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Propagation of Theobroma spp.  
by Tissue Culture

FINAL REPORT

(8th Semi-Annual Report  
4/1/90-9/30/90)

Submitted by

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to

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### I. Personnel

There were no personal changes since the 7th semi-annual report.

### II. Summary of previous results

Our previous results with micropropagation of cacao have been summarized in a paper entitled, "Increased CO<sub>2</sub> and Light Promotes In Vitro Shoot Growth and Development of Theobroma cacao L.: submitted to the Journal of the American Society for Horticultural Science. This paper is attached as appendix A.

### III. Gum

Cacao pod gum was extracted and purified using the procedure in Semi-annual report 6, page 2. After extraction, a sample was passed through a cationic exchange column, and colorimetric assays were performed on pod gums both before and after cationic exchange (Table 1).

Table 1. Analysis of cocoa pod gum before and after cationic exchange.

Component	Content (% of sample)	
	Neutral	Sugars Uronic
Crude gum	41.24	46.40
Gum after cationic exchange	50.62	38.98

#### A. Sugar analysis

Samples (2 mg) of cacao pod and stem gum as well as 3 stem gum fractions (separated by DEAE cellulose column) were hydrolyzed with ca. 5 ml liquid HF

for 2 hours. The reaction was stopped by the addition of Millipore-filtered water (total ca. 10 ml). The sample was dried at 50° C under nitrogen gas, redissolved in 0.5 ml Millipore-filtered water, and passed through a 0.2 µm filter. The samples were analysed with a Dionex HPLC with pulsed amperometric detector. Results showed that cacao gum in general contain the same type of monosaccharides as karaya (Sterculia, ), with additional arabinose and xylose (Table 2). Gum from pods is closer in composition to karaya than stem gum. The major differences are the presence of arabinose (absent in karaya) and the lower percentage of galactose. A high proportion of glucose was obtained in stem gum but for some unexplained reason glucose was not obtained in similar levels in the fractions 1, 2A and 2B, which comprises 91% of the total recovery from the DEAE column. The unfractionated stem gum did not show the presence of xylose but this sugar was observed in fraction 1 and 2A. A reexamination of the unfractionated portion at a higher sensitivity revealed the presence of xylose at low concentrations.

Table 2. Sugar comparison of various cacao gum and karaya

Gum Source	Sugar composition (% of total sugars)						
	Rha	Ara	Gal	Glu	Xyl	Gala	GluA
Karaya	35.9	0.0	21.8	1.5	0.0	27.9	12.9
Cacao pod gum	32.7	29.6	14.5	1.8	0.6	14.8	9.0
Cacao stem gum	20.3	17.0	10.4	27.9	0.0	10.8	13.6
Fraction 1	18.6	24.0	8.6	3.8	22.8	9.0	13.2
2A	22.2	11.1	18.2	3.5	26.0	6.8	12.2
2B	35.4	0.0	3.9	1.8	2.7	16.3	39.9

## B. Summary of gums

Histochemical studies demonstrated the ubiquitous presence of gums in cacao. Extraction and characterization were carried out from stems and for pods. Extraction of stems included depigmentation by boiling in 70% ethanol followed by a methanol extraction. The tissues were homogenized in distilled water, precipitated with 3 volumes of 95% ethanol, and freeze-dried. The crude gum was purified using a cationic exchange column (IR120H<sup>+</sup>), to reduce protein content and other contaminants. High levels of proteins were observed after passing the stem gum through the column suggesting that this gum is a protein polysaccharide. Further analysis using a more accurate assay are necessary before conclusions can be made about the presence of protein. Stem gum was further fractionated in a DEAE column by a salt gradient, in order to purify samples and obtain fractions with common characteristics (acidity). Both HPLC and colorimetric analyses indicated similar values for uronic sugar content (Table 3). However, there were discrepancies in the neutral sugar content obtained using these methods. This may be due to the presence of other organic compounds which would confound results from colorimetric assays.

Our results suggest that the gum of cacao could be a promising new industrial or food gum. Finding possible uses for this gum requires additional research. If an economic use is found, this could provide an additional source of revenues to the cacao industry since spent pods are essentially a waste product.

Table 3. Analysis of cocoa gum fractions after anionic exchange.

Component	Content (% of sample)						
	<u>Fraction</u>					Pod gum	Stem unfract.
	1	2A	2B	3A	3B		
<u>Colorimetric assays</u>							
Neutral sugars	54.8	45.0	45.8	22.7	40.0	46.4	62.7
Uronic sugars	28.4	12.0	27.3	0.00	16.8	39.0	29.5
<u>HPLC</u>							
Neutral sugars	77.8	81.0	43.8	NA	NA	76.20	75.6
Uronic sugars	22.2	19.0	56.2	NA	NA	23.8	24.4

#### IV. Propagation of cotyledonary nodal and mature shoots of cacao

##### A. Effect of activated charcoal and media state (liquid vs. semi-solid) on cuttings from mature shoot explants in high CO<sub>2</sub>/high light environment

Shoots pretreated with liquid media and grown in semisolid media without activated charcoal had the highest budbreak but there was no significant effect of activated charcoal on budbreak (Table 4). Activated charcoal in semi-solid medium decreased elongation. Semi-solid medium without charcoal gave the greatest elongation and highest number of leaves. This experiment suggest that the presence of activated charcoal is unnecessary for in vitro culture of mature shoots under high CO<sub>2</sub>/high light conditions with semi-solid medium. The use of a liquid medium pre-treatment did not increase elongation.

Table 4. Mature shoots (5 node cuttings) were cultured either in WPM, fructose (0.88 M) liquid medium for 4 days, or in semi-solid WPM, fructose, gelrite. They were then transferred to semi-solid WPM, fructose, gelrite, with or without activated charcoal (3 g/liter), and placed under high CO<sub>2</sub>/high light. Data after 30 days.

Pretreatment	Activated charcoal	
	-	+
	<u>Budbreak/explant</u>	
Liquid	3.9 ± 0.4 (14) <sup>2</sup>	3.4 ± 0.4 (8)
Semi-solid	2.8 ± 0.4 (13)	3.0 ± 0.3 (11)
	<u>Elongation/explant (mm)</u>	
Liquid	11.4 ± 1.6	12.6 ± 1.5
Semi-solid	16.2 ± 2.3	11.2 ± 1.8
	<u>Leaf no/explant</u>	
Liquid	1.2 ± 0.2	1.5 ± 0.4
Semi-solid	1.8 ± 0.3	1.7 ± 0.3

<sup>2</sup> number of explants in parenthesis

#### B. Single node cuttings (without fructose)

The possibility of eliminating a carbon source from tissue culture media by using high CO<sub>2</sub>/high light was attractive because it could reduce culture contamination problems associated with greenhouse explants. The growth of single node cuttings of mature shoots in liquid or semi-solid medium with or without activated charcoal was evaluated in medium without a carbon source (normally fructose). Unfortunately the control (with fructose) was lost due to contamination as a result of a thrip infestation in the culture room. The

low survival rate obtained in this experiment indicated that the presence of a carbon source is necessary for initial growth (Table 5). However the same trend from the previous experiments (Table 4) was observed i.e., charcoal in semi-solid media decreased elongation under high CO<sub>2</sub>, and no beneficial effect of the liquid pre-treatment.

Table 5. Single node cuttings from mature plants were cultured either on liquid or semi-solid WPM, without fructose and with or without activated charcoal. After 4 days all cultures were transferred to semi-solid WPM without fructose and with or without charcoal, and placed under high light/high CO<sub>2</sub>. Data after 30 days.

Pretreatment	Activated charcoal	
	-	+
	<u>Survival (total explants)</u>	
Liquid	8.8% (57) <sup>2</sup>	44.4% (45)
Semi-solid	22.9% (48)	13.3% (45)
	<u>Elongation/explant (mm)</u>	
Liquid	14	31
Semi-solid	36	7
	<u>Elongation/bud (mm)</u>	
Liquid	3.5	4.4
Semi-solid	4.0	7.0

<sup>2</sup> no of explants in parenthesis

C. Effect of salt formulation on single node cuttings

Because cacao responds better to WPM than half strength MS medium (Semi Annual Report 3, Table 5 and 7), it was hypothesized that the major difference between the two media was the partial substitution of  $\text{Ca}(\text{NO}_3)_2$  for  $\text{CaCl}_2$ . Thus, modifications of WPM were tried in order to evaluate the effect of chlorine ions on micropropagation of cacao. Medium A consisted of WPM which was modified by removing  $\text{CaCl}_2$ . Calcium was supplied by  $\text{Ca}(\text{NO}_3)_2$ ;  $\text{NH}_4\text{NO}_3$  was reduced to maintain  $\text{NO}_3$  levels, and iron concentration was doubled. Medium B was WPM without  $\text{CaCl}_2$  and  $\text{NH}_4\text{NO}_3$ , both were replaced with  $\text{Ca}(\text{NO}_3)_2$ , and casein hydrolysate (1 g/liter) was added and iron was again doubled.

WPM-A gave the highest budbreak (Table 6). There was no difference between WPM and either modified WPM (media A and B) on elongation and leaf number per explant. Surprisingly explants in half-strength MS medium had the greatest elongation due to extraordinary growth of a few explants (note high standard error).

An additional experiment was carried out because of the unexpected good results obtained using 1/2 MS (Table 6). An additional treatment was included based on the data of Flynn and Fritz (Proc. Int. Plant Tissue Culture, p 100), which reported favorable results using a modified WPM with increased concentrations of  $\text{MgSO}_4$ ,  $\text{CuSO}_4$ , inositol and thiamine, as well as a combination of sucrose and glucose as a carbon source. The growth of axillary shoots in WPM, MS/2 and modified WPM was compared under high or low  $\text{CO}_2$  (Table 7). Budbreak was the same under high and low  $\text{CO}_2$ , but high  $\text{CO}_2$  significantly increased elongation and leaf number per explant. Budbreak, elongation, and leaf number per explant were greatest with standard WPM. Modified WPM and MS/2 gave unsatisfactory results and did not differ from each other for the three variables considered. This experiment confirmed the  $\text{CO}_2$

effect but gave no evidence that caused us to change WPM as the basic salt medium.

Table 6. Effect of media modifications on budbreak and elongation of axillary shoots under high light/high CO<sub>2</sub> conditions. Data after 30 days.

Media	n	Budbreak/ explant	Elongation/ explant	Leaf no/ explant
WPM	21	0.00 ± 0.00 b	4.0 ± 0.7 b	2.9 ± 0.2 a
WPM-A	17	0.41 ± 0.15 a	3.0 ± 0.5 b	3.1 ± 0.5 a
WPM-B	19	0.05 ± 0.05 b	2.3 ± 0.3 b	3.0 ± 0.4 a
MS/2	19	0.11 ± 0.07 b	9.0 ± 2.3 a	4.0 ± 0.3 a

WPM = Woody Plant Medium

WPM-A = CaCl<sub>2</sub> replaced with Ca(NO<sub>3</sub>)<sub>2</sub>,

reduced NH<sub>4</sub>NO<sub>3</sub>, Fe doubled

WPM-B = CaCl<sub>2</sub> replaced and NH<sub>4</sub>NO<sub>3</sub> with Ca(NO<sub>3</sub>)<sub>2</sub>,

Fe doubled, casein hydrolysate added

MS/2 = half strength Murashige and Skoog salts,

normal ion and vitamins

Table 7. Comparison of MS/2, WPM, and modified WPM (Flynn and Fritz, 1990) on axillary shoot budbreak, elongation and leaf development. Data after 30 days. N=16-19.

Media	CO <sub>2</sub>	
	low	high
	<u>Budbreak/explant</u>	
WPM	0.7 ± 0.2	1.2 ± 0.4
MS/2	0.4 ± 0.1	0.5 ± 0.1
WPM mod.	0.3 ± 0.1	0.1 ± 0.1
	<u>Elongation/explant (mm)</u>	
WPM	12.7 ± 3.2	24.1 ± 5.2
MS/2	4.6 ± 1.0	7.5 ± 1.2
WPM mod.	3.1 ± 1.3	5.2 ± 1.9
	<u>Leaf no/explant</u>	
WPM	2.9 ± 0.5	3.2 ± 0.5
MS/2	1.2 ± 0.2	1.6 ± 0.2
WPM mod.	0.9 ± 0.2	1.8 ± 0.3

Main effects		N	Budbreak	Elongation	Leaf no.
CO <sub>2</sub>	Low	53	0.6 a	6.9 a	1.8 a
	High	53	0.5 a	12.4 b	2.2 a
Media	WPM	36	0.9 a	18.4 a	3.0 a
	MS/2	37	0.5 b	6.1 b	1.4 b
	WPM mod.	33	0.7 b	4.1 b	1.3 b

#### D. Effect of charcoal using axillary shoots

The effect of activated charcoal under high CO<sub>2</sub>/high light and low CO<sub>2</sub>/high light was tested using axillary shoot culture. Activated charcoal increased budbreak. Activated charcoal increased elongation under high CO<sub>2</sub>, but decreased it under low CO<sub>2</sub> (Table 8). Leaf number was decreased under high CO<sub>2</sub>. High CO<sub>2</sub> did not affect budbreak, but increased elongation and leaf number per explant. These results from activated charcoal were the opposite of results obtained from microcuttings of mature origin (see Table 4). These results suggest that activated charcoal is beneficial for axillary shoots (see Semi-annual report 5, Table 10) but not for mature shoots.

Table 8. Effect of activated charcoal (3g/liter) and high CO<sub>2</sub> on budbreak, elongation and leaf number of cacao axillary shoots. Explants were cultured on WPM, fructose (0.88 M), gelrite. Data after 30 days.

Activated charcoal	CO <sub>2</sub>	
	900 ppm	20,000 ppm
	<u>Budbreak/explant</u>	
+	0.8 ± 0.2	0.8 ± 0.2
-	0.6 ± 0.2	0.3 ± 0.2
	<u>Elongation/explant</u>	
+	4.2 ± 0.7	9.5 ± 1.7
-	5.7 ± 1.2	7.2 ± 1.7
	<u>Leaf no/explant</u>	
+	0.9 ± 0.2	1.8 ± 0.4
-	1.8 ± 0.2	2.7 ± 0.3

E. Effect of carbon source and osmotica on axillary shoots in high and low CO<sub>2</sub>

High CO<sub>2</sub> should substitute for a carbon source in the medium. But results presented above for mature shoots (see, Table 5) indicated that the lack of a carbon source limited growth of mature shoots. The role of sugar in the medium was evaluated as a carbon source (energy) and as osmotica for axillary shoots. Fructose was compared to mannitol (a non metabolizing sugar) and polyethylene glycol (PEG 6000), an osmotic agent with no biological effect. All compounds were added to the medium at an osmotic potential equivalent to 88 mM fructose. Results confirmed the beneficial effect of high CO<sub>2</sub> (Table 9). There was little effect on budbreak as previously reported but, elongation and leaf number per explant increased with high CO<sub>2</sub>. Mannitol increased budbreak at both CO<sub>2</sub> levels, but PEG and the sugar free treatments did not differ. PEG, mannitol, and the sugar free treatment did not differ for elongation and number of leaves at both CO<sub>2</sub> levels. Fructose at 1.5% gave the highest elongation and number of leaves at both CO<sub>2</sub> levels.

These results indicate that high osmotica (PEG 6000 and mannitol) do not promote explant growth under these conditions. These results indicated that the role of fructose is not as an osmotic agent, but as an energy source for the explant. We conclude that the presence of a carbon source even under high CO<sub>2</sub>/high light is beneficial for cacao micropropagation.

Table 9. Effect of osmotic component and high or low CO<sub>2</sub> on budbreak, elongation and leaf production of cacao axillary nodal shoots. Light levels were the same for all treatments. WPM, gelrite. Data after 30 days. n=15.

Osmotica	CO <sub>2</sub>	
	low	high
	<u>Budbreak/explant</u>	
No sugar	0.6 ± 0.2	0.5 ± 0.2
Fructose 0.88 M	0.4 ± 0.2	0.5 ± 0.2
PEG 6000 5.3%	0.8 ± 0.3	0.9 ± 0.4
Mannitol 0.88 M	1.2 ± 0.8	1.8 ± 0.4
	<u>Elongation/explant (mm)</u>	
No sugar	2.4 ± 0.8	2.9 ± 0.9
Fructose 0.88 M	7.8 ± 1.9	14.2 ± 3.0
PEG 6000 5.3%	1.2 ± 0.4	2.8 ± 1.6
Mannitol 0.88 M	2.3 ± 1.5	2.3 ± 0.5
	<u>Leaf no/explant</u>	
No sugar	0.4 ± 0.2	2.1 ± 0.3
Fructose 0.88 M	2.0 ± 0.4	3.9 ± 0.7
PEG 6000 5.3%	0.2 ± 0.1	0.7 ± 0.3
Mannitol 0.88 M	0.0 ± 0.0	0.2 ± 0.1

#### F. The effect of CO<sub>2</sub> on cotyledonary nodes

This experiment is a continuation of studies in cotyledonary nodes which indicated that nodal shoots require either cotyledons or roots to proliferate. In this study we investigated whether axillary shoots attached to cotyledonary nodes without cotyledons or roots would grow in high CO<sub>2</sub>. The experiment was

evaluated under a factorial of high or low CO<sub>2</sub> and high or low light. The results (Table 10) confirmed the promotive effect of cotyledons or roots on elongation. Shoots on nodes without roots and cotyledons failed to elongate. However in this experiment in contrast to Semi Annual report 6, Table 3, no promotive effect of roots plus cotyledons was observed.

Table 10. Effect of presence and absence of roots and cotyledons on axillary shoot budbreak, elongation, and leaf growth, and comparison of effect of high light and/or high CO<sub>2</sub> on the budbreak and elongation of axillary shoots when cotyledons and roots are absent. Data after 30 days. N= 5-6.

