Changes in Soluble Sugar, Starch, and Alcohol Dehydrogenase in Arabidopsis thaliana Exposed to N₂ Diluted Atmospheres

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Proper exchange of atmospheric gases is important for normal root and shoot metabolism in plants. This study was conducted to determine how restricted air supply affects foliar carbohydrates, while using the marker enzyme alcohol dehydrogenase (ADH) to report on the oxygenation status of the rootzone. Fourteen-day-old Arabidopsis thaliana (L.) Heynh. plants grown singly in 7-ml tubes containing agarified nutrient medium were placed in coupled Magenta vessels and exposed for six days to either ambient air or one of six different air/nitrogen dilutions. Redox potential of the agar medium was measured immediately after harvesting and freezing leaf tissue, and then root systems were quickly extracted from the agar and frozen for subsequent analyses. Redox potential measurements indicated that this series of gas mixtures produced a transition from hypoxia to anoxia in the root zones. Root ADH activity increased at higher rates as the redox potential neared anoxic levels. In contrast, ADH mRNA expression quickly neared its maximum as the medium became hypoxic and showed little further increase as it became anoxic. Foliar carbohydrate levels increased 1.5- to 2-fold with decreased availability of metabolic gases, with starch increasing at higher concentrations of air than soluble carbohydrate. The results serve as a model for plant performance under microgravity conditions, where absence of convective air movement prevents replenishment of metabolic gases.

Key words: Alcohol dehydrogenase — Arabidopsis thaliana — Carbohydrate metabolism.

Biological systems evolved on earth and are dependent upon gravity-mediated physical processes, such as buoyancy-driven convection, particle sedimentation, isothermal settling, diffusion, surface tension, and particle streaming for normal physiological function (Todd 1989). Today with access to spaceflight-based research, scientists can now investigate the effects of microgravity exposure on plants. Plants exposed to microgravity during spaceflight have been reported to show general signs of poor health (Halstead and Dutcher 1984, 1987) most likely due to the disruption of these gravity dependent physical processes. Specific spaceflight-induced aberrations suggest changes in metabolism associated with reduced oxygen and carbon dioxide availability. At the ultrastructural level, modifications in mitochondrial shape and size have been noted (Rubin et al. 1980, Tairbekov et al. 1980, Slocum et al. 1984, Moore 1990, Podlutsky 1992, Rasmussen et al. 1992, Kordyum 1994, Laurinavicius et al. 1994) along with a general decrease in amyloplast starch reserves (Volkman et al. 1986, Moore 1990, Podlutsky 1992, Rasmussen et al. 1992, Kordyum 1994, Laurinavicius et al. 1994). Both mitochondrial ultrastructure (Oliveira 1977) and tissue starch reserves (Hanson and Jacobsen 1984, Hurng and Kao 1993, Guglielminetti et al. 1995) have been shown to change as a result of exposure to reduced oxygen environments. Reduction in starch levels can also be the result of limiting CO₂ concentrations.

In 1993, our lab began a series of short duration experiments on growth and development in Arabidopsis under spaceflight conditions. Following a six day microgravity exposure during STS-54 mid-deck locker experiment CHROMEX-03, foliage was very low in carbohydrate compared with the ground controls (Brown et al. 1993) and analysis of root enzymes indicated that spaceflight roots had been deprived of oxygen relative to the controls (Porterfield et al. 1997). These results suggested that oxygen and carbon dioxide were less available to the plants in microgravity than in the ground controls, and can be explained by the hypothesis that the absence of convective air movement in microgravity could give rise to stagnant air layers around the plant that would then restrict replenishment of gases consumed by metabolic activity. Based on this hypothesis we set up a series of experiments that attempted to duplicate the growing conditions for CHROMEX-03, and to investigate the relationship between atmospheric gas concentrations and metabolism in Arabidopsis.

Materials and Methods

Plant growth conditions—Arabidopsis thaliana (L.) Heynh var. ‘Columbia’ plants were seeded and grown in coupled Magenta vessels (Magenta Corp., Chicago, IL) under a continuous 300 μmol m⁻² s⁻¹ PAR light source, as described by Kuang et al. (1995). At thirteen days, the plants were placed under a continuous 50-60 μmol m⁻² s⁻¹ PAR light source to simulate the light

Abbreviations: ADH, alcohol dehydrogenase; EN, Earth normal; PAR, photosynthetically active radiation.
levels in the spaceship plant growth hardware (Krikorian and Levine 1992). When the plants were fourteen days old, the Mena-
gta vessels were connected to compressed gas sources and purged
continuously at a flow rate of 32–35 ml min⁻¹ (Crispi et al. 1996).
To determine the amount of growth during a six-day run, some
fifteen day old plants (herein designated as Day-0) were harvest-
et and analyzed (9 plants per unit, 1 unit per run, 3 runs total) for
changes in dry weight and agar redox potential. Six days later,
the plants that were exposed to the control and experimental atmos-
pheres were removed from the Magenta vessels and analyzed for
changes in dry weight, agar redox potential, carbohydrate com-
position, and ADH activity and expression. For each run, tempera-
ture and relative humidity were monitored in one of the twelve
units (Crispi et al. 1996).

