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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 7/26/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose VO

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 6.234
2d semi-an 4/1/87 - 9/30/87
3d semi-an 9/30/87 - 3/31/88

Attachment

PN-ABA-554

6. 234

Propagation of Theobroma spp. by Tissue Culture.

2nd Semi-annual Report
4/1/87-9/30/87

Submitted by

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to

Agency for International Development

Washington, DC

Rec'd in SCI: JUL 2 2 1988

I. Personnel

II. *In vitro* rooting of shoots from the cotyledonary node of cacao

III. *In vitro* induction of axillary shoots from the cotyledonary node of cacao

IV. Shoot proliferation from seedling and mature shoots of cacao

   A. Detached shoots from cotyledonary nodes
   B. Detached *in vivo* grown mature shoots
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V. Somatic embryogenesis in *Theobroma grandiflorum*

I. Personnel

Sergio Filloy, graduate research assistant, left graduate school for personal reasons in May 1987 after completing the academic year. He applied for a leave of absence and returned to Costa Rica. Although he left the University in good standing and has the option to return I feel the likelihood of this occurring is slight. Sergio was a good student and I was pleased with his performance.

I have made arrangements to obtain a graduate student from Brazil who will be working towards his MS and PhD degrees.

II. *In vitro* rooting of detached shoots from the cotyledonary node of cacao

In the 1st semi-annual report, we reported that cotyledonary shoots of cacao produced *in vivo* were rooted *in vivo*, with maximum rooting at 8000 ppm IBA. An experiment was devised to determine if *in vitro* produced cotyledonary shoots could be rooted *in vitro*. Detached shoots produced *in vitro* from cotyledonary nodes were dipped in a solution of 8000 ppm IBA in 50% ethanol for 10 sec. and shoots were then cultured in test tubes containing basal medium and were supported by either vermiculite, perlite, agar, or Gelrite and evaluated after 1 month.

Rooting was optimal with agar (Table 1), but shoot appearance was best with Gelrite. This experiment will be followed to see if shoot growth *in vitro* is affected by the presence of roots.

Table 1. The effect of support media on *in vitro* rooting of shoots from cotyledonary nodes of cacao. All shoots dipped in 8000 ppm IBA in 50% ethanol.

Support	Avg no. roots	Rooted shoots (%)
Vermiculite	1.2	20
Perlite	2.6	60
Agar	2.6	100
Gelrite	1.6	40

III. *In vitro* induction of axillary shoots from the cotyledonary node of cacao

In semi-annual report 1, we reported that Thidiazuron increased cotyledonary node shoot proliferation when the cotyledon was attached. An experiment was carried out to determine if the cotyledonary nodes would continue proliferation after the cotyledons were detached. Cotyledon-less nodes of cacao, previously cultured in Thidiazuron were subcultured on basal medium with or without Thidiazuron.

After 1 month cotyledon-less nodes subcultured on Thidiazuron showed a 93% increase in number of shoots as compared to only 13.6% for nodes subcultured on Thidiazuron-free media (Table 2). This experiment indicates that the cotyledonary node continues to proliferate in the presence of Thidiazuron and that this effect is independent of cotyledons. Proliferated shoots, however, remained stunted when cotyledons were not present indicating that shoot growth requires the presence of cotyledons.

Table 2. The effect of Thidiazuron on shoot proliferation from cotyledonary nodes of cacao, cotyledons removed.

Thidiazuron (μM)	No. shoots Initial	After 1 mo.	% increase
0	4.4	5.0	13.6
0.1	4.6	8.9	93.5

An experiment was devised to determine if cotyledonary nodes would continue to respond to Thidiazuron after division. Cotyledonary nodes were either left whole, or divided into 2 or 3 pieces. Divided shoots continued to proliferate, suggesting that shoots could be multiplied by subculturing (Table 3).

Table 3. The effect of cotyledonary node division on shoot proliferation from cotyledonary nodes.