	Coty- Roots	ledons	CO <sub>2</sub>	Light	Budbreak	Elongation	Leaf no.	Total fresh weight
+	+		Low	Low	2.8 ± 0.4	8.9 ± 1.2	2.8 ± 0.9	2.9 ± 0.3
-	+		Low	Low	1.8 ± 0.3	10.8 ± 1.6	5.5 ± 1.3	2.2 ± 0.0
+	-		Low	Low	3.5 ± 0.3	4.4 ± 1.0	0.3 ± 0.3	1.8 ± 0.1
-	-		Low	Low	2.2 ± 0.4	2.3 ± 0.7	0.8 ± 0.4	0.3 ± 0.0
-	-		Low	High	3.3 ± 0.4	3.9 ± 0.8	2.5 ± 0.7	0.3 ± 0.0
-	-		High	Low	2.4 ± 0.2	5.1 ± 1.4	3.8 ± 1.2	0.5 ± 0.1
-	-		High	High	2.5 ± 0.5	5.0 ± 0.4	4.2 ± 0.8	0.5 ± 0.0

The cotyledonary nodes without roots and cotyledons were placed under a factorial combination of high or low light and high or low CO<sub>2</sub>. There was little effect of CO<sub>2</sub> and light on budbreak. However CO<sub>2</sub> increased elongation and leaf number per explant but high light was only effective in low CO<sub>2</sub>. In general high light and high CO<sub>2</sub> gave the same results. Final explant weight

of nodes was significantly increased under high CO<sub>2</sub>, indicating a definitive photosynthesis response.

G. Comparison of micropropagation systems

Recently Flynn et al. (Penn State), (Flynn, W.P., L.J. Glicenstein, and P.J. Fritz. 1990. Theobroma cacao L.: An axillary bud in vitro propagation procedure. Plant Cell Organ and Tissue Culture 20:111-117.) has published positive results of cacao micropropagation. Flynn's treatment (special medium formulation and high light) was compared to our best treatment (WPM, fructose, gelrite, high light and high CO<sub>2</sub>). Single node cuttings from 3 clones were used. The experiment was replicated three times. When results were averaged over all clones, there was no difference in budbreak between the two treatments for each trial (Table 11). Leaf number per explant was greater under high CO<sub>2</sub>/high light. Elongation was greatest with our system, except in the second replication of the experiment. This can be explained by the inclusion of activated charcoal, which has been shown to be inhibitory under high CO<sub>2</sub>/high light in semi-solid medium (Tables 4, 5). Nevertheless, in the 3 clones used in the second experiment, the elongation under high CO<sub>2</sub> was not significantly different from elongation of explants cultured under the Penn State regime. Additionally, there is a clone dependence for elongation. 'Catongo', which was used in all three replicates, showed very poor response to both methods of micropropagation. It had the poorest budbreak rate, worse elongation, and the lowest number of leaves per explant in all replicates. Clone PU-1 had the best response using either method. Clones SP-9 and UF 613 (which was used only in the third replicate) presented results very similar to clone PU-1. These trials are being continued.

Table 11. Comparison Between two methods for cacao micropropagation for three cacao clones. Penn State = modified WPM, explants on growth chamber with light and temperature control; Purdue = propagation using WPM, fructose (0.88 M), gelrite, explants placed under high CO<sub>2</sub>/high light. Data after 30 days. N=35-47.

Clone	Micropropagation system	Budbreak/ explant	Elongation/ explant	Leaf no/ explant
<u>Trial 1</u>				
PU-1	Purdue	0.7 a	3.1 a	1.0 a
	Penn State	0.6 a	2.9 a	0.2 b
Catongo	Purdue	0.2 a	0.6 a	0.05 a
	Penn State	0.4 a	0.6 a	0.1 a
SP9	Purdue	0.8 a	3.2 a	0.3 a
	Penn State	0.4 a	0.6 b	0.0 b
Main Effects	Purdue	0.6 a	2.2 a	0.5 a
	Penn State	0.5 a	1.4 a	0.1 b
<u>Trial 2</u>				
PU-1	Purdue	0.6 a	2.0 a	0.4 a
	Penn State	0.6 a	2.1 a	0.5 a
Catongo	Purdue	0.3 a	1.1 a	0.2 a
	Penn State	0.5 a	0.8 a	0.1 a
SP9	Purdue	0.6 a	1.8 a	0.3 a
	Penn State	0.5 a	2.1 a	0.2 a
Main Effects	Purdue	0.5 a	1.6 a	0.3 a
	Penn State	0.6 a	1.8 a	0.3 b

continued

Clone	Micropropagation system	Budbreak/ explant	Elongation/ explant	Leaf no/ explant
<u>Trial 3</u>				
UF613	Purdue	0.8 a	2.4 a	0.4 a
	Penn State	0.6 a	1.2 b	0.0 b
PU-1	Purdue	0.9 a	2.3 a	0.6 a
	Penn State	0.8 a	1.6 a	0.2 b
Catongo	Purdue	0.2 a	0.2 a	0.0 a
	Penn State	0.2 a	0.4 a	0.2 a
Main Effects	Purdue	0.6 a	1.7 a	0.3 a
	Penn State	0.5 a	1.1 b	0.1 b

#### H. Effect of CO<sub>2</sub> and light levels on cotyledonary nodal shoots

In order to establish optimal CO<sub>2</sub> and light for cacao axillary shoot micropropagation, two factorial experiments were conducted using three light intensities and five or six CO<sub>2</sub> concentrations. Data obtained after 30 and 60 days averaged over light levels are graphed in Fig 1 and 2. In the first experiment (Fig 1., Tables 12 and 13) there was no consistent relationship between CO<sub>2</sub> at 60 days and budbreak at 30 days, but elongation to increased with increasing levels of CO<sub>2</sub>. No trend was observed for leaf growth or for rooting. No major effect of light was observed. This experiment was repeated. In the second experiment (Fig 2., Tables 14 and 15) there was no consistent effect of CO<sub>2</sub> on budbreak, but increasing CO<sub>2</sub> increased elongation and leaf production at both 30 and 60 days. There were no consistent light effects. These experiments were flawed because it was difficult to keep CO<sub>2</sub>

levels consistent in chambers. The results suggest that the promotive effects of CO<sub>2</sub> are linear from 480 to 1800 ppm.

Table 12. Experiment 1 Cacao axillary shoots Data after 30 days

Light ( $\mu\text{mol s}^{-1}\text{m}^{-2}$ )	CO <sub>2</sub> ppm					Main effects
	633	3901	5679	8889	11462	
	<u>Budbreak</u>					
28	1.5	2.1	2.0	2.8	1.5	1.3 b
39	1.2	1.3	1.3	2.0	1.4	1.5 b
136	1.0	1.0	1.4	1.6	1.4	2.0 a
Main effects	1.3 b	1.5 ab	1.6 ab	2.2 a	1.4 ab	
	<u>Elongation (mm)</u>					
28	19.6	14.9	17.2	14.5	15.3	16.3 a
39	16.9	17.3	16.8	14.2	19.3	16.8 a
136	22.0	15.9	20.1	17.4	18.7	18.8 a
Main effects	19.7 a	17.6 a	16.0 a	18.1 a	15.4 a	

Table 13. Cacao axillary shoots Data after 60 days

Light ( $\mu\text{mol s}^{-1}\text{m}^{-2}$ )	CO <sub>2</sub> ppm					Main effects
	537	1710	4534	6320	15731	
	<u>Budbreak</u>					
28	0.2	0.6	1.0	0.4	1.0	0.6 a
39	0.7	0.7	0.9	0.9	1.3	0.9 a
136	0.4	0.3	0.8	0.4	1.3	0.6 a
Main effects	0.4 a	0.5 a	0.9 a	0.6 a	1.2 a	
	<u>Elongation (mm)</u>					
28	36.5	36.3	30.0	37.3	41.3	36.1 a
39	25.0	37.2	32.2	38.7	42.3	35.9 a
136	32.2	30.1	28.2	33.0	47.3	34.3 a
Main effects	32.4 a	34.5 a	30.3 a	36.4 a	43.8 a	
	<u>Number of leaves</u>					
28	2.6	2.5	2.7	1.9	2.0	3.1 a
39	2.5	3.1	2.2	1.6	3.4	2.6 a
136	2.9	3.9	3.5	2.4	2.9	2.4 a
Main effects	2.7 a	3.2 a	2.8 a	2.0 a	2.8 a	
	<u>Root number</u>					
28	0.0	0.1	0.4	0.4	0.3	0.2 a
39	1.0	1.0	0.4	0.4	0.5	0.6 a
136	0.0	0.2	0.3	0.3	1.2	0.2 a
Main effects	0.2 a	0.4 a	0.4 a	0.4 a	0.3 a	

Table 14. Experiment 2 Cacao axillary shoots Data after 30 days

Light ( $\mu\text{mol s}^{-1}\text{m}^{-2}$ )	CO <sub>2</sub> ppm						Main effects
	480	1300	2710	5780	18060	18830	
<u>Budbreak</u>							
28	0.3	0.0	0.9	0.1	0.1	0.6	0.3±0.1 a
39	1.2	0.4	0.6	0.1	0.6	0.2	0.5±0.1 a
136	0.4	0.5	1.5	0.9	0.4	0.1	0.6±0.2 a
Main effects	0.6±0.2 ab	0.3±0.1 b	1.0±0.3 a	0.3±0.1 b	0.4±0.1 b	0.3±0.1 b	
<u>Elongation (mm)</u>							
28	6.3	5.2	6.2	6.9	13.7	18.2	9.4±1.4 a
39	16.7	5.6	11.8	7.0	30.5	9.3	13.5±1.8 a
136	5.7	6.6	15.5	17.1	11.2	11.8	11.5±1.5 a
Main effects	9.7±1.9 b	5.8±1.0 b	11.3±2.1 b	10.4±1.7 b	18.7±3.1 a	13.1±2.5 ab	
<u>Number of leaves</u>							
28	0.9	2.2	1.7	1.3	3.1	4.0	2.2±0.2 a
39	1.2	1.4	2.5	2.5	4.2	2.5	2.4±0.2 a
136	2.4	2.5	2.5	3.3	3.4	2.5	2.8±0.2 a
Main effects	1.5±0.2 c	2.0±0.2 c	2.2±0.2 bc	2.4±0.3 bc	3.6±0.3 a	3.0±0.3 ab	

Table 15. Cacao axillary shoots Data after 60 days

Light ( $\mu\text{mol s}^{-1}\text{m}^{-2}$ )	CO <sub>2</sub> ppm						Main effects
	480	1300	2710	5780	18060	18830	
	<u>Budbreak</u>						
28	0.4	0.2	0.6	0.1	0.2	0.6	0.4±0.1 a
39	1.5	0.6	1.2	0.4	0.8	0.1	0.8±0.2 a
136	0.4	0.5	1.1	1.4	0.4	0.2	0.7±0.2 a
Main effects	0.8±0.2 a	0.4±0.1 a	1.0±0.3 a	0.7±0.2 a	0.5±0.1 a	0.6±0.1 a	
	<u>Elongation (mm)</u>						
28	7.6	7.2	9.6	11.1	13.3	32.2	13.0±1.9 b
39	24.7	11.6	16.9	13.3	43.1	12.2	19.6±2.4 a
136	8.8	7.0	14.7	17.1	12.7	14.6	12.6±1.4 b
Main effects	13.0±2.2 bc	8.6±1.4 c	13.7±2.2 bc	13.9±2.3 bc	23.3±4.2 a	19.2±3.6 ab	
	<u>Number of leaves</u>						
28	1.6	3.6	2.6	2.5	4.2	6.2	3.4±0.3 a
39	2.3	3.1	4.5	4.1	5.5	3.6	3.8±0.3 a
136	5.2	4.4	4.8	5.0	4.1	3.9	4.6±0.4 a
Main effects	3.0±0.4 a	3.7±0.4 a	4.0±0.5 a	3.9±0.4 a	4.6±0.4 a	4.5±0.5 a	
	<u>Leaf area (cm<sup>2</sup>)</u>						
28	3.7	4.7	2.0	4.1	7.3	7.7	
39	2.3	4.0	4.0	5.9	10.2	6.6	
136	9.9	7.4	7.0	6.9	6.0	8.6	

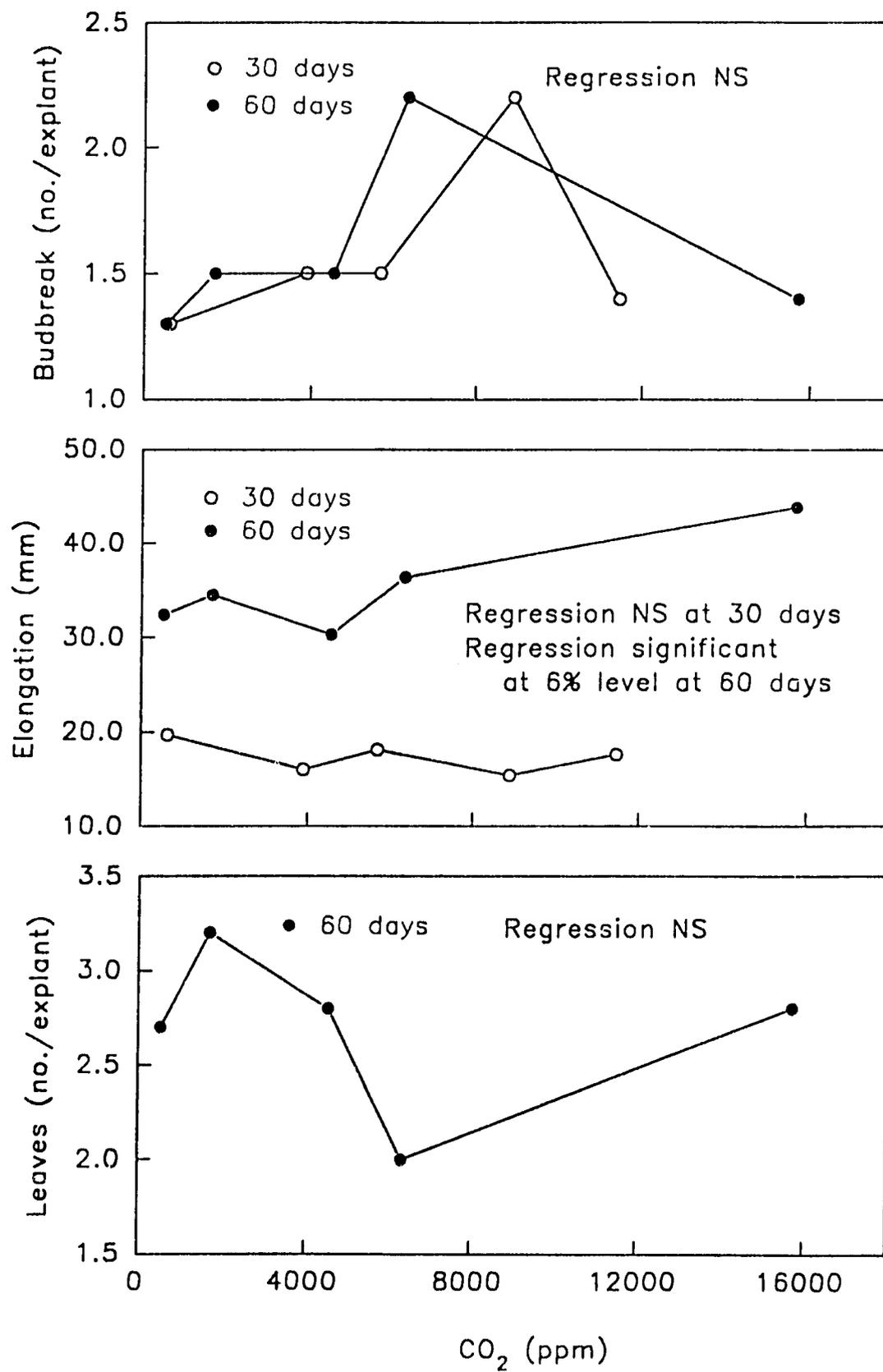


Figure 1. Effect of CO<sub>2</sub> on axillary shoot development  
 Experiment 1

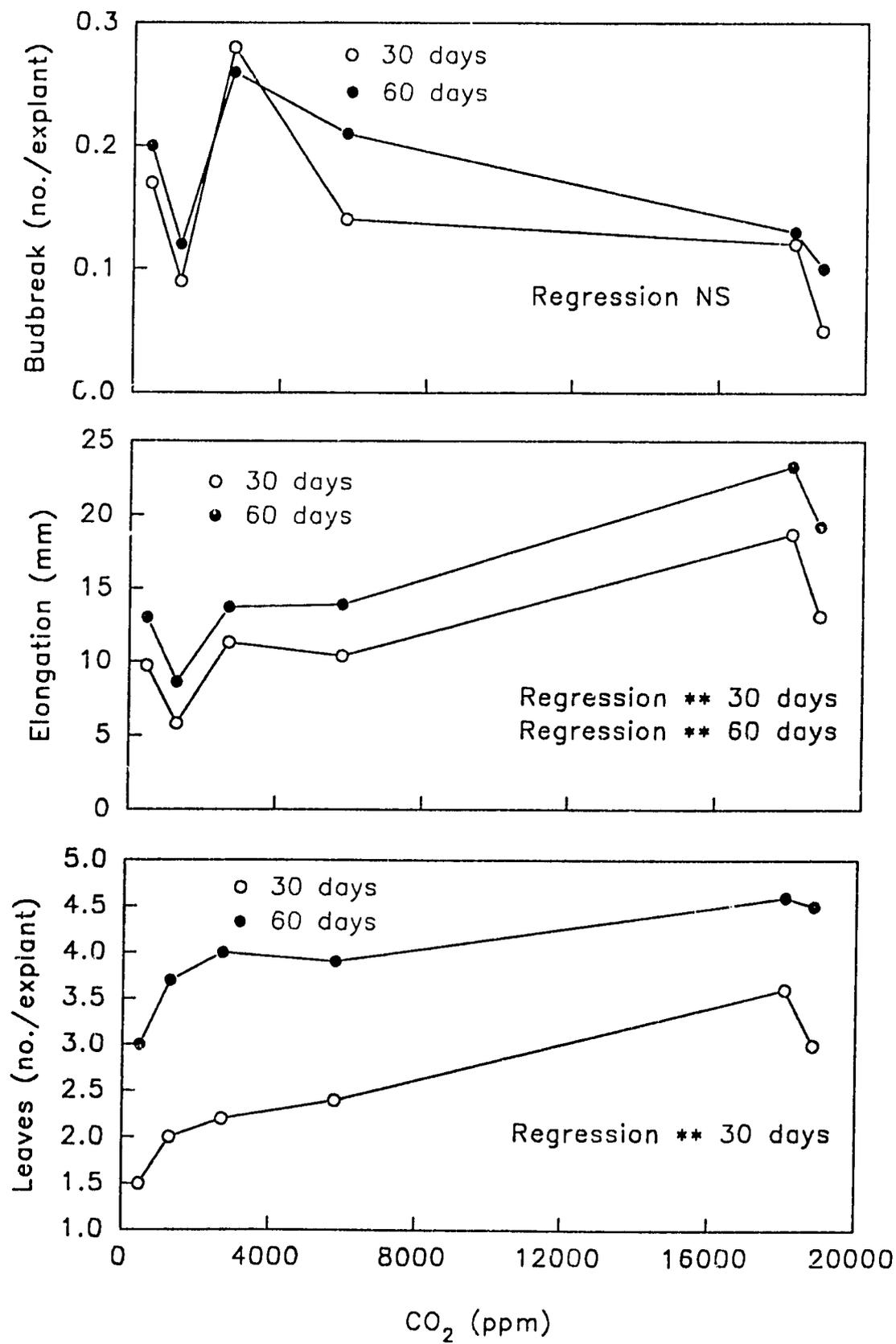


Figure 2. Effect of CO<sub>2</sub> on axillary shoot development  
 Experiment 2

## V. Somatic embryo development

### A. Cacao

Somatic embryos from embryogenic callus cultures of cacao maintained in our laboratory (BC 5, 36, and 46) were grown in liquid media on a gyratory shaker for 15 days. Embryos were then transferred to semi-solid WPM, fructose, gelrite, and placed in the high light/high CO<sub>2</sub> chamber. After 15 days 42% of somatic embryos exhibited some kind of growth. Root growth was observed in 8% of somatic embryos, and 33% presented leaf growth and development. One embryo has already developed 5 new leaves and has the appearance of a normal plant. These results indicate that somatic embryos also respond to high CO<sub>2</sub>/high light protocol.

