In Vitro Cell. Dev. Biol.—Plant 33:38–42, January 1997
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1071-2690/97 $05.00+0.00

IN VITRO PROPAGATION OF *PITHECELLOBIUM SAMAN* (RAINTREE)

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(Received 15 September 1995; accepted 20 September 1996; editor T. A. Thorpe)

Summary

Plants were obtained via organogenesis from hypocotyl explants of *Pithecellobium saman* (Jacq.) Benth. (raintree) on Murashige and Skoog (1962) medium supplemented with N^6-benzyladenine (BA). Adventitious bud induction was affected by mineral salts and benzyladenine concentrations, explant age, position of explant on the medium, and BA exposure time. The best results were obtained with explants cultured on half-strength MS medium containing 26.6 μ*M* BA, with explants of 5 and 10 d after germination placed horizontally and a 7 d-exposure period to BA. The proximal and intermediate section of hypocotyls showed the highest organogenic response. Bud development and shoot elongation was promoted by increased concentrations of activated charcoal in the culture medium. Gibberellic acid had no effect on shoot development. Rooting percentages decreased when shoots were exposed for more than 24 h to indole-3-butyric acid (IBA). Maximum rooting was obtained with 369.0 μ*M* IBA. The aerial and root systems of greenhouse plantlets were similar to those of seedlings.

Key words: Leguminosae; organogenesis; adventitious bud induction; activated charcoal; benzyladenine.

Introduction

For many years, tropical forest experts have used exotic species for reforestation programs. Unfortunately, due to pests and diseases or adaptation problems observed in many of these plantations, the interest has switched back to native species. *Pithecellobium saman* (Jacq.) Benth. (raintree) is a native hardwood, characterized by its high commercial value. It is a leguminous species which fixes nitrogen and therefore has a positive effect on soil fertility.

Although this species is still abundant in our forests, the constant deforestation process is threatening its genetic variability. One of the main problems faced by people involved with reforestation is the lack of good quality seeds. Because of commercial interest or insufficient experience, such situations have led to the use of seeds with poor genetic qualities (Salazar and Boshier, 1989). This problem is critical for tropical America's native species, because there is no appropriate area dedicated to the production of quality seeds, which most of the time are collected from any tree. Taken into account that trees have long vegetative cycles, the consequences of selecting inadequate seeds can be observed only when the trees have reached maturity, resulting in economic losses and rejection of forest plantations by the public.

Most forest species reproduce sexually through open pollination, allowing continuous variation of several characters in subsequent generations. Desirable characteristics could be maintained in the progeny by vegetative or clonal propagation of elite trees (Hartman and Kester, 1975). Tissue culture techniques offer great potential for the multiplication of forest species and can be helpful to traditional genetic improvement programs, especially in the area of genotype screening for growth rates, disease resistance, and drought tolerance. Also, it would be possible to find beneficial somatic mutations and somaclonal variations if they are present. *In vitro* techniques can contribute to the propagation of large numbers of superior individuals or so called "plus trees." In the present study we describe suitable methodology for micropropagation of *P. saman*, a hardwood.

Materials and Methods

Plant material and culture conditions. Pithecellobium saman (Jacq.) Benth. seeds stored at a temperature of 5° C at CATIE's Latin American Forest Seed Bank were used for this research. The seeds were washed with water for 30 min. They were then rinsed in 70% alcohol for 3 s and immersed for 20 min in a 5.5% commercial sodium hypochlorite solution. Finally, they were rinsed three times with sterilized distilled water in a laminar flow hood.

The seeds were germinated in the dark under aseptic conditions in agar and water-based medium. After 3 d of germination, their hypocotyls were divided into 1.5-cm-long sections and used as explants.

All experiments were conducted at 27 ± 1° C, 87 ± 3% relative humidity, under 16-h photoperiod and 55–62 μmol $m^{-2} s^{-1}$ photosynthetic photon flux density provided with fluorescent cool light.

Culture medium. The Murashige and Skoog basal medium (MS, Murashige and Skoog, 1962) was used, and each liter was supplemented with 2 mg nicotinic acid, 2 mg pyridoxine-HCl, 10 mg thiamine-HCl, 4 mg glycine, 200 mg *myo*-inositol, 3% sucrose and 0.7% agar (Agar-agar, Sigma Chemical Co., St. Louis, MO). The pH was adjusted to 5.8 with 1 *N* NaOH or HCl before autoclaving at 121° C for 20 min. Sterilized medium was dispensed into plastic petri dishes (150 × 15 mm).

