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Increased CO_2 and Light Promote in Vitro Shoot Growth and Development of *Theobroma cacao*

Antonio Figueira, Anna Whipkey, and Jules Janick
Department of Horticulture, Purdue University, West Lafayette, IN 47907

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Abstract. **Axillary shoots of cacao *(Theobroma cacao* L.), induced in vitro with cytokinins (BA or TDZ), elongated and produced leaves only in the presence of cotyledons and/or roots. Detached axillary shoots, which do not grow in 'vitro under conventional tissue culture protocols, rooted with auxin and developed normally in vivo. Detached axillary shoots from cotyledonary nodes and single-node cuttings from mature plants were induced to elongate and produce normal leaves in the presence of 20,000 ppm CO_2 and a photosynthetic photon flux density (PPFD) of 150 to 200 µmol·s^{-1}·m^{-2}. Subculture nodal cuttings continued to elongate and produce leaves under elevated CO_2 and light levels, and some formed roots. Subculture of microcuttings under CO_2 enrichment could be the basis for a rapid system of micropropagation for cacao. Chemical names used: *N* -(phenylmethyl) -1 *H* -purin-6-amine (BA); 1 *H* -indole-3-butyric 'acid (IBA); α -naphthaleneacetic acid (NAA); thidiazuron (TDZ).**

Cacao has been recalcitrant in tissue culture. Attempts to micropropagate cacao via shoot tip culture have been disappointing. Promotive factors reported include: 1) liquid medium (Adu-Ampomah et al., 1988; Blake and Maxwell, 1984; Dufour and Dublin, 1985; Orchard et al., 1979); 2) physiological stage of the explant source, i.e., either explanted during active flush (Passey and Jones, 1983) or during vegetative rest (Bertrand, 1987; Blake and Maxwell, 1984; Orchard et al., 1979); 3) frequent medium transfer (Adu-Ampomah et al., 1988; Blake and Maxwell, 1984; Legrand and Mississo, 1986); 4) decreased salt concentration (Bertrand, 1987; Dufour and Dublin, 1985); 5) increased culture vessel volume (Dufour and Dublin, 1985); 6) use of activated charcoal (Dufour and Dublin, 1985); 7) use of glucose as a carbon source (Legrand et al., 1984); and 8) explant length of 2 to 4 cm with medial bud placement (Legrand and Mississo, 1986; Litz, 1986). Despite these protocol improvements, only sporadic growth and proliferation of explanted shoots have been achieved. In many cases, shoot growth ceased after 4 to 6 weeks of culture (Adu-Ampomah et al., 1988; Blake and Maxwell, 1984; Legrand and Mississo, 1986; Legrand et al., 1984; Passey and Jones, .1983). No positive results were reported on subculture.

Recently, Flynn et al. (1990) reported bud elongation and leaf development from mature shoots cultured in vitro without exogenous growth regulators, but no data are presented comparing treatments. They reported promotive factors to include flush stage at explant excision, minimization of explant stress through careful handling, orientation of nodal explant within culture vessel, 10 h of light with a maximum of 250 µmol·s^{-1}·m^{-2} programmed to reflect diurnal flux changes, high culture vessel relative humidity, and frequent explant transfer.

Previous studies in our laboratory (Janick and Whipkey, 1985) have indicated that shoots can be induced in vitro from cotyledonary nodal tissues of cacao after epicotyl decapitation or supplementation of the basal medium with BA. Shoots elongated and developed leaves in the presence of cotyledons, but proliferated. axillary shoots, when excised from the cotyledonary node, failed to grow under standard tissue culture protocols. The objective of this study was to investigate the growth of cacao shoots in vitro, emphasizing cotyledonary axillary shoots as a model system. Emphasis on CO_2 and light was based on reports by Infante et al. (1989), Kozai (1990), and Lakso et al. (1986).