### B. Cupuassu

Somatic embryos of cupuassu (refer Semi-annual report 1) have been in culture for 3 years and continue to proliferate. Somatic embryos were treated in the same manner as cacao (above) and transferred to high CO<sub>2</sub> and high light. Cupuassu somatic embryos have responded to high CO<sub>2</sub> with apical meristem and mature leaf development but root development has not yet been obtained.

# Increased CO<sub>2</sub> and Light Promote in Vitro Shoot Growth and Development of *Theobroma cacao*

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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<sub>2</sub> and a photosynthetic photon flux density (PPFD) of 150 to 200 μmol·s<sup>-1</sup>·m<sup>-2</sup>. Subcultured nodal cuttings continued to elongate and produce leaves under elevated CO<sub>2</sub> and light levels, and some formed roots. Subculture of microcuttings under CO<sub>2</sub> 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-butylric 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<sup>-1</sup>·m<sup>-2</sup> 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<sub>2</sub> 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 nodal shoots were induced by removing the epicotyl from 1-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.<sup>z</sup>

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

<sup>z</sup>Treatment n = 30.

<sup>y</sup> ± SE.

Table 2. Effects of auxin on in vivo rooting of axillary shoots of seedling cacao.<sup>z</sup>

Auxin <sup>y</sup> concn (ppm)	Rooting (%)		
	IBA	NAA	IBA + NAA <sup>x</sup>
0	11.1 <sup>w</sup>	---	---
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	**	**	**

<sup>z</sup>Data obtained after 3 weeks (treatment n = 10).

<sup>y</sup>Auxin applied in 50% ethanol dip.

<sup>x</sup>Concentration applies to each auxin.

<sup>w</sup>Forty-four percent rooting with water alone.

NS,\*,\*\* Nonsignificant or significant at P = 0.05<sup>w</sup> or 0.01, respectively.

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for 8 sec every 8 min for 3 weeks. The soil was maintained at  $\approx 30\text{C}$  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  $0.3 \mu\text{M}$  thiamine-HCl,  $2.4 \mu\text{M}$  pyridoxine-HCl,  $0.6 \text{mM}$  *i*-inositol,  $4.1 \mu\text{M}$  nicotinic acid,  $26.6 \mu\text{M}$  glycine,  $87.6 \text{mM}$  sucrose, and  $8 \text{g}$  agar/liter; or Woody Plant Medium (WPM) (Lloyd and McCown, 1980), supplemented with  $88.8 \text{mM}$  fructose and  $2 \text{g}$  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  $4.44 \mu\text{M}$  BA and 0, 0.005, 0.01, 0.05, or  $0.1 \mu\text{M}$  TDZ applied before autoclaving. In the second experiment, cotyledonary nodes were cultured with or without roots and with or without cotyledons on WPM with  $0.05 \mu\text{M}$  TDZ. Shoots were counted and length measured 4 weeks after treatments were imposed in both experiments.

*CO<sub>2</sub> chambers.* High CO<sub>2</sub> treatments were conducted in clear acrylic chambers placed in the culture room and received compressed CO<sub>2</sub> and air mixed with a Matheson flowmeter and bubbled through distilled water to increase relative humidity. Final concentration of CO<sub>2</sub> in the chamber was maintained at 20,000 ppm. The chamber was held at a 29/25C day/night cycle.

*Diurnal CO<sub>2</sub> changes in vitro.* Test tubes (50 ml headspace) containing 10 ml semi-solid WPM supplemented with  $88.8 \text{mM}$  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  $90 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  photosynthetic active radiation (PAR) from cool-white fluorescent lamps and  $800 \text{ppm}$  CO<sub>2</sub> (ambient in culture room = low CO<sub>2</sub>) or  $200 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  PAR from very high output (VHO) cool-white fluorescent lamps and  $20,000 \text{ppm}$  CO<sub>2</sub> (high CO<sub>2</sub>). 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<sub>2</sub> and light) consisted of five tubes sampled sequentially every 2 h. Carbon dioxide concentration was measured using a Carle GC 8700 gas chromatograph with a thermal conductivity detector.

*Effect of high CO<sub>2</sub> and high light levels.* Three types of shoots were transferred to tubes with 10 ml semisolid WPM plus  $88.8 \text{mM}$  fructose and capped with polypropylene closures (Kaputs): 1) new shoots induced and elongated in vitro under low light and low CO<sub>2</sub> from five-node plagiotropic cuttings from greenhouse-grown trees; 2) axillary shoots ( $\approx 5 \text{cm}$ ) from cotyledonary nodes cultured in vitro; 3) one-node plagiotropic shoots from mature greenhouse-grown trees. The new secondary shoots were subcultured under either high light ( $150 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) and high CO<sub>2</sub> levels or low light ( $45 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) and low CO<sub>2</sub> levels. Axillary shoots or nodal cuttings were placed either under high or low CO<sub>2</sub> (as above) and high ( $200 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) or low light ( $45 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) levels obtained either by cheesecloth shading inside the CO<sub>2</sub> 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  $0.1 \mu\text{M}$  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<sub>2</sub> changes in vitro.* In tubes containing medium without cacao shoots, CO<sub>2</sub> concentrations were similar to ambient levels found in our culture room, with no appreciable diurnal difference (Table 5). In tubes with cacao shoots, CO<sub>2</sub> was depleted during the day, but returned to ambient levels at night. Carbon dioxide concentration in test tubes in the high CO<sub>2</sub> chamber ranged from 15,000 to 17,000 ppm. No significant diurnal fluctuations in CO<sub>2</sub> levels were detected from tubes in the high CO<sub>2</sub> chamber.

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

Table 3. Effect of TDZ and BA on in vitro axillary shoot proliferation from the cotyledonary node of cacao seedlings without epicotyl.<sup>2</sup>

BA ( $\mu\text{M}$ )	TDZ ( $\mu\text{M}$ )				
	0	0.005	0.01	0.05	0.1
<i>No. elongated shoots/node</i>					
0.0	$1.0 \pm 0.3^y$	$1.2 \pm 0.5$	$1.8 \pm 0.2$	$2.0 \pm 0.9$	$6.4 \pm 1.2$
4.4	$1.6 \pm 0.4$	$1.6 \pm 0.8$	$2.2 \pm 0.7$	$3.0 \pm 0.4$	$5.0 \pm 1.2$
<i>Shoot length (mm)</i>					
0.0	$27.8 \pm 2.7$	$33.5 \pm 6.4$	$46.6 \pm 7.6$	$32.5 \pm 7.3$	$22.0 \pm 2.3$
4.4	$42.8 \pm 9.4$	$57.5 \pm 6.1$	$43.8 \pm 6.5$	$48.5 \pm 3.4$	$21.8 \pm 3.8$

<sup>2</sup>Data obtained after 4 weeks (treatment n = 5).

<sup>y</sup>  $\pm$  SE.

Table 4. Effect of roots and cotyledons on budbreak and elongation of cotyledonary nodal shoots of cacao.<sup>2</sup>

Roots	Cotyledons	
	Absent	Present
<i>Budbreak/node</i>		
Absent	$2.6 \pm 0.2^y$	$3.6 \pm 0.5$
Present	$2.1 \pm 0.2$	$2.2 \pm 0.4$
<i>Elongation/explant (mm)</i>		
Absent	$5.2 \pm 0.7$	$27.8 \pm 8.8$
Present	$19.2 \pm 4.2$	$77.2 \pm 15.3$

<sup>2</sup>Data obtained after 4 weeks (treatment n = 10 to 25). Cotyledonary nodes were cultured in WPM supplemented with  $0.05 \mu\text{M}$  TDZ.

<sup>y</sup>  $\pm$  SE.

Table 5. Diurnal CO<sub>2</sub> changes in test tubes containing WPM with and without cacao axillary shoots and sealed with Kaput closures and Parafilm.

Cacao shoot	CO <sub>2</sub> (ppm ± SE) <sup>z</sup>	
	Day	Night
	<i>800 ppm CO<sub>2</sub></i>	
Absent	883 ± 62	1,002 ± 212
Present	72 ± 35	949 ± 192
	<i>20,000 ppm CO<sub>2</sub></i>	
Absent	15,860 ± 600	14,906 ± 393
Present	15,709 ± 661	17,080 ± 837

<sup>z</sup>CO<sub>2</sub> readings obtained every 2 h for 48 h; 16-h photoperiod.

Table 6. Effect of CO<sub>2</sub> and light levels on budbreak, elongation, and leaf development of axillary shoots from cotyledonary nodes of cacao.<sup>z</sup>

Light (μmol·s <sup>-1</sup> ·m <sup>-2</sup> )	CO <sub>2</sub> (ppm)	
	800	20,000
	<i>Budbreak/explant</i>	
45	3.9 ± 0.6 <sup>y</sup>	2.2 ± 0.4
200	4.0 ± 1.2	1.9 ± 0.5
	<i>Elongation/explant (mm)</i>	
45	10.2 ± 2.8	18.0 ± 4.2
200	10.0 ± 2.1	14.8 ± 3.0
	<i>Leaf area (cm<sup>2</sup>)</i>	
45	1.1 ± 0.3	2.1 ± 0.4
200	0.6 ± 0.1	1.8 ± 0.2
	<i>Leaf area/explant (cm<sup>2</sup>)</i>	
45	0.7	6.1
200	1.9	6.8
	<i>Leaf no./explant</i>	
45	0.4 ± 0.2	2.9 ± 0.5
200	2.9 ± 1.0	3.9 ± 0.5

<sup>z</sup>Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 8 to 20).

<sup>y</sup> ± SE.

high light conditions was 0.2 ± 0.2 SE as compared to 0.8 ± 0.6 for low CO<sub>2</sub>/low light levels. Shoot elongation per bud in high CO<sub>2</sub>/high light levels was 8.8 ± 1.1 compared to 3.2 ± 2.2 for low CO<sub>2</sub>/low light levels. The most striking effect of the high CO<sub>2</sub>/high light combination, however, was the normal leaf expansion and development, which was not achieved in previous experiments.

To separate the CO<sub>2</sub> effect from the light effect, a factorial experiment was conducted with cotyledonary nodal shoots, using two light levels (45 μmol·s<sup>-1</sup>·m<sup>-2</sup> or 200 μmol·s<sup>-1</sup>·m<sup>-2</sup>) and two CO<sub>2</sub> levels (Table 6). High levels of CO<sub>2</sub> 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<sub>2</sub> (Fig. 1). Leaves were smaller under high light, but the number of leaves and total leaf area per explant increased.

The same CO<sub>2</sub> 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<sub>2</sub> (Table 7). Shoot elongation and number of leaves per explant were increased by the high light level at either CO<sub>2</sub> level. High CO<sub>2</sub> increased budbreak and elongation significantly under low light; however, under high light there was no CO<sub>2</sub> effect on budbreak and the

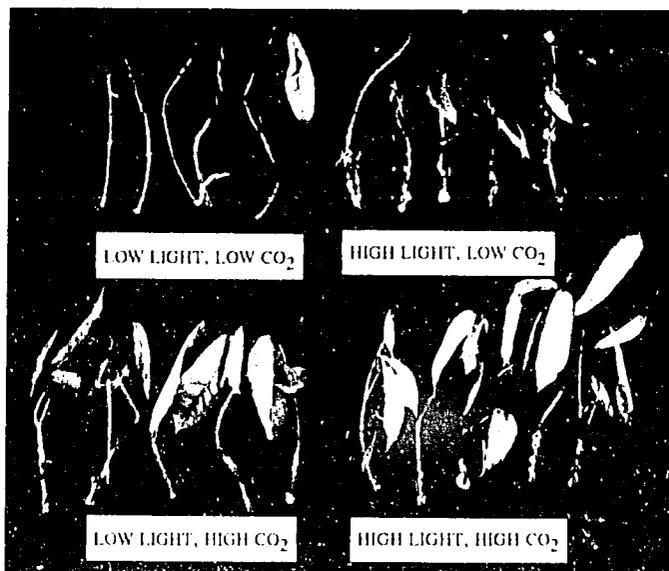


Fig. 1. Response of axillary nodal shoots of cacao to low and high levels of light and CO<sub>2</sub> (see Table 6).

Table 7. Effect of CO<sub>2</sub> and light levels on budbreak, elongation, and leaf development from nodes derived from mature trees of cacao.<sup>z</sup>

Light (μmol·s <sup>-1</sup> ·m <sup>-2</sup> )	CO <sub>2</sub> (ppm)	
	800	20,000
	<i>Budbreak/explant</i>	
45	0.4 ± 0.1 <sup>y</sup>	0.7 ± 0.1
200	1.0 ± 0.1 <sup>y</sup>	1.0 ± 0.1
	<i>Elongation/explant (mm)</i>	
45	1.3 ± 0.3 <sup>y</sup>	2.6 ± 0.4
200	3.9 ± 0.5 <sup>y</sup>	4.4 ± 0.6
	<i>Leaf no./explant</i>	
45	0.0 ± 0.0 <sup>y</sup>	0.1 ± 0.0
200	0.3 ± 0.1 <sup>y</sup>	0.8 ± 0.2

<sup>z</sup>Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 30 to 39).

<sup>y</sup> ± SE.

increase in elongation was not significant. The greatest elongation was achieved under high CO<sub>2</sub>/high light. High CO<sub>2</sub> increased the number of leaves produced under high but not under low light.

*Subculture of cotyledonary nodal shoots.* Axillary cotyledonary shoots from high CO<sub>2</sub> were dissected into nodal cuttings and recultured under high CO<sub>2</sub>/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 subcultured 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 subcultured axillary shoots under high light and high CO<sub>2</sub> 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<sub>2</sub> and high light levels.



Fig. 4. Growth of subcultured secondary shoots from mature plants induced by high CO<sub>2</sub> 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 cotyledonary 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<sub>2</sub> within culture vessels containing cacao explants indicate that CO<sub>2</sub> is being depleted during the day. The enhancement of shoot elongation and leaf development under a high CO<sub>2</sub>/high light regime in this study is consistent with the hypothesis that an increase in photosynthesis by high light levels, high CO<sub>2</sub>, 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 μmol·s<sup>-1</sup>·m<sup>-2</sup>), but we do not rule out some undetected CO<sub>2</sub> effect.

In conclusion, our results indicate that high CO<sub>2</sub> 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<sub>2</sub>/high light were observed with axillary shoots from cotyledonary nodes and nodal 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<sub>2</sub> enrichment reported by Kozai (1990) for various crops, but the CO<sub>2</sub> levels we used for cacao are considerably higher (20,000 ppm vs. 2000 to 3000 ppm). The optimum CO<sub>2</sub> 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<sub>2</sub> levels.

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## CACAO GUM: A POTENTIAL NEW ECONOMIC PRODUCT.

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### ABSTRACT:

A ubiquitous gum is present in large amounts in virtually all tissues of cacao (*Theobroma cacao* L., *Sterculiaceae*). Gum karaya, a gum produced from various *Sterculia* species in the same family, has been used in the food and medical industry, but its use has dwindled greatly because of unreliable supply. Studies were undertaken to characterize the gum of cacao derived from stems and mature pod husks. Histochemical studies located lysigenous cavities throughout the pith and cortex of stems and pericarp of pod husks. Yield averaged 1.5% of fresh weight and 8.4% of dry weight for stem gum, and 0.7% of fresh weight and 8.7% of dry weight for pod gum. Cacao pod gum was closer in composition to gum karaya than was stem gum. Both cacao gums contained the same monosaccharides as gum karaya, but with the addition of arabinose and with higher proportion of rhamnose. The major component of cacao stem gum was glucose, which is not found in either gum karaya or cacao pod gum. It also contained more glucuronic acid than either of these gums. Cacao stem gum has a higher viscosity at concentrations below 1% than gum karaya. Our results suggest that the gums extracted from pods, a waste product of the cacao industry, could be a promising source of a new industrial or food gum.