Bud induction. Explants were cultured in petri dishes containing four concentrations (25%, 50%, 75%, and 100%) of basal medium in combination with five N^6-benzyladenine (BA) concentrations (8.8, 17.7, 26.6, 35.5, and 46.6 μ*M*). Effects of factors such as explant age, position of the bud on the plant, explant orientation in the medium, or BA exposure period were studied in independent experiments in which the best BA concentration and MS salts dilution were used.

For studying the effect of explant age, we cultured hypocotyl sections 5, 10, 15, and 20 d after germination. Five-d-old hypocotyls were sectioned into proximal, intermediate and distal parts, each of which was inserted either horizontally or vertically in the culture medium.

Finally, 5-d-old hypocotyl explants placed horizontally in the medium were transferred to a basal hormone-free medium after 7, 14, 21, and 28 d of

culture so that the effect of time of exposure to BA could be studied. The optimum time, concentrations of basal medium and BA, and the best position and orientation producing the best explant were then determined.

Development of adventitious buds and shoot elongation. After 4 or 5 wk, induced buds approximately 0.3 to 1.0 cm long were placed in full-strength basal medium (without BA) containing 0, 0.5, 1.0, 2.5, or 5.0 g activated, neutralized charcoal (Merck & Co., Inc., Rahway, NJ). In another experiment the effect of 1.4, 2.8, 4.3, 5.7, 144.4, 288.8, or 433.0 μ*M* gibberellic acid (GA_3) on growth was tested. Filter-sterilized GA_3 was added to the sterilized culture medium, and buds were incubated in the dark for the first 12 d.

Rooting of shoots. After 4 wk, elongated shoots with a distinct stem were cultured for 24, 48, or 72 h in a full-strength MS medium containing four indole-3-butyric acid (IBA) concentrations (123.0, 246.0, 369.0, or 492.0 μ*M*), plus 2% sucrose and 0.05% Gelrite in 2.0 × 9.5 cm test tubes. Shoots ranged from 1.0 to 1.5 cm long with from one foliar primordium to three pairs of leaves. Twenty replicates and one experimental unit were used.

Data collection and statistical analysis. For each experiment, the percentage of explants with buds, total number of buds per explant, average number of buds per explant, and number of developed buds were recorded. The data were recorded 4 wk after culture initiation. After the rooting period of 4 wk, the rooting percentage was evaluated. Leaf number from 20 replicates and 1 experimental unit was recorded after 40 d of culture.

A completely random design with 15 replicates with 3 experimental units was used for most of the experiments. To assess the effects of strength of the medium and BA concentration on bud induction and IBA concentration on the rooting process, 4 × 5 and 4 × 3 factorial designs were used, respectively. There were four replicates and five experimental units in these experiments. The statistical treatment consisted mainly of analysis of variance (ANOVA), with Tukey's honestly significant difference criterion and regression analysis used as needed. The data requiring conversion were transformed as $x = \sqrt{x} + 0.5$ (SAS Institute, 1985).

RESULTS

Bud induction. This species showed a significant organogenic response. Adventitious bud development was observed with all MS dilutions as well as all BA concentrations. Analysis of variance indicated that the effect of the BA level in each salt dilution was significant ($P = 0.01$) for the number of buds. For MS diluted to 25%, the best BA concentration was 17.7 μ*M* whereas for a half-strength salt solution, 26.6 μ*M* BA gave better results. The MS salts diluted to 75% showed the highest number of buds with 8.8 μ*M*. A much lower response was observed for 44.4 μ*M* BA. The complete MS salt solution showed the best results with 35.5 μ*M* BA; also, at this concentration, the highest percentage of organogenic explants was observed (Fig. 1).

Effect of explant age. Explant age affected the number of buds produced: 5-, 10- and 15-d-old explants produced the highest number of adventitious buds (Table 1). Explant age did not affect other parameters. Twenty-d-old explants frequently showed hyperhydric buds. Shoot elongation was best on 5-, 10-, 15-d-old explants.

