuttings and grafting (Kennedy 1990). Compared to grafting, micropropagation techniques could 1) reduce cost; 2) greatly increase numbers of desirable clones for research studies and outplanting; and 3) assure genetic and physiological integrity of root systems by eliminating the need for grafting. Such methods are a critical component of many genetic improvement programs for higher plants, and complement both biotechnical and conventional breeding efforts (Haissig et al. 1987). The inability to induce adventitious root formation (termed ‘rooting’ hereafter) is often the limiting factor for establishing plant materials derived from tissue culture or conventional cuttings. This limitation is especially common with woody plants species wherein a reduced capacity for rooting often accompanies maturation (Davis et al. 1988).

Recently, *in vitro* shoot proliferation of green ash has been obtained from seed explants (Kim et al. 1997). In spite of successful shoot proliferation, the vegetative propagation of green ash is not optimal for efficient practical applications. A major detriment to successful vegetative propagation for green ash is the lack of well-defined rooting techniques.

This study was undertaken to develop and define *in vitro* and *ex vitro* techniques to promote root development for micropropagation systems of green ash.

Materials and methods

Plant material

Dried samaras of green ash were obtained from three individual trees growing in Harding County, South Dakota (SD1); Fall River County, South Dakota (SD2); and Norton County, Kansas (KA2) in September, 1991. Seeds were air-dried and stored in plastic bags in the dark at 4 °C for 3 years. The pericarps were removed and the apical one-quarter (containing the cotyledon tips) of each seed was removed. Seeds were surface-disinfested for 1 min in 70% ethanol and 10 min in 1.05% sodium hypochlorite (20% Clorox®), followed by five rinses in sterile, distilled water and recutting (ca. 2 mm) of the apical portion. The cut seeds containing embryos were placed in culture tubes (25×150 mm) on 15 ml agar-solidified medium (0.7% Difco Bacto) containing Murashige and Skoog (1962) salts, B5 vitamins (Gamborg et al. 1968), and 3% sucrose at pH 5.8 (MSB5). One cut-seed was placed in each test tube, and a total of 17 to 34 seeds were used for each of the three seed sources.

Three weeks after germination, radicles were removed from the germinated seedlings, and the shoot explants were placed horizontally within a Magenta GA-7 culture vessel (7.6×7.6×10.2 cm, Magenta Corp., Chicago, IL) on 60 ml of agar-solidified MSB5 medium with a combination of 10 μM 6-benzyladenine (BA) and 10 μM thidiazuron (TDZ) (Kim et al. 1994). Each culture vessel contained four cut-shoot explants. After two subcultures on the same media, induced axillary shoot tips were multiplied on MSB5 medium containing a combination of 5 μM BA, 5 μM TDZ, and 1 μM IBA (Kim 1995). Three different clones (derived from individual seedlings designated SD1009 from SD1; SD2002 from SD2; and KA2018 from KA2) were sufficiently established after six subcultures to provide clonal explant sources for *in vitro* and *ex vitro* rooting studies.

In vitro rooting experiment

Apical shoot tips of the three clones (SD1009, SD2002, and KA2018) were proliferated *in vitro* by repeated subculturing over an 8-month period. A single apical tip explant (25–35 mm in length and ca. 1 mm in stem diameter) was transferred to a rooting plug (Sorbarod Micropropagation Systems, Baumgartner Papiers SA, Switzerland) within each culture tube (Figure 1A). Culture tubes were supplied with 17 ml liquid MSB5 medium containing 3×3 factorially arranged treatments of α-naphthaleneacetic acid (NAA) (0, 5, or 10 μM) with IBA (0, 5, or 10 μM). Auxins were incorporated into the medium, and the pH was adjusted to 5.8 with 1N NaOH or HCl before

autoclaving at 121 °C and 1.2 kg cm^{-2} for 20 min. Cultures were incubated at 24±2 °C under 16-h light photoperiod with light provided by 40-watt, cool-white, fluorescent lamps at a photosynthetic photon flux of 35 to 47 μmol m^{-2} s^{-1}.

Ex vitro rooting experiment

Apical shoot tips of three clones (SD1009, SD2002, and KA2018) were collected from *in vitro* cultures multiplied over an 8-month period. Apical tip explants were trimmed to 50 mm because increased shoot length can improve survival under *ex vitro* conditions (Kim, unpublished). The basal 10 mm of each stem (ca. 1.5 mm in diameter) was treated with a 15-s dip in 1 mM NAA (aqueous), 1 mM IBA (aqueous), or distilled water (control). Auxin-dipped and control shoots were inserted into polyterra® rooting sponges (Techni-culture, Inc., Salinas, CA), placed in covered plastic trays (64×32×7 cm), and maintained under conditions of high humidity (Figure 1B). The bottom of each rooting sponge was immersed in a nutrient solution containing 1/10-strength MS salts without sucrose, pH 5.8. Nutrient solutions were replaced every week. Culture room conditions were the same as for *in vitro* rooting tests.

