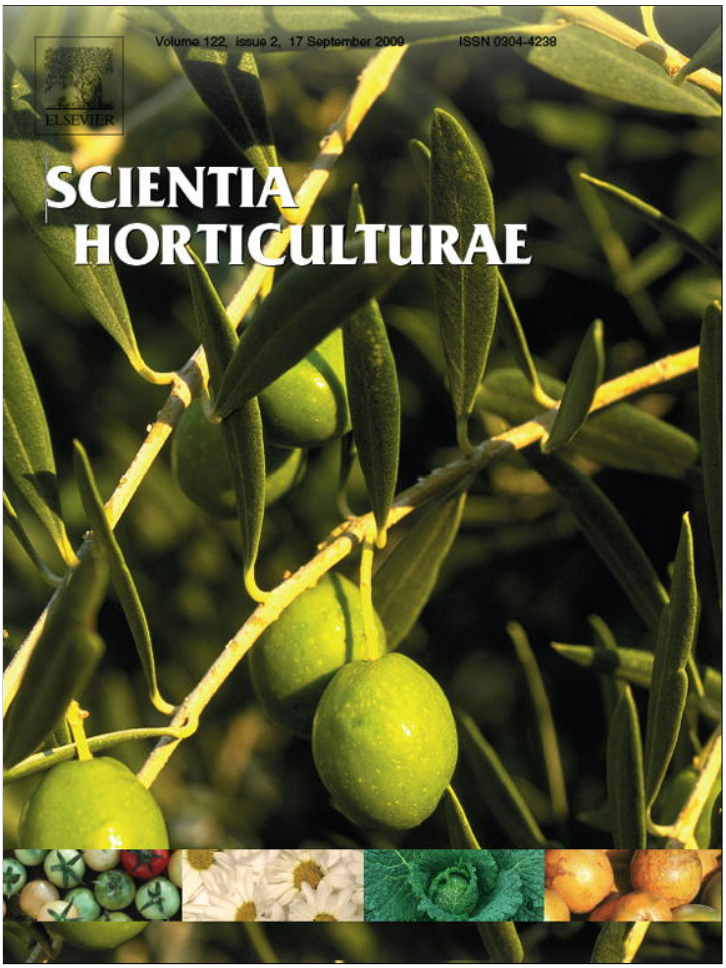

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Hypobaria and hypoxia affects growth and phytochemical contents of lettuce

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ABSTRACT

The primary objective of this research was to investigate how low pressure (hypobaria) and low oxygen (hypoxia) affect functional phytochemicals and the nutritional quality of 'Red Sails' lettuce (*Lactuca sativa* L.). Plants were grown under two levels of total gas pressure (reduced or ambient (25 or 101 kPa, respectively)) at three levels of O_2 partial pressures (low, medium or ambient (6, 12 or 21 kPa, respectively)). Hypoxia effects on nutritional and functional phytochemicals were more pronounced than hypobaria effects. Regardless of the total pressure, hypoxia, in general, enhanced leaf anthocyanin levels, enhanced total phenolic compounds, enhanced carbohydrate concentration and enhanced free radical scavenging capacity of lettuce but reduced leaf mineral concentration. Hypoxia increased the ethylene production of plants but ethylene accumulation was not the sole reason for enhanced anthocyanin production in plants grown under hypoxia. Our results suggest that low oxygen stress induces the production of protective phytochemicals and the free radical scavenging potential in lettuce, which may in turn enhance the functional value. However, further human intervention studies are needed to confirm if enhanced phytochemicals in plants have significant impact in human body.

