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STUDY OF METABOLISM AND ENERGETICS IN HYPOGRAVITY: DEGENERATIVE EFFECTS OF PROLONGED HYPOGRAVITY

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Final Report

November 1, 1976

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METABOLISM AND ENERGETICS IN HYPOGRAVITY: DEGENERATIVE EFFECTS IN A HIGHER PLANT

NASA Contract No. NAS2-8687

PERIOD OF PERFORMANCE

This Final Report summarizes activities performed on NASA Contracts NAS2-6624 and NAS2-8687 "Study of Metabolism and Energetics in Hypogravity: Degenerative Effects in a Higher Plant", for the period beginning 1 September, 1971 and ending 1 April, 1976.

INTRODUCTION

Scientific Background

The significance of the work carried out under contracts NAS2-6624 and NAS2-8687 is closely linked to research conducted under NGR 12-001-053 from 1968-71. This program, specifically addressed to the role of gravity in the formation of rigid, lignified plant cell walls hence to the development of the erect land plant body, provides a frame of reference within which the subsequent contract program may be evaluated.

Our general hypothesis as set forth in the original experiment proposal (Metabolism and Energetics in Hypogravity, ESP 057, 3/15/70, NASA-ARC) held that the plant chosen by the consortium, dwarf marigold (*Tagetes patula* cv 'Petite Gold'), would display degenerative changes in mechanical supportive systems under hypogravity because normal lignin-cellulose wall structure fails to develop. This hypothesis was, in effect, an extension to the dwarf plant of a construct based upon years of prior observation, analysis and experimentation, all summarized in the reports of NGR 12-001-053 and publications associated with that grant. This in turn, can be traced to concepts generated in the course of Extreme Environment research carried out under NASw-767 (1963-67) and NGL 12-001-052 (1967-77).

The results of "base line" studies may be highlighted as follows:

Observational Results

1) Algae do not produce lignified walls.
2) Chlorophytes, the precursor group for land plants, do not lignify

naturally but possess the potential for doing so when supplied the appropriate precursor molecules; other algal groups including Red and Brown macro-algae neither lignify themselves nor do so when fed precursors.

3) Non-vascular land plants, the Bryophytes for example, normally contain lignin in minute quantities localized in non-support component systems as hydrophobic coatings. Exceptional cases are the gametophytes of the giant mosses of New Zealand, up to ca 1 meter in height which contain as much lignin as many vascular forms — but chemically distinguishable as a polymer of more "primitive" precursors. Log lignin content vs axial height/cross-sectional area approximates a linear relationship.

4) The lower vascular plant *Equisetum* normally contains little lignin, but has evolved an alternate "solution" to support of its upright structure, namely a hollow stem which concentrates SiO_2 (ca 10% of its dry weight). Even so, a dwarf species 5-6 cm in height at maturity contains one-third the lignin found in axes ca 1 meter tall.

5) Higher vascular plant structures (for example, flower stalks of *Plantago*) also vary in lignin content in relation to dwarfism or gigantism.

6) Aquatic-Marine angiosperms derived from upright land ancestors, like green algae contain no lignin but possess (in this case, retain) the capacity for its synthesis when fed precursors.

Experimental Results

1) Seedlings of *Rhizophora*, the red mangrove reach lengths of 15-30 cm while still attached to the maternal branch. They are not normally lignified at this stage, but within their first year of growth after dropping to the swamp floor, they are typical young woody plants. When juveniles are subjected to centrifugal deformation, generating compression-tension zones, the compressional zones develop lignin within a few days and the tension zones do not. This response can be simulated by injection of membrane-disturbing chemical agents.

2) When grown at 25-100 g on the centrifuge, cucumber seedlings show reduced growth and up to ca 100% increases in lignin content. Other species, e.g. *Anagallis* show even greater degrees of elevation in lignin content.

3) When land plants such as cucumber and rye are grown from seed in water, their lignin contents are reduced by one-half or more. A second common response is the rise in peroxidase activity.

4) Cucumber seedlings grown from seed on the clinostat also show reduced lignification and (like the wheat roots in Biosatellite II) increased peroxidase activity.

Other background observations and experiments relating to lignifications how that it is also regulated by direct mechanical stimuli such as wind and by soil and atmospheric water deficits. The enzyme peroxidase, relevant because it is the final catalytic factor in the polymerization of soluble monomeric precursors to lignin, can be stimulated by a variety of so-called stress conditions including freezing, heat shock and drought.

Given all of the foregoing information it is possible to formulate a basic relationship between lignification and the evolution and maintenance of the erect land plant body.

Stated briefly, deposition of lignin in the cell wall is essential to mechanical integrity of the plant body both in terms of bulk modulus (crush-resistance) and flexural strength in the axis and its appendages; and the expression of a genetic potential for lignification is regulated by a set of environmental factors in which gravity-induced mechanical stresses play a central role.

Objectives

The most general statement of hypothesis as set forth in ESP 057 (p. 57) is "that the removal or attenuation of gravity in a plant will eventually lead to a modification or loss of those structures, products and processes which normally result from adaptive responses (to gravity)".

Out of the very general statement, there evolved a more specific set of propositions which in effect constituted operational, working hypotheses and led to definitions of observational-analytical-experimental goals.

Plants grown under hypogravity (or some mode of gravi-compensation) will contain less lignin and more peroxidase than plants grown under earth-normal gravity.

Other changes in cell wall and cell chemistry were also predicted although not detailed, but it was stipulated that some of these changes would be "degenerative" in the sense that plants so grown would, after a sufficiently long experience, suffer loss of mechanical integrity as evidenced perhaps by weaker axes. It was further expected that these effects would be most dramatic in progeny from seeds formed under hypogravity and returned to one g, that is, produced during an orbital experiment and returned to the Earth's surface for further cultivation.

It was further predicted that the clinostat would not be equivalent to satellite free fall, hence the objective of establishing clinostat performance with a selection of plant and conditions adaptable to the time, facilities and operational circumstances and constraints were introduced in the planning process and are included in the proposal document (p 80ff). These constraints were a major factor in selection of the test plant as well as in the design of baseline experiments.

Experimentation

Methods and Procedures

In addition to general procedures and techiques used in plant physiology and biochemistry a number of specialized methods or specific adaptations of existing methods were developed.

The most notable and significant of these were in the following areas:

1) Growth conditions

a) General

It is obvious that reliable baseline data require reliable controlled environment conditions for growth.

An indication of this reliability is given for four sample experimental runs (Table 1). Nevertheless, with our small populations ($n<20$), variability is inevitable, as is shown in the data for overall shoot weights and heights (Table 2). With respect to fresh weight, only experiment No.1 stands out as significantly different — and higher in value. Linear growth shows appreciably less variability, although again Expt. No.1 is distinctively high in value.

A somewhat more detailed comparison of growth data in several individual control experiments and one clinostat run indicates that some hypogravity responses can be distinguished in spite of variability (Tables 3,4).

Table 1. Growth chamber environmental data on three consecutive experiments, and one experiment (Exp. 4) in which plants were grown from seeds on the clinostat and monitored over a 21 day period. All values expressed as means. Included is an overall mean ± standard error of the mean (S.E.M.) for all runs except Exp. 4. (Temperature = Temp., Relative Humidity = R.H., Volume of Hoaglands per day = Hoaglands.

	Exp. 1 (n=20)	Exp. 2 (n=17)	Exp. 3 (n=17)	Exp. 4 (n=3)	$\bar{X}$ ± S.E.M. (n=54)
Temp.[a]	22.36	23.75	22.61	22.28	22.91 ± 0.74
Light[b] ($\mu E\ cm^{-2} sec^{-1}$)	163.00	183.06	184.96	171.00	177.01 ± 12.17
R.H.[b] (%)	65	68	65	68	66.50 ± 1.73
Hoaglands[b]	119.67	115.25	120.30	238.50	118.41 ± 2.75

[a] measured daily

[b] measured weekly

Table 2. Size and weight of marigold plants for 6 experiments

Experiment	Mean fresh weight (mg)	Mean total stem length (mm)	
1	1044 ± 194	35.1 ± 4.5	(21 days)
2	640 ± 175	27.8 ± 3.2	(21 days)
3	882 ± 418	25.6 ± 5.0	(21 days)
4	708 ± 383	23.5 ± 5.8	(21 days)
5	470 ± 169	26.5 ± 4.1	(22 days)
6	509 ± 174	27.8 ± 6.3	(22 days)

Table 3. Marigold plant growth data in three consecutive experiments measured 21 days after seeding. All values in each category are expressed as an overall mean for each experiment, and an overall mean ± S.E.M. for all three runs is listed in the last column.

