Gravity and the Stability of the Differentiated State of Plant Somatic Embryos (PEMBSIS)

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IML-2 carried an experiment to evaluate cell division and somatic embryo progression in space. Test materials comprised embryogenic cells of daylily (*Hemerocallis*), a system which has been well-characterized in this laboratory from a developmental cell biology, physiology and chromosome structure perspective. A modest test (sample size = two "petri dishes") on an earlier spaceflight (STS-57, J Mission) using the same system suggests that we are justified in using embryogenic cells of daylily as a model system for the study of space effects on cell division and development and chromosome structure in *in vitro* cultured cells in space. Cells with double nuclei and breaks were found in space samples but not in controls. Embryogenic initials are distributed on a membrane, placed on activated charcoal impregnated filter paper which is placed on a semi-solid nutrient medium held in place by a waffle-like support in a metal Petri-dish. Twelve dishes were flown in the IML-2 mission Six dishes were designed to allow fixation in space. Half (3) were fixed on flight day 12. The other dishes served as controls. Flight samples showed chromosomal damage whereas ground controls did not. Furthermore, epidermal development of flight samples was considerably poorer than ground controls. Materials fixed in space were adequate for study but quality of fixation achieved in flight was poorer than that obtained post-flight. Nevertheless, the results show that perturbation is real and not an artifact of re-entry or post-flight adaptation etc. No 1-G controls were run in Space. Hence we speak not of the "effect of μ-G on developing somatic embryos" but of the "effect of space flight factors".
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OBJECTIVE

The immediate and narrowly focussed question being asked is :-
Are mitosis and chromosome behavior in developing plant cells modified by the space environment?

BACKGROUND

Research in microgravity provides the vehicle to pursue basic plant science research activities across a broad spectrum of species. It allows concentration on those near- and intermediate-term science questions and needs which are so crucial to the development of a

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reliable data base.

Over the years, many investigators have addressed these needs from the perspective of thematic research. The long range planning activities have focussed especially on how one can use the unique charactaristics of the space environment, especially microgravity, to increase our understanding of life and its processes, and to understand how gravity affects, and has shaped, life on Earth.

Various panels and working groups have asked: How can we use gravity, primarily microgravity, to answer questions about plant development or physiology? What are the experiments needed to understand the effects of gravity on plants?

The themes may be consolidated under three broad categories. (1) to understand the role of gravity in the control of development at the whole plant, organ and cellular levels; (2) to understand the role of gravity in regulating metabolic and cellular processes in plants; (3) to understand the mechanism(s) of gravity sensing and the transduction of this information. In each of these categories, the use of variable G to manipulate and understand the thresholds is an integral part of the experimentation.

Some attention has been given over the years to such questions as:--

1) What systems, organs, organelles, substances, developmental stages, etc. exhibit sensitivity to gravity?

2) What are the systems, organs, organelles, substances, developmental stages, etc. that sense gravity?

3) What are the hypotheses that should be proposed to understand this sensitivity?

4) What hypotheses concerning plant development and physiology can be tested by manipulating gravity in the microgravity range?

5) What research is required to test these hypotheses?

6) What ground-based studies should be initiated to improve and refine the proposed hypotheses?

7) What special spaceflight conditions, facilities and equipment are required to conduct the research to test these hypotheses?

How cells manage without gravity and how they change in the absence of gravity are basic questions which only prolonged life on a Space station will enable us to answer. We know from the experience acquired so far from investigations carried out on various kinds of Space vehicles and stations, profound physiological effects can and often do occur. We need to know more of the basic biochemistry and biophysics both of cells and of whole organisms in conditions of reduced gravity. The unique environment of Space affords plant scientists an unusual opportunity to carry out experiments in microgravity but some major challenges

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must be faced before this can be done with confidence. Various laboratory activities that are routine on Earth, take on special significance and offer problems that need imaginative resolution before even a relatively simple experiment can be reliably executed on a Space Station. For example, scientists might wish to investigate whether adaptive or other changes which have occurred in the environment of Space are retained after return to Earth-normal conditions.

Gravitational plant biologists have, of course, given considerable thought to the kinds of changes in response that might result from exposing plants to μ-G in the Space environment. There are a number of situations in which orientation with respect to 1-G are already known to alter the response. Gravitropism of organs, especially roots and shoots is well known, has been extensively studied and the role of specialized cells, or statocytes is known to be central to this phenomenon. Gravitaxis has been much less extensively studied. Similarly, gravimorphogenesis, such as the formation of reaction wood, the breaking of buds and its relationship to apical dominance, or the determination of position of organ emergence or even the determination of the type of organ or cell formed have been very inadequately studied.

