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Micropropagation of *Betula platyphylla* 'Fargo' via Shoot Tip Culture and Regeneration from Leaf Tissues[1]

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Abstract

A micropropagation system was developed for mass propagation of 'Fargo'® (Dakota Pinnacle™), a newly released cultivar of Asian white birch (*Betula platyphylla)*. Shoot tips from the mature, 7-year-old tree were established on 75% strength Murashige and Skoog medium supplemented with 0.1 µM thidiazuron. The greatest shoot proliferation occurred on Woody Plant Medium supplemented with 2.2 µM benzyladenine (BA), solidified with 6.5 g/liter agar, and cultured at 24C (75F). Microshoots were rooted *in vitro* or *ex vitro* followed by establishment in the greenhouse. A system to regenerate plantlets from leaves of aseptically cultured shoots was also developed. The optimum conditions for shoot regeneration from leaves included a 2-week dark treatment before exposure to a 16/8 hour light/dark photoperiod, use of large and healthy leaf explants, and culture on the Woody Plant Medium containing 20.0 or 30.0 µM BA. The regenerated shoots proliferated on the micropropagation medium and were divided, and the resulting shoots were rooted *ex vitro* and acclimated in greenhouse conditions.

Index words: Asian white birch, *in vitro*, micropropagation, regeneration, tissue culture.

Significance to the Nursery Industry

An elite cultivar of Asian white birch, 'Fargo'® (Dakota Pinnacle™), was introduced recently. It has a dense upright growth habit, dark-green and glossy foliage, and tolerance to stressful northern environments. To make this cultivar widely available to the nursery industry, a propagation protocol was developed. Although 'Fargo'® shows above average tolerance to the bronze birch borer (*Agrilus anxius* Gory) and the birch leafminer (*Fenusa pusila* Lepeletier), further enhancement of resistance to these pests requires employing such new strategies as genetic engineering. A micropropagation protocol described herein has made 'Fargo'® available to several nurseries. In addition, a plant regeneration system was developed as a prerequisite for applying genetic engineering technology for possible further improvement of this cultivar.

Introduction

Until recently, the predominantly planted birch in landscapes was the European white birch (*Betula pendula* Roth) (6). Unfortunately, the species is susceptible to the bronze birch borer (19), making it a less than desirable landscape plant. In the northern Great Plains, the choice of birch is further limited by stresses of a harsh climate, which in turn predispose the trees to insect attack (20). The insect threat can be reduced by optimizing cultural conditions or planting other species of birch and by using genetically improved cultivars. Recently, an Asian white birch (*B. platyphylla* Sukaczev) cultivar 'Fargo'® (Dakota Pinnacle™) was selected and introduced (1). It has a dense upright growth habit, dark-green and glossy foliage, and tolerance to stressful environments, such as cold winters and dry and hot summers, high pH, heavy clay soils, and strong winds. It also shows an above average tolerance to the bronze birch borer and the birch leafminer (1). To make this cultivar widely available to nurseries, an efficient propagation protocol was needed as 'Fargo'® has not been propagated successfully using conventional vegetative propagation methods. Difficulties in propagating various species of birch are not atypical since stem cuttings do not root well (7, 13)

Propagation of birch using micropropagation systems has been accomplished in European white birch (14), *B. jacquemontii* Spach., *B. costata* Trautv, *B. albo-sinensis* Burkill, *B. ermanii* Cham., and *B. celtiberica* (18), *B. uber* Ahe Fern. (26), and *B. platyphylla* var. *szechuanica* (7). Since various species and genotypes of birch respond differently in *in vitro* conditions (13), one of the objectives of this research was to develop a micropropagation protocol that can be utilized by commercial tissue culture firms to mass produce the cultivar 'Fargo'® and make it available to the nursery trade.