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1. Introduction

The ability to produce plants under suboptimal conditions (i.e. low light, low pressure, low oxygen, low temperature) is of importance to space exploration programs to reduce engineering and material handling costs. The potential for plant growth under low pressure (hypobaria) and low oxygen (hypoxia) environments is being investigated at Texas A&M University as a part of a space exploration program. Without O_2 supplementation, hypobaric environments can lead to hypoxia, therefore, a potential limitation to plant growth under hypobaric conditions. Previous research has shown that seed germination of lettuce and wheat was not adversely affected by low pressure (50 kPa) and that seedlings grown under low pressure had greater shoot and root growth than those grown under ambient pressure (101 kPa) (He et al., 2003). Hypoxia increased ethylene production both under hypobaric and ambient pressure conditions (He et al., 2003). They also reported that growth of 'Buttercrunch' lettuce was comparable under 50 and 101 kPa total pressure and that low pressure reduced the occurrence of tip burn compared to ambient pressure (He et al., 2006). Hypobaria reduced ethylene accumulation, which resulted in better growth of seedlings than under ambient pressure (He et al., 2006). In another study, He et al. (2007) reported that biomass accumulation of 'Buttercrunch' lettuce was not affected by hypobaria (101 kPa vs. 25 kPa) but hypoxia (6 kPa oxygen partial pressure [pO_2]) reduced biomass regardless of the atmospheric pressure. There was no significant difference in biomass accumulation, photosynthesis or dark period respiration rate of lettuce plants grown under 21 or 12 kPa pO_2. These results show that food crops can be grown successfully under certain levels of hypobaric and/or hypoxic conditions.

Plants provide both nutritional (i.e. carbohydrates, proteins and lipids) and functional (i.e. secondary metabolites) phytochemicals needed to maintain good health. Epidemiological studies have reestablished the ancient wisdom that consumption of plant based foods reduces the risk of obesity and various chronic diseases such as cancer and diabetes (Fahey and Stephenson, 1999; Wargovich, 2000). Functional phytochemicals act as antioxidants by neutralizing harmful free radicals thus, protecting plant's cellular materials including DNA, proteins and lipids from oxidative damage and preventing the damage to vital functions. Functional phytochemicals are thought to act similar manner in human bodies thus, protecting cellular materials and preventing the onset of various ailments. A diet rich in natural antioxidants and anti-carcinogenic compounds is highly desirable for a healthy life, particularly for those who are constantly exposed to oxidative environments, such as in long-term space exploration missions where astronauts are constantly exposed to high levels of ionizing cosmic radiation or workers in industrial zones that results in oxidative stresses and increased rate of carcinogenesis.

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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 (Dumas et al., 2003; Beckwith et al., 2004; Engelen-Eigles et al., 2006; Kubota et al., 2006; Luis et al., 2007). Stress caused by low pressure and low oxygen could affect functional phytochemicals in a similar manner, but very few reports exists relating to how hypobaria and hypoxia influence the production of functionally important phytochemicals. Musgrave et al. (2005) reported that *Brassica rapa* L. grown onboard International Space Station (ISS) had comparable levels of leaf chlorophyll, starch, and soluble carbohydrates that were gown on earth but ISS grown plants had considerably higher levels (75%) of glucosinolates than earth grown plants. They also reported that immature seeds grown onboard ISS had more chlorophyll, starch, and soluble sugars but less proteins than earth grown plants. Levine et al. (2008) reported that hypobaria did not affect sensory characteristics, carbohydrate content, the nutrient content or the total antioxidant capacity of radish (*Raphanus sativus* L.). They reported that root glucosinolate content and leaf nitrate concentration decreased as the atmospheric pressure decreased. These results suggest that hypobaria and hypoxia may cause stress in plants that affects the production of protective phytochemicals, which may in turn affect the functional value of food crops. Low pressure plant growth facility offers an excellent opportunity to investigate hypobaria and hypoxia effects on quality of food crops. The objective of this research was to investigate how hypobaria and hypoxia affect protective phytochemicals (anthocyanin, phenolic compounds, carotenoids) and the free radical scavenging potential of food crops using lettuce as the model crop, a crop that has been identified for NASA's salad bowl program. We used 'Red Sails' because of anthocyanin pigmentation.

