DOI: 10.1007/s11099-013-0047-9 PHOTOSYNTHETICA 51 (3): 465-473, 2013

Hypobaria and hypoxia affects phytochemical production, gas exchange, and growth of lettuce

C. HE[*], D.A. JACOBO-VELÁZQUEZ[**], L. CISNEROS-ZEVALLOS[*], and F.T. DAVIES[*,+]

Department of Horticultural Sciences and Interdisciplinary Program of Molecular and Environmental Plant Sciences (MEPS), Texas A&M University, College Station, Texas 77843-2133, USA[*]
Centro de Biotecnología-FEMSA, Departamento de Biotecnología e Ingeniería de Alimentos, Tecnológico de Monterrey, Campus Monterrey, Av. Eugenio Garza Sada 2501 Sur, Monterrey, NL 64849, México[**]

Abstract

Hypobaria (low total atmospheric pressure) is essential in sustainable, energy-efficient plant production systems for long-term space exploration and human habitation on the Moon and Mars. There are also critical engineering, safety, and materials handling advantages of growing plants under hypobaria, including reduced atmospheric leakage from extraterrestrial base environments. The potential for producing crops under hypobaria and manipulating hypoxia (low oxygen stress) to increase health-promoting bioactive compounds is not well characterized. Here we showed that hypobaric-grown lettuce plants (25 kPa ≈ 25% of normal pressure) exposed to hypoxia (6 kPa pO_2 ≈ 29% of normal pO_2) during the final 3 d of the production cycle had enhanced antioxidant activity, increased synthesis of anthocyananins, phenolics, and carotenoids without reduction of photosynthesis or plant biomass. Net photosynthetic rate (P_N) was not affected by total pressure. However, 10 d of hypoxia reduced P_N, dark respiration rate (R_D), P_N/R_D ratio, and plant biomass. Growing plants under hypobaria and manipulating hypoxia during crop production to enhance health-promoting bioactive compounds is important for the health and well-being of astronauts exposed to space radiation and other stresses during long-term habitation.

Additional key words: bioprotectants; carbon assimilation; chlorophyll content; dark respiration rate; *Lactuca sativa*; low pressure; oxygen radical absorbance capacity; phytochemicals.

.

Introduction

The new Christopher Columbuses of the 21st century are the astronauts and cosmonauts who will push the envelope of long-term space exploration and human habitation. Like the early explorers or the military campaigns of Napoleon, a reliable food supply is needed for survival and sustainable advancement. It is well documented that green plants supply food, oxygen, help scrub the atmosphere of CO_2 and other volatiles, phytoremediate water, and have a significant impact on the psychological well-being of people (Paul and Ferl 2006). The development of safe, sustainable crop production systems is essential for augmenting the food supply and the gaseous environment of the crew. Besides nutritional benefits (carbohydrates, proteins, lipids, and vitamins), plants can also produce functional secondary metabolites, including protective phytochemicals (bioprotectants), particularly important for astronauts to prevent space radiation-induced chronic diseases developed after short- and long-term duration missions.

Hypobaria (low pressure) is an important component in developing sustainable, energy-efficient plant production systems. There are also major engineering and safety

Received 9 August 2012, *accepted* 11 February 2013.
[+]Corresponding author; fax: 979-845-0627; email: f-davies@tamu.edu
Abbreviations: C_A – CO_2 assimilation; Car – carotenoids; Chl – chlorophyll; DPR – dark-period respiration; FL – fluorescein; LPPG – low pressure plant growth system; ORAC – antioxidant activity; P_N – net photosynthetic rate; P_N/R_D ratio – net photosynthesis/dark respiration rate ratio; pO_2 – partial pressure of O_2; R_D – dark respiration rate; ROS – reactive oxygen species; TP – total soluble phenolics.
Acknowledgements: We thank Tom Boutton, Ray Wheeler, and Mark Tjoelker for critical review of the manuscript. Supported in part by the National Aeronautics and Space Administration (NASA) through grants No. NAG-9-1067 (Plant Growth and Metabolism at Sub-Ambient Atmospheric Pressures), and No. NAJ04HF31G (Plant Growth at Sub-Ambient Atmospheric Pressures with Control of the Partial Pressures of Constituent Gases), and the Texas AgriLife Research.

advantages of growing plants under subambient conditions for extraterrestrial base environments (Paul and Ferl 2006, He *et al*. 2009b). For a lunar mission, the ambient pressure is near 0 kPa, whereas the Martian ambient pressure varies from 0.2 to 0.9 kPa (< 1% Earth's ambient). Pressure differences between the hypobaric lunar and Martian environment, and maintaining a higher, Earth-ambient total pressure, would result in greater leakage. The reduced pressure differential between a hypobaric plant growth facility and the external environment would reduce structural requirements, permit lighter materials to be used in its construction, and improve safety issues by reducing external and internal pressure differentials (Bucklin *et al*. 2004). This would further reduce the payload volume and mass required for deployment. Furthermore, hypobaric conditions in the plant growth facility would require less buffer gas, typically N_2, to be transported or obtained *in situ* to supplement the physiologically active gases (CO_2 and O_2). Current extra vehicular activity (EVA) suit pressure is 26–30 kPa, so a reduced cabin pressure would minimize prebreathing exercises required for suiting up in the event of an emergency. This is the primary reason for considering reduced pressure by mission planners. NASA has targeted an operation pressure of ≈ 54 kPa for future space exploration for many of these reasons (Wheeler 2009).

