Regenerating Plants from in Vitro Culture of Black Locust Cotyledon and Leaf Explants

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Abstract. A protocol to achieve efficient plant regeneration from juvenile black locust (*Robinia pseudoacacia* L.) explants is described. Direct adventitious shoots were induced from cotyledon explants on woody plant medium containing 22.2 μM BA and 0.4 μM 2,4-D. Shoots developed and new shoots were induced when the explants were transferred to medium without growth regulators. The effect of dark incubation on shoot regeneration from cotyledons indicated that 15 days of darkness resulted in a high regeneration frequency (91.7%). Adventitious shoot formation also was induced from sections of in vitro-derived leaves cultured in darkness on Murashige and Skoog medium supplemented with 4.4 μM BA and 24.6 μM IBA. A shoot regeneration frequency of 89% was obtained when explants were subcultured on a medium containing 4.4 μM BA and 0.5 μM IAA. Shoots were rooted on Schenk and Hildebrandt medium with or without IBA. Plantlets were acclimatized and grown in the greenhouse. Chemical names used: *N*-(phenylmethyl)-1*H*-purin-6-amine (BA); 2,4-dichlorophenoxyacetic acid (2,4-D); indole-3-acetic acid (IAA); indole-3-butyric acid (IBA).

Black locust, an N-fixing leguminous forest tree species native to the eastern United States, is cultivated extensively as an ornamental tree in many parts of the world. This species is one of the leading candidates for woody biomass energy plantations in the United States (Ranney et al., 1988) and Europe (Mitchell, 1988). Black locust generally is propagated by seeds and via root or stem cuttings. Three in vitro regeneration pathways have been reported: axillary bud elongation and multiplication from nodal or bud explants (Barghchi, 1987; Chalupa, 1983, 1987; Davis and Keathley, 1987, 1988); differentiation of adventitious shoots from callus cultures, leaf disks, or hypocotyl explants (Brown and Sommer, 1982; Davis and Keathley, 1985; Han and Keathley, 1989); and somatic embryogenesis (Merkle and Wiecko, 1989).

One of the protocols for *Agrobacterium*-mediated gene transfer in plants involves in-

oculating leaf-disk explants with the bacterium and regenerating transformed shoots on a selection medium (Horsch et al., 1985). Davis and Keathley (1985) and Han and Keathley (1989) reported low shoot regeneration from black locust leaf disks and hypocotyl-derived callus. Therefore, developing an efficient black locust regeneration system may be a route for genetic transformation. In this paper, we report on experiments in which plantlets were regenerated from black locust cotyledon and in vitro-derived leaf explants.

Material and Methods

Plant material. Mature black locust pods were collected in Sept. 1991 from Family 698 members that were growing in a range-wide planting in Greene County, Ga. Family 698 was derived from open-pollinated seeds collected in 1981 from a tree growing in a natural stand in Tallapoosa County, Ala. Seeds were removed from pods and stored at 4°C until used. Before disinfection, seeds were scarified with concentrated H_2SO_4 for 20 min and rinsed with running tap water for 20 min. For surface disinfection, seeds were immersed in the following solutions: 70% ethanol (30 sec), 10% Roccal (National Laboratories, Montvale, N.J.) (5 min), 100% Clorox (5.25% sodium hypochlorite) (5 min), a sterile water rinse (3 min), 0.01 M HCl (3 min), and three additional sterile water rinses (3 min each). Then, seeds were imbibed overnight in sterile

distilled water. After imbibition, seeds were surface disinfested again as described above, but 50% Clorox was substituted for full-strength Clorox. Seedcoats were removed and embryos were germinated in vitro or used as a source of cotyledon explants. Germinated embryos were used to initiate shoot proliferation cultures that, in turn, were used as a source of leaf explants (see below). For germination, embryos were cultured for 30 days in tubes containing 20 ml of a sterile solution of 3% sucrose with 0.7% Phytagar (Gibco, Life Technologies, Grand Island, N.Y.). To initiate shoot proliferation, shoot-tips isolated from the germinants were cultured for 30 days on medium containing Schenk and Hildebrandt (SH) macro salts (Schenk and Hildebrandt, 1972), Murashige and Skoog (MS) micro salts (Murashige and Skoog, 1962), Gresshoff and Doy (GD) vitamins (Gresshoff and Doy, 1972), 4.4 μM BA, 3% sucrose, and 0.7% Phytagar (SH medium).