Node treatment	Total shoots Initial	After 1 mo.	% increase
Undivided	10.9	12.4	13.8
Divided	11.4	14.4	26.3

IV. Shoot proliferation from seedling and mature shoots of cacao

A. Detached shoots from cotyledonary nodes

Previous results of Janick and Whipkey (Rev. Theobroma. 15(2) 125-131; 1985), as well as results from our 1st annual report, have indicated that detached axillary shoots do not elongate or proliferate

in vitro. Usually shoots die after 6 weeks, although some shoots will survive up to 10 weeks. Ascorbic acid and glycine have been suggested to effect somatic proliferation in some woody crops. However, detached cotyledonary shoots showed no response to ascorbic acid at 100 to 400 mg/liter or glycine at 20 to 80 mg/liter (Table 4).

Table 4. The effect of ascorbic acid and glycine on bud break in shoots from cotyledonary nodes of cacao.

Treatment	Concn (mg/liter)	Total no. buds
Ascorbic acid	100	0
	200	0
	400	0
Glycine	20	0
	40	0
	80	0

A series of experiments was carried out to determine if auxin (NAA), gibberellic acid (GA_3) or anti-auxin (7-azaindole) would interact with Thidiazuron to increase bud break on detached cotyledonary shoots. In the 1st experiment Thidiazuron at 0, 0.01, 0.05, 0.1 or 1.0 μM was factorially combined with NAA (0.5 mg/liter), GA_3 (0.5 mg/liter) or NAA+GA_3 (Table 5). The results indicated that with Thidiazuron alone, optimum budbreak is achieved at 0.1 μM. There is some indication that Thidiazuron+GA_3 may be synergistic in increasing bud break of detached shoots. NAA inhibited budbreak in the presence or absence of GA_3.

Table 5. The effect of Thidiazuron, NAA and GA_3 on bud break of shoots from cotyledonary nodes of cacao.

		Total no. of buds				
	Concn	Thidiazuron (μM)				
Trt.	(mg/liter)	0.0	0.01	0.05	0.1	1.0
Thidiazuron alone		1.1	1.4	1.4	1.7	1.1
NAA	0.5	1.5	1.1	1.0	1.0	1.1
GA_3	0.5	1.2	2.1	2.2	1.2	1.2
NAA+GA_3	0.5+0.5	1.0	1.2	1.1	1.0	1.1

In another experiment, 3 concentrations of Thidiazuron were combined with 3 concentrations of 7-azaindole, an anti-auxin (Table 6). Thidiazuron increased bud break at 0.05 and 0.1 μM with the greatest response at 0.05 μM in respect to total number of buds and frequency of multiple buds. There was no positive effect of anti-auxin in increasing bud break. Usually single buds will develop over each leaf scar on a shoot. In this experiment Thidiazuron-treated shoots exhibited a marked tendency to produce multiple buds from each leaf scar. The heightened response of shoots to Thidiazuron was greater than in previous experiments which may be due either to the explants used or the fact that shoots were in test tubes in a vertical position rather than in petri plates with shoots in a horizontal position.

Table 6. The effect of Thidiazuron and 7-azaindole on bud break of shoots from cotyledonary nodes of cacao.

Thidiazuron (μM)	7-azaindole (mg/liter)	Total no. buds	Shoots with multiple buds (%)
0	0	2.3	0
	0.1	2.6	0
	1.0	2.1	0
0.05	0	4.5	70
	0.1	4.2	44
	1.0	3.2	44
0.1	0	3.9	50
	0.1	4.2	38
	1.0	3.7	50

Finally, the effect of 2 standard proliferating media, P medium and M medium, were examined.

P medium

Constituent	Concn (mg/liter)
Murashige and Skoog salts	4,300.0
folic acid	0.5
biotin	1.0
choline chloride	1.0
Ca-pantothenate	1.0
thiamine HCl	2.0
nicotinic acid	2.0
pyridoxine HCl	2.0
ascorbic acid	50.0
cyanocobalamin	0.0015
riboflavin	0.5
BA	2.0
NAA	1.0
polyvinlypyrrolidone	300.0
$FeSO_4$ $7H_2O$	27.85
sucrose	30,000.0

M medium

Constituent	Concn (mg/liter)
Murashige and Skoog salts	2,150.0
para amino benzoic acid	1.0
folic acid	0.5
biotin	0.5
choline chloride	0.5
Ca-pantothenate	0.5
thiamine HCl	1.0
nicotinic acid	2.0
pyridoxine HCl	2.0
myo-inositol	500.0
ascorbic acid	20.0
BA	4.0
malt extract	400.0
glucose	25,000.0
coconut milk	40 ml/liter

Bud break was slightly lower in P medium (Table 7), this probably reflects the presence of NAA which inhibits bud break (see Table 5).

