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Propagation of Theobroma spp.  
by Tissue Culture

3rd Semi-annual Report  
9/30/87-3/31/88

Submitted by

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to

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- I. Germplasm collection
- II. In vitro induction of axillary shoots from the cotyledonary node of cacao
- III. Shoot proliferation from seedling and mature shoots of cacao
- IV. Somatic embryogenesis in Theobroma grandiflorum

#### I. Germplasm collection

A trip to Brazil was made on September 17, 1987 in order to collect germplasm and to establish cooperative arrangements between Brazilian researchers at CEPLAC, the Brazilian research organization, and Bioplanta, a private biotechnology company interested in tropical agriculture.

I arrived in Belem on September 18 and was met by Paulo Alvim, a director of CEPLAC and Marcos Paiva, my ex-PhD student who is an employee of Bioplanta interested in both cupuacu (Theobroma grandiflorum) and cacao. We visited the research station and the experimental germplasm collection of CEPLAC. We located Fazenda Itakii where a 40 hectare farm of cupuacu including the seedless clone is being grown. There were no young fruits available but we collected seeded fruit. On October 19 we flew to Manaus where we visited CEPLAC facilities and visited research plantings of cupuacu. We also made contact with the Tropical Research Institute at Manaus. We next visited Ilhaus, headquarters of CEPLAC where I presented a seminar on my research on cacao (in Portuguese) and made arrangements to sponsor graduate training of a CEPLAC employee, Antonio Figueira. Figueira has been accepted as a graduate student and will be working on cacao propagation. He plans to arrive May 26, 1988. We located mature fruit of the seedless clone of cupuacu. We reviewed the cacao research

program as well as the tissue culture research at CEPAC. I collected mature fruits of cacao to bring back to the U.S.

I spent the last 3 days in Campinas at Bioplanta where I reviewed tissue culture propagation, mycorrhiza research, and explored Bioplanta's interest in cupuacu. A cooperative project on somatic embryogenesis was initiated with Marcos Paiva on the basis of immature embryos of cupuacu that we collected in Belem. I presented a seminar at Bioplanta on my cacao research.

## II. Effect of cotyledons on axillary bud break and shoot elongation

An experiment was designed to determine if Thidiazuron-induced nodal shoot proliferation in seedlings was effected by the presence of cotyledons or node splitting. There were three treatments:

(1) cotyledons present, (2) cotyledons absent node entire, and (3) cotyledons absent node split. Shoot proliferation was greatest with cotyledons present; however with cotyledons absent there was some bud proliferation, indicating that bud proliferation was not cotyledon dependant (Table 1). Surprisingly, twice as many shoots per node were produced in cotyledonless nodes from split than unsplit nodes, suggesting that wounding contributes to nodal bud break. Shoot elongation was only achieved in the presence of cotyledons indicating

Table 1. The effect of cotyledons and node splitting on axillary budbreak, semi-solid MS medium, sucrose.

Treatment	Budbreak (no.)
Cotyledons attached	3.1±0.8
Cotyledons detached	
node entire	1.1±0.3
node split	2.3±0.3

that shoot elongation is cotyledon dependant. We consider the cotyledonary factor responsible for shoot elongation the key to in vitro propagation of cacao. The factor may be nutritional or hormonal.

### III. Shoot proliferation from cotyledonary nodal shoots and mature shoots

In semi-annual reports 1 and 2 we reported that Thidiazuron increased shoot proliferation both from shoots derived from cotyledonary nodes of seedlings and vegetative shoots of mature plants. Although these shoots continued to proliferate in the presence of Thidiazuron, shoots failed to grow. We failed to get any response from a number of treatments including, ascorbic acid, glycine, auxin (NAA), GA<sub>3</sub>, 7-azaindole, or brief exposure to high concentrations of cytokinin (BA). The most responsive stage for shoot proliferation in mature shoots was during leaf expansion (F-2). Studies in this report concentrated on interaction of GA<sub>3</sub> and Thidiazuron, carbon source, and basal medium salts.