2. Materials methods

2.1. Low pressure plant growth (LPPG) chambers

Research was carried out at the LPPG facility at Texas A&M University. The LPPG facility is a fully automated system capable of controlling total pressure, gas composition, and nutrient supply. A detailed description of the LPPG system can be found in He et al. (2007). Briefly, the LPPG facility consisted of six cylindrical clear acrylic growth chambers, each with 55 L capacity, constructed inside a temperature controlled plant growth room. The LPPG chambers were designed to withstand pressure as low as 5 kPa. Total atmospheric pressure and the partial pressures of O_2 and CO_2 in each chamber are controlled and continuously monitored with a pressure transducer (Ashcroft K2; Dresser Instruments, Addison, TX), an oxygen sensor (MAX-250; Mextec Inc., Salt Lake City, UT), and a CO_2 sensor (GMM222; Vaisala, Helsinki, Finland), respectively and recorded at 1 min intervals. Total pressure is reduced by evacuating each chamber to target level with a rotary vane vacuum pump. Set gas compositions in individual chambers are maintained by addition of O_2, CO_2 and/or N_2 as necessary, detected by the control system. Temperature and relative humidity inside each growth chamber are continuously monitored by a HT-761 transmitter (Ohmic Instruments Co., Easton, MD) and recorded. Sylvania (400 W) metal halide lamps provide lighting to the growth room and is the light source for plants in LPPG chambers. The average light intensity inside growth chambers (at plant level) is about 600 $\mu mol\ m^{-2}\ s^{-1}$. Each chamber utilizes a counter flow heat exchanger to control humidity and condensation of excess water from air. Air from each chamber is circulated at 25 L/min, through closed stainless steel columns with steel coils for circulating chilled water (13 °C), by a diaphragm pump (Air Cadet; Cole Palmer, Vernon Hills, IL). Ethylene can be scrubbed, if necessary, by channeling circulating air through a column containing potassium permanganate. Nutrient solution can be added to self-watering pots as needed, through an air-lock system. A sealed port is provided in each chamber to sample headspace gases if manual analysis is necessary.

2.2. Plant growth

Lettuce (*Lactuca sativa* L. cv. Red Sails) seeds were germinated in 1 L pots containing washed fine grade calcined clay (particle size <1 mm, 74% porosity, 0.56 $g\ cm^{-3}$ bulk density and 2.5 $g\ cm^{-3}$ particle density) (Profile Products LLC, Buffalo Grove, IL). Pots containing imbibed seeds were kept inside a walk-in growth room maintained at 25 °C with 75% relative humidity and ≈600 $\mu mol\ m^{-2}\ s^{-1}$ PAR (irradiance at the plant level) from both fluorescent and incandescent lamps. Seeds germinated within 3–4 days. Upon germination, seedlings were fertilized with modified Hoagland's nutrient solution (pH 6.3) containing 4 mM $Ca(NO_3)_2$, 1.0 mM KH_2PO_4/K_2HPO_4, 2 mM KNO_3, 1 mM $MgSO_4$, 50 μM Fe-EDTA, 1 mM NaCl and micronutrients; 50 μM B, 10 μM Mn, 1 μM Cu, 2 μM Zn and 0.3 μM Mo.

Six to eight days after germination, 30 seedlings were transplanted into ten 4 L self-watering pots (three seedlings per pot) containing a pre-washed calcined clay medium. The reservoir of the self-watering pot was filled with modified Hoagland's nutrient solution and transplanted seedlings were allowed to grow for another 10 days in the same walk-in growth chamber. The photoperiod was 12-h, which provided a DLI of 25.9 $mol\ day^{-1}$. The same production protocol was used in all experiments described below. Seedlings were 16–18 days old when they were exposed to conditions in the following experiments.

2.3. Experiment 1: effect of hypoxia on leaf phytochemical and mineral nutrient levels at ambient atmospheric pressure (101 kPa)

At the end of 10-day growth period inside the walk-in growth chamber, six pots with uniformly grown plants were selected. These pots were then placed inside the LPPG growth chambers (one pot per chamber) and sealed. After placement, nutrient reservoir was filled with 1 L of nutrient solution through an air-lock system and refilled again with 800 mL after 5 days. All chambers were maintained at total atmospheric pressure of 101 kPa and three pO_2 levels (21, 12, and 6 kPa) were used to test hypoxia effects. Two chambers were maintained at each pO_2 level. Photoperiod was set to 12 h (09:00–21:00 h), so that plants received 25.9 mol of light per day. Carbon dioxide level inside growth chambers were maintained at a minimum set point of 100 Pa (1000 $\mu mol\ mol^{-1}$) during the photoperiod. Dark period CO_2 was not controlled. Average temperature and relative humidity inside LPPG chambers were 23 ± 3 °C, and 86 ± 3%, respectively. The experiment was repeated for a total of four replicates each treatment ($n = 4$). To avoid chamber effects, treatments were rotated among LPPG chambers. Ethylene was not scrubbed in this experiment. Ethylene levels were measured on 5th and 10th day by withdrawing a headspace gas sample (≈10 mL), at the end of dark period cycle. Approximately 1 mL of the gas sample was injected into a gas chromatograph fitted with a photoionization detector (Photovac 10 Plus, PerkinElmer Inc., Norwark, CT).