Table 7. The effect of media on bud break of shoots from cotyledonary nodes of cacao.

Medium	Total no. buds
P	1.2
M	1.6

B. Detached *in vivo* grown mature shoots

In semi-annual report 1 Thidiazuron was shown to increase bud break and survival of mature shoots with the optimum concentration 0.05 or 0.1 μM. Shoots were cultured on basal medium with 0 or 0.1 μM Thidiazuron in combination with 0.5 mg/liter GA_3 or 0.5 mg/liter NAA. Bud break was highest with 0.1 μM Thidiazuron but the response was small. The addition of GA_3 or NAA inhibited budbreak (Table 8).

Table 8. The effect of Thidiazuron, GA_3 and NAA on bud break of shoots from mature cacao trees.

Thidiazuron (0.1 μM)	GA_3 (0.5 mg/liter)	NAA (0.5 mg/liter)	Total no. buds
-	-	-	1.8
+	-	-	2.0
+	+	-	0.7
+	-	+	0.3
+	+	+	0.8

An experiment was carried out to determine if a brief exposure to a high concentration of cytokinin (6-benzyladenine or BA) would be effective in inducing bud break. Shoots were subjected to a pretreatment of 1.5, 3 or 6 hours in liquid basal medium with 1000 μM or 1.5 hours in liquid basal medium with no BA. Bud break was greatest with a 1.5 hour BA pretreatment (Table 9).

Table 9. The effect of BA pretreatment on bud break of shoots from mature cacao trees.

No. hours in 1000 μM BA	Total no. buds
0	0.6
1.5	1.5
3	1.3
6	1.3

C. Proliferation and flushing cycle

It was felt that some of the disappointing results in shoot proliferation might be due to bud stage. Two experiments were conducted

using *in vivo*-grown detached shoots at different stages in the flushing cycle. Shoots were cultured on basal medium supplemented with 0.1 ÌM Thidiazuron. Flushing cycle stages were determined from the scheme of Greathouse et al. (Greathouse, Laetsch and Phinney. 1971. Am. J. Bot. 58:281-286).

F-1 Bud burst.

F-2 Leaf expansion.

I-1 Leaf greening. No further leaf production

I-2 Leaf greening complete. Apex remains dormant.

In both experiments bud break was greatest in F-2 shoots (Table 10).

Table 10. The effect of flushing cycle on bud break of shoots from mature cacao trees.

	Total no. buds	
	Expt	
Stage	1	2
F-1	1.2	1.2
F-2	2.8	1.7
I-1	1.6	1.1
I-2	-	1.5

V. Somatic embryogenesis in *Theobroma grandiflorum*

Results of research are included in a draft of a paper prepared for publication (attached).

Somatic Embryogenesis in *Theobroma grandiflorum*

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Abstract. Somatic embryogenesis was induced in cupuacu (*Theobroma grandiflorum*) by culturing immature zygotic embryos in basal media supplemented with 2,4-D and CW. Embryos proliferated by budding and produced embryogenic callus. Cotyledonary embryos were maximized by removal of 2,4-D and replacement of sucrose with glucose. Chemical names used: buthionine sulfoximine (BSO) 2,4-dichlorophenoxyacetic acid (2,4-D), coconut water (CW), gibberellic acid (GA_3).

Cupuacu (*T. grandiflorum*) a relative of cacao (*T. cacao* L.) is a potential new fruit crop for the tropics ( ). The edible pulp arising from the outer layer of the seed integument is used for beverages and frozen desserts. A seedless clone has been selected in Brazil ( ). The objective of this study was to determine the occurrence of somatic embryogenesis from immature embryos of cupuacu.