### KEYWORDS:

cacao - gum - gum karaya - polysaccharides - *Sterculiaceae* - *Sterculia* spp - *Theobroma cacao*

### INTRODUCTION:

Lysigenous cavities filled with mucilaginous substances, which stain heavily, occur in roots (primary and secondary tissues), stems (primary and secondary tissues), flowers, and leaves (petioles, stipules, pulvini, palisade layer and midrib) of cacao (*Theobroma cacao*, *Sterculiaceae*) (Brooks & Guard, 1952). The presence of large amounts of gums in cacao tissues implies an important physiological role, which could be related to storage, osmoprotection, defense mechanisms, or other processes.

Polysaccharides of cacao were first characterized by Whistler *et al.* (1956), who found differences in hot-water-soluble polysaccharides between seed and pod husks. Blakemore *et al.* (1966) examined the hot-water-soluble fraction of husk polysaccharides and concluded that the major part of this fraction was pectic materials. Cacao pod husks (mature fruits without seeds or pulp) were examined as a source of pectin by mild acid extraction by Adomako (1972, 1975) and Berbert (1972), but

yields were low and the pectin was inferior to apple or citrus pectin in gel-forming ability.

Gum karaya, produced from various *Sterculia* species, mainly *Sterculia urens* Roxb., *Sterculiaceae*, has been used in the food and medical industry (Glicksman, 1982), but its use has diminished because its supply is variable and unreliable. Gums of cacao, a species related to karaya, might serve as a substitute.

Our original interest in cacao gums arose from its suspected role in the recalcitrance of cacao growth in vitro (Figueira *et al.*, 1989). The objectives of this study were to characterize cacao gums from stems and husks, and to evaluate their potential as a replacement for gum karaya or as a new commercial product.

#### MATERIALS AND METHODS:

Histological studies: Mature cacao pod husk sections (1 cm<sup>3</sup>) and primary and secondary stem tissues (1 cm long) were fixed in formalin-acetic acid (FAA) followed by dehydration with ethanol-~~tert~~-butanol (Sass, 1951), and embedded in Paraplast (Monoject Scientific, St. Louis). Sections were cut 15  $\mu$ m thick and then double stained with safranin-fast green for observation by light microscopy.

Chemical analysis: Freshly collected stems and husks were boiled in 70% ethanol for 30 min for depigmentation, then extracted with hot methanol. The remaining tissues were dried briefly, homogenized in distilled water, and centrifuged at 1500 g for 30 min. The supernatant was then concentrated, and gum was precipitated with 3 volumes of 95% ethanol and collected by centrifugation for 30 min at 4200 g. The pellet was redissolved in water and freeze-dried. The crude gum was purified by passing a solution of it through a cation-exchange column (Amberlite IR120[H<sup>+</sup>]) and eluting with distilled water.

Colorimetric assays for total neutral sugars (Dubois *et al.*, 1956), protein (Peterson, 1977), and uronic acids (York *et al.*, 1985) were performed on stem and pod gum before and after purification. Samples (2 mg) of cacao gums and gum karaya (Sigma) were solvolyzed with liquid HF (5 ml) for 2 h; hydrolysis was then effected by addition of Millipore-filtered water (10 ml) (J. BeMiller & M. Yadav, unpublished method). The samples were dried at 50°C under nitrogen gas, redissolved in 0.5 ml of filtered water, passed through a 0.2  $\mu$ m filter, and analyzed using Dionex (Sunnyvale, California) BioLC Gradient Pump Module HPLC with a Model Pad 2 pulsed amperometric detector (Hardy *et al.*, 1988). Samples from crude and purified preparations were digested using a hydrogen peroxide-perchloric acid procedure (Adler & Wilcox, 1985); potassium, calcium and magnesium concentrations were estimated using Varian Model Spectra AA 10 Atomic Absorption Spectrophotometer (Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia).

Rheology studies: Purified cacao stem gum solution viscosity was compared with gum karaya solution using a Brookfield (Stoughton, Massachusetts) model LVTD viscometer with the small sample adapter and spindle SCH-31. Viscosity was determined at various concentrations and spindle speeds.

#### RESULTS AND DISCUSSION:

Histology: Histochemical studies confirmed the ubiquitous presence of gums in cacao. The gum was found in lysigenous cavities as noted by Brooks & Guard (1954) which stained a deep magenta with safranin (Fig. 1). These cavities were located throughout the pith and cortex of stems, constituting a significant portion of the cross-sectional area of non-lignified shoots and were found

throughout the pericarp of husks (Fig. 1). In *Sterculia urens*, the major karaya producing species, gum ducts develop lysigenously and lysi-schizogenously (Nair & Shah, 1984). Gum formation is from the product of lysis of the epithelial cells in the pith ducts and in the cortical ducts it originates from the transformation of part of the inner tangential wall followed by the lysis of the epithelial cells. The anatomical site and mechanism of cacao gum formation is unknown.

Extraction and chemical analyses: In the present study, gum yield averaged 1.5% of fresh weight and 8.4% of dry weight of stems, and 0.7% of fresh weight and 8.7% of dry weight of husks, indicating that the gum is a significant portion of the plant dry matter. Polysaccharide yields from husks reported in the literature range from 8 to 11% dry weight basis (Blakemore *et al.*, 1966; Adomako, 1972; Berbert, 1972). Although carbohydrates play a major role controlling rhythmic shoot growth (flushing) of cacao (Machado & Hardwick, 1987), no attention had been placed on the potential role that polysaccharides play on the carbohydrate balance, or possibly on host-pathogen interaction. We have hypothesized that this gum is responsible for the recalcitrance of cacao to *in vitro* culture (Figueira *et al.*, 1989).

Colorimetric assays indicated that cacao gums are acidic protein-polysaccharides and that gums from pods are distinct from gums from stems (Table 1). Both gums have comparable amounts of neutral sugars, but the pod gum has a higher percentage of uronic acids. Uronic acid content of gum karaya ranges from 35 to 40% (Aspinall & Nasir-Ud-In, 1965; Goldstein & Alter, 1973; Glicksman, 1982). A similar amount was found in cacao pod gum. While cacao stem gum contained about 30% protein, the protein content of gum karaya is only about 1% (Anderson *et al.*, 1985).

A proposed structure of gum karaya has a main backbone chain consisting of D-galacturonic acid, L-rhamnose, and D-galactose units to which are attached single unit side chains of D-glucuronic acid or D-galactose units (Phillips *et al.*, 1980). Other structures having larger side chains have also been proposed (Goldstein & Alter, 1973). The proportions of these four monosaccharides vary with species and gum quality and type (Hirst *et al.*, 1949; Goldstein, 1954; Anderson *et al.*, 1982). The sugar composition of gum karaya obtained in the present study agreed qualitatively with previous estimates, but the proportion of monosaccharides differed. Both sources of cacao gums contained the same monosaccharides as gum karaya, but with the addition of arabinose (Table 2) and with greater amounts of rhamnose. Cacao pod gum was closer in composition to gum karaya than was cacao stem gum.

The major component of cacao stem gum was glucose, not found in any appreciable amount in cacao pod gum or gum karaya. Cacao stem gum also contained more glucuronic acid than did the other two gums. The relative proportions of monosaccharides in cacao pod gums observed in this study is similar but not identical to those reported by Whistler *et al.* (1954), Blakemore *et al.* (1966), and Adomako (1972) (Table 2). We observed glucuronic acid, previously unreported, but did not detect the presence of mannose as reported by Whistler *et al.* (1954) and Blakemore *et al.* (1966). The high levels of rhamnose and arabinose we observed differed from previous studies. Glucose and xylose were either undetected or found in reduced amounts in all studies. Uronic acids were undetected by Whistler *et al.* (1954), but observed at very high levels by Adomako (1972); this may be an artifact of the extraction method. Differences in proportions of monosaccharides are not unexpected because of the variability of source material (Glicksman, 1982) and different isolation and analyses procedures.

Crude cacao gums had a high concentration of potassium (Table 3), but purification by a cation-

exchange column successfully reduced the cationic contamination. Total ash content previously observed for cacao pod gums was 9% (Whistler *et al.*, 1956; Adomako, 1972), or 22.7% when based on dry husks (Blakemore *et al.*, 1966). Adomako (1972) identified calcium and potassium as the major elements present. Gum karaya usually occurs in a salt form containing calcium and magnesium (Kubal & Gralen, 1948). The high concentration of potassium in cacao pod husks explains the use of pod ash for soap production in Nigeria (Oduwele & Arueya, 1990).

Viscosity: The logarithm of the apparent viscosity of both cacao stem and karaya gums increased linearly with concentration (Fig. 3). However, gum karaya presented a greater rate of increase in viscosity for all levels of shear stress imposed. At 3% concentration, karaya had an apparent viscosity ranging from about 20,000 centipoises (cp) at 12 rpm to 60,000 cp at 60 rpm. Cacao stem gum had lesser rate of increase in viscosity with concentration reaching values of 500 cp at 3% concentration; at 5% concentration, the viscosity was comparable to that of a gum karaya solution at 3%. However, cacao stem gum had a higher viscosity at concentrations below 1%. Viscosity determinations of cacao pod gum are under investigation.

According to Goldstein & Alter (1972), gum karaya is water-swellable rather than water soluble and absorbs water rapidly to form viscous colloidal dispersions at low concentrations. A finely powdered form of gum karaya was used; the purified cacao stem gum was not ground finely. The rate of hydration in karaya is very dependent upon the mesh size of the gum (Glicksman, 1982). The cacao stem gum used was in the purified, i.e. the free acid, form which could have affected its viscosity.

Potential uses: Pod husks are a waste product of the cacao industry, and present a serious disposal problem. They become a significant source of disease inoculum when used as a mulch inside the plantation. Husks may be used as livestock feed, but the theobromine content reduces the proportion that can be consumed, and its use has been restricted (Wood & Lass, 1985).

Gum karaya, once an important food industry gum, has been used as an emulsifier, stabilizer and/or viscosifier for food products and as a fixative/adhesive in the pharmaceutical industry (Anderson *et al.*, 1982). In recent years, gum karaya has become relatively expensive due to tapping restrictions in India (Anderson *et al.*, 1985). A substitute gum would be of interest (Aslam *et al.*, 1978) and the gum of cacao, which is related to karaya, might serve as a substitute.

Our results suggest that water extracts of cacao stems and pods may be a promising source of a new industrial or food gum which could provide an additional source of revenue to the cacao industry. Additional research is needed to determine specific uses.

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Table 1. Preliminary analysis of cocoa gum before and after cation exchange and dialysis.

Component	Content (% of sample)			
	Crude stem gum	Stem gum after cation exchange	Crude pod gum	Pod gum after cation exchange
Neutral sugars	49	64	47	50
Uronic acids	20	20	44	39
Protein	29	30	ND <sup>2</sup>	ND

<sup>2</sup>=not determined

Table 2. Sugar comparison of cacao gum and gum karaya.

Gum source	Sugar composition (molar ratio) <sup>2</sup>							
	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA
Gum karaya	1.6	0.0	1.0	0.1	0.0	0.0	1.3	0.6
Cacao stem gum	2.0	1.7	1.0	2.8	0.0	0.0	1.1	1.4
Cacao pod gum	2.4	2.1	1.0	0.1	0.1	0.0	1.1	0.6
Cacao pod gum <sup>y</sup>	1.0	0.3	1.0	0.0	0.0	0.3	0.0	0.0
Cacao pod gum <sup>x</sup>	0.4	0.2	1.0	0.4	traces	0.3	1.3	0.0
Cacao pod gum <sup>w</sup>	0.6	0.4	1.0	traces	0.3	0.0	13.4	0.0

<sup>2</sup> All monosaccharides were standardized for galactose molar concentration

<sup>y</sup> Whistler *et al.* (1956)

<sup>x</sup> Blakemore *et al.* (1966)

<sup>w</sup> Adomako (1972)

Table 3. Cation concentration of cacao gum in percent dry weight.

Gum source	Calcium	Magnesium	Potassium	Total
Crude pod gum	0.10	0.40	9.64	10.14
Purified pod gum	0.07	0.02	0.01	0.10
Crude stem gum	0.42	1.34	3.81	5.57
Purified stem gum	0.19	0.04	0.03	0.26



Figure 1. Cross section of cacao pod husk showing darkly stained lysigenous cavities.

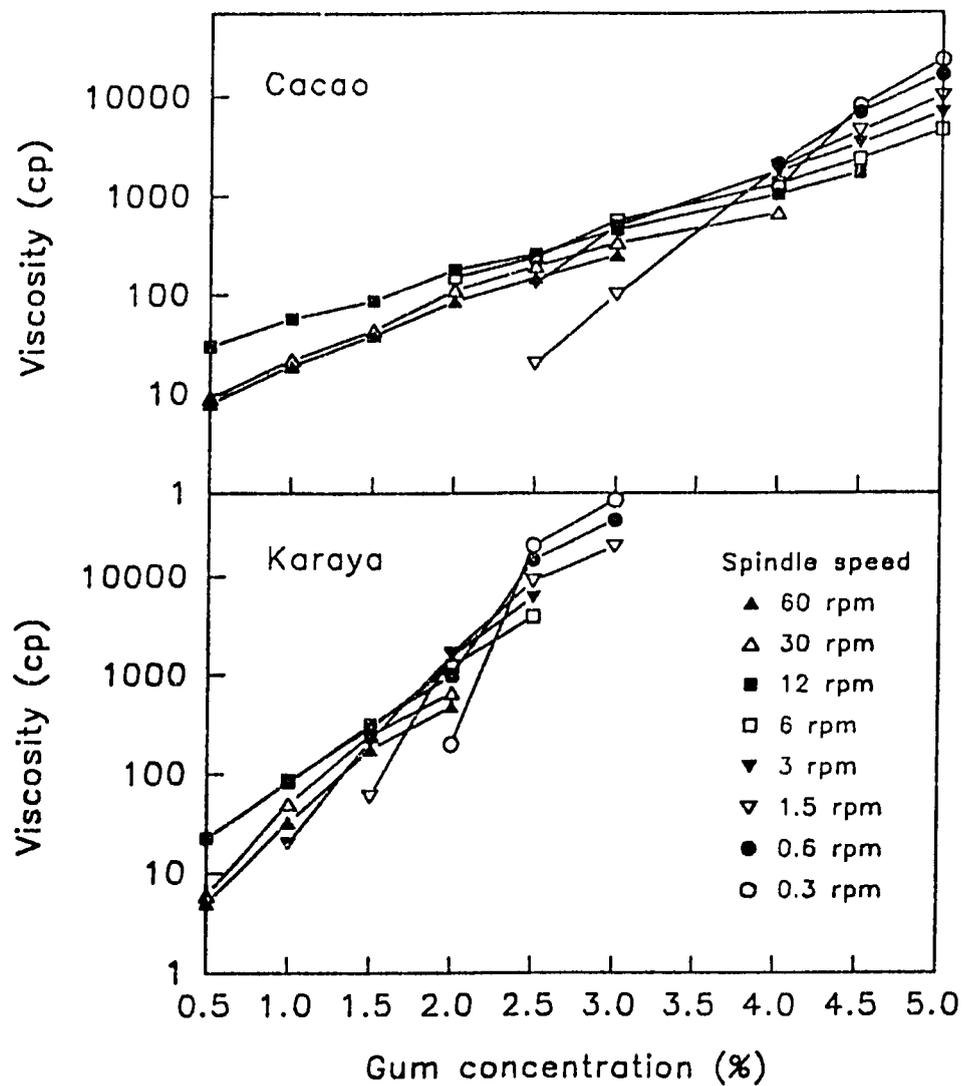


Figure 2. Changes in viscosity with concentration of cacao stem gum and gum karaya at various spindle speeds.

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## ELEVATED CO<sub>2</sub> FACILITATES MICROPROPAGATION OF *THEOBROMA CACAO* L.

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### ABSTRACT:

Cacao (*Theobroma cacao* L.) has been recalcitrant in tissue culture using conventional protocols. In vitro culture of cacao depleted CO<sub>2</sub> during the day, limiting photosynthesis. Shoot elongation and leaf development increased asymptotically after 30 days with CO<sub>2</sub> levels from ambient (470 ppm) to 28,000 ppm. The CO<sub>2</sub> effect did not occur in the dark indicating that photosynthesis stimulation is the major cause of enhanced in vitro performance. High CO<sub>2</sub> was effective in promoting shoot and root development of cotyledonary somatic embryos.

**KEYWORDS:** Micropropagation - tube closures - photoautotrophism

### INTRODUCTION:

*Theobroma cacao* L. (cacao) has been recalcitrant in tissue culture. Until recently only sporadic growth and proliferation of shoots obtained from mature plants have been achieved (Passey and Jones, 1983; Blake and Maxwell, 1984; Legrand *et al.*, 1984; Legrand and Mississo, 1986; Adu-Ampomah *et al.*, 1988). Although axillary shoots can be induced in vitro from cotyledonary nodes after removal of the epicotyl or with cytokinin supplementation, subcultured shoots failed to grow under conventional tissue culture modifications (Janick & Whipkey, 1985). Flynn *et al.* (1990) reported bud elongation and leaf development from mature shoots cultured in vitro without exogenous growth regulators, using higher photosynthetic photon flux densities. Recent results in our laboratory indicate that detached cotyledonary axillary shoots, single-node cuttings from mature plants, as well as microcuttings from elongated shoots cultured in vitro, elongate and produce leaves in vitro under elevated CO<sub>2</sub> and high light (Figueira *et al.*, 1991).

Another possibility for micropropagation of cacao exploits somatic embryogenesis. Somatic embryos can be induced from immature zygotic embryos, either directly from cotyledons (Esan, 1977; Pence *et al.*, 1979, 1980; Kong & Rao, 1982; Tahardi, 1984; Santos & Machado, 1989), or indirectly from embryogenic competent callus (Kononowicz *et al.*, 1984). Recently somatic embryogenesis was reported from mature sporophytic tissues (young bud petals and nucellus tissue) (Söndahl, 1991). However, the development of somatic embryos into autotrophic seedlings has been marginal.

Results presented in this paper indicate that elevated CO<sub>2</sub> promotes shoot elongation and leaf development of microcuttings or somatic embryos.

## MATERIALS AND METHODS:

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 removed. Embryos were germinated on Woody Plant Medium (WPM) (Lloyd and McCown, 1980), supplemented with 88.8 mM fructose and either 2 g·liter<sup>-1</sup> gellan gum (GelRite) or 8 g·liter<sup>-1</sup> agar (Sigma). The pH of the media was adjusted to 5.7 before autoclaving. Epicotyls were removed 4-6 weeks after seeds had germinated, and the axillary shoots were collected 4-6 weeks after decapitation, and used as explants in experiments described below.

