FINAL REPORT

ON

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EXPERIMENTS AND OPERATIONAL PROCEDURES FOR DEVELOPING GERM-FREE SEEDS, SEEDLINGS, PLANTS, TISSUES AND CELL LINES FOR THE LUNAR RECEIVING LABORATORY

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EXPERIMENTS AND OPERATIONAL PROCEDURES FOR DEVELOPING GERM-FREE SEEDS, SEEDLINGS, PLANTS, TISSUES AND CELL LINES FOR THE LUNAR RECEIVING LABORATORY

(Contract NAS 9-6822)

This contract was awarded to the University of Houston on July 1967 for initiating the botany program at LRL, MSC, Houston and to carry out experiments and operational procedures on various algal types, seedlings of higher plants and tissue cultures of such plants. The botanical systems included 5 algae and 12 vascular plants and studies were directed toward analysis of algal growth, seed germination, seedling development and establishment and maintenance of tissue cultures of the vascular plants. At the termination of this contract (June 30, 1968), almost all the botanical systems have been established and are being routinely maintained at both LRL and at the University of Houston. All the algal cultures were obtained from Algal collection, Indiana University, Bloomington, Indiana and have been isolated in sterile culture. Procedures for routine surface-sterilization of seeds have been worked out. The seeds of some vascular plants have been tested for germination under sterile conditions and germination data have been obtained. Callus tissue of many of the higher vascular plants (gymnosperms, dicots and monocots) have been established. Histological analysis of some of the tissues have been carried out. All the capital equipments have been delivered to LRL. The training of the NASA personnel for tissue culture techniques was achieved. In the following pages, details of these procedures and some of the data which were obtained have been given.

ALGAL MATERIALS

The following alga are presently maintained as germ-free algal cultures at the University of Houston on either solid agar media, or in liquid cultures.

Blue-green

Nostoc sp.

Anabaena sp.

Green

Chlorella vulgaris	(strain Nos., Indiana Univ. collection)
"	263
"	398
"	580
Chlorella miniata	
Trebouxia sp.	
Chlorococcum aplanosporum	
Chlamydamonas	male
"	female

Red

Porphyridium sp.

The procedures used in the cultivation of the cultures were very similar to standard microbiological techniques. All transfers were done every four weeks. These transfers were carried out inside a glove box under total asceptic conditions.

Transfers to solid agar media were done using a wire loop. Inocculum was scraped from the stock slant and transferred directly to a fresh slant. Newly innoculated tubes were covered with sterile polypropylene. This closure allows sufficient gas exchange and adequately prevents any contamination.

Liquid transfers were accomplished using sterile, disposable pipettes. 1 ml of the original culture was subcultured to a fresh tube of media. These tubes were then incubated on a wheel rotating at 2 revolutions per minute. In this horizontal position, the tubes receive aeration as well as equal light exposure. In liquid culture a cell suspension is created which can be plated to produce clones of cells which arise from single cells. These clones therefore have a uniform genetic compliment. Cultures were maintained on a 12 hr light-dark cycle.

The main part of the study centered around selection of the proper type of media for best growth for each alga. Different media were selected on the basis of available literature information. The composition of the various culture media are included in the Appendix.

Nostoc, Anabaena and Trebouxia were maintained both as cell suspensions and as colonies on solid media. The culture media used for the liquid cell suspension was Kratz and Meyer's modification of Chu's media (1955) and Hunter's micro elements A-5 (1955). The cultures were grown in screw capped tubes on a rotary wheel. Nostoc

had the best growth on this media, while Anabaena and Trebouxia grew much better on another type of media.

Anabaena, Trebouxia, Chlorella, Chlorococcum, and Chlamydamonas were found to grow best on Murshige and Skoog's media, indicated as MUK (1963). This media consists of the major and minor salt solutions with no organic additives.

Porphyridium, a salt water red alga did not grow on any of the media previously described. Because of the need for a media of higher salt concentrations, the following media was selected:

This media is a modification of that proposed by Richard Star (1964) in his catalogue of cultures of the collection at Indiana University. The original formulation contained soil extract supernatant as one of the major nutrients. Since a germ-free culture was desired, it was necessary to find another organic source to substitute for the extract. By addition of tryptone and yeast extract it was possible to obtain good growth of the alga. This modification also made the media more definable than before.

In order to establish base parameters of the algal growth, initial calculations of growth of a typical culture of Trebouxia (which is used in other experiments) was made. Cells were suspended in a known volume of solution and an inoculum was counted on a haemocytometer. Ten fields were counted which showed data as follows: 51,62,19,58,27, 18,30,77,73,288. Total = 703 and average cells/field = 70.3. Using the dilution factor, the No. of cells/ml of the suspended population was thus calculated which gave a value of 87,875 cells/ml. Such trials are initiated and followed in order to establish a routine analysis of growth of all the different species which are maintained in culture. Experiments are in progress on the other species' growth over a 3-week period (analyzed every day on alternate days).

The growth rate of Chlorella vulgaris #398 was carried out in 2 different media, v.i.z.

(1) Murashige & Skoog media (1963) indicated as MUK (major + minor with no additives).

(2) Bristol's major salt solution (1949) + Hank's salt solution.

Growth was recorded by counting algal cells in a cell counting chamber. The results indicate that Chlorella has a faster growth rate in the MUK medium than in the Bristol media as shown in Figure 1.

VASCULAR PLANTS

Seed Germination

Seed germination experiments were carried out in ten separate runs for the following plants:

Seed rice (pre-treated)

Young rice (non-treated)

Corn

Wheat

Soybean

Tobacco

Sunflower

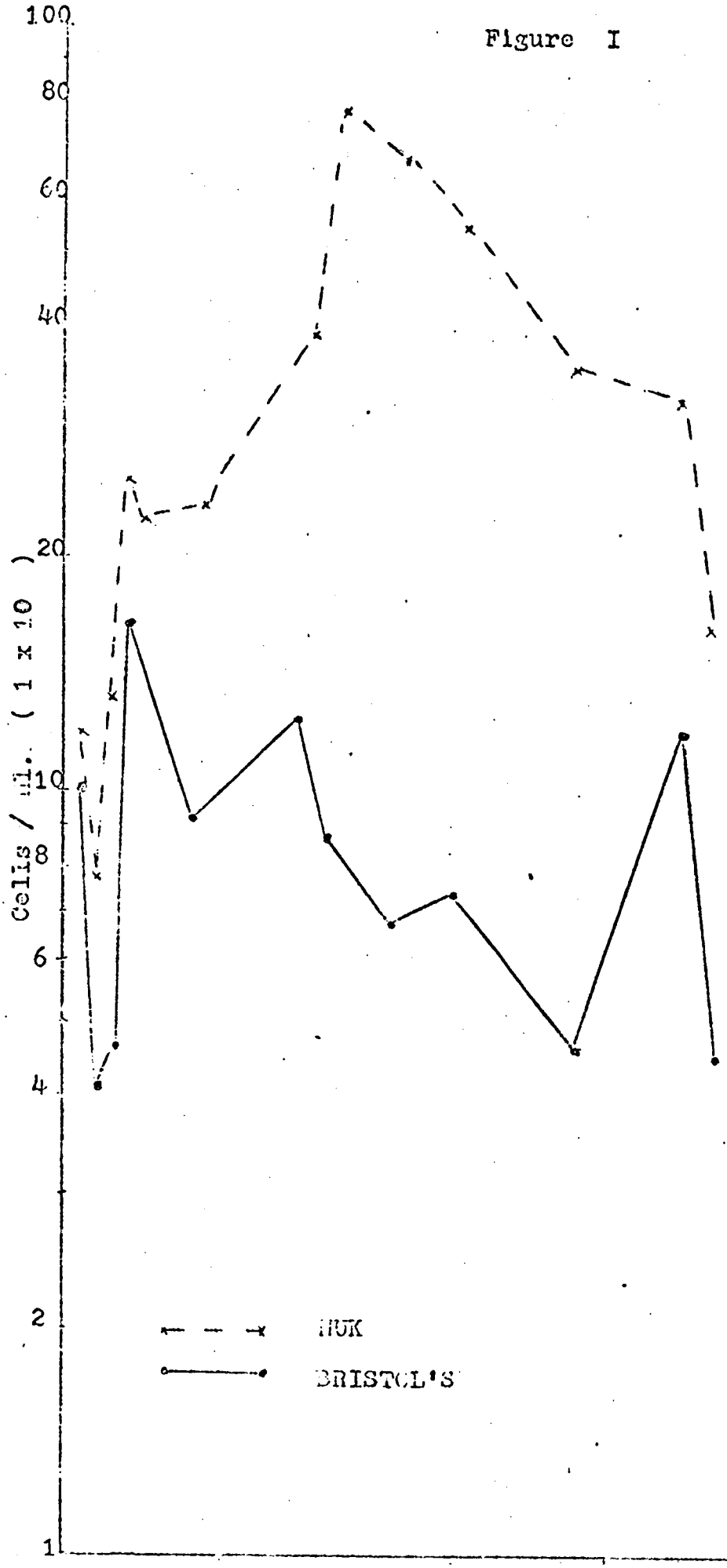
Tomato

Cabbage

The data collected from these germination experiments are represented in the graphs that follow. All seeds were surface-sterilized for 10 minutes with 10% sodium hypochlorite and were germinated in petri plates containing dishes of filter paper saturated with sterile distilled water. Pine seed germination was very slow and hence not included in the graphs.

Seed germination data are illustrated in Figures 2-3.