[1]Received for publication_____.

Journal Paper No.____ of the Purdue University Agricultural Experiment Station.

Materials and Methods

The basal medium used for all cultures consisted of Murashige and Skoog salts (1962) supplemented with 0.3 μM (0.1 mg/liter) thiamine-HCL; 2.4 μM (0.5 mg/liter) pyridoxine-HCL; 0.9 mM (100 mg/liter) i-inositol; 4 μM (0.5 mg/liter) nicotinic acid; 26.6 μM (2.0 mg/liter) glycine; 1 g/liter casein hydrolysate; 87.6 mM (30 g/liter) sucrose; and 6 g/liter agar (Sigma, St. Louis, Mo.) with pH adjusted to 5.7. All explants were cultured in plastic petri dishes (60 x 15 mm) or glass tubes (25 x 150 mm) with plastic closures (Kaputs) containing 10 ml of medium. Cultures were maintained at 26°C under low-intensity illumination (45 μ mol s^{-1} m^{-2} from cool-white fluorescent lamps) with a 16-hour photoperiod.

Initiation of somatic embryogenesis. Pods of *T. grandiflorum*, obtained from CATIE, Turrialba, Costa Rica, were surface sterilized for 30 min in a solution of 0.5% sodium hypochlorite (10% Clorox v/v) with 1 drop/liter Tween 20, rinsed 3 to 5 times in sterile distilled water, and embryos excised. Immature (7 to 15 mm) and mature (25 to 32 mm) zygotic embryos were cultured on semisolid basal medium supplemented with 1 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 10% v/v coconut water (CW).

Development of somatic embryos. Embryogenic callus and globular embryos, originating from a single zygotic embryo were explanted onto basal medium supplemented with various combinations of 0 or 10% CW; 0 or 1.0 mg/liter 2,4-D; with or without 1.5% glucose (w/v) or 3.0% sucrose; 0, 0.5 or 1.0 mg/liter gibberellic acid (GA_3); or 0, 0.01 or 0.1 mM buthionine sulfoximine (BSO) as described.

Results

None of the 15 mature zygotic embryos cultured produced somatic embryos in response to 2,4-D and CW while 10 out of 18 immature zygotic embryos (56%) produced somatic embryos. These were produced via budding as described by Pence et. al. (1979) for cacao (Fig. 1). Somatic embryos were subcultured on the medium used for embryo initiation. Seven clones continued to produce somatic embryos as well as embryogenic callus.

Clumps of embryogenic callus produced in response to 2,4-D + CW were subcultured onto basal media supplemented with factorial combinations of 0 or 1.0 mg/liter 2,4-D and 0 or 10% CW. Globular embryo production and number of cotyledonary embryos was greatest without 2,4-D or CW (Table 1).

Embryogenic callus clumps were cultured on basal media with 3% sucrose or 1.5% glucose, with or without 1.0 mg/liter 2,4-D + 10% CW. Percent globular embryos was greatest without 2,4-D and CW and was unaffected by carbon source. The number of cotyledonary embryos was reduced by 2,4-D and was greater with glucose than sucrose (Table 2).

Embryogenic callus clumps were cultured on basal media supplemented with 0, 0.01 or 0.1 mM BSO and 0 or 10 % CW. Percent globular embryos was highest at 0.1 mM BSO without CW; the number cotyledonary embryos was greatest with 0.1 mM BSO + 10 % CW (Tabl

Initiation of somatic embryos was induced by culturing immature zygotic embryos of T. grandiflorum on medium supplemented with 2,4-D + CW. These embryos became habituated to medium without 2,4-D + CW and continued to proliferate as in cacao (Pence et al., 1980).

The glucose response of somatic embryos of cupuacu was similar to that of cacao. Production of cotyledonary embryos was increased by the substitution of glucose for sucrose as observed for cacao (Elhag et al., 1987) and somatic embryo fresh weight was increased with glucose as the carbon source (Kononowicz and Janick, 1984).

The addition of BSO to culture medium enhanced the development of somatic embryos of Daucus carota L. (Earnshaw and Johnson, 1985), and appeared to increase embryogenesis in cupuacu.