	Exp. 1	Exp.2	Exp. 3	$\bar{X}$ ± S.E.M.
Number of leaves	5.0	4.82	4.58	4.80 ± 0.21
Length of leaves				
1st pair (mm)	41.4	34.1	37.6	37.7 ± 3.7
2nd pair (mm)	48.5	39.0	38.2	41.9 ± 5.8
3rd pair (mm)	36.1	21.9	18.7	25.6 ± 9.3
Hypocotyl length (mm)	13.4	13.0	10.8	12.4 ± 1.4
Total stem length (mm)	35.0	27.8	25.6	29.5 ± 5.0
Fresh wt. (mg)	1044	640	882	855.3 ± 203.3

Table 4. Plant growth data on marigolds from seed on a clinostat (Exp. 4) compared with data derived from three control groups. ($\bar{X}$ ± S.E.M.)

	Clinostat Exp. 4 (n=3)	Control $\bar{X}$ ± S.E.M.
No. of leaves	4.33 ± 7.0	4.80 ± 0.21
Length of leaves		
1st pair (mm)	18.5 ± 7.0	37.72 ± 3.67
2nd pair (mm)	22.0 ± 1.1	41.9 ± 5.8
3rd pair (mm)	26.0 ± 0	25.6 ± 9.3
Hypocotyl length (mm)	8.7 ± 1.2	12.4 ± 1.4
Total stem length (mm)	19.3 ± 4.2	29.5 ± 5.0
Fresh wt. (mg)	616.2 ± 168.5	855.3 ± 203.3

b) Novel Hypogravity Simulation

Although conventional clinostat culture of plants was routine in this study, another approach to hypogravity simulation was our so-called "bouyancy compensation" system. As noted in the review of scientific background, some ordinary land plant species can be grown in water systems. This procedure was applied to marigold by the use of containers in which water was both oxygenated and circulated by compressed air introduced from beneath. These chambers were modified from spherical-form separatory funnels (see appended offprint by Waber et al).

This technique provided bouyant support for the developing seedling but, more important was the continuous stirring effect of the aeration system. Continuous water circulation randomized orientation of the embryo within the seed and of the seedling upon emergence. After about 15 days seedling structural development created hydrodynamic drag and longer time periods in fixed position. Hence the benefits of a simple device broadly analogous to a multi-axial clinostat were available for, but under our design and scale factor condition limited to, seedling development.

c) Stray radiation effects

During endogenous ethylene experiments, marigold plants were, at times, left in plexiglass chambers for a week or longer. These plants appeared to be healthier and larger than plants grown under the same conditions except for the plexiglass chamber. This suggested that a portion of the spectrum radiated by cool-white flourescent tubes, and filtered out by plexiglass was having a deleterious effect on plant growth.

There is an appreciable amount of energy in the ultraviolet and infrared radiated by cool-white fluorescent bulbs. Table 5 indicates the percentage of total energy emitted in different spectral ranges. Plexiglass filters out IR and UV with cut-offs at λ values <400 nm and >700 nm.

A 1/4 inch "UF-3 Acrylic Plexiglass" sheet was installed in the growth rooms between light tubes and plants. "UF-3 Acrylic Plexiglass" absorbes all ultra-violet radiation, does not affect visible light transmission above 430 nm, and absorbs all infrared above 2.5μ. The effect of plexiglass screening on plant growth was sizeable. Table 6 shows effects of the filter on fresh weight of the leaves.

IR apparently causes localized heating of the plants. A 4 mm diameter thermistor was taped to the lower surface of a marigold leaf illuminated with 2cc $\mu E \cdot cm^{-2} \cdot sec^{-1}$ Room temperature was 20.1°C. The temperature of the leaf illuminated with plexiglass filtered light was 20.1°C.

Table 5. Percent of Total Energy Emitted in Different Spectral Ranges by Cool-White Fluorescent Tubes.

Spectral range (nm)	300-380	380-480	480-560	560-630	630-700	700-800	800→
% of total radiation	2	22	24	20	18	8	6

Table 6. Effect of "UF-3 Acrylic Plexiglass" Filtration of Cool-White Fluorescent Light on Marigold Fresh Weight. Light intensity was 165 $\mu E \cdot cm^{-2} \cdot sec^{-1}$.

	No plexiglass		Plexiglass				
Treatment	n	mean fresh wt. (g)	n	mean fresh wt. (g)	% increase	T^a	P
Clinostat	21	3.049 ± 1.162	6	4.579 ± 0.690	50	3.05	< .01
Rotating control	17	2.404 ± 0.903	2	4.449 ± 0.175	85	3.12	< .01
Stationary control	20	2.669 ± 0.849	6	4.315 ± 0.837	62	5.11	< .01

[a] Student's T, comparing plexiglass with no plexiglass. The difference is significant in all cases.

Publications and Reports

On Grant NGR 12-001-053

1) Siegel, S. 1969. Evidence for the presence of lignin in moss gametophytes. *A. J. Bot.* 56,175

2) Siegel, S. 1969. Phylogenetic and environmental determinants of lignification. Abstract XI *Int. Bot. Congress.* Seattle, Washington.

3) Siegel, B. and Siegel, S. 1970. Anomalous substrate specificities among the algal peroxidases. *Amer. J. Bot.* 57,285.

4) Siegel, S. 1971. Dwarfing, lignification and land plant evolution. NASA TM X-62,009. Section 8.10 p. 116.

5) Siegel, S., Carrol, P., Umeno, I. and Corn, C. 1972. The evolution of lignin: Experiments and observations. *In* Recent Adv. in Phytochemistry, vol 4,223. Appleton-Centruy-Crofts. New York.

Reports: Role of Gravitational Stress in Land Plant Evolution: The Gravitational Factor in Lignification. May 1969, December 1969.

On Contracts: NAS2-6624 and NAS2-8687. Waber, J., Williams B., Dubin, J. and Siegel, S. 1975. Changes induced in peroxidase activity under simulated hypogravity. *Physiol. Plant.* 34,18.

Reports: Study of Metabolism and Energetics in Hypogravity: Degenerative Effects of Prolonged Hypogravity. Annual Reports 30 September, 1972 through 30 November, 1975.

Thesis: Shiraki, D. 1976. The Role of Auxin and Ethylene in Dwarf Marigolds Subjected to Simulated Weightlessness*.

* A manuscript embodying the results of this study is in preparation.

Publications and Reports (cont'd.):

Thesis: Fukumoto, J. 1976. The Effect of Simulated Hypogravity on Lignification in Petite Marigolds.

Senior Honors Thesis: B. S. (Honors) Awarded, August, 1976.

2. Biochemistry

a) Cell Wall Analysis

Many aspects of cell wall biochemistry were included in the long term plan for our research program (Table 7). The termination of the program did not permit the complete evaluation of methods and procedures, however specific flow sheets were devised for the preparation of Marigold cell wall isolates to be used in determination of important mineral (Table 8) and carbohydrate constituents (Table 9).

Protein analysis was routinely run by the Lowry method using crystalline bovine serum albumin as the standard. Our original procedure for the determination of lignin was as follows:

Harvested plants are dried at 80°C for 2 or 3 days. After weighing, they are extracted with 25 ml of 95% ethanol. This mixture is brought to a boil, and the alcohol is then decanted and discarded. The residue is extracted with 25 ml of alcohol plus 2.5 ml of concentrated HCl. After boiling, the mixture is allowed to stand for one hour. The liquid is then decanted and analyzed by taking 3 ml of the extract and adding to it 3 ml of 1% solution of phloroglucinol dissolved in a 1:1 mixture of alcohol and concentrated HCl. After approximately 1 minute the O.D. of this solution is determined at 540 nm. The O.D. reading is then substituted into the following equation:

$$\frac{(\text{Volume of extract})\ (\text{O.D. at 540 nm})}{\text{dry weight (gm)}} \times 0.5 = \text{mg lignin/gm dry weight}$$

Table 7. General Scheme for Cell Wall Fractionation and Analysis

	WALL COMPONENT	DIFFERENCES	METHODS	EVALUATION
Analysis of Cell Wall Constituents	Minerals	Total Ca, Mg	Atomic Absorption	Uptake Mechanisms
	Carbohydrates	Polysaccharide qualitative and quantitative	Gas Chromatograph	Synthetic Pathways Degrative Pathways
		Monosaccharide qualitative and quantitative		
	Lignin	Qualitative	Colorimetric	Synthetic Pathways
		Deposition	Radioautography	Lignification Patterns
	Proteins	Quantitative Substrate optimum pH optimum Temperature optimum	Spectrophotometric	Synthetic Pathways Degradative Pathways
		Qualitative	Electrophoretic	

Table 8. Procedure used to Prepare Marigold Cell Walls for Calcium or Magnesium Analysis.