Clonal experiment of in vitro or ex vitro rooting

Clonal variation was evaluated using the selected treatments of NAA and/or IBA that supported the maximum responses of *in vitro* and *ex vitro* rooting. Culture materials, methods, and conditions were identical to the *in vitro* and *ex vitro* rooting experiments, respectively. For the clonal evaluations of *in vitro* rooting, apical tips of three clones (SD1009, SD2002, and KA2018) were cultured in both liquid and agar-solidified MSB5 medium supplemented with 5 μM IBA. For the clonal evaluations of *ex vitro* rooting, apical tips of three clones (SD1009, SD2002, and KA2018) were treated with 1 mM IBA dip and supplied with nutrient solution.

Data collection and statistical analysis

Five weeks after *in vitro* or *ex vitro* rooting treatments were initiated, data were collected using a hand lens and ruler. Root number, root length (mm), rooting percentage, and shoot height (mm) were determined for all experiments. For all rooting experiments, roots were counted only if they had reached a minimum length of 2 mm. The *in vitro* rooting test was conducted twice with a total of 12 replications per treatment for each clone, and the *ex vitro* rooting test was conducted twice with a total of 30 replications per

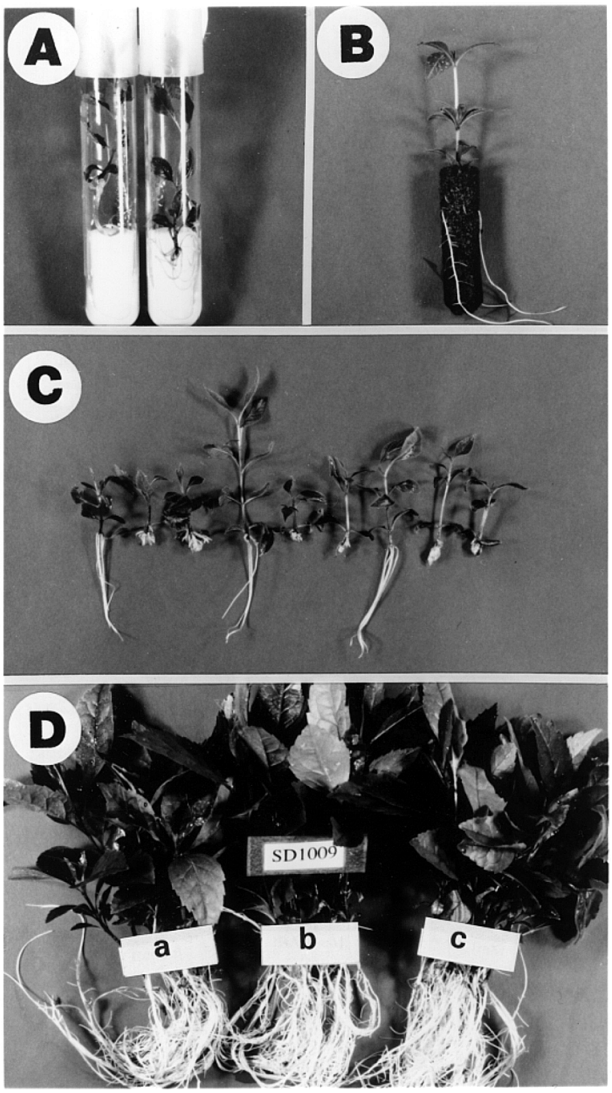


Figure 1. **A**: *In vitro* rooting of green ash clone KA2018 after 5-weeks culture in a rooting plug supplied with liquid MSB5 medium containing 5 μM IBA. **B**: *Ex vitro* rooting induced by 1 mM IBA quick-dip of green ash clone SD1009 after 5-weeks culture in a rooting sponge. **C**: Effect of NAA and IBA on *in vitro* rooting of green ash clone KA2018 after 5-weeks culture on liquid MSB5 media containing; from left to right: control (no plant growth regulator), 5 μM NAA, 10 μM NAA, 5 μM IBA, 5 μM NAA plus 5 μM IBA, 10 μM NAA plus 5 μM IBA, 10 μM IBA, 5 μM NAA plus 10 μM IBA, and 10 μM NAA plus 10 μM IBA. **D**: Effects of auxin dips on *ex vitro* rooting of green ash clone SD1009 after 5-weeks culture; (a) Control, (b) 1 mM IBA, and (c) 1 mM NAA.

