MICROPROPAGATION OF JUVENILE AND MATURE AMERICAN ELMS FROM STEM NODAL SECTIONS

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Abstract: A micropropagation system has been established for the regeneration of superior trees of *Ulmus americana* L., the American elm. The development of a reliable regeneration system is also required as a prelude to genetic transformation of selected genotypes. The effects of explant source, media formulations, and plant growth regulator concentrations on shoot regeneration of juvenile and mature American elm selections were examined. Hypocotyls, leaves, and stems taken from seedlings and stem segments taken from root cuttings of a mature tree, selection #8630, were used. Media formulations included Murashige and Skoog (MS) medium, Driver and Kuniyuki Walnut (DKW) Medium, and McCown's Woody Plant Medium (WPM). Growth regulators included benzyladenine (BA) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron or TDZ). Hypocotyls and nodal stem sections were cut and cultured on MS supplemented with either 2.2 or 4.4 μM BA. Leaves were wounded and cultured on either MS, half strength MS, DKW, or WPM containing TDZ at concentrations ranging from 0.01 to 22.5 μM. Nodal stem sections from the shoots of seedlings and mature trees and regenerated shoots within 4 weeks on both BA concentrations. The BA concentration used had a significant effect on the response of the explants. The number of stem explants proliferating shoots as well as the number of new shoots generated was larger on 2.2 μM BA than on 4.4μM BA. The 4.4μM BA concentration decreased both the quantity and quality of new shoots regenerated. Shoots were readily rooted ex vitro in soilless medium with a Rootone dip. We conclude that micropropagation and tree regeneration can be achieved from both mature American elms and from juvenile seedlings.

INTRODUCTION

Ulmus americana L., the American elm, was a common tree in the urban landscape and hardwood forest across its native North American range until the introduction of Dutch elm disease (DED). DED, caused by *Ophiostoma ulmi* (Buisman), has killed millions of trees. Since American elm seedlings have a pseudo resistance to DED in the juvenile phase, DED tolerance can only be reliably tested on mature trees. Townsend and others (1995) have recently released two DED tolerant American elms, 'New Harmony' and 'Valley Forge'. These elite trees must now be propagated for the nursery industry. A wide array of traditional methods can be used to propagate selected clones, but these techniques are difficult and often unreliable for mature trees of most hardwood species, including the American elm. Poor adventitious rooting and/or slow shoot growth are major limitations in regenerating propagules from older trees (Brand and Lineberger 1992).

A clonal propagation method with American elms is also essential for targeted DNA transformations using either *Agrobacterium*-mediated or direct DNA transfer. DNA transformation is an important approach for elm improvement because of the genetic and reproductive barriers that prevent the creation of disease-resistant trees by conventional plant breeding methods (Ager and Guries 1982). In this case, juvenility or maturity would not be a consideration, but the technical ease of successful propagation would be essential due to the relatively low fraction of genetically transformed cells produced. Until now, the only successful regeneration protocols for American elm have utilized seedling tissue with hypocotyl tissue having the highest regeneration potential. Shoot regeneration was reported from hypocotyl-derived cell suspension cultures (Durzan and Lopushanski 1975); from hypocotyl callus cultures (Karnosky and others 1982); and from cryogenically preserved hypocotyl callus (Ulrich and others 1984).

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Ho (1985) produced shoots from seedlings using epicotyl derived callus, but only a small number of shoots were recovered. George and Tripepi (1994) found that leaves had the potential to regenerate shoots, but the efficiency of regeneration was variable.