#### A. Interaction of GA<sub>3</sub> and Thidiazuron

In semi-annual report 2 there was some indication that Thidiazuron and GA<sub>3</sub> were synergistic in inducing bud break (see semi-annual report 2 Table 5). An experiment evaluated Thidiazuron from 0.01 to 0.1  $\mu$ M and GA<sub>3</sub> from 0.1 to 1.0 mg/liter. Results confirm previous studies: maximum budbreak was achieved with 0.05  $\mu$ M Thidiazuron plus 0.1 mg/liter GA<sub>3</sub>, but differences between treatments were slight (Table 2).

#### B. Effect of carbon source

Cotyledonary nodal shoots were grown in MS semi-solid and liquid medium with five sugar treatments without Thidiazuron as shown in

Table 3. In liquid media, explants did poorly with essentially no budbreak or rooting. In semi-solid medium budbreak was essentially nil in all treatments, but rooting of shoots was affected by carbon source with the greatest rooting achieved in fructose. Rooted explants from these treatments were transferred to liquid media with sucrose and Thidiazuron in order to see if the presence of roots would increase

Table 2. The effect of Thidiazuron and GA<sub>3</sub> on budbreak and callus growth of shoots from cotyledonary nodes of cacao, semi-solid, MS medium, 3% sucrose.

GA <sub>3</sub> (mg/liter)	Thidiazuron			
	0 μM	0.01 μM	0.05 μM	0.1 μM
	<u>Budbreak (no.)</u>			
0	1.6±0.3	1.5±0.3	1.7±0.3	1.3±0.2
0.1	1.8±0.3	1.6±0.3	2.3±0.5	1.6±0.3
0.5	2.0±0.6	1.7±0.2	1.9±0.3	1.4±0.2
1.0	1.2±0.1	1.0±0.2	1.4±0.3	1.3±0.2
	<u>Callus rating<sup>z</sup></u>			
0	0.5±0.2	2.0±0.2	2.1±0.2	2.9±0.1
0.1	0.7±0.2	1.3±0.2	1.9±0.3	2.0±0.2
0.5	0.5±0.2	1.6±0.2	1.8±0.1	1.9±0.2
1.0	0.6±0.2	1.7±0.2	1.9±0.2	2.3±0.2

<sup>z</sup> 0 (none) to 3 (abundant)

Table 3. Effect of carbon source on budbreak and rooting, 10 explants per treatment evaluated after 45 days, semi-solid, MS medium.

Carbon	Concn		Bud break (no.)	Rooting	
	%	μM		%	No. roots / rooted explant
Sucrose	3	88	0	30	1.3
Glucose	1.6	88	0	50	1.8
Fructose	1.6	88	0.2	70	2.4
Raffinose	5.2	88	0	30	2.0
Sucrose + Stachyose	3.0	88+	0	40	1.0

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budbreak or elongation. Thidiazuron increased budbreak but these buds did not elongate, suggesting that the presence of roots did not induce shoot elongation (data not presented).

### C. Effect of basal media salts

Five treatments (Table 4), based on three media were evaluated for

Table 4. Salt concentration of three media.

Components	Salt concentration (mg/liter)		
	Murashige and Skoog	Woody Plant Medium	Anderson's Rhododendron Medium
NH <sub>4</sub> NO <sub>3</sub>	1650.000	400.000	400.000
KNO <sub>3</sub>	1900.000		480.000
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O		556.000	
K <sub>2</sub> SO <sub>4</sub>		990.000	
CaCl <sub>2</sub>	333.000		
CaCl <sub>2</sub> ·2H <sub>2</sub> O		96.000	440.000
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O			380.000
MgSO <sub>4</sub>	181.000		
MgSO <sub>4</sub> ·7H <sub>2</sub> O		370.000	370.000
KH <sub>2</sub> PO <sub>4</sub>	170.000	170.000	
FeNaEDTA	36.700		
FeSO <sub>4</sub> ·7H <sub>2</sub> O		27.800	55.700
Na <sub>2</sub> EDTA		37.300	74.500
H <sub>3</sub> BO <sub>3</sub>	6.200	6.200	6.200
MnSO <sub>4</sub> ·H <sub>2</sub> O	16.900	22.300	16.900
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.600	8.600	8.600
KI	0.830		
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.250	0.250	0.250
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.250	0.025
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025		0.025

budbreak on nodal and mature shoots. Full strength WPM was best for budbreak although differences between treatments were slight (Table 5). Explant appearance was best with WPM and after four subcultures survival was greatest with WPM.