2.4. Experiment 2: influence of hypobaria and hypoxia on leaf phytochemical and mineral nutrient content

At the end of 10-day growth period, six pots were placed inside the LPPG growth chambers (one pot per chamber) and sealed. Plants in LPPG chambers were subjected to combination of two

atmospheric pressures (101 or 25 kPa) and two pO_2 levels (21 or 6 kPa) in 2×2 factorial design. Light and CO_2 level during photoperiod were the same as described in experiment 1. Average temperature and relative humidity inside the LPPG chambers during the experiment were 24 ± 3 °C, and 83 ± 4%, respectively. The experiment was repeated for a total of three replicates each treatment combination ($n = 3$). To avoid chamber effects, treatments were rotated among LPPG chambers. Ethylene was scrubbed by channeling circulating air through a potassium permanganate column.

2.5. Experiment 3: role of ethylene on anthocyanin accumulation under hypoxia

Six pots were placed inside the LPPG growth chambers (one pot per chamber) and sealed. Prior to sealing, a 50-mL plastic bottle containing 20-mL water and sufficient amount of 1-methylcyclopropane (1-MCP) tablets to generate 1 μmol mol^{-1} of 1-MCP gas (in 55-L chamber) was placed inside two of the six chambers (Agro-Fresh Inc., Spring House, PA, USA). Two of the remaining four chambers were injected with ≈3 mL of 20,000 μmol mol^{-1} ethylene gas to yield a final concentration of 1 μmol mol^{-1} ethylene in each chamber. The remaining two chambers were used as the control and ethylene was scrubbed by channeling circulating air through the potassium permanganate column, as previously described. Air was not channeled through the ethylene scrubber in chambers treated with ethylene or 1-MCP gas. All chambers were maintained at total pressure of 101 kPa during the experiment. Each of the two chambers within a gas treatment was maintained at set point of 21 or 6 kPa pO_2. The experiments were repeated for a total of three replicates each treatment combination ($n = 3$). To avoid chamber effects, treatments were rotated among LPPG chambers. Light and CO_2 level during photoperiod were the same as described in experiment 1. Average temperature and relative humidity inside LPPG chambers during the experiment were 25 ± 4 °C, and 86 ± 4%, respectively. Ethylene and 1-MCP were monitored on the 1st, 5th, and 10th day by withdrawing headspace gas and injecting into a digital gas chromatograph fitted with a photoionization detector (PID), as previously described.

2.6. Measurements

In each experiment, plant tops were harvested after 10 days of growth in LPPG chambers. Three plants from each treatment were wrapped separately in aluminum foil and dipped in liquid N_2 and stored at −80 °C until lyophilization. Lyophilized plants were ground in a Wiley mill to a fine powder (40 mesh) and stored at −20 °C in plastic vials containing desiccant sachets until chemical analysis. Phytochemical extraction was made in triplicate samples from each treatment combination. Fifty milligrams of ground tissue was used for each extraction.

2.7. Extraction of phenolic compounds

All extractions were made in triplicate samples from each replicate. For phenolic extraction, 15 mL of acidified 80% methanol was added to 50 mg ground tissue and extracted overnight in dark at 4 °C (Wrolstad et al., 2002). Following overnight extraction, 10 mL of chloroform was added, vortexed and centrifuged at 3000 rpm for 30 min. Supernatant was used for anthocyanin, total phenolic, and antioxidant capacity (AOC) assays. Anthocyanin content was determined using the pH-differential method (Wrolstad et al., 2002). Total phenolic content was determined using Folin-Ciocalteu reagent, and gallic acid as the standard (Singleton and Rossi, 1965). Antioxidant capacity was determined as 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, expressed as Trolox equivalents (Yamaguchi et al., 1998).