Micropropagation has been utilized to propagate a diverse group of mature woody plants including *Betula* (Brand and Lineberger 1992), *Larix x eurolepis* (Brassard and others 1996), *Malus* (Abbot and Whitely 1976), *Vitis viniferia* (Mullins and others 1979), and *Liquidambar styraciflua* (Sutter and Barker 1983). Micropropagation and shoot regeneration from tissues of mature trees have been reported for several other elm species but not for *U. americana*. Shoot multiplication from axillary buds was reported for *U. campestris* (Biondi and others 1984) and for *U. glabra* (Chalupa 1979). Lin and others (1981) were able to regenerate plantlets from a 10 year old specimen of *U. japonica*. In 1982, Chalupa demonstrated the use of root suckers as a source of explant material for the propagation of mature *U. campestri*. Proliferating shoot cultures were established from shoot tips of mature *U. procera* (Fenning and others 1993). Both Corchete and others (1993) and Cheng and Shi (1995) used nodal explants of a mature *U. pumila* to initiate shoot proliferating cultures.

The goal of this research was to develop a rapid and efficient micropropagation protocol for both seedling and mature genotypes of American elm. The data presented in this report, which include explant initiation efficiency, the mean number of shoots per primary explant, subsequent subculture regeneration, and rooting ability, indicate the feasibility of micropropagation of American elm.

MATERIALS AND METHODS

Plant Material And Explant Preparation

Cleaned and dewinged *U. americana* seed was obtained from Schumacher Seed Co., Sandwich, MA. and was stored dry in the dark at -20° C to maintain viability until needed. According to the supplier, the seed was collected from a number of trees, mixed before processing, and reported to have a 77% germination rate. Groups of 100 seeds were wrapped in bridal netting and surface sterilized for five minutes in a solution of 1.31% sodium hypochlorite and 0.5% Alconox detergent (Alconox, Inc., NY), followed by three rinses in sterile, distilled water. The seeds were germinated on slightly moistened sterile filter paper in plastic petri plates. Germination plates were sealed with plastic wrap and incubated in the dark. After 5 days, the seeds with protruding radicals were transferred to small glass jars containing 25 ml of the basal medium (described later) to complete germination.

For mature American elm propagation, root cuttings were taken from selection #8630 located at the Forest Science laboratory in Delaware, OH. This 36 year old specimen tree was first selected for elm yellows resistance (Schreiber, personal communication) and is in trials to determine DED tolerance levels. The root sections were taken in mid-November and prepared for cuttings. Root pieces were rinsed in tap water to remove soil and debris and were cut into sections 8 to 10 cm in length. Paying careful attention to polarity, the distal ends of the cuttings were dipped into a rooting hormone containing 0.3% indole-3-butyric acid (IBA), were placed in soilless media, and were maintained under culture room conditions (Schreiber 1963). Actively elongating shoot tips (2-6 cm in length) were harvested from the root cuttings, the leaf blades were removed, but the petioles were retained. The shoots were washed with a 2% Alconox solution and surface sterilized by gentle shaking in a 1.05% sodium hypochlorite and 0.5% Alconox solution for 15 minutes, then rinsed three times in sterile distilled water. Nodal segments were prepared from the shoots by trimming any damaged tissue, the petioles, and the shoot apex.

Culture Media

The basal medium consisted of Murashige and Skoog (MS) salts, (Murashige and Skoog 1962), 100 mg/l myo-inositol, 3% (w/v) sucrose, and 0.75% (w/v) Difco Bacto agar. The shoot initiation medium was basal medium supplemented with either 2.2 μM or 4.4 μM benzyladenine (BA). Additional media were used for shoot regeneration from leaves including: half strength MS salts, Driver and Kuniyuki Walnut (DKW) Medium (Driver and Kuniyuki 1984), and Woody Plant Medium (WPM) (Lloyd and McCown 1980). These were supplemented with myo-inositol, sucrose, and agar as outlined above. The plant growth regulator treatments consisted of thidiazuron

(TDZ) at 0.0, 0.01, 0.1, 1.0, 5.0, 10.0, 15.0, and 22.5 μM. Three different media were used for rooting: basal MS solidified with 0.8% (w/v) agar; MS with 1g/l activated charcoal; and MS with 2.5 μM IBA. Prior to autoclaving, the pH for the MS media was adjusted to 5.7, 5.5 for the DKW, and 5.3 for the WPM.