**Experimental system**—The experimental system consisted of
12 modified, coupled Magenta units (Magenta Corp., Chicago,
IL) arranged in three groups of four units with each group receiv-
ing a different atmospheric treatment (Crispi et al. 1996). Of the
four units receiving a particular treatment, three contained plants
(9 per unit, 27 total) and one contained the non-seeded, agar-medi-
ated control tubes (9 per unit). In all, 9 runs were completed, con-
sisting of 9 runs of the 100% Earth normal control (breathing air,
average composition: 215 mmol mol⁻¹ O₂ + 400 μmol mol⁻¹ CO₂
in N₂ and 3 runs of each of the following treatments: 4 mmol
mol⁻¹ O₂ + 8 μmol mol⁻¹ CO₂ in N₂; 34 mmol mol⁻¹ O₂ + 66
μmol mol⁻¹ CO₂ in N₂; 58 mmol mol⁻¹ O₂ + 108 μmol mol⁻¹ CO₂
in N₂; 118 mmol mol⁻¹ O₂ + 220 μmol mol⁻¹ CO₂ in N₂; 148
mmol mol⁻¹ O₂ + 276 μmol mol⁻¹ CO₂ in N₂; and, 187 mmol
mol⁻¹ O₂ + 348 μmol mol⁻¹ CO₂ in N₂). To simplify this report,
these compositions will be hereafter referred to as 2%, 16%, 27%,
55%, 69%, and 87% Earth normal (EN) atmospheres, respective-
ly. Gas handling and monitoring were conducted according to the
procedures outlined by Crispi et al. (1996).

**Analysis**—Day-0 plants (harvested and analyzed at fourteen
days old) and plants harvested at the end of the six day run were
removed from each coupled Magenta unit, divided into compo-
nent parts (leaf, floral stem, and root) and grouped by unit for
analysis. After removal and weighing of leaves and floral stems,
the redox potential of the agar medium of each tube was measured
using a needle-tipped platinum calomel redox probe (JS-30101-15,
Sargent Welch Scientific Co.). Roots were then rinsed from the
agar medium, blotted, and weighed. Both the leaf and root sam-
ples of the plants exposed to the control and experimental atmos-
pheres for six days were frozen in liquid nitrogen and stored at
-65°C until further analysis could be completed.

The leaf samples were subsequently lyophilized overnight and
ground using a mortar and pestle. Twenty five mg aliquots of each
of these samples were then placed into lock top microcentrifuge
tubes with 1.25 ml of 80% EtOH and heated at 90°C for 5 min
with intermittent vortexing. The tubes were then centrifuged at
3,000 rpm in a microcentrifuge for 10 min. This ethanol extrac-
tion step was repeated three times. The supernatant solutions
were removed and evaporated to dryness using a vacuum evaporator
and subjected to soluble sugar analysis (Jones et al. 1977). The
pellet was saved for starch analysis according to the procedures

The frozen root samples were rapidly homogenized with-
out a buffer using a Micromincer apparatus (Biospec products,
Bartlesville, OK) that was preheated to -20°C. The homogenate
was extruded into a microcentrifuge tube and a subsample was
taken for total RNA isolation (Total RNA isolation procedure
used with the EcoR1 DNA probe). The homogenate was then
in one ml of 0.14 M urea at 37°C. The reaction was allowed to
run for 40 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for
30 s) followed by 10 min at 72°C in a Perkin-Elmer thermal cy-
crater (ABI 240). The PCR products were separated by gel
ethrophoresis (1.5% agarose gel, 1 × TBE buffer at 80 vol
1% for 1 h). Competent bacteria were cultured in vitro using
the SP6 polymerase included in the BrightStar BiotinScript in
vitro transcription kit (Ambio-

**The results data were analyzed using one-
way ANOVA and/or the standard error of the means (P =
0.05) using the statistical analysis tools add-in component of
Microsoft Excel (Redmond, WA).

**Results**

**Plant growth**—Plant fresh weight gains (Fig. 1) de-
creased as the EN atmosphere was diluted with N₂. For the
leaves and stems, the 2% and 16% EN treatments were sta-
tistically different from each other and from all other treat-
ments and there were no significant differences between the
69%, 87%, and 100% EN treatments. While root fresh
weight change decreased in response to the increased dilu-
tion of the EN atmosphere, these changes in root fresh
weight were not as great as the changes in stem and leaf
fresh weight.
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Fig. 1 Mean change in fresh weight (±S.E.) for total plant, leaves, floral stems, and roots for Arabidopsis plants grown in seven atmospheric treatments. For all treatments and parameters, n=9, except for 69% (n=8 for root and total fresh weight) and 100% Earth normal (n=24 for all parameters).

Agar redox potential—To determine changes in root-zone oxygenation, agar redox potential was measured immediately after opening the chambers (Fig. 2). Both with and without plants the mean redox potential decreased in the agar-based medium as oxygen and the percent EN atmosphere decreased. The greatest plant mediated changes in redox potential were seen in 100% and 87% EN treatments. Redox potentials readings between 330 and 650 mV are considered to be hypoxic while readings below 330 mV indicate the absence of free O₂ (Marshner 1995). Based on these values, the redox data suggest that these experimental treatments represent a transition from hypoxic to anoxic conditions in the rooting medium. The medium is gradually becoming more hypoxic as the percent EN atmosphere decreases to 27% and anoxic conditions are encountered below 27% EN with plants.