Cotyledon culture. Cotyledons were excised from the embryo axis and distal and proximal ends were cut off. Cotyledon sections (≈ 4 mm long) were cultured in 60 $\times$ 15-mm petri plates containing 10 ml basal medium (BM) consisting of woody plant medium (WPM) (Lloyd and McCown, 1980) salts, GD vitamins, 3% sucrose, and 1 g-liter⁻¹ each of casein hydrolysate (CH) and myo-inositol. The pH was adjusted to 5.7, and the medium was solidified with 0.8% Phytagar before being autoclaved for 20 min at 120°C. BM was supplemented with 0.4, 4.4, or 22.2 μM BA combined with 0.5, 5.4, or 26.8 μM l-naphthaleneacetic acid (NAA) or 0.4, 4.5, or 22.6 μM 2,4-D. Three explants, adaxial surface down, were inoculated per plate and five plates were used for each treatment. Cultures were incubated under a 16-h photoperiod (100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) provided by cool-white fluorescent lamps (Sylvania F20T12/cool white; GTE Products Corp., Danvers, Mass.) or in darkness at 26 $\pm$ 2°C. In a second experiment, the effect of dark incubation was tested. Cotyledon sections were cultured on BM supplemented with 22.2 μM BA and 0.4 μM 2,4-D. Five dark pretreatments were used, during which culture plates (four plates with three explants each) were incubated in darkness for 0, 5, 15, or 30 days and subsequently transferred to a 16-h photoperiod. For the control treatment, cotyledons were maintained in darkness for the duration of the experiment. In both experiments, explants were transferred to BM without growth regulators after 45 days of culture, and morphogenic responses were recorded after 90 days.

Leaf culture. Leaf segments (≈ 3 mm long), excised from the in vitro-proliferated shoots produced as described above, were cultured in 100 $\times$ 15-mm petri plates with 25 ml BM consisting of MS salts, GD vitamins, 3% sucrose, and 0.8% Phytagar. The effect of organic N was tested by adding CH (1 g-liter⁻¹), glutamine (10 or 20 mM), or no organic N to the medium. Both N sources were added to the medium before autoclaving. Initially, the medium was supplemented with 4.4 μM BA and either 9.8 or 24.6 μM IBA. Four explants,

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abaxial surface down, were cultured per plate and six plates were used for each treatment. After 30 days, explants were subcultured to the same BM without growth regulators or supplemented with 4.4 μM BA and 0.6 μM IAA and incubated for 60 days. All plates were placed in darkness at $26 \pm 2^\circ\text{C}$.

Rooting and transfer to potting mix. To induce rooting, cotyledon- and leaf-derived shoots >1 cm long were excised and cultured under a 16-h photoperiod in tubes containing 20 ml of Phytagar-solidified SH medium without growth regulators. The effect of 2.5 μM IBA on rooting also was tested on shoots regenerated from leaf explants. After 60 days, rooted plants were transplanted to pots containing Peat-lite mix (Conrad Fafard, Agawan, Mass.). Subsequently, the plants were acclimatized for 2 months in a controlled-environment room to ambient relative humidity and transferred to the greenhouse.

Statistical analysis. All experiments were conducted twice using a completely random design. The significance of treatment effects on the caulogenic response (percentage of caulogenic explants and mean number of shoots per explant) was determined using analysis of variance. The effect of dark pretreatment duration on these responses was analyzed using polynomial regression [Systat 5.2 (Systat, 1992) and Super ANOVA 1.01 (Abacus Concepts, 1989) software]. Percentage data were subjected to arcsin transformation before statistical analysis. Variation between treatment means was analyzed using Tukey's (1953) procedure.

