Plant Cell, Tissue and Organ Culture **51:** 209–213, 1997.

Micropropagation of juvenile and mature american beech

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Received 24 December 1996; accepted in revised form 21 November 1997

Key words: beech bark disease, *Fagus grandifolia*, mature tree, rooting, tissue culture

Abstract

The purpose of this study was to micropropagate juvenile and mature American beech (*Fagus grandifolia* Ehrh.) resistant to beech bark disease. Shoot tips (from juvenile seedlings and root sprouts of mature trees) and buds from branches of mature trees, were cultured and multiplied on aspen culture medium supplemented with 0.89 μM 6-benzyladenine, 0.27 μM α-naphthaleneacetic acid, 20 g l^{-1} sucrose, and 7 g l^{-1} Difco Bacto-agar. Rooting of shoots was achieved using a 1 minute dip in 12.3 mm indole-3-butyric acid, followed by culture in a Horticube saturated with half-strength aspen culture medium containing 20 g l^{-1} sucrose. Thirteen of 33 mature genotypes were established from shoot tips taken from root sprouts, and six of 41 were established from mature buds. Plantlets of six genotypes were successfully acclimatized to a growth room for three months.

Abbreviations: AC – aspen culture medium; BBD – beech bark disease; WS – Wolter Skoog medium

Introduction

American beech (*Fagus grandifolia* Ehrh.), a slow-growing, shade-tolerant tree species native to eastern North America, is valued for its wood and as a source of food for wildlife. The fine-grained wood is used for flooring, furniture, veneer, and is also favored for fuelwood because of its high density and good burning qualities. The distinctive triangular nuts are consumed by wildlife, as well as humans. Beech bark disease (BBD), a major dieback-decline disease, causes significant mortality and wood defects in American beech. The disease develops when the beech scale insect, *Cryptococcus fagisuga* Lind., feeds on the outer bark of the tree rendering it susceptible to invasion by several species of fungi, primarily *Nectria coccinea* var. *faginata* Lohman, Watson, and Ayers, and *N. galligena* Bres. (Houston, 1994). In forest stands, where BBD is endemic, a few trees remain insect- and disease-free. These trees, although found in relatively low numbers, have been shown to be resistant to *C. fagisuga* (Houston, 1983). Resistant trees often occur in groups. Isozyme genetic studies have shown such trees are either clones derived from root sprouts, families of seedling origin, or combination of clones and families (Houston and Houston, 1987).

The factors that influence root sprouting and survival in American beech are not completely understood (Jones and Raynal, 1987, 1988), thereby making it difficult to stimulate sprouting routinely of disease-resistant trees. Rooting of adventitious shoots produced via root sprouts has been limited (Reid, 1984). *In vitro* propagation would provide an alternative means to propagate selected American beech genotypes with resistance to BBD.

Some success with *in vitro* propagation of mature European beech (*F. sylvatica* L.) has been reported (Ahuja, 1984; Chalupa, 1985; Meier and Reuther, 1994; Nadel et al., 1991a,b). To our knowledge, there have been no reports on *in vitro* propagation of *F. grandifolia*. This communication reports on a study, initiated with non-selected juvenile seedlings, in order to determine a suitable culture medium to

micropropagate mature American beech with known BBD-resistance.

Materials and methods

Explant initiation and micropropagation on two media were studied using explants from actively growing shoots from seedlings, root sprouts from mature trees, and forced shoots from dormant mature trees. Actively growing shoots (10 cm long) from beech seedlings, aged 3 months to 2 years, were stripped of leaves and rinsed in tap water for 30 min. Shoots were then surface disinfested in 0.5% (v/v) sodium hypochlorite containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) for 15 min, followed by three rinses in sterile, deionized water. Shoot tips (1 cm long) were excised and placed upright in 25×95 mm culture vials (one shoot tip per vial) containing 9 ml of either a Wolter and Skoog (WS) medium (1966) or Aspen culture (AC) medium (Ahuja, 1983) supplemented with 0, 0.44, or 4.44 μM 6-benzyladenine (BA) and 0, 0.05 or 0.54 μM α-naphthalenenacetic acid (NAA), 20 g l^{-1} sucrose, and 10 g l^{-1} Difco Bacto-agar. The pH of the media was adjusted to 5.6 before autoclaving at 121 °C for 20 min. All explants were transferred to fresh media at 3-week intervals. As explants increased in size, they were transferred to 50×70 mm glass jars containing 30 ml of media. All cultures were incubated at 25 °C with a 16-h photoperiod provided by cool-white fluorescent lamps (65 μmol m^{-2} s^{-1}). The experiment was repeated and a total of 24 shoot tips per treatment were cultured. After 12 wk, the cultures were evaluated for growth and survival. The culture medium was then modified in order to optimize a medium that would support continuous, vigorous growth with good elongation of shoot tips, and multiplication coming more from axillary versus adventitious proliferation. Aspen culture medium was supplemented with 0, 0.89 or 2.22 μM BA and 0, 0.05 or 0.27 μM NAA, 20 g l^{-1} sucrose and 7 g l^{-1} Difco Bacto-agar.