Figure I



GERMINATION OF DICOT SEEDS

Figure 2

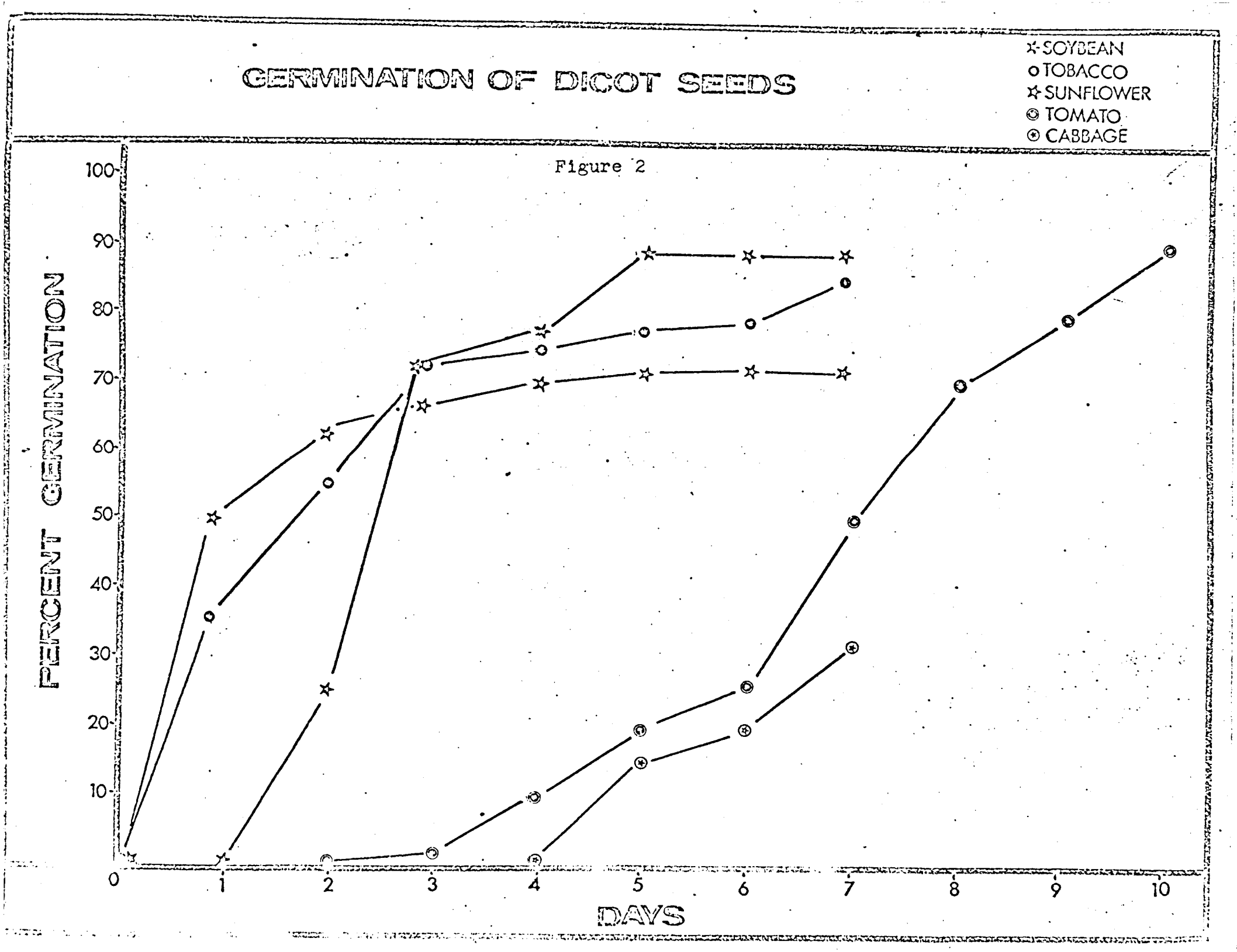
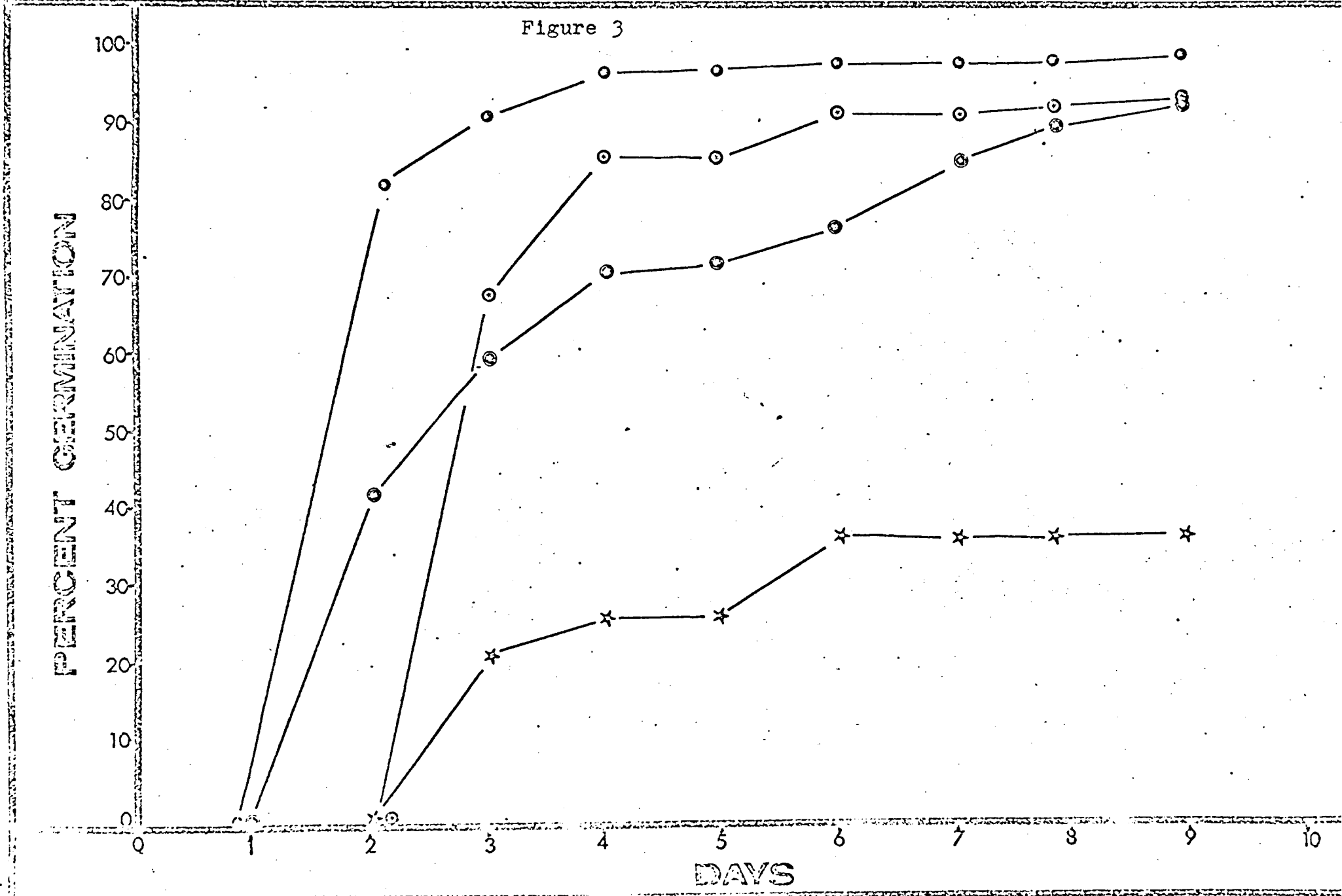


Figure 3


GERMINATION OF MONOCOT SEEDS
SEED RICE
CORN
YOUNG RICE
WHEAT
PERCENT GERMINATED
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
DAYS

VASCULAR PLANTS

Seedling Development

Seedling development experiments have been carried out for the following plants:

Pine	Sugar cane
Sunflower	Cabbage
Potato	Soybean
Tomato	Wheat
Tobacco	Rice
	Corn

No data was compiled on seedling development. Seedlings were grown on White's medium in 25 x 100 mm growth tubes. All seeds were surface-sterilized for ten minutes with 10% sodium hypochlorite. Sterile techniques were followed in all experiments.

Tissue Culture

Tissue cultures of plants were established from either newly-germinated seedlings or by routine subcultures of already established strains. Details of procedure for initiating callus cultures of plants and composition of the various media which were used are included in the Appendix. In some cases where special emphasis has been made to the successful establishment of culture, the composition of the media has been added in the text. Cultures were grown in appropriate media to which agar was added for solid cultures. In the case of liquid cultures, agar was omitted and 40 ml. of nutrient media was poured into each 125 ml. Erlenmeyer flask before autoclaving. The liquid cultures were left on a gyrorotatory shaker (135 rpm) in the culture room.

Cultures were maintained in a culture room at 25 ± 1°C with illumination for 12 hours per day. In the case of rice cultures, they were maintained in the dark. For growth experiments, callus tissue of approximately uniform initial weight was removed aseptically with a sterile spatula and transferred into fresh media. The standard error of the mean dry weight was calculated when possible. An average of 10 flasks for each experimental medium was set up in some experiments and growth measurements were made at the end of 7,14,21 and 28 days. Fresh weight and dry weight were obtained. The following plants were studied.

Oryza sativa (Rice)

Zea mays (Corn)

Glycine soja (Soybean)

Nicotiana tabacum (Tobacco)

Helianthus annus (Sunflower)

ORYZA SATIVA - (Rice)

Attempts were made to establish callus tissue from rice root; the procedure used by Yatazawa et al. (1967) was followed. Rice seeds were surface-sterilized for 20 minutes with 25% sodium hypochlorite and then washed twice in sterilized distilled water. The seeds were then soaked overnight in the sterilized distilled water. The seeds were sterilized again in 15% sodium hypochlorite for 10-15 minutes and were germinated in petri-plates containing filter paper saturated with sterile distilled water. These were germinated in the dark for 5 days.*

*In spite of these procedures, contamination rate was very high. Therefore, seeds were de-husked and surface-sterilized and allowed to germinate.

The five-day old rice seedlings were transplanted aseptically on a Heller's solid medium of the following composition:

Major salts:		Heller (1953)
KCl	750 mg/l	White (1954)
$Ca\ Cl_2.2H_2O$	75 mg/l	
$Na\ NO_3$	600 mg/l	
$Mg\ SO_4.7H_2O$	250 mg/l	
$Na\ H_2\ PO_4.H_2O$	125 mg/l	

Minor salts:	
$Mn\ SO_4$	0.1 mg/l
$Zn\ SO_4$	.1 mg/l
$H_3\ BO_3$	.1 mg/l
KI	0.01 mg/l
$Cu\ SO_4.5H_2O$	0.03 mg/l
$Al\ Cl_3$	0.03 mg/l
$Ni\ Cl_2.6H_2O$	0.03 mg/l
$Fe\ Cl_3$	1 mg/l

Additional Supplements:	
Glycine	3 ppm
Tryptophane	60 ppm
Thiamine Hydrochloride	0.5 ppm
Nicotinic Acid	0.5 ppm
Pyridoxine	0.5 ppm
Sucrose	2%
Agar	0.6%

Different growth factors were also added to the Heller's medium. These were combined as in the following:

(1) 2, 4-D

(2) 2, 4-D + Coconut milk

(3) 2, 4-D + Coconut milk, NAA

(4) 2, 4-D + NAA Yeast extract

(5) 2, 4-D + Yeast extract

(6) 2, 4-D + Yeast extract, coconut milk

The growth factors such as 2,4-D were at concentration of 2.0 ppm. NAA was at the concentration of one ppm, yeast extract at 0.5% and coconut milk was at 10%. All cultures were incubated in darkness at $30^{o}C$.

Three to four weeks after incubation, the hypocotyl root axis of the germinated seedlings proliferated to form callus tissue. Then callus tissues grew actively on further subcultures of Heller's medium with 0.5% yeast extract, 10% coconut milk and NAA. The tissues grew very loosely and became spread out over the surface of the media. These cultures were maintained in the dark at room temperature. Liquid cultures were also established. Growth rate studies were initiated. In one typical growth experiment, uniform inoculums of callus tissue of rice were transferred to 10 culture vessels. After 4 weeks of growth, both fresh weight and dry weight measurements were obtained. They are as follows:

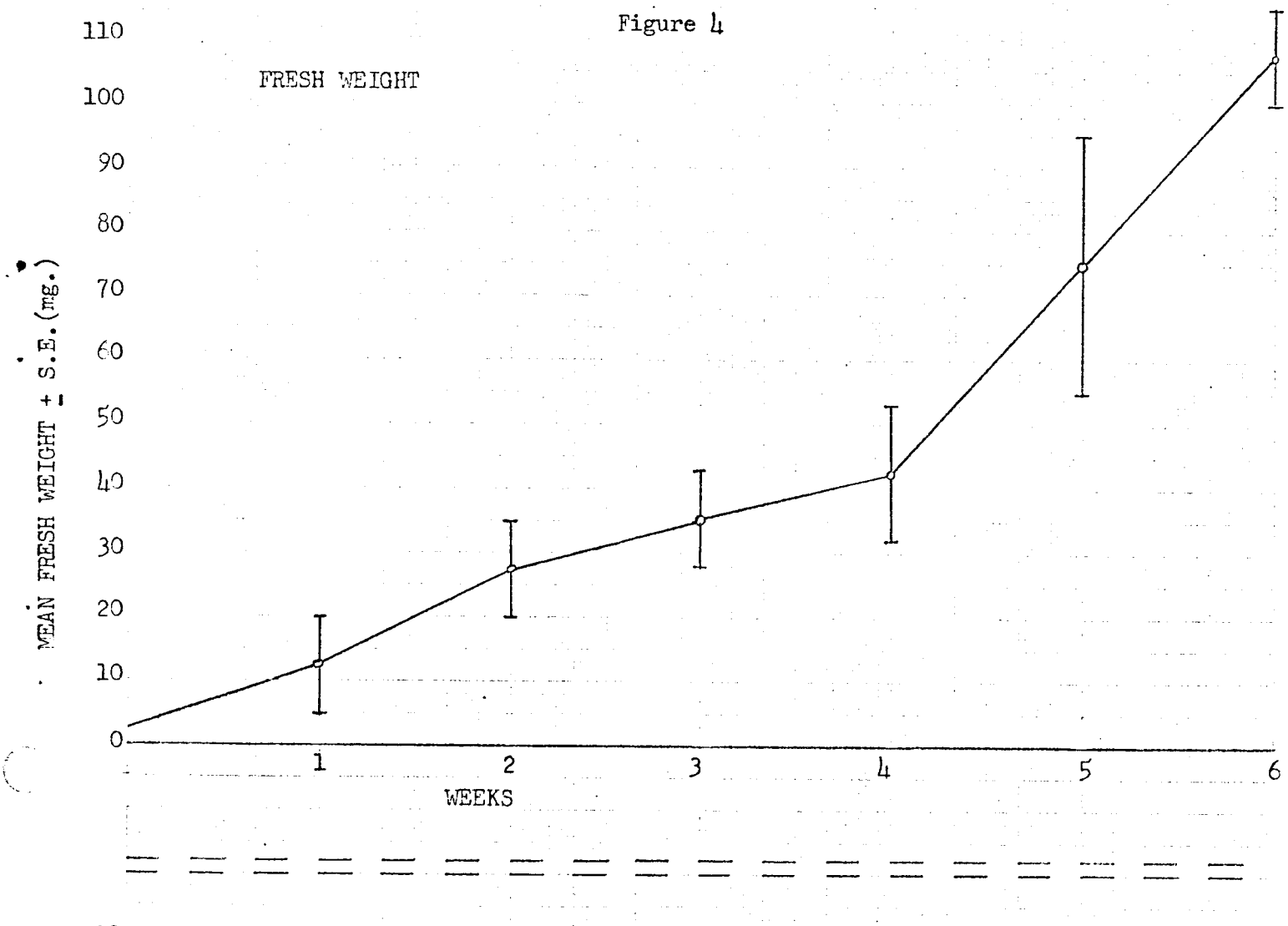
Fresh weight	2.5930 gms.	± 0.4321
Dry weight	0.1883	± 0.0151

At present, efforts are directed to obtain large quantities of stock materials so that growth measurements on a weekly basis can be obtained.

ZEA MAYS - (Corn)

Seeds of Zea mays, variety A-204 (Asgrow Seed Co., San Antonio, Texas) were surface-sterilized and allowed to germinate in petri plates containing moist filter paper. Three to four days after germination, adventitious roots were cut as 3-5 cm. segments and transferred to White's medium (BBL) supplemented with 1×10^{-6} of both 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid and 10% coconut milk. The explants were maintained in this agar medium and were incubated in diffuse light (200-300 ft. candles) at $25 \pm 1^{\circ}C$. After 2-3 weeks of incubation, swollen nodules were observed at regular intervals along the root axis. These swollen nodules eventually proliferated into callus tissue. Histological serial sections at this stage revealed proliferations of actively dividing cells in the periphery of the nodules surrounding older mature cells toward the central portion of the nodules. Isolation of a number of these spherical nodules into fresh media resulted in an actively growing callus culture. If the cultures were allowed to grow without frequent subcultures, roots were formed which in turn showed nodule formation along the root axis. Some of the newly-formed roots showed proliferations of new callus tissue. After establishment of the callus tissue, wet and dry weight measurements were taken at weekly intervals for six weeks. The initial inoculum was weighed and transferred to fresh media Ten replicates per week were used for the growth measurements. Various media differing either in the constituents of the major and minor salts (White, Murashige & Skoog, Heller, modified Bonner & Devirian) or in minor variations of additional supplements (coconut milk, yeast extract, dextrose, kinetin) were tested for growth of the callus tissue. Best growth was obtained in a medium supplemented with coconut milk. When yeast extract was substituted for coconut milk, the cultures failed to

Figure 4


FRESH WEIGHT
MEAN FRESH WEIGHT ± S.E.(mg.)
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
WEEKS

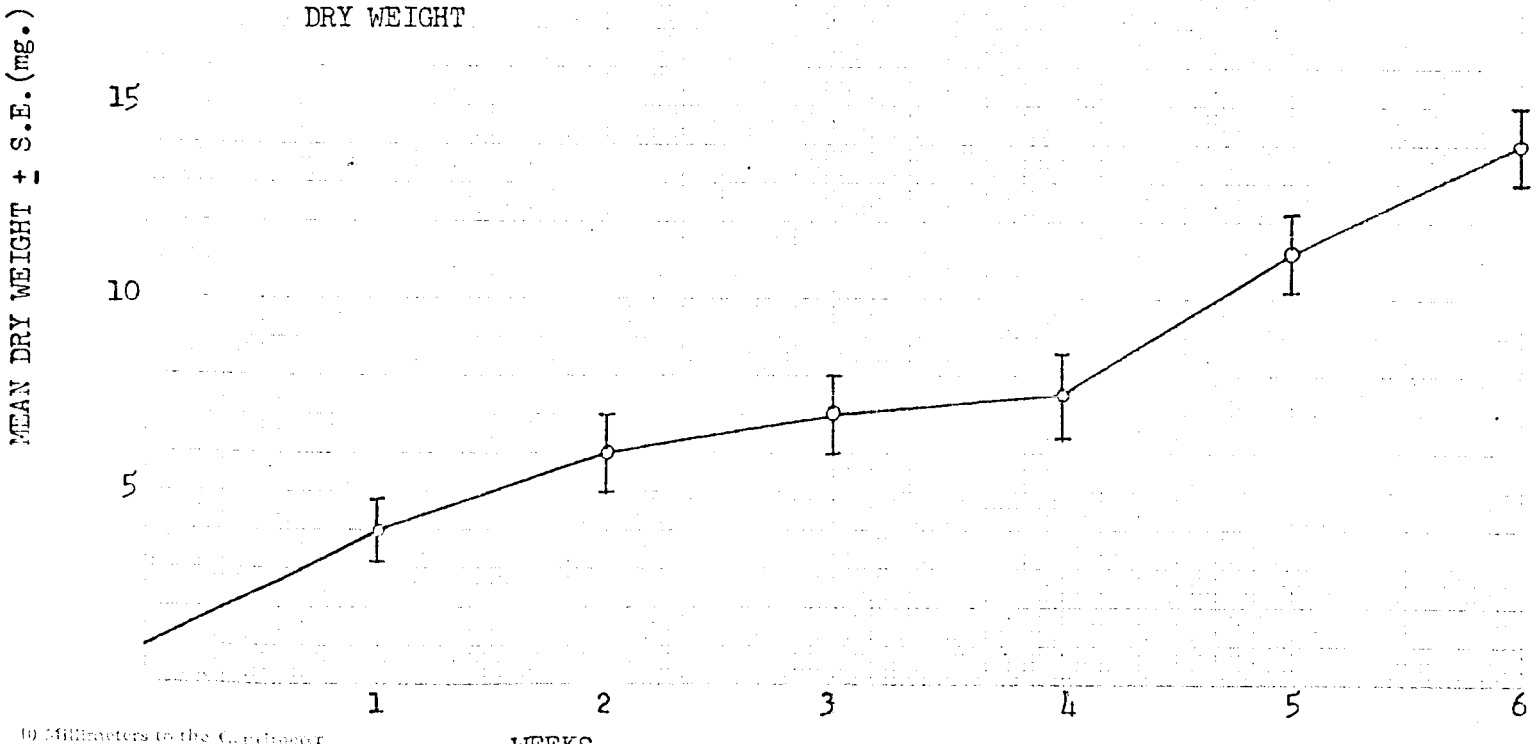
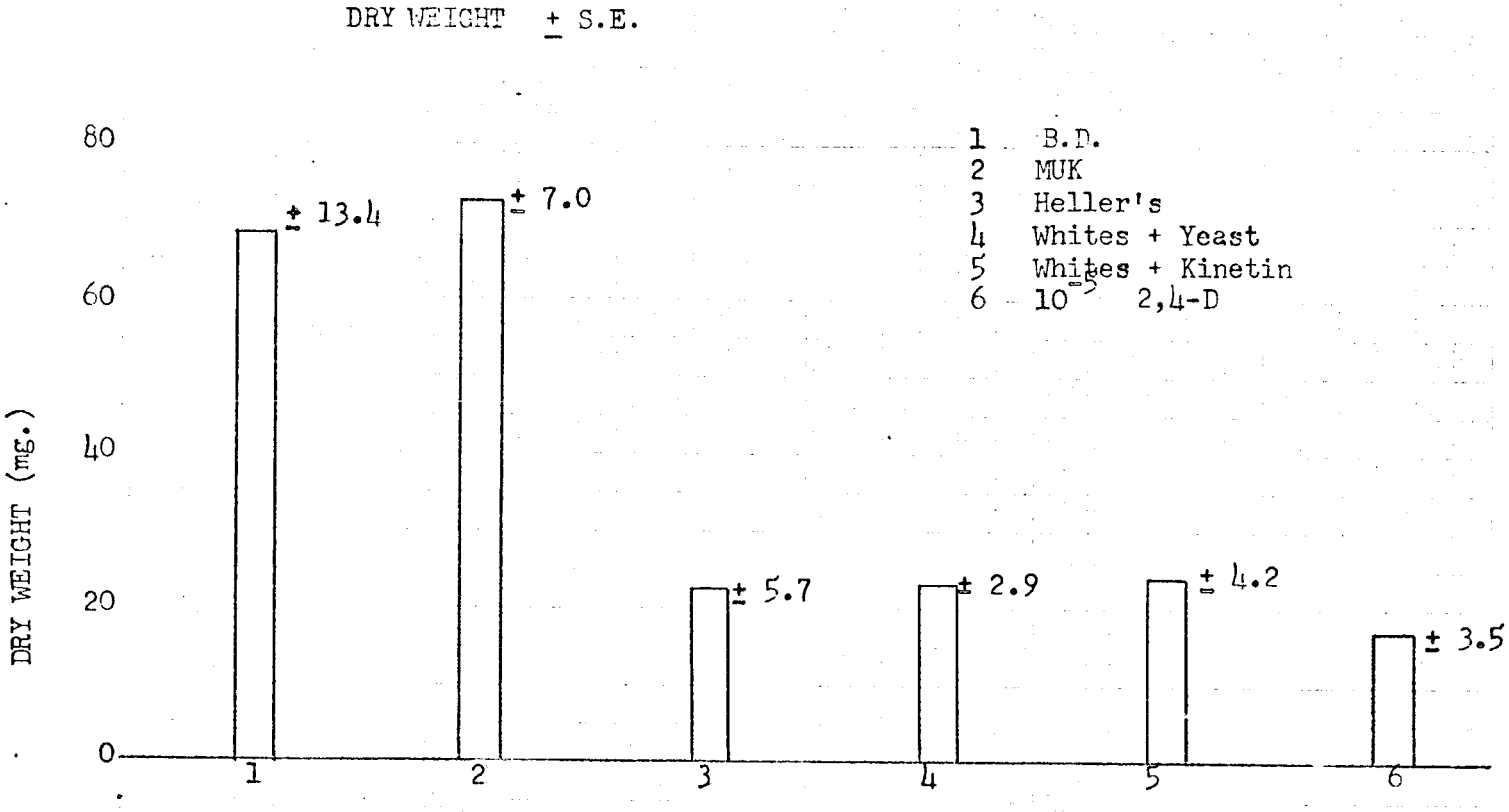
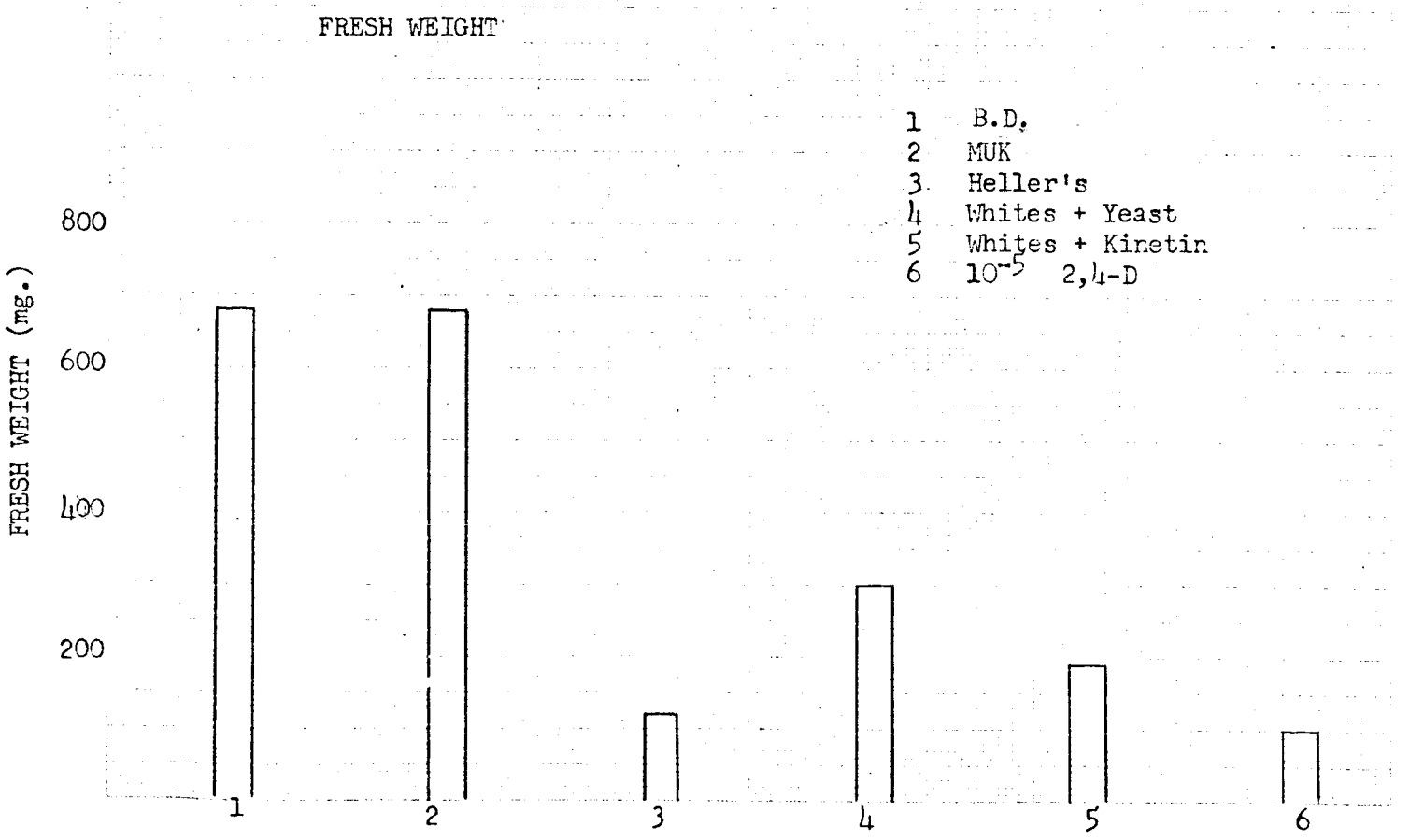