Shoot Proliferation

Cultures were maintained at 25° ± 2° C under cool white fluorescent lights with a 16 hour photoperiod. The regeneration capacity of 4 week old seedling leaves, hypocotyls, and nodal stem segments was tested, while the roots and cotyledons were discarded. In a preliminary study, the hypocotyl and nodal stem segments were placed on shoot initiation medium with either 2.2 or 4.4 μM BA at a density of one explant per glass jar containing 25 ml of medium. In subsequent seedling studies, 8 to 10 hypocotyl or stem segments were used per GA7 vessel (Magenta Corp., Chicago, IL) containing 50 ml of medium with 25 or 30 replicates per treatment.

Nodal segments from #8630 were placed horizontally at a density of two to four segments per GA7 vessel on MS with either 2.2 or 4.4 μM BA for shoot proliferation. The newly formed shoots were routinely subcultured on MS with 2.2 μM BA at a density of nine explants per GA7 vessel every 4 weeks for further shoot proliferation.

Using the George and Tripepi (1994) protocol, seedling leaves were taken from the fourth, fifth, sixth and seventh nodes and were wounded by cutting across the mid-vein and piercing the lamina. Leaves were cultured on one of the four different basal media with one of the seven different TDZ concentrations or a control without TDZ for a total of 32 treatment combinations. The leaves were plated abaxial side up at a density of four leaves per plate with 15 replicate plates per treatment. The plates were placed in complete darkness for 14 days and then moved to the culture shelf conditions described above. Explants were examined for 8 weeks for shoot regeneration.

Rooting And Plantlet Production

Healthy shoots approximately 4 cm in length with fully expanded leaves from both the seedling and #8630 cultures were placed in one of five rooting treatments. Rooting treatments consisted of three in vitro treatments on modified MS media described above and two ex vitro treatments. For the in vitro treatments, nine microcuttings per replicate per treatment were aseptically placed vertically in approximately one centimeter of media in a GA7 vessel. For the ex vitro treatments, microcuttings were washed under running water to remove any adhering medium. The basal end of the cutting was dipped in water and either placed into the Redi-Earth or dipped into Rootone (Rigo Co., Buckner, KY) and then placed into the Redi-Earth. The microcuttings were stuck in 3 inch by 5 inch aluminum trays with plastic lids with 25 microcuttings per tray.

The microcuttings were rooted under culture room conditions. After 4 weeks, the microcuttings were inspected and rooting percentages recorded. Acclimation of rooted microcuttings was completed by repotting the plantlets into cell packs containing soilless medium and placing them under intermittent mist (6 seconds mist every 6 minutes) and 60% light exclusion for 10 days followed by 14 days under 60% light exclusion without mist. The acclimated plants were maintained in the greenhouse under full sun following standard horticultural practices.

Data Analysis

Data were taken after 4 weeks on the number of explants that initiated shoot regeneration and the number of shoots per regenerating explant for both the seedling and #8630 experiments. The data were analyzed using the SAS General Linear Model (GLM) procedure (SAS Institute 1992) to assess treatment differences and shoot regeneration responses. The means and percentages were separated using both T-tests (LSD) and Duncan's multiple Range Test.

RESULTS

Shoot Proliferation From Seedling Explants

The differences in shoot proliferation response of the three explant sources were quite apparent. The preliminary experiment indicated that shoot proliferation, which could have resulted from axillary, adventitious, or both sources, was initiated from stem nodal segments but not from hypocotyl tissue. Further, explants on the lower BA concentration produced an average of 4.5 shoots per responding explant while those on the higher BA concentration produced 1.8 shoots per responding explant (data not shown). In the second experiment, both BA levels tested induced shoot proliferation on the stem explants (Table 1), but 2.2 μM BA was significantly better than 4.4 μM BA in both the percentage of responding explants and the mean number of new shoots per responding explant. Of the explants tested, 78.5% responded on the medium containing 2.2 μM BA while 39.0% responded to the medium containing 4.4 μM BA. Nodal segments exposed to 2.2 μM BA produced on average 2.3 shoots, while 1.7 shoots were produced on medium with 4.4 μM BA