Hypobaric environments are typically associated with hypoxia, particularly, when total gas pressure is reduced below 50 kPa. Hence there is a need to supply sufficient partial pressures of O_2 to avoid hypoxia in plants under hypobaric conditions. Earlier studies have demonstrated that seed germination and seedling growth are possible at hypobaric conditions (Musgrave *et al*. 1988, Schwartzkopf and Mancinelli 1991, He *et al*. 2003). It is also a common observation that plants can be grown at high altitude, where pressures are well below 70 kPa (Gale 1972, Davies *et al*. 2005), although the invariable association between increasing altitude, decreasing temperature, and change in light quality confounds the issue of the effect of pressure alone. The question here is whether the rates of plant growth and morphogenesis compare closely with those at ambient pressure. A major potential limitation to plant growth under hypobaria is that oxidative phosphorylation can become limited if the partial pressure of O_2 (pO_2) is reduced (Drew 1997). To test limits of hypobaria, seedlings germinated and grew during a week-long study at 6 kPa total gas pressure, provided the atmosphere that was composed predominately of oxygen (pO_2 = 5 kPa; ≈ 83% O_2); but at lower total pressures and therefore less O_2, seeds failed to germinate (Schwartzkopf and Mancinelli 1991). He *et al*. (2007) reported that biomass accumulation of 'Buttercrunch' lettuce was not affected by hypobaria (25 kPa), but hypoxia (6 kPa pO_2) reduced plant gas exchange (photosynthesis, dark-period respiration) and biomass regardless of the total atmospheric pressure. Plant production could be done in separate hypobaric facilities with the crew using supplementary oxygen supply while tending the space crops. Hence, there is the rationale for growing plants at 25 kPa total pressure.

Plants produce functional phytochemicals that act as antioxidants by neutralizing harmful free radicals, thus protecting cell DNA, proteins, and lipids from oxidative damage (Wargovich 2000). A diet abundant in natural antioxidants and anticarcinogenic compounds is highly desirable for astronauts living in oxidative environments and exposed to ionizing cosmic radiation. Production of functional phytochemicals is influenced by both genetic and environmental factors that induce stress in plants. Light, temperature, salt, and water stress have been shown to enhance functionally important phytochemicals in various crops (Cisneros-Zevallo 2003, Dumas *et al*. 2003, Beckwith *et al*. 2004, Kubota *et al*. 2006).

Stresses caused by hypobaria and hypoxia could affect functional chemicals in a similar manner. There are differing reports on how hypobaria influences the production of functionally important chemicals. Levine *et al*. (2008) reported that hypobaria (33 kPa) at normal oxygen (21 kPa pO_2) had no effect on total antioxidant activity of radish. Stutte (personal communication) found that hypobaria (33 kPa) at normal oxygen level enhanced anthocyanin concentration, but reduced biomass of two lettuce cultivars from 27–41%. Rajapakse *et al*. (2009) reported that hypoxia (6 kPa pO_2) under both ambient pressure and hypobaric (25 kPa) conditions increased the production of protective phytochemicals, including phenolic compounds such as leaf anthocyanins, but there was a 25% reduction in biomass.

While it is important to increase bioprotectants, it is equally critical to maintain photosynthesis and plant biomass production under hypobaria for crop production. We hypothesized that hypobaric-produced lettuce plants could be subjected to hypoxic stress during the final stages of production to enhance bioprotectants without loss of biomass.

Materials and methods

Low pressure plant growth system (LPPG) is a fully automated system, capable of controlling pressure and gas concentrations in ambient or reduced pressure growth chambers (He *et al*. 2006, 2007). The LPPG system consisted of six growth chambers designed to operate at pressures as low as 5 kPa (Fig. 1). The six chambers were housed in an environmentally controlled growth room. Total pressures, and partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) were controlled and monitored during experiments. The LPPG was a semiclosed system, since O_2, CO_2, and N_2 were added and controlled. Temperature was recorded, although not controlled directly by

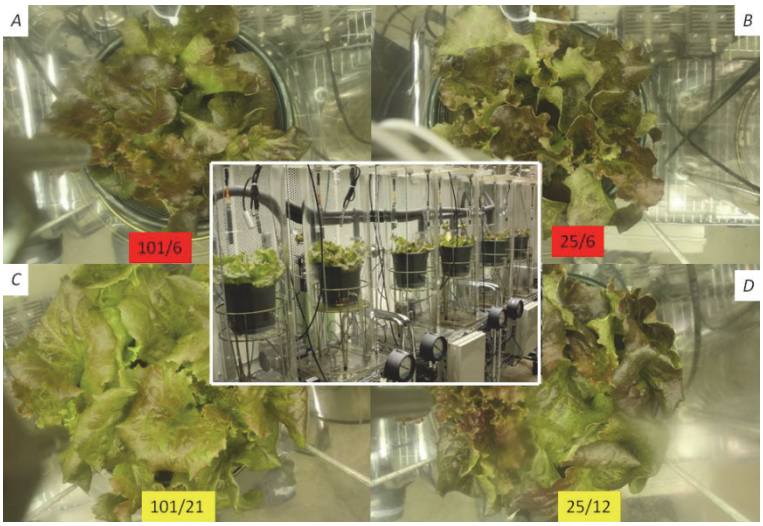

Fig. 1. 'Red Sails' lettuce plants (34 d old at termination) in a 10-d study. Plants were exposed to (*A*) hypoxia at ambient total pressure (101/6 kPa pO_2), (*B*) hypoxia under hypobaria (25/6 kPa pO_2), (*C*) ambient pressure and oxygen 101/21 kPa O_2), and (*D*) (25/12 kPa pO_2). *Insert* is of the six chamber LPPG system.

the LPPG system. Temperature control and lighting were provided by placing the LPPG system in an independent plant growth room. The pressure and control systems of each chamber were independent so that conditions could be set to satisfy statistical experimental designs. The LPPG operating system and parameters measured are explained in greater detail in He *et al*. (2007). There was also an ethylene scrubbing system to remove endogenous ethylene generated by lettuce plants. The system included a stainless steel column filled with potassium permanganate to strip ethylene from the air as it was circulated over the cooling coils and returned to a given chamber. There were no mixing fans in the chambers. We used mixing fans in earlier prototypes of the chamber, but we found there was sufficient mixing of air, since the air was recycled for controlling humidity and ethylene (He and Davies 2012).