Glass homogenize individual plants in 10 ml McIlvaine's citric acid phosphate buffer (pH 7)

↓

centrifuge 10 min. at 300 g

↓ → supernatant

3X wash pellet with buffer, centrifuge

↓ → supernatant

wash pellet with 1:1 chloroform:methanol, centrifuge

↓ → supernatant

3X wash pellet with acetone, centrifuge

↓ → supernatant

2X wash pellet with water, centrifuge

↓ → supernatant

lyophilize precipitate, weigh

↓

incubate in pronase in pH 7.9 Sorenson's phosphate buffer (0.67M) at 25°C for 48 hours, centrifuge

↓ → supernatant

2X wash pellet with water, centrifuge

↓ → supernatant

(all supernatants above →) pool, analyze for cytoplasmic Ca + Mg.

lyophilize pellet, weigh

↓

incubate inpectinase (1 mg/ml) in pH 4.5 acetate buffer for 24 hours at 45°C, centrifuge

↓ → supernatant

2X wash pellet with water, centrifuge

↓ → supernatant

Pool, analyze for Ca + Mg associated with pectins.

lyophilize pellet, weight, analyze for Ca + Mg remaining with the cell wall residue.

Table 9. Cell Wall Isolation and Preparation Procedure for Carbohydrate Analysis.

Glass homogenize 2-3 plant (*ca* 2 gm) in 10 ml cold distilled water

↓

Centrifuge 10 min. at 300 g

↓ → Discard supernatant

2X Wash pellet with water, centrifuge 10 min. at 300 g

↓ → Discard supernatant

1X Wash pellet with 1:1 chloroform:methanol, centrifuge

↓ → Discard supernatant

3X Wash pellet with acetone, centrifuge

↓ → Discard supernatant

2X Wash pellet with water, centrifuge

↓ → Discard supernatant

Lyophilize pellet, weigh

↓

Incubate in pronase in pH 7.9 Sorenson's phosphate buffer (.067M) at 25°C for 48 hours, centrifuge

↓ → Discard supernatant

2X Wash pellet with water, centrifuge

↓ → Discard supernatant

Lyophilize pellet, weigh

↓

Incubate pellet in pectinase (1 mg/ml) in pH 4.5 acetate buffer for 24 hours at 45°C, centrifuge

↓

2X Wash pellet with water, centrifuge

↓ → Save supernatant, analyze for sugars

Lyophilize pellet, weigh

↓

Add 2 ml of 2N *TFA to about 40 mg lyophilized pellet, hydrolyze in sealed vial for 1 hour at 121°C, cool

↓

Air dry

↓

React with Tri Sil-Z

↓

Analyze for sugars

* Trifluoroacetic Acid

This procedure proved unsatisfactory because of the color caused by extraneous cell components. A new procedure for lignin analysis involving pre-extraction of these extraneous components was therefore devised:

New Lignin Determination Procedure

1) Fresh plant ground in cold Sorenson's buffer, pH 7.0.

2) Centrifuge at 18,000 g for 20 minutes: supernatant is then discarded.

3) Pellet resuspended in 50 volumes of Sorenson's buffer pH 7 overnight.

4) Mixture centrifuged again at 18,000 g for 20 minutes — supernatant is discarded.

5) Pellet is resuspended for 5 minutes in 50% ETOH which was brought to a boil.

6) Centrifuge at 18,000 g for 20 minutes — supernatant discarded.

7) Resuspend in Benzene-ETOH (2:1 v/v) which has been brought to a boil. Pellet broken up with metal spatula and mixed with "Vortex Genie" mixer and centrifuged at 18,000 g for 20 minutes supernatant discarded 3x.

8) Resuspend pellet in ∿30 ml KOH-ETOH (95%) and heat in water bath (70 - 95°). Agitate occasionally with vortex mixer over a 30 minute period.

9) Centrifuge at 18,000 g for 20 minutes. Supernatant decanted and brought up to equal volume with KOH-ETOH. Pellet used for cellulose.

10) Take 10 ml aliquots in centrifuge tubes and add equal volumes of 12 N HCl. Spin down KCl precipitates at 18,000 g for 10 minutes.

11) Put supernatants in Spec 20 cuvettes. Zero Spec 20 with d.H_2O and take O.D. reading at 540 nm.

12) Add phloroglucinol to samples and dissolve by shaking. Take O.D. reading at 30 minutes. Lignin activity is computed with respect to known concentrations of vanillin.

b) Enzymology

In the course of our program, three enzymes related to cell wall chemistry were examined. These enzymes, their activities, and operational significance are:

Cellulase

Hydrolysis of cellulose

Wall degradation in senescence (e.g. abscission)

Phenylalanine δ-Deaminase

Deamination of tyrosine and phenylalanine respectively to their cinnamic acids.

Biogenesis of monomeric lignin precursors.

Peroxidase

Catalysis of oxidations by H_2O_2 — donor substrates include phenols, aromatic amines, indoles (including auxins), ascorbic acid and thiols.

Activation of hydroxycinnamic acid derivatives by conversion to monomer radicals which are then polymerized to lignin in the wall matrix. Transformations of indole auxins and various natural products such as alkaloids, quinones.

Using the standard soluble substrate, carboxymethyl cellulose and viscometric assay, not cellulase activity was detected in seedlings and plants up to floral bud stage. Analytical methods were standardized against fungal cellulase, hence its absence may be biologically significant, not a technical problem. Efforts to demonstrate α-deaminase activity were also unsuccessful, although the analytical procedures are essentially straight forward and simple.

Attention was therefore directed toward the determination of peroxidase activity both as the enzyme involved directly in lignification and as a general indicator of disturbances in or displacements of environmental norms.

The peroxidase activity of the seedlings maintained in the various environments was determined according to the following scheme:

Cell Free Extract: Plants were homogenized in 0.1 M phosphate buffer pH 6.1 (1 seedling/ml) and then centrifuged at 30,000 X g for 30 minutes. The resulting supernatant served as the stock enzyme preparation.

Substrate: 5 X 10^{-3} M guaiacol and H_2O_2 in 0.1 M phosphate buffer pH 6.1.

Procedure: To 3 ml of the substrate was added 0.1 ml of the stock enzyme preparation (or a suitably diluted portion of the stock) and the linear increase in absorbance at 470 nm was then followed in a B and L Spectronic 20.

Lowry protein determinations were also made on these stock cell-free preparations, and activity data were expressed in ΔO.D. units (Δ Absorbance) per minute per mg protein.

Special procedures were adopted for the determination of wall-bound peroxidase as opposed to the cytoplasmic fraction.

Wall fractions were washed in 0.07 M phosphate buffer, pH 7, at 0.05°C by up to 7 centrifugations (8,000 g, 10 min.) and resuspensions as required to remove all traces of non-bound enzyme.

The washed walls were then supended in 1 M Ca-nitrate and incubated at 6°C for 24 hr. The supension was centrifuged at 10,000 g for 10 minutes and the supernatant used for assay of bound enzyme.

c) Ethylene

Our involvement with ethylene and related problems resulted from decisions made at the October 1974 meeting of the Plant Hypogravity Consortium at ARC. Unexpectedly, this special endeavor, intended as a short term additional commitment, occupied us during the final 18 months of the project and in our judgement, rightly so. We feel not only that aspects of the ethylene problem have been newly revealed, but also that a "wider biology" of *Tagetes* seeds and plants requires additional study.

The fact that mercury perchlorate ($Hg(ClO_4)_2$) can bind and release ethylene under the appropriate conditions has been used for the development of a quantitative method for detection of this biologically active gas.

Mercury and tellurium are unique in the realm of metal-ligand coordination chemistry because they form addition compounds with olefins distinct from the loose pi-complexes formed by ions such as Ag. Hg-salts are known to react with unsaturated aliphatic and aromatic alcohols and unsaturated acids. The structure, stereochemistry and kinetics of Hg-olefin bonding has been a controversy until recently, when sophisticated physical methods have finally clarified and elucidated its nature. The main features of the reversible reactions involved are as follows:

In the transition state, Hg exhibits carbonium ion positive charge localization characteristics, although an actual carbonium ion intermediate has been ruled out.

The addition conformation and the existence of the alcohol and ether have been proven on the basis of proton magnetic resonance spectra data.