Materials and Methods

In vivo production and rooting of cotyledonary axillary shoots. Axillary cotyledonary nodel shoots were induced by removing the epicotyl from l-month-old cacao seedlings grown in the greenhouse. Excised axillary shoots and epicotyls (8 cm long) were dipped for 10 sec in various concentrations of IBA and/or NAA in 50% ethanol as described (Tables 1 and 2). Shoots were transferred to a 1 soil : 1 perlite mixture (v/v) and misted

Table 1. Rooting of main and axillary shoots of 1-month-old seedlings of cacao in vivo, 3 weeks after treatment with 4000 ppm IBA plus 4000 ppm NAA in 50% ethanol.[z]

Shoot type	Rooting (%)	Roots/ cutting	Root length (mm)
Main	50	6.8 ± 1.2[y]	21.0 ± 1.8
Axillary	80	6.7 ± 0.9	17.4 ± 1.6

[z]Treatment n = 30.
[y]± SE.

Table 2. Effects of auxin on in vivo rooting of axillary shoots of seedling cacao.[z]

Auxin[y] concn (ppm)	Rooting (%)		
	IBA	NAA	IBA + NAA[x]
0	11.1[w]	---	---
40	37.5	20.0	33.3
400	33.3	33.3	37.5
2000	62.5	77.8	77.8
4000	83.3	70.0	62.5
8000	100.0	50.0	---
Significance			
Linear	**	*	NS
Quadratic	**	**	**

[z]Data obtained after 3 weeks (treatment n = 10).
[y]Auxin applied in 50% ethanol dip.
[x]Concentration applies to each auxin.
[w]Forty-four percent rooting with water alone.
[NS,*,**]Nonsignificant or significant at $P = 0.05$ or 0.01, respectively.

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for 8 sec every 8 min for 3 weeks. The soil was maintained at ≈ 30C for 3 weeks with heating cables.

In vitro shoot proliferation from cotyledonary nodes. Mature pods obtained from greenhouse-cultivated trees grown from seed were washed with tap water and flamed with 95% ethanol in a laminar flow hood. Seeds were extracted, and the mucilaginous seed coat was removed. Embryos were germinated on either half-strength MS salts (Murashige and Skoog, 1962) supplemented with 0.3 µM thiamine-HCl, 2.4 µM pyridoxine-HCl, 0.6 mM *i* -inositol, 4.1 µM nicotinic acid, 26.6 µM glycine, 87.6 mM sucrose, and 8 g agar/liter; or Woody Plant Medium (WPM) (Lloyd and McCown, 1980), supplemented with 88.8 mM fructose and 2 g gellan gum/liter (GelRite). The pH of both media was adjusted to 5.7 before autoclaving.

Epicotyls were removed 4 to 6 weeks after seeds had germinated. In the first experiment, roots were removed and explants were transferred to fresh WPM as formulated previously and supplemented with 0 or 4.44 µM BA and 0, 0.005, 0.01, 0.05, or 0.1 µM TDZ applied before autoclaving. In the second experiment, cotyledonary nodes were cultured with or without roots and with or without cotyledons on WPM with 0.05 µM TDZ. Shoots were counted and length measured 4 weeks after treatments were imposed in both experiments.

CO_2 chambers. High CO_2 treatments were conducted in clear acrylic chambers placed in the culture room and received compressed CO_2 and air mixed with a Matheson flowmeter and bubbled through distilled water to increase relative humidity. Final concentration of CO_2 in the chamber was maintained at 20,000 ppm. The chamber was held at a 29/25C day/night cycle.

Diurnal CO_2 changes in vitro. Test tubes (50 ml headspace) containing 10 ml semi-solid WPM supplemented with 88.8 mM fructose (with or without leafy axillary shoots originated from cotyledonary nodes) were capped with polypropylene closures (Bellco Kaputs, Vineland, N. J.) in which silicone septa had been inserted, and wrapped with flexible plastic (Parafilm). Treatments included 16-h photoperiod of 90 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetic active radiation (PAR) from cool-white fluorescent lamps and 800 ppm CO_2 (ambient in culture room = low CO_2) or 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAR from very high output (VHO) cool-white fluorescent lamps and 20,000 ppm CO_2 (high CO_2). Gas (1 ml) from inside the tubes was extracted with a syringe every 2 h for 48 h. Each of the four treatments (with or without explants, high or low CO_2 and light) consisted of five tubes sampled sequentially every 2 h. Carbon dioxide concentration was measured using a Carle GC 8700 gas chromatography with a thermal conductivity detector.