Mature explant material was obtained from beech trees growing in Maine, Massachusetts, and West Virginia, previously selected for resistance to BBD (Houston, pers. comm.). In the spring and fall (1992 and 1993) root segments from 33 selected trees were dug, cut into 15–25 cm long segments, and placed horizontally in sand or growing medium (Metro Mix 700) with the proximal end of the root segment protruding. Roots were placed in the greenhouse under a 16-h photoperiod, and flats (placed on bottom-heat pads) were watered every other day. Root sprouts, at least 3 cm long that developed on the root segments, were excised, disinfested in 0.5% (v/v) sodium hypochlorite for 5 min, rinsed in sterile water, and shoot tips cultured as previously described for the juvenile seedling material. The culture medium was AC medium supplemented with 0.89 μM BA, 0.27 μM NAA, 20 g l^{-1} sucrose, and 7 g l^{-1} Difco Bacto-agar.

In late winter, branch samples were collected from 41 selected mature trees and forced in a solution of 20 g l^{-1} sucrose plus 0.2 g l^{-1} 8-hydroxyquinoline. Branch samples were covered with plastic bags to conserve moisture loss and placed under a 16-h photoperiod (65 μmol m^{-2} s^{-1}). The solution was changed and branch ends cut every 3–4 days. As soon as the dormant buds opened, they were excised, disinfested and cultured as previously described for seedling shoot tips. The culture medium was the same as used with the sprouts from root segments.

In preliminary rooting trials, a number of treatments and methods were attempted using approximately 2 cm-long shoots from *in vitro* cultures established from juvenile seedlings. Shoots were pulsed for 7–10 days on full-, half- or quarter-strength AC medium supplemented with 0.89 μM BA, 0–49 μM indole-3-butyric acid (IBA), 20 g l^{-1} sucrose and 7 g l^{-1} Difco Bacto-agar. Cultures were placed in the light (65 μmol m^{-2} s^{-1}) or in the dark during the pulse treatment, prior to transfer to light on the same culture media without IBA. In another rooting trial, the basal end of shoots was given a 1 min dip in 12.3 mM IBA, cultured on above media strengths and placed in light or dark for 7–10 days, prior to transfer to fresh media and cultured in light. The addition of 5 g l^{-1} activated charcoal or a 1 vermiculite: 1 perlite medium (v/v) to various strength media was also tested.

Rooting of *in vitro*-generated shoots, from juvenile or mature explants, was achieved by dipping the basal end of a microshoot into 12.3 mM IBA for 1 min, and placing this shoot into a LC-1 Horticube [a foam-like rooting medium] (Smithers-Oasis; Kent, OH) saturated (100 ml) with half-strength AC medium containing 20 g l^{-1} sucrose in a Magenta GA7 vessel. The cultures were incubated at 25 °C with a 16-h photoperiod provided by cool-white fluorescent lamps (65 μmol m^{-2} s^{-1}). When roots protruded through the Horticube (1–2 months), rooted plantlets were potted in a mix containing 2 field soil: 1 sand: 1 peat: 1 perlite (by volume) and acclimatized to the growth room.