Plant growth conditions: Lettuce, cv. 'Red Sails', was germinated in 20 cm × 14 cm-diameter plastic pots that were filled to within 1 cm of the top with fine-grade calcined clay (*Profile Greens*, *Profile Products LLC*, Buffalo Grove, Illinois, USA) (particle size < 1 mm, 74% porosity, 0.56 g mL^{-1} bulk density, and 2.5 g mL^{-1} particle density). The inert, calcined clay was prewashed with deionized water and allowed to drain thoroughly before sowing. After the seeds were germinated, plants were supplied with a modified Hoagland's nutrient solution (pH 6.3) containing 4.0 mM $Ca(NO_3)_2$, 1.0 mM KH_2PO_4/K_2HPO_4, 2.0 mM KNO_3, 1.0 mM $MgSO_4$, 50 mM Fe as Fe-EDTA, 1 mM NaCl, and micronutrients: 50 mM B, 10.0 mM Mn, 1.0 mM Cu, 2.0 mM Zn, and 0.3 mM Mo. Ten days after imbibition, three seedlings were transplanted to 20 cm × 14 cm-diameter, self-watering pots (*S-series* pots, *Apollo Plastics LTD*, Mississauga, Ontario, Canada) with a volume of 4 L, filled with prewashed calcined clay. The reservoir of the self-watering pots can hold 1 L nutrient solution. The lettuce seedlings were allowed to grow in normal atmospheric pressure in a controlled growth chamber for another 11 d. Only containers with uniform, 21-d-old plants were selected and transferred to the LPPG chambers for the treatments, so plants were 31-d-old at the termination of 10 d studies. Each chamber contained one pot with 3 seedlings. There were 3 replicated chambers (n = 3). Additional nutrient solution was delivered to the reservoir of the pot as needed during the duration of the experiment without disturbing the total atmospheric pressure of a given chamber. Irradiation was approximately 600 µmol m^{-2} s^{-1} at canopy level inside of the pressure chambers provided by *Sylvania 400 W* metal halide (*M400U*) lamps with a 12 h light /12 h dark phase, maximum/minimum temperature of 26.1 ± 0.6/20.0 ± 0.1°C, and maximum/minimum RH of 91.2 ± 3.7 (dark period)/83.7 ± 2.7% (light period). Supplementary CO_2 was added during the light cycle to maintain a minimum set point level of 100 Pa pCO_2. Without supplementary CO_2 during the light period, CO_2 levels would fall within several hours to a CO_2 compensation point of around 2–4 Pa CO_2 (20–40 µL L^{-1} equivalent at 101 kPa) at 21 kPa pO_2.

Plants were exposed to 6 treatments:

Total pressure [kPa]	pO_2 [kPa]
101 (ambient TP)	21 (normal O_2, 10 d) 6 (hypoxia, 10 d) 21 (7 d) – 6 (3 d)
25 (low TP)	12 (10 d) 6 (10 d) 12 (7 d) – 6 (3 d)

In previous studies (He *et al.* 2006, 2007, 2009a,b), P_N, R_D, and biomass production were statistically the same between 101/21 and 25/12 kPa pO_2.

Net photosynthetic rate (P_N) was measured by rate of CO_2 uptake (draw-down) during the light-period cycle

(Wheeler 1994, He *et al*. 2007). CO_2 accumulated in the chambers during the dark-period cycle (without supplementary CO_2). To determine P_N, slopes of the regression lines of CO_2 concentration over time (min) in each chamber were determined during the light cycle. The pCO_2 were measured by CO_2 sensors within each chamber independently at 1-min intervals during the light cycle. The change of pCO_2 in each chamber was related to P_N during the light-period cycle. The minimum CO_2 concentration set-point was 100 Pa (pCO_2) for all the treatments in ambient and low pressure chambers. When CO_2 levels were drawn down (assimilated by plants in the chamber) to 100 Pa, which was equivalent to 1,000 µmol mol^{-1} at 101 kPa total pressure, the system added supplementary gas to maintain the set point level CO_2 in each chamber through the remaining duration of the light-period cycle. The rates of P_N were obtained during the first 100 min (10 min after lights were turned on during the light cycle). Hence, at 1-min intervals, there were 100 measurements taken in determining the daily P_N in each chamber (one container per chamber with three seedling plants per container) per treatment. There were three replicated chambers (n = 3) per treatment.

Dark respiration rate (R_D) was measured by CO_2 accumulation in chambers during the dark period (Wheeler *et al*. 1994, Richards *et al.* 2006, He *et al*. 2007). The pCO_2 data were measured by CO_2 sensors within each chamber independently at 1-min intervals during the dark period. During the dark-period cycle, no supplementary CO_2 was added to chambers and CO_2 was allowed to rise without maximum set points. To avoid any residual effects of the light cycle, data during the first 10 min of the dark cycle were not used for calculation. Hence, there were 710 × 1-min interval measurements taken nightly for R_D in each chamber per treatment. R_D was determined by the slopes of the regression equations of accumulated CO_2 concentration in each chamber during the dark-period cycle. There were three replicated chambers, n = 3.

Extraction of total soluble phenolics (TP) and anthocyanins: The external leafs of lettuce plants (~ 3–5 leaves) were used for phytochemicals determinations. The leaves were cut into two equal parts and evenly separated into two portions. One portion (~ 5g) was homogenized with methanol (20 mL), while the other portion (~ 5g) was homogenized with an ethanolic solvent (0.225 N HCl in 95% ethanol) using an *Ultra-Turrax* homogenizer (*IKA Works*, *Inc*., Wilmington, NC, USA). Extracts were centrifuged at 29,000 × g for 15 min at 4ºC. The total soluble phenolics (TP) and antioxidant activity (ORAC value) were determined from the clarified methanolic extracts, whereas the clarified ethanolic extracts were used for anthocyanins content determinations.

Determination of TP: TP were determined with the method of Swain and Hillis (1959). Methanolic extracts (15 µL) were diluted with nanopure water (240 µL) in a microplate well, followed by the addition of 0.25 N Folin-Ciocalteu reagent (15 µL); 96 well microplates were used for TP determinations. The mixtures were incubated 3 min and then, 1 N Na_2CO_3 (30 µL) was added. The final mixtures were incubated for 2 h at room temperature and in the dark. Spectrophotometric readings at 725 nm were collected using a plate reader (*Synergy HT*, *Bio-Tek Instruments*, Vermont, USA). Total phenolics concentrations were expressed as mg of chlorogenic acid equivalents per g and per pot of fresh tissue.