In addition to these categories of inquiry, one can expect as yet unidentified situations where the "Earth normal" (1-G) condition is required--that is, μ-G might be expected to eliminate a response, i.e. have a qualitative effect. These are essentially unidentified or unknown at present; indeed virtually all aspects of plant physiology and development are potential candidates. It will not be a trivial matter to disclose these situations and to validate them using rigorous scientific methods.

Last but not least are instances where μ-G would be expected to alter a response, i.e. have a quantitative effect. For example, the extent and nature of lignification might be expected to be different in μ-G because a system undergoing lignification would be less G-"loaded". Also in this category, and minimally understood at present, is the area of investigation which asks the question "What effect does μ-G have on cells that are not specialized for G-sensing?" And, "What effect does μ-G have on developmental, physiological and reproductive processes?" etc. (See Table 1 on the following page).

In my own work carried out a Stony Brook, pure science issues have been dealt with, but the thematic approach referred to above has also provided for an optimal progression of experiments aimed at defining and isolating various operational problems related to intermediate-term experiments on plant systems.

The more immediate work derives its impetus from two kinds of space flight findings made by us. The first is that when embryogenic somatic cells of carrot were exposed to a μ-G environment in small petri dishes in a Soviet Biosatellite for nearly 3 weeks, the broad events of asexual embryogenesis occurred but the progression from one developmental stage to another was slowed down, specifically at the point where advanced embryonic forms with a distinct root and shoot pole was to have developed. Access, somewhat later, to roots of seedlings grown on US Shuttle missions as "mid-deck experiments" by scientists doing space investigations with objectives that did not directly require their using all available roots (and hence the "extra" roots might otherwise have been discarded), enabled me as a "guest investigator" to study the chromosomal status of roots of oats and sunflower. Evaluation or monitoring of karyological status was used as an indicator of general cell "health" and status of the plants.

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Table 1 Some expected changes in plant response as a result of exposure to μ-G in the Space environment. (From Krikorian, in press).

A. Situations in which orientation with respect to 1-G is already known to alter the response
gravitropism
organs, especially roots and shoots
specialized single cells, e.g. statocytes
gravitaxis
gravimorphogenesis
formation of reaction wood
bud break/apical dominance
determination of position of organ emergence
determination of the type of organ or cell formed
epinasty
B. Unidentified situations where "Earth normal" (1-G) is required--that is, μ-G might be expected to eliminate a response, i.e. have a qualitative effect
unknown at present, "everything" is a potential candidate
C. Situations where μ-G would be expected to alter a response, i.e. have a quantitative effect
lignification?
unknown at present, e.g. What effect does μ-G have on cells that are not specialized for G-sensing?
What effect does μ-G have on developmental, physiological and reproductive processes? etc.

Karyological disturbances including chromosome breakage, deletions and translocations were encountered, along with much-reduced levels of cell division in oats and sunflower but not in mung bean (*Vigna radiata*) where, however, level of cell division in root tips was still much reduced. These findings were interpreted as cell level indications of potentially substantive problems to be overcome before growth of plants in space could be reliably carried out.

Procedures were developed for a mid-deck locker experiment which would yield root tips for subsequent cytological examination, especially for metaphase chromosome aberration assay. Roots of a chromosomally well-characterized liliaceous monocotyledon (*Hemerocallis*, a garden daylily, 2n = 22), and a composite dicotyledon, *Haplopappus gracilis*, were initiated under aseptic conditions from rooted shoots which were trimmed on Earth less than one day prior to lift-off, i.e. at the latest possible moment. The two species were grown in NASA's Plant Growth Unit (PGU) equipped with an air exchange system (AES), a device that permitted mid-deck cabin air to be passed through a HEPA filter and then through the individual growth chambers (so-called plant growth chambers, PGCs) which in turn were inserted into the PGU. We have developed daylily as a model system capable of going from embryogenic cells to fully differentiated, clonal plants which can survive in the field, and have worked on it extensively. Along with the daylily, *Haplopappus gracilis* was

