Novel Applications of Plant Tissue Culture and Conventional Breeding Techniques to Space Biology Research

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Abstract

In the minds of many, plant cell and tissue culture may now be viewed as a well-developed technology. It has been all but reduced to a tool to solve practical problems and contribute to plant improvement. While this is so in some instances, it is definitely not so in trying to utilize and integrate plant cell and tissue culture into the projected needs of Space biology programs. This is not merely because of the challenges posed by the unusual physical environment of Space such as microgravity, or the constraints posed by Spaceflight protocols but is due to our inadequate knowledge of the factors that control plant growth and development, including systems as they grow in vitro. The premature (and in many cases unrealistically optimistic) initial assessments of what is really known about what happens in aseptic culture belies the many fundamental developmental and physiological questions that still need to be answered before plant "tissue culture technology" can be meaningfully integrated into sophisticated programs and exploited. Nevertheless, opportunities exist to use existing methods. More challenging will be to see novel ways in which to integrate tissue culture strategies into Space biology activities. In the solving of outstanding problems and in the developing of novel approaches, it is anticipated that this knowledge will enable a fuller capability for tissue culture management and utilization schemes here on Earth. Recognition of the still-unanswered basic science questions needing imaginative resolution should in fact stimulate viewing the whole problem in a new and more realistic light. Perhaps "conventional" or "traditional" tissue and cell culture is "dead" (or ought to be dead) but this is a perfect time for the birth of a new era in the use of in vitro systems to study growth and development. This era should, in part, be characterized by a rigorous establishment of what happens when specific media components and environmental parameters are systematically varied. This is literally an area that can enable one with modest resources to "kill two birds with one stone", i.e. basic and applied.

I. Introduction

Retirements inevitably bring to mind new evaluations and in Europe especially, where retirement from Academic life is still somewhat earlier than in the USA, it is especially commonplace that new opportunities often surface for experienced scientists. I myself retired very recently so it is natural that I should reflect on my own career even as I write this paper for a volume being published in honor of Professor Karl-Hermann Neumann, a Senior Scientist whom I have known since our Graduate School days. Indeed, some valuable lessons for others still ‘in full- time service’ may perhaps be drawn from my own attempts over many years to "re-tread" one small

subsection of an area that frequently is seen by non-specialists merely as an “enabling technology" - ready for full application. Professor Neumann will hopefully put his own "old wine into new bottles" after his retirement and in so doing, give many others the benefit of his extensive experience in plant cell and tissue culture. After all, all "retirees"-- real or theoretical -- will be sure to ask and assess for themselves “what did my hard work over many years ‘really’ mean?"

When I was a young and naive scientist, the futuristic aspects of Space plant biology seemed to offer an ideal opportunity which afforded me a chance to participate in the development of tissue culture technologies intended for a novel, even glamorous, environment. An added attraction was that even as one worked in the context of ‘Space and Gravitational Biology’, one simultaneously contributed to the development of a more mature outlook on what various nascent biotechnologies could offer for the benefit of mankind here on Earth. This aspiration, indeed it could be viewed as a need, encouraged me to test, some would say even stretch, the basic science aspects of plant cell and tissue culture to the full extent of my capabilities. The need to ‘put things together’ and make bits and pieces of tissue culture methodology work as a co-ordinated whole was very attractive for it allowed great scope and encouraged vision even as it gave intellectual pleasure and no small degree of satisfaction (cf. Krikorian and Levine, 1991; Krikorian, 1998, 2000).

In this essay an attempt will be made to highlight some of the challenges that I have encountered, and lessons that I have learned. But first, some sort of stage needs to be set for those who are largely uninitiated in this seemingly esoteric area. By the time the reader comes to the end of the paper it will hopefully be apparent that the objectives are not at all esoteric but indeed are part of a unified ‘whole’.

II. Conventional Views of Plant Biology in General and Plant Tissue and Cell Culture in Particular

Many now hold that there is a more-than-adequate grasp of the relevant basic phenomenological aspects of plant cell and tissue culture. This view inevitably leads to the contention that practice of the technology must now perforce be relegated to ‘horticulturists’ and that concerted efforts of ‘basic plant scientists’ must now be directed exclusively to understanding mechanisms–this means full understanding at the molecular level. Nevertheless, some investigators like myself with broad interests have persistently contended that attention must be placed on disclosing, categorizing and ultimately understanding the many outstanding intricacies of all the significant processes involved. From the outset, I understood fully that problems of adapting and applying nominally well-established biotechnologies to the problems of Space Biology would not be trivial and that any imagined line between ‘horticulture’ and ‘basic plant science’ was purely arbitrary and self-serving. Only with an understanding much fuller than was then available would make it possible to utilize technologies fully and in a sustainable fashion. Since the projected uses and concepts were very rudimentary indeed, it was expected that this would take time and would be an evolving technology. Work done by the Soviets was considered rather ‘secret’ and details were often lacking so one had little reliable data base to draw upon (cf. Halstead and Dutcher, 1987 for a review that the Russians themselves said was a better review of what had been done in Space using plants than was readily available to them in the Russian language!). It

may come as a surprise to some that all the essentials of applied “tissue culture”were in place and being practiced in the 1930s! (see Krikorian, 1997; Arditti and Krikorian, 1996).

Nowadays and regrettably, funds for basic, non-applied research are increasingly becoming limited to those studies restricting themselves to molecular biological approaches. Plants like Arabidopsis thaliana (Cruciferae), long appreciated as useful in the study of genetics because of the relatively short life cycle (Meyerowitz, 1994) are even perceived by an astonishing number of scientists as holding ‘the’ key to understanding all important mechanisms in virtually all higher plants. This is supposedly so because its genome nominally encompasses some 90% plus of what is thought to be needed in both basic and practical aspects such as improved agriculture through genetic engineering (Wilson, 2000). But the fact remains that Arabidopsis is a simple plant and accordingly cannot serve convincingly as a model for more anciently evolved and more complicated plant groups.

Let it be clear that I do not dispute that progress on all fronts is necessary and that the use of molecular methods has already contributed greatly to our understanding (cf. e.g. Raghavan, 2000). But ‘all’ the answers are not available yet - not by far, nor will they be I predict in the foreseeable future. I am, however, realistic enough to maintain that there is a need for ever-more imaginative and innovative schemes and projects and initiatives if funds are to be made available so that the still much-needed basic physiological and conventional biochemical work can continue – especially as it applies to plant cell and tissue culture.

Stated succinctly, there remains much to be done in plant cell and tissue culture that is outside the immediate scope of applied horticulture on the one hand, and molecular biology and molecular genetics on the other. Molecular approaches are doomed to be sterile if there is inadequate understanding of the ‘biology’ or ‘natural history’ nominally motivating them. Again, one will be quick to ask: “Who will fund this research aimed at taking full advantage of all modern methods?” Indeed, is a place for plant cell and tissue culture in such schemes justified?

Fortunately, in the USA, the National Aeronautics and Space Administration (NASA) has until recently been a source of funding for some plant research that might otherwise would have been relegated to chance funding. Whether the privileged position that I enjoyed vis a vis tissue culture for space research will be sustained in the future is a moot point. As usual, availability of funds is always being challenged by those who argue for a different perspective. In the course of this essay it will emerge that significant findings on cell and tissue culture systems were facilitated through our addressing specific Spaceflight requirements that in retrospect literally ‘forced’ us to solve long-ignored questions. Had these requirements not existed the work may well have taken alternative turns.

III. Biology in Space: Some Basic Questions

The major issues confronting the US Space Life Sciences research program may be reduced to three questions. (1) Is there a fundamental effect of the Space environment on living systems?; (2) How may we best utilize Space to probe questions of more general importance to the broad field of biology?; (3) How may we best develop and use the foundation of knowledge and understanding that will make long-term manned Space habitation possible and free from major

risks? The settling of these intertwined, and somewhat inconsistent viewpoints and issues using a wide range of organisms will become increasingly important to the International Space program in the years ahead. Few experienced plant biologists should doubt that aseptically cultured plants and plant cells and tissues offer opportunities for study, albeit very challenging ones, in these contexts. In fact, some have gone so far as to say that non-aseptic experiments should not be performed in Space and that even entire Spacecraft should be sterilized for long term missions such as explorations of Mars (Greenberg, 2001).

Whether the answer to the first question posed above turns out to be positive, i.e. some fundamental distinguishing feature(s) of growth and development of plants at near-0 G compared with 1 G emerges; or negative, i.e. no such fundamental feature(s) emerge, either result could have major consequences for any contemplated protracted use of plants in Space. This would apply whether the utilization of plants in a context of so-called 'Space Agriculture', or as components of 'Controlled (earlier referred historically to as "closed") Environment Life Support Systems' (acronym CELSS) (Corey and Wheeler, 1992; Nielsen et al., 1996; Gregory et al., 2000) or as test subjects in experiments which aim to use the unique features of Space to study the effects of gravity on plant growth via "G-unloading" (cf. Keefe and Krikorian, 1983; Krikorian and Levine, 1991, Zimmermann et al.,1988 and refs. there cited.).

The broad concepts on which any effort along the lines just summarized is to be based may pardonably be reduced to the following generalities: (1) That there may be some direct effect of micro-gravity, or the lack of it, upon fundamental processes and/or genetic makeup of cells and organisms; and (2) Except for gravitational effects, there is no significant difference between the Space environment and the environment of Earth insofar as plants interact with it.

How cells manage without gravity and how they change in the absence of gravity are basic questions which only prolonged life on a facility such as 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, including Platforms and Stations such as the no longer-existing Soviet Space Station 'Mir' [Peace], profound physiological effects can and often do occur (Nechitailo and Mashinsky, 1993; Tripathy et al., 1996). More needs to be known about the basic biochemistry and biophysics both of cells and of whole organisms in conditions of reduced gravity. 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 will even have to investigate whether adaptive or other changes which have occurred in the environment of Space are retained after return to Earth-normal conditions. Otherwise, one will perforce end up having an isolated and parochial discipline under the aegis of 'Space Plant Biology'. Limitation of research specifically to the micro-G environment would surely endanger any research effort. Some sort of connection with Earth-normal biology is crucial.

Gravitational plant biologists have, of course, given considerable thought to the kinds of changes in response that might result from exposing plants to micro-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, gravi-morphogenesis, 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 (Sack, 1991, 1998; Digby and Firn, 1995).

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, micro-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 (Barlow, 1995).

Last but not least are instances where micro-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 micro-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 micro-g have on cells that are not specialized for G-sensing?" And, "What effect does micro-G have on developmental, physiological and reproductive processes?" etc. (Krikorian, 1996a and b, 1998).