Table 1. Analysis of variance for *in vitro* rooting responses of three green ash clones.

Source	df	Root number		Root length		Shoot height	
		F	P>F	F	P>F	F	P>F
Clone	2	28.89	0.0001	219.90	0.0001	160.30	0.0001
NAA	2	191.68	0.0001	1451.09	0.0001	160.83	0.0001
IBA	2	13.06	0.0001	179.69	0.0001	26.46	0.0001
Clone∗NAA	4	14.77	0.0001	202.82	0.0001	21.26	0.0001
Clone∗IBA	4	12.94	0.0001	28.95	0.0001	1.58	0.1806
NAA∗IBA	4	7.19	0.0001	172.31	0.0001	16.23	0.0001
Clone∗NAA∗IBA	8	6.79	0.0001	28.74	0.0001	2.83	0.0049
Error	285						
Total	311	EMS = 28.19		EMS = 16.17		EMS = 37.52	

treatment for each clone. The clonal experiment was conducted twice with a total of 12 replications per clone (*in vitro* rooting) or with a total of 30 replications per clone (*ex vitro* rooting). A replication consisted of one explant in a culture tube or rooting sponge, and all experiments used a completely randomized design. Mean number of roots, mean length of roots, and mean height of shoots were analyzed for all experiments.

The analysis of variance (ANOVA) for treatment means was performed using the General Linear Model (GLM) procedure (SAS 1996) to account for unequal sample sizes. Variation among treatment means of each clone and clone means was analyzed using Tukey's Honestly Significant Difference Test ($\alpha = 0.05$).

Results

In vitro rooting experiment

Auxin concentration and clone significantly affected *in vitro* root number, root length, and shoot height (Table 1). Significant interactions occurred among NAA and IBA, clone and NAA, or clone, NAA, and IBA for all test variables (Table 1). A significant clone by IBA interaction was apparent for root number and root length (Table 1). These interactions indicate that NAA or IBA and clone effects should be examined together under *in vitro* conditions.

The mean number of *in vitro* roots was highest when shoot-tip explants were cultured on 10 μM NAA (SD1009) or a combination of 5 μM NAA and

10 μM IBA (SD2002 and KA2018) (Table 2). The longest roots and tallest shoots developed when explants were cultured on medium supplemented with 5 μM IBA for all three clones (Table 2). In the absence of NAA or IBA, no roots were produced by SD1009 or SD2002 explants. However, 91% of KA2018 explants produced roots without exogenously applied auxin, and these roots grew well (Table 2).

The first roots were visible 10 to 15 days after initiating auxin treatment *in vitro*. Many root initials (<0.5 mm in length), particularly those induced by a single treatment of NAA or a combination of NAA and IBA, frequently ceased growth and subsequently developed callus tissue (data not shown). Roots that did emerge occurred at the internodal region of the stem, usually immediately above the nodes. In addition, roots induced by NAA alone or a combination of NAA and IBA were thickened and short (less than 5 mm long) (Figure 1C; Table 2). No inhibition of root growth was apparent with single IBA treatments (Table 2). Maximal root elongation (more than 15 mm) *in vitro* occurred when 5 μM IBA was incorporated into liquid MSB5 medium (Figure 1C; Table 2).

Clonal experiment of in vitro rooting

The clone of green ash significantly affected the number of roots ($p < 0.01$ for liquid, $p < 0.001$ for solidified, df = 2), root length ($p < 0.001$ for liquid, $p < 0.05$ for solidified, df = 2), and shoot height ($p < 0.001$ for liquid, $p < 0.001$ for solidified, df = 2) under *in vitro* conditions using liquid or agar-solidified MSB5 with 5 μM IBA (Figure 2). For liquid medium, clone KA2018 produced the most roots (Figure 2A), the longest roots (Figure 2B), and the tallest shoots (Figure 2C), although clone SD2002 had a similar mean root length (Figure 2B). For agar-solidified medium, a significant difference was observed between all test variables of KA2018 and SD1009 (Figure 2). Clone SD1009 was significantly lower for all test variables in both liquid and agar-solidified medium (Figure 2).

Ex vitro rooting experiment

Under *ex vitro* conditions, both auxin treatment and clone significantly affected root number, root length, and shoot height (Table 3). No significant interactions were evident between clone and auxin for any of the test variables (Table 3). Roots usually emerged 14 to 21 days after axillary shoots were placed into polyterra® rooting sponges (Figure 1B). After 35 days, 100% of the shoots had rooted with or without auxin treatments.

