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Effects of thidiazuron and benzyladenine on axillary shoot proliferation of three green ash (*Fraxinus pennsylvanica* Marsh.) clones

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Abstract

Mature seeds of three green ash (*Fraxinus pennsylvanica* Marsh.) clones, SD1009 (South Dakota origin), SD2002 (South Dakota origin), and KA2018 (Kansas origin) were cut to remove the apical portion and germinated on Murashige and Skoog (1962) salts with B5 vitamins (Gamborg et al., 1968) (MSB5) medium without plant growth regulators. Stable axillary shoot establishment was achieved for all three clones by subculture on MSB5 medium containing a combination of 5 μM thidiazuron (TDZ), 5 μM 6-benzyladenine (BA), and 1 μM indole-3-butyric acid (IBA). Following shoot establishment, axillary shoots were placed on MSB5 medium containing a single treatment of TDZ (1, 5, 10, 20, or 40 μM) or BA (1, 5, 10, 20, 40, or 80 μM). Concentration of TDZ and BA significantly affected shoot biomass (total dry weight of axillary shoots), with 10 μm TDZ or 40 μm BA providing maximum shoot proliferation with all three clones. Significant clonal differences also were noted in the proliferation of axillary shoots, with clone SD1009 exhibiting the highest axillary shoot proliferation. Axillary shoots were rooted under *ex vitro* conditions and acclimatized to the greenhouse.

Abbreviations: BA - 6-benzyladenine; IBA - indole-3-butyric acid; MSB5 - Murashige and Skoog (1962) salts with B5 vitamins (Gamborg et al., 1968); TDZ - thidiazuron

Introduction

Green ash (*Fraxinus pennsylvanica* Marsh.), a deciduous broad-leaved tree, grows widely across North America, especially in riparian zones. Because the species can tolerate a range of environmental conditions, it is one of the most successful and commonly planted broad-leaved trees in the North American Great Plains (Solomon et al., 1993). Although its timber is used in many products such as tool handles and baseball bats, it serves primarily for environmental protection such as in shelterbelts, wildlife plantings, riparian buffer strips, and strip-mine revegetation (Solomon et al., 1993).

Little practical experience has been acquired in propagating *Fraxinus* clones for conservation forestry, and most ornamental forms have been cloned by grafting onto seedling rootstocks (Hammatt and Ridout, 1992). Micropropagation could provide a 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). Previous experience with tissue culture of *Fraxinus* species is limited, although plantlets have been obtained from surface-disinfested buds of white ash (*F. americana* L.) seedlings (Navarrete et al., 1989; Preece et al., 1987). This process has been more recently used for common ash (*F. excelsior* L.; Hammatt, 1994; Hammatt and Ridout, 1992) and flowering ash (*F. ornus* L.; Arrillaga et al., 1992). Plantlets also have been obtained from somatic embryogenesis using cut-

seed explants of white ash (Bates et al., 1992; Preece et al., 1989).

The objectives of this research were to establish clonal materials of selected green ash genotypes and determine suitable plant growth regulator type and concentration for *in vitro* shoot proliferation of green ash seedlings.

Materials and methods

Plant material

Mature green ash samaras were collected from 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.

Culture conditions

Culture media contained Murashige and Skoog (1962) salts with B5 vitamins (Gamborg et al., 1968) (MSB5), 3% sucrose, and 0.7% agar (Difco Bacto). Before autoclaving, plant growth regulators (BA, IBA, and/or TDZ) were added and pH was adjusted to 5.8 with 1N NaOH or HCl. Culture tubes (25×150 mm) contained 15 ml medium, and Magenta GA-7 culture vessels (76×76×102 mm, Magenta Corp., Chicago, IL; termed 'culture vessel') contained 60 ml medium. After capping with autoclavable lids, medium was autoclaved at 121 °C and 1.2 kg cm^{-2} for 20 min. Subcultures were made every 5 weeks. Cultures were incubated at 24±2 °C under a 16-h photoperiod with light provided by 40-watt, cool-white, fluorescent lamps at a photosynthetic photon flux of 35 to 47 μmol $m^{-2}s^{-1}$.

Culture establishment

The pericarps were removed and the apical one quarter (containing the cotyledon tips) of each seed were 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 of the apical portion (ca. 2 mm) (hereafter termed 'cut seed'). The embryo-containing cut seeds were placed in culture tubes on MSB5 medium without plant growth regulators. Three weeks after germination, radicles were removed from seedlings, and explants were placed horizontally within culture vessels on MSB5 medium with a combination of 10 μM TDZ and 10 μM BA (Kim et al., 1994). After two subcultures on the same medium, induced axillary shoot tips were transferred to MSB5 medium containing a combination of 5 μM TDZ, 5 μM BA, and 1 μM IBA (Kim, 1995).

