Plant Regeneration from Leaf Tissues of Siberian Elm

James A. Kapaun and Zong-Ming Cheng

Department of Plant Sciences, North Dakota State University, Fargo, ND 58105

Additional index words. auxin, adventitious shoot formation, cytokinin, organogenesis, *Ulmus pumila*

Abstract. Plants were regenerated from leaf tissue of greenhouse-grown seedlings of Siberian elm (*Ulmus pumila* L.). Shoot regeneration was induced on Murashige and Skoog (MS) medium containing 5 to 10 μM of BA. Up to 55% of the leaf explants formed shoots with an average of 2.4 shoots per explant. Addition of 2.5 or 5 μM of IBA failed to enhance regeneration. Thidiazuron at 0.5 or 1.0 μM also induced shoot regeneration, but the shoots failed to elongate as well as shoots regenerated from media containing BA. Incubation in darkness for 7, 14, or 21 d had little effect in promoting shoot regeneration, except that incubation for 21 d increased shoot regeneration on the medium with 5.0 μM BA. Genotypes differed in shoot regeneration potential, with regeneration frequencies ranging from 13% to 55%. Regenerated shoots were micropropagated on Driver and Kuniyuki Walnut medium. Ninety percent of microcuttings rooted directly in potting soil. This regeneration system will be valuable for genetic transformation and cell selection of Siberian elm. Chemical names used: 6-benzylaminopurine (BA); indole-3-butyric acid (IBA); *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (thidiazuron, TDZ).

Siberian elm, a medium-sized tree native to northeastern Asia, is resistant to Dutch elm disease [*Ophiostoma ulmi* (Buism.) Nannf.] and has been widely planted in windbreaks of the northern Great Plains. Susceptibility of Siberian elm to 2,4-dichlorophenoxyacetic acid (2,4-D), a herbicide used extensively to control broadleaf weeds in cereal crops and turfgrass, has discouraged its further use (Quam et al., 1991). The lack of 2,4-D resistance in elm germplasm makes it difficult to breed Siberian elm for resistance to the herbicide. A 2,4-D degrading gene, *tfdA*, has been isolated from a soil bacterium, *Alcaligenes eutrophus* (Streber et al., 1987). The gene, encoding 2,4-dichlorophenoxyacetate monooxygenase, has been successfully transferred to tobacco (*Nicotiana tabacum* L.) (Lyon et al., 1989; Streber and Willmitzer, 1989) and cotton (*Gossypium hirsutum* L.) (Bayley et al., 1992) to confer resistance. This gene might be transferred to Siberian elm to develop 2,4-D resistance and permit use of the tree in future windbreak plantings.

For genetic transformation of Siberian elm, it is a prerequisite to develop a regeneration system. For elms, plant regeneration has been mostly with American elm (*U. americana* L.)

(Bolyard et al., 1991; Cheng et al., 1992; Durzan and Lopushanski, 1975; George and Tripepi, 1994; Ulrich et al., 1984). Benzylaminopurine (BA) at 5.0, 10.0, or 15.0 μM effectively induced shoot regeneration from leaf tissues of in vitro-grown (Cheng et al., 1992) or 2-year-old greenhouse-grown seedlings (George and Tripepi, 1994). Thidiazuron (TDZ) at 0.1–22.5 μM also induced regeneration from leaf explants of greenhouse-grown seedlings (Bolyard et al., 1991; George and Tripepi, 1994). Plants have been regenerated from English elm (*U. procera* S.) (Fenning et al., 1993), Chinese elm (*U. parvifolia* Jacq.) (Bolyard et al., 1991), and a hybrid elm (*Ulmus* $\times$ Pioneer) (Fink et al., 1986). A micropropagation system has been developed to multiply mature Siberian elm (Cheng and Shi, 1995; Corchete et al., 1993), and preliminary results show that Siberian elm can also be regenerated (Cheng et al., 1992). The objective of this research was to develop a plant regeneration system for Siberian elm for future application of genetic transformation.