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chosen because it, too, has well-characterized chromosomes--the lowest number among higher plants, 2n = 4 --and we have done a lot of work on it *in vitro*. Both systems offered additional advantages at the so-called operational level because plant materials can be well-managed, for instance they can be cloned, manipulated biologically in ways not possible with seedlings and grown in the low light--an environment which is characteristic of the PGU. Three distinct but clonal *Haplopappus* population types were used: (1) capitulum (flowering head)-derived tissue culture plantlets; (2) two lines of aseptic seedling clones initiated from pre-sterilized, germinated seeds, and (3) apex-derived tissue culture plantlets. These clonal *Haplopappus* populations were selected because of differences in gross morphology, growth rates, branching pattern, frequency of floral bud initiation and mode of root production (e.g. adventitious vs. regenerative direct). Since it has not yet been possible to achieve fixation in flight with materials from the plant growth chambers of the PGU, fixation (with colchicine for metaphase arrest) for chromosome studies was perforce performed so as to study cells in their first division on Earth. Control fixations on both flight and "ground" materials were also performed with direct fixations as well to eliminate any potential role of an enhanced sensitivity response of μ-G-exposed root meristems to colchicine--i.e. to avoid any sort of artifact. Briefly, the results showed that even though shoot growth and root production occurred, flight samples generally performed worse than ground controls and there were significant negative effects on chromosomes in both species. Dosimetry data, as in other US Shuttle missions, indicated that levels were low (order of 5 mrad/day) and thus well below those expected to have any effect on chromosome structure.

Roots of haplopappus clones that were characteristically of the most mature phenotype (capitulum-origin) showed lower levels of damage when contrasted with those that were more immature, i.e. from trimmed (rootless at the initiation of the space experiment) seedling clones. The daylily showed chromosomal damage both in root tips and basal meristems (leaf base areas). The number of roots produced by daylily in the flight because of the flight duration was fewer than those produced by *Haplopappus* but we could look at daylily leaf base meristems to supplement root tip data (basal meristems are found primarily in the monocots). There were statistically significant ($p < 0.01$) differences between each of the five flight PGCs. An especially interesting feature of that experiment was that each population of plantlets in PGC #3 showed much reduced levels of chromosomal damage. We have no way of knowing why this was so. In theory, PGC 3 should have been a replicate of PGC 4 and PGC 5. Because of hardware idiosyncrasies that need not be gone into here, we have hypothesized that there was an increased air flow in PGC 3 and that might have acted as counter-measure to the aberrations encountered. Specifically, the more drying brought about by increase water evaporation, the less damage. Because there is no way to simulate on Earth the μ-G environment for longer than the briefest of periods, it would be essential to carry out a microgravity experiment in space to resolve that issue using a rather complicated, impractical protocol that one cannot envision being possible in these early stages of co-operative experimentation on MIR or the mid-deck of the Space Shuttle or even the Space lab.

I concluded some time ago that as a result of our flight experience, which has been both satisfying and frustrating, that experiments designed to be done in space in NASA's Plant Growth Unit, an apparatus originally designed to grow small seedlings for what is termed

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"experiment-specific" (that is, not generic) purposes, are difficult to manage aseptically, a condition which I maintain is absolutely necessary to get unequivocal data, and is very labor-intensive to use even for ground based work. Moreover, some important environmental parameters such as air flow and water delivery, are, or at least were under the original design, impossible to measure, monitor, maintain and or even document with precision. This is recognized by NASA, of course, and the plan is to modify and incrementally improve the apparatus or to design new ones so as to minimize these problems for future experiments. But this will require considerable testing and validation before any new hardware is rendered and validated as reliable. Even then, one is still confronted with the problem of studying cellular mechanisms with an intact plant. The advantage of cell cultures is apparent. But this is dependent on demonstrating that cultures can be reliably dealt with in the context of a space experiment. This is not a trivial matter [5].

Totipotent cells cultures are able to provide all the advantages of a developing plant system. Cells taken from the various organs of the higher plant body can be nurtured and grown on nutrient media so as to elicit morphogenetic capacity and to stimulate cell divisions in such as way as to get structures that can make organized growing regions of shoot, root and even to yield an entire embryo, a so-called somatic embryo. Thus, one is not only working at the cellular level but also at the level of cells that are morphogenetically competent and able to express this competence provided the conditions are permissive.

We are trying to use aseptically cultured, embryogenic cells of the garden daylily (*Hemerocallis*) as a model for the study of development in higher plants both on earth and in the space environment (microgravity). The use of cells which can develop and pass through all stages of embryogenesis rather than seedlings or fully mature plants is preferred since the material is clonal, doesn't take up much room and it is considerably easier to achieve the kind of environmental control over the system necesary to separate direct from indirect effects of microgravity.