Skeptics and critics has emphasized that there is no fundamental or constitutive short-term, or acute effect of gravity, or absence of it, upon plants and their cells. Or, stated another way, it is irrelevant to consider the question seriously for manned spacecraft and Space Stations such as the one being presently assembled because there will always exist a certain 'above-threshold G environment' associated with either on-orbit maneuvering or human activity (Keefe and Krikorian, 1983; Krikorian and Levine, 1991). Conversely, in terms of the practical functioning and growth of plants, there is a profound effect of the altered physical environment associated with micro-gravity. This boils down to somewhat semantic arguments of "direct" vs. "indirect" effects of micro-G. On this simplistic argument, the "direct" effect is mediated, only to a minor extent, through such phenomena as the sensing and orientation response. (It is a truism that changes in organisms during the last 500 million years are due largely to the rearrangements of basically similar building blocks-cells-by gene regulation mechanisms and not by the creation of new genes. In other words, basic metabolic patterns were already in place and new kinds of organisms were produced by rearrangements of the same biochemistry in different building blocks. I contend that microgravity/space must have effects on processing of cues of various sorts and the first step is to define what these effects are –Markert and Krikorian, 1993.)

On this line of argument, much more important are the indirect effects of the physical environment. One may ask, for example, what is unique about the low G environment with respect to Space experiments involving fluids? In the first place, although the laws of classical physics such as momentum and mass conservation, energy conservation, Maxwell's equations etc. still apply, the relative importance of the G force to other forces changes. Also, the fact that experiments are being conducted in a non-inertial frame becomes more important and the effects of variation of g become more important. Moreover, in the near-weightless environment of Space (here weightlessness is defined as $10^{-6 \text{ to } -4}$ G), there are no convective currents, no buoyancy, and surface tension dominates. Consequently, because of the dominance of surface tension/molecular attraction, fluids tend to deposit in unexpected or undesirable ways on plant surfaces and growth media, greatly impeding air movement. The exchange of energy and metabolic gases between the plant and its liquid or gaseous environment will be radically different in space because of the absence of gravity-driven convection. Diffusion, convection, active mixing, and asymmetrical distribution of particles all will be different in Space. But the fact is that information on the precise nature of these phenomena is sparse and has been the subject of only limited study and that by engineers in a non-biological context (Benedikt, 1960; Claaseen and Spooner, 1994). There is no way of predicting what the exact nature of the convective environment in micro-G will be. Studies on Earth indicate that absence of G-driven convection will have considerable impact on the normal gas and heat exchange phenomena that are important to "normal" plant physiological function. Even now, there is reasonable, albeit circumstantial, evidence that there may be effects of the lack of gravity-driven convection that have significant impact on plant growth (Musgrave et al., 1997). Altered aeration and gas exchange in the environment of roots may well provide one component of an explanation for observed anomalies in space-grown materials such as cell structure, atypical cytological characteristics such as chromosomes which are ruptured or otherwise altered, and poor mitochondrial development (Nechitailo and Mashinsky, 1993).

Similarly, biological manifestations of these indirect effects may range from far reaching to insignificant depending on the biology of the system, and the culture environment. It logically follows that if equipment for experimentation such as plant growth chambers either for basic science experiments or for practical functions are to be designed properly, it is

important to characterize as rigorously as possible the interaction between micro-G, other physical environmental parameters, and plant responses (cf. e.g. Salisbury, 1991 a and b; Nielsen et al., 1996).

It will come as no surprise that NASA has recognized for some time that a number of issues regarding plant growth in Space need resolution (Table 1). Emphasis, of necessity, has thus far been placed on study of short term effects or adaptations or responses to Space. This should be distinguished from long term adaptations compatible with a true biology of plants in Space.

Table 1: Some Expected Changes in Plant Response as a Result of Exposure to micro-G in the Space Environment

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, micro-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 micro-G would be expected to alter a response, i.e. have a quantitative effect:
Lignification?
Unknown at present, e.g. What effect does micro-G have on cells that are not etc. specialized for G-sensing, or on developmental, physiological and reproductive processes?

Some effort has gone into evaluating directional responses as a result of reduced G, i.e. tropisms (Brown, 1996). Altered tropistic responses are not, however, the most important effects plants will show when grown in the reduced gravity environment of Space. They probably have little direct significance for early or primary development of plants in Space but they still may have indirect effects (Digby and Firn, 1995).

Nevertheless, marked changes in plant appearance due to disturbances in orientation in "above"-ground or "below"-ground organs, in epinasty of roots and leaves, and diminution of nutational movements all have been reported (Dutcher et al., 1994; Nechatailo and Mashinsky, 1993). Since phenotype is of adaptational significance, this will have effects on biomass. It is necessary to study qualitatively and quantitatively the patterns that relate changes in plants to parameters that have significance for growth, development and metabolism. There are many questions of importance to plant growth in Space. Changes in biochemistry, reflected in such effects as replacement of cellulose with hemicellulose and associated anatomical disturbances due to ‘G-unloading’ on support tissues such as lignin may be expected. Modifications in water transport mechanisms and a consequent change in vasculature may also be predicted (Sachs, 1991).

All the above indicates that the way in which things are exposed, ‘grown’ if you will, in the Space environment will impact tremendously the results.

Understandably, the objective of an experiment normally dictates the way in which it should be grown. But I have already emphasized that the database for growing plants in Space is minuscule and the near-term and increasingly urgent requirement is to move step-wise towards developing systems for the reliable growth of plants in Space (Salisbury, 1991a and b; Corey and Wheeler, 1992; Musgrave et al., 1997.)

IV. The Controlled Ecological Life Support Systems (CELSS) Challenge

A controlled environment agriculture is often seen as a critical component of man's ability to achieve a “permanent presence” in Space. This permanent presence will at the outset necessarily be acute--say order of three to six months--but it will increase in duration to chronic, very much longer levels, eventually becoming even multi-generational (Pirie, 1980; Mitchell, 1993). As part of the human support capabilities, there will be a need for advanced life support systems. A CELSS as now envisioned by many in the USA, Europe and Japan as well, will rely on biological means, whereas an "open" one will emphasize a purely chemical approach and ‘resupply’ parameters. Various forms of plant life are central to the whole support effort but plants will also predictably play roles other than as a source of nutrition, and a means of atmospheric control or waste processing (cf. e.g.Wolverton et al., 1983, 1984). This role has been, for lack of a better description or heading, categorized under “Human Factors”. The mere presence of, or access to plants in the ultimately monotonous and relatively confined environment of even large Space facilities, and even the supplementation of a perhaps otherwise highly processed and unimaginative diet with a few fresh greens will undoubtedly have a positive effect on the human psyche. In fact a ‘Salad machine’ has been proposed (Kliss and MacElroy, 1990).

If one views the overall problems in the context of food production, waste processing and their control and management, then the question arises as to what extent can newer plant biotechnologies play a role in enhancing the efficiency of, or managing, these kinds of anticipated activities? The next section addresses these issues.

V. Background to the Use of Cultured Cells and Tissue

Readers will appreciate that activities by tissue culturists over the past twenty-five years have dramatically increased capabilities for problem solving. There now is a massive literature that seeks to provide the interface between innovative breeding and various plant propagation and management practices, and the challenges and opportunities posed by particular species (Lindsey, 1992; Vasil and Thorpe, 1994; Bajaj, 2000). The most obvious of the potential uses of plant tissue culture technology in a CELSS setting involves relatively rapid multiplication of higher plants (Keefe and Krikorian, 1983).

Table 2 provides a list of various in vitro strategies which can lead to multiplication of desired ‘bio-specimens’, whatever the intended use. Whereas the term "tissue culture" or in vitro culture is largely used in a generic sense, and hence does not necessarily connote a precise strategy, the enumeration in Table 2 provides in greater detail a number of the different strategies than one can adopt in an effort aimed at yielding increased numbers of propagules or plantlets. These range from the relatively well-established procedures of (1) fostering various levels of branching or shoot development by releasing (usually hormonally) various corelational controls normally in place in the intact plant body; (2) inducing organized growth or direct organogenesis from excised organ or tissue systems without an intermediate and extensive callus stage; (3) fostering de novo organized growth in the form of shoots and roots from callus systems; to (4), the much less predictable stimulation of the formation of somatic or non-zygotic embryos from so-called morphogenetically competent cells and tissues (cf. Soh and Bhojwani, 1999).

Table 2: Strategies for Multiplication of Higher Plants in vitro.

- Shoots from terminal, axillary or lateral buds
  . shoot apical meristems (no leaf primordia present)
  . shoot tips (leaf primordia or young leaves present)
  . buds
  . nodes
  . shoot buds on roots
- Direct organogenesis
  . adventitious shoot and/or root formation on an organ or tissue explant without an intervening callus
- Indirect organogenesis
  . adventitious shoot and/or root formation on a callus
- Somatic embryogenesis
  . direct formation on a primary explant
. 'direct' formation from embryo-equivalent cells grown in suspension or on semi-solid media
- Direct plantlet formation via an organ of perennation formed in vitro
- Micrografting
- Ovule culture
- Embryo rescue
- Mega-and microspore culture
- Infection with a crown gall plasmid genetically altered to give teratoma-like tumors

Additionally, plantlets can be generated directly from organs of perennation which are sometimes controllably and precociously inducible in vitro. Similarly, micro- grafting performed in vitro can effectively lead to more plants in some cases. Ovule culture can in certain cases rescue or lead to plants that would otherwise be lost, and embryo culture can provide the same capability. Mega-and microspore (pollen) culture can likewise lead to materials that reflect either the genotype of the female or male germline respectively. A much more tenuous method uses infection with a genetically altered plasmid of the crown gall micro-organism (Agrobacterium tumefaciens) that lead to teratoma-like (tumorous) structures from which whole plants can ultimately be recovered (cf. Ream and Gelvin, 1996).

All these strategies can, then, be invoked with varying levels of efficiency and technical finesse towards the end of multiplying plantlets in vitro. No useful purpose is served here in extensively discussing each of these strategies. The works referred to above address the specifics and provide detailed perspectives on the precise range of capabilities. In theory at least, virtually all plant species are amenable to manipulation. Nevertheless, it follows that there are a very large number of parameters that must be taken into consideration if any of these strategies is to be optimized in the Space environment, just as the many environmental parameters must be addressed in depth here on Earth (see Kozai et al., 1992 and Fig.1). Convective mass transfer and liquid atomization are but two examples of major problems that must be overcome in Space (see Prince et al., 1991 and Bayvel and Orzechowski, 1993 respectively.)