2.8. Extraction and separation of carotenoids and chlorophyll

Carotenoids and chlorophyll were extracted using 50:25:25 (v/v) hexane:acetone:methanol. Ten milliliters of solvent mixture was added to 50 mg ground tissue and extracted overnight in the dark at 4 °C. Following overnight extractions, 5 mL of water was added, vortexed and centrifuged at 4 °C for 30 min. Equal volume (2-mL) of hexane and N-N-dimethylformamide (DMF) was added to 200-μL of supernatant, vortexed and allowed to separate for 15 min in dark. Absorbance of hexane fraction was recorded at 450 nm and carotenoids were quantified as β-carotene equivalent using extinction coefficient for β-carotene (Wrolstad et al., 2002). For chlorophyll determination, absorbance of DMF fraction was recorded at 664 and 647 nm and chlorophyll was estimated as described by Moran (1982).

2.9. Total carbohydrate determination

Total carbohydrate content was determined following the Somogyi–Nelson method (Somogyi, 1952). Briefly, 5 mL of 80% methanol and 10 mL of sodium acetate buffer (pH 4.5) were added to 50 mg ground tissue and boiled for 2 h. Upon cooling, 1 mL invertase (5 units/mL) and 1 mL amyloglucosidase (50 units/mL) were added and incubated at 45 °C for 3 days. Total carbohydrates were expressed as glucose equivalents.

2.10. Leaf elemental analysis

Leaf macro- and micro-elements mineral were analyzed by the Clemson University Agricultural Service Laboratory.

2.11. Experimental design and data analysis

There were six LPPG chambers. Therefore, each experiment was repeated to obtain three or four replicates ($n = 3$ or 4) depending on the experiment. Each LPPG chamber contained a pot with three plants. Phytochemical extractions were performed on triplicate samples from each treatment/replicate combination. All data were subjected to analysis of variance (ANOVA) and means were compared using LSD or PDIFF ($P = 0.05$) procedure of SAS (SAS Inc., Cary, NC).

3. Results

3.1. Effect of pO_2 on phytochemical content at ambient atmospheric pressure

Shoot fresh weight was not different between plants grown at 21 and 12 kPa pO_2 but it was lower (≈26%) in plants grown at 6 kPa pO_2 (data not shown). However, shoot dry weight was similar for all pO_2 levels (Table 1). Leaf anthocyanin, total phenolics, carbohydrate and mineral contents were affected by hypoxia (Tables 1 and 2). Plants grown at 6 and 12 kPa pO_2 had more red pigmentation than those grown at 21 kPa pO_2 (Fig. 1A and B). Consistent with visual observations, leaf anthocyanin concentration of plants grown at 6 and 12 kPa pO_2 was about 75% greater than that of plants grown at 21 kPa pO_2 (Table 1). There was no significant difference in leaf anthocyanin concentration between 6 and 12 kPa pO_2 grown plants. Total leaf anthocyanin content per pot followed a similar pattern. Total phenolic concentration of plants grown at 6 and 12 kPa pO_2 was about 18% greater than that of plants grown at 21 kPa pO_2. Total phenolic content was not affected by the pO_2. Plants grown at 6

Table 1
Leaf dry weight, anthocyanin concentration, total anthocyanin content, total phenolic concentration and content, ratio of anthocyanin:total phenolics, free radical scavenging activity, and carbohydrate concentration and content of 'Red Sails' lettuce grown for 10 days at ambient total gas pressure (101 kPa) under 6, 12 or 21 kPa partial pressure of oxygen (pO_2). Ethylene was not scrubbed from the chambers. Each number is the average of 12 measurements (triplicate measurements from each replicate). Mean separation within columns by LSD at $P = 0.05$. Means with same letter, within a column, are not significantly different. Concentrations are based on dry weight. Each pot had three lettuce plants therefore, total content is presented as mg/pot.