It will be apparent that the questions posed are broad and fundamental.

The immediate and narrowly focussed question being asked is :-

Are mitosis and chromosome behavior in developing plant cells modified by the space environment?

METHODS

Initiation and maintenance of embryogenic suspensions

Embryogenic suspensions of a diploid daylily clone (*Hemerocallis* cv. "Autumn Blaze") were derived from vegetative shoot apex using methods that have been outlined elsewhere [6), 7), 11)]. The process of generating the suspension originated in this case with an apex excised from a greenhouse grown plant on 3 November 1988, and placed in 50 ml of liquid medium in a 250 ml erlenmeyer flask and rotated on a horizontal shaker at ca 100 rpm. The initial nutrient medium consisted of Murashige and Skoog salts and "vitamins" [13)], 100 μM iron EDTA [14)], supplemented with coconut water 10 % v/v and 9 μM 2,4-D [2 mg/l], 560 μM inositol, and 87.6 mM sucrose adjusted to pH 4.5 with 1 N

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KOH solution. Routine medium sterilization was by autoclaving for 20 minutes at 126 °C and 103 kPa. Cultures were grown in darkness at 22 °C and 40 % RH and the callus culture was reduced to a finely sievable suspension by 15 December 1989. Thereafter, cultures were maintained in the same medium except that kinetin 4.6 μM [1 mg/l] was used in place of coconut water and pH was lowered to 4.5. Serial subcultures were made at 3 week intervals in nipple culture flasks [8] using material sieved through a number 60 screen (230 micrometer pore size) (Selector Tissue Sieves, E-C Apparatus, St. Petersburg, FL).

Preparation of embryogenic cells and inoculation onto semi-solid medium in Flight-Qualified Petri Dish-type Chambers

A highly responsive inoculum capable of yielding somatic embryos was obtained by passing the suspension sequentially through number 40, 60, 80, 100, 200 and 400 mesh sieves (380, 230, 190, 140, 74 and 37 μm pore size respectively) and washed with DS 5 a medium [17] . Settled cells of the final 200 to 400 fraction were distributed by pipetting 0.20 ml aliquots onto each of four 3 cm long washed, single-thickness Spectrapor membrane strips (Fisher Scientific, Cat. No. 8 667B, size. 64 INF), placed on a 8.2 cm diameter circle of activated charcoal-impregnated filter paper (# 508, Schleicher and Schuell, Keene, NH). The charcoal paper rested on 75 ml of DS 5 a nutrient medium made semi-solid with 1.2 % w/v washed National Formulary grade agar [16] .

NASDA's Spaceflight-Qualified "Plant Cell Culture Chamber"

The embryogenic daylily cells prepared and inoculated in this way were allowed to incubate in an anodyzed aluminum petri-dish type chamber made available by the National Aeronautics and Space Development Agency of Japan (NASDA). The bottom of the dish is outfitted with a honeycomb insert (secured in place by means of a spring) to prevent dislodging of the semi-solid substrate. There is Velcro on the dish by which attachment may be achieved. An opening which permits aseptic ventilation via a 25 mm diameter TF-450 Gelman filter (HT-450 Gelman No. 66221 actually used) is located in the lid. An O-ring lubricated with silicone lubricant ensures a good seal.

Plant Cell Fixation Chambers were utilized as well. These chambers allow fixative to be introduced into the dish.

A synchronous ground control was carried out in identical dishes; additional synchronous ground controls were performed using 100 x 15 mm presterilized plastic petri dishes containing a final volume of approximately 45 ml of medium. These plastic petri dishes were wrapped with a thin strip of Parafilm to secure the lid.

Post-flight or Analysis after Recovery

Chromosome analysis is carried out by examining materials fixed with the mitotic arresting agent (a so-called cytostatic) colchicine. These analyses rely mainly on squashes of randomly selected embryos pulled off their support surfaces in the petri dishes. Structure of chromosomes is determined.