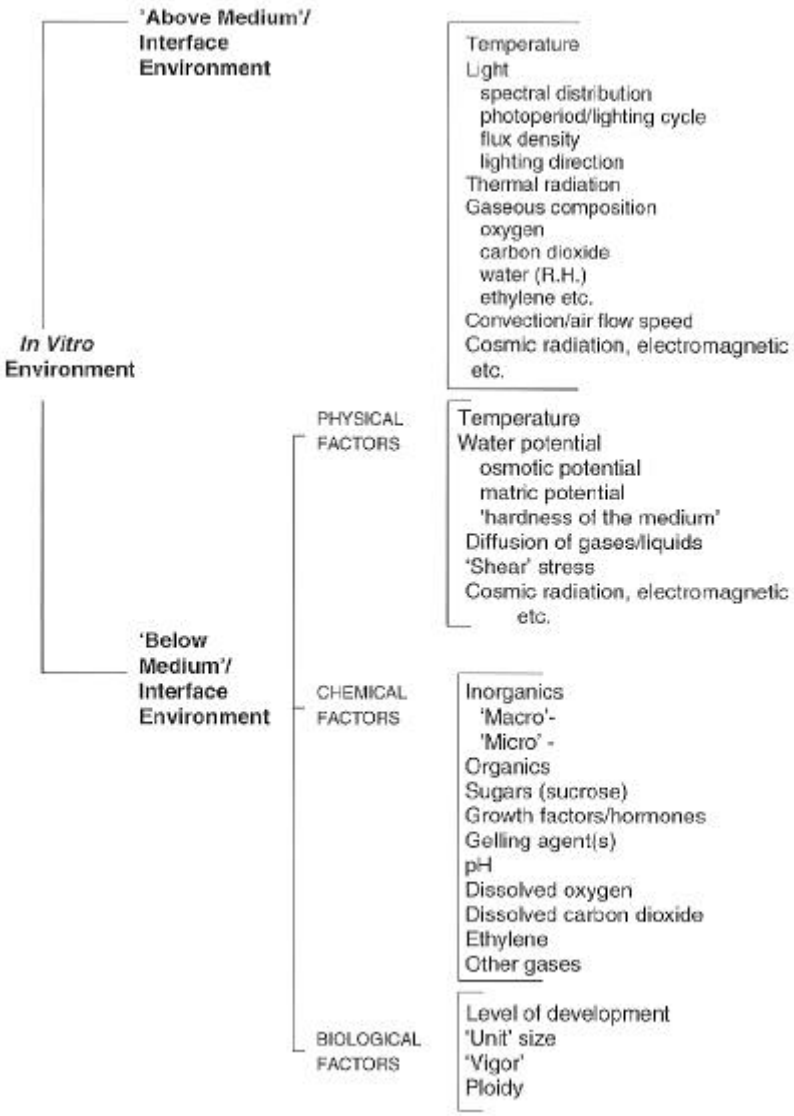

Figure 1: Some macro- and micro-environmental parameters affecting development and growth in vitro (Inspired by Kozai et al., 1992).

VI. Gravitational and Developmental Studies Using Higher Plant Cells in the Context of Modern Biotechnologies

Plant biotechnology is, of course, a broad, complex field, and there is considerable overlap of basic science, technique and technology into areas usually termed "industrial biotechnology" and "chemical engineering". It is moreover, a rapidly moving field. Even so, numerous opportunities still exist for integrating and, indeed, taking advantage of a number of distinctive research perspectives or capabilities which are currently of special interest to various Space Agencies. At the same time, these same research opportunities and approaches, if better integrated and co-ordinated, have high potential for broadening the science and technology base and strengthening interdisciplinary approaches here on Earth.

This is sure to optimize 'science return' and to contribute more effectively to a increasing our basic understanding of a host of questions with both basic and biotechnological importance. Integrating the two areas will require that practitioners from various disciplines work together to identify the critical needs and capabilities of each.

One especially salient example follows:

Bioreactors

The culture of plant cells in bioreactors is increasingly being seen as having substantial potential in the biotechnology industry for the production of high cost biochemicals, enzymes, and other distinctive secondary products etc. On Earth, because of gravity, the content of a bioreactor must be mixed in order to obtain a proper distribution of nutrients, oxygen, temperature and pH environment. This mixing creates a harsh hydrodynamic shear environment detrimental to sensitive plant cells. If not mixed properly, cells tend to congregate, and by zone sedimentation, fall to the bottom of the bioreactor. Furthermore, the requirements for oxygenation creates a foaming in the bioreactor which also tends to perturb and otherwise damage cells. These factors limit the concentration and density of the bioreactor nutrient culture medium. On Earth, it is known that concentration and density of the solution are directly linked to the optimal performance of bioreactors; the higher the density, the more cost effective the bioreactor 'run' (Doran, 1993; Wilson and Hilton, 1995).

In microgravity, zone sedimentation disappears, which should reduce the aggregation of cultured cells. Moreover, only gentle mixing is required to distribute nutrients and oxygen. These factors should permit higher concentrations and densities to be achieved in a low G environment. Additionally, since the cells do not need to maintain the same surface forces that they require in Earth-normal gravity, they can divert more energy sources for growth and differentiation and in theory, at least, the biosynthesis of more product, or even novel products the production of which would be unpredictable (Cogoli and Tschopp, 1982; Cogoli and Gmünder, 1991). Because one can impose variable gravity on these cell systems, one has the means to test the consequences of increasing or decreasing G on secondary product biosynthesis.
Some work has already been carried out on plant cells in a Bioreactor setting in Spaceflight. Results indicate that metabolism, productivity and differentiation characteristics of a variety of cells is altered. This might be due to decreased cell interactions (contacts) when cells are freely suspended (Krikorian, 1996a). Clearly, there are many opportunities to study these responses and these are sure to lead to a better understanding of the mechanisms by which plant cells control production of secondary metabolites and other cell products. With this knowledge, control of enhanced, sustained production of product by plant cells might be possible. See Durzan (2000) for a detailed analysis of metabolic engineering and specific plant biosyntheses in a Space environment.

There is, then, a great need to pursue specific biosyntheses under various controlled microenvironments. The study of mechanisms in the context of bioreactors is rudimentary and only when much more is known will one be able to move forward. Sophistication of available cell culture chambers or bioreactors has steadily been improving and opportunities for increasing our knowledge base is virtually unlimited. Capability to study inter- and intracellular, even subcellular gaseous environments, combined with study of compartmentalization of various key signaling events and sensing components within cells, as well as the targeted manipulation of pools and nutrients with novel agents such as chelators, all provide means to disclose and understand mechanisms.

The use of molecular markers such as monoclonal antibodies to bind to specific sites and the cloning and analysis of cDNA encoding a specific synthesis could provide yet another level of detail in pursuing the nature of basic control mechanisms. Study of inducible control of gene expression in cultured plant cells in a Space environment is certain to disclose new and unexpected findings (Reynolds, 1999).

VII. Gravitational Biology and Mechanisms which Control the Differentiation and Development of Plant Cells, Tissues and Organs in vitro: Relevance to Emerging Plant Biotechnologies

One of the major constraints to progress in genetic engineering of higher plants continues to be the ever-present lack of a complete understanding of the controls which permit cells to express their innate potential to multiply and embark on a pathway of development that approximates that of zygotic cells (Thorpe, 1995; Neumann, 1995; Raghavan 1997; Soh and Bhojwani, 1999). A major objective is to recover plants from such cells via a developmental pathway involving production of somatic embryos for use not only as an end unto itself for basic research, but in a Life Support System, and as means to store germplasm for a multitude of purposes in Space (Keefe and Krikorian, 1983; Krikorian, 1996a). Yet another impetus for this kind of work is that the understanding of what controls totipotency and genetic expression is often fundamental to operations wherein new genetic material may be introduced into plant cells (Soh and Bhojwani, 1999; Raghavan, 1997, 2000).

Significantly, NASA has for some time been concerned with problems of so-called plant “gravimorphogenesis” and a major question has been whether proper polarity can be achieved in single cells in an environment where gravity signals have been "eliminated or erased ". Early on in my Space biology investigations, the seemingly cogent argument was made that totipotent cultured cells offered substantial merit for studying the question whether polarity could be established in the absence of G vector - or more accurately in an environment where the G vector was more or less neutralized, as in a clinostat (Hoshizaki, 1973; Brown, 1996; Krikorian, 1998). The argument went along the following lines. If one, in theory at least, could work towards determining thresholds for any number of parameters, it should be equally interesting to ascertain yet another type of threshold level. To me, a particularly interesting question was "What constitutes ‘the’ or ‘a’ minimal G-responsive unit from the perspective of development? The theory of totipotency has, of course, historically been predicated on the view that individual cells are capable of giving rise to entire plants and it seemed perfectly reasonable to ask the question whether free cells could establish polarity and continue to develop in Space.

The cell that is the classical example of this presumed need to establish polarity, perforce, is the fertilized egg or zygote which can grow and develop into an embryo and from that sprout into a whole plant (Vroemen et al., 1999). Why not, merely then, take seeds and use them as experimental objects to determine development thresholds? One could I argued, indeed, do this but one would be determining whether an embryo could develop or progress from one stage to another, under a measurable G load. Because the embryo is generally very well developed in most seeds, one cannot study very early developmental events using seeds which are separated from the parent plant (the sporophyte). If one wanted to use early stages of development, one had to have the fertilized egg in place (in situ) in the embryo sac of the ovule (which will develop into the seed) or, one had to have lots of isolatable, fertilized egg cells that can grow. The earlier in development the better, since from the earliest stage, all

else grows. To have as complete a picture as possible, one obviously wanted to be able to expose a broad spectrum of stages to the Space and a range of hypo-G environments. (Again the indispensability of access to a centrifuge for use in Space became apparent.) These are not easy points on a curve to determine, however, because what one is essentially being challenged to do is to expose fertilized egg cells removed from the embryo sac of a higher plant and to allow them to develop under controlled conditions into a fully mature embryo - passing through all the classical stages of embryogeny, all the while assessing their performance at specific G levels. Each of the stages would have to be pre-determined to be amenable to mass collection, in the first place, so as to have ample supply for experimentation. Then, assuming one had enough of them at any of several stages of development, one had to be able to grow them on Earth. This assumes one has the skills and understands the requisite nutrient requirements to bring the developing embryos through to their full level of growth. Then one has to be able to "package" them for flight experimentation. All this was a major challenge - and given the state of the technology, it was impossible to do. Hence the argument that totipotent cells would provide a more tractable substitute for zygotes. [It had not been convincingly shown that embryogenic cultures grown in suspensions were already zygote-equivalents, that is they were already determined (Krikorian, 2000). I vacillated on this point of exactly when determination occurred. That was because of several unresolved parameters. Had I known this for certain no Space flight experiments would have been proposed using cultured plant cells! Or, at least the question asked would have been posed in a different context.]