Table 1. The effect of seedling explant source and BA concentration on shoot initiation and multiplication

Explant source	Culture medium	No. responding/ no. cultured	Percentage of explants responding	Mean no. of new shoots /responding explant ± SE
Hypocotyl	MS 2.2 μM BA	1/270	0.4	0
	MS 4.4 μM BA	0/252	0.0	0
Stem	MS 2.2 μM BA	204/258	78.5***	2.3 ± 0.6**
	MS 4.4 μM BA	71/184	39.0	1.7 ± 0.7

*** Percentages are significantly different at $\alpha = 0.001$.
** Mean numbers are significantly different at $\alpha = 0.01$

The results of the preliminary experiment indicated that the hypocotyl tissue responded poorly to the conditions used. When the experiment was repeated with a larger number of explants, the trends remained consistent. Only one out of the 540 hypocotyls tested produced any callus and it failed to undergo shoot organogenesis. The other hypocotyls darkened and senesced without further development.

In an effort to replicate the results of George and Tripepi (1994), seedling leaves were used as an explant source. Wounded leaves began to proliferate callus within 1 week of plating on all TDZ concentrations tested. During the 2 week dark period, friable and creamy white callus formed on the wound sites, along the midrib, and at the base of the petiole. After exposure to light, the callus began to darken and by the fifth week was dark brown and no growth was apparent. A second type of callus was sporadically observed on the explants plated on 10 and 15 μM TDZ concentrations. This callus was hard, green, and grew rapidly in the light where it quickly covered the softer callus. The various media formulations did not appear to affect callus formation. This protocol was repeated four separate times but unlike George and Tripepi, no shoot organogenesis was observed from the leaf tissue itself or from either type of callus.

Shoot Proliferation From #8630

While shoot proliferation was observed from #8630 explants cultured on both BA concentrations, the 2.2 μM BA was significantly better than 4.4 μM BA in both the percentage of responding explants and the mean number of new shoots per responding explant (Table 2). An overall average number of 3.9 shoots were produced per explant on 2.2

μM BA, while only 0.6 shoots were produced per explant on 4.4 μM BA. The shoots produced on medium containing 4.4 μM BA were smaller with shorter internodes and decreased leaf area.

The response of the nodal sections cultured from the initial cambium sprouts of #8630 root cuttings differed from the subsequent cuttings from the same root segments. While the percentage of explants proliferating shoots was not significantly different, the number of new shoots per explants was highly significant. Explants harvested 1/25/96 produced a range of 3-16 new shoots per responding explants on 2.2 μM BA while explants placed on 4.4 μM BA produced callus but did not proliferate shoots. Explants from the other harvest dates produced fewer shoots. The responses of the subcultured nodal sections of #8630 were similar to the those of the seedlings.

Table 2. The effect of BA concentration on shoot initiation and multiplication of #8630 explants

Culture medium	Date of initiation	Total no. of explants	Percentage of explants responding	Mean no. of new shoots/responding explant ± SE
MS 2.2 μM BA	1/25/96	17	47.6 a[1]	9.1 ± 4.1 b
	2/14/96	58	61.1 a	2.9 ± 2.0 c
	3/19/96	17	55.5 a	1.9 ± 1.7 c
	4/03/96	18	55.6 a	2.5 ± 1.5 c
Overall		110	55.7 *[2]	3.9 ± 3.6 **[3]
MS 4.4 μM BA	1/25/96	12	8.3	0.0
	2/14/96	28	50.0	0.8 ± 0.7
Overall		40	28.1	0.6 ± 0.7

[1] Means separation within a column followed by the same letter are not significantly different using Duncan's multiple range test at P = 0.05.
[2] Overall percentages are significantly different at α= 0.05.
[3] Overall mean numbers are significantly different at α = 0.01.

Repeated Subculture

The results of subculturing from seedling-derived and #8630-derived shoots are presented in Figure 1. The results were similar for both seedlings and #8630 shoots. An average of 3.0 new shoots was observed for every seedling nodal explant used, while an average of 3.6 new shoots was observed for every #8630 nodal explant used. There were no significant changes in the extent or quality of shoot production during continued subculture of the #8630-derived shoots.

