

Induction of hypoxic root metabolism results from physical limitations in O₂ bioavailability in microgravity

J. Liao^a, G. Liu^a, O. Monje^b, G.W. Stutte^b, D.M. Porterfield^{a,c,*}

^a Department of Biological Sciences, University of Missouri-Rolla, 105 Schrenk Hall, 1870 Miner Circle, Rolla, MO 65409, USA

^b Dynamac Corporation, Kennedy Space Center, FL 32780, USA

^c Department of Electrical and Computer Engineering, University of Missouri-Rolla, Rolla, MO 65409, USA

Received 19 October 2002; received in revised form 8 July 2003; accepted 11 July 2003

Abstract

Numerous spaceflight experiments have noted changes in the roots that are consistent with hypoxia in the rootzone. These observations include general ultrastructure analysis and biochemical measurements to direct measurements of stress specific enzymes. In experiments that have monitored alcohol dehydrogenase (ADH), the data shows this hypoxically responsive gene is induced and is associated with increased ADH activity in microgravity. These changes in ADH could be induced either by spaceflight hypoxia resulting from inhibition of gravity mediated O₂ transport, or by a non-specific stress response due to inhibition of gravisensing. We tested these hypotheses in a series of two experiments. The objective of the first experiment was to determine if physical changes in gravity-mediated O₂ transport can be directly measured, while the second series of experiments tested whether disruption of gravisensing can induce a non-specific ADH response. To directly measure O₂ bioavailability as a function of gravity, we designed a sensor that mimics metabolic oxygen consumption in the rhizosphere. Because of these criteria, the sensor is sensitive to any changes in root O₂ bioavailability that may occur in microgravity. In a KC-135 experiment, the sensor was implanted in a moist granular clay media and exposed to microgravity during parabolic flight. The resulting data indicated that root O₂ bioavailability decreased in phase with gravity. In experiments that tested for non-specific induction of ADH, we compared the response of transgenic *Arabidopsis* plants (ADH promoted GUS marker gene) exposed to clinostat, control, and waterlogged conditions. The plants were grown on agar slats in a growth chamber before being exposed to the experimental treatments. The plants were stained for GUS activity localization, and subjected to biochemical tests for ADH, and GUS enzyme activity. These tests showed that the waterlogging treatment induced significant increases in GUS and ADH enzyme activities, while the control and clinostat treatments showed no response. This work demonstrates: (1) the inhibition of gravity-driven convective transport can reduce the O₂ bioavailability to the root tip, and (2) the perturbation of gravisensing by clinostat rotation does not induce a non-specific stress response involving ADH. Together these experiments support the microgravity convection inhibition model for explaining changes in root metabolism during spaceflight.

© 2004 COSPAR. Published by Elsevier Ltd. All rights reserved.

Keywords: Spaceflight; Plant; Root; Hypoxia; Oxygen

1. Introduction

Plants encounter sources of undocumented stress during spaceflight. Recent experiments have sought to understand these responses by monitoring the expression and activity of the stress-induced enzyme alcohol

dehydrogenase (ADH). The first of this work was conducted in two separate flight experiments, where plants were exposed to microgravity during 6 or 11 days of spaceflight (Porterfield et al., 1997). Post-flight analysis of the *Arabidopsis thaliana* root samples included measurement of ADH activity, localization, and expression. When flown for 6 days, root ADH enzyme activity increased 90% in the spaceflight roots. Analysis of the root materials from the 11-day experiment indicated that an 89% increase in enzyme activity was associated with a

* Corresponding author. Tel.: +1-573-341-6336; fax: +1-573-341-4821.

E-mail address: mporterf@umr.edu (D.M. Porterfield).