pO_2 (kPa)	Leaf dry weight (g)	Anthocyanin conc. (mg g^{-1})	Total anthocyanin content (mg/pot)	Phenolic conc. (mg g^{-1})	Total phenolic content (mg/pot)	Anthocyanin: total phenolics (%)	Free radical scavenging activity (μmol g^{-1})	Carbohydrate conc. (mg g^{-1})	Total carbohydrate content (mg/pot)
21	5.2 a	2.2 b	10.4 b	32.0 b	159.0 a	6.8 b	208 b	178.4 b	833.1 b
12	5.2 a	3.5 a	16.8 a	36.8 ab	183.1 a	9.3 a	252 a	197.4 b	919.7 b
6	4.5 a	4.2 a	18.0 a	39.0 a	169.8 a	10.5 a	265 a	288.3 a	1167.3 a

Table 2
Leaf mineral concentration of 'Red Sails' lettuce grown for 10 days under ambient total gas pressure (101 kPa) at 6, 12 or 21 kPa partial pressure of oxygen (pO_2). Ethylene was not scrubbed from the chambers. Each number is the average of 4 measurements. Mean separation within columns by LSD at $P = 0.05$. Means with same letter, within a column, are not significantly different. Concentrations are based on dry weight.

pO_2 (kPa)	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})	S (mg g^{-1})	Zn (μg g^{-1})	Mn (μg g^{-1})	Fe (μg g^{-1})	Cu (μg g^{-1})
21	40.8 a	5.7 a	71.6 a	5.4 a	1.9 a	2.7 a	30.0 a	862 a	153.0 a	4.3 a
12	35.1 b	5.6 a	61.5 a	5.0 a	1.8 a	2.5 a	22.8 b	851 a	143.3 a	3.3 a
6	32.8 b	4.6 a	48.7 b	3.6 b	1.3 b	1.8 b	21.5 b	612 b	129.8 a	2.8 a

and 12 kPa pO_2 had a greater percentage of anthocyanin in total phenolic fraction than the plants grown at 21 kPa pO_2. The DPPH free radical scavenging activity of plants grown at 6 or 12 kPa pO_2 was greater (24%) than plants grown at 21 kPa pO_2. There was no significant difference in these parameters between plants grown at 6 and 12 kPa pO_2. Plants grown at 6 kPa pO_2 had significantly more total carbohydrate concentration (54%) than those grown at 12 or 21 kPa pO_2. There was no significant difference in carbohydrate concentration of plants grown under 12 or 21 kPa O_2. Total carbohydrate content followed a similar pattern but the magnitude of increase was not as great as the increase in concentration. Total chlorophyll or β-carotene levels were not significantly affected by pO_2 (data not shown). In general, leaf mineral nutrient concentration decreased as the pO_2 decreased (Table 2). Levels of K, Ca, Mg, S and Mn were significantly ($P < 0.05$) lower in plants grown at 6 kPa pO_2 than those grown at 12 or 21 kPa pO_2, while plants grown at 21 kPa had the greatest level of N and Zn.