For the seedling population, shoot proliferation seemed to improve with repeated subculture. The second passage of subculturing produced an average of 4.1 shoots per responding explant compared to an initial average of 2.3 shoots per responding explant. This may indicate a selection for plants that will grow better in culture. For the #8630, shoot proliferation did not appear to be affected by repeated subculture. An average of 3.5 new shoots developed for every explant used and this was consistently observed.

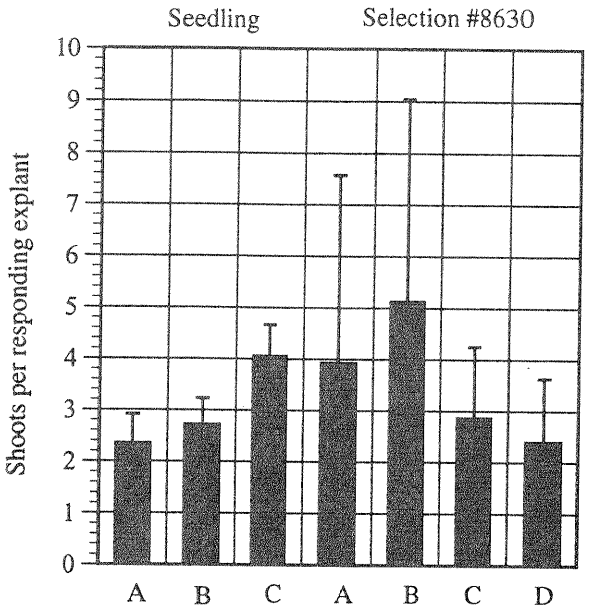

Figure 1. Comparison of shoot multiplication rates between the seedling population and #8630. Histograms indicate the mean number of shoots from initiation and subsequent subcultures on MS supplemented with 2.2 μM BA. Vertical lines indicate ± SE; **A:** initiation; **B:** first subculture; **C:** second subculture; **D:** third subculture.

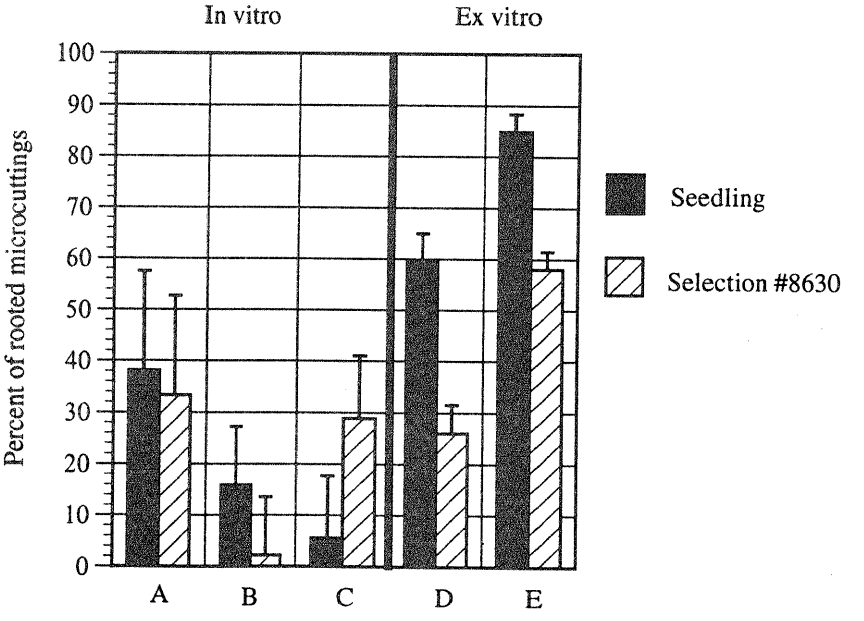

Figure 2. Comparison of microcuttings rooted from the seedling population and #8630. Histograms indicate the mean percentage of rooted microcuttings observed within 4 weeks of treatment. Vertical lines indicate ± SE; **A:** MS with 0.8% Agar; **B:** MS with 1 g/L activated charcoal; **C:** MS with 2.5 μM IBA; **D:** Redi-Earth without Rootone; **E:** Redi-Earth with Rootone.