Materials and Methods

Leaves from 1- to 3-month-old seedlings of Siberian elm were used as explants. In the first two experiments, leaves were taken from seedlings derived from two seed sources. Source 1 seed was collected from a single tree in Fargo, N.D. Source 2 seed came from several trees in Bismarck, N.D. Only the Fargo seed source was used in the last two experiments. Plants were grown in potting medium of sphagnum peat moss and perlite (Sunshine Mix No. 1; Fisons Horticulture, Vancouver, B.C., Canada), maintained in a greenhouse at $24 \pm 2^\circ\text{C}$ under a 16-h photoperiod, and were watered once a week with

fertilizer (16N–17P–17K) (Technigro Plus; Fisons Horticulture, Mississauga, Ontario, Canada).

Leaves, about two-thirds expanded (second or third leaf from the tip), were excised and surface-disinfected in 15% commercial bleach solution (0.8% NaOCl) with 0.1% (v/v) detergent (Dial; The Dial Corp., Phoenix, Ariz.) for 10 min. Explants were then rinsed three times with sterile distilled water. The leaves were cut transversely into 5-mm-wide strips and placed abaxially side in contact with the medium in 15 $\times$ 100-mm petri plates (25 mL medium per plate). The medium consisted of MS basal medium (Murashige and Skoog, 1962), the appropriate plant growth regulators, and 3.0% (w/v) sucrose. Media were solidified with 0.3% (w/v) Phytigel (Sigma Chemical Co., St. Louis). The pH was adjusted to 5.8 before autoclaving for 15 min at 121°C . Cultures were kept at $24 \pm 2^\circ\text{C}$ in a culture room with a 16-h photoperiod at $\approx 54 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Explants were transferred to fresh medium after 3 weeks in all experiments.

Four sets of experiments were conducted. Expt. 1 examined the interaction between BA, indole-3-butyric acid (IBA), and two seed sources. For each seed source, explants were cultured on media containing a factorial combination of BA (0.0, 5.0, 10.0, or 15.0 μM) and IBA (0.0, 2.5, or 5.0 μM). Experiment 2 examined the effect of TDZ on shoot regeneration. Explants were cultured on medium containing 0.0, 0.1, 0.25, 0.5, or 1.0 μM TDZ. In expt. 3, the interaction of an initial dark incubation with BA was examined. Explants were cultured on medium containing 5.0, 10.0, or 15.0 μM BA, and exposed to darkness for 0, 7, 14, or 21 d before they were transferred to a 16-h photoperiod. Experiment 4 examined the effect of donor plant on shoot regeneration. Leaf explants from six genotypes from the Fargo seed source were cultured on MS medium containing 10 μM BA.

Each treatment had four replications (plates), except for the first trial of the BA/IBA experiment, which had two. Each plate contained eight explants, and all experiments were conducted twice. The percentage of explants regenerating and the number of shoots regenerated from each responding explant were evaluated 2 and 4 weeks after initiation; only data from 4 weeks are presented. Percentage data were arcsin transformed and then subjected to analyses of variance (ANOVA) using the general linear models procedure with the protected Fisher's LSD mean separation technique (SAS, 1990). Data from the two separate experiments and two seed sources were combined, since results were similar in percentage of explants showing regeneration and the number of shoots from regenerating explants between two separate experiments, and from two separate seed sources.

The regenerated shoots were excised from leaf explants and transferred to a micropropagation medium. The medium consisted of DKW basal medium (Driver and Kuniyuki, 1984) supplemented with 2.0 μM

Received for publication 3 Sept. 1996. Accepted for publication 24 Oct. 1996. This research was supported by the U.S. Dept. of Agriculture Forest Service grant 28-C2-802 and McIntire-Stennis Project ND6207. We thank Drs. P. McClean and H. Cross for reviewing the manuscript. We also thank Wayne Sargent for helpful suggestions and assistance. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

BA, 3.0% (w/v) sucrose, and solidified with 0.3% (w/v) Phytigel. Forty microshoots, 2–3 cm long, were excised and placed directly into autoclaved potting medium (Sunshine Mix No. 2; Fisons Horticulture). The potting medium was contained in test tube caps placed in Magenta GA7 vessels to maintain high humidity.