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1) Chromosome Methods

Relevant fractions of unorganized cell suspensions were collected by sieving through a filtering pan, rinsing with the DS 5 a basal medium (no sugar but with 560 μM inositol and "vitamins"), and resuspending in 0.2 % w/v colchicine (Calbiochem-Behring) solution in $CaCl_2.2H_2O$ in water and allowing it to remain for about 3.5 hours at 4 °C in darkness in preparation for metaphase analysis. Somatic embryos at various stages of development and plantlets generated from them but maintained *in vitro* were handled in the same way. Samples were rinsed with BMS prior to fixation in cold absolute alcohol-glacial acetic acid (3:1, v/v) for a total of 3 changes and allowed to stand for 48-72 hours. Hydrolysis was done by 1 N HCl at 58-60 °C for 15-20 minutes. Staining was done by a modified Feulgen procedure for 1-2 hours, soaked in tap water from 15 minutes to overnight in a refrigerator to remove excess stain and squashed in acetocarmine [10]. Photographs of chromosomes were made on a Zeiss Photoscope using Kodak Technical Pan film (TP 135). Counts of cells in division were carried out at using a 40 X lens and photographed under oil immersion. Five to ten representative cells at metaphase with well spread and favorably condensed chromosomes were selected for measurement and comparison within and between cultures. All measurements were made with a compass on 3340 X glossy photographic enlargements of the chromosomes that were mounted on millimeter graph paper. Long arm, short arm, total chromosome length and centromeric index measurements were used to construct idiograms. Relative length was determined by dividing the length of the total haploid genome times 100. The terminology used to describe chromosome morphology and type follows that of Levan et al. (1964). Controls consisted of karyotyped, diploid normal embryogenic suspensions and plantlets derived from them using the same regeneration methods.

2) Assessment of Embryogenic Response

Somatic embryo production was qualitatively scored at the end of the experiment after petri dish inoculation and at weekly intervals in the controls up to 4 weeks. A positive score for somatic embryogenesis consisted of recognition of various stages of somatic embryogenesis characteristic for daylily (cf. refs. 17 and 11). Germination of somatic embryos and rearing them to rooted plantlets *in vitro* was done by transferring randomly selected embryos of different stages of development to Schenk and Hildebrandt (1972) medium (hereafter referred to as BSH).

Some Details of the Spaceflight Mission

Embryogenic cells of a diploid daylily clone (*Hemerocallis* cv. Autumn Blaze) grown in suspension culture on the basal salts of Murashige and Skoog supplemented with 3 % sucrose and 10 % v/v coconut water and 2 mg/l 2,4-D were sieved so as to obtain a 200-400 (74 to 37 micrometer size range) fraction. Test dishes were inoculated at the Stony Brook laboratory and taken in a reduced temperature cooler (12 °C) by commercial airline to Kennedy Space Center where they were maintained in a darkened incubator at 22 °C. "Late access loading" enabled the dishes, appropriately packed and cushioned, to be installed into a

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mid-deck locker of the Shuttle orbiter some 13 hours before scheduled lift-off.

The IML-2 experiment was launched at 12:43 p.m. on July 8, 1994 (EDT); landing was 6:38 a.m. on July 23, 1994 (EDT), thus making it 14 days 17 hours and 55 minutes. The shuttle was STS-65 (Columbia). Orbital altitude was 300 km (circular orbit) at 28.5 degrees inclination.

The experiment was largely passive but also involved introduction of fixative into three of the dishes with buffered glutaraldehyde. That means that only half of the dishes that could accommodate fixation in space were fixed (three out of six); three out of the six that could have been fixed were not fixed. Six dishes that could not accommodate fixation remained fixed.

After recovery, materials were flown by commercial carrier to New York, taken to the Stony Brook laboratory and processed. The materials were photographed and fixed within nine hours of landing and we thus were able to catch cells well before their first division cycle could be completed on Earth.

FLIGHT RESULTS

Adherence to lift-off and recovery schedules made this experiment relatively convenient to carry out.

We were able to demonstrate by means of this experiment that the embryogenic daylily system can provide a very useful model for the study of plant growth and development in space.

A major potential problem entailing the use of plant cells for plant space biology experimentation was obviated in this experiment. It is well known that plant cells grown in vitro offer special challenges to investigators due to their inherent nature. For example, the common procedure utilized by many investigators interested in animal and microbial cell is to purchase cell lines from commercial sources. Plant cell investigators do not have this option for a number of reasons and hence need always to initiate and grow their cells in their own laboratories. When plant cells are grown on a large scale, as for space biology experimentation, this creates logistical problems. These logistical problems involve need in many instances for highly specialized growing equipment and facilities, the long lead time needed to generate, select and maintain cell lines with known characteristics makes it impractical to set up operations at a place like Hangar L, Kennedy Space Center. Moreover, the very nature of plant cell cultures makes it necessary to have a certain amount of redundancy as to availability of cells in a particular physiological or morphological state for a given timeframe of experimentation. Given the realities of space flight and the potential uncertainty of time of lift-off etc. a host of potential difficulties emerge in connection with plant cell use. For the present, these are best dealt with by growing cells in the investigator's laboratory and setting up the experiment in the laboratory and then by one means or another, transporting the cells in a state of quiescence or arrest to the place of lift-off [5].