CO<sub>2</sub> chambers: Elevated CO<sub>2</sub> treatments were conducted in clear acrylic chambers which were supplied with compressed CO<sub>2</sub> and air mixed with a Matheson flowmeter. The CO<sub>2</sub> concentration in the chambers was measured using a Carle GC 8700 gas chromatograph with a thermal conductivity detector. The chambers were held at 31°C/24°C day/night cycle and illuminated as described with VHO cool white fluorescent lamps. Relative humidity inside the chambers ranged from 50 to 70%.

Diurnal CO<sub>2</sub> 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, and silicone septa were inserted in various types of closures: (1). polypropylene closures (Kaputs, Bellco Glass, Vineland, New Jersey, USA); (2). same closure, but wrapped with flexible plastic (Parafilm, American National Can, Greenwich, Connecticut, USA); (3). SunCaps (Nichimen Co, Tokyo, Japan) a plastic film containing a 8 mm diameter patch of microporous polypropylene, affixed to tubes with two rubber bands; (4). Star\*Pac (Agristar Inc. New Orleans, Louisiana, USA) a non-porous polyethylene film, affixed to tubes with two rubber bands. Treatments included 16 h photoperiod of 90 μmol·s<sup>-1</sup>·m<sup>-2</sup> photosynthetic active radiation (PAR) from cool-white fluorescent lamps and ambient CO<sub>2</sub> (= low CO<sub>2</sub>) or 200 μmol·s<sup>-1</sup>·m<sup>-2</sup> PAR from VHO cool white fluorescent lamps and 20,000 ppm CO<sub>2</sub> (= high CO<sub>2</sub>). Gas (1 ml) from inside the tubes was extracted with a syringe every 2 h for 48 h. Each type of closure had 4 treatments (with or without explants, high or low CO<sub>2</sub> and light), consisting of 5 tubes sampled sequentially every 2 h. CO<sub>2</sub> concentration was measured using a Carle GC 8700 gas chromatograph with a thermal conductivity detector.

Effect of CO<sub>2</sub> concentration and illumination: In the first experiment cotyledonary axillary shoots (ca. 5 cm) were transferred to tubes with 10 ml semi-solid WPM plus 88.8 mM fructose and capped with polypropylene closures (Kaputs). The tubes were then placed under six different CO<sub>2</sub> levels [average CO<sub>2</sub> concentrations measured at 470 ppm (growth room ambient); 2,000 ppm; 5,200 ppm; 9,300 ppm; 18,900 ppm and 28,000 ppm] and four photosynthetic photon flux densities (44, 53, 68 and 120 μmol·s<sup>-1</sup>·m<sup>-2</sup>) obtained by cheese-cloth shading inside the CO<sub>2</sub> chamber. CO<sub>2</sub> levels were monitored daily. In the second experiment cotyledonary axillary shoots were placed in chambers with ambient CO<sub>2</sub> or high CO<sub>2</sub> (20,000 ppm) either under high light (120 μmol·s<sup>-1</sup>·m<sup>-2</sup>) or complete darkness (wrapped in aluminum foil). Data on shoot elongation and leaf development were obtained four weeks after initiating treatment.

Effect of CO<sub>2</sub> on somatic embryo development: An embryogenic clone BC 46 was used, that had been maintained over 10 years (Pence *et al.*, 1979) on basal media containing 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, 1000 mg·liter<sup>-1</sup> casein hydrolysate, and 87.6 mM

sucrose, and 8 g liter<sup>-1</sup> agar (Sigma). Proembryogenic callus which no longer developed beyond the globular stage, was transferred to liquid medium (1/2 MS + 116.8 mM sucrose + 10% coconut water), to induce cotyledonary structures. Cotyledonary embryos were transferred after 30 days to semi-solid WPM containing 88.8 mM fructose and grown under high (20,000 ppm) and low CO<sub>2</sub> (ambient) regimes.

## RESULTS AND DISCUSSION:

Diurnal CO<sub>2</sub> changes in vitro: There was little diurnal variation in CO<sub>2</sub> concentration within the tubes without cacao shoots for both CO<sub>2</sub> regimes (Table 1). The presence of cacao shoots decreased the CO<sub>2</sub> content inside the tubes under ambient CO<sub>2</sub> conditions for all closures during the day, except SunCap, but at night under the same conditions, all closures allowed CO<sub>2</sub> to return to levels similar to the control (absence of cacao shoot). Extreme CO<sub>2</sub> depletion during the day occurred with Kaputs plus Parafilm, indicating that this closure provided the greatest barrier to diffusion of CO<sub>2</sub>. There was no difference in CO<sub>2</sub> concentration due to the presence or absence of shoots at ambient CO<sub>2</sub> with SunCap closures, indicating that this closure did not restrict CO<sub>2</sub> exchange, which agrees with Kozai (1990). Under high CO<sub>2</sub> conditions, the presence of shoots only marginally decreased CO<sub>2</sub> during the day, and CO<sub>2</sub> returned to control levels during the night, except for SunCap. CO<sub>2</sub> levels with Star\*Pac or Kaput closures were higher under high CO<sub>2</sub> than in tubes closed with SunCap or Kaput plus Parafilm. These results suggest that under ambient CO<sub>2</sub> levels, cacao axillary shoots deplete CO<sub>2</sub> during the day to levels below the compensation point, limiting photosynthesis. Under low CO<sub>2</sub> conditions, SunCap allowed greater CO<sub>2</sub> exchange and prevented CO<sub>2</sub> build up during the dark period. However, at CO<sub>2</sub> levels of 20,000 ppm, Star\*Pac and Kaput allowed more CO<sub>2</sub> exchange than the other closures.

Effect of CO<sub>2</sub> concentration and illumination: In a previous study using detached axillary cotyledonary shoots and single-node cuttings from mature plants, we reported a beneficial effect of elevated CO<sub>2</sub> (20,000 ppm) on shoot elongation and leaf development (Figueira *et al.*, 1991). However, the CO<sub>2</sub> levels were much higher than the levels (1,000 to 4,500 ppm) employed by Lakso *et al.* (1986); Infante *et al.* (1989) and Kozai (1990). The effect of CO<sub>2</sub> concentration on shoot elongation and leaf development was tested with six CO<sub>2</sub> concentration from ambient to 28,000 ppm for 30 days.

Shoot and leaf development increased asymptotically as CO<sub>2</sub> increased from ambient to 28,000 ppm (Fig. 1). The CO<sub>2</sub> effects in Fig. 1 are averaged over four light levels, whose effects were not significant during this period. This response to increasing levels of CO<sub>2</sub> also suggests that an additional effect besides photosynthesis was involved, as CO<sub>2</sub> fixation is assumed to be optimum much below 2,000 ppm if absorption was as efficient as in field-grown plants. The response to high levels of CO<sub>2</sub> may simply be due to the low absorption of CO<sub>2</sub> into the relatively leafless shoot. The indirect effect of CO<sub>2</sub> permeability of closures is not a factor here because only Kaputs without a film wrapper was used. CO<sub>2</sub> levels inside the tubes during the day were slightly lower than chamber CO<sub>2</sub> levels but paralleled chamber differences (data not shown). The inhibition of photorespiration could also account for the beneficial effect of increasing CO<sub>2</sub>, but net photosynthesis measurements have not yet been made.

High CO<sub>2</sub> concentrations can prevent or delay ethylene responses when ethylene concentration is below 1 ppm, probably by competitive inhibition (McKeon & Yang, 1987). The possible ethylene inhibition effect of high CO<sub>2</sub> can be inferred from results from a second experiment, where axillary cotyledonary shoots were placed under high or low CO<sub>2</sub> at high light or dark conditions, in order to

determine if the CO<sub>2</sub> effect could be separated from photosynthesis. In high light, high CO<sub>2</sub> increased the number of leaves and elongation, but darkness decreased the number of leaves at both CO<sub>2</sub> levels and shoot elongation at high CO<sub>2</sub> (Table 2). These results imply that the major CO<sub>2</sub> effect is due to either enhanced CO<sub>2</sub> assimilation or to reduction in photorespiration, although some synergistic effect of high CO<sub>2</sub> on ethylene inhibition and photosynthesis cannot be discounted. Insufficient gas exchange and ethylene accumulation have been suggested as factors responsible for the recalcitrance of cacao in vitro (Blake & Maxwell, 1984; DuFour & Dublin, 1985; Etienne, 1988; Lemerrier *et al.*, 1990), but no direct evidence has been presented due to lack of suitable experimental procedures. The common observation of excessive callus formation together with prominent stem lenticels in cacao tissue cultures suggests that ethylene is present.

Our present hypothesis is that the recalcitrance of cacao to tissue culture is a nutritional response that may be due to the presence of gums (Figueira *et al.*, 1989). The benefits of high CO<sub>2</sub> are assumed to be result of a shift to autotrophic nutrition, but the response to levels above saturation point suggests other beneficial effects.

Effect of CO<sub>2</sub> on somatic embryos development. High CO<sub>2</sub> plus high light promoted shoot elongation, leaf and root development of cotyledonary somatic embryos (Table 3 and Fig. 2); secondary embryogenesis was unaffected. Wang & Janick (1984, 1985) induced radicle development in somatic embryos by renewing liquid media and demonstrated that somatic embryo leachates inhibited lettuce seed germination. The inhibitor was shown not to be abscisic acid but resembled *p*-coumaric acid (Wang & Janick, 1985). Conversion of somatic embryos to seedlings was enhanced by suppression of cotyledons (Novak *et al.*, 1986; Adu-Ampomah *et al.*, 1987; Duhem *et al.*, 1989), use of liquid medium (Adu-Ampomah *et al.*, 1987), and inclusion of high levels of activated charcoal (Duhem *et al.*, 1989), protocol modifications all compatible with the hypothesis of an inhibitor. The explanations for our positive results with high CO<sub>2</sub> included: (1) a photosynthetic effect; (2) ethylene inhibition; (3) inhibitor reduction. Evidence for the latter is the report by Kobayashi *et al.* (1991) that increased CO<sub>2</sub> decreased total phenolic content in *Thalictrum* cell cultures.

In summary our results indicate that subculture of microcuttings or development of somatic embryos derived from sporophytic tissue under CO<sub>2</sub> enrichment could be the basis for a rapid system of micropropagation for cacao.

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Table 1. Diurnal CO<sub>2</sub> changes in culture tubes capped with various closures and containing or lacking axillary cotyledonary shoots of cacao in response to low and high CO<sub>2</sub>.

Cacao shoot	CO <sub>2</sub> concentration within sealed tubes (ppm)			
	Closure			
	Kaput	Kaput+ Parafilm	Star*Pac	Suncap
	<u>Ambient CO<sub>2</sub></u>			
	<u>Day</u>			
Absent	688±95	823±65	605±151	523±22
Present	289±40	71±36	139±39	523±26
	<u>Night</u>			
Absent	566±44	827±93	458±10	463±13
Present	670±98	949±205	716±70	509±18
	<u>High CO<sub>2</sub> (20000 ppm)</u>			
	<u>Day</u>			
Absent	20893±404	15824±656	21248±154	16362±346
Present	18729±37	15709±688	20080±372	13457±423
	<u>Night</u>			
Absent	21286±648	15126±421	21435±208	14761±1490
Present	19086±694	17080±904	21088±526	13124±258

Table 2. Effect of CO<sub>2</sub> and light on shoot elongation and leaf development of axillary cotyledonary shoots of cacao.

Illumination ( $\mu\text{mol}\cdot\text{s}^{-1}\text{m}^{-2}$ )	CO <sub>2</sub> (ppm)	
	500	20000
	<u>Shoot elongation (mm)</u>	
0	3.9±2.2	1.8±0.6
135	1.9±0.5	6.1±2.1
	<u>Number of leaves</u>	
0	0.6±0.2	0.5±0.2
135	1.4±0.3	2.8±0.3

Table 3. Effect of CO<sub>2</sub> on development of cacao somatic embryos.

CO <sub>2</sub> (ppm)	Shoot development (%)	Root development (%)	Secondary embryos (%)
500	21±8	3±3	41±9
20000	50±10	7±5	51±10

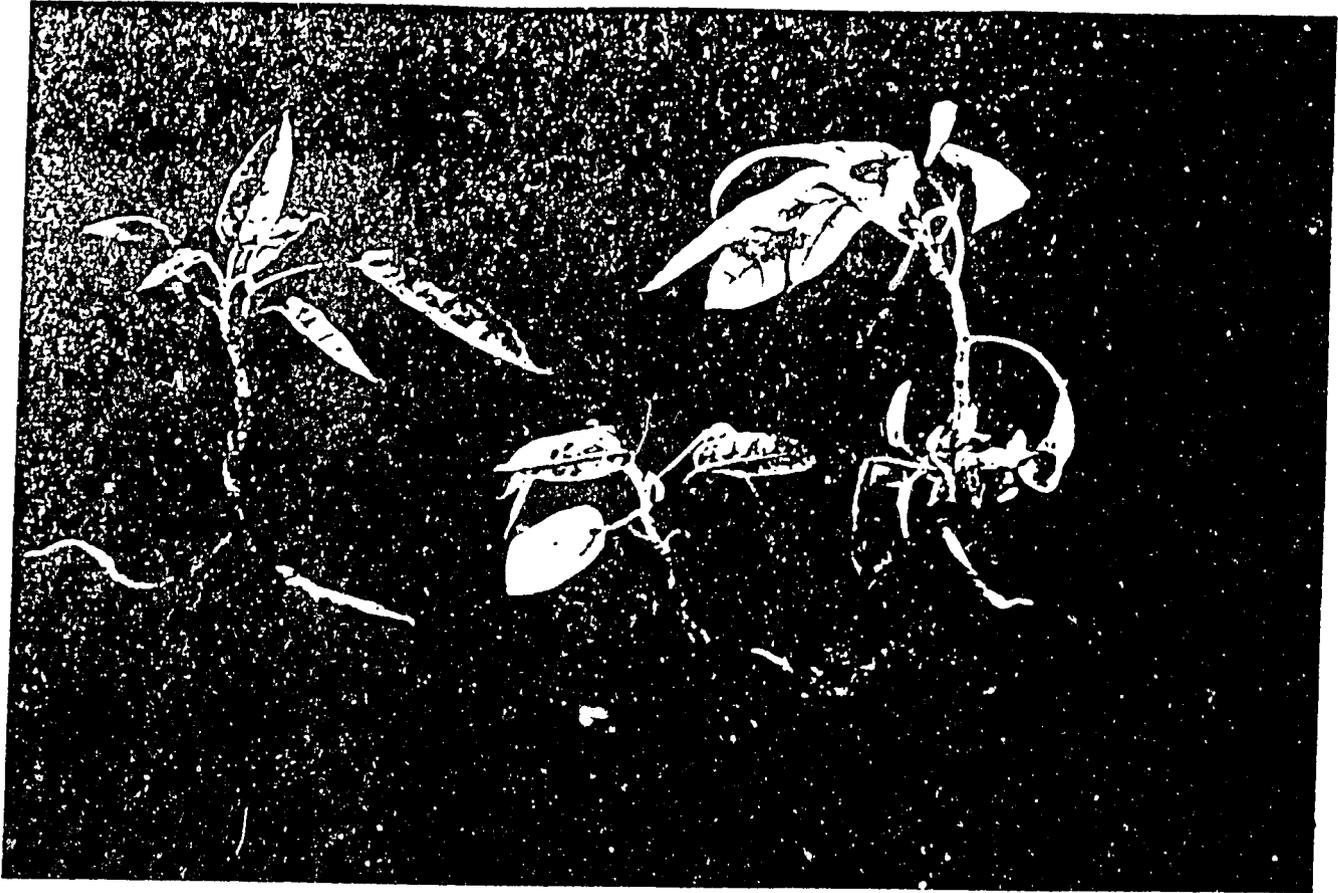


Figure 2. Development of somatic embryos in response to high CO<sub>2</sub>.

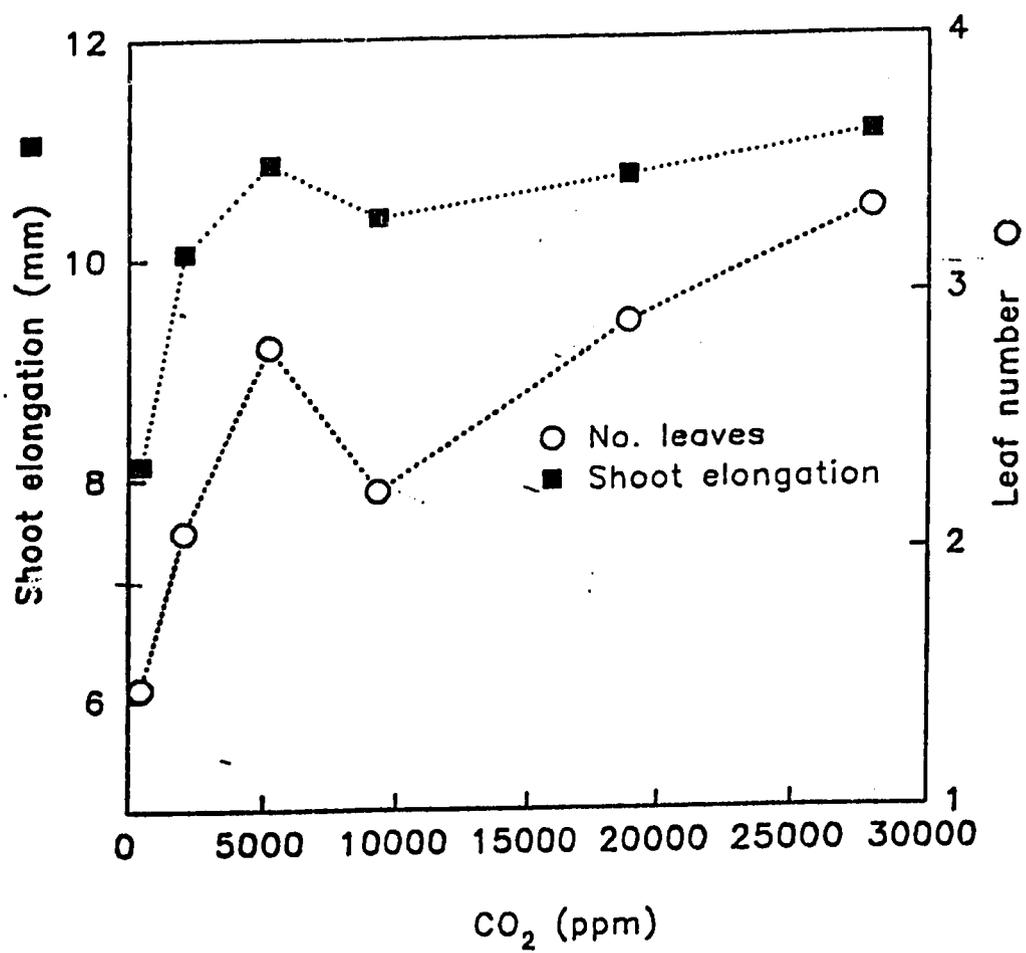


Figure 1. Response of axillary cotyledonary shoots of cacao to CO<sub>2</sub> concentration after 30 days. The responses are averaged over four light levels (44, 53, 68 and 120  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ).

