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Space, the final frontier: A critical review of recent experiments performed in microgravity

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Abstract

Space biology provides an opportunity to study plant physiology and development in a unique microgravity environment. Recent space studies with plants have provided interesting insights into plant biology, including discovering that plants can grow seed-to-seed in microgravity, as well as identifying novel responses to light. However, spaceflight experiments are not without their challenges, including limited space, limited access, and stressors such as lack of convection and cosmic radiation. Therefore, it is important to design experiments in a way to maximize the scientific return from research conducted on orbiting platforms such as the International Space Station. Here, we provide a critical review of recent spaceflight experiments and suggest ways in which future experiments can be designed to improve the value and applicability of the results generated. These potential improvements include: utilizing in-flight controls to delineate microgravity versus other spaceflight effects, increasing scientific return via next-generation sequencing technologies, and utilizing multiple genotypes to ensure results are not unique to one genetic background. Space experiments have given us new insights into plant biology. However, to move forward, special care should be given to maximize science return in understanding both microgravity itself as well as the combinatorial effects of living in space.

Keywords

Experimental controls; International Space Station; Microgravity; Space biology; Next-generation sequencing

1. Introduction

Utilization of plant growth facilities on orbiting space platforms such as the International Space Station (ISS) provides researchers with an opportunity to perform novel and exciting experiments in a multitude of plant science disciplines. These facilities also allow for assessing the potential of plants to have a role in long-distance space travel or extra-planetary habitation. Microgravity also provides a unique environment to assess plant physiological responses related to gravity, such as the gravitropic and phototropic responses. The conditions of microgravity are generated via freefall conditions, where the speed of the object matches the effect of gravity, effectively canceling gravity's pull. This microgravity environment can be achieved in multiple ways, including drop-towers, parabolic flights of

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airplanes, sounding rockets or orbiting space platforms (e.g., the ISS), and has been previously reviewed [1]. The study of physiological responses to a prolonged microgravity environment had not been possible prior to the spaceflight era, and plants had not been exposed to a reduced gravity vector throughout natural history. Many reviews have given updates on the state of plant space biology at the time of the publication [2–6].

In addition to studies in basic plant biology, space research provides an opportunity to study bioregenerative life support. A bioregenerative life support system must be developed to provide renewable oxygen and a source of food to astronauts for long distance space travel or interplanetary habitation to be possible. Plants have the potential to fulfill these roles; however, research is still needed to further enhance our understanding of how plants respond to the unique or challenging conditions of spaceflight. Some of these conditions include limited growth space, exposure to high levels of cosmic radiation [7–9], lack of a unilateral gravity vector, reduced nutrient sustainability, among many others. An additional challenge involves limited access and available space on orbiting platforms, resulting in long waiting time during or between experiments. Additionally, if a space experiment is unsuccessful, repeating the experiment is extremely difficult due to the logistical and financial constraints. It is for these reasons that thoughtful planning is imperative to maximize the scientific return of space flight experiments. Here, we take a critical look at recent space experiments and provide suggestions for increasing the scientific return on experiments performed in microgravity environments. We limit our considerations to experiments performed in true microgravity (or free fall) conditions and do not consider analogs to microgravity such as clinostats or random positioning machines [reviewed in 10].

2. Importance of controls in space experiments

Many experiments conducted upon orbiting space platforms (International Space Station, NASA's Space Shuttle, Sounding Rockets, etc.) lack important 1-*g* in-flight controls. These controls are used to simulate the gravity of Earth and are created using centrifuges on board the orbiting platforms. Not having this control leads to difficulty in distinguishing between true microgravity effects and effects derived from other aspects of space flight (radiation exposure, vibration, etc.). Additionally, many spaceflight effects can be the product of improper hardware design or conditions, which often result in deleterious effects such as poor gas exchange, ethylene buildup (due to the lack of convection in space) as well as the lack of adequate temperature control, among others. In fact, some older facilities, such as the Biorack on the space shuttle, have shown the utility of a 1-*g* in-flight control to distinguish deleterious environmental effects of spaceflight from microgravity such as the build-up of ethylene gas (Fig. 1) [11]. To account for these secondary (or indirect) spaceflight effects, the use of 1-*g* in-flight controls can be implemented.

NASA-developed spaceflight hardware to grow plants on board the International Space Station includes the Biological Research in Canisters (BRIC) and Petri Dish Fixation Unit (PDFU) system [12] as well as the Advanced Biological Research System (ABRS) [13]. Neither of these facilities is capable of providing a 1-*g* in-flight control because of their design. In addition, the Russian space agency Roscosmos utilizes a greenhouse facility termed Lada, where they have previously demonstrated the ability to produce multiple

generations of pea plants [14]. However, this facility also lacks 1-*g* in-flight control capability. In contrast, an example of hardware that allows for 1-*g* in flight control is the European Modular Cultivation System (EMCS) [6]. The EMCS also provides gas exchange and ethylene removal, which also help to mitigate non-microgravity induced spaceflight effects. However, the EMCS is not without its limitations, which include the ability to house only relatively small plants or seedlings.