Effect of hypocotyl position on plant and orientation on medium. The total number of buds, percentage of explants with buds, and the total number of developed buds per explant in the proximal and intermediate sections were significantly different from those in the distal section (Table 2). The explant's orientation on the medium affected the time required for bud formation, the number of buds, and bud development (data not shown). In a horizontal orientation, buds were visible after 24 to 27 d of culture, whereas in a vertical orientation it took about 37 to 42 d of culture. Buds developed from the explant's upper side. Seventy percent of explants were organogenic in the horizontal orientation and only 20% in the vertical position. The number of adventitious buds observed from horizontal and vertical orientations was 28 and 16, respectively.

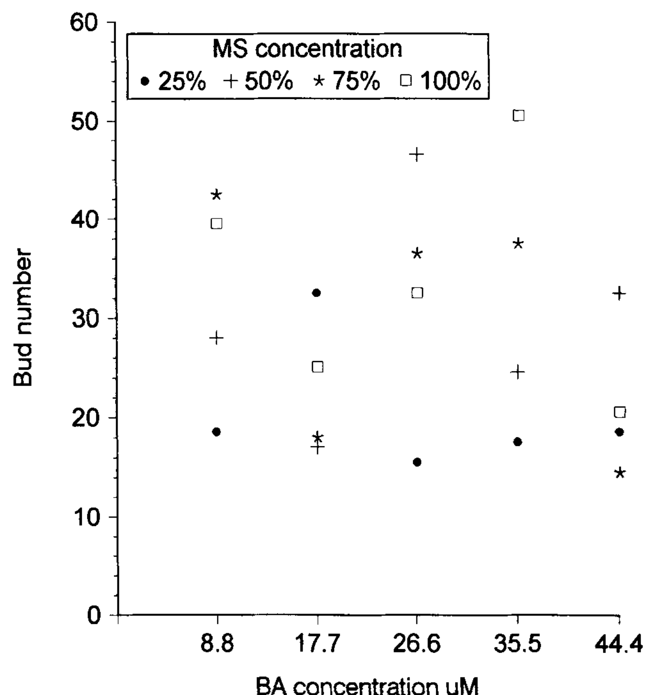

FIG. 1. Means of the total number of buds formed on *P. saman* hypocotyl explants and effect of BA and MS concentrations. Data were collected at the end of induction phase (30 d). Statistical differences between factors interaction were significant (analysis of variance, $P = 0.01$).

TABLE 1

EFFECT OF AGE OF EXPLANT ON BUD INDUCTION OF *P. SAMAN*[a]

Age (days)	Percent Explants with Buds	Total Number of Buds	Average Number of Buds Per Explant	Total Number of Developed Buds
5	66.6	124	5.9	7.0
10	66.6	117	6.2	9.0
15	57.6	112	6.9	3.3
20	66.6	85[b]	3.3	4.0

[a]Explants were cultured in half-strength MS + 26.6 μ*M* BA. Data for all parameters were collected after 4 weeks.
[b]Means significantly different at $P = 0.05$.

TABLE 2

EFFECT OF THE HYPOCOTYL SECTION ON PLANT ON BUD REGENERATION EFFICIENCY OF *P. SAMAN*

Hypocotyl[a] Section	Percent Explants with Buds	Total Number of Buds	Average Number of Buds Per Explant	Total Number of Developed Buds
Proximal	53.0 z[b]	76.0 z	4.8 z	8.0 z
Intermediate	46.6 z	72.0 z	5.0 z	13.0 z
Distal	20.0 y	26.0 y	4.3 z	2.0 y

[a]Explants were cultured on half-strength MS + 26.6 μ*M* BA. Data for all parameters were collected after 4 weeks.
[b]Means followed by different letters are significantly different at $P = 0.05$.

TABLE 3

EFFECT OF EXPOSURE PERIOD TO BA ON BUD INDUCTION IN EXPLANTS OF *P. SAMAN*[a]

Period (days)	Percent Explants with Buds	Total Number of Buds	Average Number of Buds Per Explant	Total Number of Developed Buds
7	53.3	53.0	3.5	8.0[b]
14	30.0	49.0	5.5	3.0
21	43.3	53.0	4.7	5.0
28	40.0	51.0	4.5	4.0

[a]Explants were cultured on half-strength MS + 26.6 μM BA. Data for all parameters were collected after 4 weeks.
[b]Means significantly different at $P = 0.05$.