### D. Interaction of media and Thidiazuron

The interaction of media (WPM and ARM), salt strength, and the presence or absence of Thidiazuron was tested with mature and nodal shoots using sucrose as the carbon source. The response of nodal shoots

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Table 5. Effect of basal media composition and concentration on budbreak and explant survival, semi-solid medium, 3% sucrose.

Media	Budbreak (no.)	Survival (%)			
		1	2	3	4
		<u>Nodal shoots</u>			
MS	0.5	70	50	40	20
1/2MS	0.0	90	60	50	50
WPM	0.6	100	100	90	70
1/2WPM	1.0	90	90	70	70
ARM	0.3	90	90	80	60
		<u>Mature shoots</u>			
MS	1.0	70	60	10	0
1/2MS	1.1	60	20	0	-
WPM	2.1	90	80	70	60
1/2WPM	1.1	60	50	30	30
ARM	1.7	100	90	50	30

was greatest with full strength WPM and Thidiazuron (Table 6). This confirms previous experiments; cacao shoots respond best to full

Table 6. Interaction of basal media and Thidiazuron, semi-solid medium, 3% sucrose.

Media	Budbreak (no.)	
	Thidiazuron	
	0.0 $\mu$ M	0.05 $\mu$ M
	<u>Nodal</u>	
WPM	0.5 (10) <sup>z</sup>	3.5 (10)
1/2WPM	0.4 (10)	1.7 (10)
ARM	0.2 (10)	2.3 (8)
1/2ARM	0.7 (10)	1.8 (10)
	<u>Mature</u>	
WPM	2.6 (5)	2.1 (7)
1/2WPM	3.0 (2)	0.6 (7)
ARM	2.3 (6)	2.0 (7)
1/2ARM	2.3 (6)	1.8 (2)

<sup>z</sup> (no. uncontaminated shoots)

strength WPM and that Thidiazuron increases budbreak. Half strength ARM performed the same as half strength WPM. Results from mature shoots were confounded by high contamination rates in this experiment, but indicated no response to Thidiazuron

#### E. Effect of carbon source and salt strength

This experiment was designed to test an interaction between sugar source and WPM media at full and half strength; all treatments contained 0.05  $\mu\text{M}$  Thidiazuron. Gelrite was used based on the observation of improved shoot appearance (see semi-annual report 1, Table 1). Budbreak was very high in this experiment (Table 7). Results were difficult to

Table 7. Effect of salt strength and carbon source on budbreak, semi-solid medium.

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Budbreak (no.)		
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WPM	Sucrose	Fructose
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<u>Budbreak (no. axillary buds)</u>		
1X	2.8	3.8
1/2X	4.5	4.3
<u>Elongation (% explants)</u>		
1X	30	20
1/2X	10	10
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interpret. Fructose increased budbreak at half strength WPM but there was little difference at full strength WPM. Half strength WPM was better than full strength contrary to results in Table 6. WPM at full strength seemed to promote shoot elongation. This was the first time that stem elongation was deemed obvious enough to record. This experiment has been repeated and results will be presented in the next semi-annual report.

#### F. Effect of Thidiazuron, GA<sub>3</sub>, and IBA on bud elongation

Shoots from a number of treatments: WPM with and without Thidiazuron, 1/2 WPM with and without Thidiazuron, ARM with and without Thidiazuron, 1/2 ARM with Thidiazuron, and MS with fructose and Thidiazuron were grown in five treatments: Thidiazuron alone, IBA alone, GA<sub>3</sub> alone, Thidiazuron plus GA<sub>3</sub>, and Thidiazuron plus IBA. Elongation was observed again but was not influenced by growth regulators (Table 8). It would appear that WPM is inducing some stem elongation but surprisingly there was no evidence of a GA<sub>3</sub> effect on elongation.

Table 8. Effect of Thidiazuron, IBA, and GA<sub>3</sub> on budbreak and elongation, semi-solid WPM, 1.6% fructose.