If one adheres to the line of reasoning started above and brings it to its logical conclusion, we can come to the viewpoint that the degree of sensitivity or responsiveness to G varies at different degrees of organization or stages of developmental complexity. Experiments which impose varying G force levels on different degrees of "developed material" should permit us to pinpoint the degree of prior organization, if any, at which salient problems might arise in the Space environment. This should teach us, my argument continued, what are the minimal G forces required for normal plant development (and physiology), and through centrifugation in Space, the maximum G force that be tolerated before a gravitropic response is elicited. By "erasing G signals" one could investigate what happened.

Indeed the very first experiment using embryogenic cells (then simplistically referred to merely as ‘totipotent’ cells) of a higher plant in Space was performed on the Soviet unmanned satellite BioKosmos in 1975. Initially the conclusion was drawn that somatic embryogenesis proceeded unhindered in the weightless environment (Krikorian et al., 1981). Somewhat later, a more sophisticated analysis of data showed that there was in fact a blockage in the normal progression of somatic embryogenesis from ‘free’ cells in vitro beyond the globular stage and that there was a failure to polarize (Krikorian, 1991, 1998). More recent investigations using Dactylis glomerata have confirmed and extended our pioneer observation. That Spaceflight reduces somatic embryogenesis from primary explants as well as from already-embryogenically determined cultured cells derived from embryogenic suspensions is not surprising (Conger et al., 1998). Opportunities to probe in greater depth whether this constitutes a limitation of specific receptors to function in low G or whether it is ‘merely’ a reflection of the technology of the system being tested will be at once apparent (see more on this later).

Additional to work on somatic embryogenesis, and from the perspective of bioreactors and stage-specific biosynthesis, should constitute a unique opportunity to ascertain whether specific syntheses can be sustained in Space in ways not possible on Earth. As of yet, there is no way to non-invasively stop somatic embryo development at a specific stage, and hence the

conventional approach to the study of stage-specific biosynthesis has been to mechanically isolate the stage in question being sought. This is much harder to do than might be apparent. Synchronization is no simple problem.

Use of pH and Reduced Nitrogen to Control Development of Embryogenic Cells

Conceptual approaches related to Spaceflight experiment implementation in the context of seeking an understanding as to whether there are developmentally-related limitations to gravity sensing, i.e. “Is there a morphologically 'minimum' unit required for g sensing?” has led to the appreciation that as-simple-as possible-controls must be sought in embryogenic or developmental models. In the course of this work, an innovative and important mechanism based on pH was discovered in my laboratory which controls transition from preglobular stage embryos to globular and later stage somatic embryos. The mechanism, whatever the basis of it is, is not restricted to a few species for it has been shown to be operative in each of the test systems we studied (cf. Smith and Krikorian, 1990a and b, l991, 1992).

Briefly, we showed that embryogenic cultures of carrot and other plants can be initiated and maintained with continuous multiplication using a hormone-free medium. In the course of this work we also showed that high frequency production or initiation of embryogenic globules from wounded zygotic embryos is dependent upon the use a medium with a pH above 4.5 and NH_4^+ as the sole nitrogen source. However, maintenance with continued multiplication of unorganized, embryogenic cell masses requires that the pH of hormone-free, NH_4^+-containing medium be maintained at or fall to 4 during each culture period. If the medium is buffered at or above pH 4.5, embryogenic globules continue to develop into later embryo stages (Smith and Krikorian 1989, 1990a and b).

In the case of carrot cultures, preglobular stage embryos - that is to say the stage most reminiscent of the zygote - before the proembryo or globular stage embryo forms - can thus be kept "cycling" and increasing in number provided the pH is kept low. If the pH is elevated, the somatic embryos continue their development and proceed through the "normally expected" stages of embryogenesis - globular, heart, torpedo and cotyledonary (or the equivalent in a monocotyledonous system).

The "simple" parameter of pH under these circumstances should not be viewed as a sort of second messenger but merely a case of providing an inappropriate, non-stressful environment for embryogenic progression. For years one has heard and read the dogma that the "best" pH for a cell culture medium is such and such (usually around pH 5.6-5.8 or so). Clearly this now needs to be qualified and a further statement made about what situation one is talking about. If one wants more preglobular stage somatic embryos (or embryogenic cells or cell clusters), then the pH should be kept low (below pH 4.5). (It should again be emphasized that the low pH does not confer embryogenic status or capacity on the cells. The pH works only on cells that are already in the embryogenic mode. It is a modulating agent---an important one--not an inducing one (Smith and Krikorian, 1990a; Krikorian and Smith, 1992) . Another factor, long appreciated as important, involves nitrogen supply--be it ammonium or nitrate. Reduced nitrogen (ammonium or casein hydrolysate or glutamine) will support continued somatic embryo development - i.e. stages beyond preglobular stage somatic embryos; nitrate will not and should therefore not be used alone to support continued embryo development after somatic embryo induction-initiation has begun. This too is an area that requires investigation in terms of permissive metabolism (Smith and Krikorian, 1989).

No doubt there are many more controlling factors similar to pH and type of nitrogen that will be encountered (cf. Krikorian, 2000).

Thus, the need to study the control mechanisms in this system still provides a wide range of opportunities to study effects on plant growth of distinctive environments such as that of Space, and on Earth provides a much-needed tool to improve responses in hitherto seemingly ‘non-embryogenic’ systems that have usually been described as being too "recalcitrant" or "fastidious" in their requirements to be induced to be embryogenic, i.e. express their embryogenic potential. Higher plant biotechnology is dependent upon reliable means to manipulate and manage developing plant cells in vitro and knowledge from such studies should go far to providing a better understanding of what controls expression of morphogenetic potential at various stages of the culture process–ranging from the primary explant to sustained subculture.

The role of somatic embryogenesis biotechnology as it relates to higher plants in the operation and management of a Controlled Ecological Life Support System (CELSS) will be apparent. An important point here is that opportunities to disclose new control mechanisms can emerge in the course of studies that are not necessarily directed towards a specific goal.

VIII. Emergence of a ‘New’ Perspectives on Somatic Embryogenesis

At Stony Brook, we made significant progress towards developing procedures for somatic embryogenesis in Space using daylily and carrot as models. In the course of this work we (1) developed and refined exquisitely sensitive systems; (2) increasingly appreciated that our long standing, healthy skepticism of many of the nominal truisms associated with what has come to be called "tissue culture technology" was totally justified.

Specifically, in the process of developing and defining our embryogenic cultures, we advanced to conceptualizing our results in a framework of either fostering or limiting of embryogenic progression at key points in the process. This amounted to providing of, or avoidance of what are normally thought of as stresses or insults at what we preferred to call "phenocritically sensitive or vulnerable" stages of differentiation and development. Significantly, our work was largely done in the context of developing sustainable cultures for Spaceflight purposes, extending their performance potential through many days in states of "suspended animation" in anticipation of Spaceflight initiation, and overcoming normally severe limitations to progression of free embryogenic cells in predominantly liquid environments, in contrast to progression in/on semi-solid (Smith and Krikorian, 1992; Krikorian, 1999).

Our focus on the smallest of totipotent cell units disclosed an essential and unappreciated feature of the somatic embryogenic process. The should provide a much-needed stimulus for re-directing current cell biological and molecular work (Komamine et al., 1992; Pennell et al., 1992; Mordhorst et al., 1998). Stated in its barest bones essential, the ‘new’ perspective is this: inefficiencies or recalcitrance of a system in the context of plant cell and tissue culture biotechnology should be viewed in a cell biological and developmental context as failed responses due to stresses and inappropriate environmental conditions limiting the progression of embryo development. The smaller and less developed advanced the unit, the greater the vulnerability to stress. This elegantly simple and precise focus on what is happening in growing, developing ‘embryogenic cell cultures’, again more precisely developing somatic embryos, should allow detailed investigation at a number of levels going from the single cell

stage to few cell stage to the multicellular level. It offers a way of studying what stress ‘really’ is so far as an embryogenic system is concerned (Krikorian, 1996d).

My early views on somatic embryogenesis in Space and establishing whether polarity could be established in low G have been given above. But that represents a good example of trying to use a system to answer a very basic question without first fully understanding the constraints of the system. It was nominally a well-established fact of life that totipotent cells which were morphogenetically competent could be induced to embark upon a course of embryogenic development. It was only a number of years later that in the course of developing ever-improved somatic embryo systems for use in Space experimentation that we showed in the Stony Brook laboratory that embryogenic cultures of daylily, indeed probably all plants, are already determined in the primary culture stage, well before a culture is perpetuated through subculture. The fact that cannot be over-emphasized, is that maintenance culture conditions ‘simply’ perpetuate a determined state in the form of a proliferative collection of initials limited in their development but undergoing what might be termed a ‘forced regenerative polyembryony’. Under these conditions, the initials do not progress beyond the first few divisions before the newly formed cells ‘separate’ (that is, cells within a developing embryo are shifted out of their normal position, ultimately leading to a detachment or fragmentation of cells and groups of cells from the embryogenic unit). A kind of repetitive embryogenesis is thus fostered and variously-sized polyembryonic fragments are produced. Regeneration is dependent on a permissive environment. So far as suspensions are concerned, somatic embryos are formed only as a consequence of the ‘direct’ development or advancement of pre-existing somatic embryo initials. Proembryogenic masses (PEMs) do not exist according to their more usual, classical as it were, definition (Thorpe, 1995). They are more accurately described as clusters of budding somatic embryo initials with varying capacity for development or as polyembryonic fragments of somatic tissue. All this emphasizes that established embryogenic suspensions cannot, by their very nature, constitute model systems for the study of the induction of somatic embryogenesis since these events would have already occurred (Krikorian, 2000).

The new facts which we have found, and their potential to redirect perspective and experiment planning, should assist scientists and plant tissue culturists in their efforts to generate and better control embryogenic systems. The finding that totipotent cells in suspension are already somatic embryos or zygote-equivalents and not ‘undifferentiated’ cells needing to be induced to the embryogenic state by any of a variety of ill-defined or empiric means proves that the long existence of a common belief is by no means infallible evidence of its correctness. It is proverbial that habit dies hard, and the judicial attitude is far from universal. From the outset, the very discovery of somatic embryogenesis has had a certain ambivalence associated with it. For many years the field has been fraught with many uncertainties and considerable empiricism, even failure to recognize what was actually happening in the cultures being worked with (Krikorian and Simola, 1999).