TABLE 4

EFFECT OF ACTIVATED CHARCOAL CONCENTRATION ON LEAF FORMATION FROM BUDS OF *P. SAMAN*

	Activated Charcoal Concentration (mg l^{-1})				
	0	0.5	1.0	2.5	5.0
Average number of leaves[a]	1.0	2.0	2.1	3.3	4.5
Transformed mean	1.2	1.5	1.6	1.9	2.2

[a]The shoots were elongated first on MS + activated charcoal for 4 weeks, then on half-strength MS + 26.6 μM BA after 4 weeks. Data are mean values from 20 replicatons.

TABLE 5

STATISTICAL ANALYSIS OF DATA FROM EXPERIMENTS ON EFFECT OF ACTIVATED CHARCOAL CONCENTRATION ON LEAF FORMATION FROM BUDS OF *P. SAMAN*

Source of Variation	df	Mean Squares	F value
Concentration	4	0.71818832	6.65**
Error	96	0.10797281	
Corrected total	99		

**Significant at $P = 0.01$.

Effect of BA exposure period. The BA exposure period did not influence adventitious bud formation. Primordium differentiation was observed after 25 d of culture and no visual differences were detected between treatments. The only statistically significant result was the total number of developed buds after 7 d of exposure to BA (Table 3).

Effect of activated charcoal and GA_3 concentrations on development of adventitious buds and shoot elongation. Adventitious bud development was better when activated charcoal was added to the culture medium. Without activated charcoal, plantlets showed slower, less vigorous growth. There was a positive effect of activated charcoal concentration on the average number of leaves per plant (Tables 4 and 5). When explants with developed adventitious buds were cut into sections containing *de novo* buds, then placed in a culture medium with the best activated charcoal concentration (5 g/l), more buds gradually developed. After subculture, other buds developed. Best results were obtained when buds were transferred to fresh medium containing activated charcoal every 15 to 22 d. GA_3 had no effect on *P. saman* bud elongation. With the GA_3 concentrations tested, buds did not develop; on the contrary, a translucent callus was formed at the base of each explant. Some buds exhibited weak development of one or two malformed leaves.

Rooting: Effect of concentration and IBA exposure period. IBA was an effective root inducer for *P. saman* (Fig. 2). Effect of the exposure period to IBA was highly significant at $P = 0.01$, but IBA concentrations and exposure period–concentration interaction was not. The exposure period showed a significant ($P = 0.05$) negative lineal tendency on the percentage of shoots that rooted. The highest rooting percentage (63%) was observed with a 24-h exposure to 369 μM IBA and the lowest (11%) with a 72-h exposure to 492 μM IBA (Fig. 3). Because differences between the various concentrations were not significant, it was not possible to find an optimum concentration; however, with 492.0 μM of IBA, necrotic spots were often observed at the base of the stem. This was more frequently observed in buds exposed for 72 h to IBA. Necrosis was accentuated with 72 h exposure to IBA. At 123.0, 246.0, and 396.0 μM IBA, roots were thicker, longer, and more branched.

Growth of plantlets in the greenhouse. After 8 wk, rooted shoots were directly transferred to *ex vitro* conditions in mixtures of soil–sand–coffee trash (2:1:1) under nonsterile conditions and irrigated daily. Most of the plantlets lost their *in-vitro*-formed leaves following transfer to soil, but new leaves formed subsequently, and they grew normally with survival percentages of up to 95%. Plants showed higher development when they did not drop their leaves on transfer. During greenhouse growth, shoot leaf number and branch expansion of plantlets was comparable to that of seedlings (Fig. 4 *a*). After 3 mo., leaf number was higher ($P = 0.01$) in the micropropagated plants than in seedlings.

The root systems of both plant types were similar. Roots of *in vitro* plantlets also developed nodules, which is typical of legumes (Fig. 4 *b*).

DISCUSSION

Pithecellobium saman (Jacq.) Benth. showed a high degree of organogenic response for all MS salt dilutions and BA concentrations.

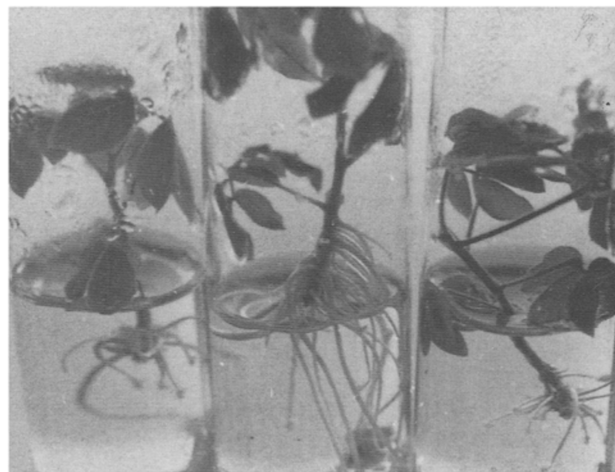

FIG. 2. Rooted shoots of *P. saman* after 8 wk on auxin-containing MS medium plus 2% sucrose.