Thidiazuron (0.05 $\mu$ M)	IBA (1.0 $\mu$ M)	GA <sub>3</sub> (1.0 $\mu$ M)	Budbreak (no.)	Elongation (% explants)
-	-	+	2.3	40
-	+	-	5.8	40
+	-	-	5.6	40
+	-	+	3.5	50
+	+	-	4.6	30

#### IV. Somatic embryogenesis of cupuacu

Previous studies had suggested that somatic embryogenesis in cupuacu was improved when glucose replaced sucrose as the carbon source. Embryogenic callus of cupuacu was grown on three concentrations of sucrose and four concentrations of glucose. Data from Table 9 indicate that embryogenesis can occur in cupuacu with either sucrose or glucose. Cotyledonary embryos were highest with 1% sucrose. Results from this experiment were unexpected in that the number of cotyledonary embryos was consistently higher with sucrose than with glucose, the opposite of results reported in semi-annual report 2 (V Table 2).

Table 9. Effect of carbon source and concentration on somatic embryogenesis, semi-solid, MS medium.

Carbon	%	No. cotyledonary embryos	Percent globular embryos
Sucrose	1.0	8.9	92
	3.0	5.6	94
	6.0	5.3	97
Glucose	0.5	3.0	92
	1.5	3.7	94
	3.0	4.3	96
	6.0	2.3	81

**PROLIFERATION AND ROOTING OF COTYLEDONARY NODAL SHOOTS OF CACAO**

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Cotyledonary nodes of cacao (*Theobroma cacao* L.) cultured in semisolid medium based on Murashige and Skoog salts supplemented with vitamins, casein hydrolysate, and 3% sucrose increased axillary shoot production in response to 4.4  $\mu$ M 6-benzylamino purine (BA) and 0.005-1.0  $\mu$ M thidiazuron after removal of the main shoot. Thidiazuron was more effective than BA in increasing the number of shoots per node. Main and axillary shoots of cacao seedlings grown *in vivo* rooted in a soil-perlite (1:1) medium in response to treatment of the base with IBA and/or NAA at 2000-8000 ppm in 50% ethanol for 10 seconds with maximum response achieved with IBA at 8000 ppm. Detached axillary shoots derived from the cotyledonary node failed to grow or proliferate *in vitro* with standard media manipulation including reduced salt concentration, BA, zeatin, or thidiazuron, gelrite or agar, or cotyledonary extracts.

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**INFLUENCE OF CALCIUM AND AGAR ON VITRIFICATION AND SHOOT-TIP NECROSIS IN ORCHID CULTURES**

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Shoot-tip necrosis and severe vitrification was observed in culture (*Cymbidium Allippa* MHL.) cultures on Murashige and Skoog medium containing 0.6% B27 agar. Calcium concentrations in explants cultured on media containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, and 20  $\mu$ M increased with increasing levels of Ca in the medium. Increasing medium Ca levels reduced growth, especially at 30  $\mu$ M Ca. To study the influence of Ca and agar on these physiological problems, explants were cultured on media containing 3, 9 and 18  $\mu$ M Ca and B27 agar levels of 0.6, 0.9 and 1.2%. Shoot proliferation and growth decreased with an increase in agar and Ca levels in the medium. Increasing Ca concentrations reduced or eliminated shoot-tip necrosis and vitrification, with the incidence being more pronounced at higher agar concentrations. These results indicate that increasing Ca is not only related to shoot-tip necrosis, but also appears to be involved with tissue vitrification.

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***IN VITRO* SHOOT PROLIFERATION OF "MM-106" APPLE AND "CARLIANNA 2c24" PLUM**

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Shoot tips of "MM-106" apple rootstock and "Carlianna 2c24" plum rootstock were established on Woody Plant Medium (WPM) containing 58.4  $\mu$ M sucrose and 1.5  $\mu$ M agar. Primary shoot proliferation and elongation was obtained when cultures were initiated on media containing 10  $\mu$ M 6-benzylamino purine (BA), 1  $\mu$ M gibberellin acid (GA3) and 0.05  $\mu$ M indolebutyric acid (IBA); and then transferred to media with 6  $\mu$ M GA3. When subcultured shoot segments were placed on media containing 2  $\mu$ M BA, 1  $\mu$ M GA3 and 0.05  $\mu$ M IBA; it was found that the response of "MM-106" was improved by rising the sucrose concentration from 0.4% to 11.2% mM; while addition of 283.8  $\mu$ M ascorbic acid had a negative effect on "Carlianna 2c24". Shoot proliferation and elongation in "Carlianna 2c24" subcultured shoot segments was best when placed first on media supplemented with 2  $\mu$ M BA, 10  $\mu$ M GA3 and 0.05  $\mu$ M IBA; and then transferred to same media but containing 1  $\mu$ M BA.