Although 'Fargo'® shows above average tolerance, it is not resistant to the bronze birch borer and leafminer (1). To improve this cultivar further for resistance to these pests, genetic transformation would be a method of choice because it allows inserting a single gene into the plant genome without disrupting the overall genetic makeup. One of the prerequisites for genetic engineering is the capability of regenerating plants from somatic tissues. Plant regeneration has been reported for European white birch using leaf explants (9, 21, 23, 25) and catkin tissue (22). Regeneration has also been achieved in European white birch by somatic embryogenesis (8, 17). However, plant regeneration for Asian white birch has not been reported. Therefore, another objective of this research was to develop a regeneration system for 'Fargo'® for possible improvement by genetic transformation, as reported recently in Japanese white birch [*B. platyphylla* var *japonica* (Miq.) Hara] (15).

Materials and Methods

Micropropagation. Shoot tips, 2–4 cm (approximately 1 in) long, were collected in July from actively and inactively growing shoots of the originally selected mature stock plant (7 years old) in the birch experimental plot in Fargo, ND.

[1]Received for publication February 20, 2000; in revised form March 3, 2000. This research was supported in part by McIntire-Stennis Project ND6209 and North Dakota Centennial Decade Windbreak Project 4673. We thank W. A. Sargent for assistance and C. Lee, L. Dahleen, and A. Lafta for reviewing the manuscript.

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The entire shoot tips were surface sterilized in an 0.4% sodium hypochlorite solution containing 0.1% Tween 20 for 10 min and rinsed three times with sterile distilled water. Three initiation media were Murashige and Skoog (MS) basal salts (16) at 100%, 75%, and 50% strength, respectively, plus MS vitamins, 2% sucrose, and 0.1 µM thidiazuron (TDZ). Media were adjusted to pH 5.7, solidified with 0.65% Difco Bacto agar (Difco Co., Detroit, MI, #0140-01-0) and autoclaved at 121C (250F) for 15 min. Fifteen randomly chosen actively or inactively growing shoot tips were used for each of three media. One shoot tip was placed vertically into one test tube with 12.5 ml of medium. Cultures were maintained at 24 ± 2C (75 ± 4F) under a 16 hr photoperiod provided by cool-white fluorescent light tubes. Light intensity was approximately 36.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ measured at the shelf level.

The newly produced entire shoots were used for subculture and shoot proliferation in baby food jars. The basal proliferation medium consisted of the woody plant medium (WPM) salts (11) supplemented with MS vitamins, 2% sucrose and 0.65% Difco Bacto agar (except in experiment 3, see below) at pH 5.7 before autoclaving. Experiment one examined the effect of concentration of benzyladenine (BA) at 0, 0.4, 1.1, 2.2, 4.4, or 5.3 µM on shoot proliferation. Experiment two examined the effect of temperature on shoot proliferation with cultures being placed in growth chambers at 20, 22, 24, or 26C (68, 72, 75 or 79F), respectively. The third experiment examined the effect of gelling agents on shoot proliferation. Three gelling agents used were: 0.25% Phytagel (Sigma Co., St. Louis, MO, #P8169), 0.65% Sigma agar (#A1296), and 0.65% Difco Bacto agar. All experiments were run in completely random designs, with each treatment consisting of three baby food jar replicates, each jar containing three shoots. All experiments were repeated once. The light condition was the same as that in the initiation experiment. The cultures were transferred to fresh medium every 30 days and all new shoots formed were excised. The duration of the BA and temperature experiments was 60 days, and that of the gelling agent experiment was 90 days.