Again, while none of the above was directly related to Space biology, the findings from our research emerged in the course of attempting to develop and streamline procedures for testing in Space. In fact there had been, one might say, a preoccupation of trying to work with absolutely minimum units—‘free’ cells. I feel fortunate to have had the instigation to view the systems we worked on from a fresh perspective that fostered a particular mind-set and thus opened our eyes, so to speak, to unconventional interpretations as to what was needed for a well-controlled and manageable experiment. (Admittedly this all took a fair amount of time, some might say too long a period. The thesis of my last Graduate Student, Dr. Joel

Weidenfeld contains all the details and reference may be made to that pending full publication of the work, Weidenfeld, 2000.)

IX. Phenotype is Affected in Unexpected Ways by Environment: The Case of Ethylene and Morphology of Cultured Tissues

In the case of aseptically cultured daylily shoots, use of ancymidol (α-cyclopropyl-α-[p-methoxy-phenyl]-5-pyrimidine methyl alcohol) has shown that the compound has significant value in keeping plants short, with an optimum somewhere between 3 and 10 mg/liter. Growth has been repeatedly tested and shown to be suppressed over a 10 week period but normal growth resumes upon return to ancymidol-free medium. This strategy has had considerable value in enabling us to handle propagules of daylily more efficiently, especially in those situations where the capacity of a growing environment (vessel) in accommodating a large plant is limited. Cultures can be kept short, the ancymidol can be removed, the plant resumes 'normal' growth and the test conditions can be imposed.

The role of ethylene gas in maintaining and initiating transition to mature phenotype in daylily was discovered in the course of studies aimed at evaluating the effect of ethylene gas on growth of cultures in a sealed environment. It transpired that a phase change, typified by a very obvious change in phenotype, was effected by the presence of ethylene at a specific level. The phase change obtained led to a phenotype that is typical of the mature plant (Smith et al., 1989). It will be appreciated that a major problem in cloning operations of various plants by in vitro means is that they are frequently juvenile and require a period of growth (that is empirically determined) before they function as the adolescent or mature phenotype (Vasil and Thorpe, 1994; Soh and Bhojwani, 1999). In materials that are important in certain biotechnologies and agriculture, a better understanding of phase change and transition from juvenility to maturity is required before proper utilization of the given technology may be implemented.

Understandably, by far the most attention has been given to crystallizable chemicals and growth regulators as they apply to controlling mechanisms of higher plant growth but gases can play important roles as well (Kanellis et al., 1997). Since better understanding of closed or controlled environments in association with plant growth was, and is still needed, it was believed that this kind of activity would serve multiple purposes.

Again, chance or serendipity helped to shed some light on an important problem. Populations of miniature plantlets with fan-shaped growth form had been encountered a number of times in suspension-derived and suspension-derived but protoplast-generated embryogenic cells placed on semi-solid media. There was several particularly noteworthy features about these miniature, mature forms. All plantlets originating from a given stock or common culture did not follow one or other growth habit. Thus, we had no reason to believe that there was anything inherent within a given cell line or culture or its prior origin in terms of whether it came from cells or protoplasts, for obviously pre-disposing it to form fans. But, if several members of a population within a given culture vessel conspicuously showed the fan habit, close scrutiny showed that they all had the special feature. The same is true of juvenile populations. Mixtures of fans and juvenile forms were never seen. Because of asynchronous development of the plantlets in culture, especially on agar media, there is inevitably some variation in size within a culture vessel. Not only was there contrasting leaf morphology, but in the root system as well. In the juvenile state, the roots are fewer, thinner and longer. In the "mature" plantlets, the roots are more numerous, fleshy, thicker and more fibrous, much like

those of much older plants. Attempts to maintain the mature fan-shaped plantlets after removal from culture vessels were never successful. Plantlets consistently shown new leaf growth of the juvenile form and, when removed and planted in soil in due course, usually after a year or so, they embarked upon the course of growth that ultimately yielded fans. The possibility that the different growth forms were the result of localized micro-environmental influences within our growth chambers had been examined. Light and temperature were not responsible for the growth form differences. The hypothesis that “maturity-inducing/sustaining” substances(s) may have been produced and released into the medium by fan plantlets, and that mature growth might thus be prompted in juvenile plants was also been tested. When juvenile forms were placed aseptically on media in jars from which fan plantlets had been removed, and vice versa, the juvenile forms remained juvenile and the fan forms soon produced new juvenile growth. To our disappointment, juvenile forms never grew into fan forms under in vitro conditions--even when a substantial period of time had elapsed and one might normally expect fans. This may perhaps be due to depletion of nutrients and the dramatic slowing down of growth in cultures which have been maintained for many months on the same medium. (It does not follow, of course, that shoots with “mature” characteristics cannot arise in culture from "juvenile" meristems. Several daylily hybridizers, commercial growers and enthusiasts when questioned about ever having encountered the absence of, or a curtailment of the normal juvenile phase in seedling material stated, however, that they had never seen a mature form before its time.)

All tests involving opening of a culture vessel, even without subsequent removal of contents, resulted in the rapid reversal of the fan habit; juvenile forms remained unaffected. This suggested that a gas was being released to the environment whenever a jar was opened. (The occurrence of fans seemed to be correlated with the exceptionally tightly sealed jars.) Suspicions that a volatile or gaseous component of the environment might be involved were strengthened by a simple experiment. The mere loosening or "cracking" of the lid and re-tightening was sufficient to result in reversion of fans to the juvenile form. (Within a couple of days the reversal is complete.) To make a long story short, ethylene releasing substances, and inhibitors of ethylene production were tested and the change in phenotype was shown to be due to ethylene accumulation in the culture vessel (Smith et al., 1989).

Years ago, Hussey and Stacey (1981) called attention to an “ethylene morphology” in potato clones multiplied in sealed culture vessels. Nodes from plantlets generated in sealed vessels gave rise, however, to 'normal' shoots when transferred to loosely sealed vessels. In daylily, unlike potato, the form change involves size, it is clearly juvenile to mature, and it is reflected in root morphology as well. This all figures significantly in the improvement of vegetatively propagated plants (Abbott and Atkin, 1987).

The fact that the form change has never been shown to be permanent, that is that the mature fan shape reverts to the juvenile form, emphasizes that under the conditions tested, the form change is physiological in nature rather than epigenetic. Clearly, stabilization of the mature form could provide useful information on those events associated with determination phenomena. In any case, our work showed that the daylily shoot apex is not necessarily determined or programmed to produce leaf primordia in a fixed mode merely because it has reached a certain volume, size or age. (This was shown to be the case in Musa clones as well the apex of the raceme (male ‘floral’ bud)) was excised and grown in vitro (Krikorian et al., 1993, 1999).

Abnormalities of Plant Materials Exposed to Space, Cytogenetic Profiling and Somaclonal Variation

Yet another aspect of our work is intimately associated with observations made in Space-grown materials. Experiments first performed as mid-deck locker of the Space Shuttle on seedlings and later on aseptic propagules and still later on cell-culture-derived propagules early showed that chromosomal changes can occur that cannot be accounted for by the radiation environment; the measured radiation has been too low, even insignificant, to account for the changes.

Changes include chromosome rearrangements that are due to breaks at apparent "hot spots" that in turn lead to changes in ploidy and karyotype in ways that cannot as yet be duplicated on Earth. They may lead to permanently altered genotypes (Krikorian, 1996b).

All the evidence available indicates that perturbations in cell division are a major manifestation of stress (Krikorian, 1996d, 1999). The level of the stress effects are dependent on the particulars of the system, especially the 'developmental state and biology'. [Even so, we recognize that the same concerns of experimentation in Space vis à vis gravitational controls expressed above hold for radiation experiments in Space and that this important issue will also need to be resolved experimentally. Similarly, and unfortunately, Space tests which have disclosed that Spaceflight can have adverse effects on plant cell function such as division as evidenced by reduction in the level and fidelity of cell division were limited by necessarily imperfect controls onboard the Spacecraft. Recognizing this limitation brought on by unavailability of appropriate centrifuges in Space, disturbances ranging from slight to extreme - have been found at the level of the nucleus and chromosomes (Krikorian et al., 1992; Krikorian, 1996b, 1998; Conger et al., 1998).]
Binucleate cells in systems that are normally uninucleate, chromosomal deletions, translocations, aneusomaty, microchromosomes, bridges etc. (all changes that are not generally "removed" through diplontic selection in seed-producing species and certainly not in vegetatively propagated ones) as well as cells with massive chromosome fragmentation have been encountered in cells of Space-grown somatic embryos, Space-generated roots on tissue culture-derived plantlets, Space-generated roots on cloned seedlings and in roots of seedlings (Kann et al., 1991; Levine and Krikorian, 1996). Nevertheless, all test specimens, despite the fact that they were clonal or near-isogenic, have not always shown these effects although they were nominally in the same "hardware" or growing environment (Krikorian et al., 1992; Krikorian, 1999 and in process).
Careful scrutinizing of all the data (Krikorian, 1999) indicates that there are several interacting components as to the nature of the responses encountered. What we have learned about vulnerability or responsiveness of embryogenically competent free cells at specific "phenocritically sensitive" stages provided an appealing framework for hypothesis development and testing. It also has significance for understanding some problems generally recognized by cell and tissue culture workers–namely somaclonal variation.

Somaclonal variation is generally appreciated as brought on by a number of conditions. Less emphasis has been placed on its being brought on by various aspects of the culture process (cf. e.g. van Harten 1998). In connection with the need to be absolutely certain that materials exposed by us to the Space environment were chromosomally 'perfectly normal' prior to exposure, thus eliminating any potential criticism that results were due to imperfections in the materials exposed in the first instance, extra-ordinary precautions were taken to study the chromosomal profile during each stage of the culture process.