With the 1 mM IBA treatment, root length and shoot height were significantly different than with 1 mM NAA and control treatments (Table 3). A

Table 2. Effects of NAA and IBA on *in vitro* rooting responses of three green ash clones after 5 weeks on liquid MSB5 medium[1].

Clone	Auxin level (μM)		Rooted shoots	Mean number of roots	Mean root length (mm)	Mean shoot height (mm)
	NAA	IBA	(%)			
SD1009	0	0	0	0d[2]	0f	39.3 (2.1)bc
		5	100	4.3 (0.5)cd	19.8 (0.7)a	46.2 (1.4)a
		10	100	3.0 (0.3)d	13.9 (0.8)b	41.2 (1.0)ab
	5	0	100	11.3 (1.8)bc	2.3 (0.3) de	31.6 (1.3)de
		5	83	16.8 (2.9)ab	3.8 (0.6)cde	34.9 (1.4)cd
		10	83	6.1 (1.4)cd	1.8 (0.4)ef	32.9 (1.0)de
	10	0	100	20.3 (1.4)a	4.8 (0.3)c	30.2 (1.3)de
		5	92	18.3 (2.7)ab	4.3 (0.5)cd	31.9 (1.3)de
		10	92	16.1 (1.8)ab	4.7 (0.5)c	28.9 (0.7)e
SD2002	0	0	0	0e	0c	33.8 (1.4)cd
		5	100	4.0 (0.3)de	51.3 (1.9)a	48.5 (1.1)a
		10	100	3.8 (0.3)de	45.7 (1.0)b	43.3 (1.5)ab
	5	0	91	5.7 (1.0)cde	2.3 (0.3)c	36.0 (1.9)bcd
		5	91	13.3 (1.7)b	2.7 (0.4)c	40.6 (2.5)bc
		10	91	28.5 (3.1)a	2.5 (0.3)c	39.4 (1.7)bc
	10	0	82	8.1 (1.3)bcd	2.4 (0.4)c	34.5 (1.7)cd
		5	91	11.7 (1.7)bc	2.7 (0.3)c	35.5 (1.8)cd
		10	91	9.5 (1.5)bcd	2.5 (0.3)c	31.0 (0.8)d
KA2018	0	0	91	3.3 (0.5)c	35.3 (5.2)b	50.8 (2.5)b
		5	100	5.8 (0.2)c	53.0 (1.6)a	74.1 (2.9)a
		10	100	5.8 (0.8)c	46.2 (1.4)a	67.5 (3.1)a
	5	0	100	15.8 (1.3)b	4.1 (0.3)c	42.7 (2.2)bc
		5	100	17.5 (1.3)ab	4.1 (0.2)c	44.8 (1.7)bc
		10	100	23.8 (0.9)a	5.0 (0.3)c	47.0 (2.8)bc
	10	0	100	21.3 (2.1)ab	4.3 (0.7)c	42.1 (1.1)bc
		5	100	18.5 (2.0)ab	4.1 (0.2)c	40.0 (1.9)c
		10	91	21.5 (2.6)ab	4.3 (0.5)c	37.4 (1.8)c

[1]Each number represents the mean (and standard error of the mean) of 12 replications.

[2]Data within a column of each clone followed by same letters are not significantly different according to Tukey's Honestly Significant Difference Test (α = 0.05).