Antioxidant activity (ORAC value): The antioxidant activities were determined with the oxygen radical absorbance capacity (ORAC) assay. The ORAC values were obtained with the procedure of Wu *et al*. (2004) for hydrophilic ORAC with slight modifications described by Villarreal-Lozoya *et al*. (2007). The methanolic extracts were diluted (1:50) with a 75 mM phosphate buffer solution (PBS, pH 7.4). The diluted extracts (25 µL) were loaded into the wells of a clear-bottom 96-well black plate (*Corning*, New York, USA) and incubated at 37ºC for 45 min prior to analysis. Fluorescein sodium salt (FL) was used as protein probe and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as free radical source. FL stock solution (FL_1) was prepared by diluting FL powder (112.5 mg) in PBS (50 mL). A second FL solution (FL_2) was made by diluting FL_1 (100 µL) in PBS (10 mL). FL_1 and FL_2 were stored under dark conditions at 4°C until used. Prior to analysis, a FL_3 solution was prepared by diluting FL_2 (400 µL) in PBS (25 mL). AAPH solution was prepared by dissolving AAPH pellets (260 mg) in PBS (10 mL) previously incubated at 37°C for at least 45 min. Fluorescence readings were done in a plate reader (*Synergy HT*, *Bio-Tek Instruments*, Vermont, USA). After priming the injectors, FL_3 (200 µL) was injected in each well followed by the injection of the AAPH solution (75 µL). Fluorescence readings were taken every minute during 50 min using excitation and emission wavelengths of 485 nm and 520 nm, respectively. The loss of fluorescence was recorded and processed by analytical software package *KC-4 v. 3.4* (*Bio-Tek Instruments*, Vermont, USA). Samples were compared against a trolox standard and a blank curve made from normalized data using the area under the curve. Results were expressed as µmol of trolox equivalents per g and per pot of fresh tissue.

Anthocyanin content was determined with a modification of the method described by Fuleki and Francis (1968). Hexane (15 mL) was added to the cleared ethanolic extract to remove carotenoids. The mixture was gently shaken on a separation funnel and allowed to settle for phase formation. The ethanol rich phase was recovered and used for anthocyanin content determinations. Spectrophotometric readings at 535 nm were taken, subtracting absorbance at 700 nm. Anthocyanin contents

were expressed as mg of cyanidin 3-glucoside equivalent per g and per pot of fresh tissue, using a molar extinction coefficient of 25,965 M^{-1} cm^{-1} and a molecular mass of 449 g mol^{-1}.

Extraction and determination of chlorophyll (Chl) and carotenoids (Car) content: Chl and Car from lettuce leaves (~ 1 g) were extracted with acetone (3 mL) in the presence of $MgCO_3$ (200 mg) using a mortar and pestle. The turbid pigment extracts were transferred to a centrifuge tube and the mortar was rinsed with acetone (1.5 mL). Acetone was added to the centrifuge tube to obtain a final volume of 5 mL. The extract was centrifuged at 500 × *g* for 5 min. Spectrophotometric readings were taken at 470 nm, 663.2 nm, and 646.8 nm and the Chl and Car contents were calculated as described by Lichtenthaler and Buschmann (2001).

Statistical analysis: Replication was achieved by repeating treatments under the same conditions over time. Low and ambient total pressure chambers were run concurrently. Chambers were alternated during treatments between low and ambient total pressure at hypoxic and nonhypoxic conditions to avoid any chamber effects. There were no chamber effects. For a given experimental run, irradiance in all chambers was 600 µmol m^{-2} s^{-1}. All reported data were pooled from repeated independent treatments. There were 6 chambers, so replications of treatments were run with combinations of total pressure and partial pressure of pO_2 and repeated as needed under the same environmental conditions. There were three plants per container and one container per chamber, with each container as a single replicate. There were three replicates per treatment (n = 3). *ANOVA* was also conducted to determine main effects and interactions, with mean separation by *Duncan´*s test (p<0.05) for plant gas exchange, biomass, and bioprotectant analysis.

Results

Plant growth and gas exchange: Exposure of ambient and hypobaric lettuce plants to hypoxia for 10 d reduced P_N, R_D, and the ratio of P_N/R_D (a measurement of photosynthetic efficiency) (Figs. 2,3; Table 1). 10 d of hypoxia caused a 41 and 26% reduction in fresh biomass of ambient total pressure and hypobaric plants, respectively (Table 2). However, when plants were exposed to hypoxia for just the final 3 d of the production cycle, the gas exchange and growth were comparable with nonhypoxic plants (Figs. 2,3; Tables 1,2). Hypobaric plants had 18% greater biomass than ambient pressure plants, when both were exposed to hypoxia during the final 3 d of production. Hypobaria had no adverse effect on plant gas exchange or growth. Lettuce plants at 101/21 and 25/12 kPa pO_2 had comparable photosynthesis, dark-period respiration, and subsequent plant biomass. Hence, plants could be exposed to hypoxia during the end of the production cycle without adverse effects to photosynthesis, dark-period respiration or growth.

Health-promoting bioactive compounds: The control, nonstressed 101/21 kPa pO_2 lettuce plants, had among the lowest levels of anthocyanins, phenolics, and antioxidant activity (Table 2). The anthocyanin concentration of plants grown under hypoxia increased by 2.6-fold for 101/6 and 2.0-fold for 25/6 kPa pO_2, compared with plants grown under nonhypoxic conditions (Table 2). However, this increase in anthocyanin concentration was attributed to a dilution effect, since the fresh mass of plants grown under hypoxia (6 kPa pO_2) for 10 d was significantly lower than in plants grown under 101/21 and 25/12 kPa pO_2. To determine if real synthesis occurred and not just an increase in concentration due to smaller-sized plants, the concentration of anthocyanins was expressed as mg g^{-1}, compared to total mg $plant^{-1}$ (3 lettuce seedlings per container per chamber, with 3 replicated chambers, n = 3). The total anthocyanin con-