The second order reaction above has a reaction velocity constant of 5.1×10^{3} molecules^{-1} sec^{-1}, $\Delta H = 9.2$ kcal/mole.

Excess of H^{+} prevents hydrolysis of $Hg(ClO_4)_2$, although the reaction was found to be independent of H^{+} concentration.

The stability of the Hg-C_2H_4 compound is temperature sensitive, 0°C or lower being required.

The olefin can be recovered from an aqueous perchlorate solution by halo-acids. Ethylene concentrations above 0.5 ppm can be detected by direct injection of an air sample into the gas chromatograph without previous concentration in mercuric perchlorate. With direct injection however, the danger exists of detecting other substances with closely similar or identical column retention times.

The only gas with a retention time closely similar to ethylene, likely to be present in our growth rooms and the experiments performed, is carbon monoxide. When the Poropac 80/100 stainless steel column is used at conditions optimum for ethylene detection (see Table 10), carbon monoxide has a retention time identical to that of ethylene, but sensitivity limits for the two gases differ greatly. The minimum carbon monoxide concentration detectable under the conditions used is about 1 ppt (10^{-3}). In the range of ethylene concentrations detected by direct injection for the same conditions (0.5 ppm to 10 ppm), a concentration of 1 or more ppt would have to be present to produce a reading above blank valves. Since humans are sensitive to CO at concentrations in the ppm range, we can safely say there was not sufficient CO present in our growth rooms to affect ethylene determinations. When the sensitivity

Table 10. Optimum Conditions for Ethylene Detection Using a Varian Gas Chromatograph Model 1840 and Poropac 80/100 Stainless Steel Column[a].

Gas flow rates:

N_2: 25 ml/min.

H_2: 25 ml/min.

Temperatures:

column: 90 C

detector: 270 C

injector: 270 C

Attenuation: 16

Range: 10^{-12}

Sample size: 1 ml

Column characteristics:

length: 6'

outer diameter: 1/8"

liquid phase: none

[a] Column supplied by Varian Inc.

is decreased to detect higher concentrations of ethylene by direct injection, it is also decreased for carbon monoxide. When the mercuric perchlorate trap is used, there is no possibility of concentrating carbon monoxide as it is not subject to mercuric perchlorate binding.

The concentration of ethylene by mercuric perchlorate is affected by the flow rate of the air sample through the solution and by the temperature of the solution. Data presented in Table 11 indicate that recovery of ethylene is more than doubled when bubbling rate is decreased by half. When standard ethylene samples are bubbled through mercuric perchlorate solutions at temperatures above 0°C, the complex formed is unstable and breaks down releasing the ethylene. At room temperature we were not able to recover any ethylene from standard ethylene air samples due to the instability of the complex formed.

The possibility that the marigold plant itself could produce ethylene was tested. Nine, twenty-nine day old healthy plants were placed in a 60 liter plexiglass chamber. Pots were wrapped in parafilm to prevent possible evolution of ethylene into the chamber air by microorganisms present in the growth medium. Air sampled before sealing the chamber indicated that no background ethylene was present. The chamber was placed in the growth room under the light bank so that the plants received standard illumination. Temperature in the plexiglass chamber was about 22°C. Relative humidity in the chambers was not controlled.

Table 11. The Effect of Flow Rate on the Recovery of Ethylene from Mercuric Perchlorate. The air volume indicated was drawn into a syringe, then bubbled by hand through 30 ml of mercuric perchlorate contained in a test tube. The test tube was maintained in an ice bath at 0°C during and after bubbling.

		Flow Rates			
		Slow (5 ml/min.)		Fast (10 ml/min.)	
Ethylene Concentration	Air Volume sampled	Concentration recovered	% recovery	Concentration recovered	% recovery
5 ppm	10 ml	1.66 ppm	33.2	0.616 ppm	12.3
5 ppm	50 ml	26.91 ppm	107.0[a]	8.620 ppm	34.5

[a] The 7% recovered in excess of that introduced is due to experimental error.

After 24 hours, air in the chamber was sampled again for ethylene. One liter of air was drawn out through a rubber septum in the end of the chamber and bubbled through mercuric perchlorate. No ethylene was detected. The plants remained healthy for several days in the chamber beyond the test period.

Possible evolution of ethylene by stressed marigold plants was also tested. Twenty-nine day old plants were cut off at the surface of the soil. The cut portions of the stems were wrapped with cotton soaked in Hoaglands solution. Two plants were placed in each of 3 jars. After 24 hours, air, in the jars was sampled through rubber septa in the plexiglass lids. Results are presented in Table 12. Air samples were injected directly into the gas chromatograph.

It is not known whether ethylene found in the jars was produced by the plant itself or by microorganisms present in the plant material. A variety of common microorganisms are known to produce ethylene or cause the production of ethylene by host plants they infect. Of 228 species of fungi reported 25% produced ethylene. *Aspergillus clavatus* produced an astounding 514 ppm (in a 10 ml container) during 24 hours in culture. Bacteria have also been found to produce significant quantities of ethylene.

Microorganisms have been present in our growth rooms, mainly as co-inhabitants of the soil medium used for culturing marigold plants, since we began growing plants in plastic pots. Root rot fungus (*Pythium* sp.) has been recovered in culture from specimens of marigold plants grown in these growth chambers.

Table 12. Evolution of Ethylene by Stressed Marigold Plants

Jar number	Ethylene production nl/g
1	42.83
2	33.31
3	61.06

The following fungi were cultured from rediearth and seed coats of marigold seeds maintained in seed storage jars.

Cladosporium

Alternaria

Penicillium sp.

Aspergillus niger

Fusarium sp.

Actinomycetes (various)

Yeasts (unidentified)

Other fungi were present in culture but did sporulate and were not identified.

Suspecting that the major source of fungi in growth chambers was the marigold seed itself, the room, its equipment and the watering system were sterilized with 800 ppm Roccal. Rediearth was autoclaved to ensure its sterility. About 2 weeks after planting, fungal hyphae could be seen growing out from the seed coat. Saboraud dextrose agar plates exposed to the air in the chambers developed only actinomycete growth.

Various sterilization techniques utilizing 95% ethanol and 2% chlorox were tested in all attempt to surface sterilize seed coats. Effectiveness was tested by placing treated seeds on Saboraud dextrose agar plates. When seed coats were effectively sterilized, the seeds were no longer viable or produced abnormal stunted plants. With less harsh sterilization procedures, seeds germinated and grow normally on rediearth, but fungi were invariably present.

When sterilization technique was ineffective, fungal growth was evident on the agar in 2 or 3 days. At about 4 days roots emerged from the seeds and several days later, in spite of extensive fungal growth, hypocotyls emerged. The seedlings did not grow beyond the cotyledon stage on agar in the presence of fungi. That they do germinate and appear healthy for 10 days or longer suggests compatibility between the seedling and the fungi present on its seed coat. This relationship warrants further investigations.

Several fungi (*Aspergillus* and *Cladosporium* for example), isolated from seed coats and from used rediearth, were cultured in sealed flasks on agar and rediearth. We were not able to demonstrate conclusively the production of ethylene by these fungi because both the rediearth and the agar medium produced ethylene (32 ppm and 54 ppm ethylene respectively). When flasks were allowed to air out through cotton stoppers for a week, then resealed, no ethylene was detected in the cultures while the agar control had 0.1 ppm present 24 hours after resealing. These results do not, by any means, indicate that microorganisms present in the growth chambers are not producing ethylene. Only the most prolific fungi were tested and the tests did not include a variety of substrates and conditions. It is known that ethylene production varies with substrate and environmental conditions. Also, we were not able to reproduce the exact conditions in the growth room in culture flasks.

Equipment and materials used on a regular basis in the growth rooms were tested as possible ethylene sources. Each was sealed in a 460 ml jar for 24 hours. Air samples were injected directly into the gas chromatograph. Table 13 presents the results. (Smoking is not allowed in the growth rooms. but tobacco smoke was tested to determine how much ethylene it might contain).

The discovery that clinostat motors can produce ethylene suggests the probable presence of the gas in the facilities used by other members of the consortium to conduct clinostat experiments.

Table 13. Ethylene Production by Growth Room Equipment and Materials. n = 1 except where indicated otherwise.

Item	Ethylene detected after 24 hours	
0.25 rpm clinostat motor [a]	474.57 nl/hr	
Hoaglands and headers	20.3 ppm	n = 2
3 inch screened pot	0	
Dried soil containing roots	0	
Cotton gauze with algae	0	
Pipe smoke	4842	n = 2

[a] Temperature rose to about 30°C with the motor running inside the test jar.