After six subcultures, three different clones (designated SD1009 from SD1; SD2002 from SD2; and KA2018 from KA2) were sufficiently established to provide clonal explant sources for shoot proliferation studies.

Shoot proliferation

TDZ effect - Experiment I

Axillary shoots (ca. 25 mm long) were placed within culture vessels on MSB5 medium with a 3×5 factorial arrangement of clones (SD1009, SD2002, or KA2018) and TDZ (1, 5, 10, 20, or 40 μM).

BA effect - Experiment II

Axillary shoots (ca. 25 mm long) were placed within culture vessels on MSB5 medium with a 3×6 factorial arrangement of clones (SD1009, SD2002, or KA2018) and BA (1, 5, 10, 20, 40, or 80 μM).

Statistical analysis

Experiments I and II

After 5-weeks growth *in vitro*, data were collected on axillary shoot (more than 10 mm long) number and shoot biomass (mg; total dry weight of axillary shoots per culture vessel). Experiments were conducted three times with a total of nine replications for each clone-TDZ combination (15 combinations) or clone-BA combination (18 combinations) treatment using a completely randomized design. A replication consisted of four axillary shoots in a culture vessel. The mean number of axillary shoots per culture vessel, mean dry weight per axillary shoot, and mean shoot biomass per culture vessel were analyzed as test variables for both experiments. Before drying for shoot biomass measurement, axillary shoots were excised from initial explants and any associated callus was excluded. Dry weight per axillary shoot is the shoot biomass per culture vessel divided by the number of axillary shoots per culture vessel. Analysis of variance of the treatment means was performed using the General Linear Model (GLM) procedure (SAS, 1996) to account for unequal sample sizes. If applied TDZ or BA concen-

trations had significant effects on test variables, then contrasts were estimated for linear and quadratic components of either TDZ or BA effects. Because TDZ or BA treatment concentrations were not increased at constant increments, coefficients for testing the contrasts of linear and quadratic components of TDZ or BA effects were measured using the Interactive Matrix Language (IML) procedure (SAS, 1985). The response of each genotype was examined at each of the five TDZ or six BA levels using Tukey's Honestly Significant Difference Test (α = 0.05).

Results

Germination

Germination percentage and epicotyl length were significantly different among seed sources ($p < 0.01$) on MSB5 medium without plant growth regulators. KA2 and SD2 seed sources had higher germination percentages than did SD1, and KA2 seed source produced longer epicotyls than SD1 and SD2 (Table 1).

Shoot proliferation

TDZ effect

Significant differences occurred among clones ($p< 0.01$, df = 2) and TDZ ($p< 0.01$, df = 4) treatments for mean shoot biomass per culture vessel; however, data did not fit the regression models tested (Table 2). The highest shoot biomass was obtained on 10 μM TDZ for all clones (Table 2). Significant interaction occurred between clone and TDZ concentration ($p< 0.05$, df = 8) for shoot biomass. Means of the 15 TDZ-clone combinations showed that clone SD1009 produced as much or more shoot biomass than clone SD2002 or KA2018 at all TDZ concentrations (Table 2).

Significant differences occurred in the number of axillary shoots ($p< 0.01$, df = 4) and dry weight per axillary shoot ($p< 0.01$, df = 4) in response to different TDZ concentrations. Significant clonal differences were also observed for axillary shoot number ($p< 0.01$, df = 2) and dry weight per axillary shoot ($p< 0.01$, df = 2). Significant interactions were evident between clone and TDZ concentration for number of axillary shoots ($p< 0.01$, df = 8) and dry weight per axillary shoot ($p< 0.01$, df = 8). The most axillary shoots were stimulated by TDZ concentrations of 10 μM (SD1009 and SD2002) or 40 μM (KA2018) (Table 3). The number of axillary shoots and dry weight per axillary shoot showed a linear response for clone SD2002 (Tables 3, 4). For clone KA2018, the number of axillary shoots was best described by a quadratic model (Table 3). The greatest dry weight per axillary shoot occurred when shoot explants were cultured on TDZ concentrations of 1 μM (SD1009) or 5 μM (SD2002 and KA2018) (Table 4). Higher concentrations (10 μM or more) of TDZ resulted in reduced dry weight of individual axillary shoots for all three clones (Table 4).