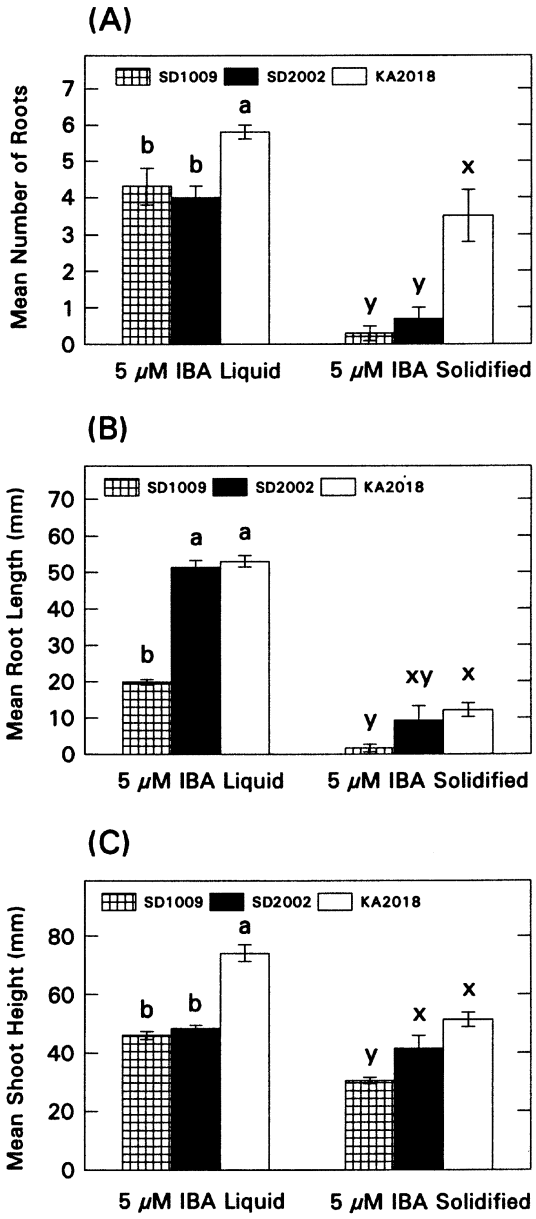


Figure 2. Effects of three green ash clones (SD1009, SD2002, and KA2018) on *in vitro* rooting after 5-weeks culture on liquid or agar-solidified MSB5 medium containing 5 μM IBA. Experiments were conducted twice with a total of 12 replications using a completely randomized design. A replication consisted of one explant in a culture tube. The vertical T-lines on the bar graph represent the standard error. Response levels labelled with the same letters are not significantly different according to Tukey's Honestly Significant Difference (α = 0.05).

Table 3. Analysis of variance for *ex vitro* rooting responses of three green ash clones.

Source	df	Root number		Root length		Shoot height	
		F	P>F	F	P>F	F	P>F
Auxin dip	2	4.11	0.0182	16.73	0.0001	17.63	0.0001
Clone	2	11.76	0.0001	144.37	0.0001	59.32	0.0001
Clone∗Auxin dip	4	0.28	0.8922	0.52	0.7204	1.98	0.0995
Control vs. Others	1	7.85	0.0057	16.89	0.0001	18.55	0.0001
Control vs. NAA	1	4.57	0.0341	2.42	0.1221	2.94	0.0881
Control vs. IBA	1	7.41	0.0072	31.38	0.0001	33.41	0.0001
NAA vs. IBA	1	0.29	0.5896	15.85	0.0001	15.96	0.0001
Error	165						
Total	173	EMS = 0.80		EMS = 319.95		EMS = 226.19	

significant difference occurred between root number in response to 1 mM IBA and control (Table 3). The greatest mean number of roots occurred when shoot-tip explants were treated with a 15-s dip in 1 mM IBA for all clones (Figure 3A). For all clones, roots were longest and shoots were tallest when explants were dipped in 1 mM IBA (Figure 3B; 3C).

Clonal experiment of ex vitro rooting

Under *ex vitro* conditions, the clone of green ash significantly affected the number of roots, root length, and shoot height in response to a 1 mM IBA dip ($p < 0.05$, df = 2) (Figure 3). Clone SD1009 produced the longest roots and tallest shoots, whereas clone KA2018 produced the shortest roots and the shortest shoots after a 1 mM IBA dip (Figure 3B, 3C). The mean root length and shoot height were significantly different among the clones (Figure 3B, 3C), and a significant difference was observed between mean root numbers of SD1009 and SD2002 after a 1 mM IBA dip (Figure 3A).

Discussion

Previous research on *Fraxinus* spp. has shown that NAA and IBA are effective auxins to stimulate rooting of micropropagated shoots. With white ash (*F. americana* L.), Preece et al. (1987) and Navarrete et al. (1989) observed that a combination of 5 μM NAA and 5 μM IBA in agar-solidified MS medium resulted in the greatest number of roots per shoot without the formation of thickened, brittle roots. Common ash (*F. excelsior* L.) exhibited maximal root