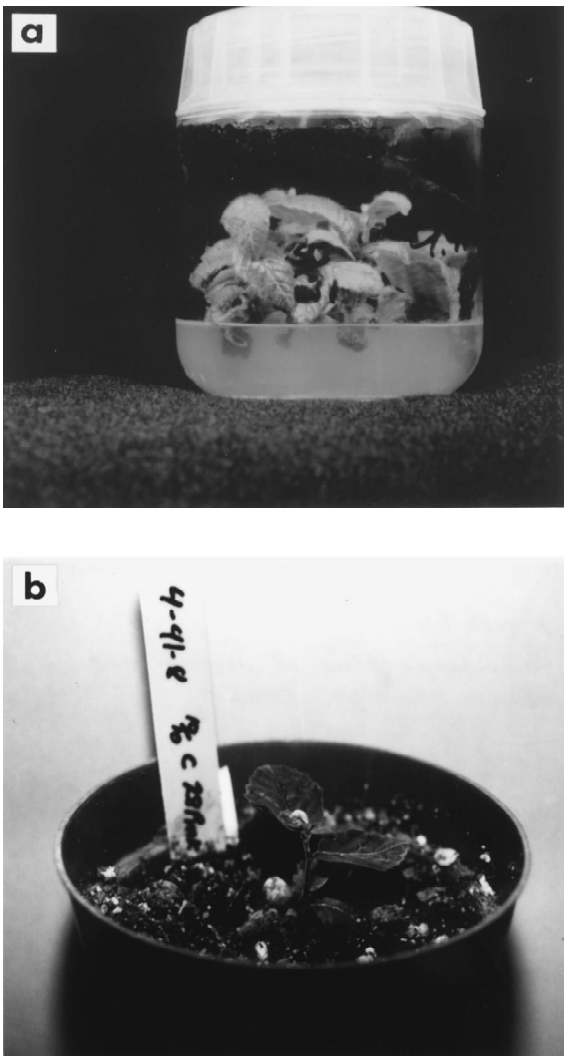

Figure 1. (*a*) Multiplication phase of shoot tip from root sprout obtained from root segment of a mature American beech tree. (*b*) Rooted plantlet of American beech.

Results

Shoot tips, from juvenile seedlings, cultured on AC or WS media supplemented with BA and NAA initially responded in a similar fashion. Shoot tips elongated slightly and adventitious buds began to form at the base. However, within 6 wk shoots on WS media became chlorotic, and by 9 wk all cultures were dead. Shoot tips cultured for 12 wk on AC medium without BA and 0–0.54 μM NAA produced little or no growth response. Shoot tips cultured on AC medium containing 0.44 or 4.44 μM BA, elongated and an average multiplication rate (number of new shoots per primary explant over 12 weeks) of 1.1 and 1.5, respectively, was observed at all NAA concentrations. Explants cultured on 4.44 μM BA produced a large amount of callus at the base of shoots. Aspen culture medium supplemented with 0.89 μM BA, 0.27 μM NAA, 20 g l^{-1} sucrose, and 7 g l^{-1} Difco Bacto-agar resulted in more vigorous new growth with a healthy green color, and an average multiplication rate of 1.3 (Figure 1a). Mean shoot length after 12 wk on this medium was 1.8 cm, and more shoots were derived via axillary proliferation than with the original medium (data not shown).

From mature beech trees selected for resistance to BBD, 24 genotypes (73%) produced root sprouts, and 13 genotypes (39%) were micropropagated. The number of shoot tip explants cultured varied for each genotype, depending upon the number of sprouts regenerated from root segments (Table 1). The culture of forced buds from mature branches, however, was less successful. Initially, 10 genotypes out of 41 were responsive to *in vitro* culture, but over time cultures declined and only six (15%) could be maintained. Bud explants that did survive grew slowly and produced shoots with thicker stems than those from root sprouts.

In preliminary rooting experiments, rooting of shoots was extremely limited. Only 3% rooting was achieved when shoots (from juvenile seedlings) were cultured on half-strength AC medium containing 49 μM IBA or given a 1 min dip in 12.3 mM IBA and placed in the dark for 7 days, prior to transfer to growth regulator-free media in the light. The addition of activated charcoal or the vermiculite:perlite mix to half-strength AC medium with IBA increased rooting percentages to 25–43% and 12–14%, respectively. However, when rooted shoots were transferred to a potting mix for acclimatization to the growth room (Figure 1b), shoots began to decline and eventually all plantlets died.

The best rooting percentages and overall plantlet survival was achieved using the Horticube treatment. Rooting percentages ranged from 48–97% for shoots derived from cultured shoot tips of root sprouts (Table 1). Shoots obtained from mature bud explants of tree #1-12-6, #1-20-6-51, #1-20-13-132, #4-41-C2, #27WV, and #28WV, had rooting percentages of 50, 54, 90, 97, 93 and 48%, respectively.

Discussion

Root sprout explants taken from mature American beech trees, selected for resistance to BBD, were es-

Table 1. Response of mature *Fagus grandifolia* (American beech) to root sprouting, shoot tip culture, rooting, and plantlet survival.