Shoot biomass was significantly different among clones in response to 1, 10, or 20 μM TDZ ($p < 0.01$, df = 2) (Table 2). Axillary shoot number was significantly different among clones in response to 5, 10, or 20 μM TDZ ($p < 0.01$, df = 2) (Table 3). Dry weight of individual axillary shoots was significantly affected by clone only in response to 1 μM TDZ ($p < 0.01$, df = 2) (Table 4).

BA effect

A significant difference occurred among clones ($p< 0.01$, df =2) and BA ($p< 0.01$, df = 5) treatment for mean shoot biomass per culture vessel. Shoot biomass showed a quadratic response with the maximal point at 40 μM BA for all three clones, but the rate of increase varied among clones (Table 2). No significant interaction occurred between clone and BA concentration ($p = 0.2770$, df = 10).

Significant differences occurred in the number of axillary shoots ($p< 0.01$, df = 5) and dry weight per axillary shoot ($p< 0.01$, df = 5) in response to different BA concentrations. Significant clonal differences were also observed for axillary shoot number ($p< 0.01$, df = 2) and dry weight per axillary shoot ($p< 0.01$, df =2). Significant interactions occurred between clone and BA concentration for both number of axillary shoots ($p< 0.01$, df = 10) and dry weight per axillary shoot ($p< 0.05$, df = 10). The number of axillary shoots gave quadratic and linear responses for clone SD2002 and KA2018, respectively (Table 3). BA concentration affected dry weight per axillary shoot at linear level for both clone SD1009 and SD2002 (Table 4). The most axillary shoots were stimulated by BA concentrations of 40 μM (SD1009) or 80 μM (SD2002 and KA2018) (Table 3). The greatest dry weight per axillary shoot occurred when shoot explants were cultured on BA concentrations of 5 μM (SD2002 and KA2018) or 10 μM (SD1009) (Table 4).

Shoot biomass was significantly different among clones in response to 5, 20, 40, or 80 μM BA ($p<$

Table 1. Germination and epicotyl length of three green ash genotypes after 3 weeks on MSB5 medium without plant growth regulators[1]

Seed source	Germination (%)	Epicotyl length (mm)
SD1	92	24.9 (8.2)
SD2	100	24.7 (7.4)
KA2	100	36.6 (9.6)

[1]Each number represents the mean (and standard error of the mean) of 17-36 seed explants.

Table 2. Effect of TDZ and BA concentrations on mean of shoot biomass per culture vessel of three green ash clones after 5-weeks culture on MSB5 medium[1]

Clone treatment[2]		Mean of shoot biomass per culture vessel (mg)		
		SD1009	SD2002	KA2018
TDZ (μM)	1	75.8 (11.9)a[3]	62.9 (9.4)a	25.0 (6.0)b
	5	103.3 (7.3)	81.4 (17.0)	75.6 (10.7)
	10	157.8 (13.9)a	85.0 (15.5)b	78.6 (17.9)b
	20	90.0 (9.8)a	34.7 (9.8)b	62.2 (10.9)ab
	40	53.3 (14.7)	36.4 (4.8)	43.8 (5.7)
Contrast[4]		NS	NS	NS
BA (μM)	1	50.0 (20.9)	52.5 (8.4)	13.8 (3.8)
	5	67.8 (8.0)a	85.7 (4.8)a	38.8 (8.8)b
	10	84.4 (16.8)	86.4 (11.4)	46.3 (10.3)
	20	131.5 (12.7)a	93.9 (12.4)ab	71.1 (9.3)b
	40	151.3 (14.5)a	129.4 (21.7)ab	81.5(12.3)b
	80	142.2 (14.6)a	88.0 (20.7)b	71.1 (6.8)b
Contrast[4]		Q**	Q**	Q**

[1]Each number represents the mean (and standard error of the mean) of 9 replications.
[2]Separate experiments were conducted for clone by TDZ and BA concentrations.
[3]Data within a row among clones followed by same letters are not significantly different according to Tukey's Honestly Significant Difference Test ($\alpha = 0.05$).
[4]Q = quadratic, NS = not significant.
**Significant at the $\alpha = 0.01$ level.

0.05, df = 2) (Table 2). Axillary shoot number was significantly different among clones in response to 10 or 40 μM BA ($p < 0.01$, df = 2) (Table 3). Dry weight of individual axillary shoots was significantly affected by clone in response to 10 or 80 μM BA ($p < 0.05$, df = 2).

Discussion

Preece et al. (1995) reported that *in vitro* seed germination can eliminate the need to overcome dormancy, and provide high seed germination and growth from immature and mature seeds of several ash genotypes. That technique also was quite effective for axenic germination of the three seed sources used in this study.