A Selected Ethylene Bibliography

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Results

The experimental findings presented here fall into two major groups consistent with the operational history of the Plant Hypogravity Consortium. First are those concerned with growing marigolds from seed under conditions presumed to simulate hypogravity. Such experiments are consistent with the original program set forth in ESP 057. It was early decided, however, that there might be a special value in 21-day plants because epinastic sensitivity is first apparent at that stage. This decision together with the information that our efforts should take into consideration a probable 7-day first orbital experiment, effected a shift toward growing plants to the 21 day stage and then studying their responses to 24 hour to 7 day clinostat treatments.

Accordingly, a second experimental set representing 21-day plants after 24 hours on the clinostat is also presented.

This second set cannot be compared with the first and although there are indeed experimental findings of interest, it obviously was not designed with cell wall biochemistry, especially lignification and associated biomechanical responses in mind.

1) Cell Wall Composition

Cell Wall carbohydrate analysis had not been carried out fully at the termination of the project. Nevertheless, worthwhile baseline data and a few experimental results were obtained with respect to carbohydrates and lignin, as well as calcium content.

Wall-bound Ca-levels were found to be insensitive to light over a range of intensities affecting growth markedly (Table 14 and 15).

Table 14. Fresh weights of Marigold plants grown at different light intensities for 21 days.

Light Intensity $\mu E.cm^{-2}.sec^{-1}$	Mean Fresh Weight mg/plant
30.8 ± 1.4	60.3 ± 11.0
90.0 ± 5.0	319.1 ± 78.9
116.0 ± 4.0	448.7 ± 129.2
164.3 ± 1.6	628.5 ± 183.5

Table 15. Calcium content of marigold plants grown at varied light intensities.

Plants grown at varied light intensities for 21 days

light intensity $\mu E \cdot cm^{-2} \cdot sec^{-1}$	calcium content ppm (fr. wt.)
30	703 ± 142 (n = 4)
90	683 ± 73 (n = 4)
116	703 ± 121 (n = 4)
164	753 ± 145 (n = 4)

On the clinostat, the fall of *ca* 25% in wall calcium observed, took place both on vertical and horizontal rotation (Table 16) hence may be a general response to vibrational stress factors.

Major wall polysaccharide fractions display a modest light dependency (Table 17). Cellulose and pectin each decrease slightly, bringing about a somewhat larger relative increase in the hemicellulosic fraction. Although this change of about 30% in the hemicelluloses is of marginal significance, additional evidence for these polysaccharides as an environmentally responsive group is shown by changes in five main sugar components (Table 18). Here with increasing light intensity, glucose, galactose and mannose appear to decrease, whereas xylose and arabinose increase in proportion.

On the clinostat, again the cellulose and pectin fractions change little if at all, whereas hemicelluloses undergoes a modest increase (Table 19). This change if significant may also reflect contributions from vibrational stress to the "hypogravity" response.

Additional indications that both vibrational and hypogravitational factors influence the highly sensitive hemicellulose fraction is suggested by changes in its component sugars (Table 20). Present data are insufficient for analysis of the changes observed. It is clear however that the hemicelluloses are environmentally sensitive. These changes are consistent with the known interconversions and biogenetic relations among the monosaccharides:

Table 16. Calcium content (ppm on fresh weight basis) of marigolds grown under the indicated environmental conditions.

21 day old, stationary control	1207 (n=12)
21 day old, clinostat [a]	919 (n=4)
21 day old, clinostat + 2 days [b]	917 (=4)

[a] Plants grown from seed on a rotating clinostat.

[b] Plants grown as stationary controls for 21 days and then placed on a rotating clinostat for 2 days.

Table 17. Effect of Light Intensity on Cell Wall Carbohydrate content of 21 Day Marigolds Grown from Seed.

Intensity $\mu E \cdot cm^{-2} \cdot sec^{-1}$	Analysis (mg/g)		
	Cellulose	Pectin	Hemicellulose
82	565	301	134
124	568	293	149
165	541	284	175

Table 18. Effect of light Intensity upon Hemicellulose Composition in 21 Day Marigolds Grwon from Seed.

Intensity $\mu E \cdot cm^{-2} \cdot sec^{-1}$	Composition (%)				
	D-Glucose	D-Galactose	D-Mannose	D-Xylose	D-Arabinose
82	28	18	∿1	27	20
124	17	8	4	7	60
165	16	11	5	11	57

Table 19. Effect of Clinostat Culture on Cell Wall Carbohydrate Content in 21 Day Marigolds.

Condition	Analysis (mg/g)		
	Cellulose	Pectin	Hemicellulose
Stationary Control	584	301	115
Clinostat	564	292	144
Rotating Control	575	293	130

Table 20. Effect of Clinostat Culture upon Hemicellulose Composition in 21 Day Marigolds.

Condition	Composition (%)				
	D-Glucose	D-Galactose	D-Mannose	D-Xylose	D-Arabinose
Stationary Control	12	11	7	19	52
Clinostat	16	7	10	23	44
Rotating Control	18	10	10	18	42

glucose $\longleftrightarrow$ galactose glucose $\longrightarrow$ xylose	4-epimerase dehydrogenase, decarboxylase
glucose $\longleftrightarrow$ mannose mannose $\longrightarrow$ arabinose	2-epimerase dehydrogenase, decarboxylase
xylose $\longleftrightarrow$ arabinose	xylose epimerase

The study of lignification was not as intensive in marigold as in earlier work with cucumber. The general operational plan placed priorities upon examination of areas such as cell wall carbohydrate that had been neglected in the earlier investigations reviewed above. This, in conjunction with our involvements with ethylene physiology delayed some of the obvious in-depth studies projected.

Nevertheless, the experiments carried out show responses of some interest. In aerated liquid media, seedlings grow more slowly and contain markedly less lignin (Table 21). Under these conditions no protein synthesis takes place the value of *ca* 37 mg/seedling being about the same level found in the seeds. In contrast, the controls show an increase from the seed level to *ca* 100 mg/seedling.

On the clinostat, a rather different picture appears (Table 22). Thus relative to static control, there is 16% more lignin in plants grown under gravi-compensation. Relative to rotating controls, however, there is 19% less lignin in the usual clinostat mode. Protein content follows a similar pattern giving *ca* 190 mg/seedling in the rotating control; 140 mg/seedling on the clinostat; and 100 mg/seedling for static controls. Thus, *ca* 27% less protein was found clinostat-grown seedlings compared with rotating controls.

Table 21. The lignin Content of Marigold Seedlings Cultured in Circulating Nutrient Liquid ("Buoyancy Compensation").

Incubation from Seed Days	Shoot Length cm		Lignin mg/g dry wt.	
	Control	Liquid	Control	Liquid
7	2.3 ± 0.4	1.7 ± 0.2	trace	none
10	2.9 ± 0.4	2.0 ± 0.8	13 ± 3	3 ± 1
16	3.3 ± 0.5	2.1 ± 0.8	25 ± 5	8 ± 3

Table 22. The Lignin Content of 16 Day Clinostat Grown Marigolds

Growth Condition	Lignin Content		
	mg/g Dry wt.	Relative	
		a=100	c=100
a. Static Control (n-22)	25 ± 5	100	70
b. Clinostat (n=14)	29 ± 6	116	81
c. Rotating Control (n=14)	36 ± 6	114	100

With longer growth periods on the clinostat, these differences in lignin tend to disappear as the clinostat level approximates the elevated level of the rotating control. Plant growth from day 14 to day 21 is most conspicuous in terms of the "fanning out" of leaf area, yielding a highly dissected structure with many surfaces and edges.

2) Peroxidase

One of the early points established in our investigation was the rise in peroxidase activity both in aerated liquid media and on the clinostat. The activity parameter is here expressed in terms of rate of change in absorbance (under conditions specified above in Methods and Procedures) on a protein basis: ΔO.D. $\cdot$ Min$^{-1}\cdot\mu$g protein^{-1} and we can conveniently define activity units (U_{px}) so that 1 $U_{px} = 10^{-2}$ O.D.$\cdot$Min$^{-1}\cdot\mu$g Protein^{-1}.

No peroxidase activity was detected in dry marigold seed. Although seeds of some plant species contain the enzyme, synthesis sommonly follows the placement of the seed in growth conditions.

In circulating liquid media marigold peroxidase activities rise rapidly and within 6 days reach 275% of controls (Table 23). After 17 days these seedlings attain *ca* 330% of control enzyme activity.