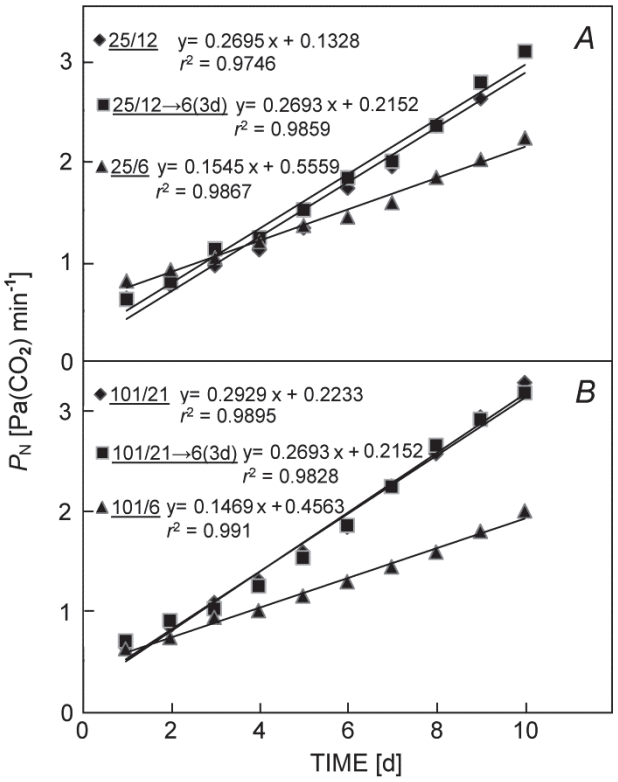

Fig. 2. Effect of hypobaria and hypoxia on net photosynthetic rate (P_N) of *A*: 25 kPa and *B*: 101 kPa plants, of 'Red Sails' lettuce (n = 3). Plants were exposed to 25/12, 25/6, and 25/12 → 25/6 kPa pO_2 during final 3 d of a 10-d study, or 101/21 (ambient), 101/6, and 101/21 → 101/6 kPa pO_2 during final three days of production.

centration and accumulation were, respectively, 2.7-fold and 2.8-fold greater in hypobaric plants exposed to hypoxia during the final 3 d, than the nonstressed 101/21 kPa pO_2 plants of comparable size, supporting increased biosynthesis and not a dilution effect, since plants were of equal fresh mass. There were no differences in anthocyanin concentration and accumulation of 101 kPa plants exposed to 3 d of hypoxia.

Similarly increased biosynthesis in hypobaric-hypoxic stressed (last 3 d) lettuce plants occurred with increased concentrations of total phenolics, antioxidant activity, and Car, which were, respectively, 3.2-fold, 2.7-fold, and 1.3-fold greater than in plants grown under ambient conditions (101/21 kPa pO_2) (Table 2). However, the greatest reduction of total Chl and Car occurred in 101 kPa plants exposed to hypoxia for 10 d, due to reduced total plant biomass (Table 2). Chl concentration and total Chl were the highest in hypobaric plants exposed to 3 d of hypoxia.

Discussion

This research demonstrated that the combined effect of hypobaria and hypoxia increased the bioprotectant content of lettuce plants, while maintaining plant biomass production. It is the first report to show that hypobaric plants exposed to hypoxia during the final days of production have enhanced phytochemical production without adversely affecting photosynthetic rates and plant biomass. Clearly, there was an enhanced effect of subjecting hypobaric plants to short-term hypoxic stress to increase bioprotectant production, which was greater than long-term hypoxia. This finding is of particular importance to long-duration space habitation – producing plants under more efficient hypobaric systems and manipulating hypoxia to enhance fresh food resources for the crew.

While P_N was not affected by total pressure, 10 d of hypoxia reduced P_N, R_D, and P_N/R_D ratio, as reported previously (He *et al*. 2007). The P_N/R_D ratio is an indication of energy available for photosynthesis and growth, including how efficiently carbohydrates are utilized during DPR, and it has been used to predict growth performance (Smith *et al*. 1995). The pCO_2 during the light period was maintained at a saturating level of 100 Pa (1,000 µl L^{-1}), nearly 3-fold normal air, so photorespiration was not an issue and hypoxia decreased C_A.

A potential limitation to plant growth under hypobaria is, if pO_2 is reduced, that oxidative phosphorylation can become limited (Wargovich 2000). The 41 and 26% growth reduction, respectively, under 101 and 25 kPa with 'Red Sails' lettuce at 6 kPa pO_2 during the 10-d cycle is nearly identical to the 39 and 27%, respectively, growth reduction of 'Butter Crunch' lettuce (He *et al*. 2007). As previously reported, hypoxia increased anthocyanins and phenolics, but there was a 25% growth reduction in 'Red Sails' under ambient total pressure (Rajapakse *et al*. 2009). A goal of the current research was to maintain similar biomass of 3-d hypoxia-exposed 'Red Sails' plants compared with nonhypoxic plants, which was achieved.

So why did hypoxia not adversely affect photosynthesis and growth during the final 3 d of production? One reason might be that lettuce seedlings were sufficiently large so that even though the gaseous environment of the chamber was 6 kPa pO_2 (only 29% of O_2 of ambient, normal air), internal leaf mesophyll pO_2 from photosynthesis could have been higher during the light period. Another advantage of low pressure is that gaseous diffusion increases with the reduction of boundary layer resistance (Rygalov *et al*. 2004). Changes in pressure affect gas diffusion rates and surface boundary layers and change convective transfer capabilities and water evaporation rates. This could enhance O_2 and CO_2 levels in leaf mesophyll cells. Acclimation of leaves to hypobaria and hypoxia might also occur more quickly, since a 30% increase in biomass production typically occurs during the final 3 d of production.