Previous research on *Fraxinus* species has demonstrated that TDZ and BA are effective plant growth regulators for axillary shoot proliferation. Navarrete et al. (1989) observed that 3 or 10 μM TDZ stimulated axillary shoot proliferation in white ash. They also showed that optimal axillary shoot proliferation with adequate shoot elongation rates occurred when 3 μM TDZ, 1 μM BA, and 1 μM IBA were incorporated

Table 3. Effect of TDZ and BA concentrations on mean number of axillary shoots per culture vessel of three green ash clones after 5-weeks culture on MSB5 medium[1].

Clone treatment[2]		Mean number of axillary shoots per culture vessel		
		SD1009	SD2002	KA2018
TDZ (μM)	1	2.9 (0.5)	3.8 (0.3)	2.8 (0.5)
	5	8.3 (0.9)a[3]	4.1 (0.3)b	4.8 (0.6)b
	10	10.0 (1.1)a	5.8 (0.5)b	7.0 (0.6)b
	20	8.9 (0.9)a	5.0 (0.7)ab	7.2 (0.7)b
	40	5.4 (1.0)	5.7 (0.5)	8.3 (0.8)
Contrast[4]		NS	L*	Q**
BA (μM)	1	1.9 (0.6)	3.8 (0.6)	1.9 (0.5)
	5	2.8 (0.4)	4.4 (0.6)	2.5 (0.6)
	10	2.2 (0.5)b	5.4 (0.5)a	4.0 (0.4)a
	20	7.1 (0.8)	6.7 (0.6)	5.7 (0.7)
	40	14.3 (1.1)a	9.4 (1.1)b	6.4 (0.8)b
	80	10.9 (0.7)	9.8 (0.8)	10.7 (0.8)
Contrast[4]		NS	Q**	L**

[1]Each number represents the mean (and standard error of the mean) of 9 replications.
[2]Separate experiments were conducted for clone by TDZ and BA concentrations.
[3]Data within a row among clones followed by same letters are not significantly different according to Tukey's Honestly Significant Difference Test (α = 0.05).
[4]L = Linear, Q = quadratic, NS = not significant.
*Significant at the α = 0.05 level.
**Significant at the α = 0.01 level.

into MS medium. Using shoot-tip and nodal explants of common ash, Hammatt and Ridout (1992) obtained axillary shoot proliferation on Driver and Kuniyuki (DKW) medium (Driver and Kuniyuki, 1984) containing 22.2 μM BA. With flowering ash, Arrillaga et al. (1992) obtained *in vitro* shoot proliferation using a combination of 22.2 or 44.4 μM BA with 0.5 μM NAA incorporated into Heller medium (HM; Heller, 1953). Under the conditions of this study, all three clones of green ash produced maximal axillary shoot biomass on MSB5 medium with 10 μM TDZ or 40 μM BA (Table 2). Shoot biomass is a determinant of both axillary shoot number and dry weight, thus it serves as a more accurate indicator of effective shoot proliferation than axillary shoot number alone.

Nutrient requirements are an important consideration for *in vitro* culture of *Fraxinus*. Earlier work has shown that various *Fraxinus* species may possess unique nutritional requirements, as white ash, common ash, and flowering ash exhibit suitable axillary shoot proliferation on MS, DKW, and HM media, respectively (Arrillaga et al., 1992; Hammatt and Ridout, 1992; Navarrete et al., 1989). Using shoot tips of white ash, Preece et al. (1987) showed that consistent axillary shoot proliferation was induced by 22 or 45 μM BA in Woody Plant Medium (Lloyd and McCown, 1980). These BA levels, however, also resulted in extensive callus formation. Callus production is frequently an essential step in the regeneration of adventitious organs, yet growth of callus is believed to inhibit axillary shoot proliferation in woody plant tissue culture (Huetteman and Preece, 1993). Hammatt and Ridout (1992) observed that 22.2 or 44.4 μM BA in MS medium caused a high proportion of shoot mortality in common ash. In contrast to the seed sources of white ash used by Preece et al. (1987), callus did not adversely affect shoot proliferation at similar or higher levels of BA (20, 40, or 80 μM) with green ash genotypes in this study. And, shoot mortality observed by Hammatt and Ridout (1992) was not evident in any of the three green ash clones tested. Because optimal axillary shoot proliferation of *Fraxinus* likely depends on species-specific physiological conditions and nutritional requirements, additional studies are needed to

Table 4. Effect of TDZ and BA concentrations on mean dry weight per axillary shoot of three green ash clones after 5-weeks culture on MSB5 medium[1].