DRY WEIGHT
MEAN DRY WEIGHT ± S.E.(mg.)
15
10
5
1
2
3
4
5
6
WEEKS

Figure 5





grow. Kinetin caused a much more compact, but friable callus. Neither dextrose nor 2,4-D at 1 x 10^{-5} had any stimulatory effect on growth Root differentiation was extremely abundant in the modified Bonner & Devirian media. Cytological examination of the tissues revealed various stages of cell division. Analyses of the metaphase and anaphase stages of cell division in cells indicated a diploid condition. Growth data on corn tissue cultures are shown in Figs. 4-5.

Cultures of corn tissue are maintained both at LRL and at the University of Houston.

GLYCINE SOJA (Soybean)

NICOTIANA TABACUM (tobacco)

HELIANTHUS ANNUS (sunflower)

Tissue culture of tobacco, sunflower and soybean were tested for their growth in four different medium. The medium used were:

a. White's medium

b. Heller's medium

c. Murashige and Skoog's medium

d. Modified BD medium

2,4-dichlorophenoxy acetic acid (2,4D) α-naphthalene acetic acid (NAA) both at 1 x 10^{-6}M and coconut milk (10%) are used in the medium as growth factors. Data on F.W. and D.W. are shown in Figures 6-11.

It is evident that the modified BD medium is a very good medium for growth. All the different tissues (tobacco, sunflower and soybean) grown on this medium turned green after 3-6 days of culture. This indicates chlorophyll synthesis in the cells.

Figure 6

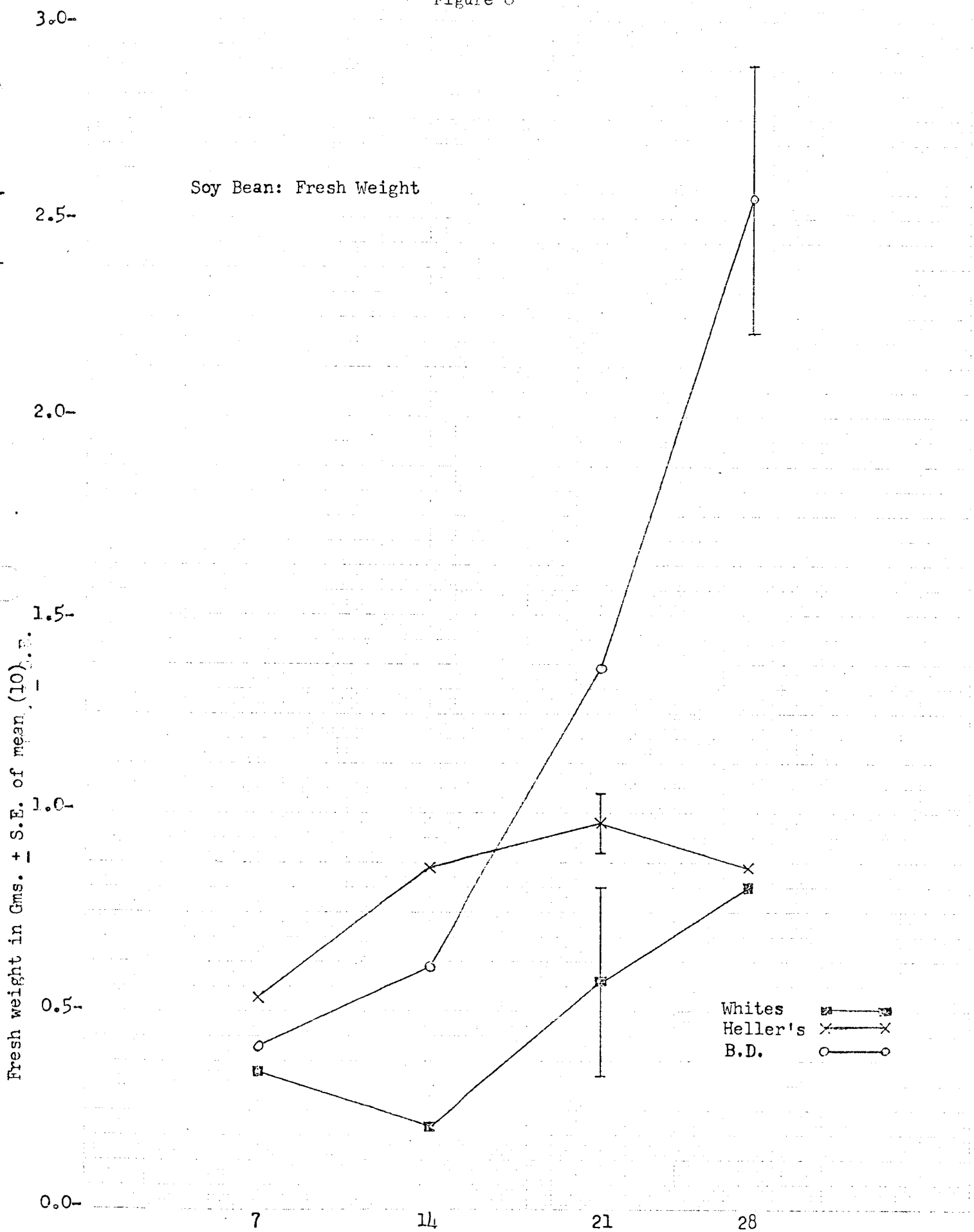

Soy Bean: Fresh Weight
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fresh weight in Gms. ± S.E. of mean (10)
7
14
21
28
Age in Days
Whites
Heller's
B.D.

Figure 7

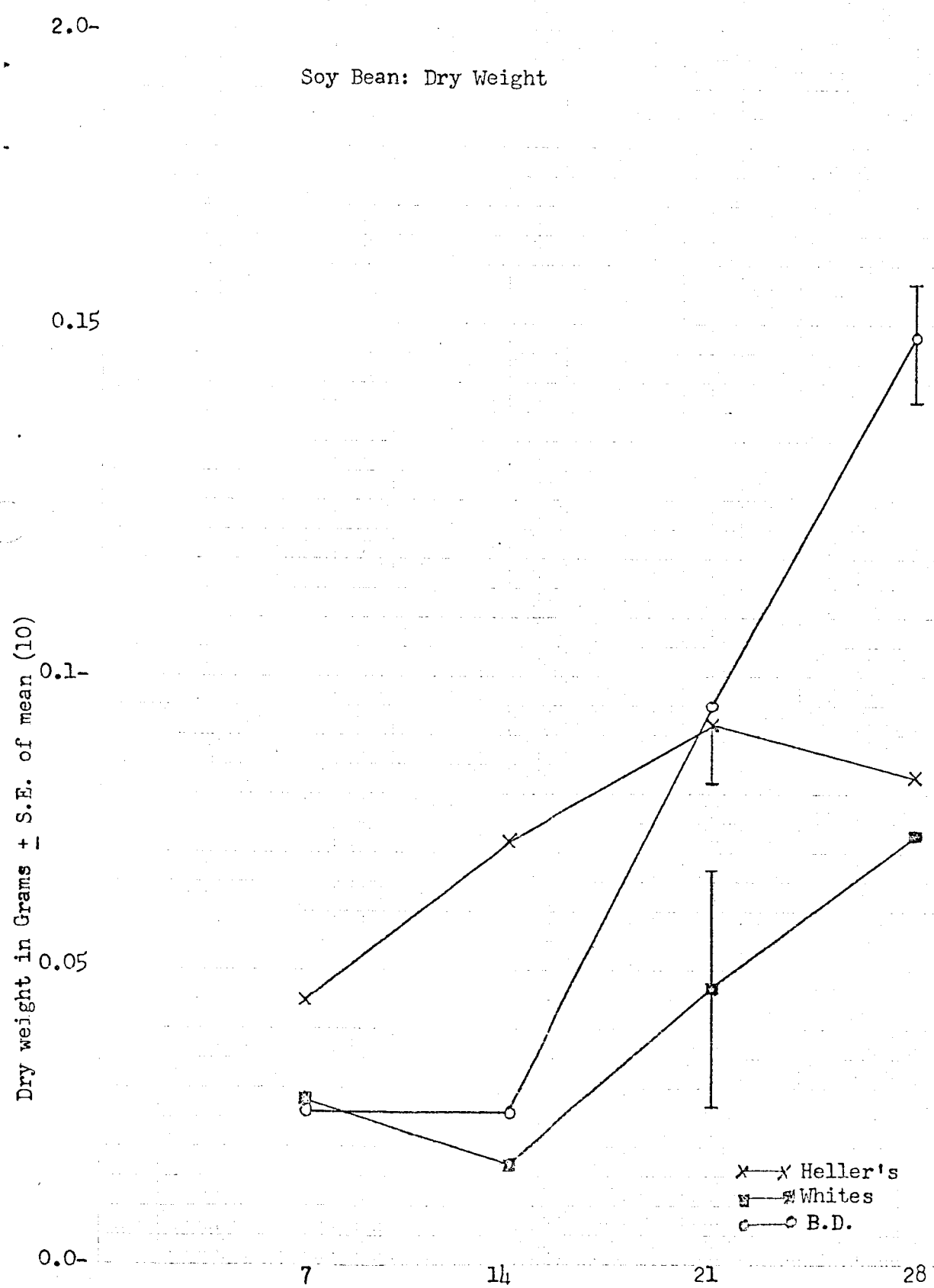

2.0-
Soy Bean: Dry Weight
0.15
0.1-
0.05
0.0-
Dry weight in Grams + S.E. of mean (10)
7
14
21
28
Age in Days
Heller's
Whites
B.D.