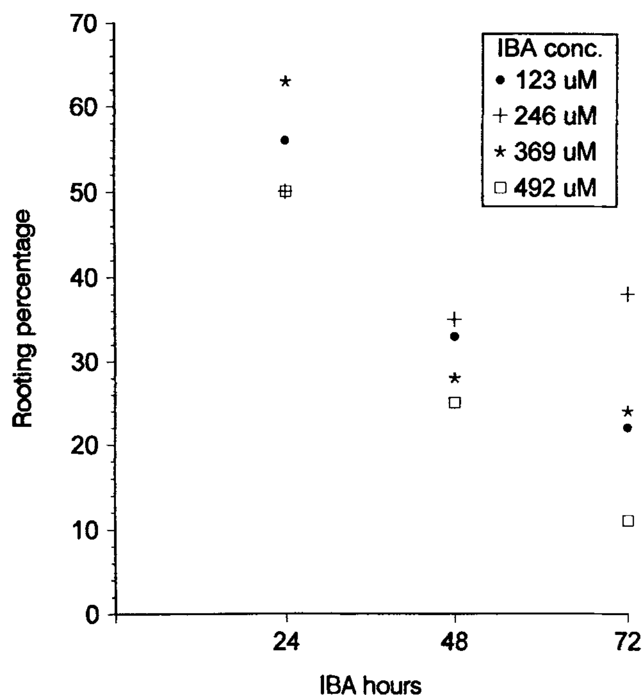

FIG. 3. Effects of the period and concentration of IBA on adventitious buds from cultured hypocotyl explants of *P. saman*. Statistical differences between different period of exposure were significant (analysis of variance, $P = 0.01$).

When the MS salt concentration increased, the need for BA also increased in order for regeneration to occur. The optimum BA concentration ranged between 26.6 and 35.5 μM with 50% and 100% salt dilution, respectively. In *P. jeringa*, Rajadurai et al. (1987) tested different cytokinins, and BA was found to be most effective for promoting bud proliferation. In *P. scalare*, the best culture medium for bud elongation from buds of an adult tree was modified MS, supplemented with BA; epirrhizal sprouts showed bud elongation on MS with naphthaleneacetic acid and BA (Castillo de Meier and Bovo, 1989). Also, BA was required for sustained proliferation of cell colonies of protoplasts from *P. dulce*, and differentiation of shoots from protoplast-derived calluses occurred on MS medium with BA and indole-3-acetic acid (Saxena and Gill, 1987).

Physiological factors such as explant age, the section of the hypocotyl used as explant, and its orientation on the medium play an important role in *P. saman* organogenesis. Explants taken between 5 and 15 d after germination were more organogenic than those taken after 20 d. In other studies, explant age has been found to be a very important factor for regulation of organogenesis, because juvenile tissues have shown a higher regeneration rate aptitude (Villalobos et al., 1984; Webb et al., 1988; Goldfarb et al., 1991; Martínez Pulido et al., 1991). Regarding the section and explant's orientation, polarity was observed within the explant since the distal part of the hypocotyl was less responsive for adventitious bud development, and horizontal placement on the medium favored both the speed of production and number of adventitious buds. In *Pinus strobus*, Webb et al. (1988) observed that bud formation was highly polar and originated mostly in the apical portion of the hypocotyl, and there was basipetal loss of caulogenic capacity. Phillips (1980) has indicated that morphological polarity derives from the polarized transport of growth substances such as auxins.

P. saman hypocotyl explants required only 7 d of exposure to a culture medium containing BA for adventitious bud induction. These results agree with the findings of other authors, e.g., Villalobos et al. (1984), who observed that in *Pinus radiata*, 3 d of exposure to BA was enough to start the morphogenetic process. Also, Martínez Pulido et al. (1991) observed that BA exposure period affected bud number, quality, and growth rate in *Pinus canariensis* cotyledonary explants, with the optimum exposure ranging between 2 and 3 wk.