The IML-2 experiment gave us an opportunity to test this concept of preparing material in the P.I.'s laboratory and transporting it to Kennedy Space Center. Similarly, it deemed

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useful to assess the possibility of processing material at Stony Brook after recovery, rather than at the landing or recovery site. This has the potential of enabling the investigator to be able to work in familiar surrounding with the option of being able to make on-site, even last minute decisions as to how to handle materials. Again, it would be very impractical to prepare for all eventualities at the recovery site, especially if the primary landing site was changed to the "secondary landing site". In these days of limited budgets, it makes it far more practical to have personnel accessible who may in fact not be "paid for" by the flight project. In short there are a number of valid reasons for seeking to work with plant cells in a form of "remote" scientific activities.

The experiment was a success from the perspective of being able to carry out the cell preparation and somatic embryo evaluation after recovery at Stony Brook. Thus the concept of carrying out a successful experiment via a "remote" science principal investigator was validated.

Fixation in Space using the Plant Cell Fixation Chambers was achieved. While the subjective evaluation of the fixation carried out in space indicates that the fixation is not as good as that carried out on earth using more sophisticated methods, it shows that fixation in space can be achieved with some degree of confidence provided adequate precautions are taken. This precautionary proviso is given here since we did not have deuate and timely access to the plant cell fixation chamber (i.e. the fixable metal petri dishes) with enough lead time to work out all details of the fixation. Moreover, there is considerable concern over the utilization of glutaraldehyde-based fixatives in the space environment for a variety of valid safety concerns.

Results of the examination of material fixed in space using a single glutaraldehyde fixative demonstrated that the cytological changes and chromosomal aberrations encountered by us in previous spaceflight missions are real. That is they are not apparently not due to various situations that might accompany re-entry. Since they are present in both ground fixed and space fixed material, then it seems justified that we "save ourselves" the immediate "bother" of becoming pre-occupied with space-based fixation. The results are essentially the same, although the earth-based fixations are more elegant, easier to interpret, and hence much easier to work with. The outcome of this is that one will have to resolve issues of cytological "elegance" and temper it against expediency and practicality as to what is more readily feasible in the space environment.

1) The responses included a substantial number of binucleate cells in flight samples. These binucleate cells are not uniformly present throughout individual somatic embryos that were scored but were present among normal, uninucleate cells. The ground control samples are uniformly uninucleate.

2) Since our methodology for karyotype analysis involves treatment of cells and somatic embryos with colchicine, we have been cautious to eliminate the possibility of an increased sensitivity of the flight-exposed cells to colchicine, leading to a doubling of the nuclei. We can effectively eliminate this interpretation since both flight and ground controls were fixed as well **without colchicine** and the presence of double nuclei in flight samples holds, whereas no ground controls ever showed double nuclei.

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3) Serial sampling and examination of flight samples after recovery, beyond what we refer to as sampling 1 (i.e. the first sampling after recovery) indicates that the number of binucleate cells diminish in some samples but do not disappear in any. This could be interpreted as adaptation and it may be that plant cells adapt to space.

4) We are rearing some embryos into plantlets of both flight and controls to see the outcome. This information is too early to report despite the "final" nature of this report. Daylily simply takes several years to rear to maturity.

5) In addition to the condition of double nuclei, aberrations in chromosome structure such as breaks, micronuclei and microchromosomes have been encountered as well. See Table 2 for detailed results.

Table 2 Summary of scorings of daylily (lumped without regard to embryo stage from 542,086 unambiguous division figures in 231 individual somatic embryos.
Note: some features may be present more than once in a given cell.

	Flight Fixed (Direct) [%]	Flight (Unfixed in Space but Direct Fixed on Earth) [%]	Ground Control (Direct Fixation)	Ground Control (Colchicine Fixation)
dividing cells	1.4	1.8	6.1	6.5
micronuclei	6.1	4.2	-	-
double nuclei	4.3	4.7	-	-
bridges	2.0	1.6	-	-
breaks	3.6	4.2	-	-
severe fractures	1.8	3.0	-	-
"ring" chromosomes	0.3	1.0	-	-