Rooting And Plantlet Production

As illustrated in Figure 2, the most effective treatment for rooting American elm microcuttings was Redi-Earth with a Rootone dip. Within 2 weeks, leaf abscission and shoot death were observed on microcuttings of both #8630 and the seedling population placed on in vitro rooting medium with activated charcoal. A similar response was observed for the seedling microcuttings on medium containing 2.5 μM IBA. Rooting percentages were affected by the rooting treatment. For the seedling microcuttings, all in vitro treatments rooted at less than 40% while the ex vitro treatments showed significantly higher rooting percentages. Eighty-five percent of the seedling-derived microcuttings rooted when stuck in Redi-Earth after a Rootone dip while 59% rooted without Rootone treatment. The trends for the #8630-derived shoots were similar, but the percentages of rooted microcuttings were generally lower than the seedling-derived microcuttings.

DISCUSSION

Nodal stem tissue from both juvenile and mature American elm selections were adequate starting materials for micropropagation. Axillary shoots have been shown to be the most reliable explant source to produce clonal plants from tissue culture (Brand and Lineberger 1992). Chalupa (1982) also demonstrated that stump and root sprouts were good sources of explant material and had a higher regeneration potential than explants taken from other parts of a mature tree, *U. glabra*. Previous work with above ground tissues from #8630 have consistently failed to produce propagules despite various techniques including softwood cuttings, hardwood cuttings, and grafts. Root cuttings and the micropropagation of shoots originally derived from root cuttings have resulted in the clonal propagation of this American elm.

In the current study, shoot regeneration was achieved on MS medium supplemented with either 2.2 μM or 4.4 μM BA, but explants on the lower concentration of BA produced longer shoots with larger leaves. Both Corchete and others (1993) and Cheng and Shi (1995) found that lower concentrations of BA stimulated shoot formation on explants of *U. pumila* better than higher concentrations of BA in their respective studies. No obvious mutants were observed from the shoots regenerated in vitro and all shoots were approximately the same size prior to harvest as microcuttings. As a group, the ex vitro treatments rooted significantly better than the in vitro treatments. Even those in vitro treatments which resulted in similar levels of rooted cuttings were less desirable than the ex vitro treatments due to the relatively low cost and ease of the latter method. The best rooting was achieved using a soilless mix and a Rootone dip.

Only one of the three seedling tissues utilized, showed repeatable shoot proliferation. The hypocotyl explants failed to form callus or undergo shoot organogenesis on MS with either BA concentration. This result differs from Durzan and Lopushanski (1975) and Ulrich and others (1984), who observed both callus production and shoot regeneration from seedling hypocotyl tissue. Differences in growth may be the result of genotype effects, differences in seedling age, or the differences in plant growth regulator concentrations, particularly their use of an auxin during callus initiation.

Seedling leaf explants failed to support shoot proliferation in culture. George and Tripepi (1994) reported significant levels of shoot initiation from seedling leaf explants under similar conditions using leaves from 2 year old half-sib seedlings from an open-pollinated Delaware-2. They reported that some leaf explants supported shoot proliferation on DKW supplemented with 7.5, 15.0, or 22.5 μM TDZ, but not all genotypes responded. Both the age and the genotype of George and Tripepi's explants differed from the seedling used in this study. Despite the failure of shoot proliferation from leaf tissues in these experiments, different methods of shoot regeneration from leaf tissue are being examined as a preliminary step to plant transformation.

Note: RAPD DNA profiles (Kamalay and Carey. 1995. J. Environ. Hort. 13: 155-159) of the cuttings propagated from the #8630 roots do not match those taken from the leaves of the mature tree. At the time of this writing, this discrepancy cannot be explained but further research is being conducted to answer this question.

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