On the clinostat, the pattern of response is parallel but lesser in extent, giving 152% of control after 17 days (Table 24). The rotating control remains, however at a peroxidase level slightly but not significantly lower than in control seedlings.

Table 23. Peroxidase Activity in Marigolds Cultured in Circulating Nutrient Liquid.

Incubation Days	Peroxidase Activity (U_{px}) Control	Liquid Culture
0	0	0
6	0.4 ± 0.2	1.5 ± 0.4
10	1.4 ± 0.4	3.5 ± 1.0
17	2.5 ± 0.7	8.3 ± 1.7

Table 24. The Peroxidase Activity of Clinostat Grown Marigolds.

Condition	Activity (U_{px}) on Day		
	6	10	17
Static Control	0.4 ± 0.2	1.4 ± 0.4	2.5 ± 1.7
Clinostat	1.0 ± 0.2	1.8 ± 0.6	3.8 ± 0.5
Rotating Control	0.3 ± 0.2	1.2 ± 0.5	2.5 ± 0.5

Culture conditions also affect the organ distribution of enzyme activity (Table 25). By normalizing activity in the hypocotyl the typical gradient (Static Control) is evident and disturbance in this pattern, although most extreme (reversed) in liquid-grown seedlings is also apparent in other modes.

When the other culture conditions are normalized to the static control, the extreme response is again evident in liquid culture and the opposed directions of clinostat and rotating control responses are readily apparent.

3) Short-Term Ethylene-Peroxidase Relations

The brief exposure to rotation of marigolds grown for 21 days under stationary conditions yields a quite different pattern of responses.

This is in part occasioned by profound changes with increasing maturity between day 15-17 and day 21. These include, for example a decrease in total peroxidase and an apparent shift from 85-93% cytoplasmic enzyme to *ca* 95% wall bound. In addition, some indications of response such as changes in lignin and wall polysaccharides are minimal and cannot be reliably measured over 24-48 hour periods against the high baselines established during the preceding 21 days.

Those changes that have proved amenable to measure include peroxidase, ethylene and, for comparative purposes, epinastic growth curvatures (Table 26).

Plants receiving 24 hours of rotation after 21 days of stationary growth are unchanged in peroxidase. Cytoplasmic fractions in all cases fell within

Table 25. Relative Organ Distribution of Peroxidase Activity in 16 Day Liquid and Clinostat-Grown Marigolds.

Condition	a) Relative to Hypocotyl Activity		
	Shoot	Hypocotyl	Root
Static Control	107	100	230
Liquid Medium	0	100	35
Clinostat	125	100	200
Rotating Control	100	100	150
	b) Relative to Static Control		
	Shoot	Hypocotyl	Root
Static Control	100	100	100
Liquid Medium	0	2470	433
Clinostat	100	133	133
Rotating Control	40	67	50

Table 26. Effect on Biological Responses of 24 hr. Clinostat Treatment of Marigolds after 21 Days of Stationary Growth.

Response	Stationary Control	Clinostat	Rotating Control
Peroxidase (U_{px})			
cytoplasmic	0.28 ± 0.05	0.30 ± 0.01	0.29 ± 0.08
wall	4.95 ± 0.40	4.33 ± 0.99	3.48 ± 0.43
Ethylene			
Rate $nl \cdot g^{-1} \cdot hr^{-1}$	0.075	0.280	0.052
Conc. 24 hr. nl/l	1.42	4.59	0.90
Epinasty-2nd leaf Pair			
Δ° from axis	11.3 ± 3.8	36.2 ± 2.0	6.2 ± 1.0

a few percent of one another, and wall peroxidase activities showed only minimal deviations. If any change in the wall fractions exists in these data, it is in the rotating control, not clinostat, group. In contrast, ethylene rises sharply under clinostat but not rotating control conditions and epinastic response parallels ethylene.

When 21 day plants were exposed to exogenous ethylene at levels consistant with the concentrations produced (as in Table 26), both cytoplasmic and wall peroxidase activities were affected as was the nastic response. The pattern of response, however, did not correspond to that found when ethylene was allowed to accumulate.

These findings suggest that longer term responses to rotation expressed during development and those evolved by "shock" treatment may be related with difficulty at best. This is consistent with other well known plant responses to short *vs.* long-term application of light and temperature.

Table 27. Effects of Exogenous Ethylene on Peroxidase Activity and Epinasty in 21 Day Marigolds (Stationary Growth) After 24 hours.

		Ethylene Introduced nl/1	
	0	1.0	10.0
Peroxidase			
cytoplasmic	0.31 ± 0.05	0.42 ± 0.03	0.40 ± 0.05
wall	2.0 ± 1.0	2.7 ± 0.3	0.8 ± 0.3
Epinasty-2nd leaf pair			
Δ° from axis	4.5 ± 0.9	-14*	43 ± 7

* hyponastic response

Significance of This Research

1) Experimental

In spite of the number of years of effort represented by this report, a great deal of time and resources were invested in attempting the development of uniform and standard procedures by the consortium as a whole. Thus many areas of actual research were short of complete or definitive stages of accomplishment when the project ended. For this reason, comprehensive, detailed discussion of all facets of this study identified above would be premature. Nevertheless, there are some areas which warrant firm conclusions and others in which trends and directions are reasonably well indicated.

It is well to begun a consideration of the significance of these studies by recognizing the limitations upon gravitational experimentation. Ultimately these limits must be expressed in terms of interactions between molecules and aggregate molecular systems at all higher levels of organization. Thus we recognize that even in our experiments in liquid systems, the only factor limiting gravi-perception at the intracellular level is continuous disorientation of the seed and seedling, not the "relief" provided by buoyancy. Dedolph and Dipet in 1971 (Plant Physiol 47,756) proposed to design a new type of clinostat "on which intracellular conditions can be rendered virtually identical to those of plants in satellite free fall regardless of plant size or duration of experiment". They also recognized, however, the need for "occasional satellite free fall experiments for verification". We add that these must include long-term exposures and large plants in statistically acceptable numbers.

Even earlier — in 1969 — Cook (Math. Biosci 5,353) formulated a mathematical model for the disorientation process and described its implementation electromechanically. Even here a limit to the size of organism was recognized.

Such approaches may in time replace satellite free fall in the study of plant responses to hypogravity. At present, however, this is not proven; and such proof must take into account the inevitable biological problems of statistics and individuality.

Whatever opportunities may arise for genuine orientation-free culture of plants on the Earth's surface in the future, we are now constrained by clinostats which are themselves numerically limited and far from pure rotational instruments in practice.

In the main these imperfections are expressed in terms of vibration, thus creating perturbations that we can recognize in differences between horizontal- and vertical-axis rotation. And when dealing with plants slender in form (e.g. cucumber seedlings) rotator vibration may be the major perturbation. When, however, young marigolds beyond the first two weeks are used, then the leaf system with its surfaces and edges creates an additional disturbance of an aerodynamic character. The potential for error in torque generated by aerodynamic drag is considerable when it is recalled that plant growth and the differentiation and placement of appendages (leaves) show spirality and, at least interspecifically, chirality (cf. D'arcy Thompson, "On Growth and Form").

Recognizing the limitations of the clinostats that have produced the existing body of knowledge about experimental hypogravity — and continue to do so — the results presented above can now be examined for whatever significance they possess.

The major findings in this program have been summarized in terms of response parameters; the responses; their directions under experimental gravity regimes, and the conclusions (i.e. "basis for response") in each case (Table 28).

This evaluation indicates that wall hemicelluloses, lignin, tissue protein and peroxidase all respond to hypogravity during growth. Flotation and clinostat regimes are quantitatively different but directionally consistent. Most of these responses require some degree of correction for vertical axis rotational-vibrational effects.

It is not clear that peroxidase changes significantly during 24 hour excursions on the clinostat, but wall bound enzyme is affected by rotation — and that response is opposite in direction to those seen in plants grown from seed on the clinostat. Ethylene production seems to be clearly tied to gravity, however many other stress or injury factors also stimulate its biosynthesis.

Thus, insofar as they could be tested during the tenure of this project, the original hypotheses still apply:

1) Lignification decreases and peroxidase increases during growth under hypogravity.

2) Other wall components are selectively altered.

3) Other response parameters (e.g. protein, ethylene) are also affected.

4) Vibrational (and aerodynamic) factors associated with rotation complicate the use of real clinostats and limit their value making satellite free-fall experimentation essential.

Thus a sizeable body of knowledge involving land plant evolution, the nature of the aquatic environment, the space environment and the principles of biomechanics are further interlocked and supported by our findings.