In a study of *Arabidopsis* gene expression at low atmospheric pressure, less than one-half of the 200 genes dramatically up- or down-regulated by hypobaria were similarly affected by hypoxia, suggesting that the

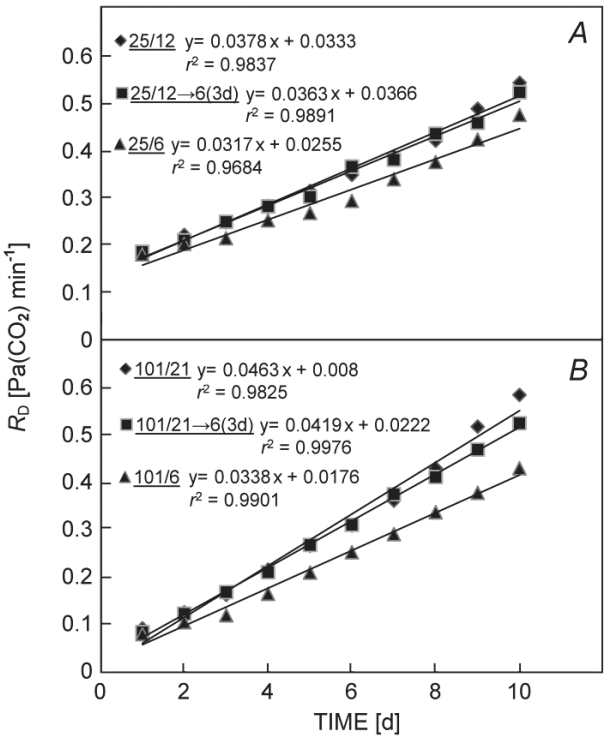

Fig. 3. Effect of hypobaria and hypoxia on dark respiration rate (R_D) of *A*: 25 kPa and *B*: 101 kPa 'Red Sails' lettuce (n = 3). Plants were exposed to 25/12, 25/6, and 25/12 → 25/6 kPa pO_2 during final 3 d of a 10-d study, or 101/21 (ambient), 101/6, and 101/21 → 101/6 kPa pO_2 during final 3 d of production.

Table 1. Effect of total gas pressure and partial pressure of O_2 (pO_2) on net photosynthetic rate (P_N), dark respiration rate (R_D), and P_N/DPR ratio of lettuce during final day of 10-d production (n = 3). Treatments with a small cross ($^+$) were subjected to 6 kPa of oxygen partial pressure (pO_2) for the final 3 d of production. *Different letters* within a column indicate that values are significantly different by *Duncan*'s test (p<0.05). NS – nonsignificant, $^*p$<0.05, $^{**}p$<0.01.

Total pressure [kPa]	pO_2 [kPa]	P_N [Pa(CO_2) min^{-1}]	R_D [Pa(CO_2) min^{-1}]	P_N/R_D ratio
101	21	3.26^a	0.51^a	6.4^a
	6	1.99^b	0.37^b	5.4^b
	21–6$^+$	3.23^a	0.46^{ab}	7.0^a
25	12	3.04^a	0.44^{ab}	6.9^a
	6	2.19^b	0.37^b	5.9^b
	12–6$^+$	3.04^a	0.42^{ab}	7.2^a
Significance				
Total pressure		NS	NS	NS
pO_2		**	*	**
TP × pO_2		NS	NS	NS

Table 2. Effects of total gas pressure (TP) and oxygen partial pressure (pO_2) on plant fresh mass (FM), leaf anthocyanin concentration, total phenolics concentration, antioxidant activity, total chlorophyll concentration, and total carotenoid concentration of 'Red Sails' lettuce (n = 3). Treatments with a small cross ($^+$) were subjected to 6 kPa of oxygen partial pressure (pO_2) for the final 3 d of production. Anthocyanin concentration is reported as mg of cyanidin-3-glucoside equivalents. Total phenolics concentration is reported as mg of chlorogenic acid equivalents. Antioxidant activity is reported as µg of trolox equivalents. *Different letters* within a column indicate that values are significantly different by *Duncan's* test (p<0.05). NS – nonsignificant, $^*p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001.

TP [kPa]	pO_2[1] [kPa]	FM [mg g^{-1}]	Anthocyanins [mg g^{-1}]	Anthocyanins [mg $plant^{-1}$]	Phenolics [mg g^{-1}]	Phenolics [mg $plant^{-1}$]	Antioxidant activity [µg g^{-1}]	Antioxidant activity [mg $plant^{-1}$]	Chlorophyll [mg g^{-1}]	Chlorophyll [mg $plant^{-1}$]	Carotenoids [mg g^{-1}]	Carotenoids [mg $plant^{-1}$]
101	21	67.7^a	0.16^b	10.3^b	1.3^c	91.2^b	13.6^b	921^b	1.15^b	77.7^b	0.27^c	17.9^b
	6	40.1^c	0.41^a	16.8^b	3.7^a	149.3^b	45.0^a	1,795^{ab}	1.26^{ab}	50.6^c	0.30^{bc}	12.3^c
	21–6$^+$	56.2^{ab}	0.20^b	15.0^b	1.7^{bc}	94.6^b	27.0^{ab}	1,477^{ab}	1.23^{ab}	69.3^b	0.28^{bc}	15.6^{bc}
25	12	62.7^{ab}	0.22^b	13.7^b	2.8^{abc}	174.8^b	28.5^{ab}	1,705^{ab}	1.28^{ab}	78.5^b	0.30^{bc}	18.7^b
	6	46.2^{bc}	0.43^a	19.7^{ab}	3.2^{ab}	150.7^b	38.2^a	1,785^{ab}	1.37^{ab}	62.7^{bc}	0.32^{ab}	14.8^{bc}
	12–6$^+$	68.4^a	0.43^a	28.6^a	4.1^a	269.8^a	36.7^a	2,496^a	1.45^a	98.1^a	0.36^a	24.2^a
Significance												
TP		NS	NS	*	*	**	NS	NS	*	**	NS	*
pO_2		**	*	*	**	**	*	NS	NS	***	**	**
TP × pO_2		NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS

response to hypobaria is unique and more complex than an adaptation to hypoxia, inherent to hypobaric environments, *i.e.*, hypobaria does not equal hypoxia (Paul *et al.* 2004).