Our data from Space suggests that cells of species with large chromosomes and DNA content with variously located centromeres (e.g. not predominantly metacentric) (like daylily) show signs of considerable perturbation. Although data has been drawn from only a few species,

cells of polyploids with large chromosomes that are essentially metacentric (like hexaploid bread wheat) show very few disturbances (cf. Tripathy et al., 1996 for other results with wheat.) . The younger the somatic embryo in terms of its developmental progression, the more sensitive it is. The more advanced, the less (carrot and daylily). The more polyploid the system the more resistant to perturbation it seems to be but the higher the DNA level in the nucleus the more sensitive it seems to be (daylily 2n vs 4n). Cells of species with small chromosomes and low DNA content like mung bean and carrot show far fewer mitotic anomalies, sometimes even none. Samplings from intact, well-defined meristems like root tips of haplopappus (2n = 4) derived from germinated seedlings show fewer aberrations than those from de novo-generated root initials produced from aseptically-generated propagules or stem cuttings. Somatic embryos produced from small developing embryogenic initials tend to show more abnormalities than cells of materials that were more-developed or advanced at the time of their exposure (carrot and daylily). The smaller the responding embryogenic initial, the greater the vulnerability to perturbation and hence they show more "damage" in space (daylily). Embryogenic cells dispersed in a semi-solid medium tend to show less perturbation than those in liquid or on semi-solid media (carrot) (Krikorian, 1996c). These details and conceptual framework should go far towards enabling the resolution of some of the discrepancies that have been emphasized in the course of attempting a consistent interpretation of results from various bits of flight data (cf. e.g. Halstead and Dutcher, 1987). All this suggests that well-controlled experiments are now finally within sight of being performed provided we take into account "all" of the variables. Most of these variables were not apparent before we achieved the present level of sophistication in the initiation, development and analysis of our embryogenic systems using free cells.

If we interpret our "Space chromosome" results in a context of additive or synergistic response to stresses in combination with specific features of "biology" and "developmental stage", and wherein a level of stress sufficient to elicit stress symptoms is not reached under ground control conditions, the picture becomes significantly clearer. It becomes even easier to appreciate if we include the fact that behavior of fluids is dramatically altered in space . Because of the dominance of surface tension/molecular attraction, and absence of gravity-driven convection, fluids tend to deposit in unexpected or undesirable ways on plant surfaces and growth media, having many affects including greatly impeded air movement (Krikorian and Levine, 1991; Levine and Krikorian, 1992a,b). In this new context of level of developmental complexity and stage sensitivity, we were able to define the parameters of stress sensitivity and response, and to resolve its basis in a cell biological context. This should later facilitate better study in a more molecular context. It would be very attractive if Space effects could be explained on the basis of additive, known effects of stress since it means that one could avoid stress by a proper matching of objectives with plant material. This would be equivalent of employing proper counter-measures, as they are called. If we view our Space findings in this context, what is happening in the Space environment is that additive levels of stresses not normally experienced on Earth and which result from "Spaceflight conditions" additively or synergistically combine to foster the reaching of critical threshold levels of the stress-inducer, whatever its exact nature(s) may be. (It would still remain of course to be determined whether several things are going on in the Space environment, including synergism between radiation and microgravity, or responses to electromagnetic disturbances etc., or whether there are a "merely" a number of hitherto inadequately un- or underappreciated, hence uncontrolled, variables the effects of which are being manifested in the course of an experiment in Space.)
All the above will contribute to our understanding of growth regulation at various levels of complexity ranging from free cells in vitro to tissues to whole plants.

X. Final Analysis

A reader informed in plant tissue work will appreciate at once that developing and implementing plant tissue and cell culture technologies for use in Space research is sure to be no mean task. All plant-related technologies, are indeed, doubly difficult to put in place and implement, since the basic aspects need to be understood well enough to render a given system reliable and cost-effective.

Some years ago plant tissue cultures were examined, albeit superficially, from the perspective of possibly providing edible material for use in a Space setting (Byrne and Koch, 1962; Hildebrandt et al., 1963). Given the then state of the art, and in view of the food preference biases and economic constraints inherent in generation of unconventional food, even algae (Krauss, 1962; Lembi and Waaland, 1988), no-one has since then seriously given thought to direct use of a higher plant tissue culture as a food source. A consideration which had not then surfaced relates to the somewhat now routine use of low levels of growth substances (some of which, like the auxins, are active as herbicides in much higher doses) in initiating and maintaining higher plant cells and tissues in vitro. There is no doubt that the Food and Drug Administration-type regulations and health considerations nowadays would preclude direct consumption of tissue cultures so grown as human food because of the potential of cultures to retain and/or complex these growth substances. Eliminating potentially toxic residues by processing procedures could solve the problem but would add yet one more detail to be contended with. Even so, one should at least mention that there are some tissue culture and molecular biology strategies on the horizon which could well eliminate this drawback (Smith and Krikorian, 1989, 1990a). From the perspective of what technology is currently available or likely to become available in the moderately near-term, it seems likely that the most reasonable position for various national Space agencies is to view tissue culture--more precisely that aspect of it which deals with multiplication from pre-formed or organized starting materials--only from the viewpoint of a potential management tool. Micropropagation, as it is called, does have some substantial merit in a Controlled Ecological Life Support System setting. Not unreasonable uses of plant tissue culture for food production could even include such things as tomato fruits being generated from flowers produced from thin layers grown in vitro (Compton and Veilleux, 1991, 1992).

The scope of information currently available renders brief analysis of the systems presently available difficult. Table 3 attempts to summarize the kinds of activities that tissue culture techniques can facilitate. Near term, intermediate and long range implications are provided.

Table 3: Range of Activities that Currently Available Tissue Culture Techniques Can Facilitate

RESEARCH WITH POTENTIAL FOR NEAR TERM IMPACT
Culture techniques can facilitate:
- Rapid multiplication of select specimens
- Elimination of virus and specific pathogens
- Virus indexing
- Germplasm introduction and evaluation
- Germplasm collection, preservation and management
- Production of polyploids, haploids, somaclonal variants for new crop production and use in breeding, etc.
- Elimination of certain breeding barriers
  - . in vitro fertilization in ovulo
  - . embryo rescue and/or storage
  - . androgenesis
  - . gynogenesis

RESEARCH WITH INTERMEDIATE IMPACT

All the above in more recalcitrant species, plus

Selection for complex traits such as tolerance to stress

. biotic - diseases and pests

. abiotic – temperature, salt, herbicides

In vitro mutation breeding

Cryopreservation

RESEARCH WITH LONG RANGE IMPLICATIONS

All the above in still more recalcitrant species, plus

Genetic engineering

. transformation by selectable genes etc.

. organelle transfer

. wide crosses-somatic hybridization

Understanding controls in developmental and physiological processes

Since no-one can predict the time scale for the impacts listed, suffice it to say that research developments could in fact change significantly the ordering presented in Table 3. A fact that will readily be obvious is, however, that "real genetic engineering," that is bona fide, substantive and controlled manipulation of the higher plant genome for anything other than single-gene traits (i.e. polygenic traits) by recombinant DNA techniques is, in my opinion at least, some time off in the future. Also, it should be stressed that few informed investigators view any of the methods as providing directly materials which are useful as products ready for introduction into the agricultural or horticultural "pipeline." Instead, and especially in those cases where tissue culture-derived or "genetically-engineered" lines are envisioned, they are more properly viewed as the starting point for further genetic manipulation via more "conventional" plant breeding technologies. These processes will shorten the time needed for implementing change in traits etc. but the whole business will more a matter of degree than in kind.

However, be all that as it may, even a casual examination of Table 4 will disclose the range of benefits that could accrue to a Controlled Ecological Life Support System program. The question therefore becomes reduced to the level of commitment to bring about feasibility. Table 4 provides an overview of some of the advantages and disadvantages of in vitro systems for Space research and development. Like many other tasks which are specific to Space agency needs, the systems would, for the most part, have to be developed for the given purpose or activity mode.

Obviously, there are some exceptions to this statement. For instance, potato can easily be micropropagated, even by the uninitiated, on a simple, hormone-free medium using a very simple procedure (cf. Espinosa et al., 1984). Wheeler et al., (1986) have used the method to initiate potato plants for their Space-related work. One can readily envision use of such a system to manage activities in Space, provided the detailed parameters for plant life support are worked out.

Cryopreservation could likewise provide, especially in the context of long duration missions, a management potential for relatively efficient germ plasm storage. Unfortunately, cryopreservation as it is currently carried out normally does not provide the high degree of reliability that would be warranted in these cases (cf. Kartha and Engelmann, 1994; Benson, 1995). And, long-term storage of germplasm in vitro without these special precautions is fraught with difficulties although one might well be surprised with the stability that can be achieved without them (Sayavedra-Soto et al., 2000).

Although many of the problems that have to be dealt with are still research-problems, many others are ripe for development. Fine tuning development problems are still to be achieved but there is not a very big gap between initiating a plant tissue culture program which is highly predictable and ultimately what one would call a "process technology", and a "product technology". While these are still largely explorative (cf. Heinstein and Emery, 1988) the time has come to move forward with more confidence.

The encouraging aspect should be that one can predict with confidence that sooner or later all of the items in Table 3 will be possible. On the other hand, and as pointed out in Table 4 below (under "disadvantages") gaining access to the advantages of these activities will not be a casual or facile strategy.

Table 4: Excised Organ, Tissue, Cell and Protoplast Systems in a Space Setting

Advantages

1. Offers a broad range of capabilities, especially access to the controlled level of prior differentiation, growth and development, depending on the system adopted.
2. Once adopted, "tissue cultures" provide a standardized way of acquiring information - e.g. in flight acquisition of engineering data for plant growth systems etc.
3. Offer several options for clean "management" of biomaterials. Enables a "Modular Approach" to both basic and applied research.
4. Tissue culture systems provide the necessary interface between molecular biology and bio-engineering and these will ultimately permit a fuller understanding of plant development, growth, productivity etc., so crucial to a effort.

Disadvantages

1. Labor-intensive as “tissue culture” is generally and currently practiced. But effort beginning to be automated (cf. Holdgate and Zandvoort, 1992).
2. Development of experimental material is not a casual activity. It is a process not an event.
3. All species are not yet equally amenable to the full range of manipulations. Technologies, if they are to be generally applicable, obviously cannot be restricted to only certain species.

Similarly, it will be understood that conventional breeding or at least an awareness of what is available from traditional breeders will also have a role to play in a Controlled Ecological Life Support System effort. Plants that are smaller or "miniaturized" and hence require smaller space for growth, have shorter life cycles or specific stages of development, or have features such as low light or other specific physiological needs will obviously be useful in research.

XI. Outlook

Modest but realistic plans should be encouraged by the various Space agencies as soon as possible to develop tissue culture capabilities so that carefully orchestrated Controlled Ecological Life Support System-specific “tissue culture” work can be undertaken. In each instance, a detailed and thoughtful plan could be delineated within the framework of a sustained, long-term effort.

In those cases where specific pathogen-free or indexed propagules are seen to be of direct benefit, micropropagation should be undertaken and integrated. It will be important to start off modestly--e.g. with potato nodal or similar cuttings and to analyze systematically and at each step along the way the benefits that accrue from the use of in vitro methods as a tool.