# Increased CO<sub>2</sub> and Light Promote in Vitro Shoot Growth and Development of *Theobroma cacao*

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*Additional index words.* micropropagation, cacao

**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<sub>2</sub> and a photosynthetic photon flux density (PPFD) of 150 to 200 μmol·s<sup>-1</sup>·m<sup>-2</sup>. Subcultured nodal cuttings continued to elongate and produce leaves under elevated CO<sub>2</sub> and light levels, and some formed roots. Subculture of microcuttings under CO<sub>2</sub> 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-butylric 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.

cacao shoots in vitro, emphasizing cotyledonary axillary shoots as a model system. Emphasis on CO<sub>2</sub> 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 nodal shoots were induced by removing the epicotyl from 1-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.<sup>a</sup>

Shoot type	Rooting (%)	Roots/cutting	Root length (mm)
Main	50	6.8 ± 1.2 <sup>b</sup>	21.0 ± 1.8
Axillary	80	6.7 ± 0.9	17.4 ± 1.6

<sup>a</sup>Treatment n = 30.

<sup>b</sup> ± SE.

Table 2. Effects of auxin on in vivo rooting of axillary shoots of seedling cacao.<sup>a</sup>

Auxin <sup>b</sup> concn (ppm)	Rooting (%)		
	IBA	NAA	IBA + NAA <sup>c</sup>
0	11.1 <sup>**</sup>	---	---
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	**	**	**

<sup>a</sup>Data obtained after 3 weeks (treatment n = 10).

<sup>b</sup>Auxin applied in 50% ethanol dip.

<sup>c</sup>Concentration applies to each auxin.

<sup>\*</sup>Forty-four percent rooting with water alone.

NS, \*\*, \* Nonsignificant or significant at P = 0.05 or 0.01, respectively.

REC'D IN R&D/R

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<sup>-1</sup>·m<sup>-2</sup> 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

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for 8 sec every 8 min for 3 weeks. The soil was maintained at  $\approx 30\text{C}$  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  $0.3 \mu\text{M}$  thiamine-HCl,  $2.4 \mu\text{M}$  pyridoxine-HCl,  $0.6 \text{ mM}$  *D*-inositol,  $4.1 \mu\text{M}$  nicotinic acid,  $26.6 \mu\text{M}$  glycine,  $87.6 \text{ mM}$  sucrose, and  $8 \text{ g}$  agar/liter; or Woody Plant Medium (WPM) (Lloyd and McCown, 1980), supplemented with  $88.8 \text{ mM}$  fructose and  $2 \text{ g}$  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  $4.44 \mu\text{M}$  BA and 0, 0.005, 0.01, 0.05, or  $0.1 \mu\text{M}$  TDZ applied before autoclaving. In the second experiment, cotyledonary nodes were cultured with or without roots and with or without cotyledons on WPM with  $0.05 \mu\text{M}$  TDZ. Shoots were counted and length measured 4 weeks after treatments were imposed in both experiments.

*CO<sub>2</sub> chambers.* High CO<sub>2</sub> treatments were conducted in clear acrylic chambers placed in the culture room and received compressed CO<sub>2</sub> and air mixed with a Matheson flowmeter and bubbled through distilled water to increase relative humidity. Final concentration of CO<sub>2</sub> in the chamber was maintained at 20,000 ppm. The chamber was held at a 29/25C day/night cycle.

*Diurnal CO<sub>2</sub> changes in vitro.* Test tubes (50 ml headspace) containing 10 ml semi-solid WPM supplemented with  $88.8 \text{ mM}$  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  $90 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  photosynthetic active radiation (PAR) from cool-white fluorescent lamps and 800 ppm CO<sub>2</sub> (ambient in culture room = low CO<sub>2</sub>) or  $200 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  PAR from very high output (VHO) cool-white fluorescent lamps and 20,000 ppm CO<sub>2</sub> (high CO<sub>2</sub>). 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<sub>2</sub> and light) consisted of five tubes sampled sequentially every 2 h. Carbon dioxide concentration was measured using a Carle GC 8700 gas chromatograph with a thermal conductivity detector.

*Effect of high CO<sub>2</sub> and high light levels.* Three types of shoots were transferred to tubes with 10 ml semisolid WPM plus  $88.8 \text{ mM}$  fructose and capped with polypropylene closures (Kaputs): 1) new shoots induced and elongated in vitro under low light and low CO<sub>2</sub> from five-node plagiotropic cuttings from greenhouse-grown trees; 2) axillary shoots ( $\approx 5 \text{ cm}$ ) from cotyledonary nodes cultured in vitro; 3) one-node plagiotropic shoots from mature greenhouse-grown trees. The new secondary shoots were subcultured under either high light ( $150 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) and high CO<sub>2</sub> levels or low light ( $45 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) and low CO<sub>2</sub> levels. Axillary shoots or nodal cuttings were placed either under high or low CO<sub>2</sub> (as above) and high ( $200 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) or low light ( $45 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) levels obtained either by cheesecloth shading inside the CO<sub>2</sub> 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  $0.1 \mu\text{M}$  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<sub>2</sub> changes in vitro.* In tubes containing medium without cacao shoots, CO<sub>2</sub> concentrations were similar to ambient levels found in our culture room, with no appreciable diurnal difference (Table 5). In tubes with cacao shoots, CO<sub>2</sub> was depleted during the day, but returned to ambient levels at night. Carbon dioxide concentration in test tubes in the high CO<sub>2</sub> chamber ranged from 15,000 to 17,000 ppm. No significant diurnal fluctuations in CO<sub>2</sub> levels were detected from tubes in the high CO<sub>2</sub> chamber.

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

Table 3. Effect of TDZ and BA on in vitro axillary shoot proliferation from the cotyledonary node of cacao seedlings without epicotyl.<sup>†</sup>

BA ( $\mu\text{M}$ )	TDZ ( $\mu\text{M}$ )				
	0	0.005	0.01	0.05	0.1
<i>No. elongated shoots/node</i>					
0.0	$1.0 \pm 0.3^y$	$1.2 \pm 0.5$	$1.8 \pm 0.2$	$2.0 \pm 0.9$	$6.4 \pm 1.2$
4.4	$1.6 \pm 0.4$	$1.6 \pm 0.8$	$2.2 \pm 0.7$	$3.0 \pm 0.4$	$5.0 \pm 1.2$
<i>Shoot length (mm)</i>					
0.0	$27.8 \pm 2.7$	$33.5 \pm 6.4$	$46.6 \pm 7.6$	$32.5 \pm 7.3$	$22.0 \pm 2.3$
4.4	$42.8 \pm 9.4$	$57.5 \pm 6.1$	$43.8 \pm 6.5$	$48.5 \pm 3.4$	$21.8 \pm 3.8$

<sup>†</sup>Data obtained after 4 weeks (treatment n = 5).

<sup>y</sup>  $\pm$  SE.

Table 4. Effect of roots and cotyledons on budbreak and elongation of cotyledonary nodal shoots of cacao.<sup>†</sup>

Roots	Cotyledons	
	Absent	Present
<i>Budbreak/node</i>		
Absent	$2.6 \pm 0.2^y$	$3.6 \pm 0.5$
Present	$2.1 \pm 0.2$	$2.3 \pm 0.4$
<i>Elongation/explant (mm)</i>		
Absent	$5.2 \pm 0.7$	$27.8 \pm 8.8$
Present	$19.2 \pm 4.2$	$77.2 \pm 15.3$

<sup>†</sup>Data obtained after 4 weeks (treatment n = 10 to 25). Cotyledonary nodes were cultured in WPM supplemented with  $0.05 \mu\text{M}$  TDZ.

<sup>y</sup>  $\pm$  SE.

Table 5. Diurnal CO<sub>2</sub> changes in test tubes containing WPM with and without cacao axillary shoots and sealed with Kaput closures and Parafilm.

Cacao shoot	CO <sub>2</sub> (ppm ± SE) <sup>a</sup>	
	Day	Night
	800 ppm CO <sub>2</sub>	
Absent	883 ± 62	1,042 ± 212
Present	72 ± 35	949 ± 192
	20,000 ppm CO <sub>2</sub>	
Absent	15,860 ± 600	14,906 ± 393
Present	15,709 ± 661	17,080 ± 837

<sup>a</sup>CO<sub>2</sub> readings obtained every 2 h for 48 h; 16-h photoperiod.

Table 6. Effect of CO<sub>2</sub> and light levels on budbreak, elongation, and leaf development of axillary shoots from cotyledonary nodes of cacao.<sup>a</sup>

Light (μmol·s <sup>-1</sup> ·m <sup>-2</sup> )	CO <sub>2</sub> (ppm)	
	800	20,000
	<i>Budbreak/explant</i>	
45	3.9 ± 0.6 <sup>y</sup>	2.2 ± 0.4
200	4.0 ± 1.2	1.9 ± 0.5
	<i>Elongation/explant (mm)</i>	
45	10.2 ± 2.8	18.0 ± 4.2
200	10.0 ± 2.1	14.8 ± 3.0
	<i>Leaf area (cm<sup>2</sup>)</i>	
45	1.1 ± 0.3	2.1 ± 0.4
200	0.6 ± 0.1	1.8 ± 0.2
	<i>Leaf area/explant (cm<sup>2</sup>)</i>	
45	0.7	6.1
200	1.9	6.8
	<i>Leaf no./explant</i>	
45	0.4 ± 0.2	2.9 ± 0.5
200	2.9 ± 1.0	3.9 ± 0.5

<sup>a</sup>Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 8 to 20).

<sup>y</sup> ± SE.

high light conditions was 0.2 ± 0.2 SE as compared to 0.8 ± 0.6 for low CO<sub>2</sub>/low light levels. Shoot elongation per bud in high CO<sub>2</sub>/high light levels was 8.8 ± 1.1 compared to 3.2 ± 2.2 for low CO<sub>2</sub>/low light levels. The most striking effect of the high CO<sub>2</sub>/high light combination, however, was the normal leaf expansion and development, which was not achieved in previous experiments.

To separate the CO<sub>2</sub> effect from the light effect, a factorial experiment was conducted with cotyledonary nodal shoots, using two light levels (45 μmol·s<sup>-1</sup>·m<sup>-2</sup> or 200 μmol·s<sup>-1</sup>·m<sup>-2</sup>) and two CO<sub>2</sub> levels (Table 6). High levels of CO<sub>2</sub> 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<sub>2</sub> (Fig. 1). Leaves were smaller under high light, but the number of leaves and total leaf area per explant increased.

The same CO<sub>2</sub> 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<sub>2</sub> (Table 7). Shoot elongation and number of leaves per explant were increased by the high light level at either CO<sub>2</sub> level. High CO<sub>2</sub> increased budbreak and elongation significantly under low light; however, under high light there was no CO<sub>2</sub> effect on budbreak and the

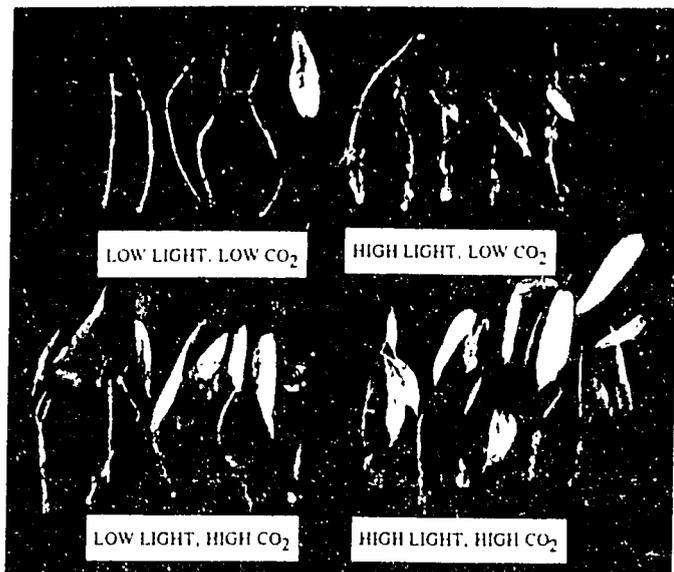


Fig. 1. Response of axillary nodal shoots of cacao to low and high levels of light and CO<sub>2</sub> (see Table 6).

Table 7. Effect of CO<sub>2</sub> and light levels on budbreak, elongation, and leaf development from nodes derived from mature trees of cacao.<sup>a</sup>

Light (μmol·s <sup>-1</sup> ·m <sup>-2</sup> )	CO <sub>2</sub> (ppm)	
	800	20,000
	<i>Budbreak/explant</i>	
45	0.4 ± 0.1 <sup>y</sup>	0.7 ± 0.1
200	1.0 ± 0.1 <sup>y</sup>	1.0 ± 0.1
	<i>Elongation/explant (mm)</i>	
45	1.3 ± 0.3 <sup>y</sup>	2.6 ± 0.4
200	3.9 ± 0.5 <sup>y</sup>	4.4 ± 0.6
	<i>Leaf no./explant</i>	
45	0.0 ± 0.0 <sup>y</sup>	0.1 ± 0.0
200	0.3 ± 0.1 <sup>y</sup>	0.8 ± 0.2

<sup>a</sup>Shoots were cultured on semi-solid WPM. Data obtained after 4 weeks (treatment n = 30 to 39).

<sup>y</sup> ± SE.

increase in elongation was not significant. The greatest elongation was achieved under high CO<sub>2</sub>/high light. High CO<sub>2</sub> increased the number of leaves produced under high but not under low light.

*Subculture of cotyledonary nodal shoots.* Axillary cotyledonary shoots from high CO<sub>2</sub> were dissected into nodal cuttings and recultured under high CO<sub>2</sub>/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 subcultured 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 subcultured axillary shoots under high light and high CO<sub>2</sub> 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<sub>2</sub> and high light levels.



Fig. 4. Growth of subcultured secondary shoots from mature plants induced by high CO<sub>2</sub> 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 cotyledonary 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<sub>2</sub> within culture vessels containing cacao explants indicate that CO<sub>2</sub> is being depleted during the day. The enhancement of shoot elongation and leaf development under a high CO<sub>2</sub>/high light regime in this study is consistent with the hypothesis that an increase in photosynthesis by high light levels, high CO<sub>2</sub>, 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 μmol·s<sup>-1</sup>·m<sup>-2</sup>), but we do not rule out some undetected CO<sub>2</sub> effect.

In conclusion, our results indicate that high CO<sub>2</sub> 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<sub>2</sub>/high light were observed with axillary shoots from cotyledonary nodes and nodal 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<sub>2</sub> enrichment reported by Kozai (1990) for various crops, but the CO<sub>2</sub> levels we used for cacao are considerably higher (20,000 ppm vs. 2000 to 3000 ppm). The optimum CO<sub>2</sub> 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<sub>2</sub> levels.

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## CACAO GUM: A POTENTIAL NEW ECONOMIC PRODUCT.

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### ABSTRACT:

A ubiquitous gum is present in large amounts in virtually all tissues of cacao (*Theobroma cacao* L., *Sterculiaceae*). Gum karaya, a gum produced from various *Sterculia* species in the same family, has been used in the food and medical industry, but its use has dwindled greatly because of unreliable supply. Studies were undertaken to characterize the gum of cacao derived from stems and mature pod husks. Histochemical studies located lysigenous cavities throughout the pith and cortex of stems and pericarp of pod husks. Yield averaged 1.5% of fresh weight and 8.4% of dry weight for stem gum, and 0.7% of fresh weight and 8.7% of dry weight for pod gum. Cacao pod gum was closer in composition to gum karaya than was stem gum. Both cacao gums contained the same monosaccharides as gum karaya, but with the addition of arabinose and with higher proportion of rhamnose. The major component of cacao stem gum was glucose, which is not found in either gum karaya or cacao pod gum. It also contained more glucuronic acid than either of these gums. Cacao stem gum has a higher viscosity at concentrations below 1% than gum karaya. Our results suggest that the gums extracted from pods, a waste product of the cacao industry, could be a promising source of a new industrial or food gum.

### KEYWORDS:

cacao - gum - gum karaya - polysaccharides - *Sterculiaceae* - *Sterculia* spp - *Theobroma cacao*

### INTRODUCTION:

Lysigenous cavities filled with mucilaginous substances, which stain heavily, occur in roots (primary and secondary tissues), stems (primary and secondary tissues), flowers, and leaves (petioles, stipules, pulvini, palisade layer and midrib) of cacao (*Theobroma cacao*, *Sterculiaceae*) (Brooks & Guard, 1952). The presence of large amounts of gums in cacao tissues implies an important physiological role, which could be related to storage, osmoprotection, defense mechanisms, or other processes.

Polysaccharides of cacao were first characterized by Whistler *et al.* (1956), who found differences in hot-water-soluble polysaccharides between seed and pod husks. Blakemore *et al.* (1966) examined the hot-water-soluble fraction of husk polysaccharides and concluded that the major part of this fraction was pectic materials. Cacao pod husks (mature fruits without seeds or pulp) were examined as a source of pectin by mild acid extraction by Adomako (1972, 1975) and Berbert (1972), but

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yields were low and the pectin was inferior to apple or citrus pectin in gel-forming ability.