Induction of roots from microshoots was undertaken in both *in vitro* and *ex vitro* conditions. *In vitro* rooting was performed using 20% standard WPM salts and 0.5 µM indolebutyric acid (IBA) as described by Jansson and Welander (7) . A total of 623 microshoots were rooted over a three-month period in baby food jars, each with 5 shoots. Rooted shoots were transferred to flats filled with Jiffy Mix (Jiffy Mix, Shippegan, Canada) and covered with clear plastic top. After one week, the covers were gradually lifted during a 3-week period and the survival rate was recorded. *Ex vitro* rooting was done in flats with non-sterile Jiffy Mix medium. The basal portion (approximately 2 mm, or 0.1 in) of microshoots (2–3 cm, approximately 1 in long) were quick-dipped (2–3 sec) in distilled water (24 shoots), 40 ppm indolebutyric acid (IBA) (60 shoots), or α-napthaleneacetic acid (NAA) solution (62 shoots), and studded in the medium. Flats were covered with clear plastic tops to maintain high humidity inside. After 2 weeks, the cover on each tray was gradually lifted during a 3-week period. The rooted plants were transferred to Sunshine Mix #1 (Fisons Western Corp., Vancouver, Canada) and grown in the greenhouse under a 16-hr photoperiod.

Regeneration. Leaves about 1 cm (0.4 in) wide were excised from stock shoots growing in double Magenta GA7 vessels (Magenta Corp., Chicago, IL) as explants for regeneration experiments. The leaf was cut across the midrib with scissors, but still attached, and was placed abaxial side up on the medium. The basal regeneration medium consisted of WPM salts, MS vitamins, 2% sucrose, and 0.65% Difco Bacto agar. The initial regeneration experiment examined all combinations of NAA at 0, 3, 6, 9 µM and BA at 0, 7.5, 15.0, 22.5 µM. The plates were maintained at 24C (75F) under a 16-hr photoperiod with the light intensity of approximately 36.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the shelf level. The second experiment compared media with a factorial combination of NAA (0, 4, 8 µM) and BA (0, 10, 20 µM), and the third experiment tested the effect of BA (0, 10, 20, 30 µM) versus TDZ (0, 1, 2, 3 µM) on shoot regeneration. In both of these experiments, the plates were placed in the dark for 2 weeks prior to exposure to a 16/8 hr photoperiod as above. Each treatment had two petri dishes (15 × 100 mm, (0.6 × 4.7 in)) (replicates), each containing four explants. All experiments were run in a complete randomized design, and were repeated once.

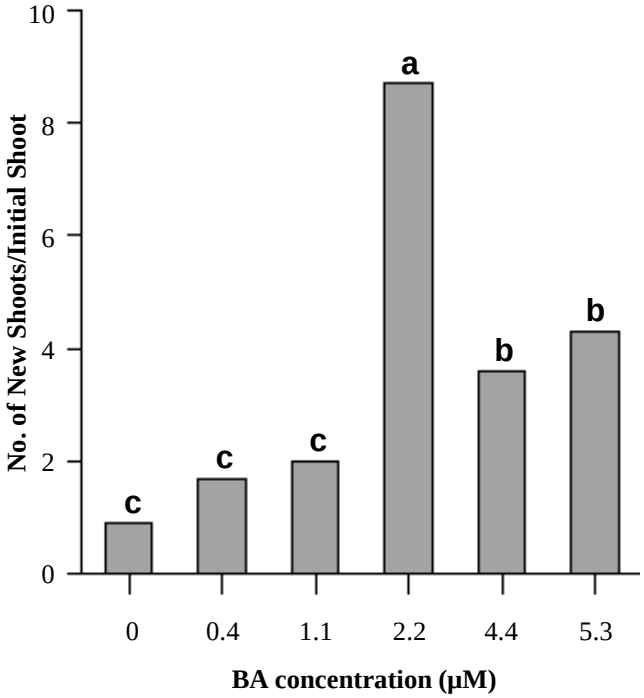

Fig. 1. Effect of BA concentrations on microshoot proliferation of 'Fargo'® birch. Data were combined from two experiments (n = 18). Only shoots longer than 0.75 cm (0.3 in) were counted and mean separation with LSD after analysis of variance ($P \leq .05$).

Statistical analysis. The data were subjected to analysis of variance and mean separation with the least significant difference (LSD, $P \leq .05$), either using the general linear procedure, SAS (24) (Fig. 1, Fig. 2), or using MSTAT 4.0 (Michigan State University, East Lansing, MI) (Table 1).