Results

Regeneration frequency and the number of shoots regenerated from responding Siberian elm explants were similar between the two seed sources in the first two sets of experiments (data not shown). However, regeneration frequency varied greatly within the treatments in all experiments, with standard errors of 8% to 24% (Table 1).

In the experiment examining the interaction of BA and IBA, callus formation was visible 1 week after elm leaf explants were placed on the media containing plant growth regulators. Two distinct types of calli formed on single explants; one was white and compact and the other was light green and crystalline. The green calli formed mostly at the cut midrib and secondary veinal regions. More calli appeared to have formed on explants on media containing 2.5 or 5.0 μM IBA (data not shown). Shoots initiated from explants on all media containing BA in ≈ 2 weeks (Fig. 1A). Shoots arose primarily from the light green, crystalline calli and the surrounding tissues. Shoots failed to form on media without BA (Table 1).

TDZ had a significant effect on Siberian elm shoot regeneration. Very few calli and no shoots formed on explants on the medium containing 0.0, 0.1, or 0.25 μM TDZ. Calli formed in 2 weeks and shoots emerged 3 weeks after leaf explants were inoculated on medium containing 0.5 or 1.0 μM TDZ; the frequencies of explants forming shoots were 18% and 41%, respectively. Explants on media supplemented with TDZ formed shoots about 1 week later than those on the media with BA.

Shoot regeneration frequency was unaffected by darkness when explants were cultured on medium containing 10.0 or 15.0 μM BA, except for those on medium containing 10 μM BA with a 7-d dark treatment (Table 2). Incubation in darkness for 14 d or more enhanced shoot regeneration from explants on medium with 5.0 μM BA. Shoot regeneration frequency increased from 20% without a dark treatment to 50% with 3 weeks of darkness. Explants cultured in darkness appeared to produce more callus than those cultured in light. Shoot regeneration potential differed among the six clonal seedlings. Leaf explants from seedling 2 had the highest shoot regeneration (55%), whereas seedling 5 had the lowest (13%) (Table 2).

In all experiments, explants started to turn brown after 3 weeks. However, senescence of explants and calli was delayed by 1 to 2 weeks when explants were placed in darkness. All explants and the white, compact calli senesced after 6 weeks, even though they were transferred to fresh media after 3 weeks. Regener-

Table 1. The effects of IBA and BA on adventitious shoot formation from greenhouse-grown leaf explants of two seed sources of Siberian elm.

IBA (μM)	BA (μM)	Shoot formation	
		Regeneration frequency (%) $\pm$ SE ²	Shoots formed/responding explant $\pm$ SE
0.0	0.0	0 e	0 e
	5.0	34 $\pm$ 24 bc	1.5 $\pm$ 0.7 cd
	10.0	46 $\pm$ 17 a	2.1 $\pm$ 0.9 ab
	15.0	46 $\pm$ 18 a	2.5 $\pm$ 1.1 a
2.5	0.0	0 e	0 e
	5.0	23 $\pm$ 8 cd	1.1 $\pm$ 0.5 d
	10.0	27 $\pm$ 13 b–d	1.2 $\pm$ 0.8 d
	15.0	30 $\pm$ 14 b–d	1.3 $\pm$ 0.6 cd
5.0	0.0	0 e	0 e
	5.0	19 $\pm$ 14 d	1.3 $\pm$ 0.3 cd
	10.0	28 $\pm$ 21 b–d	1.4 $\pm$ 0.4 cd
	15.0	37 $\pm$ 16 ab	1.7 $\pm$ 0.5 bc
Significance			
BA concentration			
Linear		***	***
Quadratic		*	NS
IBA concentration			
Linear		NS	NS
Quadratic		NS	NS

²Percentage data were arcsin transformed.