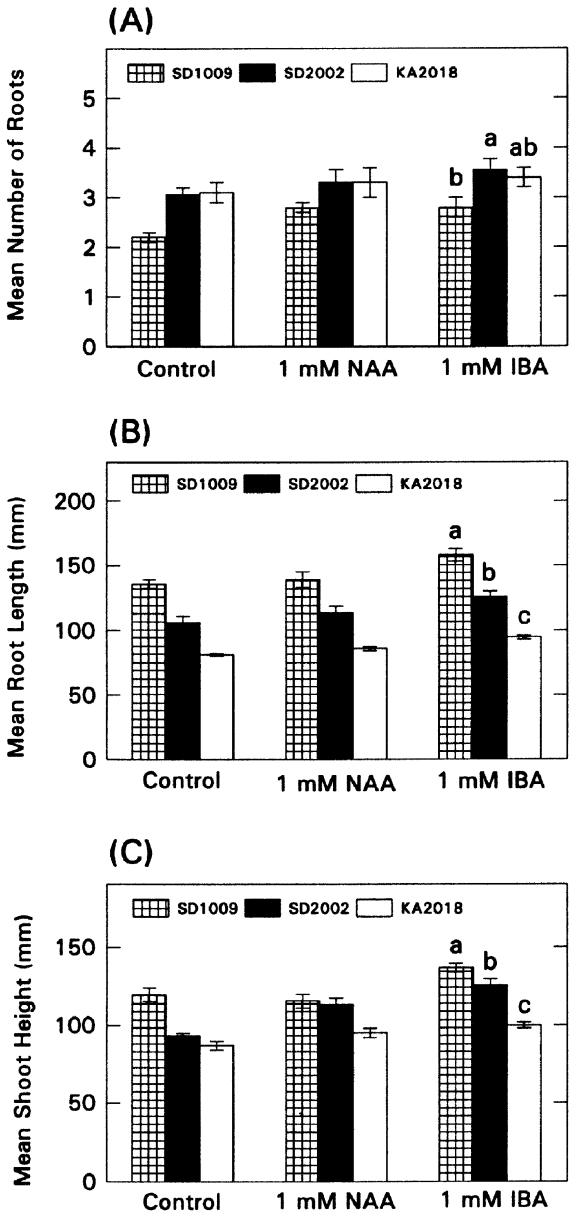


Figure 3. Effects of auxin dips on *ex vitro* rooting of three green ash clones (SD1009, SD2002, and KA2018) after 5-weeks culture. Experiments were conducted twice with a total of 30 replications using a completely randomized design. A replication consisted of one explant in a rooting sponge. The vertical T-lines on the bar graph represent the standard error. Response levels labelled with the same letters are not significantly different according to Tukey's Honestly Significant Difference ($\alpha = 0.05$).

formation when shoots were cultured on agar-solidified, half-strength Woody Plant Medium (WPM) (Lloyd and McCown 1980) containing 2.45, 4.9, or 9.8 μM IBA (Hammatt and Ridout 1992). With flowering ash (*F. ornus* L.), Arrillaga et al. (1992) showed that roots were obtained using liquid Rugini medium (Rugini 1986) with a combination of 0.4 μM BA and 2.5 μM NAA. However, previous studies have not yet defined an efficient *in vitro* rooting system for practical applications with green ash. Under the conditions of this study, all three clones exhibited effective rooting in response to 5 μM IBA incorporated into liquid MSB5 medium (Table 2). Data suggests that optimal rooting response may occur between 0 and 5 μM IBA. In addition, rooting of some genotypes may benefit from testing NAA at concentrations below 5 μM.

Nutrient requirements and substrates are important factors for root formation by *Fraxinus* spp. White ash, common ash, and flowering ash exhibit optimal rooting on agar-solidified MS (Navarrete et al. 1989); agar-solidified, half-strength WPM (Hammatt and Ridout 1992); and liquid Rugini media (Arrillaga et al. 1992), respectively. In a rooting experiment with these three clones of green ash, agar-solidified MSB5 medium minimally supported root development (Figure 2) and the formation of short roots accompanied by callus tissue (data not shown). Rooting plugs supplied with liquid MSB5 medium provided a more favorable substrate for root development by the three clones (Figure 2). Improved rooting with the use of rooting plugs is perhaps attributable to improved aeration, which is strongly enhanced by this procedure. Aeration was previously shown to play an important role in raspberry root development using foam substrates (Gebhart 1985). Rooting plugs with liquid medium may provide a suitable balance between aeration and moisture availability (humidity) at the base of the shoot-tip explant, and thereby promote root initiation and development.

Differences among *in vitro* rooting responses of three clones are perhaps related to differences in endogenous auxin or cytokinin levels. Control over the type of organ produced in culture is governed by the balance of exogenous and endogenous growth regulators (Christianson and Warnick 1988). Cells of the same plant can have different endogenous levels of plant growth regulators, as well as variation in transport and sensitivity to plant growth regulators (Minocha 1987). It is reasonable to postulate that responses to exogenous or endogenous auxins also will vary with clone. Further work toward understanding clonal differences in rooting responses may also provide progress toward improving rooting efficiencies of recalcitrant genotypes.