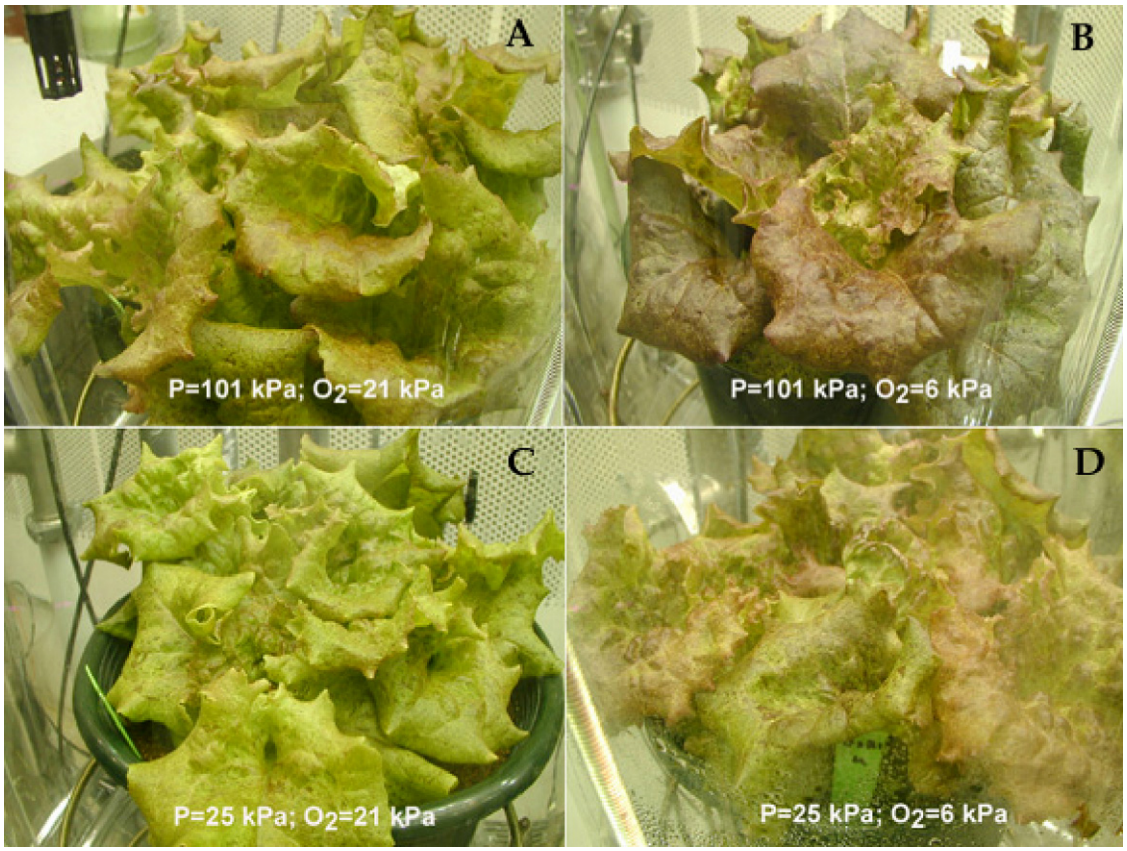

Fig. 1. Effect of total pressure and oxygen partial pressure on growth of Red Sails lettuce. Picture are plants grown at (A) 101/21 kPa total pressure/pO_2; (B) 101/6 kPa total pressure/pO_2; (C) 25/21 kPa total pressure/pO_2; (D) 25/6 kPa total pressure/pO_2.

Table 3
Effects of total gas pressure and oxygen partial pressure (pO_2) on leaf dry weight, anthocyanin concentration/content, total phenolic concentration/content, ratio of anthocyanin:total phenolics, free radical scavenging activity, total carbohydrate concentration/content and leaf chlorophyll concentration/content of 'Red Sails' lettuce. Ethylene was scrubbed from the chambers. Each number is the average of nine measurements (triplicate measurements from each replicate). Mean separation within columns by PDIFF of SAS at $P = 0.05$. Means with same letter, within a column, are not significantly different. Concentrations are based on dry weight. Each pot had three lettuce plants therefore, total content is presented as mg/pot.

Total pressure (kPa)	pO_2 (kPa)	Leaf dry weight (g)	Anthocyanin conc. (mg g^{-1})	Total anthocyanin content (mg/pot)	Phenolic conc. (mg g^{-1})	Total phenolic content (mg/pot)	Anthocyanin: total phenolics (%)	Free radical scavenging activity (μmol g^{-1})
101	21	3.5 a	1.6 bc	4.7 b	25.9 b	90.9 a	6.0 bc	192 b
101	6	2.8 a	2.9 a	8.4 a	29.3 a	80.9 ab	9.8 a	229 a
25	21	1.2 b	1.0 c	1.3 c	25.5 b	29.8 c	4.1 c	182 b
25	6	1.9 b	2.1 b	5.4 b	27.4 ab	53.9 bc	7.6 b	201 b

Total pressure (kPa)	pO_2 (kPa)	Carbohydrate conc. (mg g^{-1})	Total carbohydrate content (mg/pot)	Chlorophyll conc. (mg g^{-1})	Total chlorophyll content (mg/pot)
101	21	169 b	666 ab	9.1 ab	31.5 a
101	6	254 a	814 a	9.4 a	24.9 ab
25	21	124 b	162 c	8.0 c	9.9 c
25	6	166 b	363 bc	8.4 bc	17.5 bc

Table 4
Effect of total gas pressure and oxygen partial pressure (pO_2) on leaf mineral concentration of 'Red Sails' lettuce. Ethylene was scrubbed from chambers. Each number is the average of three measurements. Mean separation within columns by PDIFF of SAS at $P = 0.05$. Means with same letter, within a column, are not significantly different. Concentrations are based on dry weight.