A recent study utilized the BRIC–PDFU system to assess the effects of microgravity on root hair development in *Arabidopsis* [15]. Seedlings were grown for 2 weeks in the BRIC system on board the space shuttle, followed by transcription profiling comparing spaceflight and ground controls. The authors acknowledged the limits of the BRIC–PDFU system—such as potential hypoxic environment and lack of ethylene removal, and suggest confounding variables may be present in the expression profiles of seedlings grown within the system. The lack of an in-flight control may potentially result in an increase in falsely identifying genes that are related to microgravity exposure, but these genes also could instead be the results of another spaceflight effect (e.g., ethylene exposure). Additionally, the potential non-microgravity spaceflight effects in the BRIC–PDF system are increased by the lack of ability to initiate germination in flight (seeds germinate on the ground prior to launch), exposing the germinating seedlings to the stresses of vehicle launch. The BRIC–PDF system was utilized with subsequent RT-qPCR and microarray experiments to analyze differences in transcription between cell cultures and whole plants flown in microgravity conditions for 12 days [16]. There was a much more dramatic transcription response in cell cultures when compared to ground control, however the lack of a 1-*g* in-flight control limits the ability to determine if the cell culture response is due to microgravity or other spaceflight influences or due to the stresses of launch.

Another study compared the results using the Biological Research in Canisters and Petri Dish Fixation Unit hardware in spaceflight using two controls, both of which were on the ground. Compared to a control in standard laboratory petri dishes, the utilization of the Biological Research in Canisters hardware resulted in reduced endodermal cell size (Table 1) [17]. The experiment further illustrates the importance in separating the direct effects of spaceflight compared to indirect (secondary) effects, including the influence of flight hardware.

There may be similar limitations in studies conducted in the ABRs. For instance, the ABRs were used to study the effects of spaceflight effects on the proteome of *Arabidopsis* plants grown on the International Space Station [18]. Over 1500 tissue-specific (leaf or root) proteins were expressed in spaceflight samples when compared to ground controls. However, because of the hardware system, no 1-*g* in-flight control was available, limiting the ability to decipher microgravity effects versus other spaceflight effects. The lack of a 1-*g* in-flight control is not uncommon in the NASA hardware suite. Few facilities provide the ability to perform a 1-*g* in-flight control, leaving investigators with limited options available to perform important spaceflight controls.

The EMCS was used for transcriptome analysis of *Arabidopsis* seedlings exposed to microgravity [19]. The experiment had an on-board centrifuge for 1-*g* in-flight controls, as

well as the 1 *g* ground controls. More than 200 genes were differentially regulated at least 2-fold between ground and spaceflight. *Oryza sativa* calli flown aboard the Chinese Shenzhou 8 spacecraft identified 157 genes differentially expressed between 1-*g* in-flight controls versus 1-*g* ground controls, while 678 were differentially expressed 1 *g* and microgravity flight samples [20]. These results suggest that spaceflight effects, not directly related to microgravity, potentially influence a large suite of genes, solidifying the need for 1-*g* in-flight controls if accurate identification of true microgravity responsive genes is to be achieved. Additionally, a study of *Arabidopsis* calli grown in another Chinese space experiment revealed substantial differences in protein expression between ground controls, 1-*g* in-flight control, and microgravity effects [21]. The study found that when comparing differently expressed proteins, the plants in the in-flight 1-*g* control had 30% of these proteins in common with the microgravity treatment. However, when comparing microgravity-grown calli to ground controls, the number of differentially expressed proteins in common dropped to 15%. To help mitigate the issue with spaceflight effects versus microgravity effects, future hardware development should focus on the inclusion of components that allow for creation of simulated gravity, thereby permitting investigators to include 1-*g* in-flight controls in their experimental design.

However, it must be noted that current facilities for 1-*g* in-flight control are not without their own limitations. For example, the size of the hardware that holds the sample can affect the gravity gradient along the radial access of rotation [22]. Thus, larger sample containers will experience a larger gradient of gravity, exposing portions of the plant to dissimilar gravity. In addition, inertial shear forces created by the centrifuge act in the direction of rotation and introduce a stimuli not experienced by 1-*g* ground controls [23]. This issue is particularly troublesome for studies of free-floating specimen such as aquatic plants. Shear force can be minimized by increasing the radius of the centrifuge; however, no such facility currently exists on the International Space Station [24].