Because tissue cultures are small, compact, relatively well controlled, and can be grown in large numbers, tissue culture capability provides an opportunity to select plants for use in growth chamber experiments. The idea here is that in vitro systems grown in modes conducive to eliciting change (so-called culture-associated or somaclonal variation), could well provide a convenient means of identifying plantlets or plants that can cope more efficiently within a given chamber or closed environment. Since there will be a long term need to determine the various levels of flexibility and/or constraints in growing and managing plants in the Space environment, such tissue culture-generated plants could play an important role in the operations. Certainly, in seeking an answer as to how to get a design for a "test bed" or facilities for model testing, one can use cultured tissues in addition to seed-generated plantlets. One major advantage that cultured systems afford is that by being miniaturized they

can facilitate obtaining answers to questions on such matters as: How do you provide the needed support for plants in Space?; What is the photosynthetic capacity of plants in Space?; Will plants produce acceptable yields in Space? etc. (cf. e.g. Bugbee, 1999).

As in all efforts, success with tissue cultures will depend on:

- -Investigators and support personnel with adequate scientific background and training
- -Financial resources
- -A bureaucracy sympathetic to research with a "lead-time" to "development" and administrators able to facilitate implementation of programs
- -Recognition not only of capabilities but limitations of "tissue culture" techniques
- -Teamwork at individual, institutional, local, national and international level
- -Maximum integration with other disciplines
- -Frequent and open communication

From the perspective of more traditional breeding, the most reasonable recommendation in the initial stages of such work would be that no special attempt be made to incorporate breeding -whether mutational (van Harten, 1998) or conventional (Baker, 1986;Richards, 1997)- directly into such programs. Instead, as needs arise or are anticipated, specific investigators or experts on a given species could be sought out with the view of ascertaining what "amenable" Space-biology plant germ plasm already exists, or what would be required to generate or "design" a variety or form that could serve a stated objective more effectively. In all this, however, it will be apparent that there will be trade-offs. The adage that "one is constantly seeking models which are 'models' for something other than themselves" should constantly be kept in mind.

References

Abbott, A.J. and R.K. Atkin, (Eds.) (1987): Improving Vegetatively Propagated Crops. Academic Press, London.

Arditti, J. and A.D. Krikorian (1996): Orchid micropropagation: the path from laboratory to commercialization and an account of several unappreciated investigators. Botanical Journal of the Linnean Society 122, 185-241.

Bajaj, Y.P.S. (Ed.) (2000): Biotechnology in Agriculture and Forestry. 44. Transgenic Trees. Springer-Verlag, Berlin.

Baker, R. J. (1986): Selection Indices in Plant Breeding. CRC Press, Boca Raton, FL.

Barlow, P.W. (1995): Gravity perception in plants: a multiplicity of systems derived by evolution? Plant, Cell and Environment 18, 951-962.

Bayvel, L. and Z. Orzechowski (1993): Liquid Atomization. Taylor & Francis. Washington, D.C.

Benedikt, E.T. (Ed.) (1960): Weightlessness- Physical Phenomena and Biological Effects. Plenum Press, NY.

Benson, E.E. (1995): Cryopreservation of shoot-tips and meristems. Pages 121-132. In: Cryopreservation and Freeze-drying Protocols, (J. G. Day and M. R. McLellan, eds.), Methods in Molecular Biology 38. Humana Press, Totawa, NJ.

Brown, A.H. (1996): Gravity related features of plant growth behavior studied with rotating machines. Journal of Gravitational Physiology 31, 69-74.

Bugbee, B. (1999): Engineering plants for spaceflight environments. American Society for Gravitational and Space Biology Bulletin. 12, 67-74.

Byrne, A.F. and R.B. Koch (1962): Food production by submerged culture of plant tissue cells. Science 135, 215-216.

Claassen, D.E. and B.S. Spooner (1994): Impact of altered gravity on aspects of cell biology. International Review of Cell Biology 156, 301-373.

Cogoli, A. and F.K. Gmünder (1991): Gravity effects on single cells. Advances in Space Biology in Medicine 1, 183-248.

Cogoli, A. and A. Tschopp (1982): Biotechnology in space laboratories. Advances in Biochemical Engineering 22, 1-50.

Compton, M.E. and R.E.Veilleux (1991): Shoot, root and flower morphogenesis on tomato inflorescence explants. Plant Cell, Tissue and Organ Culture 24, 223-231.

Compton, M.E. and R.E. Veilleux (1992): Thin cell layer morphogenesis. Horticulture Reviews 14, 239-264.

Conger, B.V., Z. Tomaszewski Jr., J.K. Macdaniels and A. Vasilenko (1998): Spaceflight reduces somatic embryogenesis in orchardgrass (Poaceae). Plant, Cell and Environment 21, 1197-1203.

Corey, K.A. and R.M. Wheeler (1992): Gas exchange in NASA's biomass production chamber. BioScience 42, 503-509.

Digby, J. and R.D. Firn (1995): The gravitropic set-point angle (GSA): the identification of an important developmentally controlled variable governing plant architecture. Plant, Cell and Environment 18, 1434-1440.

Doran, P.M. (1993): Design of reactors for plant cells and organs. Advances in Biochemical Engineering Biotechnology 48, 115-168.

Durzan, D.J. (2000): Metabolic engineering of plant cells in a Space environment. Biotechnology and Genetic Engineering Reviews 17, 349-383.

Dutcher, F. R., Hess, E. L. and Halstead, T. W. 1994. Progress in plant research in space. Advances in Space Research 14, 159-171.

Espinoza, N., R. Estrada, P. Tovar, J. Bryan and J.H. Dodds (1984): Tissue Culture Micropropagation, Conservation, and Export of Potato Germplasm. International Potato Center (CIP). Lima, Peru.

Greenberg, R. and B.R. Tufts (2001): Infecting other worlds. American Scientist 89, 296-299.

Goins, G.D., H.G. Levine, C.L. Mackowiak, R.M. Wheeler, J.D. Carr and D.W. Ming (2000): Comparison studies of candidate nutrient delivery systems for plant cultivation in Space. SAE (Society of Automotive Engineers Technical Paper Series 972304) 7 pages.

Halstead, T.W. and F.R. Dutcher (1987): Plants in space. Annual Review of Plant Physiology 38, 317-345.

Heinstein, P. and A. Emery (1988): Processes with plant cell cultures. In: Biotechnology (H.-J. Rehm and G. Reed, eds.), Vol. 6b, pp. 213-248. VCH, NY.

Hildebrandt, A.C., J.C. Wilmar, H. Johns and A.J. Riker (1963): Growth of edible chlorophyllous plant tissues in vitro. American Journal of Botany 50, 248-254.

Holdgate, D.P. and E.A. Zandvoort (1992): Automated micropropagation and the application of a laser beam for cutting. Pages 297-311. In: Transplant Production Systems : Proceedings of the International Symposium on Transplant Production Systems, Yokokama, Japan, 21-26 July 1992; (K. Kurata and T. Kozai, eds.). Kluwer Academic Publishers, Dordrecht.

Hoshizaki, T. (1973): Influence of gravitational forces on plants. Environmental Biology and Medicine 2, 47-79.

Hussey, G. and N.J. Stacey (1981): In vitro propagation of potato (Solanum tuberosum L.). Annals of Botany 48, 787-796.

Kanellis, A.K. et al. (Eds.) (1997): Biology and Biotechnology of the Plant Hormone Ethylene. Kluwer Academic, Dordrecht and Boston.

Kann, R.P., S.A. O'Connor, H.G. Levine, and A:D. Krikorian (1991): Generation and multiplication of plantlets from callus derived from capitulum of Haplopappus gracilis (Nutt.) Gray and their karyotype analysis. Plant Science 75, 245-255.

Kartha, K.K. and F. Engelmann (1994): Cryopreservation and germplasm storage. In: Plant Tissue Culture, (I. K. Vasil and T.A. Thorpe, eds.), pp. 194-230; Kluwer Academic Publishers, Dordrecht, Netherlands.

Keefe, J.R. and A.D. Krikorian (1983): Gravitational biology on the space platform. Paper presented in the 13th Intersociety Conference on Environmental Systems. 11-13 July 1983, San Francisco, CA. SAE (Society of Automotive Engineers Technical Paper Series 831133) 23 pages.

Kliss, M. and R.D. MacElroy (1990): Salad machine: a vegetal production unit for long duration missions. Paper presented in the 20th Intersociety Conference on Environmental Systems 9-12 July, Williamsburg, Va. SAE (Society of Automotive Engineers Technical Paper Series 901280) 23 pages.

Komamine, A., R. Kawahara, M. Matsumoto, S. Sunabori, T. Toya, A. Fujiwara, M. Tsukahara, J. Smith, M. Ito, H. Fukuda, K. Nomura and T. Fujimura (1992): Mechanisms of somatic embryogenesis in cell cultures: Physiology, Biochemistry, and Molecular Biology. In Vitro Cell Dev. Biol. 28, 11-14.

Kozai, T., K. Fujiwara, M. Hayashi and J. Aitken-Christie (1992): The in vitro environment and its control in micropropagation, In: Transplant Production Systems. (K. Kurata and T. Kozai, eds.) pp. 247-282. Kluwer Academic Publishers, Dordrecht and Boston.

Krauss, R.W. (1962): Mass culture of algae for food and other organic compounds. American Journal of Botany 49, 425-435.

Krikorian, A.D. (1991): Embryogenic plant cells in microgravity. American Society for Gravitational and Space Biology Bulletin 4, 65-72.

Krikorian, A.D. (1996a): Strategies for “minimal growth maintenance” of cell cultures: A perspective on management for extended duration experimentation in the microgravity environment of a space station. Botanical Review 62, 41-108.

Krikorian, A.D. (1996b): Embryogenic somatic cell cultures of daylily (Hemerocallis): A system to probe spaceflight-associated mitotic disturbances. In: Plants in Space Biology (H. Suge, ed.), pp. 111-126. Institute of Genetic Ecology, Tohoku University, Sendai.

Krikorian, A.D. (1996c): Gravity and the stability of the differentiated state of plant somatic embryos (PEMBSIS). In: IML-2 Final Report of IML-2 Experiments, pp. 163-178. Space Experiment Department, Office of Space Utilization Systems, National Space Development Agency of Japan. NASDA-TMR-960004.

Krikorian A.D. (1996d): Space stress and genome shock in developing plant cells. Physiologia Plantarum 98, 901-908.

Krikorian, A.D. (1997): Commercializing micropropagated ferns. Singapore National Academy of Sciences (SNAS) Journal 24/24, 84-96.

Krikorian, A.D. (1998): Plants and somatic embryos in Space: What have we learned? American Society for Gravitational and Space Biology Bulletin 11(no.2), 5-14.

Krikorian, A.D. (1999): Somatic embryos of daylily in space. Advances in Space Research (32nd COSPAR Scientific Assembly, Nagoya, Japan) 23, 1987-1997.