136% increase in ADH mRNA. Dwarf wheat and *Brassica rapa* root samples were analyzed and also indicated that ADH activity increased significantly as a result of exposure to the spaceflight environment (Porterfield et al., 2000). For dwarf wheat root, ADH activity increased nearly 3-fold, while in *B. rapa* the increase was 6-fold when compared with controls. *B. rapa* was also studied during the Collaborative Ukrainian Experiment (Stout et al., 2001) and included plants at various stages of development. Some plants were germinated in orbit, while others were pre-grown for 13 days before launch. To better understand if the changes in the ADH activity were due to induction of hypoxic fermentative metabolism, ADH and pyruvate decarboxylase (PDC) activities were measured. In roots of plants that were in the vegetative stage, ADH increased by 50% while PDC activity increased by only 9%. In the older flowering plants, root ADH and PDC activities increased by almost 5-fold and 1.5-fold, respectively.

To understand qualitative changes in root ADH gene expression a transgenic *Arabidopsis* plant, transformed with a DNA construct composed of the β -Glucuronidase (GUS) gene driven by the *Arabidopsis* ADH promoter, was flown in a flight experiment. In the transgenic *Arabidopsis* plants exposed to microgravity the cytochemical staining patterns of GUS activity indicated the ADH promoted gene was activated in the root tips of the plant during spaceflight (Paul et al., 2001). This is similar to the results observed in previous experiments (Porterfield et al., 1997; Porterfield et al., 2000; Stout et al., 2001) obtained using direct cytochemical localization of ADH activity.

Whole plant GUS staining patterns of spaceflight exposed plants were also compared to hypoxic controls (Paul et al., 2001). The plants that were exposed to rootzone flooding/hypoxia expressed ADH in the root tips and the shoot apex, which is consistent with previous reports. Dolferus et al. (1994) showed this identical pattern of ADH gene expression in rootzone flooded *Arabidopsis* plants using the ADH promoted GUS gene. In comparison to ground hypoxia controls, the spaceflight exposed plants differed in the lack of expression in the shoot apex. This indicates that either the signal transduction between the roots and shoots is disrupted in microgravity, or ADH induction in the roots is an anomaly and not indicative of true root system hypoxia.

In addition to hypoxia and anoxia, ADH can also be induced by salt, temperature, and soil drying (Dolferus et al., 1994; Jarillo et al., 1993; Naidoo et al., 1992; Russell and Sachs, 1992). In all of the spaceflight experiments that have been conducted, control treatment plants were exposed to growth conditions that were identical to those experienced during spaceflight, except for microgravity and acceleration. Therefore, none of the known stress factors explain the changes in ADH activity observed in these spaceflight experiments. The

fact that ADH activity increases can be elicited by other types of environmental stress does support the interpretation that ADH increases may be the result of a non-specific stress response (Paul et al., 2001), possibly related to the plant's inability to detect a gravity stimulus.

The work presented here was designed to differentiate between two competing hypotheses to explain spaceflight induction of ADH. The biophysical oxygen limitation (BOL) hypothesis states the ADH is induced in microgravity due to spaceflight hypoxia caused by the alteration of normal gravity dependent behaviors in liquids and gases. Fundamental to this is the disruption of gravity-dependent buoyancy-driven thermal convection. Without such mechanisms to facilitate gas exchange for metabolism, the bioavailability of O₂ to the roots would be limited to diffusional flux. Alternatively, the observed root ADH changes could result from a non-specific stress response initiated by the plants inability to sense gravity. Since ADH can be induced by other forms of environmental stress, it is possible that it could be induced non-specifically as part of a general response. Furthermore the plants inability to orient growth by gravity sensing might initiate such a non-specific response. We have tested these hypotheses in a series of two different experiments. First, we developed a new sensor technology to measure root oxygen bioavailability, and used it during a KC-135 flight experiment to determine if microgravity does inhibit gas exchange. We also conducted experiments with transgenic *Arabidopsis* plants (ADH promoted GUS reporter gene) to determine if disruption of gravity sensing by clinostat rotation can induce ADH.