Results and Discussion

Micropropagation. Shoot tip explants initiated new shoots slowly. All shoot tips on media with either 50% or 100% strength MS salts became necrotic after 6 weeks and no new shoots were produced. Eight of the 15 explants on the 75%

MS medium exhibited shoot elongation, and three formed multiple shoots from the lower portion of the explants. These newly formed shoots began to grow vigorously, while most original explants became chlorotic. After 6 weeks, these vigorously growing shoots and the original explants that still appeared green were transferred to fresh medium consisting of WPM salts supplemented with 4.4 µM BA. One month after transfer, one additional shoot tip in the 75% MS medium developed small new shoots.

The proliferation experiments were started after sufficient shoots were available. BA concentration had a significant effect on shoot proliferation (Fig. 1). In the absence of BA, few new shoots produced and roots formed on many shoots. The greatest proliferation occurred on medium containing 2.2 µM BA, with an average of 8.7 new shoots greater than 0.75 cm (0.3 in), long enough to be transferred to *in vitro* rooting medium. At the highest level of BA tested (5.3 µM), shoots were inconsistent in size, variably vitrified, and somewhat chlorotic.

The temperature also had a significant effect on shoot proliferation. At 20C (68F) and 22C (72F), proliferation rates were lower than that at 24C (75F) (Fig. 2). At 26C (79F), cultures grew vigorously but developed chlorotic or necrotic shoots and leaves in 2 weeks, which reduced shoot quantity and quality.

Shoot proliferation was affected by gelling agent (Table 1). Shoots on Phytagel-solidified medium vitrified rapidly, then became chlorotic and necrotic, producing no new shoots. Shoots on both Difco Bacto agar and Sigma agar media produced a similar number of new shoots.

Microshoots rooted easily *in vitro* with an overall rooting rate of 95%. Roots started to appear after 7 days, and extensive roots were evident after 14 days. The *in vitro* rooted shoots had an overall survival rate of 80% when they were transferred to soil-less medium. For *ex vitro* rooting, no significant differences were detected, with rooting rates of 83%, 76% and 88% for the water control, 40 ppm IBA or NAA solutions, respectively. From all rooting experiments over several months, more than 600 plants were transferred to the greenhouse. Only three plants with variegated leaves were observed, no other plants showed abnormal morphology.

Regeneration. In the first experiment, without a dark treatment, no shoot formation occurred over the 2 months even after transfer to fresh media (data not presented). Roots formed from leaf explants on all media without BA, while prolific greenish/yellow calli were elicited from leaves on media with high BA and NAA (data not presented). In the second experiment with a 2-week dark treatment, shoot regeneration occurred only on media supplemented with 10 and 20 µM BA, with 50% and 100% of the leaf tissues regenerated shoots, respectively. No shoots formed from leaves on media without BA or on media with both BA and NAA (Data not presented). Shoots produced on medium with 20 µM BA were more consistent in size and vigor than those formed on medium with 10 µM BA. Different cytokinins also had a significant effect on shoot regeneration (Table 2). BA was more effective in inducing shoot formation than TDZ at the concentrations tested. Medium with 20 or 30 µM BA consistently produced numerous, large shoots. Shoot regeneration occurred on media containing 1 and 2 µM TDZ, but it was much slower than that occurred on BA media (7 weeks vs. 4 weeks). The regenerated shoots were multiplied using