The ability of immature zygotic embryos to produce somatic embryos introduces the possibility of propagating seedless clones of cupuacu from zygotic embryos rescued before they abort.

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Table 1. Effect of 2,4-D and CW on development of somatic embryos of *T. grandiflorum*.

2,4-D (mg/liter)	CW 0%	CW 10%
	Globular embryos (% total area)	
0	95.5±1.1	84.7±4.1
1.0	50.5±5.6	81.5±2.9
	Cotyledonary embryos	
0	4.2±0.8	2.7±0.8
1.0	0.0±0.0	0.4±0.2

Table 2. Effect of carbon source, 2,4-D and CW on development of somatic embryos of *T. grandiflorum*.

1mg/liter 2,4-D 10% CW	Carbon source: 3%	Carbon source: Sucrose 1.5%	Carbon source: Glucose
	Globular embryos (% total area)		
-	86.2±3.7	83.8±4.9	
+	67.0±5.4	73.0±5.8	
	Cotyledonary embryos		
-	3.1±0.6	5.0±1.1	
+	0.4±0.2	0.0±0.0	

Table 3. Effect of BSO and CW on development of somatic embryos of *T. grandiflorum*.

BSO (mM)	CW 0%	CW 10%
	Globular embryos (% total area)	
0	90.6±2.2	85.6±3.1
0.01	94.2±1.1	91.6±2.0
0.1	86.8±3.5	82.0±6.0
	Cotyledonary embryos	
0	7.8±1.3	7.5±1.6
0.01	5.7±1.2	6.3±0.9
0.1	3.4±0.5	8.7±1.4

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Propagation of *Theobroma* spp. by Tissue Culture

3rd Semi-annual Report
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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

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program as well as the tissue culture research at CEPLAC. 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_3, 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_3 and Thidiazuron, carbon source, and basal medium salts.

A. Interaction of GA_3 and Thidiazuron

In semi-annual report 2 there was some indication that Thidiazuron and GA_3 were synergistic in inducing bud break (see semi-annual report 2 Table 5). An experiment evaluated Thidiazuron from 0.01 to 0.1 μM and GA_3 from 0.1 to 1.0 mg/liter. Results confirm previous studies: maximum budbreak was achieved with 0.05 μM Thidiazuron plus 0.1 mg/liter GA_3, 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_3 on budbreak and callus growth of shoots from cotyledonary nodes of cacao, semi-solid, MS medium, 3% sucrose.

GA_3 (mg/liter)	Thidiazuron 0 μM	0.01 μM	0.05 μM	0.1 μM
		Budbreak (no.)		
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
		Callus rating[z]		
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

[z] 0 (none) to 3 (abundant)

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

Carbon	Concn %	Concn μM	Bud break (no.)	Rooting %	Rooting 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 +	3.0	88+			
Stachyose	0.02	0.3	0	40	1.0

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.

	Salt concentration (mg/liter)		
Components	Murashige and Skoog	Woody Plant Medium	Anderson's Rhododendron Medium
NH_4NO_3	1650.000	400.000	400.000
KNO_3	1900.000		480.000
$Ca(NO_3)_2 \cdot 4H_2O$		556.000	
K_2SO_4		990.000	
$CaCl_2$	333.000		
$CaCl_2 \cdot 2H_2O$		96.000	440.000
$NaH_2PO_4 \cdot H_2O$			380.000
$MgSO_4$	181.000		
$MgSO_4 \cdot 7H_2O$		370.000	370.000
KH_2PO_4	170.000	170.000	
FeNaEDTA	36.700		
$FeSO_4 \cdot 7H_2O$		27.800	55.700
Na_2EDTA		37.300	74.500
H_3BO_3	6.200	6.200	6.200
$MnSO_4 \cdot H_2O$	16.900	22.300	16.900
$ZnSO_4 \cdot 7H_2O$	8.600	8.600	8.600
KI	0.830		
$Na_2MoO_4 \cdot 2H_2O$	0.250	0.250	0.250
$CuSO_4 \cdot 5H_2O$	0.025	0.250	0.025
$CoCl_2 \cdot 6H_2O$	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

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

Media	Budbreak (no.)	Survival (%) Subculture 1	2	3	4
		Nodal shoots			
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
		Mature shoots			
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 μM	0.05 μM
	Nodal	
WPM	0.5 (10)[z]	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)
	Mature	
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)

[z] (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 μM 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.