Krikorian, A.D. (2000): Historical insights into some contemporary problems in somatic embryogenesis. In: Somatic Embryogenesis in Plants. (S. Mohan Jain, P. K. Gupta and R. J. Newton, eds.) Volume 6, 17-49. Kluwer Academic Publishers, Dordrecht.

Krikorian, A.D., F. R. Dutcher, C.E. Quinn and F.C. Steward (1981): Growth and development of cultured carrot cells and embryos under space flight conditions. In: Advances in Space Research Volume l, 117-127. W.R. Holmquist (Ed.). Pergamon Press, Oxford, NY.

Krikorian, A.D., H. Irizarry, S.S. Cronauer-Mitra and E. Rivera (1993): Clonal fidelity and variation in plantain (Musa AAB) regenerated from vegetative stem and floral axis tips in vitro. Annals of Botany 71, 519-535.

Krikorian, A.D., H. Irizarry, R. Goenaga, M.E. Scott and B.E.L. Lockhart (1999): Stability in plant and bunch traits of a French-type dwarf plantain micropropagated in vitro from the floral stem tip and five lateral corm tips of a single mother plant: Good news on the tissue culture and bad news on banana streak virus. Scientia Horticulturae 81, 159-177.

Krikorian, A.D. and H.G. Levine (1991): Development and growth in Space. Pages 491-555. In: Plant Physiology: A Treatise (R.G.S. Bidwell and F.C. Steward, eds.) Vol. X. Academic Press, NY.

Krikorian, A.D., H.G. Levine, R.P. Kann and S.A. O'Connor (l992): Effects of spaceflight on growth and cell division in higher plants. In: Advances in Space Biology and Medicine, (S.L. Bonting, ed.), Vol 2, pp. 181-209. JAI Press, Greenwich, CT.

Krikorian, A.D. and L.K. Simola (1999): Totipotency, somatic embryogenesis and Harry Waris (1893-1973). Physiologia Plantarum 105, 348-345.

Krikorian, A.D. and D.L. Smith (l992): Somatic embryogenesis in carrot (Daucus carota. In: Plant Tissue Culture Manual: Fundamentals and Application, (K. Lindsey, ed.), pp. PTCM-A9 1-32, Kluwer Academic Publishers, Dordrecht.

Lembi, C.A. and J.R. Waaland (1988): Algae and Human Affairs. Cambridge University Press, Cambridge.

Levine, H.G. and A:D. Krikorian (l992a): Chromosomes and plant cell division in space: Environmental conditions and experimental details. Advances in Space Research 12, 73-82.

Levine, H.G. and A.D. Krikorian (1992b): Shoot growth in aseptically cultivated daylily and haplopappus plantlets after a 5-day spaceflight. Physiologia Plantarum 86, 349-359.

Levine, H.G. and A.D. Krikorian (l996): Root growth in aseptically cultivated plantlets of Haplopappus gracilis after a five day spaceflight. Journal of Gravitational Physiology 31, 17-27.

Lindsey, K. (1992): Plant Tissue Culture Manual. Kluwer Academic Publishers, Dordrecht.

Markert, C.L. and A.D. Krikorian (1993): Life: Origin and evolution on earth; How can we escape? In: "A Scientific Role for Space Station Freedom - How Space Platforms Can Serve Research in Life Sciences". AIAA-92-1348. Presented at a Symposium at AIAA [American Institute of Aeronautics and Astronautics Space Programs and Technologies Conference] Huntsville, Alabama 24 March 1992. NASA Technical Memorandum 4502, pp. 35-39.

Meyerowitz, E. M. (Ed.) (1994): Arabidopsis. Monograph 27. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mitchell, C.A. (1993): The role of bioregenerative life-support systems in a manned future of space. Transactions of the Kansas Academy of Sciences 96, 87-92.

Mordhorst, A.P., K.J. Voerman, M.V. Hartog, E:A. Meijer, J. van Went, M. Koorneeff and S. De Vries (1998): Somatic embryogenesis in Arabidopsis thaliana is facilitated by mutations in genes repressing meristematic cell divisions. Genetics 149, 549-563.

Musgrave, M.E., A. Kuang and S.W. Matthews (1997): Plant reproduction during spaceflight: importance of the gaseous environment. Planta 203, S177-S-184.

Nechitailo, G.S. and A.L. Mashinsky (1993): Space Biology. Studies at Orbital Stations. Translated by N. Lyubinov. Mir Publishers, Moscow.

Neumann, K.-H. (1995): Pflanzliche Zell- und Gewebekulturen. Eugen Ulmer Verlag, Stuttgart.

Nielsen, S.S., M.A. Belury, K.P. Nickel and C.A. Mitchell (1996): Plant nutrient composition altered with controlled environments for future space life-support systems. In: Progress in New Crops, (J. Janick, ed.), pp. 624-632, ASHS Press, Arlington, VA.

Pennell, R.I., S. De Vries and K. Roberts (1992): Identification of a transitional state in the developmental pathway to carrot somatic embryogenesis. Journal of Cell Biology 119, 1371-1380.

Pirie, N.W. (1980): Space victualling. Endeavour, N.S. 4, 74-77.

Prince, C.L., V. Bringi and M.L. Shuler (1991): Convective mass transfer in large porous biocatalysts: plant organ cultures. Biotechnology Progress 7, 195-199.

Raghavan, V. (1997): Molecular Embryology of Flowering Plants. Cambridge University Press, Cambridge and NY.

Raghavan, V. (2000): Developmental Biology of Flowering Plants. Springer Verlag, NY.

Ream, W. and S.B. Gelvin (1996): Crown gall: Advances in Understanding Interkingdom Transfer. American Phytopathological Society, St. Paul, MN.

Reynolds, P.H.S. (Ed.) (1999): Inducible gene Expression in Plants. CABI Publishing, Oxford and NY.

Richards, A.J. (1997): Plant Breeding Systems. 2nd ed. Stanley Thomas, London.

Sachs, T. (1991): Pattern Formation in Plant Tissues. Cambridge University Press, Cambridge.

Sack, F.D. (1991): Perception of gravity by plants. International Review of Cytology 127, 193-252.

Sack, F.D. (1998): Autotropism, automorphogenesis and gravity. Physiologia Plantarum 102, 328-335.

Salisbury, F.B. (1991a): Lunar farming: Achieving maximum yield for the exploration of space. Hort. Science 26, 827-833.

Salisbury, F.B. (1991b): Some challenges in designing a Lunar, Martian, or microgravity CELSS. Acta Astronautica 27, 211-217.

Sayavedra-Soto, L., A.D. Krikorian and M. Reporter (2000): Long term stability of electromanipulated protoplasts of Glycine max var. Acme and a Catharanthus roseus mutant. Journal of Plant Physiology 156, 137-140.

Smith, D.L., K. Kelly and A.D. Krikorian (1989): Ethylene-associated phase change from juvenile to mature phenotype of daylily (Hemerocallis) in vitro. Physiologia Plantarum 76, 466-473.

Smith, D.L. and A.D. Krikorian (1989): Release of somatic embryogenic potential from excised zygotic embryos of carrot and maintenance of proembryonic cultures in hormone-free medium. American Journal of Botany 76, 1832-1843.

Smith, D.L. and A.D. Krikorian (1990a): pH Control of carrot somatic embryogenesis.. In: Progress in Plant Cellular and Molecular Biology. Proceeding's, VII International Congress on Plant Tissue and Cell Culture, (H.J.J. Nijkamp, L.H.W. Van der Plas and J. van Aartrijk, eds.), pp. 449-453, Kluwer Academic, Dordrecht, Netherlands.

Smith, D.L. and A.D. Krikorian (1990b): Low external pH replaces 2,4-D in maintaining and multiplying 2,4-D-initiated embryogenic cells of carrot. Physiologia Plantarum 80, 329-336.

Smith, D.L. and A.D. Krikorian (1991): Growth and maintenance of an embryogenic cell culture of daylily (Hemerocallis) on hormone-free medium. Annals of Botany 67, 443-449.

Smith, D.L. and A.D. Krikorian (l992): Low external pH prevents cell elongation but not multiplication of embryogenic carrot cells. Physiologia Plantarum 84, 495-501.

Soh, Woong-Young and S.S. Bhowjwani, (Eds.) (1999): Morphogenesis in Plant Tissue Cultures. Kluwer Academic Publishers, Dordrecht.

Thorpe, T.A. (Ed.) (1995): In Vitro Embryogenesis in Plants. Kluwer Scientific, Dordrecht and Boston.

Tripathy, B.C., C.S. Brown, H.G. Levine and A.D. Krikorian (1996): Growth and photosynthetic responses of wheat plants grown in Space. Plant Physiology 110, 801-806.

van Harten, A.M. (1998): Mutation Breeding. Theory and Practical Applications. Cambridge University Press, Cambridge.

Vasil, I.K. and T.A. Thorpe. (Eds.) (1994): Plant Tissue Culture. Kluwer Academic, Dordrecht.

Vroemen, C., S. De Vries and R. Quatrano (1999): Signalling in plant embryos during the establishment of the polar axis. Cell & Developmental Biology 10, 157-164.

Weidenfeld, J. (2000): Towards Defining a Functional System for Somatic Embryogenesis. Ph.D. Thesis, State University of New York at Stony Brook, Stony Brook, New York. 297 pages.

Wheeler, R.M., K.L. Steffen, T.W. Tibbitts and J.P. Palta (1986): Utilization of potatoes for life support systems II. The effect of temperature under 24-H and 12-H photoperiods. American Potato Journal 63, 639-647.

Wilson, P.E.G. and M.G. Hilton (1995): Plant cell bioreactors. Pages 413-439. In: Bioreactor System Design, (J.A. Asenjo and J. Merchuk, eds.), Marcell Dekker, NY.

Wilson, Z.A. (Ed.) (2000): Arabidopsis: A Practical Approach. Oxford University Press, Oxford.

Wolverton, B.C., R.C. McDonald and W.R. Duffer (1983): Microorganisms and higher plants for waste water treatment. Journal of Environmental Quality 12, 236-242.

Wolverton, B.C., R.C. McDonald and E.A. Watkins (1984): Foliage plants for removing indoor air pollutants from energy-efficient homes. Economic Botany 38, 223-228.

Zimmermann, U., R. Schnettler and K. Hannig (1988): Biotechnology in space: Potentials and perspectives.. In: Biotechnology. Vol. 6b. Special Microbial Processes, (H.- J. Rehm and G. Reed, eds.), pp. 639-672 VCH Verlagsgesellschaft, Weinheim.