DISCUSSION

There are many as yet unanswered questions about plant cell development in microgravity. We know, of course, that all development entails an orderly progression of cellular events both in terms of time and geometry (dimensional space). Work done by us on Soviet Kosmos 782 and 1129 biosatellites using morphogenetically competent carrot cells which could undergo somatic embryo formation showed that while the broad events of asexual embryogenesis could occur, the transition from one stage to another was slowed down. The system used for the Kosmos experiments involved the generation of embryogenic cells, their induction on Earth to produce proembryos, and their subsequent

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exposure to space conditions to evaluate their capability of expressing their capacity to form later stage somatic embryos. The fidelity of the developmental pathway of cells to proembryos to later stages of embryogeny could thus be scored. Similarly, the broad temporal aspects could be traced. The experimental design had its limitations however--especially insofar as the temporal aspects were concerned. There was no onboard fixation; neither was a 1 G centrifuge available on the 1129 flight. Moreover, we had at that time no capability of performing karyotype analysis on carrot since the chromosomes are so small.

Table 3 shows that the same kind of results were obtained with daylily during the IML-2 flight. The percentage of embryogenic initials that advanced in space was less than that achieved on earth. Furthermore, the level of epidermal fidelity was much reduced in the flight samples. This suggests that the Kosmos results were, in fact, more reliable than might have been supposed. (I never felt completely comfortable with the Kosmos results since there was a substantial lag between preparation of cells and lift-off, and because we had minimal control over the disposition of the materials once they left our hands in Moscow.

Table 3 Summary of scorings of daylily somatic embryos developed in space (with regard to embryo stage and development of epidermal "finish") from 1,000 individual somatic embryos. Figure in brackets following values for "stage" indicate the level of development of the epidermal "finish" (i.e. integrity of the epidermal layer). Note that space grown embryos do not have as good a finish as those that are generated on earth. This means that the cells that make up the epidermis have not stabilized and are perturbed by some spaceflight-associated factor.

	Flight Fixed (Direct) [%];	Flight (Unfixed in Space but Direct Fixed on Earth) [%]	Ground Control (Direct Fixation)	Ground Control (Colchicine Fixation)
Stage 1	221 [15]	165 [13]	101 [37]	97 [42]
Stage 2	327 [22]	401 [20]	226 [53]	200 [55]
Stage 3	400 [48]	403 [43]	331 [85]	359 [87]
Stage 4	52 [53]	31 [51]	342 [93]	344 [90]

Since that period when our knowledge of plant development in space was virtually non-existent, other pieces of information have emerged. A particularly interesting feature of plant growth in space that we have encountered involves varying manifestations of perturbation(s) to the cell division process. Modifications in cell division, detected at the level of the chromosomes--ranging from slight to extreme--have been found in a number of instances. But whereas most species examined have shown the perturbations, all test specimens have not. This has raised questions in our mind as to whether mechanisms of information transfer or signalling through the various parts of the cell occurs normally in space. We have wondered, for instance, whether the chromosomal or cell division changes we have observed are the outcome of indirect effects that are manifested only if a certain

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constellation of parameters or conditions are fulfilled. These conditions could, for instance, be brought about by subtle interactions between cell surfaces or among any of the special features of the space environment such as altered metabolic gas levels or modified water relations. We have also wondered whether small aseptically cultured, developing cell units might respond differently from "whole plants" or if there are thresholds of sensitivity in terms of both G level and time, i.e. is there a finite period of cell division disturbance prior to an adaption to the low G or space environment? Obviously, cells must be able to divide normally and to partition their genetic information (via chromosomes) with high fidelity if sustained and efficient plant growth is to occur in space. In short, plant cells need to get their signals straight and to process them accurately.

Alterations in the karyology of somatic embryos developed in space clearly occurred in the IML-2 mission somatic embryos.

It is becoming increasingly apparent that the results are real. Centrifuge 1-G controls will have to be run to ascertain whether the effects are due to μ-G or due to spaceflight related factors.

The following questions emerge:-

Q: Are any of the chromosome effects noted attributable to radiation damage?
A: Radiation dosimetry data obtained in the past by radiation specialists on our behalf from passive radiation detectors (thermoluminescent detectors) flown in the actual flight package have been interpreted as representing levels "typical" of the level expected for shuttle flights of the duration and orbital characteristics of the mission flown. The same is true of other missions using plants studied by Krikorian and his group. The levels encountered (which are on the order of 10 mrad tissue absorbed dose or dose rate of 10 mrad/day) would not seem sufficient to account for the degree of chromosomal damage observed.

Specific information from the IML-2 data are not yet available to us in digested form.