Figure 8

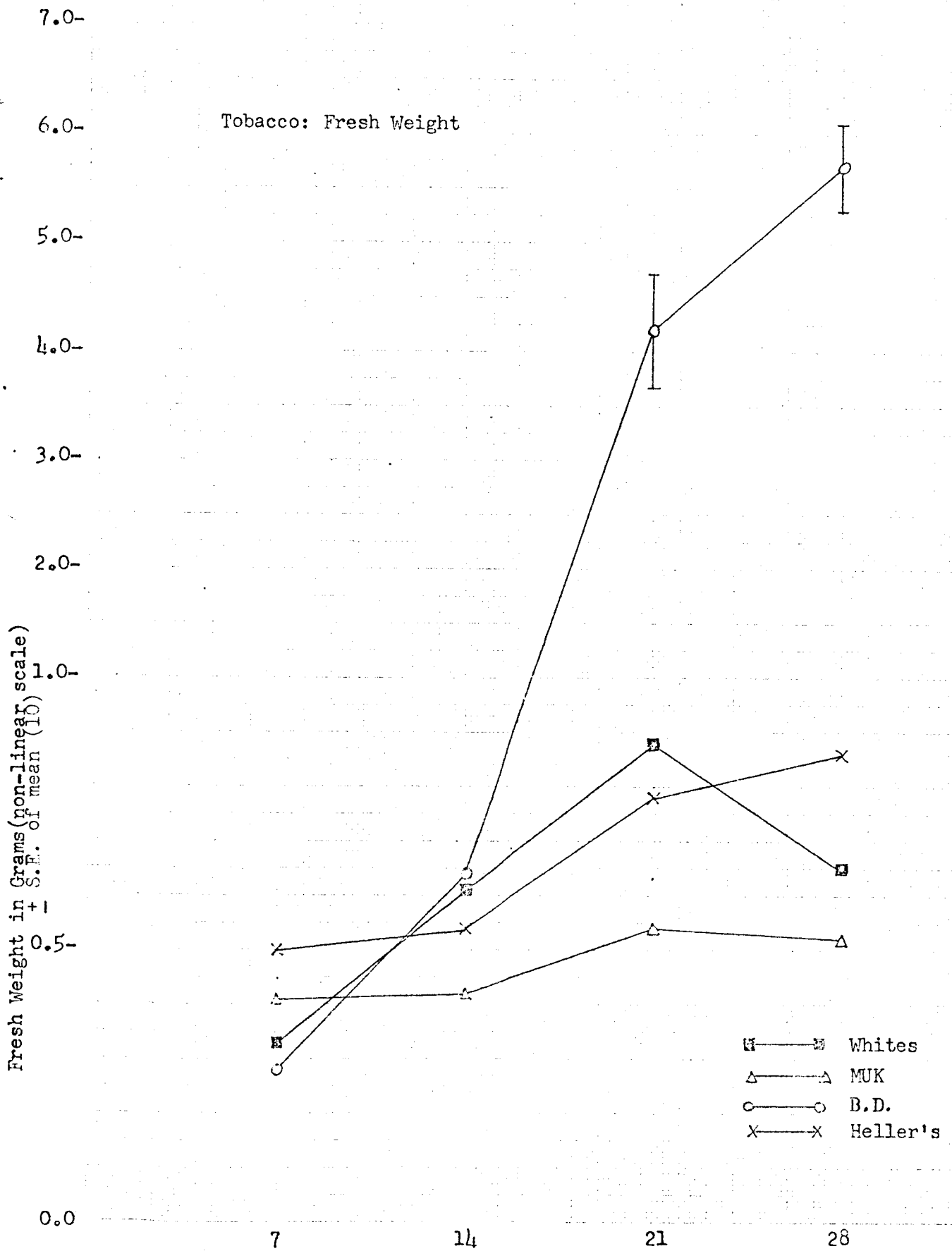

Tobacco: Fresh Weight
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.5
0.0
Fresh Weight in Grams (non-linear scale)
S.E. of mean (±1)
7
14
21
28
Age in Days
Whites
MUK
B.D.
Heller's

Figure 9

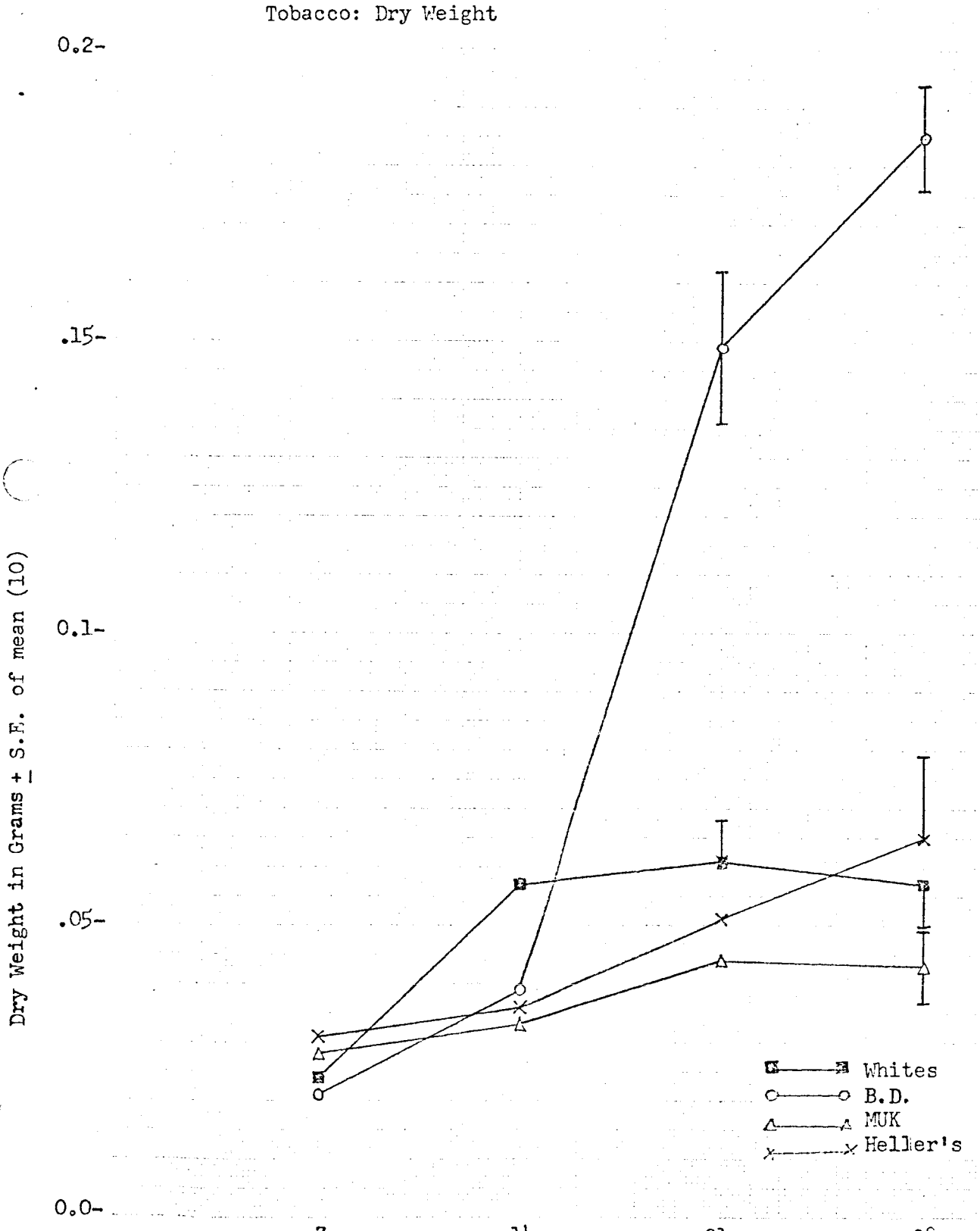

Tobacco: Dry Weight
0.2-
.15-
0.1-
.05-
0.0-
Dry Weight in Grams + S.E. of mean (10)
7
14
21
28
Age in Days
Whites
B.D.
MUK
Heller's