Gum karaya, produced from various *Sterculia* species, mainly *Sterculia urens* Roxb., *Sterculiaceae*, has been used in the food and medical industry (Glicksman, 1982), but its use has diminished because its supply is variable and unreliable. Gums of cacao, a species related to karaya, might serve as a substitute.

Our original interest in cacao gums arose from its suspected role in the recalcitrance of cacao growth in vitro (Figueira *et al.*, 1989). The objectives of this study were to characterize cacao gums from stems and husks, and to evaluate their potential as a replacement for gum karaya or as a new commercial product.

#### MATERIALS AND METHODS:

**Histological studies:** Mature cacao pod husk sections (1 cm<sup>3</sup>) and primary and secondary stem tissues (1 cm long) were fixed in formalin-acetic acid (FAA) followed by dehydration with ethanol-tert-butanol (Sass, 1951), and embedded in Paraplast (Monoject Scientific, St. Louis). Sections were cut 15  $\mu$ m thick and then double stained with safranin-fast green for observation by light microscopy.

**Chemical analysis:** Freshly collected stems and husks were boiled in 70% ethanol for 30 min for depigmentation, then extracted with hot methanol. The remaining tissues were dried briefly, homogenized in distilled water, and centrifuged at 1500 g for 30 min. The supernatant was then concentrated, and gum was precipitated with 3 volumes of 95% ethanol and collected by centrifugation for 30 min at 4200 g. The pellet was redissolved in water and freeze-dried. The crude gum was purified by passing a solution of it through a cation-exchange column (Amberlite IR.120[H<sup>+</sup>]) and eluting with distilled water.

Colorimetric assays for total neutral sugars (Dubois *et al.*, 1956), protein (Peterson, 1977), and uronic acids (York *et al.*, 1985) were performed on stem and pod gums before and after purification. Samples (2 mg) of cacao gums and gum karaya (Sigma) were solvolyzed with liquid HF (5 ml) for 2 h; hydrolysis was then effected by addition of Millipore-filtered water (10 ml) (J. BeMiller & M. Yadav, unpublished method). The samples were dried at 50°C under nitrogen gas, redissolved in 0.5 ml of filtered water, passed through a 0.2  $\mu$ m filter, and analyzed using Dionex (Sunnyvale, California) BioLC Gradient Pump Module HPLC with a Model Pad 2 pulsed amperometric detector (Hardy *et al.*, 1988). Samples from crude and purified preparations were digested using a hydrogen peroxide-perchloric acid procedure (Adler & Wilcox, 1985); potassium, calcium and magnesium concentrations were estimated using Varian Model Spectra AA 10 Atomic Absorption Spectrophotometer (Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia).

**Rheology studies:** Purified cacao stem gum solution viscosity was compared with gum karaya solution using a Brookfield (Stoughton, Massachusetts) model LVTD viscometer with the small sample adapter and spindle SCH-31. Viscosity was determined at various concentrations and spindle speeds.

#### RESULTS AND DISCUSSION:

**Histology:** Histochemical studies confirmed the ubiquitous presence of gums in cacao. The gum was found in lysigenous cavities as noted by Brooks & Guard (1954) which stained a deep magenta with safranin (Fig. 1). These cavities were located throughout the pith and cortex of stems, constituting a significant portion of the cross-sectional area of non-lignified shoots and were found

throughout the pericarp of husks (Fig. 1). In *Sterculia urens*, the major karaya producing species, gum ducts develop lysigenously and lysi-schizogenously (Nair & Shah, 1984). Gum formation is from the product of lysis of the epithelial cells in the pith ducts and in the cortical ducts it originates from the transformation of part of the inner tangential wall followed by the lysis of the epithelial cells. The anatomical site and mechanism of cacao gum formation is unknown.

Extraction and chemical analyses: In the present study, gum yield averaged 1.5% of fresh weight and 8.4% of dry weight of stems, and 0.7% of fresh weight and 8.7% of dry weight of husks, indicating that the gum is a significant portion of the plant dry matter. Polysaccharide yields from husks reported in the literature range from 8 to 11% dry weight basis (Blakemore *et al.*, 1966; Adomako, 1972; Berbert, 1972). Although carbohydrates play a major role controlling rhythmic shoot growth (flushing) of cacao (Machado & Hardwick, 1987), no attention had been placed on the potential role that polysaccharides play on the carbohydrate balance, or possibly on host-pathogen interaction. We have hypothesized that this gum is responsible for the recalcitrance of cacao to in vitro culture (Figueira *et al.*, 1989).

Colorimetric assays indicated that cacao gums are acidic protein-polysaccharides and that gums from pods are distinct from gums from stems (Table 1). Both gums have comparable amounts of neutral sugars, but the pod gum has a higher percentage of uronic acids. Uronic acid content of gum karaya ranges from 35 to 40% (Aspinall & Nasir-Ud-In, 1965; Goldstein & Alter, 1973; Glicksman, 1982). A similar amount was found in cacao pod gum. While cacao stem gum contained about 30% protein, the protein content of gum karaya is only about 1% (Anderson *et al.*, 1985).

A proposed structure of gum karaya has a main backbone chain consisting of D-galacturonic acid, L-rhamnose, and D-galactose units to which are attached single unit side chains of D-glucuronic acid or D-galactose units (Phillips *et al.*, 1980). Other structures having larger side chains have also been proposed (Goldstein & Alter, 1973). The proportions of these four monosaccharides vary with species and gum quality and type (Hirst *et al.*, 1949; Goldstein, 1954; Anderson *et al.*, 1982). The sugar composition of gum karaya obtained in the present study agreed qualitatively with previous estimates, but the proportion of monosaccharides differed. Both sources of cacao gums contained the same monosaccharides as gum karaya, but with the addition of arabinose (Table 2) and with greater amounts of rhamnose. Cacao pod gum was closer in composition to gum karaya than was cacao stem gum.

The major component of cacao stem gum was glucose, not found in any appreciable amount in cacao pod gum or gum karaya. Cacao stem gum also contained more glucuronic acid than did the other two gums. The relative proportions of monosaccharides in cacao pod gums observed in this study is similar but not identical to those reported by Whistler *et al.* (1954), Blakemore *et al.* (1966), and Adomako (1972) (Table 2). We observed glucuronic acid, previously unreported, but did not detect the presence of mannose as reported by Whistler *et al.* (1954) and Blakemore *et al.* (1966). The high levels of rhamnose and arabinose we observed differed from previous studies. Glucose and xylose were either undetected or found in reduced amounts in all studies. Uronic acids were undetected by Whistler *et al.* (1954), but observed at very high levels by Adomako (1972), this may be an artifact of the extraction method. Differences in proportions of monosaccharides are not unexpected because of the variability of source material (Glicksman, 1982) and different isolation and analyses procedures.

Crude cacao gums had a high concentration of potassium (Table 3), but purification by a cation-

exchange column successfully reduced the cationic contamination. Total ash content previously observed for cacao pod gums was 9% (Whistler *et al.*, 1956; Adomako, 1972), or 22.7% when based on dry husks (Blakemore *et al.*, 1966). Adomako (1972) identified calcium and potassium as the major elements present. Gum karaya usually occurs in a salt form containing calcium and magnesium (Kubal & Gralen, 1948). The high concentration of potassium in cacao pod husks explains the use of pod ash for soap production in Nigeria (Oduwele & Arueya, 1990).

**Viscosity:** The logarithm of the apparent viscosity of both cacao stem and karaya gums increased linearly with concentration (Fig. 3). However, gum karaya presented a greater rate of increase in viscosity for all levels of shear stress imposed. At 3% concentration, karaya had an apparent viscosity ranging from about 20,000 centipoises (cp) at 12 rpm to 60,000 cp at 60 rpm. Cacao stem gum had lesser rate of increase in viscosity with concentration reaching values of 500 cp at 3% concentration; at 5% concentration, the viscosity was comparable to that of a gum karaya solution at 3%. However, cacao stem gum had a higher viscosity at concentrations below 1%. Viscosity determinations of cacao pod gum are under investigation.

According to Goldstein & Alter (1972), gum karaya is water-swellable rather than water soluble and absorbs water rapidly to form viscous colloidal dispersions at low concentrations. A finely powdered form of gum karaya was used; the purified cacao stem gum was not ground finely. The rate of hydration in karaya is very dependent upon the mesh size of the gum (Glicksman, 1982). The cacao stem gum used was in the purified, i.e. the free acid, form which could have affected its viscosity.

**Potential uses:** Pod husks are a waste product of the cacao industry, and present a serious disposal problem. They become a significant source of disease inoculum when used as a mulch inside the plantation. Husks may be used as livestock feed, but the theobromine content reduces the proportion that can be consumed, and its use has been restricted (Wood & Lass, 1985).

Gum karaya, once an important food industry gum, has been used as an emulsifier, stabilizer and/or viscosifier for food products and as a fixative/adhesive in the pharmaceutical industry (Anderson *et al.*, 1982). In recent years, gum karaya has become relatively expensive due to tapping restrictions in India (Anderson *et al.*, 1985). A substitute gum would be of interest (Aslam *et al.*, 1978) and the gum of cacao, which is related to karaya, might serve as a substitute.

Our results suggest that water extracts of cacao stems and pods may be a promising source of a new industrial or food gum which could provide an additional source of revenue to the cacao industry. Additional research is needed to determine specific uses.

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Table 1. Preliminary analysis of cocoa gum before and after cation exchange and dialysis.

Component	Content (% of sample)			
	Crude stem gum	Stem gum after cation exchange	Crude pod gum	Pod gum after cation exchange
Neutral sugars	49	64	47	50
Uronic acids	20	20	44	39
Protein	29	30	ND <sup>‡</sup>	ND

<sup>‡</sup>=not determined

Table 2. Sugar comparison of cacao gum and gum karaya.

Gum source	Sugar composition (molar ratio) <sup>‡</sup>							
	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA
Gum karaya	1.6	0.0	1.0	0.1	0.0	0.0	1.3	0.6
Cacao stem gum	2.0	1.7	1.0	2.8	0.0	0.0	1.1	1.4
Cacao pod gum	2.4	2.1	1.0	0.1	0.1	0.0	1.1	0.6
Cacao pod gum <sup>‡</sup>	1.0	0.3	1.0	0.0	0.0	0.3	0.0	0.0
Cacao pod gum <sup>*</sup>	0.4	0.2	1.0	0.4	traces	0.3	1.3	0.0
Cacao pod gum <sup>‡</sup>	0.6	0.4	1.0	traces	0.3	0.0	13.4	0.0

<sup>‡</sup> All monosaccharides were standardized for galactose molar concentration

<sup>‡</sup> Whistler *et al.* (1956)

<sup>\*</sup> Blakemore *et al.* (1966)

<sup>‡</sup> Adomako (1972)

Table 3. Cation concentration of cacao gum in percent dry weight.

Gum source	Calcium	Magnesium	Potassium	Total
Crude pod gum	0.10	0.40	9.64	10.14
Purified pod gum	0.07	0.02	0.01	0.10
Crude stem gum	0.42	1.34	3.81	5.57
Purified stem gum	0.19	0.04	0.03	0.26

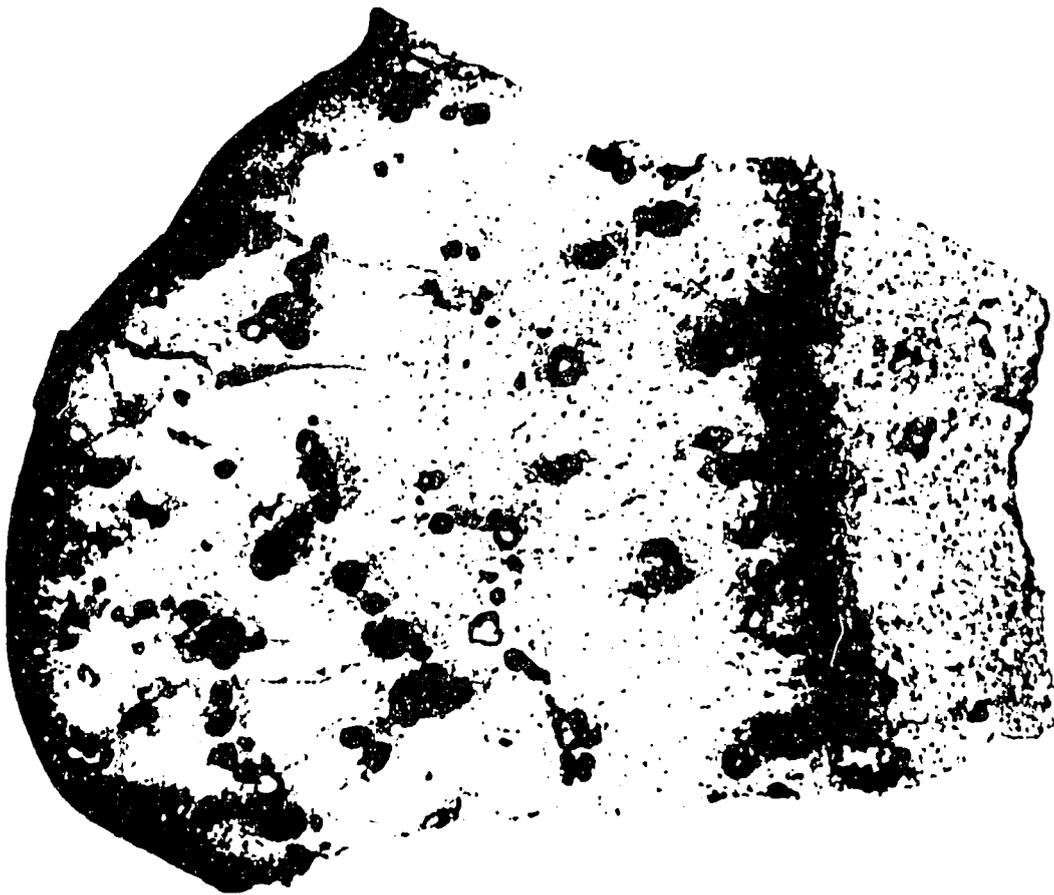


Figure 1. Cross section of cacao pod husk showing darkly stained lysigenous cavities.

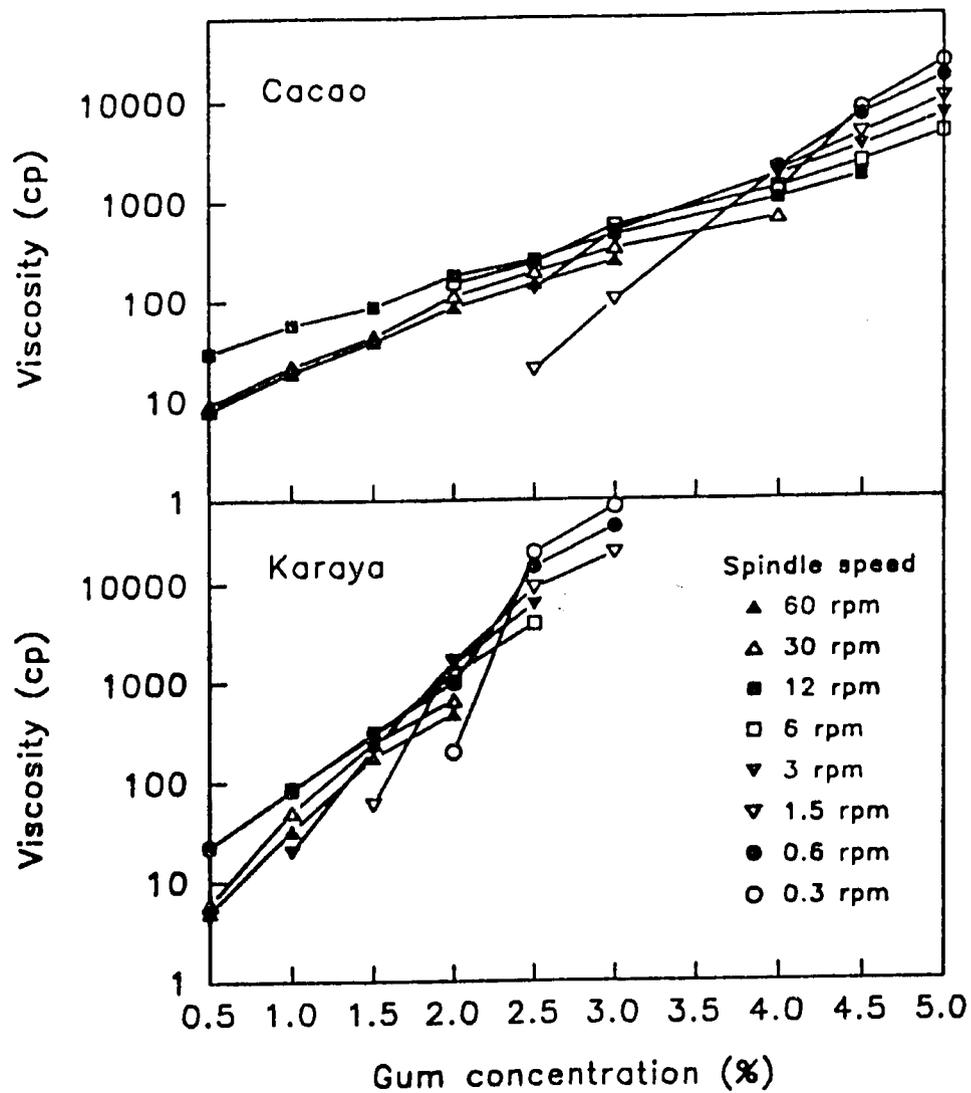


Figure 2. Changes in viscosity with concentration of cacao stem gum and gum karaya at various spindle speeds.

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ELEVATED CO<sub>2</sub> FACILITATES MICROPROPAGATION OF *THEOBROMA CACAO* L.