In another study, hypobaric lettuce plants reached the CO_2 compensation point sooner than ambient total pressure plants, regardless of pO_2 or irradiance (He *et al.* 2009b). This suggests that hypobaric plants use CO_2 more efficiency than ambient pressure plants. The work of He *et al.* (2009b) agrees with Richards *et al.* (2006), who reported that photosynthesis of 40 Pa pCO_2 plants was greater at hypobaric than ambient pressure, but when pCO_2 was in a nonlimiting range (70 to 100 Pa), photosynthesis was insensitive to hypobaria in a short-term, 16-h study of *Arabidopsis*. In the current study, lettuce plants were exposed to 100 Pa CO_2 (≈ 1,000 µmol mol^{-1}(CO_2) under normal air).

So why did the combination of hypobaria and hypoxia enhance production of bioprotectants? It is well known that abiotic stresses induce the accumulation of reactive oxygen species (ROS) in plants (Fukao and Bailey-Serrer 2004). ROS affect plant growth and are signaling molecules associated with the stress-induced activation of the phenylpropanoid metabolism (Dixon and Pavia 1995). It is likely that hypobaric lettuce plants accumulate ROS at low levels, and thus plant growth and phenolics biosynthesis are not affected. However, we hypothesize that hypoxia applied during the last 3 d of the production cycle on hypobaric lettuce plants increased ROS concentrations at levels that altered the cellular redox potential in lettuce, triggering the activation of the phenylpropanoid metabolism and thus enhancing the biosynthesis of anthocyanins. It is likely that the hypobaric lettuce plants exposed to hypoxia during the last 3 d of the production cycle, were sufficiently developed to acclimate to the oxidative stress. Hence,

anthocyanin concentration and total content were enhanced and plant biomass was maintained.

Rajapaske *et al*. (2009) reported that hypoxia alone (under ambient total pressure) induced the production of protective phytochemicals, including leaf anthocyanin and total phenolics in lettuce. The increase of anthocyanin was high as compared to total phenolic compounds at low pO_2, suggesting that hypoxia favored the anthocyanin biosynthetic pathway. They also reported an increase in ethylene and a 25% reduction in fresh biomass of ambient pressure plants exposed to hypoxia (6 kPa pO_2). Ethylene has been shown to increase the activity of phenylalanine ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway, which leads to the production of phenolic compounds and anthocyanins. However, in the current study, ethylene was removed with potassium permanganate, so ethylene never accumulated. In spaceflight environments (International Space Station, Russian MIR), ethylene can accumulate to very high levels (Campbell *et al.* 2001), which can depress plant growth and greatly reduce photosynthesis (He *et al*. 2009a, He and Davies 2012); hence, the need is to maintain a low ethylene atmosphere in space crop production facilities.

Another advantage of hypobaric plants was greater leaf Chl and Car than in ambient pressure plants. Lettuce leaves phenotypically responded to hypobaria by producing higher Chl concentrations, with the highest leaf Chl occurring in hypobaric plants under short-term hypoxic conditions. Hypobaric storage has been reported to promote Chl retention in a large number of plant species (Burg 2004). While concentrations of Chl and Car were generally the same among hypobaric plants, there was greater total production in plants exposed to hypoxia during the final 3 d. However, when hypobaric plants were exposed to hypoxia for a prolonged period of 10 d, Chl contents were comparable with those of ambient, nonhypoxic plants. He *et al*. (2007) also reported that hypobaric plants had higher Chl than ambient pressure plants, however, ethylene was allowed to naturally accumulate, while it was removed in the current research.

In summary, hypobaria and hypoxia are essential for controlled environment agricultural (CEA) crop production for long-term, human habitation on the Moon and Mars. The combined effect of hypobaria and hypoxia during the final 3 d of production, enhanced the antioxidant activity, stimulated greater synthesis of anthocyanins, total phenolics, and Car without reduction in plant biomass. While 10 d of hypoxia increased the concentration of bioprotectants, there was reduced plant biomass and subsequently no significant differences with the control (ambient pressure and pO_2) in the total amount of anthocyanins, phenolics, and antioxidant activity. Hypobaria enhanced Chl synthesis. The results showed that bioprotectants can be increased by exposing hypobaric plants to hypoxia during the end of the production cycle, without loss of biomass. Increasing health-promoting bioactive compounds is particularly important for the well-being of astronauts exposed to space radiation and other stresses during habitation.

References

Beckwith, A.G., Zhang, Y., Seeram, N.P. *et al*.: Relationship of light quantity and anthocyanin production in *Pennisetum setaceum* (cvs Rubrum and Red Riding Hood). – J. Agr. Food Chem. **52**: 456-461, 2004.

Bucklin, R.A., Fowler, P.A., Rygalov, V.Y. *et al*.: Greenhouse design for the Mars environment: Development of a prototype deployable dome. – Acta Hort. **659**: 127-134, 2004.

Burg, S.P.: Postharvest Physiology and Hypobaric Storage of Fresh Produce. – CAB International, Wallingford, Oxfordshire 2004.

Campbell, W.F., Salisbury, F.B., Bugbee, B. *et al*.: Comparative floral development of Mir-grown and ethylene-treated earth-grown Super Dwarf wheat. – J. Plant Physiol. **158**:1051-1060, 2001.

Cisneros-Zevallos, L.: The use of controlled post-harvest abiotic stresses as a tool for enhancing the nutraceutical content and adding-value to fresh fruits and vegetables. – J. Food Sci. **68**: 1560-1565, 2003.

Davies, F.T., Calderon, C.M., Huaman, Z., Gomez, R.: Influence of a flavonoid (formononetin) on mycorrhizal activity and potato crop productivity in the highlands of Peru. – Sci. Hort. **106**: 318-329, 2005.

Dixon, R.A., Paiva, N.L.: Stress-induced phenylpropanoid metabolism. – Plant Cell **7**: 1085-1097, 1995.

Drew, M.C.: Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. – Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 223-250, 1997.

Dumas, Y., Dadomo, M., Lucca, G.D., Groller, P.: Effects of environmental factors and agricultural techniques on antioxidant content of tomatoes. – J. Sci. Food Agr. **83**: 369-382, 2003.