Tree No.	(State)[1]	No. root segments[2]	No. sprout clusters[3]	No. shoot tips surviving culture[4]	Total no. shoots multiplied *in vitro* (Over a 3yr period)	No. rooted shoots/no. attempted[5]	Rooting (%)	No. plants survived[6]
1-10-6	(ME)	27	6	4	47	23 / 31	74	0
1-12-7	(ME)	37	6	4	53	32 / 45	71	1
1-20-7-65	(ME)	18	6	20	64	42 / 48	88	0
1-20-16-1	(ME)	27	7	30	48	31 / 40	78	0
4-41-1	(MA)	16	14	4	196	104 / 180	58	0
4-41-8	(MA)	14	1	3	100	65 / 84	77	3
7	(WV)	12	15	5	124	74 / 112	66	0
8	(WV)	10	4	5	120	93 / 108	86	1
13	(WV)	8	2	5	144	84 / 128	66	1
22	(WV)	27	13	6	112	93 / 96	97	4
27	(WV)	14	20	2	104	89 / 96	93	3
28	(WV)	13	1	3	54	26 / 42	62	0
98	(WV)	12	4	4	124	54 / 112	48	0
Mean ± s.e.		18.1±2.4	7.6±1.7	7.3±2.3	99.2±12.4	62.3±8.1/ 86.3±12.1	74.2±4.0	1.0±0.4

[1]ME, Maine; MA, Massachusetts; WV, West Virginia.
[2]Root segments were placed horizontally in sand or growing medium (Metro Mix 700) for production of adventitious shoots.
[3]Root sprout clusters: one to seven shoots (average of two) arising from the same origin on a root segment.
[4]Shoot tips were cultured and multiplied on aspen culture (AC) medium (Ahuja, 1983) supplemented with 0.89 μM 6-benzyladenine and 0.27 μM α-naphthaleneacetic acid.
[5]Rooting was achieved by dipping the basal end of shoots into 12.3 mM indole-3-butyric acid for 1 min, followed by culture in a Horticube saturated with half-strength AC medium containing 20 g l^{-1} sucrose.
[6]Plantlet survival recorded after 3 months in the growth room.

tablished *in vitro* and rooted plantlets produced. Aspen culture (AC) medium containing 0.89 μM BA and 0.27 μM NAA proved to be a suitable medium for establishing and multiplying cultures, and a 1 min dip in 12.3 mM IBA induced rooting. However, optimum conditions have yet to be determined. Ahuja (1984) reported differentiation of a few shoots from bud explants, taken from a mature tree of *F. sylvatica* var. *purpurea*, by culture on Woody Plant medium (WPM) supplemented with 2.22 μM BA, 0.11 μM NAA, and 0.09 μM 2,4-dichlorophenoxyacetic acid. Chalupa (1985), using shoot tips and nodal segments from juvenile seedlings of *F. sylvatica*, reported multiple shoot production on several media containing 0.44–2.7 μM BA and 0.49–0.98 μM IBA. Nadel et al. (1991a,b) found AC medium superior for the culture of buds from adult trees of *F. sylvatica*. Aspen culture (AC) medium supplemented with indole-3-acetic acid, gibberellic acid, and zeatin (all at 5 μM) enhanced shoot elongation and leaf growth (Nadel et al., 1991a). Meier and Reuther (1994) were able to establish about 14% of their 51 grafted mature *F. sylvatica* genotypes *in vitro*, which closely correlates with our results (15%) for *F. grandifolia*. Woody plant medium containing 1.5% fructose and 4.5 μM BA proved to be more suitable for establishing cultures of *F. sylvatica* mature genotypes, over several other media formulations tested (BTM, Chalupa, 1981; GD, Gresshoff & Doy, 1972; MS, Murashige and Skoog, 1962; SH, Schenk & Hildebrandt, 1972). Rooting (65–75%) was achieved by dipping shoots into 4.92 mM IBA for 30 s, followed by culture on WPM without growth regulators (Meier and Reuther, 1994). Studies using sprouts from roots of *F. sylvatica* have not been reported, perhaps because unlike *F. grandifolia* this species does not usually produce root sprouts in nature (Houston, pers. comm.).

In our study both juvenile and mature genotypes could be propagated *in vitro*. The selection of genotypes greatly influenced success of *in vitro* propagation of *F. grandifolia*. Although only two culture media formulations were tested in this study, the suitability of different basal media seems to depend upon genotype and age and type of explant chosen for cul-

ture. Further experimentation with a wider variety of basal media formulations may allow for propagation of other genotypes selected for resistance to BBD. Further studies to facilitate the transfer of rooted plantlets to soil and ultimately to forest situations is also necessary.

Acknowledgements

We thank Dr. Barbara M. Reed (USDA-ARS) for critical review of the manuscript and Dr. Darroll D. Skilling for support.

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