Because the explant size, environmental culture conditions, and auxin treatments were quite different, direct comparisons of root development between *in vitro* and *ex vitro* rooting studies are difficult. The *ex vitro* rooting

method, however, resulted in favorable root development and shoot growth without producing the callus and brittle roots observed under *in vitro* rooting conditions (Figure 1; Figure 3). In a previous study on white ash, Bates et al. (1992) observed that a quick dip in 1 mM IBA produced approximately 80% *ex vitro* rooting of micropropagated shoots. The three clones of green ash responded similarly in this study with maximal root formation, favorable root development, and suitable shoot growth also occurring when explants were dipped in 1 mM IBA (Figure 1D; Figure 3).

Growth regulator and nutritional fluctuations are necessary to stimulate the proper developmental sequences of tissue cultured plantlets from bud initiation through stem elongation, and finally rooting (Mott and Amerson 1981). Environmental factors such as light quality, photoperiod, CO_2 concentration, O_2 concentration, pH, osmotic potential, relative humidity, temperature, plant growth regulators, and nutrients can have dramatic effects on rooting processes and are relatively well understood, at least in terms of modifying root production (Haissig et al. 1992).

Root formation is also influenced by genotype and environmental interactions (Davis et al. 1988). In contrast to environmental influences, genotypic variability in rooting potential is less well understood. The three clones of green ash used in the present study exhibited variable root development depending on environmental factors. For example, clone SD1009 exhibited the lowest root development under *in vitro* conditions, whereas it displayed the highest rooting response under *ex vitro* conditions.

The techniques described here to induce a high frequency of roots of satisfactory quality in these seed-derived clones has potential application for green ash propagation; however, these techniques have not yet been applied to mature trees. The production of clonal plant materials is especially useful for studies to determine the genetic versus environmental bases of observed phenotypic variation in a given trait. Similar *in vitro* and *ex vitro* rooting procedures could possibly be applied to other species that are normally regarded as difficult-to-propagate.

Acknowledgements

This research was supported by a Cooperative Research Agreement (No. 28-C3-737) to the Department of Forestry, Fisheries, and Wildlife, University of Nebraska-Lincoln from the USDA Forest Service, Rocky Mountain Research Station, National Agroforestry Center. Additional research funding was provided by the University of Nebraska Center for Biotechnology Research Associateship Program and the Landscape Plant Development Center, Chanhassen, MN, USA. The authors thank Drs. Carol M. Schumann and Jianwei

Zhang for providing valuable comments, Mr. Jimmy Martinez for the photography, Mr. Richard Carman for technical assistance, and the USDA Forest Service, Rocky Mountain Research Station Biometrics Unit (Rudy King and Jill Williams) for statistical review. Use of trade names in this paper does not constitute endorsement by the USDA Forest Service.

References

Arrillaga, I., Lerman, V. and Segura, J. 1992. Micropropagation of juvenile and adult flowering ash. Jour. Amer. Soc. Hort. Sci. 117: 346–350.

Bates, S., Preece, J.E., Navarrete, N.E., Van Sambeek, J.W. and Gaffney, G.R. 1992. Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white ash (*Fraxinus americana* L.). Plant Cell Tiss. Org. Cult. 31: 21–29.

Bradshaw, H.D., Jr. and Stettler, R.F. 1995. Molecular genetics of growth and development in *Populus*. IV. Mapping QTLs with large effects on growth, form, and phenology traits in a forest tree. Genetics 139: 963–973.

Christianson, M.L. and Warnick, D.A. 1988. Organogenesis *in vitro* as a developmental process. HortSci. 23: 515–519.

Davis, T.D., Haissig, B.E. and Sankhla, N. 1988. Adventitious Root Formation by Cuttings. Advances in Plant Sciences Series, Vol. 2. Dioscorides Press, Portland, OR. 315 p.

Gamborg, O.L., Miller, R.A. and Ojama, K. 1968. Nutrient requirements of suspension cultures of soybean roots cells. Exp. Cell Res. 50: 151–158.

Gebhart, K. 1985. Development of a sterile cultivation system for rooting of shoot tip cultures (red raspberries) in duroplast foam. Plant Sci. 39: 141–148.

Haissig, B.E., Nelson, N.D. and Kidd, G.H. 1987. Trends in the use of tissue culture in forest improvement. Bio/Technology 5: 52–56, 59.

Haissig, B.E., Davis, T.D. and Riemenschneider, D.E. 1992. Researching the control of adventitious rooting. Physiol. Plant. 84: 310–317.

Hammatt, N. and Ridout, M.S. 1992. Micropropagation of common ash (*Fraxinus excelsior*). Plant Cell Tiss. Org. Cult. 13: 67–74.