2. Materials and methods

2.1. Root oxygen bioavailability sensor

Given the fact that the oxygen concentration does not change in microgravity, a standard oxygen concentration sensor could not be used, as it would not detect changes in non-diffusionary transport. Instead, a sensor was constructed that simulates the relative oxygen consumption activity and geometry of a growing root tip. Because of these design criteria we would expect that this root oxygen bioavailability (ROB) sensor would be sensitive to changes in convective oxygen transport, as would an actual root. For these experiments, a generic ROB sensor was constructed according to the diagram in Fig. 1. A gold wire with a diameter of 500 μ m is used as the cathode and a silver wire with a diameter of 250 μ m, electroplated with chloride ions, is used as a reference. During operation the cathode is polarized to -750 mV and the resulting current is related to oxygen transport and availability in the surrounding media.

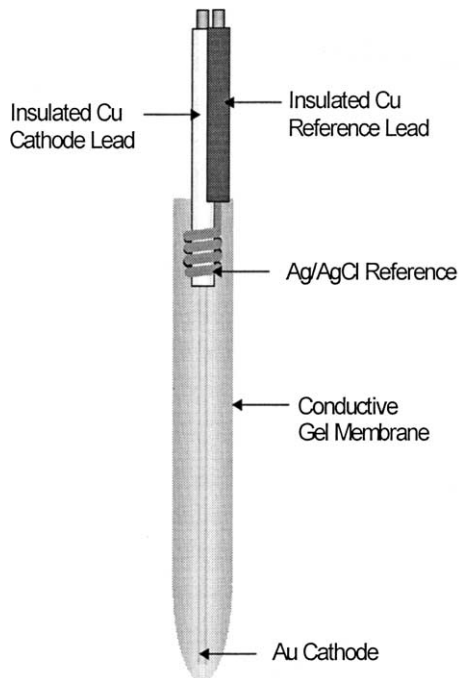


Fig. 1. Construction of a root oxygen bioavailability sensor. Both the reference and cathode are contained within a common solution and membrane allowing the sensor to operate in both aqueous and aerial environments. The gel membrane system and the geometry of the cathode are designed to allow the sensor to consume oxygen in a manner that simulates the activity of an actual root.

This design is a modification of a Clark-style electrode; however with some significant innovations. Both the Clark and Whalen style electrodes are constructed to be stir-insensitive by application of a gas permeable membrane over a very small cathode. This effectively increases the resistance of the electrode to a level where the consumption of oxygen in the electrode can no longer significantly impact the concentration of oxygen in the medium that is being measured (Schneiderman and Goldstick, 1978). In other words, it is diffusion limited and thereby stir-insensitive. This modification, which renders the electrode stir-insensitive, is necessary to insure that the electrode does not actually change what it is supposed to be measuring. In microgravity the actual concentration of oxygen does not change, but what does potentially change is the activity or bioavailability. So the requirement of the application to microgravity research requires a drastically different approach. The ROB sensor is designed to be stir-sensitive, just as an actual root is. This insures the sensor is sensitive to changes in oxygen activity, or bioavailability, as well as direct changes in oxygen concentration. Again, it is the changes in bioavailability that we are concerned with in microgravity. This modification is achieved by increasing the cathodic surface area and by using a new conductive gel membrane system. The gel membrane is

constructed using a 5% polyacrylimide gel made with 100 mM KCl.

Basic calibration experiments with these sensors demonstrate that the sensor does report changes in oxygen bioavailability associated with mechanical convection and oxygen concentration (Fig. 2). Later experiments were conducted to directly measure root oxygen bioavailability as a function of gravity during a KC-135 microgravity experiment (Monje et al., 2000). During the experiment the KC-135 flew four groups of 10 parabolas. The sensor was embedded in a container with water saturated arcillite plant growth medium and the sensor readings were recorded along with data from an accelerometer.

2.2. Clinostat experiments

Transgenic *Arabidopsis* plants containing the ADH promoted GUS construct were graciously provided by Rudy Dolferus (Dolferus et al., 1994). Dry, vernalized seeds were sterilized in microcentrifuge tubes with a 60 s wash in 70% (v/v) ethanol, followed by a wash in a 30% (v/v) bleach solution (1.576% sodium hypochloite) with approximately 1 μ l/ml triton X-100 surfactant for 15 min. The bleach solution was removed in a laminar flow hood with a sterile transfer pipette, and then the seeds were rinsed 8–10 times with sterile water.