Table 28. A Diagnostic Summary of Marigold Performance Data on Experimental Regimes.

Parameter	Response to Test Condition			Basis for Response	
Long Term Growth	Flotation	Clino.	Rotation	Hypo-G	Vibration
Cell Wall					
Calcium	--	Decr.	Decr.	No	Yes
Cellulose	--	N.S.	N.S.	No	No
Pectin	--	N.S.	N.S.	No	No
Hemicellulose					
Total	--	Incr.	Incr.	Yes	Yes
Sugars	--	Change	Change	Yes	Yes
Lignin	Decr.*	Decr.	Incr.	Yes	Yes-Opposite
Tissue					
Protein	Decr.	Decr.	Incr.	Yes	Yes-Opposite
Peroxidase	Incr.	Incr.	N.S.	Yes	No
24 Hour Treatment of Pre-Grown Plants					
Peroxidase					
Cytoplasmic	--	N.S.	N.S.	No	No
Wall	--	Decr.	Decr.	?	Yes
Ethylene	--	Incr.	N.S./Decr.	Yes	Yes?

* Decr. = decrease in amount or concentration

Incr. = increase

N.S. = no significant dhange

2) Technical Problems

The significance of this project has been considered primarily in terms of tests of hypothesis related to the cell wall and secondarily in terms of other experimental findings.

There is a third area here somewhat loosely termed "technical", concerned with problems that have only become apparent as a result of special experimental procedures and conditions. These operational findings are important because they may lead to discovery of new relationships as well as hidden complexities and sources of error.

The most important of these are:

The UV-IR effect and the screening properties which some plastic materials used in the fabrication of chambers possess. The emission characteristics of fluorescent tubes outside of the visible range and the presumably varied filter properties of plastic materials must obviously be taken into account in the design of experiments.

The problems encountered with rotation have already been discussed and it is sufficient to point out that not only must hypogravity effects be corrected for mechanical-vibrational components, but they also open the possibilities for interactions not amenable to simple numerical correction. Further, aerodynamic drag may create torque problems that amplify assymetries related to spirality and chirality intrinsic to plant growth.

The existence of uncontrolled and often unsuspected sources of ethylene in facilities and equipment must be eliminated or controlled — and certainly more closely monitored — in future experiments. It is also evident that the plant's ability to produce ethylene and to respond to it must be taken into account especially when experiments involve small enclosed spaces.

Physiological ethylene problems could have been anticipated: even obscure sources of ethylene contamination might have been considered earlier. Fungal symbionts in seeds are another matter, however. The use of "symbiont" here is deliberate and is applied because we are dealing with a microflora intimately associated with the inner seed coat layers, not a casual surface contamination. Even the most conservative assessment of the presence of these fungi (and possibly bacteria) means that the seed upon placement in culture is subject to the influence of gaseous and soluble metabolites from associated microbiota, as well as those from its own life processes.

Innovative procedures based upon selective inhibition are needed to control such microflora without jeopardizing embryo viability — and monitoring is essential.

The foregoing argue strongly for a major reassessment of the validity and adequacy of the design concepts and operations associated with ground based hypogravity research. Although these reservations and qualifications may not apply to short-term phenomena such as geotropism, they most certainly affect long-term growth, development and metabolism.

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Changes Induced in Peroxidase Activity under Simulated Hypo-gravity

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Abstract

A new method of inducing and maintaining a hypo-gravity environment is described. Using this new technique (buoyancy compensation), the effect of reduced gravitational stress on dwarf marigolds (*Tagetes patula*) was studied. It was found that the specific activity of peroxidase was elevated significantly in gravity compensated seedlings. This effect, discovered with the buoyancy compensators, was confirmed with plants grown on traditional clinostats.

Introduction

"Klinostats" have been used to study plant geotropism since 1872 when Sachs (1882) introduced the instrument into the laboratory as a gravity-compensating research tool. By 1904, enough information on the growth behavior of plants on clinostats had been gathered to demonstrate a number of practical limitations of the instrument as a gravity-compensating device (Newcombe 1904). The limitations noted, such as the need for evenness of rotation speed, the avoidance of centrifugal effects *etc.*, have been generally alleviated by later workers studying the conditions of clinostat rotation necessary to eliminate the unidirectional stimulus of gravity (Gordon 1963, Larsen 1953, 1962).

This laboratory has been engaged in the study of the degenerative effects of hypo-gravity (hypo-g) culture on the dwarf marigold, *Tagetes patula*. In conjunction with this research, we have also been evaluating an alternative method of hypo-g induction and maintenance, in the belief that experiments using the classical clinostat would be complemented by the new method, allowing the observation and evaluation of hypo-g effects that are not revealed using traditional clinostat methodology alone.

The alternative method of hypo-g induction and maintenance that we are proposing is essentially a buoyancy or flotation technique. This technique takes advantage of the gravity compensating effect of a liquid supporting medium and then neutralizes the directionality of the gravitational vector that is left by slowly and constantly rotating and tumbling the plants. This apparatus (Figure 1) is simply an elaboration and refinement of a similar device used by Siegel *et al.* (1972) to study lignification in cucumbers.

This paper reports the results of preliminary experiments using this technique of hypo-g induction and maintenance

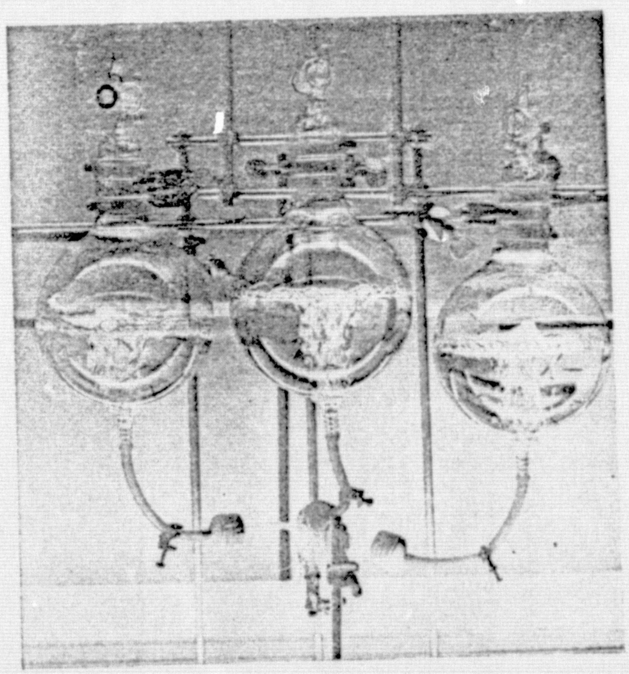

Figure 1. *A set of three buoyancy compensators for hypo-gravity experiments.* Seeds and seedlings continuously circulated (*i.e.* no net orientation) by bubbling millipore filtered air through the system.

and comparing the results obtained with those from traditional clinostats.

Methods and Materials

Seeds of the dwarf marigold, *Tagetes patula* L. cv. Petite Gold, were surface-sterilized by soaking for 5 min in 60% ethanol. The seeds were then placed in a Petri dish with sterile distilled water and allowed to germinate. When the seedlings were large enough for handling ($\simeq$3 days) they were transferred to either the buoyancy compensators containing sterile half-strength Hoagland's solution or to 25 mm × 150 mm culture tubes containing a sterile mixture of 50:50 vermiculite peat moss moistened with Hoagland's solution.

Air was filtered through 0.22 μm filters and bubbled into the chambers from the bottom after transfer of the seedlings to the buoyancy compensator. The rate of air flow ($\simeq$650 cm^3/min) was sufficient to rotate the plants without damaging them. The culture tubes were inserted onto two 24" (61 cm) diameter styrofoam discs, rotating at 1 r/min. One disc of plants was set on a vertical axis, as a rotating control while the other disc served as the horizontal axis clinostat. The position of the culture tubes ranged from 16 cm to 28 cm from the axis, resulting in calculated *g*-forces ranging from $10^{-4} \times g$ to $10^{-6} \times g$.

The plants were grown under a 16 h light/8 h dark cycle with a light intensity of 9700 to 10,800 lux. The temperature ranged from 25°C (light) to 19°C (dark).

At the time of analysis, the complete plant was homogenized in a 0.07 *M* pH 6.0 phosphate buffer, and centrifuged at $32{,}000 \times g$ for 30 min. The peroxidase activity of the whole plant was determined utilizing 0.1 ml aliquots of the cell-free supernatant fraction and a 5×10^{-3} *M* guaiacol plus H_2O_2 substrate (Saunders *et al.* 1964). Protein concentrations were determined according to the method described by Lowry *et al.* (1951).