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**RAPID *IN VITRO* ROOTING OF APPLE**

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*In vitro* rooting of woody plants is often slow with variable results. 'Jonathon', 'McIntosh', 'M7A', and 'MM106' apples were multiplied on a modified Murashige & Skoog (MS) medium. Shoot tips 2.5 cm long were placed on a

modified MS medium with IBA at one of the following concentrations: 0, 1, 2, or 4  $\mu$ g l<sup>-1</sup>. No other plant growth regulators were added. After one week on the above media, half the shoots were transferred to the same rooting medium without IBA. Four days after transfer, many shoots began producing numerous strong roots. Most of the cultivar x IBA concentration combinations had 100% rooting. Shoots not transferred produced abundant callus with few roots.

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**Anatomical and Morphological Changes During Adventitious Root Formation in *Prunus Serotina* Ehrh.**

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Anatomical and morphological changes during development and growth of adventitious roots in black cherry (*Prunus serotina*) were described using electron, transmission electron and light microscopy. Observations were made on axillary shoots derived from cultured vegetative buds of 50-year-old black cherry after being micropropagated for more than one year. Root formation was stimulated by a low salt medium supplemented with 5  $\mu$ M IBA and 87 mM sucrose in darkness.

**135 ORAL SESSION (Abstr. 734-741)  
VEGETABLE CROPS POST-HARVEST II: STORAGE**

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**STORAGE METHODS FOR ONIONS**

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Storage methods for onion, *Allium cepa* L. cv. Granex were evaluated during 1983, 1984 and 1985. Onions were stored in an air conditioned room; at 5°C in air, 5% CO<sub>2</sub>-3% O<sub>2</sub>-92% N<sub>2</sub> or 10% CO<sub>2</sub>-3% O<sub>2</sub>-87% N<sub>2</sub>; and at 1°C in air, 5% CO<sub>2</sub>-3% O<sub>2</sub>-92% N<sub>2</sub>. Relative humidity of storage at 5°C and 1°C was maintained at 70-85%. Marketable bulbs decreased 12 to 25% per month when stored at room temperature. About 99% of the onions were marketable after 28 weeks of storage at 1°C in 5% CO<sub>2</sub>-3% O<sub>2</sub>-92% N<sub>2</sub>. More than 92% of these bulbs remained in a marketable condition after an additional 3 weeks in air at room temperature.

Bulb quality as indicated by lower sugar concentrations and greater pungency decreased during storage. Bulb quality decreased most rapidly when onions were stored in air at 1°C and least rapidly when onions were stored in high CO<sub>2</sub>-low O<sub>2</sub> atmospheres at either 1°C or 5°C.

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**CHANGES IN SHOOT GROWTH AND THEIR RELATIONSHIP WITH CO<sub>2</sub> LEVEL AND pH OF THE CELL SAP IN ONION BULBS**

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Changes in shoot growth were related with CO<sub>2</sub> level in internal atmosphere and pH of the cell sap in bulbs of a short day onion 'Texas Grano 1015Y'. Shoot growth was fastest at 13°C but was negligible at 1°C and 34°C. However, after bulbs were transferred to 27°C, shoot growth in bulbs stored at 13°C and 20°C was significantly suppressed but that at 1°C and 34°C was promoted. Respiration rate was highest at 13°C and 20°C during storage. CO<sub>2</sub> level in bulbs was increased with storage temperatures but not directly related with respiration rates. Shoot growth was not inhibited by elevated CO<sub>2</sub> level as high as 8% by sealing bulbs and a hypothesis that shoot growth was suppressed by high CO<sub>2</sub> level in bulbs at high temperature was rejected. pH in the sap was not related with CO<sub>2</sub> content in inner scales, but was increased by 0.2 units at 1°C compared to 30°C in outer scales. It was postulated that pH change by storage temperature controlled shoot growth by modifying distribution of hormones between cytoplasm and vacuoles, and CO<sub>2</sub> level in bulbs.

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