Effect of high CO_2 and high light levels. Three types of shoots were transferred to tubes with 10 ml semisolid WPM plus 88.8 mM fructose and capped with polypropylene closures (Kaputs): 1) new shoots induced and elongated in vitro under low light and low CO_2 from five-node plagiotropic cuttings from greenhouse-grown trees; 2) axillary shoots (≈5 cm) from cotyledonary nodes cultured in vitro; 3) one-node plagiotropic shoots from mature greenhouse-grown trees. The new secondary shoots were subculture under either high light (150 $\mu mol \cdot s^{-1} \cdot m^{-2}$, and high CO_2 levels or low light (45 $\mu mol \cdot s^{-1} \cdot m^{-2}$) and low CO^2 levels. Axillary shoots or nodal cuttings were placed either under high or low CO_2 (as above) and high (200 $\mu mol \cdot s^{-1} \cdot m^{-2}$) or low light (45 $\mu mol \cdot s^{-1} \cdot m^{-2}$) levels obtained either by cheesecloth shading inside the CO_2 chamber or by using non-VHO lamps with a lower lamp : area ratio. All data were obtained 4 weeks after initiating treatment. Leaf area was obtained using a LI-COR (LI-COR, Lincoln, Neb.) leaf-area meter.

Results

In vivo rooting of axillary cotyledonary shoots. Rooting was obtained from 50% of epicotyls and 80% of axillary shoots dipped in a solution of IBA and NAA, 4000 ppm each (Table 1). In a second study, using different auxin concentrations, optimum rooting was obtained from 8000 ppm IBA (Table 2). Rooted shoots were transferred to soil and grew into normal plants.

In vitro shoot proliferation from cotyledonary nodes. In the first experiment, proliferation of axillary nodal shoots was induced by BA or TDZ. Maximum proliferation was achieved with 0.1 µM TDZ alone (Table 3). Shoots elongated and produced leaves (data not presented) in the presence of cotyledons at all treatments, although high cytokinin concentrations inhibited shoot elongation.

The second experiment was carried out to determine the effect of cotyledons and roots on axillary shoot proliferation and elongation. There was little effect of treatment on budbreak. The presence of either roots or cotyledons promoted elongation; maximum elongation occurred when both roots and cotyledons were present (Table 4).

Diurnal CO_2 changes in vitro. In tubes containing medium without cacao shoots, CO_2 concentrations were similar to ambient levels found in our culture room, with no appreciable diurnal difference (Table 5). In tubes with cacao shoots, CO_2 was depleted during the day, but returned to ambient levels at night. Carbon dioxide concentration in test tubes in the high CO_2 chamber ranged from 15,000 to 17,000 ppm. No significant diurnal fluctuations in CO_2 levels were detected from tubes in the high CO_2 chamber.

Effect of light and CO_2 on shoot budbreak and elongation. In a preliminary experiment, secondary shoots were cultured in high CO_2 receiving PPFD of 150 $\mu mol \cdot s^{-1} \cdot m^{-2}$, or in low CO_2 receiving 45 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Budbreak per explant in high CO_2/

Table 3. Effect of TDZ and BA on in vitro axillary shoot proliferation from the cotyledonary node of cacao seedlings without epicotyl.[z]

BA (µM)	TDZ (µM) 0	0.005	0.01	0.05	0.1
	No. elongated shoots/node				
0.0	1.0 ± 0.3[y]	1.2 ± 0.5	1.8 ± 0.2	2.0 ± 0.9	6.4 ± 1.2
4.4	1.6 ± 0.4	1.6 ± 0.8	2.2 ± 0.7	3.0 ± 0.4	5.0 ± 1.2
	Shoot length (mm)				
0.0	27.8 ± 2.7	33.5 ± 6.4	46.6 ± 7.6	32.5 ± 7.3	22.0 ± 2.3
4.4	42.8 ± 9.4	57.5 ± 6.1	43.8 ± 6.5	48.5 ± 3.4	21.8 ± 3.8

[z]Data obtained after 4 weeks (treatment n = 5).
[y]± S E.