Root ADH—The changes in root ADH activity and expression show inverse correlation with the decreasing availability of oxygen in the agar medium, although the ADH mRNA response curve did not correspond with the changes in ADH activity. The greatest increases in ADH activity occurred as the percent EN atmosphere dropped below 55% (Fig. 3), and the greatest increase in ADH mRNA was associated with atmospheres above 55% EN, and reached a peak somewhere around the 27% EN environment.

Shoot carbohydrates—Carbohydrate responses to the changing atmospheric conditions were complex (Fig. 4). Starch increased as the percent EN atmosphere decreased from 100% to 55%, but below 55% EN the starch levels

Fig. 2 Mean redox potential (±S.E.) of agar based media, with and without Arabidopsis plants, in seven atmospheric treatments.

Fig. 3 ADH activity and expression (±S.E.) in Arabidopsis roots in response to changes in Earth normal atmosphere and redox potential. n=3 for all reported values.

Fig. 4 Starch and soluble sugars (±S.E.) in Arabidopsis leaves in response to changes in the EN atmosphere. For 100% EN n=9, for all others n=3.
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were significantly lower than the control plants. Soluble sugars (glucose, sucrose, and fructose) did not change as the atmosphere was diluted down to 16% with N₂. The 2% EN plants exhibited a dramatic increase in all soluble sugars as compared to all other treatments.

Discussion

These experiments are unique because both CO₂ and O₂ concentrations were reduced in concert with one another. The consequences of decreasing metabolic gases on plant growth are significant (Fig. 1). These changes were more significant in the leaf and stem fresh weights than in the roots. This is most likely due to the fact that, even in the control plants, the root environment was hypoxic (Fig. 2) due to the growth medium used, while the aerial atmosphere represented ambient conditions.

Since the plant root systems were already adapted to low oxygen conditions even before the experimental treatments were begun, we were able to look at ADH activity and expression during this transition from hypoxic to anoxic conditions (Fig. 2). As the percent EN atmosphere and oxygen availability decreased, ADH activity increased (Fig. 3), and the increases in ADH activity were the greatest when the percent EN atmosphere dropped below 55%. This is in contrast to the response shown by ADH expression. While ADH mRNA levels did increase as oxygen decreased (Fig. 2, 3), the greatest increases in ADH expression were associated with higher oxygen concentrations (Fig. 3). The biggest change in ADH expression occurred as the gaseous environment decreased from 100% EN to 87% EN and expression reached its peak around 16% EN. Since the probe contained no intron sequences this suggests that in Arabidopsis post-transcriptional processes may become limited or there is a post-transcriptional control system for the ADH gene.

Carbohydrate concentrations (Fig. 4) may be responding to both the changes in CO₂ and O₂ in the leaves, and changes in root oxygenation. Between 100 and 55% EN atmospheres the increase in starch concentration levels may be due to an increased affinity for CO₂ by Rubisco (Azcón-Bieto et al. 1981) and/or a decrease in sucrose demand in the roots associated with the reduced oxygen levels. The dramatic decrease in starch concentrations associated with treatments below 55% EN is not due to CO₂ concentrations dropping below the compensation point of Arabidopsis because it has been shown that the CO₂ compensation point is lowered when oxygen levels are decreased (Azcón-Bieto et al. 1981). Instead it is more probable that root metabolism and mineral nutrient acquisition have been affected in such a manner that mineral nutrient deficiencies in the leaves are now affecting shoot carbon metabolism. At 2% EN atmosphere soluble sugar concentrations are significantly higher than in all of the other treatments. In leaves starch synthe-

sis and conversion into sugars are under very tight regulatory control. It is possible that this regulation may not be affected until oxygen concentrations drop enough to restrict the metabolically active process of phloem loading in the source leaves and unloading in the sinks.

When comparing data from this study with results obtained from CHROMEX-03, none of the treatments entirely reproduced the spaceflight results. In microbiogravity, where convective currents are lacking, the resulting boundary layers surrounding various parts of a plant organ would have differing levels of oxygen and carbon dioxide based on the metabolic processes of that particular plant organ. For example, in the light, the boundary layer surrounding a leaf during photosynthesis would have reduced CO₂ and elevated O₂ concentrations relative to the ambient atmosphere. Around the root the opposite situation would occur, with O₂ concentrations decreasing and CO₂ concentrations increasing as a result of cellular respiration. Nevertheless, the results obtained in this study demonstrate that growth and metabolism can be controlled by altering the concentrations of oxygen and carbon dioxide, and that atmospheres below 27% EN are inhibitory to both processes.

The results also allow us to make predictions about what the actual in vivo conditions were during the spaceflight experiment. Experiments based on these results utilizing dual environment atmospheric chambers to treat roots and shoots independently may be needed to reproduce the observed spaceflight responses.

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References

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