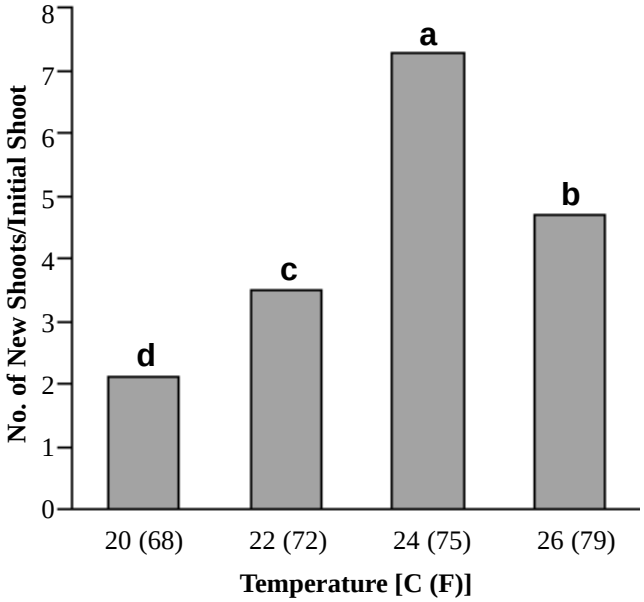

Fig. 2. Effect of temperatures on shoot proliferation of 'Fargo'® birch. Data were combined from two experiments (n = 18). Only shoots longer than 0.75 cm (0.3 in) were counted and mean separation with LSD of 0.89 after analysis of variance ($P \leq .05$).

micropropagation procedures described earlier, rooted, and successfully transplanted to the greenhouse like the micropropagated shoots.

Results demonstrated that 'Fargo'®, a newly introduced Asian white birch cultivar, can be effectively propagated *in vitro*. This protocol appears to be effective for commercial production, overcoming the difficulty with conventional propagation techniques. The step of *in vitro* rooting can be eliminated to reduce the cost as shown with other micropropagated birch (7, 14). Inhibition of shoot proliferation by Phytagel for this plant is in opposition to those observed in some other woody plants, like *Eucalyptus grandis* W. Hill ex Meaiden (12) and Siberian elm (*Ulmus pumila* L.) (3). The exact reasons for such inhibition in birch shoot proliferation are unknown. It may be due to the deficiency or toxicity of some microelements in Phytagel (3, 5).

Out of more than 600 plants transferred to the greenhouse, three showed variegated leaves and reduced chlorophyll, which may have been due to pre-existing variations. These types of phenotypic variations can be readily selected against (14). No other morphological variations were noticed, suggesting that the micropropagation protocol can be used to propagate 'Fargo'® while maintaining the true genotype.

Table 1. Effect of gelling agents on microshoot proliferation of 'Fargo'® birch.

Gelling agent	Mean number of new shoots/original shoot[z]
0.25% Pytagel	0.0b
0.65% Bacto agar	24.8a
0.65% Sigma agar	31.9a

[z]Data were combined from two experiments (n = 18). Only shoots longer than 0.75 cm (0.3 in) were counted and mean separation with LSD after analysis of variance ($P \leq .05$).

Table 2. Comparison of BA and TDZ concentrations on shoot regeneration from leaf explants of 'Fargo'® Birch.

Plant growth regulator	Conc. (μM)	Explants forming shoots[z] (%)	Weeks required for shoot formation
BA	0	0.0	No regeneration
	10	87.5	4
	20	100.0	4
	30	100.0	4
TDZ	0	0.0	No regeneration
	1	37.5	7
	2	12.5	7
	3	0.0	No regeneration

[z]The data were combined from two experiments, n = 16.

Microshoots can be rooted easily *in vitro* or *ex vitro.* Further improvement for *ex vitro* rooting and survival rates may be possible by very careful humidity control, fungicide application, and selection of the optimal rooting medium.

Shoot regeneration was best achieved with 20 or 30 μM BA and incubation in the dark for 2 weeks. Requirement of a dark treatment for shoot regeneration is similar to that for European white birch (9), and several other woody plants (2, 4, 10). Inhibition of shoot formation by auxin has also been observed in European white birch (9).

In conclusion, a successful micropropagation protocol was developed for 'Fargo'® birch. This has made the cultivar available to several commercial nurseries. This protocol of plant regeneration from leaf tissues may make it possible for further genetic improvement via cell selection and genetic transformation.

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