Table 4. Effect of roots and cotyledons on budbreak and elongation of cotyledonary nodal shoots of cacao.[z]

Roots	Cotyledons: Absent	Present
	Budbreak/node	
Absent	2.6 ± 0.2[y]	3.6 ± 0.5
Present	2.1 ± 0.2	2.3 ± 0.4
	Elongation/explant (mm)	
Absent	5.2 ± 0.7	27.8 ± 8.8
Present	19.2 ± 4.2	77.2 ± 15.3

[z]Data obtained after 4 weeks (treatment n = 10 to 25). Cotyledonary nodes were cultured in WPM supplemented with 0.05 µM TDZ.
[y]± SE.

Table 5. Diurnal CO_2 changes in test tubes containing WPM with and without cacao axillary shoots and sealed with Kaput closures and Parafilm.

Cacao shoot	CO_2 (ppm ± SE)[z] Day	Night
	800 ppm CO_2	
Absent	883 ± 62	1,042 ± 212
Present	72 ± 35	949 ± 192
	20,000 ppm CO_2	
Absent	15,860 ± 600	14,906 ± 393
Present	15.709 ± 661	17.080 ± 837

[z]CO_2 readings obtained every 2 h for 48 h; 16-h photoperiod.

Table 6. Effect of CO_2 and light levels on budbreak, elongation, and leaf development of axillary shoots from cotyledonary nodes of cacao.[z]

Light ($\mu mol \cdot s^{-1} \cdot m^{-2}$)	CO_2 (ppm) 800	20.000
	Budbreak/explant	
45	3.9 ± 0.6[y]	2.2 ± 0.4
200	4.0 ± 1.2	1.9 ± 0.5
	Elongation/explant (mm)	
45	10.2 ± 2.8	18.0 ± 4.2
200	10.0 ± 2.1	14.8 ± 3.0
	Leaf area (cm^2)	
45	1.1 ± 0.3	2.1 ± 0.4
200	0.6 ± 0.1	1.8 ± 0.2
	Leaf area/explant (cm^2)	
45	0.7	6.1
200	1.9	6.8
	Leaf no./explant	
45	0.4 ± 0.2	2.9 ± 0.5
200	2.9 ± 1.0	3.9 ± 0.5

[z]Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 8 to 20).
[y]± S E.

high light conditions was 0.2 ± 0.2 SE as compared to 0.8 ± 0.6 for low CO_2/low light levels. Shoot elongation per bud in high CO_2/high light levels was 8.8 ± 1.1 compared to 3.2 ± 2.2 for low CO_2/low light levels. The most striking effect of the high CO_2/hiigh light combination, however, was the normal leaf expansion and development, which was not achieved in previous experiments.

To separate the CO_2 effect from the light effect, a factorial experiment was conducted with cotyledonary nodal shoots, using two light levels (45 $\mu mol \cdot s^{-1} \cdot m^{-2}$ or 200 $\mu mol \cdot m^{-1} \cdot m^{-2}$) and two CO_2 levels (Table 6). High levels of CO_2 significantly decreased budbreak and increased elongation, as in the previous experiment, while light intensity had no effect on budbreak or elongation. Average area per leaf and per explant was greater with high CO_2 (Fig. 1). Leaves were smaller under high light, but the number of leaves and total leaf area per explant increased.

The same CO_2 and light levels were used with single-node plagiotropic cuttings from mature cacao trees. The high light level increased budbreak in low and high CO_2 (Table 7). Shoot elongation and number of leaves per explant were increased by the high light level at either CO_2 level. High CO_2 increased budbreak and elongation significantly under low light; however, under high light there was no CO_2 effect on budbreak and the

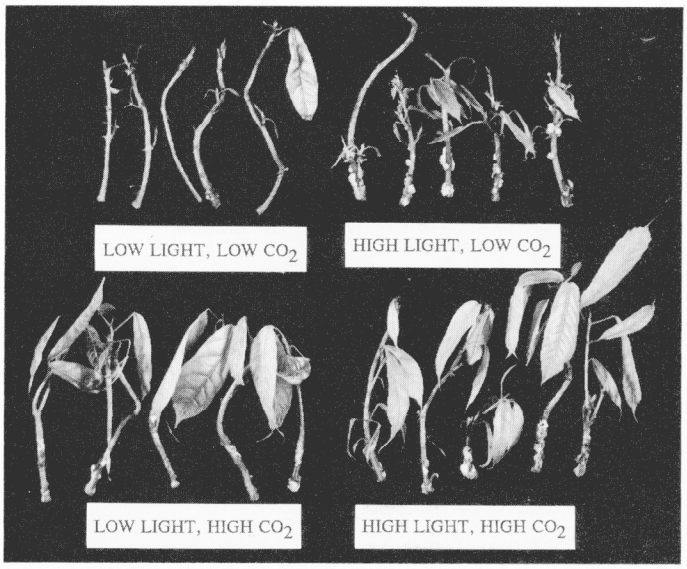

Fig. 1. Response of axillary nodal shoots of cacao to low and high levels of light and CO_2 (see Table 6).