Clone treatment[2]		Mean dry weight per axillary shoot (mg)		
		SD1009	SD2002	KA2018
TDZ (μM)	1	31.1 (4.9)a[3]	16.1 (1.5)b	8.9 (1.6)b
	5	14.2 (2.2)	18.6 (2.4)	19.5 (5.1)
	10	16.6 (1.5)	14.6 (2.5)	10.7 (2.1)
	20	10.3 (0.8)	6.6 (1.3)	9.1 (1.7)
	40	10.5 (2.2)	6.4 (0.6)	5.7 (1.0)
Contrast[4]		NS	L**	NS
BA (μM)	1	22.2 (10.4)	14.8 (1.4)	5.6 (1.5)
	5	27.5 (4.7)	22.8 (3.2)	18.8 (4.5)
	10	45.8 (13.9)a	15.7 (1.1)ab	11.0 (2.0)b
	20	21.1 (3.7)	14.6 (2.4)	13.1 (1.8)
	40	11.1 (1.3)	15.1 (3.5)	13.7 (2.5)
	80	13.3 (1.3)a	9.0 (1.8)ab	7.2 (1.1)b
Contrast[4]		L*	L**	NS

[1]Each number represents the mean (and standard error of the mean) of 9 replications.
[2]Separate experiments were conducted for clone by TDZ and BA concentrations.
[3]Data within a row among clones followed by same letters are not significantly different according to Tukey's Honestly Significant Difference Test (α = 0.05).
[4]L = Linear, NS = not significant.
*Significant at the α = 0.05 level.
**Significant at the α = 0.01 level.

optimize nutrition and plant growth regulators for specific genotypes.

In this study, shoots arising from node-associated callus in the medium at the explant base were termed 'short shoots' (basal shoots less than 10 mm long), and were not counted as axillary shoots because it was difficult to distinguish whether short shoots were of axillary or adventitious origin. Nevertheless, short- shoot numbers appeared to increase with increasing TDZ and BA test concentrations for all three clones (data not shown). One objective of this study was to establish clonal materials of specific genotypes. Axillary shoot material, which is easier to handle, was more useful than short shoots for subsequent rooting and plantlet establishment. Because adventitious shoots may have an increased frequency of somaclonal variation (Huetteman and Preece, 1993), the clonal fidelity of short shoots is more questionable than that of axillary shoots.

TDZ is one of several substituted ureas such as N-N′-diphenylurea (Mok et al., 1980) and N-(2-chloro-4-pyridyl)-N′-phenylurea (Fellman et al., 1987) that have been investigated for cytokinin activity (Huetteman and Preece, 1993; Lu, 1993). Compared to most other compounds with cytokinin activity, lower concentrations of TDZ can stimulate axillary shoot proliferation in many woody plants, whereas higher TDZ concentrations may result in the formation of both axillary and adventitious shoots (Chalupa, 1988; van Nieuwkerk et al., 1986). Therefore, direct comparisons between TDZ and the other amino purine cytokinins at equimolar concentrations are difficult to analyze statistically (Huetteman and Preece, 1993). High rates of shoot proliferation, often desirable for efficient micropropagation, may include both axillary and adventitious shoots. If clonal fidelity is desired, however, TDZ or other cytokinins must be used at levels that stimulate only axillary shoot growth, thereby avoiding potential somaclonal variants derived from adventitious shoots. Under the conditions of this study, critical TDZ or BA levels for stable shoot proliferation were 10 μM TDZ or 40 μM BA, respectively. Levels above 10 μM TDZ or 40 μM BA perhaps would be useful to elicit somaclonal variation if desired.

The clone often profoundly affects explant performance. Using shoot explants cultured on medium containing TDZ, Preece et al. (1991) reported that significant differences in axillary shoot proliferation were apparent among silver maple clones within a provenance. Under the conditions of this study, the clone also significantly influenced axillary shoot proliferation. Clonal differences in shoot biomass were most apparent between clones SD1009 and KA2018 (Table 2). Because cells within the same plant can have different endogenous levels of plant growth regulators and additional variation in receptor affinity or cellular sensitivity to plant growth regulators (Minocha, 1987), it is reasonable to expect that *in vitro* responses will vary with clone. More studies on diverse genotypes are required to further characterize genotypic variation of green ash responses to *in vitro* conditions.

In this study, clone SD1009 exhibited the highest *in vitro* response for axillary shoot proliferation. This trend continued with subsequent root formation and plantlet establishment (data not shown). These results suggest that clone SD1009 has further potential to serve as clonal plant material for *in vitro* studies of pest and stress resistance of green ash. The studies described in this paper will provide useful techniques for the micropropagation of other clones of green ash, including selected mature trees.

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