Results and Discussion

Cotyledon cultures. In 5 days, explants had enlarged and elongated. Morphogenic responses required the presence of growth regulators in the culture medium. Callus, roots, and shoots formed within 15 to 20 days after culture initiation. In general, darkness increased callus and root formation, and BA levels >0.4 μM inhibited this organogenic response (data not shown). In most cases, shoot formation occurred directly from intact surfaces and cut areas of the explants (Fig. 1). Occasionally, indirect shoot organogenesis, via callus, was observed. Transferring explants to a medium without growth regulators induced additional shoots to develop.

Dark incubation significantly enhanced caulogenesis (percentage of explants forming shoots and mean number of shoots per explant) on cotyledons (Tables 1 and 2). Analysis of variance for both responses also showed a significant three-way interaction among auxin type, auxin concentration, and BA concentration (Table 2). Thus, irrespective of illumination conditions, BA levels >0.4 μM increased caulogenic percentages from explants cultured on media containing the lowest auxin concentration (0.5 μM NAA or 0.4 μM 2,4-D). This promotive effect was not altered significantly by higher NAA concentrations. In contrast, the highest 2,4-D concentration (22.6 μM) drastically reduced the caulogenic response (10.0% vs. 56.6%) when cotyledons were cul-

tured with 22.2 μM BA (Table 1). In the same way, none of the BA and NAA combinations tested significantly affected the mean number of shoots per explant, while combinations of BA and 2,4-D (4.4 and 22.6 or 22.2 and 4.5 μM , respectively) significantly lowered this number compared to that obtained in the presence of 0.4 μM 2,4-D (1.1 vs. 7.8 and 3.0 vs. 8.0 shoots) (Table 1). Shoot length was variable and ranged from 0.5 to 8 cm (data not shown). Shoots grown in darkness were etiolated and about six times longer than those grown in light.

The effect of darkness of various durations on adventitious shoot formation from cotyledons was best described by a cubic model ($P \leq 0.0015$, $r^2 = 60.1\%$) for the caulogenic re-

sponse, with an optimum at 15 days in darkness (91.7%). Dark periods longer than 15 days decreased this effect, with equal responses at 30 and 90 days. In contrast, data for the mean number of shoots per explant did not fit any of the regression models tested (Table 3). Initial darkness enhanced caulogenesis in other legumes (Barwale et al., 1986; Mohamed et al., 1992). Two weeks of darkness also increased caulogenesis in *Malus* spp. leaf cultures (Fasolo et al., 1989; Korban et al., 1992).

Leaf culture. As with the cotyledons, caulogenesis occurred on the surface of the explants or in cut areas. Adventitious buds were macroscopically visible after 20 days of culture. Callus and root formation were observed only on leaves cultured on media lacking glutamine.

Fig. 1. Adventitious shoot formation on a black locust cotyledon explant cultured under a 16-h photoperiod on woody plant medium. Bar = 1 mm.

Table 1. Effect of growth regulators and illumination conditions on the caulogenic response of black locust cotyledon cultures. Culture time was 90 days. Values are means of five experimental replications with three explants each.