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ABSTRACT:

Cacao (*Theobroma cacao* L.) has been recalcitrant in tissue culture using conventional protocols. In vitro culture of cacao depleted CO<sub>2</sub> during the day, limiting photosynthesis. Shoot elongation and leaf development increased asymptotically after 30 days with CO<sub>2</sub> levels from ambient (470 ppm) to 28,000 ppm. The CO<sub>2</sub> effect did not occur in the dark indicating that photosynthesis stimulation is the major cause of enhanced in vitro performance. High CO<sub>2</sub> was effective in promoting shoot and root development of cotyledonary somatic embryos.

KEYWORDS: Micropropagation - tube closures - photoautotrophism

INTRODUCTION:

*Theobroma cacao* L. (cacao) has been recalcitrant in tissue culture. Until recently only sporadic growth and proliferation of shoots obtained from mature plants have been achieved (Passey and Jones, 1983; Blake and Maxwell, 1984; Legrand *et al.*, 1984; Legrand and Mississo, 1986; Adu-Ampomah *et al.*, 1988). Although axillary shoots can be induced in vitro from cotyledonary nodes after removal of the epicotyl or with cytokinin supplementation, subcultured shoots failed to grow under conventional tissue culture modifications (Janick & Whipkey, 1985). Flynn *et al.* (1990) reported bud elongation and leaf development from mature shoots cultured in vitro without exogenous growth regulators, using higher photosynthetic photon flux densities. Recent results in our laboratory indicate that detached cotyledonary axillary shoots, single-node cuttings from mature plants, as well as microcuttings from elongated shoots cultured in vitro, elongate and produce leaves in vitro under elevated CO<sub>2</sub> and high light (Figueira *et al.*, 1991).

Another possibility for micropropagation of cacao exploits somatic embryogenesis. Somatic embryos can be induced from immature zygotic embryos, either directly from cotyledons (Esan, 1977; Pence *et al.*, 1979, 1980; Kong & Rao, 1982; Tahardi, 1984; Santos & Maclado, 1989), or indirectly from embryogenic competent callus (Kononowicz *et al.*, 1984). Recently somatic embryogenesis was reported from mature sporophytic tissues (young bud petals and nucellus tissue) (Söndahl, 1991). However, the development of somatic embryos into autotrophic seedlings has been marginal.

Results presented in this paper indicate that elevated CO<sub>2</sub> promotes shoot elongation and leaf development of microcuttings or somatic embryos.

## MATERIALS AND METHODS:

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 removed. Embryos were germinated on Woody Plant Medium (WPM) (Lloyd and McCown, 1980), supplemented with 88.8 mM fructose and either 2 g liter<sup>-1</sup> gellan gum (GelRite) or 8 g liter<sup>-1</sup> agar (Sigma). The pH of the media was adjusted to 5.7 before autoclaving. Epicotyls were removed 4-6 weeks after seeds had germinated, and the axillary shoots were collected 4-6 weeks after decapitation, and used as explants in experiments described below.

CO<sub>2</sub> chambers: Elevated CO<sub>2</sub> treatments were conducted in clear acrylic chambers which were supplied with compressed CO<sub>2</sub> and air mixed with a Matheson flowmeter. The CO<sub>2</sub> concentration in the chambers was measured using a Carle GC 8700 gas chromatograph with a thermal conductivity detector. The chambers were held at 31°C/24°C day/night cycle and illuminated as described with VHO cool white fluorescent lamps. Relative humidity inside the chambers ranged from 50 to 70%.

Diurnal CO<sub>2</sub> 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, and silicone septa were inserted in various types of closures: (1). polypropylene closures (Kaputs, Bellco Glass, Vineland, New Jersey, USA); (2). same closure, but wrapped with flexible plastic (Parafilm, American National Can, Greenwich, Connecticut, USA); (3). SunCaps (Nichimen Co, Tokyo, Japan) a plastic film containing a 8 mm diameter patch of microporous polypropylene, affixed to tubes with two rubber bands; (4). Star\*Pac (Agristar Inc. New Orleans, Louisiana, USA) a non-porous polyethylene film, affixed to tubes with two rubber bands. Treatments included 16 h photoperiod of 90  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  photosynthetic active radiation (PAR) from cool-white fluorescent lamps and ambient CO<sub>2</sub> (= low CO<sub>2</sub>) or 200  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  PAR from VHO cool white fluorescent lamps and 20,000 ppm CO<sub>2</sub> (= high CO<sub>2</sub>). Gas (1 ml) from inside the tubes was extracted with a syringe every 2 h for 48 h. Each type of closure had 4 treatments (with or without explants, high or low CO<sub>2</sub> and light), consisting of 5 tubes sampled sequentially every 2 h. CO<sub>2</sub> concentration was measured using a Carle GC 8700 gas chromatograph with a thermal conductivity detector.

Effect of CO<sub>2</sub> concentration and illumination: In the first experiment cotyledonary axillary shoots (ca. 5 cm) were transferred to tubes with 10 ml semi-solid WPM plus 88.8 mM fructose and capped with polypropylene closures (Kaputs). The tubes were then placed under six different CO<sub>2</sub> levels [average CO<sub>2</sub> concentrations measured at 470 ppm (growth room ambient); 2,000 ppm; 5,200 ppm; 9,300 ppm; 18,900 ppm and 28,000 ppm] and four photosynthetic photon flux densities (44, 53, 68 and 120  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) obtained by cheese-cloth shading inside the CO<sub>2</sub> chamber. CO<sub>2</sub> levels were monitored daily. In the second experiment cotyledonary axillary shoots were placed in chambers with ambient CO<sub>2</sub> or high CO<sub>2</sub> (20,000 ppm) either under high light (120  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) or complete darkness (wrapped in aluminum foil). Data on shoot elongation and leaf development were obtained four weeks after initiating treatment.

Effect of CO<sub>2</sub> on somatic embryo development: An embryogenic clone BC 46 was used, that had been maintained over 10 years (Pence *et al.*, 1979) on basal media containing MS salts (Murashige and Skoog, 1962) supplemented with 0.3  $\mu\text{M}$  thiamine-HCl, 2.4  $\mu\text{M}$  pyridoxine-HCl, 0.6 mM *i*-inositol, 4.1  $\mu\text{M}$  nicotinic acid, 26.6  $\mu\text{M}$  glycine, 1000 mg liter<sup>-1</sup> casein hydrolysate, and 87.6 mM

sucrose, and 8 g liter<sup>-1</sup> agar (Sigma). Proembryogenic callus which no longer developed beyond the globular stage, was transferred to liquid medium (1/2 MS + 116.8 mM sucrose + 10% coconut water), to induce cotyledonary structures. Cotyledonary embryos were transferred after 30 days to semi-solid WPM containing 88.8 mM fructose and grown under high (20,000 ppm) and low CO<sub>2</sub> (ambient) regimes.

## RESULTS AND DISCUSSION:

Diurnal CO<sub>2</sub> changes in vitro: There was little diurnal variation in CO<sub>2</sub> concentration within the tubes without cacao shoots for both CO<sub>2</sub> regimes (Table 1). The presence of cacao shoots decreased the CO<sub>2</sub> content inside the tubes under ambient CO<sub>2</sub> conditions for all closures during the day, except SunCap, but at night under the same conditions, all closures allowed CO<sub>2</sub> to return to levels similar to the control (absence of cacao shoot). Extreme CO<sub>2</sub> depletion during the day occurred with Kaputs plus Parafilm, indicating that this closure provided the greatest barrier to diffusion of CO<sub>2</sub>. There was no difference in CO<sub>2</sub> concentration due to the presence or absence of shoots at ambient CO<sub>2</sub> with SunCap closures, indicating that this closure did not restrict CO<sub>2</sub> exchange, which agrees with Kozai (1990). Under high CO<sub>2</sub> conditions, the presence of shoots only marginally decreased CO<sub>2</sub> during the day, and CO<sub>2</sub> returned to control levels during the night, except for SunCap. CO<sub>2</sub> levels with Star\*Pac or Kaput closures were higher under high CO<sub>2</sub> than in tubes closed with SunCap or Kaput plus Parafilm. These results suggest that under ambient CO<sub>2</sub> levels, cacao axillary shoots deplete CO<sub>2</sub> during the day to levels below the compensation point, limiting photosynthesis. Under low CO<sub>2</sub> conditions, SunCap allowed greater CO<sub>2</sub> exchange and prevented CO<sub>2</sub> build up during the dark period. However, at CO<sub>2</sub> levels of 20,000 ppm, Star\*Pac and Kaput allowed more CO<sub>2</sub> exchange than the other closures.

Effect of CO<sub>2</sub> concentration and illumination: In a previous study using detached axillary cotyledonary shoots and single-node cuttings from mature plants, we reported a beneficial effect of elevated CO<sub>2</sub> (20,000 ppm) on shoot elongation and leaf development (Figueira *et al.*, 1991). However, the CO<sub>2</sub> levels were much higher than the levels (1,000 to 4,500 ppm) employed by Lakso *et al.* (1986); Infante *et al.* (1989) and Kozai (1990). The effect of CO<sub>2</sub> concentration on shoot elongation and leaf development was tested with six CO<sub>2</sub> concentration from ambient to 28,000 ppm for 30 days.

Shoot and leaf development increased asymptotically as CO<sub>2</sub> increased from ambient to 28,000 ppm (Fig. 1). The CO<sub>2</sub> effects in Fig. 1 are averaged over four light levels, whose effects were not significant during this period. This response to increasing levels of CO<sub>2</sub> also suggests that an additional effect besides photosynthesis was involved, as CO<sub>2</sub> fixation is assumed to be optimum much below 2,000 ppm if absorption was as efficient as in field-grown plants. The response to high levels of CO<sub>2</sub> may simply be due to the low absorption of CO<sub>2</sub> into the relatively leafless shoot. The indirect effect of CO<sub>2</sub> permeability of closures is not a factor here because only Kaputs without a film wrapper was used. CO<sub>2</sub> levels inside the tubes during the day were slightly lower than chamber CO<sub>2</sub> levels but paralleled chamber differences (data not shown). The inhibition of photorespiration could also account for the beneficial effect of increasing CO<sub>2</sub>, but net photosynthesis measurements have not yet been made.

High CO<sub>2</sub> concentrations can prevent or delay ethylene responses when ethylene concentration is below 1 ppm, probably by competitive inhibition (McKeon & Yang, 1987). The possible ethylene inhibition effect of high CO<sub>2</sub> can be inferred from results from a second experiment, where axillary cotyledonary shoots were placed under high or low CO<sub>2</sub> at high light or dark conditions, in order to

determine if the CO<sub>2</sub> effect could be separated from photosynthesis. In high light, high CO<sub>2</sub> increased the number of leaves and elongation, but darkness decreased the number of leaves at both CO<sub>2</sub> levels and shoot elongation at high CO<sub>2</sub> (Table 2). These results imply that the major CO<sub>2</sub> effect is due to either enhanced CO<sub>2</sub> assimilation or to reduction in photorespiration, although some synergistic effect of high CO<sub>2</sub> on ethylene inhibition and photosynthesis cannot be discounted. Insufficient gas exchange and ethylene accumulation have been suggested as factors responsible for the recalcitrance of cacao in vitro (Blake & Maxwell, 1984; Dufour & Dublin, 1985; Etienne, 1988; Lemercier *et al.*, 1990), but no direct evidence has been presented due to lack of suitable experimental procedures. The common observation of excessive callus formation together with prominent stem lenticels in cacao tissue cultures suggests that ethylene is present.

Our present hypothesis is that the recalcitrance of cacao to tissue culture is a nutritional response that may be due to the presence of gums (Figueira *et al.*, 1989). The benefits of high CO<sub>2</sub> are assumed to be result of a shift to autotrophic nutrition, but the response to levels above saturation point suggests other beneficial effects.

Effect of CO<sub>2</sub> on somatic embryos development. High CO<sub>2</sub> plus high light promoted shoot elongation, leaf and root development of cotyledonary somatic embryos (Table 3 and Fig. 2); secondary embryogenesis was unaffected. Wang & Janick (1984, 1985) induced radicle development in somatic embryos by renewing liquid media and demonstrated that somatic embryo leachates inhibited lettuce seed germination. The inhibitor was shown not to be abscisic acid but resembled *p*-coumaric acid (Wang & Janick, 1985). Conversion of somatic embryos to seedlings was enhanced by suppression of cotyledons (Novak *et al.*, 1986; Adu-Ampomah *et al.*, 1987; Duhem *et al.*, 1989), use of liquid medium (Adu-Ampomah *et al.*, 1987), and inclusion of high levels of activated charcoal (Duhem *et al.*, 1989), protocol modifications all compatible with the hypothesis of an inhibitor. The explanations for our positive results with high CO<sub>2</sub> included: (1) a photosynthetic effect; (2) ethylene inhibition; (3) inhibitor reduction. Evidence for the latter is the report by Kobayashi *et al.* (1991) that increased CO<sub>2</sub> decreased total phenolic content in *Thalictrum* cell cultures.

In summary our results indicate that subculture of microcuttings or development of somatic embryos derived from sporophytic tissue under CO<sub>2</sub> enrichment could be the basis for a rapid system of micropropagation for cacao.

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Table 1. Diurnal CO<sub>2</sub> changes in culture tubes capped with various closures and containing or lacking axillary cotyledonary shoots of cacao in response to low and high CO<sub>2</sub>.

Cacao shoot	CO <sub>2</sub> concentration within sealed tubes (ppm)			
	Closure			
	Kaput	Kaput+ Parafilm	Star*Pac	Suncap
	<u>Ambient CO<sub>2</sub></u>			
	<u>Day</u>			
Absent	688±95	823±65	605±151	523±22
Present	289±40	71±36	139±39	523±26
	<u>Night</u>			
Absent	566±44	827±93	458±10	463±13
Present	670±98	949±205	716±70	509±18
	<u>High CO<sub>2</sub> (20000 ppm)</u>			
	<u>Day</u>			
Absent	20893±404	15824±656	21248±154	16362±346
Present	18729±37	15709±688	20080±372	13457±423
	<u>Night</u>			
Absent	21286±648	15126±421	21435±208	14761±1490
Present	19086±694	17080±904	21088±526	13124±258

Table 2. Effect of CO<sub>2</sub> and light on shoot elongation and leaf development of axillary cotyledonary shoots of cacao.

Illumination ( $\mu\text{mol}\cdot\text{s}^{-1}\text{m}^{-2}$ )	CO <sub>2</sub> (ppm)	
	500	20000
	<u>Shoot elongation (mm)</u>	
0	3.9±2.2	1.8±0.6
135	1.9±0.5	6.1±2.1
	<u>Number of leaves</u>	
0	0.6±0.2	0.5±0.2
135	1.4±0.3	2.8±0.3

Table 3. Effect of CO<sub>2</sub> on development of cacao somatic embryos.

CO <sub>2</sub> (ppm)	Shoot development (%)	Root development (%)	Secondary embryos (%)
500	21±8	3±3	41±9
20000	50±10	7±5	51±10



Figure 2. Development of somatic embryos in response to high CO<sub>2</sub>.

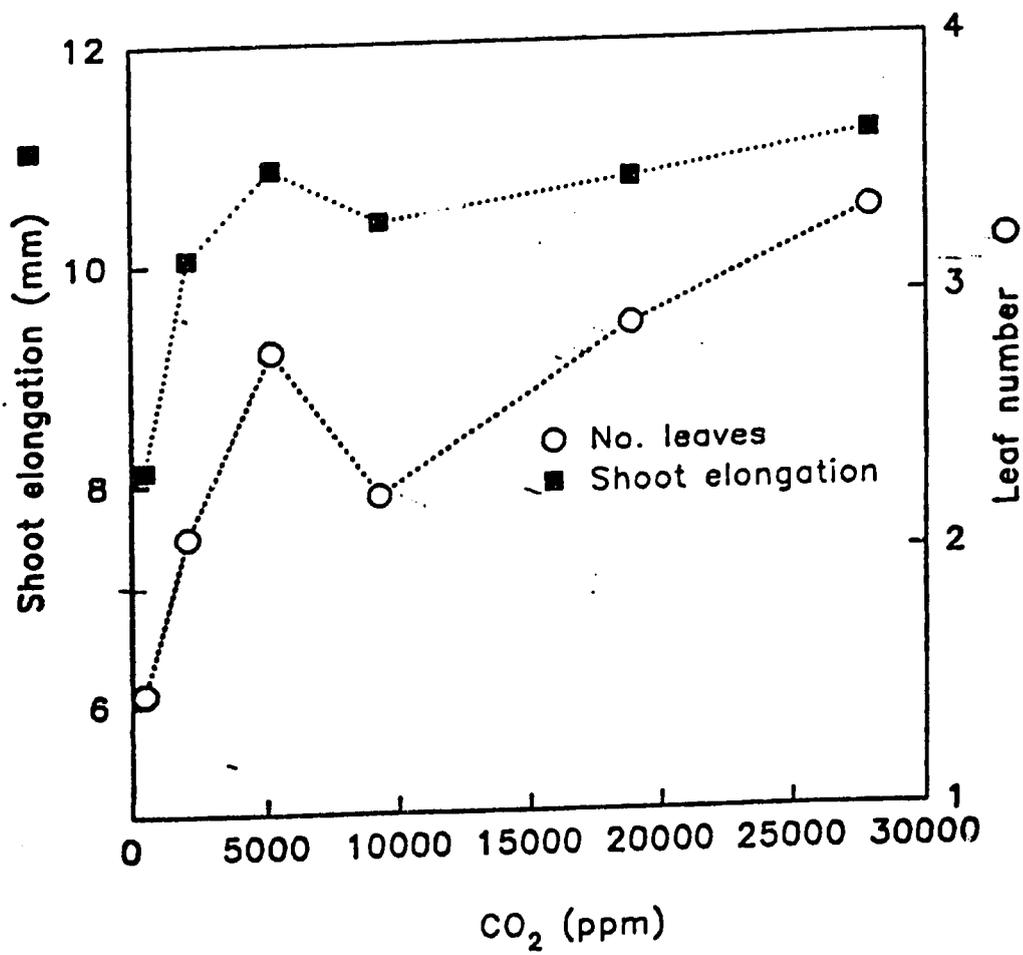


Figure 1. Response of axillary cotyledonary shoots of cacao to CO<sub>2</sub> concentration after 30 days. The responses are averaged over four light levels (44, 53, 68 and 120  $\mu\text{mol s}^{-1}\text{m}^{-2}$ ).

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