The seedlings were germinated and the plants grown on the vertical surface in petri plates containing solid phytigel media containing Murashige and Skoog basic

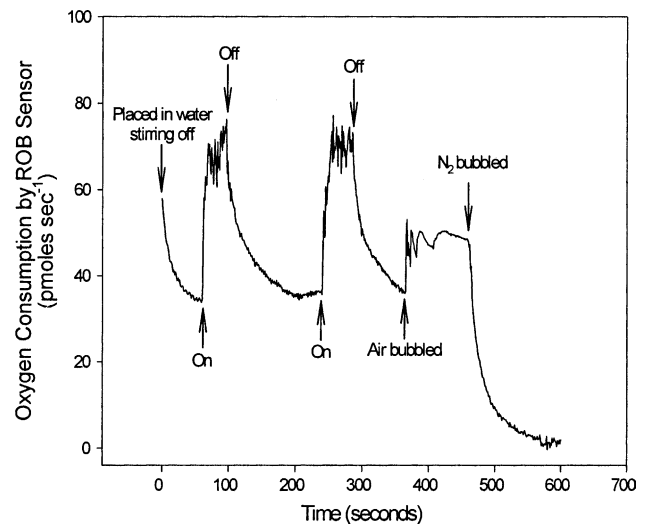


Fig. 2. The use of a root oxygen bioavailability sensor to measure and report changes in convection based oxygen transport in an aqueous medium. Note the difference between oxygen availability in stirred (rapid mechanical convection) and air bubbled (moderate mechanical convection) conditions. For this particular experiment, a generic sensor was used to test basic construction techniques and the performance of the conductive gel membrane system.

salts according to the procedures described by Paul et al., 2001). Plants were grown in continuous light (300–400 $\mu\text{mol m}^{-2} \text{s}$) at 24–26 °C prior to the experiments.

During the experiment the plants were exposed to one of three treatments that included: (1) clinostat rotation of 1 rpm, (2) root zone hypoxia accomplished by flooding with nutrient solution to submerge the roots, and (3) control plants that were maintained in the vertical position without flooding. The three sets of plants were assayed at 0, 12, 24, 36 and 48 h. For the enzyme activity assays, the roots were homogenized in extraction buffer (50 mM NaPO_4 [pH 7.0], 10 mM EDTA, 0.1% sarkosyl, 0.1% Triton X-100, and 10 mM β -mercaptoethanol). The homogenate was centrifuged in a microcentrifuge for 15 min, and the supernatant was used for both the GUS and ADH assays. GUS was measured using the procedures outlined by Jefferson et al. (1987), while the ADH assays were performed according to the method described by Xie and Wu (1989).

3. Results

The ROB sensor technology was used in the KC-135 flight experiment to test the buoyancy-driven convection hypothesis. The objective was to make direct physical measurements of the changes in oxygen bioavailability that may occur in a root matrix due to microgravity inhibition of bioavailability. The ROB sensor biosimulates the relative oxygen consumption activity and geometry of a growing root tip. Because of these design criteria the ROB sensor is sensitive to changes in connective oxygen transport, in a manner that is equivalent to an actual root. The results of this experiment aboard the KC-135 show that oxygen bioavailability is directly modulated in phase with the microgravity/gravity profiles measured by the accelerometer on the KC-135 aircraft (Fig. 3). During the experiment, data was recorded while the KC-135 flew four groups of 10 parabolas. Upon initiation of the first parabola the sensor output immediately responded by showing a marked decrease