Activated charcoal had a positive effect on bud development. GA_3 showed no effect, confirming a previous report by Rajadurai et al. (1987) for *P. jeringa*. The specific effect of activated charcoal on plant development is not well understood, but it has been associated with the absorption of compounds from cultivated tissues or from the agar that could inhibit growth (Fridborg et al., 1978; George and Sherrington, 1984). An important property is that it adsorbs growth reg-

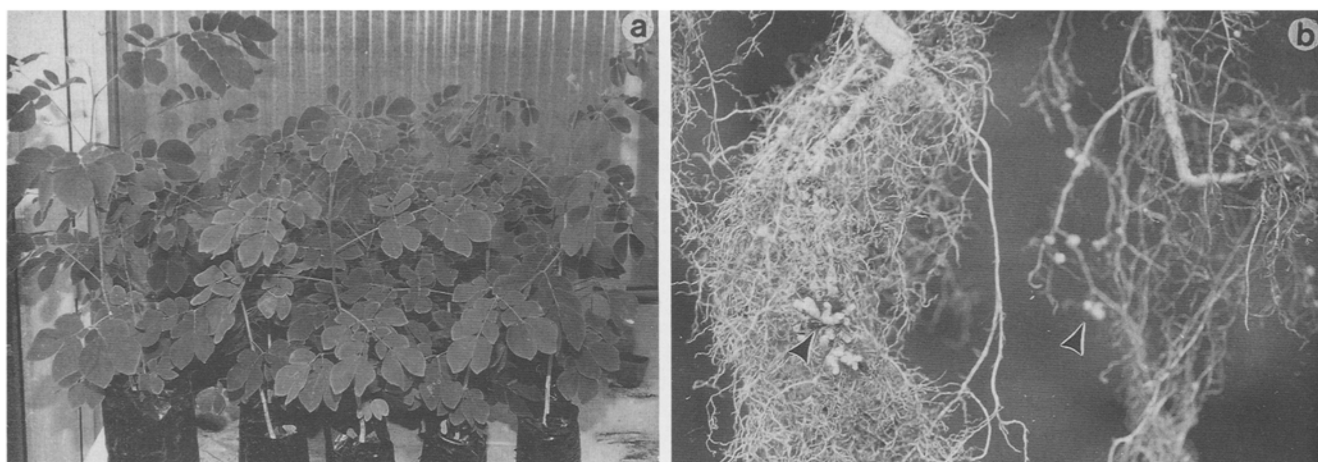

FIG. 4. *a*, Plants of *P. saman* regenerated in vitro grown in soil for 4 mo. under greenhouse conditions. *b*, Root system of regenerated plant (*left*) showing nodules as in seedlings (*arrowhead*).

ulators from the culture medium, especially auxins. Some authors suggest that activated charcoal contains polyamines that can play a very relevant role in the development of morphogenetic events (*see* Dumas and Monteuuis, 1995).

Root's were produced in the presence of IBA, although no statistical differences were found between IBA concentrations tested. On the contrary, the statistical differences found between the three exposure periods prove that very long exposure to high IBA concentrations affect rooting negatively.

The plants showed good growth under greenhouse conditions, and their development was not affected. Normal growth was shown by their many roots, nodules, and leaf area.

In conclusion, it was possible to develop an efficient propagation protocol via organogenesis in *P. saman*, a commercially valuable species of Costa Rica. Seeds were germinated for 5 d and from these, hypocotyl explants were obtained. For bud induction, proximal and intermediate sections of hypocotyl were placed horizontally on one-half strength MS with 26.6 μM BA, 3% sucrose, and 0.7% agar for 28 d. We optimized bud development and shoot elongation by transferring the shoot buds onto full-strength MS containing 5 g l^{-1} activated charcoal. Rooting was obtained on MS medium with 2% sucrose and 0.05% Gelrite. Sixty-three percent rooting was obtained with 369 μM IBA for a 24-h exposure period. Plantlets adapted readily to greenhouse conditions and survival was 95%. With the protocol for plant regeneration from cultured hypocotyl tissue described here, shoots form in 9 wk or less, rooted shoots in 8 wk, and complete plantlets in 3 or 4 mo.

Acknowledgments

This work was supported by Centro Agronómico Tropical de Investigación y Enseñanza (CATIE). The authors also thank Dr. Indra Harry for her advice.

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