Table 7. Effect of CO_2 and light levels on budbreak, elongation, and leaf development from nodes derived from mature trees of cacao.[z]

Light ($\mu mol \cdot s^{-1} \cdot m^{-2}$)	CO_2 (ppm) 800	20,000
	Budbreak/explant	
45	0.4 ± 0.1[y]	0.7 ± 0.1
200	1.0 ± 0.1[y]	1.0 ± 0.1
	Elongation/explant (mm)	
45	1.3 ± 0.3[y]	2.6 ± 0.4
200	3.9 ± 0.5[y]	4.4 ± 0.6
	Leaf no./explant	
45	0.0 ± 0.0[y]	0.1 ± 0.0
200	0.3 ± 0.1[y]	0.8 ± 0.2

[z]Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 30 to 39).
[y]± SE.

increase in elongation was not significant. The greatest elongation was achieved under high CO_2/ligh light. High CO_2 increased the number of leaves produced under high but not under low light.

Subculture of cotyledonary nodal shoots. Axillary cotyledonary shoots from high CO_2 were dissected into nodal cuttings and recultured under high CO/high light. Each axillary shoot averaged *5.6* explants. After 1 month, 80% of explants produced growing buds, some of which developed leaves, and roots (Fig. 2). Similar results were obtained with shoots from mature trees (Figs. 3 and 4).

Discussion

The difficulty of getting cacao to grow and proliferate shoots in vitro has been a common observation of many researchers (see introduction). One exception to this generalization has been cotyledonary nodal tissue, but the growth of axillary shoots appeared to be cotyledon-dependent, and subculture cotyledonary axillary shoots failed to grow (Janick and Whipkey, 1985). Our present results indicate that axillary shoots of cacao grow normally in vivo and that roots can partially substitute for cotyledons in promoting elongation of axillary shoots in vitro.

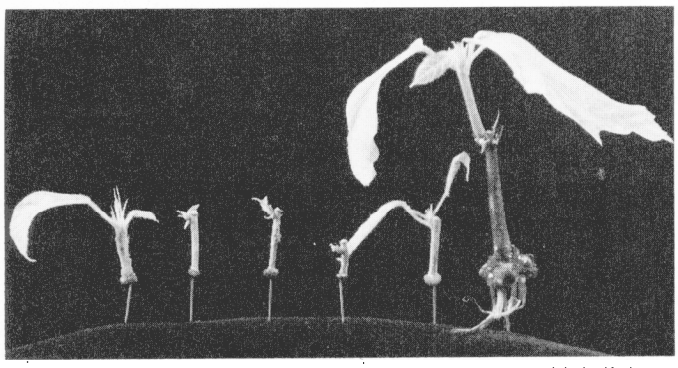

Fig. 2. Growth of subculture axillary shoots under high light and high CO_2 levels after 4 weeks. The six cuttings were obtained from a single elongated shoot. Note rooting of the basal microcutting.

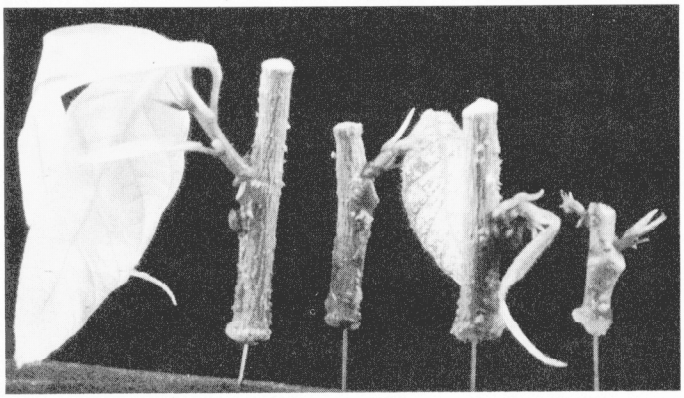

Fig. 3. Budbreak and normal leaf development from nodal cuttings of mature plants induced by high CO_2 and high light levels.