Figure 10

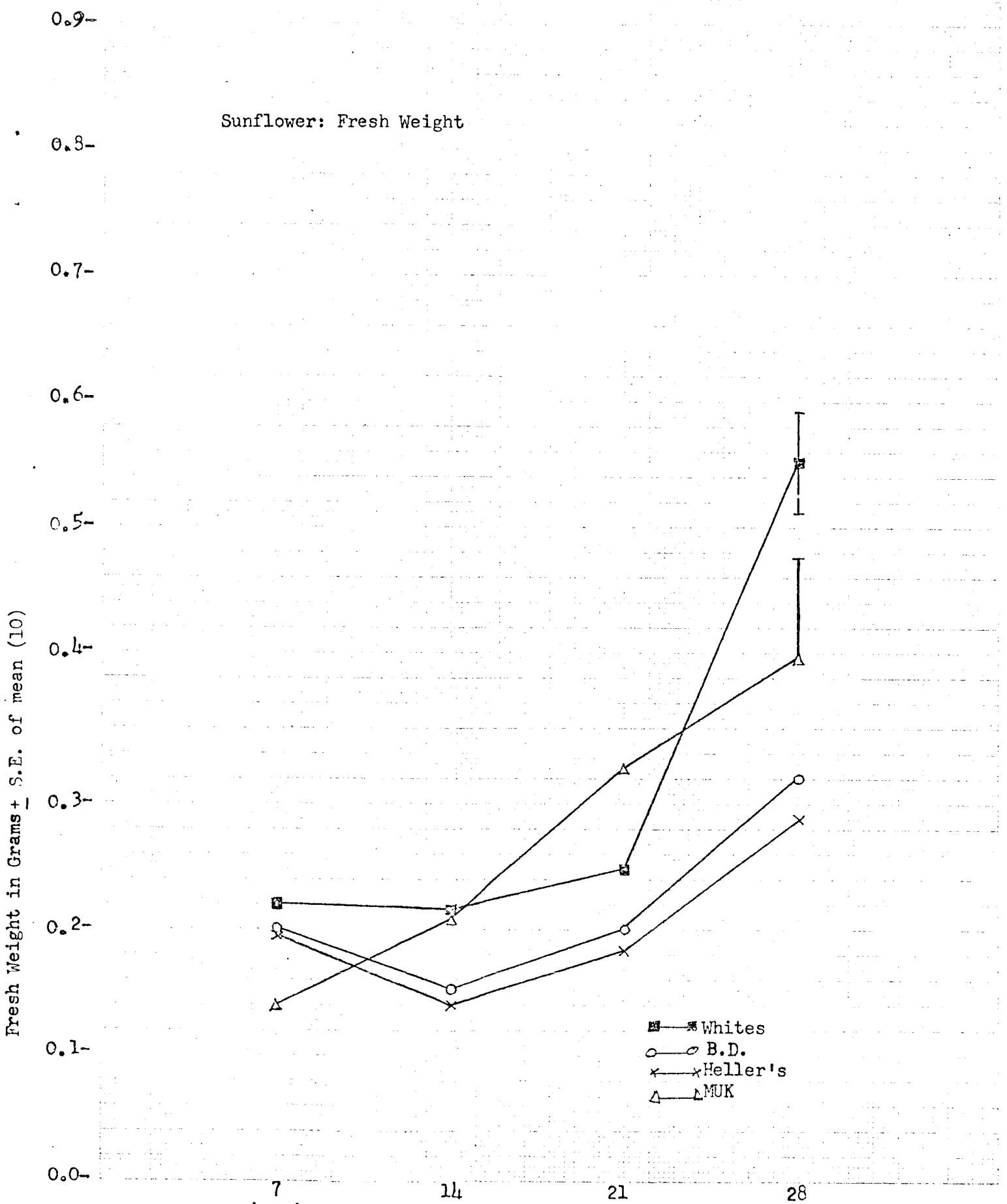
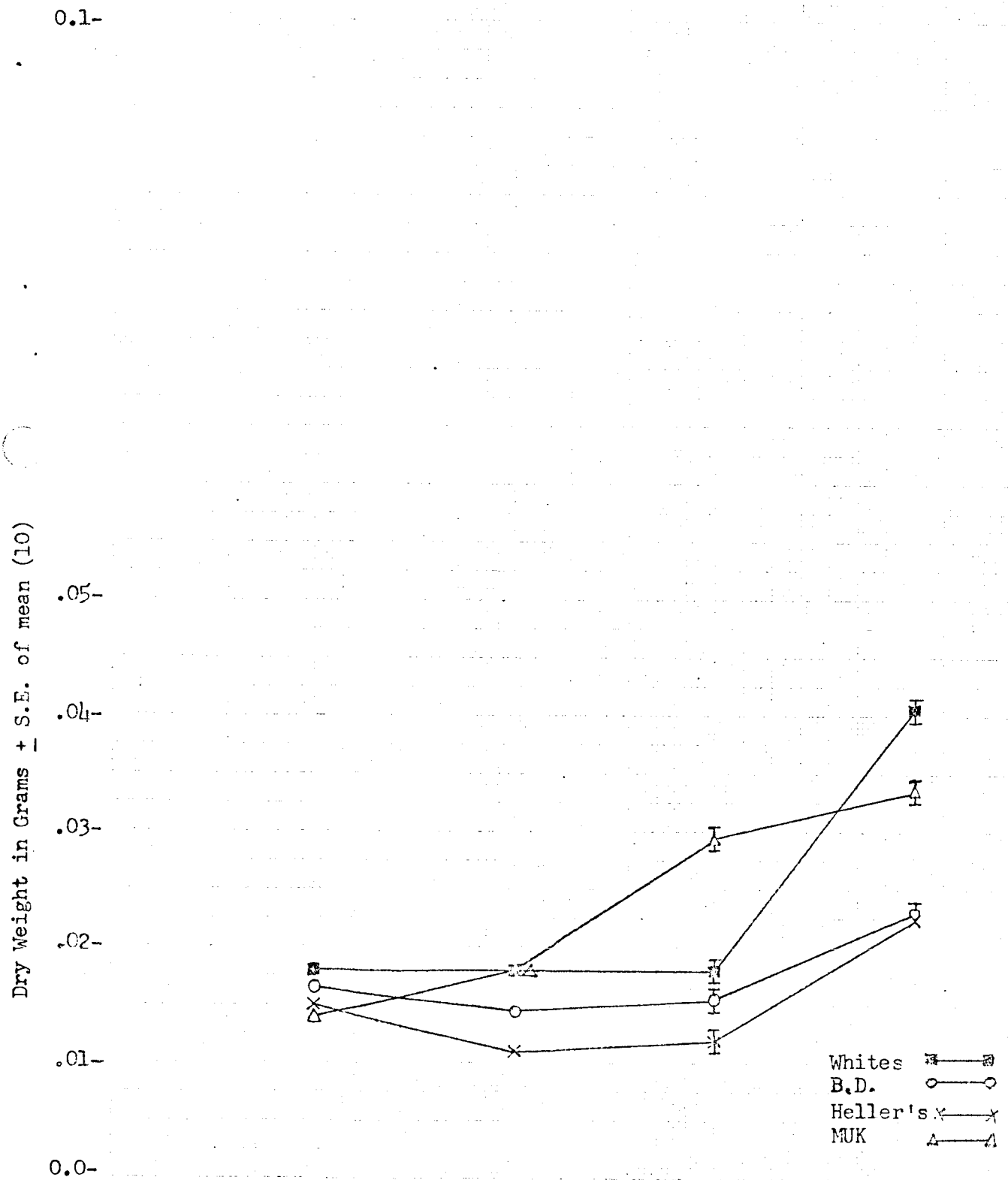

Sunflower: Fresh Weight
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Fresh Weight in Grams ± S.E. of mean (10)
7
14
21
28
Age in Days
Whites
B.D.
Heller's
MUK

Figure 11

Sunflower: Dry Weight



Tissue cultures have also been established on the following plants.

Pinus strobus (Pine)

Lycopersicon esculentum (Tomato)

Brassica oleracea (Cabbage)

Solanum tuberosum (Potato)

Vicia faba (Broad bean)

Haplopappus gracilis

The cultures are growing in either White's media or in modified BD medium supplemented with 1 x 10^{-6}M 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA) and coconut milk (10%) or yeast extract (1-3 gms/l). Since these cultures have only been established and since there is not enough of stock materials, no growth experiments have been carried out thus far.

Equipment: Equipments for developing the botanical systems were acquired and have been delivered to LRL. Few of the capital items are retained at the University of Houston because of the continuation of the work on a new contract. The following items have been obtained

Analytical balance
Toploading balance
Table model Centrifuge
pH meter
Multi-magnestir and hot plate
Magnestir
Stirring hot plate
Sterilizer for instruments
Rotary pressure or vacuum pump
Spectronic 20
Colony counter
Supermixer
Rinco Evaporator
Automatic pipetting machine for media preparation
Desiccator
Incubator bath
Soxhlet apparatus and extraction stand
Kjeldahl digestion apparatus
Vacuum oven
Pipette washer and accessories
Incubator shaker
Virtis homogenizer
Gyrorotatory shaker
Instruments
Disintegrator
Slide Projector
Test tube rotator

Training of NASA Personnel:

Mr. Walter Horne who was recruited as a NASA personnel was trained in this laboratory for the first 5-6 months when the LRL was getting ready for occupation. Mr. Horne was trained in the methods of maintaining biological systems under total asceptic conditions. He was instructed in many of the microbiological and tissue culture techniques. During the whole year, this laboratory was in communication with the botany laboratory at LRL and was collaborating towards developing botanical systems for the lunar mission.

This collaboration has resulted in submitting two papers at Biological Sciences meetings, one at the annual meetings of the Tissue Culture Association at Puerto Rico in June 1968 and the second one at the Annual meetings of the American Institute of Biological Sciences at Columbus, Ohio in September 1968. The abstracts of both the papers are included in the Appendix.

In conclusion, the contractor is pleased to report that the project has been successfully carried out. Most of the botanical systems which are needed for the Lunar Receiving Laboratory have been obtained and background information on some of these botanical systems has been gained. An active working laboratory has been established at LRL. A NASA personnel was trained in this laboratory and results derived from this collaboration have been reported in Annual Biological Sciences meetings. They will be published in scientific journals.

APPENDIX

MAINTENANCE OF ALGAL CULTURES

[Adopted in part from STARR, R. Amer. J. Bot. 51:1037-1038 (1964)]

The following paragraphs describe the general methods of cultivation.

ILLUMINATION--Illumination is provided by a series of slimline 40-w cool-white fluorescent tubes, a 16-hr light period alternating with an 8-hr dark period. For rapid multiplication in liquid cultures, an intensity of 400-500 ft-c is used. At this intensity, liquid cultures usually reach their optimum state for study within 1-2 weeks, depending on the species and the condition of the inoculum employed. Liquid soil-water cultures, which are used in the maintenance of many bacterially contaminated strains, are moved after their initial multiplication period to areas receiving light of 50-100 ft-c intensity. Too much light is often harmful to aging cultures.

Algal stocks on agar media are illuminated after transfer with an intensity of 250 ft-c for 6 or 7 days until good growth has been obtained. Such transfers are then moved to areas with an illumination level of 50-75 ft-c.

PERIODS OF TRANSFER--Cultures are transferred at different intervals depending on the species. More delicate, bacteria-free species on agar are kept in cotton-plugged tubes at 20 C, receiving 50-75 ft-c illumination on a 16-8-hr light-dark cycle. Such cultures are transferred routinely every 2 months. The more hardy bacteria-free species are kept in screw-cap tubes. After the initial illumination following transfer, as described in the section above, the cultures are allowed to mature for several weeks at 20 C, receiving light of an intensity of 50-75 ft-c on a 16-8-hr light-dark cycle. The cultures are then placed in a 10-C room with incandescent illumination of 50 ft-c intensity on a 6-18-hr light-dark cycle.

The caps are left slightly loosened to allow for some exchange of air. Under these conditions, transfers are made routinely every 6 months, although for many species 12 or 18 months would be sufficiently frequent.

Most non-bacteria-free cultures are maintained in soil-water media and, depending on the species, are transferred at 10-day, 1-,2-,3-,4-, or 6-month intervals. It cannot be emphasized too strongly that stock cultures should be kept in dim light after their initial growth period.

MEDIA--The cultures are maintained on one of the several media, formulae of which are given below. These media should be sterilized by autoclaving in the usual manner, except as otherwise indicated. The medium on which a culture is maintained is not necessarily the best medium for the production of populations with normal morphology. Cultures maintained on agar often exhibit normal morphology only after transfer to a liquid medium such as soil-water medium.

The following are compositions of some of the common culture media for algae.

(1) Bristol's Solution (as modified by H. C. Bold, Bull. Torrey Bot. Club 76:101-108, 1949)

Six stock solutions, 400 ml in volume, are employed. Each contain one of the following salts in the amounts listed:

$NaNO_3$	10.0 g
$CaCl_2$	1.0 g
$MgSO_4.7H_2O$	3.0 g
K_2HPO_4	3.0 g
KH_2PO_4	7.0 g
NaCl	1.0 g

10 ml of each stock solution are added to 940 ml of Pyrex-distilled water. To this is added a drop of 1.0% $FeCl_3$ solution. Two ml

of minor elements solution (Trelease and Trelease, American Jour. Bot. 22:520-542, 1935) may also be added. Solidify with 15 g of agar per liter, if desired.

(2) Cyanophycean Agar. For each 1000 ml of medium required:

KNO_3	5.0 g
K_2HPO_4	0.1 g
$MgSO_4.7H_2O$	0.05 g
Fe Ammonium Citrate	10 drops of 1% solution

The above should be added to 1000 ml of Pyrex-distilled water. Solidify with 15 g of agar.

(3) Euglena Medium. To 1000 ml of Pyrex-distilled water add:

Sodium acetate	1.0 g
Beef extract	1.0 g
Tryptone	2.0 g
Yeast extract	2.0 g
Calcium chloride	0.01 g

If desired, the above medium may be solidified by adding 15 g of agar.

(4) Malt Agar. For each 500 ml of medium required:

Pyrex-distilled water	500.0 ml
Malt extract	15.0 g
Agar	7.5 g

(5) NBB Agar. For each 500 ml of medium required:

Pyrex-distilled water	500.0 ml
Sodium acetate	0.25 g
Beef extract	0.25 g
Tryptone	0.25 g
Agar	7.5 g

It has been found that 50 ml of the supernatant from soil-water medium added to the above formula stimulates growth in certain

species.