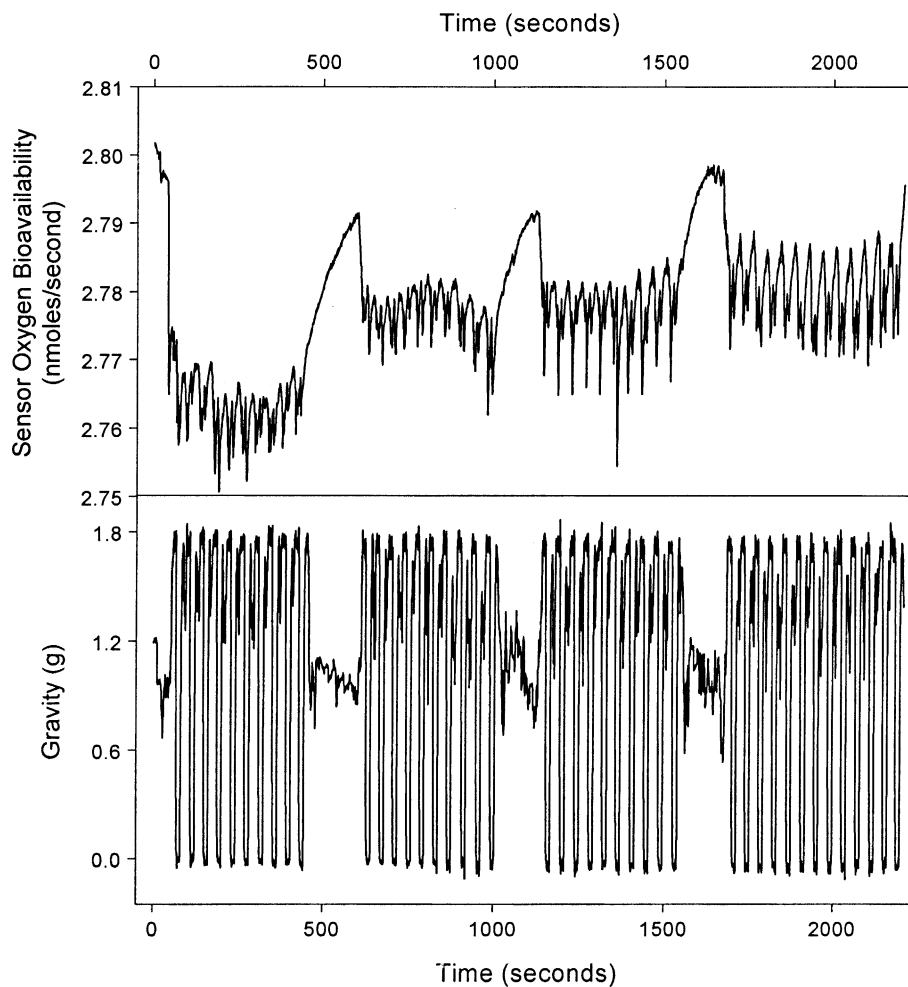


Fig. 3. Data showing the direct measurement of root oxygen bioavailability (top panel) as a function of gravity (bottom panel) during a KC-135 microgravity experiment. During the experiment data was recorded while the KC-135 flew four groups of 10 parabolas.

in output. The output of the probe then shows 10 individual cycles corresponding to the 10 parabolas followed by a return to 1 g and the slow recovery of the sensor back to the original ambient 1 g value. This pattern was repeated during three more groups of 10 parabolas that show similar responses. These are direct physical measurements, which show that, for a root growing in a partially wet clay matrix nutrient delivery system, the impact of inhibiting the convective component of oxygen transport to the root surface is substantial.

To determine if disruption of gravity sensing could initiate a non-specific stress response involving ADH, we conducted clinostat experiments using transgenic *Arabidopsis* plants. In the experiment we exposed plants to 48 h of clinostat rotation, rootzone hypoxia, or static control conditions and measured resulting GUS and ADH activity. GUS activity (Fig. 4, Panel A) increased significantly in roots exposed to hypoxia and was statistically different from both the clinostat and control plants. The clinostat and control did differ statistically in

the 12 and 24 h sample but not in the 36 and 48 h materials. ADH activity (Fig. 4, Panel B) also increased as a result of rootzone flooding. In contrast the clinostat and control plants were statistically the same as one another at all sampling times and did not indicate that there was any stress response in the tissue. We also examined the qualitative changes in whole plant ADH promoted GUS staining (data not shown). The analysis indicated that there was no GUS staining in the roots or shoots of either the control or clinostat rotated plants. In comparison the hypoxically treated plants expressed staining in both the root and shoot tips.