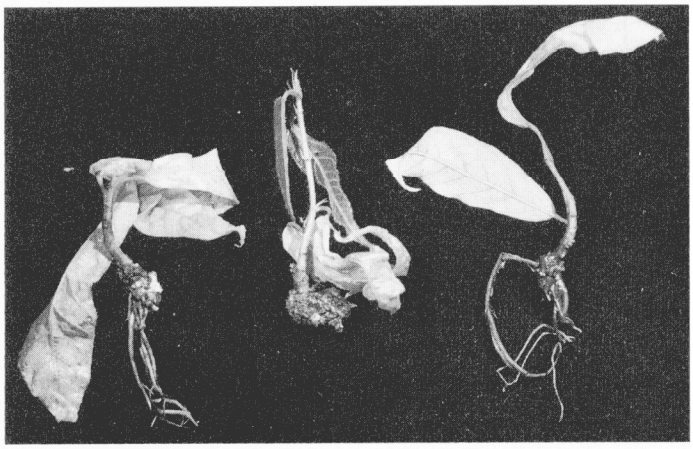

Fig. 4. Growth of subculture secondary shoots from mature plants induced by high CO_2 and high light levels.

This substitution suggests that the lack of growth of axillary shoots in vitro is due to nutrition rather than the absence of any cotyledonay promotive factor. It seems unlikely that the promotive factor of roots is cytokinin, because treatment with various kinds, concentrations, and times of application of cytokinins has failed to be promotive (Adu-Ampomah et al., 1988; Bertrand, 1987; Blake and Maxwell, 1984; Dufour and Dublin, 1985; Janick and Whipkey, 1985; Legrand and Mississo, 1986; Litz, 1986; Orchard et al., 1979; Passey and Jones, 1983).

A polysaccharide gum is ubiquitous in all tissues of cacao (Adomako, 1972; Blake and Maxwell, 1984; Blakemore et al., 1966; Brooks and Guard, 1952; Legrand et al., 1986; Orchard et al., 1979; Passey and Jones, 1983; Whistler et al., 1956). Lack of growth of cacao in tissue culture under conventional systems may be due to interference (either physical or chemical) by this gum (Figueira et al., 1989). The growth of rooted cotyledonary axillary shoots in vivo and the absence of growth in vitro are compatible with the hypothesis that shoots are not being properly nourished either from the medium or from photosynthesis.

Studies of diurnal changes in CO_2 within culture vessels containing cacao explants indicate that CO_2 is being depleted during the day. The enhancement of shoot elongation and leaf development under a high CO_2/high light regime in this study is consistent with the hypothesis that an increase in photosynthesis by high light levels, high CO_2, or both, is responsible for the improved performance of cacao in vitro (Infante et al., 1989; Kozai, 1990; Lakso et al., 1987).

The positive results for in vitro propagation-of cacao axillary buds recently reported by Flynn et al. (1990) are puzzling, because no single factor was implicated as essential. We suggest the main factor in their results is due to a high light level (daily average of 175 $\mu mol \cdot s^{-1} \cdot m^{-2}$), but we do not rule out some undetected CO_2 effect.

In conclusion, our results indicate that high CO_2 and high light levels enhanced in vitro shoot elongation and leaf development of cacao shoots and microcuttings. Apparently, improved photosynthesis overcomes the inability of cacao to respond to in vitro conditions. The benefits of high CO_2/high light were observed with axillary shoots from cotyledonary nodes and nodel cuttings from mature trees as well as subcultures from these shoots. Enhanced rooting of axillary and mature shoots has been observed under this regime. This study confirms the beneficial effects of CO_2 enrichment reported by Kozai (1990) for various crops, but the CO_2 levels we used for cacao are considerably higher (20,000 ppm vs. 2000 to 3000 ppm). The optimum CO_2 levels for cacao remains to be determined. Our results suggest that a system for rapid micropropagation of cacao should be feasible with high light and high CO_2 levels.

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