(6) Porphyridium Agar. For each 500 ml of medium required:

Pyrex-distilled water	200.0 ml
Natural sea water	250.0 ml
Soil-water supernatant	50.0 ml
Yeast extract	0.5 g
Tryptone	0.5 g
Agar	7.5 g

(7) Proteose Agar. For each 1000 ml of medium required:

Bristol's solution ([1], above)	1000.0 ml
Proteose peptone	1.0 g
Agar	15.0 g

(8) Soil Extract Agar. For each 1000 ml of medium required:

Bristol's solution ([1], above)	960.0 ml
Soil-water supernatant	40.0 ml
Agar	15.0 g

(9) Soil-water Medium. (E. G. Pringsheim, Jour. Ecology 33:193-204, 1946) Variations of this medium are for non-sterile culture, especially for isolation purposes and for growing algae in order to secure "normal" growth forms. Success with soil-water media depends on the selection of a suitable garden soil. This soil should be of medium, but not too great, humus content and should not have been recently fertilized with commercial fertilizers. Soils with a high clay content are usually not the most suitable for most organisms.

A variety of soil-water media can be made using a basic formula to which are added certain additional materials. The basic soil-wate medium is made by placing ¼-½ in. of garden soil in the bottom of a

test tube and then adding Pyrex-distilled water until the tube is full. The tube is then plugged with cotton and steamed (not autoclaved) for 1 hr on 2 consecutive days. A few algae such as Spirogyra grow well in this basic medium. For most presumptively phototrophic algae which thrive in an alkaline medium, a small pinch of powdered $CaCO_3$ is placed in the bottom of the test tube before the soil and water are added.

10. Kratz and Meyer's modification of Chu;s media:

Am. J. Bot. 42:282 (1955)

	Stock	Final conc. mg/l
KNO_3	40 g/200 ml	4000 mg/l
K_2HPO_4	10 g/100 ml	1000 mg/l
$MgSO_4.7\ H_2O$	12.5 g/100 ml	250 mg/l
Na citrate	16.5 g/100 ml	165 mg/l
$Ca(NO_3)_2.4\ H_2O$	25 g/100 ml	25 mg/l
$Fe_2(SO_4)_3.6\ H_2O$	2 g/200 ml	4 mg/l

Add 1 ml of Hunter's A-5 microelements/liter of media; adjust pH = 6.8

A-5 microelements: (Am. J. Bot. 25: p. 322) (1938).

	Stock	Final conc. mg/l
H_3BO_3	2.86 g/l	2.86 mg/l
$MnCl_2.4\ H_2O$	1.81 g/l	1.81 mg/l
$ZnSO_4.7\ H_2O$	0.222 g/l	.222 mg/l
$CuSO_4.5\ H_2O$	0.079 g/l	.079 mg/l
MoO_3 (85%)	0.0177 g/l	.0177 mg/l

PROCEDURES FOR INITIATING CALLUS CULTURES OF

VARIOUS PLANT MATERIALS

(Use sterile tools throughout; re-sterilize after each use).

GAMETOPHYTES FROM FERN SPORES:

Shake spores in 15% Clorox soln. for 5-10 min., pour into sterile filter, rinse twice with sterile water, streak on medium.

TISSUE CULTURES:

FROM FLESHY ROOTS OR TUBERS: Scrub clean. Immerse in 15% Clorox for about 15 min. Transfer to sterile water for a few min. Drain in sterile paper. Pare away surface, cut into discs of ca. 5 mm. thickness. Punch out inocula and place on medium.

FROM STEMS:

HERBACEOUS (e.g. tobacco): Sterilize surface with alcohol swab. Peel off outer part, cut up inner part in sterile paper into suitable sized pieces and place on medium upside down.

WOODY (e.g. Parthenocissus): Wash with detergent soln. Immerse in 15% Clorox for about 10-15 minutes, and then into sterile water for a few minutes. Transfer into sterile papers, shave off killed tissue, cut into suitable pieces and place on medium upside down (base end up).

FROM SEEDS (embryos): First dissect seed to determine position of embryo. If internal (e.g. Pine) remove seed coats, sterilize in 15% Clorex 5-10 min. Soak in sterile water for a few hours. In sterile petri dish dissect out embryos, place all or parts on medium. In seeds with exposed embryos, do not remove seed coat but wash seeds

in detergent soln., sterilize in Clorox soln. and proceed as above. In all of the above procedures, the time and concentration of the sterilization in Clorox solution are critical; you want to kill the surface contaminants without killing the cells that are to be grown. If the plant materials are killed, then try lower concentration of Clorox and/or shorter time. If you get contaminants in cultures, try increasing these factors. Each new material is a new case.

TRANSFER OF ESTABLISHED CULTURES: When size of culture has increased several times, remove into sterile petri dish, cut into large pieces and place on fresh media.

STERILE MATERIALS: (for autoclave can use pressure cooker at 15 lbs.)

PAPER ROLLS: Towels stacked flat, then loosely rolled and wrapped. Autoclave at 250^{o} and 15 lbs. for 1 hr., or in 350^{o} oven for at least one hour.

PETRI PLATES: Wrapped and sterilized as above.

MEDIA: Autoclave in covered vessels for 20-30 min. Allow pressure to come down slowly to prevent boiling over.

WATER: Same as medium.

POLYETHYLENE CAPS: Store in alcohol (70%). Drain in sterile paper before use.

TOOLS: (Forceps, Scalpels, cork borers, etc.). Soak working end in alcohol. Carefully flame off excess alcohol and place between sterile papers to cool before use.

The point to be emphasized throughout is that of sterile technique. The actual handling of tissues to be cultures should be done in as sterile environment as possible. Working surfaces should be wiped off with alcohol periodically when working. Expose cultures and open media to open air as briefly as possible.

CULTURE MEDIA AND SOLUTIONS:

CULTURES	MINERAL SALTS	SUCROSE	AGAR	OTHER ADDITIVES OR COMMENTS
Fern gametophytes	Knop's solution or Knudson's soln. Ferric citrate	¼%	1%	Acidify with dil HCl to pH5.5 Slant media for spores
Plant tissues	Knop's solution or White's solution or modified salt soln. depending on particular tissue + Minor elements + Fe	2-3%	1%	Auxin generally 2,4-D or NAA at 1×10^{-6} M (1 ppm) B-vitamins 10-15% coconut milk when needed
Plants as seedlings starting from seeds	"	2-3%	1%	Omit the auxin 2,4-D or NAA Add IAA or Gibberellic acid concentrations o 1-10 ppm
	"	2-3%	1%	
Plant embryos (excised)	Simple salt solution	2%	0.8%	
Tobacco tissue	Modified Murashige & Skoog nutrient medium	3%	0.8%	2,4-D, IAA or kinetin at concentration ranges of 1×10^{-6} M

It is seen that all the above media contain minor and major elements (details on next page), ferric citrate or ferric chloride, sucrose and agar. pH is generally adjusted to 5.5 before addition of sugar. The use of coconut milk is avoided in order to have a completely defined media. In the case of some tissues, it will be difficult to maintain the callus growth without coconut milk or other indefined growth factor like yeast extract, malt extract, etc.

Auxins which are generally used are:
IAA = Indoleacetic acid
NAA = -naphthalene acetic acid
2,4-D = 2.4-dichlorophenoxyacetic acid
GA = Gibberellic acid
Kin.= Kinetin

MEDIA FOR
PLANT TISSUE CULTURE

Stock solutions are generally made using distilled deionized water. Absolute care should be taken to see that the salts dissolve thoroughly. Stock solutions can be maintained in ice-box for 1-2 months.

1. KNUDSON"S SOLUTION (x4, dilute 1:3)--Major elements:

		Final concentration
$Ca(NO_3)_2.4\ H_2O$	8.0 gm	500 mg/liter
$(NH_4)\ SO_4$	4.0	250 mg/liter
$MgSO_4.7\ H_2O$	2.0	125 mg/liter
Water to make	4 liters	

Minor elements

$MnCl_2.4\ H_2O$	2500 mg	
H_3BO_3	2000	
$ZnSO_4.7\ H_2O$	50	
$CoCl_2.6\ H_2O$	30	
$CuCl_2.2\ H_2O$	15	
$Na_2MoO_4.2\ H_2O$	25	
Water to make	1000 ml	Use 0.5 ml per liter of final medium

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2. KNOP'S SOLUTION (x 4. dilute 1:1)

Major elements

$Ca(NO_3)_2.4\ N_2O$	4.0 gm
KNO_3	1.0
$MgSO_4.7\ H_2O$	1.0
KH_2PO_4	1.0
Water to make	4 liters

Minor elements

Use same composition as above, i.e. 0.5 ml per liter

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3. WHITE'S MEDIUM (1954)

	Stock	Final
$Ca(NO_3)_2$	2.0 gm/l	200 mg/l
Na_2SO_4	2.0	200
KNO_3	0.8	80
KCl	0.65	65
NaH_2PO_4	0.165	16.5
$MgSO_4$	36.0	360
$MnSO_4$	0.45	4.5
$ZnSO_4$	0.15	1.5
H_3BO_3	0.15	1.5
Kl	0.075	0.75
$CuSO_4$	0.002	0.02
Na_2MoO_4	0.021	0.21
$Fe_2(SO_4)_3$	0.25 gm/l	2.5 mg/l
Glycine	0.3	3.0
Thiamine	0.01	0.1
Pyridoxine	0.01	0.1
Nicotinic acid	0.05	0.5

White's solution is generally used to start new materials in tissue cultures. This composition has been modified and adjusted to suit particular requirements of the investigator and at times called Modified White's Solution.

Since tobacco tissues are favorite materials for a number of tissue culture investigations, various culture medium have been used in the last few years. Since 1963, Murashige and Skoog (1963) have developed a salt solution in which tobacco tissue cultures grow and maintain a very active growth rate. Various modifications have been made again to suit particular needs.

4. MURASHIGE AND SKOOG MEDIUM (MUK medium)

	Final concentration
NH_4NO_3	1650 mg/l
KNO_3	1900
$CaCl_2.2\ H_2O$	440
$MgSO_4.7\ H_2O$	370
KH_2PO_4	170
$FeCl_3.6\ H_2O$	1.5
H_3BO_3	6.2
$MnSO_4.4\ H_2O$	22.3
$ZnSO_4.4\ H_2O$	8.6
Kl	0.83
$Na_2MoO_4.2\ H_2O$	0.25
$CuSO_4.5\ H_2O$	0.025
$CoCl_2.6\ H_2O$	0.025
Glycine	3.0
Niacin	0.5
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Myo-inosital	10.0

5. MODIFIED BONNER-DEVIRIAN MEDIUM (modified BD medium)

			Stock	Final	
Soln.	A	NH_4NO_3	82.5 g/l	1650 mg/l	use 20 ml/l
		KNO_3	95.0 g/l	1900	
"	B	$CoCl_2.2\ H_2O$	88.0 g/l	440	" 5
"	C	$MgSO_4.7\ H_2O$	74.0 g/l	370	" 5
		KH_2PO_4	34.0 g/l	170	
"	D	Na_2EDTA	7.45 g/l	35	" 5
		$FeSO_4$	5.57 g/l	28	
"	E	$MnSO_4\ H_2O$	900 mg/200 ml	4.5	
		$ZnSO_4.7\ H_2O$	300	1.5	
		H_3BO_3	300	1.5	
		$CuSO_4.5\ H_2O$	8	0.04	
		$Na_2MoO_4.2\ H_2O$	50	0.25	
		$CoCl_3.6\ H_2O$	1	0.005	
		$AlCl_3$	0.6	0.003	
		$CaCl_2.6\ H_2O$	0.6	0.003	
		KI	0.2	0.001	
		Glycine		3.0	
		Niacin		0.5	
		Thiamine HCl		HC 0.1	
		Pyridoxine HCl		0.1	
		Myo-inosital		10.0	

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Volume 55 July, 1968 Number 6, Part 2

AMERICAN JOURNAL OF BOTANY

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PROGRAM WITH ABSTRACTS OF PAPERS TO BE PRESENTED AT THE MEETINGS OF THE BOTANICAL SOCIETY OF AMERICA AND CERTAIN AFFILIATED GROUPS AT THE OHIO STATE UNIVERSITY

Columbus

SEPTEMBER 2–6, 1968

Abstracts of papers to be presented to the Phycological Section will be published in the Journal of Phycology

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embryo were excised from the ovule. The zygotic embryo was discarded, whereas the nucellus was planted on a medium supplemented with either a combination of adenine sulfate (25 mg/liter), NAA (0.5 mg/liter) and orange juice (5%) or malt extract (500 mg/liter). In about 4 weeks some of the nucellar explants developed embryos. Histologic preparations confirmed the nucellar origin of adventive embryos. When transferred to a fresh medium of the same composition, the embryos developed into seedlings with well developed roots and a few leaves. Subsequently, the seedlings were transferred to vermiculite + Hoagland's nutrient solution where they continued their growth.