Q: Might there be synergism between microgravity and radiation?
A: It has been suggested by some investigators that there may be synergism. However, unless there is major synergism between microgravity and rather low levels of radiation, it seems unlikely that radiation alone is responsible for the chromosome effects encountered. The only way to be certain though is to use shielding, and to re-run the experiments.

Q: Have any plants flown in space and showing chromosomal and cell division perturbations and damage been grown to maturity after recovery?
A: The daylily material from the IML-2 flight needs to be "grown out" and reared to maturity. But material from earlier flight shows that there is a correlation between what the plants "look like" (so-called phenotype) after they are grown out and the extent of initial damage experienced at the somatic embryo stages.

Q: Is it not true that cells with damaged chromosomes will die and be deleted from the "pool" of cells that divide (and hence one might raise the question whether this is all

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interesting but academic and hence really a moot point)?
A: Yes, indeed cells showing extreme damage will die. Gross chromosome aberrations themselves are probably unimportant since the majority are removed by so-called diplontic selection (that is during meiosis in sexual reproduction). But there is very strong evidence that most agents or conditions that increase chromosome aberrations also increase frequency of point mutations. I would doubt seriously that this does not occur in flight-grown specimens though we have not yet analyzed for that in a detailed way since the species we have been concerned with so far are not the best ones to pursue that point. All the indications are that a mutational event(s) occur in the space material.

Q: What is the cause of the observed imperfections in the cell division process?
A: At this juncture we are not certain of the cause or causes of the anomalies although we are quite certain that they are real and not artifacts of analysis. Hormonal perturbations, failure or imperfections in processing of various "communicative" signals between cells, and modifications to plant cell water relations are all candidate "causes". The level of cytological perturbations encountered have varied considerably from flight to flight, ranging to severe to quite modest. This suggests that subtle, and apparently un- or under-appreciated factors have not been taken fully into account from flight experiment to flight experiment. A major variable that has existed in the experimentation thus far is level of development, species and duration of spaceflight exposure. Steps are being taken to test these systematically and with increased appreciation of TOTAL control.

The "bottom line" is that plants can tolerate and even accumulate chromosome aberrations; less severe chromosome aberrations or modifications to the genome such as variation in number of chromosomes, translocations and inversions etc. are totally tolerated and passed on.

The question arises as to whether the changes observed are representative of a sort of space adaptation. The changes may represent modifications to enable survival. Alternatively, the basis of the damage in the first place may be due to DNA repair mechanisms are adversely affected in space.

[So far, Krikorian refers to the changes are being brought about by space flight factors, not microgravity. Centrifuge controls need to be done in space and thus far this has not been possible!]

CONCLUSIONS

1) The IML-2 experiment, while a modest experiment, shows real promise in enabling us to extend our earlier findings on atypical nuclear and chromosome behavior in space-grown plant materials.

2) We believe we can demonstrate convincingly through tests of this sort that cultured, embryogenic cells can serve as models for the study of development in higher plants in

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space environments and in microgravity. This means it will be considerably easier to achieve the kind of control over the system necessary to separate direct from indirect effects and thus enable us to move towards resolving the still many outstanding questions. We hope that some of these can be resolved in future flights

The level and fidelity of division achievable in higher plant cells on Earth, even when they are randomly oriented and the effects of G are neutralized by means of rotating clinostats, is not sustained in their counterparts during or immediately after Space flight. Karyological changes in root cells of Shuttle grown oats, mung bean and sunflower, daylily (*Hemerocallis*) and *Haplopappus gracilis* have been encountered in seedlings and aseptic tissue cultured propagules. Significant chromosomal damage (between 3 and 30 %) that alters subsequent cell division potential perforce leads to reconfigured cell division centers. Less extreme karyotype changes (deletions and translocations) modify genetic make-up and continued development. Declining cell division and chromosomal damage and mitotic disturbances are not explainable on the basis of radiation dosimetry data. The apparent malfunctioning of spindles and phragmoplasts (future cell wall sites) in these same cells suggests a perturbation in the interdependence of differentiated microtubule configurations in mitosis. Clearly, Space flight can significantly affect level of cell division and chromosome partitioning in plant cells. Whether this is a manifestation of altered signal processing or is a "stress" response to reduced gravity remains to be tested. Since plant cells undergo virtually no movement during development and the plane of cell division and subsequent enlargement determine morphology, modification in either should have important consequences for plant development during protracted exposure to Space.

Clearly more experimentation is in order to resolve outstanding issues.

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