3. New technologies to study gene expression

As noted above, there has been considerable focus on the effects of spaceflight on the plant genome, largely, how microgravity conditions changes gene and protein expression. Most of these experiments utilized microarray technology to identify changes in gene expression. These microarray experiments have provided a wealth of data on the effects of microgravity on the genome. Still, they are limited in a variety of ways. First, a microarray chip must be available for your model organism, and development of a custom microarray chip can be costly. In addition, development of microarrays also requires an existing knowledge about the organism's genome. Second, genes with high sequence identity may result in non-specific hybridization to the microarray, resulting in high background levels and thereby leading to an inaccurate measure of gene expression. Microarray analysis is not as sensitive for identifying low copy number transcripts when compared to sequencing technologies [25]. Lastly, comparing expression levels across different experiments is challenging in part due to a difficulty in normalization methods [26].

The previously mentioned proteomics study [18] compared protein expression to gene transcription (via microarray) of a previous, similar experiment. Not surprisingly, there was

little correlation between the two types of analyses, with only 34 of the 546 genes being differentially expressed at both transcript and protein levels. These results are likely due to the difficulties that arise with normalization of microarray data across different experiments. There was a significant correlation between transcript and protein abundance in these 34 genes exhibiting differential expression.

The first study to use next-gen sequencing techniques (mRNA-Seq) for gene expression analysis on plants exposed to microgravity compared the differences in transcription of Mizuna (*Brassica rapa* var. *nipposinica*) grown on the ISS to plants in 1 *g* ground controls [27]. Twenty genes specific to the reactive oxygen species (ROS) pathway were upregulated, which is a common response to biotic and abiotic stress. With the genomic resources available for common space flight model species (*Arabidopsis*, *Oryza*, *Brassica*, etc.), there is now an opportunity to increase the scientific data return via utilization of next-gen technologies.

In addition to transcriptomics, next generation sequencing provides the capability to study epigenomics as well, such as gene methylation and histone modifications [28,29]. DNA methylation is an epigenetic mechanism employed within the cell to regulate gene expression, as well as silence transposable elements [30]. Often, regulation via DNA methylation is a response to environmental factors, affecting overall gene expression. Methylated genes are then replicated and subsequently methylated via DNA methyl-transferases. Bisulfate sequencing, a next generation sequencing technique, provides the capability to detect DNA methylation at a single-base resolution [31,32]. Along with DNA methylation, histone modifications are shown to be an epigenetic regulator of gene expression [33]. Chromatin immunoprecipitation sequencing (ChIP-seq) fragments the DNA associated with histones and utilizes next gene sequencing techniques to identify histone locations within the genome [29,34]. ChIP-seq technology is still in its infancy, and is not without its challenges [34]. However, epigenetic sequencing techniques such as ChIP-seq and bisulfate sequencing can provide valuable insight into how a novel environment such as microgravity can affect epigenetic processes, and, thereby, differential gene expression.

4. The use of diverse genotypes and species

The concept of genotype or ecotype-specific responses to microgravity environments is another component of spaceflight experiments that has been largely unexplored. The vast majority of experiments conducted in true microgravity conditions focus on one, or a few, selected genotypes in the model plant and very small *Arabidopsis thaliana*. These genotypes are often selected because they are well characterized, grow robustly in the lab, and often have sequenced genomes. The focus on a single genotype is largely due to the need for extensive replication requiring the use of small plants due to space limitations on board orbiting space platforms. However, results from microgravity experiments must be scrutinized due to the limited number of genotypes utilized and the potential for genotype-specific results. For example, a mutation to the *fla* gene delayed flowering in *Arabidopsis* ecotype Landsberg erecta (LER), but this same altered phenotype was not present in *fla* mutants of ecotype Columbia erecta (COL) [35]. Similarly, a study conducted on two different ecotypes of *Arabidopsis* (Columbia and Cape Verde Islands) exposed to elevated

CO₂ levels revealed that the magnitude of various biological responses, including photosynthesis, starch accumulation, nitrogen content and differential gene expression, varied between the two ecotypes [36]. Ecotypic differences like this are well documented, and have shown to influence flowering time [37], disease resistance [38] as well as oxidative stress tolerance [39], all of which are important aspects of a bioregenerative life support system and potential food source.

Additionally root phenotypes such as meristem zone, root elongation zone and root mature cell length all differed by up to 100% in distinct *Arabidopsis* ecotypes [40]. The root elongation zone has a central role in root phototropic and gravitropic responses. Thus, it would not be unreasonable to suggest that these root responses could vary significantly among different genotypes. The concept of genotype-specific results is further supported in a study which demonstrated differences in the blue-light-based phototropic responses in roots and shoots of seedlings of the COL, LER, and C24 ecotypes of *Arabidopsis* [41].

The growth and development of four genotypes of *A. thaliana* were compared in an older spaceflight study. Some interesting differences in growth were observed (Table 2) [42]. Most space-flight studies in recent years have focused on single genotypes of *A. thaliana* [15,17,19,43,44]. Comparing results to develop a complete physiological picture is difficult due to the use of multiple ecotypes.