Kennedy, H.E., Jr. 1990. Green ash (*Fraxinus pennsyvanica* Marsh.), pp. 348–354. In: Burns, R.M. and Honkala, B.H. (Tech. coords.) Silvics of North America: 2. Hardwoods. Agriculture Handbook 654. U.S. Department of Agriculture, Forest Service, Washington, DC.

Kim, M.-S., Schumann, C.M., Cregg, B.M. and Klopfenstein, N.B. 1994. Micropropagation and regeneration systems of green ash (*Fraxinus pennsylvanica* Marsh), Abstract, p. 74. In: Michler, C.H., Michael, R.B., Cullen, D., Nance, W.N., Sederoff, R.R. and Slavicek, J.M. (Eds.) Applications of Biotechnology to Tree Culture, Protection, and Utilization. USDA Forest Service, North Central Forest Experiment Station, Gen. Tech. Rep. NC-175.

Kim, M.-S., Schumann, C.M. and Klopfenstein, N.B. 1997. Effects of benzyladenine and thidiazuron on axillary shoot multiplication of three green ash (*Fraxinus pennsylvanica* Marsh) clones. Plant Cell Tiss. Org. Cult. 48(1): 45–52.

Kim, M.-S. 1995. Micropropagation of Green Ash (*Fraxinus pennsylvanica* Marsh.): A comparative Study of Three Genotypes. M.S. thesis, University of Nebraska, Lincoln. 98 p.

Libby, W.J. and Ahuja, M.R. 1993. Micropropagation and clonal options in forestry, pp. 425–442. In: Ahuja, M.R. (Ed.) Micropropagation of Woody Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Lloyd, G. and McCown, B. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latitulia*, by use of shoot-tip culture. Comb. Proc. Int. Plant Prop. Soc. 30: 421–427.

Minocha, S.C. 1987. Plant growth regulators and morphogenesis in cell and tissue culture of forest trees, pp. 50–60. In: Bonga, J.M. and Durzan, D.J. (Eds.) Cell and Tissue Culture in Forestry, Vol. I. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Mott, R.L. and Amerson, H.V. 1981. A Tissue Culture Process for the Clonal Production of Loblolly Pine Plantlets. N. C. Agr. Res. Serv. Tech. Bull. No. 271, pp. 1–14.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497.

Navarrete, N.E., Van Sambeek, J.W., Preece, J.E. and Gaffney, G.R. 1989. Improved micropropagation of white ash (*Fraxinus americana* L.), pp. 146–149. In: Rink, G. and Budelsky, C.A. (Eds.) Proceedings of the Seventh Central Hardwood Conference, March 5–8. Carbondale, IL.

Newcombe, G. and Bradshaw, H.D., Jr. 1996. Quantitative trait loci conferring resistance in hybrid poplar to *Septoria populicola*, the cause of leaf spot. Can. J. For. Res. 26: 1943–1950.

Newcombe, G., Bradshaw, H.D., Jr., Chastagner, G.A. and Stettler, R.F. 1996. A major gene for resistance to *Melampsora medusae* f. sp. *deltoidae* in a hybrid poplar pedigree. Phytopathology 86: 87–94.

Preece, J.E., Christ, P.H., Ensenberger, L. and Zhao, J. 1987. Micropropagation of ash (*Fraxinus*). Comb. Proc. Intl. Plant Prop. Soc. 37: 366–372.

Rugini, E. 1986. Olive (*Olea europaea* L.), pp. 253–267. In: Bajaj, Y.P.S. (Ed.) Biotechnology in Agriculture and Forestry. Vol. 1. Trees I. Springer Verlag, Berlin, Heidelberg.

Solomon, J.D., Leininger, T.D., Wilson, A.D., Anderson, R.L., Thompson, L.C. and McCracken, F.I. 1993. Ash Pests: A Guide to Major Insects, Diseases, Air Pollution Injury, and Chemical Injury. USDA Forest Service, Gen. Tech. Rep. SO-96, New Orleans, LA. 45 p.

SAS Institute. 1996. SAS/STAT User's Guide. 6.11 Edition. SAS Institute, Inc., Cary, NC.

Steiner, K.C., Williams, M.W., DeHayes, D.H., Hall, R.B., Eckert, R.T., Bagley, W.T., Lemmien, W.A., Karnosky, D.F., Carter, K.K. and Cech, F.C. 1988. Juvenile performance in a range-wide provenance test of *Fraxinus pennsylvanica* Marsh. Silvae Genet. 37: 104–111.