Growth regulators			Explants forming shoots (%)			Mean no. shoots/explant		
BA	2,4-D	NAA	Photoperiod			Photoperiod		
			Dark	(16 h)	Mean ²	Dark	(16 h)	Mean ²
---	---	---	0.0	0.0	0.0	0.0	0.0	0.0
0.4	---	0.5	0.0	0.0	0.0 a	0.0	0.0	0.0 a
	---	5.4	0.0	0.0	0.0 a	0.0	0.0	0.0 a
	---	26.8	6.7	0.0	3.3 ab	0.2	0.0	0.1 a
	0.4	---	6.7	6.7	6.7 a-c	0.2	0.2	0.2 a
	4.5	---	40.0	30.0	35.0 a-e	1.0	0.7	0.8 ab
	22.6	---	50.0	20.0	35.0 a-e	2.3	0.8	1.5 ab
4.4	---	0.5	46.6	26.6	36.6 b-e	5.4	2.3	3.8 a-c
	---	5.4	40.0	6.7	23.3 a-e	2.5	0.8	1.6 ab
	---	26.8	53.3	20.0	36.6 b-e	2.8	1.6	2.2 ab
	0.4	---	60.0	30.0	45.0 de	12.5	3.2	7.8c
	4.5	---	40.0	20.0	30.0 a-e	6.7	1.0	3.8 a-c
	22.6	---	13.3	26.6	19.9 a-e	1.4	0.8	1.1 ab
22.2	---	0.5	40.0	6.7	23.3 a-e	2.1	0.2	1.1 ab
	---	5.4	66.6	20.0	43.3 b-e	5.6	1.5	3.4 a-c
	---	26.8	66.6	13.3	39.9 b-e	7.7	2.8	5.2 bc
	0.4	---	80.0	33.3	56.6 e	10.0	6.0	8.0 c
	4.5	---	36.6	59.2	48.2 de	3.9	2.1	3.0 ab
	22.6	---	20.0	0.0	10.0 a-d	4.2	0.0	2.1 ab

²Interaction of auxin type $\times$ auxin concentration $\times$ BA concentration. Mean separation in columns by Tukey's test ($P \leq 0.05$). Treatments without growth regulators were omitted from the analysis.

Table 2. Analysis of variance for Table 1 data.^a

Source of variation	df	Mean square	
		Caulogenesis (%)	Mean no. shoots
Photoperiod (A)	1	12792.3**	278.3**
Auxin type (B)	1	3411.8*	65.8**
Auxin level (C)	2	308.6 ^{NS}	40.3*
BA level (D)	2	7713.6**	202.7**
A × B	1	1061.9 ^{NS}	13.8 ^{NS}
A × C	2	154.0 ^{NS}	3.7 ^{NS}
A × D	2	1285.0 ^{NS}	52.2**
B × C	2	1526.1 ^{NS}	80.9**
B × D	2	2086.7*	3.0 ^{NS}
C × D	4	1728.8*	33.4**
A × B × C	2	677.8 ^{NS}	7.9 ^{NS}
A × B × D	2	1128.0 ^{NS}	13.2 ^{NS}
A × C × D	4	690.8 ^{NS}	18.2 ^{NS}
B × C × D	4	1991.6**	43.7**
A × B × C × D	4	804.2 ^{NS}	7.3 ^{NS}
Error	144	483.1	9.4

^aTreatments without growth regulators were omitted from the analysis.

^{NS},*,**Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

Table 3. Effect of dark incubation on shoot formation in black locust cotyledon cultures. Cotyledons were cultured on woody plant medium supplemented with 22.2 μ M BA and 0.4 μ M 2,4-D for 45 days and then transferred to medium without growth regulators. Values are means of four replications with three explants each. Data were recorded after 90 days.

Dark incubation (days)	Caulogenic explants (%)	Mean no. shoots/explant $\pm$ SD
0	25.0	2.0 $\pm$ 1.6
5	50.0	2.4 $\pm$ 1.7
15	91.7	6.6 $\pm$ 0.8
30	75.0	2.5 $\pm$ 0.8
90	75.0	4.5 $\pm$ 2.0
Contrast	C	NS

^{NS}Nonsignificant; C = cubic.

The percentage of leaf explants forming shoots and the mean number of shoots per explant significantly decreased on media containing glutamine (Tables 4 and 5). Irrespective of the organic N source, adding 4.4 μ M BA and 0.6 μ M IAA to the subculture medium significantly enhanced the caulogenic response (35.3% vs. 58.7%). This BA- and IAA- supplemented medium also increased the mean number of shoots that formed on explants initially cultured with 24.6 μ M IBA and 4.4 μ M BA (Tables 4 and 5). Adding glutamine to the culture medium also resulted in fewer adventitious shoots in Hinoki cypress (*Chamaecyparis obtusa* Endle.) (Ishii, 1986). In contrast, glutamine benefited shoot elongation and proliferation in other legumes (Dhawan and Bhojwani, 1985; Tabone et al., 1986). Wright et al. (1987) reported that pyroglutamic acid (a compound formed as a result of the chemical transformation of glutamine during autoclaving) stimulated caulogenesis on soybean [*Glycine max.* (L.) Merr.] leaf cultures, while filter-sterilized glutamine inhibited regeneration.