	Budbreak (no.)	
WPM	Sucrose	Fructose
	Budbreak (no. axillary buds)	
1X	2.8	3.8
1/2X	4.5	4.3
	Elongation (% explants)	
1X	30	20
1/2X	10	10

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_3, 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_3 alone, Thidiazuron plus GA_3, 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_3 effect on elongation.

Table 8. Effect of Thidiazuron, IBA, and GA_3 on budbreak and elongation, semi-solid WPM, 1.6% fructose.

Thidiazuron (0.05μM)	IBA (1.0μM)	GA_3 (1.0μM)	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 uM 6-benzylamino purine (BA) and 0.005-1.0 uM 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 max[illegible] 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 QUINCE CULTURES

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Shoot-tip necrosis and severe vitrification was observed on Quince C (Cydonia oblonga Mill.) cultures on Murashige and Skoog media containing 0.6% Phytagar. Calcium concentrations in explants cultured on media containing [illegible] uM BA, 1.2% Phytagar and Ca levels [illegible] 30 mM increased with increasing levels of Ca in the medium. Increasing medium Ca levels reduced growth, especially at 30 mM Ca. To study the influence of Ca and agar on these physiologic problems, explants were cultured on media containing 3, 9 and 18 mM Ca and Phytagar 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 [illegible] being more pronounced at higher agar concentrations. These results indicate that Ca medium [illegible] 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 "MARIANNA 2624" PLUM

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Shoot tips of "MM-106" apple rootstock and "Marianna 2624" plum rootstock were established on Woody Plant Medium (WPM) containing 58.4 mM sucrose and [illegible] g l-1 agar. Optimum shoot proliferation and elongation was obtained when cultures were initiated on media containing 1[illegible] µM 6-benzylamino purine (BA), 1 µM gibberellic acid (GA3) and [illegible] µM indolebutyric acid (IBA); and then transferred to media with [illegible] µM GA3. When subcultured shoot segments were placed on media containing 8 µM BA, 1 µM GA3 and [illegible] µM IBA; it was found that the response of "MM-106" was improved by raising the sucrose concentration from [illegible] to 11[illegible] mM; while addition of 283.8 µM ascorbic acid had a negative effect on "Marianna 2624". Shoot proliferation and elongation in "Marianna 2624" subcultured shoot segments was best when placed first on media supplemented with 8 µM BA, 10 µM GA3 and 0.05 µM IBA; and then transferred to same media but containing 1 µM 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 mgl^{-1}. 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.

6.234

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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 micropropogated for more than one year. Root formation was stimulated by a low salt medium supplemented with 5 uM 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 1986. Onions were stored in an air conditioned room; at 5°C in air, 5% CO_2-3% O_2-92% N_2 or 10% CO_2-3% O_2-87% N_2; and at 1° in air or 5% CO_2-3% O_2-92% N_2. 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_2-3% O_2-92% N_2. 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_2-low O_2 atmospheres at either 1° or 5°C.

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CHANGES IN SHOOT GROWTH AND THEIR RELATIONSHIP WITH CO_2 LEVEL AND pH OF THE CELL SAP IN ONION BULBS

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Changes in shoot growth were related with CO_2 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° and 34°C. However, after bulbs were transferred to 27°C, shoot growth in bulbs stored at 13° and 20°C was significantly suppressed but that at 1° and 34°C was promoted. Respiration rate was highest at 13° and 20°C during storage. CO_2 level in bulbs was increased with storage temperatures but not directly related with respiration rates. Shoot growth was not inhibited by elevated CO_2 level as high as 8% by sealing bulbs and a hypothesis that shoot growth was suppressed by high CO_2 level in bulbs at high temperature was rejected. pH in the sap was not related with CO_2 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_2 level in bulbs.

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