In addition to diverse genotypes, diversity in terms of plant species will be important in constructing a bioregenerative life support system [2,3]. To date, the majority of species studied upon orbiting platforms such as the International Space Station have been model species such as *A. thaliana*, *B. rapa*, and other low biomass species which are well-fitted to the tight confines available to date. However, as part of a bioregenerative life support system, plant will be required to provide a source of food for astronauts, as well as sufficient photosynthesis to continually provide a renewed source of oxygen. For this to occur, larger, edible crops will likely be necessary to create a fully functional life support system. It must be noted, however, that when utilizing crops for bioregenerative life support, genetic diversity is again crucial. Utilization of multiple species and/or genotypes can help to mitigate the potential for disease to affect the life support system.

5. Conclusions

Recent experiments in plant space biology have provided a wealth of knowledge pertaining to physiology, genetics and genomics of plants grown under microgravity conditions. Despite any limitations we may cite, these space experiments clearly have provided unique insights into many areas of plant biology. Nevertheless to move forward, it is important to utilize sufficient controls (when possible) to decouple true microgravity effects from other spaceflight effects to obtain the full value of science conducted on board orbiting spacecraft. Special care also should be given to understanding microgravity itself as well as the combinatorial effects of living in space. In addition, scientific return can be maximized by moving to new, next-gen technologies that are more sensitive and widely applicable than microarrays. Lastly, experiments should aim to expand to multiple eco/genotypes for plants studied under microgravity conditions. This approach would help to ensure results are not

specific to a single genetic background. Fortunately, we are aware of upcoming experiments on the International Space Station that are addressing some of these critical issues.

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Fig. 1.

Adequate controls delineate hardware and microgravity effects. Hypocotyls of *Arabidopsis thaliana* Landsberg seedlings from the Biorack spaceflight project and the controls. The in-flight centrifuge provided a valuable control, which showed a clear environmental effect from the space experiment, rather than a true microgravity effect. Figure is modified from [7]. A. Exaggerated hypocotyl hook from a seedling grown in-flight in microgravity (F- μg). B. Seedling with an exaggerated hypocotyl from the in-flight, 1- g control performed with the on-board centrifuge (F-1 g). C. Seedling with a normal hypocotyl from the ground control (G-1 g). Scale bar = 0.5 mm.

Table 1

Comparison of endodermal cells in petioles of *Arabidopsis* seedlings in microgravity versus the controls. Results are from a spaceflight experiment (using the BRIC hardware) and controls. Seedlings from the spaceflight experiment (FL) were compared to a ground control (GC), which both were in spaceflight hardware. To assay any potential effects from growth in spaceflight hardware, we performed another control by growing seedlings in Petri dishes in standard laboratory conditions (termed the hardware control, HC). Endodermal cells were significantly smaller in seedlings grown in spaceflight hardware (FL and GC) compared to those in the HC. The change in the shape of the cell was one parameter that appears to be a true microgravity effect.

Measurement	Mean $\pm$ Standard error		
	Flight (FL)	Ground (GC)	Hardware control (HC)
Length (μm)	62.7 ^a $\pm$ 3.3	59.8 ^a $\pm$ 2.7	88.9 ^b $\pm$ 8.7
Perimeter (μm)	166.7 ^a $\pm$ 8.4	134.7 ^a $\pm$ 8.2	246.4 ^b $\pm$ 24.0
Area (μm^2)	657.6 ^a $\pm$ 63.5	697.6 ^a $\pm$ 44.6	2032.8 ^b $\pm$ 518.6
Roundness [#]	0.41 ^a $\pm$ 0.02	0.26 ^b $\pm$ 0.01	0.28 ^b $\pm$ 0.01

The values are means (μm) $\pm$ standard error, except for the roundness value[#] (in which 1.0 = perfect circle). Means followed by different letters indicate a significant difference ($P < 0.05$) as indicated by an ANOVA/Tukey-Kramer post-test. Sample size was 10 seedlings for each treatment. Table is modified from [17].

Table 2

Mean length (mm $\pm$ SE) of *Arabidopsis* seedlings following 81 h of growth during a spaceflight experiment using the Biorack hardware. The values in parentheses are the number of seedlings. Means followed by different letters indicate statistical differences as determined by an ANOVA/Tukey test ($P < 0.05$). Table is modified from [42].

ECOTYPE	Flight-81 h
Columbia (Col)	3.4 ^a $\pm$ 0.2 (20)
Wassilewskija (Ws)	5.4 ^b $\pm$ 0.3 (24)
Landsberg (Ler)	5.6 ^b $\pm$ 0.3 (19)
C24	4.8 ^b $\pm$ 0.3 (18)