NS, *, **Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively ($n = 96$).

Fig. 1. (A) Callus and shoot formation from a Siberian elm leaf explant 3 weeks after inoculation on a regeneration medium; (B) an elongated shoot excised from a leaf explant 8 weeks after inoculation on regeneration medium; (C) elongated shoots under micropropagation; (D) plant acclimated to ambient environments. Bars = 1 cm.

ated shoots elongated to 5 to 15 mm after 6 to 8 weeks of culture (Fig. 1B). These shoots were excised and multiplied on the micropropagation medium (Fig. 1C). Ninety percent of the microshoots placed directly in potting medium rooted and were successfully acclimated in the greenhouse within 6 weeks after transfer to the ambient environment (Fig. 1D).

Discussion

Successful plant regeneration from leaf explants derived from two seed sources indicates that the regeneration system we tested is reliable and might be applicable to Siberian elm from other geographic locations. The response of leaf explants from Siberian elm to

Table 2. Shoot regeneration from leaf explants of six Siberian elm seedling genotypes after 4 weeks in culture.

Seedling	Shoot formation	
	Regeneration frequency (%) $\pm$ SE ^z	Shoots formed/ responding explant $\pm$ SE ^z
1	33 bc	1.2 a
2	55 a	1.4 a
3	25 cd	1.0 a
4	33 bc	1.1 a
5	13 d	0.6 b
6	47 ab	1.3 a

^zMean separation within the column by Fisher's LSD at $P \leq 0.05$ ($n = 64$).

BA and IBA was similar to that of leaf explants from other tree species investigated in our laboratory, such as American elm (Cheng et al., 1992), Asian white birch (*Betula platyphylla*) (Schnurr and Cheng, 1994), and hackberry (*Celtis occidentalis* L.) (Cheng et al., 1994). BA at 5.0, 10.0, or 15.0 μ M was sufficient for inducing shoot regeneration. Auxin (IBA) promoted callus formation and was unnecessary for shoot regeneration. Shoot formation from Siberian elm was also induced by TDZ, an effective cytokinin for adventitious shoot induction in American elm (Bolyard et al., 1991; George and Tripepi, 1994), Chinese elm (Bolyard et al., 1991), and several other woody plants (for review, see Huetteman and Preece, 1993). Regenerated Siberian elm shoots from explants on TDZ-supplemented media had difficulty in elongating, which is similar to that observed in American elm (George and Tripepi, 1994) and other woody plants (Huetteman and Preece, 1993; Lu, 1993).

Dark treatment is beneficial for plant regeneration in diverse woody plant species (Cheng and Reisch, 1989; Chevreau et al., 1989; Liu et al., 1983). In our experiment with Siberian elm, darkness only enhanced shoot regeneration when explants were placed on media with a relatively low concentration of BA. Increased regeneration may have resulted from slower breakdown of BA during dark incubation.

Shoot regeneration can vary depending on the genotype of the donor plant. Difference in shoot regeneration has been found in many woody plant species (Chevreau et al., 1989; Cousineau and Donnelly, 1991; George and Tripepi, 1994). This variation was evident in our first experiments. Since explants were isolated from a mixed seedling population, all segments of one leaf formed shoots, whereas all segments of another leaf might be incapable of forming shoots, even in the same petri plate. Explants from all donor plants formed shoots but regeneration frequency and the num-

ber of shoots regenerated varied greatly among the seedlings.

These experiments demonstrate that plant regeneration from seedling leaves of Siberian elm can be obtained readily and reliably at a moderate frequency. Regenerated shoots were incorporated into a micropropagation system (Cheng and Shi, 1995) to multiply the number of shoots. These microshoots rooted easily and were placed in a greenhouse for further growth. Additional experiments are needed to maximize efficiency of the system. This regeneration system will be valuable for genetic transformation and cell selection of Siberian elm.

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