Rooting and transfer to potting mix. In cotyledon- and leaf-derived shoots, roots occasionally formed on the base and on the elongated stems of individual shoots after 30 days on medium without growth regulators. Seventy-one percent to 73% of shoots cultured on SH medium without growth regulators rooted after 60 days. Adding 2.5 μ M IBA to the

rooting medium slightly increased the percentage of rooting in shoots obtained from leaf-explant cultures (83.9%). The mean number of roots per rooted shoot was variable, ranging from 1 to 6. All transplants survived during acclimatization.

Our study achieved direct caulogenesis from black locust cotyledon and leaf explants. Because different media and treatments were

used, the results from the two groups of explants are not comparable. The procedures described herein improved the number of caulogenic explants obtained compared with those previously obtained with leaf (Davis and Keathley, 1985) or hypocotyl (Han and Keathley, 1989) explants of this species. Differences could be attributed to the genotype, illumination conditions, and culture media used. The higher shoot regeneration frequency reported here should be useful in experiments to produce transgenic black locust plants.

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Table 4. Effect of growth regulators and organic N sources on shoot formation in black locust leaf cultures. Explants were cultured for 30 days on MS medium containing one of several organic N sources and supplemented with IBA and BA, then transferred to the same medium without growth regulators or with BA and IAA. Values are means of three replications with four explants each.

Growth regulators (μM)		Organic N source ^z				Mean ^y
Initial culture	Subculture	None	CH	G (10 mM)	G (20 mM)	
Caulogenic explants (%)						
BA/IBA (4.4/9.8)	None	76.7	66.7	25.0	0.0	
	BA/IAA (4.4/0.6)	78.3	58.3	58.3	33.6	
BA/IBA (4.4/24.6)	None	61.1	52.8	0.0	0.0	
	BA/IAA (4.4/0.6)	88.9	72.2	50.0	30.6	
	Mean ^x	76.2 b	62.5 b	33.3 a	16.0 a	
Mean no. shoots/explant						
BA/IBA (4.4/9.8)	None	1.6	1.0	1.8	0.0	1.1 a
	BA/IAA (4.4/0.6)	3.7	1.8	2.3	0.5	2.1 a
BA/IBA (4.4/24.6)	None	1.7	1.0	0.0	0.0	0.7 a
	BA/IAA (4.4/0.6)	9.1	6.4	1.8	1.7	4.8 b
	Mean ^x	4.0 b	2.5 ab	1.5 a	0.6 a	

^aCH = casein hydrolyzate; G = glutamine.

^bInteraction of growth regulators in the initial culture $\times$ growth regulators in the subculture medium. Mean separation for each entry by Tukey's test ($P \leq 0.05$).

^cEffect of N source.

Table 5. Analysis of variance for Table 4 data.

Source of variation	df	Mean square	
		Caulogenesis (%)	Mean no. shoots
Organic N source (A)	3	5997.0**	26.8**
Growth regulators in initial medium (B)	1	234.0 ^{ns}	15.2*
Growth regulators in subculture medium (C)	1	6248.1**	77.5**
A × B	3	159.1 ^{ns}	9.6 ^{ns}
A × C	3	477.4 ^{ns}	9.0 ^{ns}
B × C	1	487.5 ^{ns}	28.8**
A × B × C	3	129.2 ^{ns}	3.5 ^{ns}
Error	80	271.0	3.6

^{ns},*,**Nonsignificant or significant at $P \leq 0.05$ or 0.01 , respectively.

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