Fukao, F., Bailey-Serrer, J.: Plant response to hypoxia – is survival a balancing act? – Trends Plant Sci. **9**: 449-456, 2004.

Fuleki, T., Francis, F. J.: Quantitative methods for anthocyanins. 1. Extraction and determinationm of total anthocyanin in cranberries. – J. Food Sci. **33**: 72-77, 1968.

Gale, J.: Availability of carbon dioxide for photosynthesis at high altitudes: theoretical considerations. – Ecology **53**: 494-497, 1972.

He, C., Davies, F.T.: Ethylene reduces plant gas exchange and growth of lettuce grown from seed to harvest under hypobaric and ambient total pressure. – J. Plant Physiol. **169**: 369- 378, 2012.

He, C., Davies, F.T., Lacey, R.E. *et al*.: Effect of hypobaric conditions on ethylene evolution and growth of lettuce and wheat. – J. Plant Physiol. **160**: 1341-1350, 2003.

He, C., Davies, F.T., Lacey, R.E.: Hypobaric conditions effect gas exchange, ethylene evolution and growth of lettuce for advanced life support systems (ALS). – Habitation **11**: 49-61, 2006.

He, C., Davies, F.T., Lacey, R.E.: Separating the effects of

hypobaria and hypoxia on lettuce: growth and gas exchange. – Physiol. Plant. **131**: 226-240, 2007.

He, C., Davies, F.T., Lacey, R.E.: Ethylene reduces gas exchange and growth of lettuce plants under hypobaric and normal atmospheric conditions. – Physiol. Plant. **135**: 258-271, 2009a.

He, C., Davies, F.T., Lacey, R.E.: Hypobaria, hypoxia and light affect gas exchange, and the CO_2 compensation and saturation points of lettuce (*Lactuca sativa*) L. – Botany **87**: 712-721, 2009b.

Kubota, C., Thomson, C.A., Wu, M., Javanmardi, J.: Controlled environments for production of value-added food crops with high phytochemical concentrations: lycopene in tomato as an example. – HortScience **41**: 522-525, 2006.

Levine, L.H., Bisbee, P.A., Richards, J.T. *et al*.: Quality characteristics of the radish grown under reduced atmospheric pressure. – Adv. Space Res. **41**: 754-762, 2008.

Lichtenthaler, H.K, Buschmann, C.: Extraction of photosynthetic tissues: Chlorophylls and carotenoids. – In: Wrolstad, R.E., Acree, T.E., Decker, E.A. *et al*. (ed.): Handbook of Food Analytical Chemistry, Current Protocols in Food Analytical Chemistry. Pp. 165-170. John Wiley &Sons, Madison 2001.

Musgrave, M.E., Gerth, W.A., Scheid, H.W., Strain, B.R.: Growth and mitochondrial respiration of mungbeans *(Phaseolus aureus* Roxb.) germinated at low pressure. – Plant. Physiol. **86**: 19-22, 1988.

Paul, A.L., Ferl, R.F.: The biology of low atmospheric pressure - implications for exploration mission design and advanced life support. – Gravit. Space Biol. Bull. **19**: 3-18, 2006.

Paul, A.L., Schuerger, A.C., Popp, M.P., Richards, J.T., Manak M.S., Ferl R.I.: Hypobaric biology: Arabidopsis gene expression at low atmospheric pressure. – Plant Physiol. **134**: 215-223, 2004.

Rajapakse, N.C., He, C., Cisneros-Zevallos, L., Davies, F.T.: Hypobaria and hypoxia affects growth and phytochemical contents of lettuce. – Sci. Hort. **122**: 171-178, 2009.

Richards, J.T., Corey, K.A., Paul, A.L. *et al*.: Exposure of *Arabidopsis thaliana* to hypobaric environments: implications for low-pressure bioregenerative life support systems for human exploration missions and terraforming on Mars. – Astrobiology **6**: 851-866, 2006.

Rygalov, V.Y., Fowler, P.A., Wheeler, R.M., Bucklin, R.A.: Water cycle and its management for plant habitats at reduced pressures. – Habitation **10**: 49-59, 2004.

Schwartzkopf, S.H., Mancinelli, R.L.: Germination and growth of wheat in simulated Martian atmospheres. – Acta Astronaut. **25**: 245-247, 1991.

Smith, B.N., Lytle, E.M., Hansen, L.D.: Predicting plant growth rates from dark respiration rates: an experimental approach. – In: Roundy, B.A., McArthur, E.D., Haley, J.S. *et al.* (ed.): Proceedings: Wildland Shrub and Arid Land Restoration Symposium. Oct 19-21, 1993, Las Vegas, Nevada. Gen Tech Rep. INT-GTR-315. USDA For. Serv., Intermountain Research Station, Ogden, Utah, US. Pp.243-245, 1995.

Swain, T., Hillis, W.E.: The phenolics constituents of Prunus domestica. I. The quantitative analysis of phenolics constituents. – J. Sci. Food Agr. **10**: 63-68, 1959.

Villarreal-Lozoya, J.E., Lombardini, L., Cisneros-Zevallos, L.: Phytochemical constituents and antioxidant capacity of different pecan [*Carya illinoinensis* (Wangenh.) K. Koch] cultivars. – Food Chem. **102**: 1241-1249, 2007.

Wargovich, M.J.: Anticancer properties of fruits and vegetables. – HortScience **35**: 573-575, 2000.

Wheeler, R.M.: Roadmaps and Strategies for Crop Research for Bioregenerative Life Support Systems: A Compilation of Findings from NASA's Advanced Life Support Meetings. NASA Kennedy Space Center Biological Sciences Office. NASA/TM – 2009-214768, 2009.

Wheeler, R.M., Mackowiak, C.L., Sager, J.C., Yorio, N.C., Knott , W.M.: Growth and gas exchange by lettuce stands in a closed, controlled environment. – J. Amer. Soc. Hort. Sci. **119**: 610-615, 1994.

Wu, X., Beecher, G.R., Holden, J.M. *et al*.: Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. – J. Agr. Food. Chem. **52**: 4026-37, 2004.