Fifteen seedlings from the 10 test-tube-cultured seedlings and buoyancy compensators were used for each protein and peroxidase assay. Each assay is represented by one point in Figures 2 and 3.

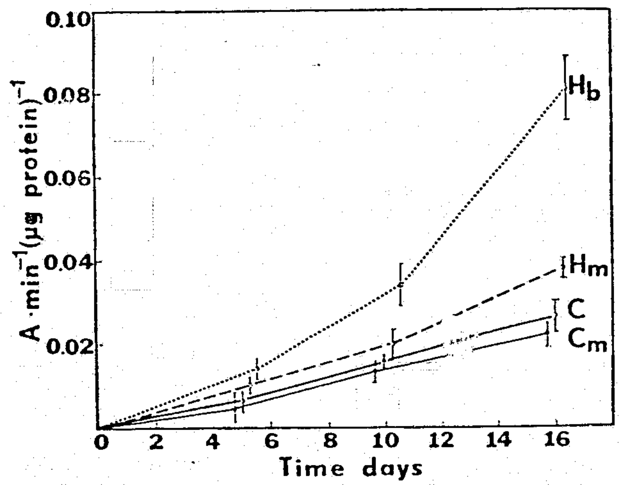

Figure 2. *Peroxidase activity* [$\Delta A \times min^{-1} \times (\mu g\ protein)^{-1}$] *vs. number of days in the indicated gravitational environments.* In this figure and all subsequent figures, H_m refers to hypo-gravity (mechanical clinostat), C_m to rotational control (mechanical clinostat), H_b to hypo-gravity (buoyancy compensators) and C to stationary control. All reported averages are ±SD.

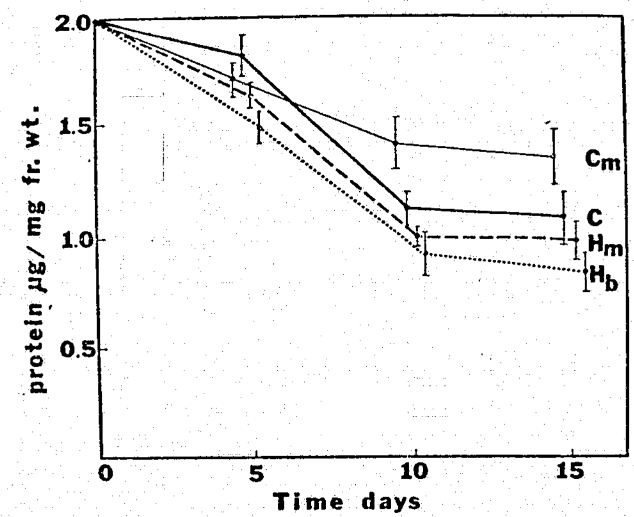

Figure 3. *The protein content* (μg protein/mg fresh weight) *is plotted against time of exposure to the indicated gravitational environment.*

Results

Plants grown in an environment of reduced gravitational stress have a higher peroxidase activity than the control plants grown under normal $1 \times g$ conditions. Figure 2 illustrates the increase in peroxidase activity with time exhibited by the hypo-g cultured seedlings. This hypo-g effect, initially discovered in the buoyancy compensator cultured material, was confirmed by parallel determinations using plants grown on the mechanical clinostat.

Preliminary investigations of the mechanism of this effect suggest that it is not due to a general increase in protein synthesis. Figure 3 shows that the Lowry-positive protein content of the tissue is decreasing with time as the peroxidase activity is increasing. Nevertheless, it is clear from Figures 2 and 3 that hypo-g culture has marked metabolic effects on the plants, and that these effects become amplified with increasing time in an environment of reduced gravitational stress.

It is interesting and somewhat paradoxical that the metabolic alterations attested to by Figures 2 and 3 are not clearly expressed in the gross morphological characters of root and shoot length (Figure 4). In the case of the buoyancy compensator cultured material, however, the elongation of the

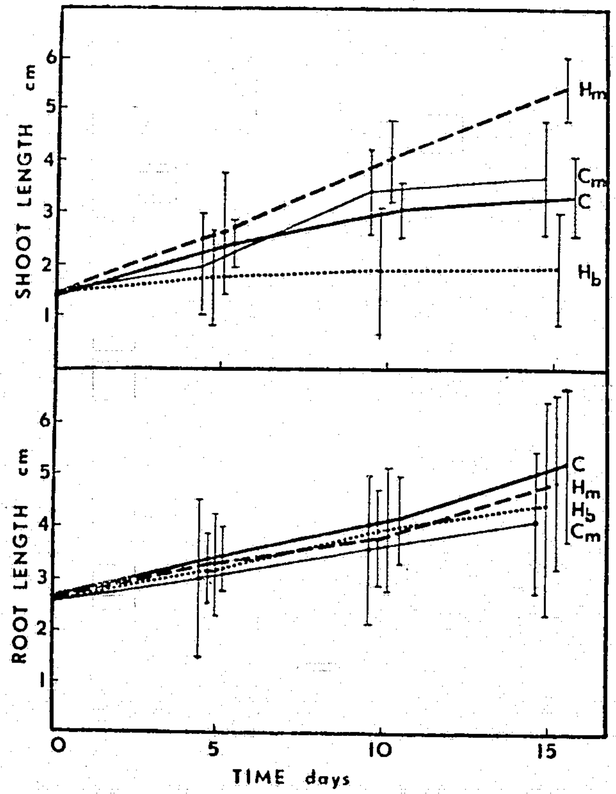

Figure 4. *Root length and shoot length* (cm) *plotted against time in the indicated gravitational environment.* No significant differences in the roots were discernible. No effect on shoot growth was found except in the buoyancy compensated seedlings (H_b) where the elongation of the internodes is clearly inhibited.

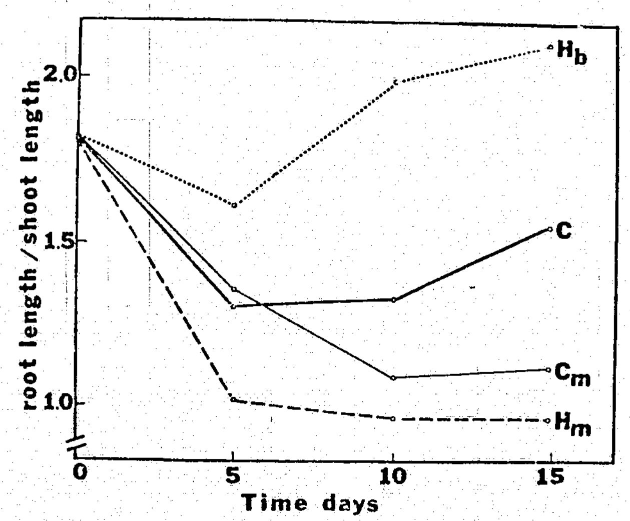

Figure 5. *Time course of the average root length to shoot length ratio of the dissimilarly grown seedlings.* The inhibition of the internodes of the buoyancy compensated seedlings in relation to their roots is demonstrated.

shoot internodes in relation to the roots is clearly inhibited, as evident in Figure 5. Whether or not this inhibition is a true effect of hypo-g, or is a consequence of the culture conditions, has not been determined.

Discussion

The validity and feasibility of using a buoyant culture technique to induce and maintain a hypo-gravity environment have been established. The results obtained using this technique correlate well with results obtained from experiments using the mechanical clinostat. The buoyancy culture method has proven to be of value in sorting out true hypo-g effects from those effects due to vibrational stress, a persistent problem with mechanical clinostats. Although long-term experiments are not yet feasible with this new method, it could be invaluable in short-term tracer and hormone experiments.

In connection with the Biosatellite program, Conrad (1968, 1971) reported changes in peroxidase activity in response to reduced gravitional stress. We have considerably expanded and refined this observation. There is, however, a basic difference between the two results: our effect is based on the results of relatively long-term experiments, while Conrad's data were collected after only 45 h of hypo-g exposure. Over the entire period of observation, peroxidase activity served as a reliable marker of the hypo-g condition. Our confidence in this claim is based on replicability within and reproducibility of experiments and the presence of the same response in whichever mode of gravity-compensation was under investigation.

In conclusion, the increase in peroxidase activity with time in plants grown on horizontal clinostats or in buoyancy compensators represents a response of the plant tissue to an environment of reduced gravitational stress. We are currently attempting to further characterize this response, especially in regard to mechanism, using both modes of compensation.

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