4. Conclusions

The ROB sensor was designed to measure oxygen bioavailability as opposed to concentration. This is important because the absolute oxygen concentration is not influenced by gravity. The sensor biosimulates the general consumption parameters of an actual root and is dependent upon non-diffusionary, gravity dependent mechanism to transport oxygen within the soil medium. The sensor reported that there were indeed measurable changes in oxygen bioavailability in microgravity. These changes occurred in phase with the data obtained from the accelerometer. The clinostat experiments allowed us to determine if ADH can be induced as part of a non-specific stress response resulting from the plants' inability to sense gravity. The results indicate that this was not the case as neither quantitative nor qualitative measurements of ADH and GUS activity revealed any significant difference between clinostat and stationary control plants. Furthermore both of these treatments were statistically different from plants exposed to rootzone hypoxia.

These experiments sought to better understand the nature of ADH induction in spaceflight exposed roots by directly testing two conflicting hypotheses to explain this phenomenon. Based on the results presented here we can conclude that the induction of ADH in spaceflight is not part of a non-specific stress responses, but instead is indicative of true spaceflight hypoxia caused by microgravity induced biophysical limitations.

What is the nature of the biophysical limitations of space and why does it induce spaceflight hypoxia in the roots? The exchange of small molecules, including ions, gases, and water, between plants and the environment are subject to the physical behavior of molecules outside of the plant. Gravity exerts significant influence on this process and thereby mediates much of the exchange of small molecules by the plant. Buoyancy driven thermal convection is driven by gravity and directs bulk movement of fluids and gases in the environment. Without this process availability of gases or dissolved solutes becomes limited to that which can be provided by

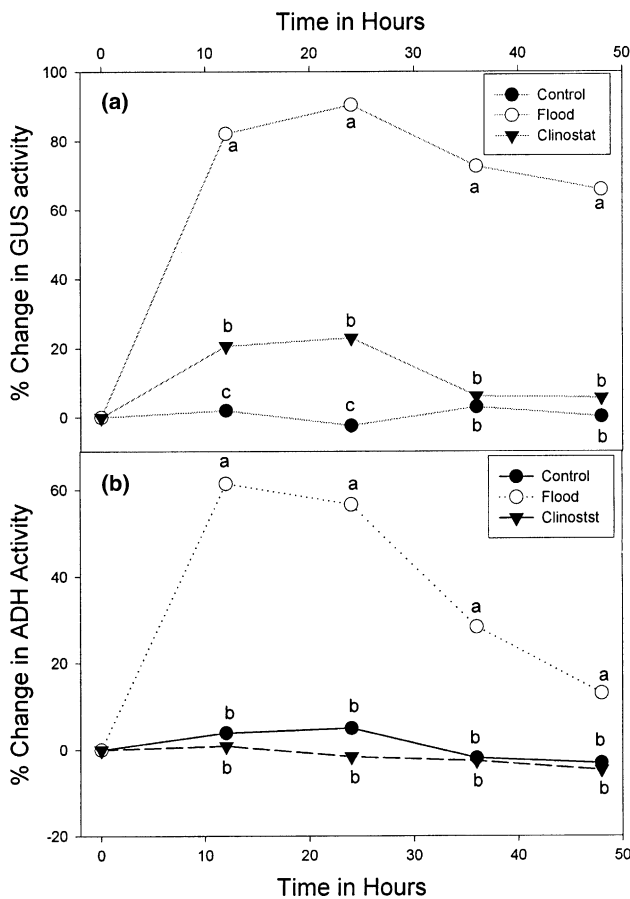


Fig. 4. GUS and ADH root activities in plants exposed to clinostat, flooding or control conditions. For each sampling three replicate measurements of five roots each were conducted. The data was analyzed by ANOVA ($P \leq 0.05$) and statistically significant values are indicated by different letters.