9:15 **Blakely, L. M., Helen K. Jennings, and Beryl B. Turner.** California State Polytechnic College, Pomona—GENERAL BEHAVIOR OF CELL AND ORGAN CULTURES OF HAPLOPAPPUS RAVENII—Cultured cells of stem origin of *H. ravenii* (a close relative of *H. gracilis* but with $n = 4$) show promising characteristics for studies on the physiology of growth of higher plant cells. Now 10 months in culture, the cells display a rapid growth rate, do not differentiate organs, produce dense cell suspensions in liquid medium, develop prominent chloroplasts in the light, and when plated on an agar medium 40% or more of 1–5-celled units grow into multi-cellular colonies within 7 days. Studies in progress are aimed at further improving the growth rate, cell separation, and plating efficiency through manipulation of the nutrient medium and the environment. These cells have displayed the behavior described above for the past 5 months, and their nuclei are now aneuploid about the 4*n* number. In early passages when most cells had 2*n* or 4*n* nuclei, there was little cell separation and adventitious roots were produced prolifically. Just before roots ceased being produced by the main cell line, a subline was started using root tips or root segments as transfer inocula. The nuclei of root-tip cells by that time were all tetraploid. Placed in a medium containing auxin (routinely used in maintaining the root subline), these very slender roots branch freely, and after a lag period the branches elongate at a rate of about 1 cm per week. Large mats of roots are thus produced during a monthly subculture period. Factors influencing root development in this subline are being investigated.

9:30 **Davidson, D., and W. R. Sharp.** Case Western Reserve University, Cleveland—CALLUSES AND TERATOMAS IN COLCHICINE-TREATED PLANTS—Shoot apices of cabbage and tobacco plants were treated with lanolin paste containing 2% colchicine. Several changes were noted in the treated plants. Those dealing with the long term changes in particular will be considered. In treated cabbages growing shoots had formed within 7–10 days. After 3 months calluses appeared on these plants. The calluses were first formed close to the site of treatment, but they have continued to form on growing shoots, where they grow out of leaf scars. Some calluses gave rise to root-like outgrowths, but these never exceeded 5 mm in length. Six months after the original colchicine treatment, shoots developed from calluses; some plants have produced several shoots. These growth changes are similar to those found in genetic tumors. The induction of abnormal growth cannot be attributed solely to the colchicine treatment since similar changes have occurred in cabbage plants that were decapitated when other plants were treated with colchicine. Tobacco (*N. glauca-langsdorffii*, amphidiploid plants) has also been treated with colchicine-lanolin paste. Teratomatas and lateral branches have formed on treated plants. The teratomatas have formed both at the apex of the treated shoot and below the first leaf. It is well known that this particular abnormality would develop on these hybrid plants after flowering, or can be induced by irradiating them. The fact that they are developing on young plants with 5–8 fully formed leaves that are also giving rise to lateral branches indicates that the change induced by colchicine is operative at a site unrelated to that involved in the induction of polyploid cells. These results suggest that a change occurs in the ability of shoot apices to control lateral bud formation. Such a change may be related to an effect on levels of growth factors. This possibility will be discussed.

9:45 **Norstog, K.** Northern Illinois University, DeKalb—PHYSICAL FACTORS IN RELATION TO DEVELOPMENT OF CULTURED BARLEY EMBRYOS—Barley embryos excised when slightly less than 20% of their full-term length (i.e., about 5 mm) responded variously to culture on media differing only in sugar concentration, or when grown under differing intensities of light, or at differing temperatures, or combinations of these. Precocious germination—the assumption of a phase in which cellular elongation is a predominant growth expression—occurred at low temperature (20–25 C) in dark or low light intensity (20 ft-c), and was more characteristic of embryos cultured on low-sugar media (0.1–0.15 M sucrose). Embryological growth—a phase in which cellular division is predominant and cellular enlargement is minimal—occurred at higher temperature (25–30 C) and at higher light intensities (100–200 ft-c), and with media having high sucrose levels (0.3–0.35 M). Intermediate responses were noted when any or all of these factors (sugar concentration, temperature, light) were moderate (0.2 M, 25 C, 20–50 ft-c). Continuous culture at a temperature of 35 C was lethal. The inhibition of precocious germination by high light, high sugar, or high temperatures was reversed both in light- and dark-grown culture by addition of 1–5 mg/liter gibberellic acid to the medium. Precocious germination in vitro is not caused exclusively by low sugar concentration as formerly supposed, since it may be suppressed by culture at high light and temperature (200 ft-c, 30 C). It appears to be related to the action of gibberellin or gibberellin-like substances.

10:15 **Venketeswaran, S., and W. H. Horne.** University of Houston, and Brown and Root-Northrop, Lunar Receiving Laboratory, Houston, Texas—ISOLATION AND DEVELOPMENT OF CORN-TISSUE CULTURE FROM DIPLOID ROOTS—Adventitious root explants of field corn (*Zea mays* var. A-204) have been isolated and maintained as tissue culture in a salt-sucrose medium containing 1×10^{-6} M of both 2,4-dichlorophenoxyacetic acid and α-napthalene acetic acid. This medium was usually supplemented with 10% coconut milk. The explants initiated masses of proliferating cells resulting in swollen nodules at regular intervals along the original root explant. Isolation of these proliferations resulted in the formation of large masses of undifferentiated, homogeneous cells with a relatively high growth rate, These occasionally gave rise to further adventitious rootlets which repeatedly proliferated into masses of callus cells. Histological examination indicated rapid cell divisions in the periphery of the swollen nodules and in the callus cells. Electronmicrographs of the cells revealed normal cellular details and organelles. Among these were numerous double-membraned organelles containing darkly stained storage products of possible starch or lipid composition. Detailed investigations are in progress and will be presented.

10:30 **Bajaj, Y. P. S., A. W. Saettler, and M. W. Adams.** Michigan State University, East Lansing, and Crops Research Division, U. S. D. A.—THE EFFECT OF IONIZING AND NON-IONIZING RADIATIONS ON BEAN TISSUE CULTURES—Tissue cultures of *Phaseolus vulgaris* L. ('Common Light Red Kidney' and 'Redkote') have been raised in agar-solidified and in liquid media containing White's minerals, sucrose (3%), 2,4-D (1 ppm) + kinetin (0.1 ppm) and yeast extract (1,000 ppm), and the effect of gamma and ultraviolet radiations on their growth has been studied. Three-day-old suspension cultures containing free cells, aggregates of cells, and small callus

Schedule and Abstracts of Papers Presented at the Nineteenth Annual Meeting of the Tissue Culture Association

SAN JUAN, PUERTO RICO
JUNE 10 - JUNE 13, 1968
THE PUERTO RICO SHERATON HOTEL

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id formation in Cathar-
cultures.[1] Betty Patter-
son and David P. Carew, College of Pharmacy, University of Iowa, Iowa City.

Suspension cultures of *Catharanthus roseus* grown in a modified Wood and Braun medium have been continuously subcultured for three years and growth values were determined. The tissues grow well in media with and without NAA and kinetin.

Liquid medium as well as callus tissue harvested from agar medium was subjected to alkaloid analysis. Prior to extraction, the liquid medium was concentrated under reduced pressure and the callus from agar medium was lyophilized. The liquid concentrate and the lyophilized tissue were separately extracted with selected solvents to yield eight fractions. The general alkaloid distribution in each fraction was studied using two-dimensional thin-layer chromatography. Fractions which contained several alkaloids were then subjected to column chromatography using deactivated alumina. Elution was effected with a series of solvents (benzene, chloroform and methanol) and 70 5.0 ml fractions were collected. Thin-layer chromatographic analysis of each 5.0 ml fraction was carried out using three different solvent systems. Through comparisons with known compounds and on the basis of data in the literature, the following alkaloids were found to be present in both the liquid medium and the tissue: akuammicine, lochneridine, lochrovine, cavincidine, sitsirikine, dihydrositsirikine. Mitraphylline was found only in the liquid medium and pericalline was defected only in the tissue.

[1] Supported in part by grant HE-05290 from the National Institutes of Health.

53. **Studies on tissue cultures of higher plants for exposures to lunar material.**[1] S. Venketeswaran and Walter H. Horne, University of Houston, and Brown & Root-Northrop, Lunar Receiving Laboratory, Houston, Texas.

Tissue cultures of several economically important plants (corn, rice, soybean, sunflower, tobacco, tomato, cabbage, potato, citron, pine, and sugarcane) have been established from hypocotyl and root explants and are being maintained for exposure to lunar material after the first manned flight to the lunar surface. Initial growth parameters of the tissue cultures, germination of the various seeds and growth of the seedlings under sterile, controlled conditions have been obtained for comparison with the lunar material challenge. The tissue cultures maintain an active growth in White's medium supplemented with 1 x 10^{-6} M of both 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA) and 10% coconut milk. Of particular importance is a strain of corn tissue which has been derived from the lateral root axis. Proliferation occurred from regions of the lateral root resulting in swollen nodules of tissues at regular intervals along the root axis. Cytological analyses show that the tissues have thus far maintained a diploid condition. Preliminary electron microscope observations on the cells reveal a parietal layer of cytoplasm with normal structure of nucleus and other organelles. The growth characteristics of the different tissues under specific culture conditions at the Lunar Receiving Laboratory and analyses of the response of these tissues under simulated runs are in progress and will be discussed.

[1] Supported by Contract (order) No. NAS 9-6822 from the National Aeronautics and Space Administration.

54. **Cytological characteristics of ten-year-old rye grass endosperm tissue cultures.** Knut Norstog, Wendell E. Wall, and Gary P. Howland, Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois; Department of Botany, University of North Carolina at Raleigh, North Carolina; Biology Department, Yale University, New Haven, Connecticut.

A clone of perennial rye grass (*Lolium perenne* L.) endosperm tissue culture, isolated in 1955, was studied after ten years of periodic subculturing. Cytological, morphological, and growth-rate data were obtained, and demonstrate that the tissue has remained essentially triploid (3n = 21). On the basis of chromosome counts in 392 cells, the prevalent numbers were 21 (76 counts) and 22 (77 counts). The range of chromosome numbers was 18–50. Surprisingly only two cells were 18, four cells were 19, and ten cells were 20 in chromosome number. A karyotype analysis suggests that the tissue is statistically triploid but not morphologically triploid in a strict sense, since seven triplets of homologs are no longer in existence. Dicentric chromosomes and fragmented chromosomes are present. The growth rate of the tissue has diminished by about 30% in ten years, and its ability to synthesize starch is reduced.

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