diffusion. This is especially important in plants, fungi, bacteria, and any other biological systems that cannot mechanically ventilate to drive gas exchange. The impact of diffusion limited exchange is significant. For example, a candle flame will take on a spherical shape and will burn for only 30–40 s (Ross et al., 1991) before being extinguished by lack of available oxygen. Without gravity, oxygen availability to the flame becomes diffusion limited and does not provide for adequate environmental exchange. Similar limitations in mass transport and exchange do occur in biological systems exposed to microgravity, as shown in physical mathematical models (Porterfield, 2002).

Acknowledgements

This work was supported by funding from the Missouri Research Board, and the USDA/NRICGP (2001-35100-10751) to DMP.

References

- Dolferus, R., Jacobs, M., Peacock, W.J., Dennis, E.S. Differential interactions of promoter elements in stress responses of the *Arabidopsis* ADH gene. *Plant Physiol.* 105, 1075–1087, 1994.
- Jefferson, R.A., Kavanagh, T.A., Bevan, M.W. GUS fusion: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907, 1987.
- Jarillo, J.A., Leyva, A., Salinas, J., Martinez-Zapater, J.M. Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidopsis thaliana*, a chilling-tolerant plant. *Plant Physiol.* 101, 833–837, 1993.
- Monje, O., Porterfield, D.M., Stutte, G.W. Boundary layers around plant leaf and root tissues depend on gravity. *Grav. Space Biol. Bull.* 14, 25, 2000.
- Naidoo, G., McKee, K.L., Mendelssohn, I.A. Anatomical and metabolic responses to waterlogging and salinity in *Spartina alterniflora* and *S. patens* (Poaceae). *Am. J. Bot.* 79, 765–770, 1992.
- Paul, A.L., Daugherty, C.J., Bihn, E.A., Chapman, D.K., Norwood, K.L.L., Ferl, R.J. Transgene expression patterns indicate that spaceflight affects stress signal perception and transduction in *Arabidopsis*. *Plant Physiol.* 126, 613–621, 2001.
- Porterfield, D.M., Matthews, S.W., Daugherty, C.J., Musgrave, M.E. Spaceflight exposure effects on transcription, activity, and localization of alcohol dehydrogenase in the roots of *Arabidopsis thaliana*. *Plant Physiol.* 113, 685–693, 1997.
- Porterfield, D.M., Barta, D.J., Ming, D.W., Morrow, R.C., Musgrave, M.E. Astroculture root metabolism and cytochemical analysis. *Adv. Space Res.* 26, 315–318, 2000.
- Porterfield, D.M. The biophysical limitations in physiological transport and exchange in plants grown in microgravity. *J. Plant Growth Regul.* 21, 177–190, 2002.
- Ross, H.D., Sotos, R.G., T'ien, J.S. Observation of candle flames under various atmospheres in microgravity. *Combust. Sci. Technol.* 75, 155–160, 1991.
- Russell, D.A., Sachs, M.M. Protein synthesis in maize during anaerobic and heat stress. *Plant Physiol.* 99, 615–620, 1992.
- Schneiderman, G., Goldstick, T.K. Oxygen electrode design criteria and performance characteristics: recessed cathode. *J. Appl. Physiol.* 45, 145–154, 1978.
- Stout, S.C., Porterfield, D.M., Briarty, L.G., Kuang, A., Musgrave, M.E. Evidence of rootzone hypoxia in *Brassica rapa* L. grown in microgravity. *Int. J. Plant Sci.* 162, 249–255, 2001.
- Xie, Y., Wu, R. Rice alcohol dehydrogenase genes: anaerobic induction, organ specific expression and characterization of cDNA clones. *Plant Mol. Biol.* 13, 53–68, 1989.