Total pressure (kPa)	pO_2 (kPa)	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})	S (mg g^{-1})	Zn (μg g^{-1})	Mn (μg g^{-1})
101	21	44.8 ab	6.1 a	66.1 a	5.5 b	2.2 b	2.9 ab	35.0 b	1028 b
101	6	37.8 b	4.5 b	52.5 b	4.4 c	1.6 c	2.0 c	22.3 c	817 c
25	21	49.1 a	6.3 a	64.7 a	6.9 a	2.7 a	3.1 a	46.3 a	1331 a
25	6	42.2 ab	6.2 a	62.0 a	5.7 b	2.1 b	2.7 b	33.5 b	1060 b

3.2. Effect of total pressure and pO_2 on phytochemical content

Plants grown at 101/6 kPa total pressure/pO_2 had the most red pigmentation (Fig. 1B) while plants grown at 25/21 kPa total pressure/pO_2 had the least red pigmentation (Fig. 1C). Plants grown at 101/21 and 25/6 kPa total pressure/pO_2 were in between in coloration (Fig. 1A and D). Overall, plants grown at 25/21 kPa total pressure/pO_2 appeared to be pale green color (Fig. 1C). Apart from pigmentation differences, appearance of plants was normal and was of acceptable quality in all treatments. However, the leaves of plants grown under 25 kPa total pressure were not as firm as the leaves of plants grown under 101 kPa total pressure (data not shown). In general, plants grown at 101 kPa total pressure had greater dry biomass than plants grown at 25 kPa total pressure (Table 3). Dry biomass was not significantly different between plants grown at 6 and 21 kPa pO_2 at either total pressure levels. Plants grown at 25 kPa total pressure and 21 kPa pO_2 had the least biomass accumulation.

In general, anthocyanin production, free radical scavenging activity and carbohydrate accumulation were lower in plants grown at 25 kPa total pressure than in plant grown at ambient total pressure (Table 3). Total pressure did not significantly affect the total phenolic concentration, but total phenolic content was lower under 25 kPa total pressure than ambient total pressure. The ratio of anthocyanin to total phenolic was generally lower under 25 kPa total pressure than 101 kPa total pressure. Consistent with previous observations, low pO_2 (6 kPa) generally increased anthocyanins, total phenolics, free radical scavenging activity, and carbohydrate levels, regardless of the total pressure. Increase in anthocyanin was greater than the increase of other phenolic compounds at low pO_2, so that the ratio of anthocyanin to total phenolics increased when grown at low pO_2 both under low and ambient total pressure. Low total pressure generally reduced the leaf chlorophyll concentration and this was also evident from lighter green appearance of leaves inside low pressure chambers. Partial pressure of O_2 had no effect on chlorophyll concentration.

Table 5
Effect of ethylene or 1-methylcyclopropane (MCP) at 1 μmol mol^{-1} on anthocyanin, total phenolic, and β-carotene concentrations in 'Red Sails' lettuce grown under 6 or 21 kPa oxygen partial pressures (pO_2) at ambient total gas pressure (101 kP). Ethylene was scrubbed from two control chambers. Ethylene was not scrubbed from ethylene or MCP treated chambers. Each number is the average of nine measurements (triplicate measurements from each replicate). Mean comparison within a column by PDIFF of SAS at $P = 0.05$. Means with same letter, within a column, are not significantly different. Concentrations are based on dry weight.

pO_2 (kPa)	Ethylene treatment	Headspace ethylene level[a] (μmol mol^{-1})	Anthocyanin conc. (mg g^{-1})	Total phenolic conc. (mg g^{-1})	Anthocyanin: total phenolics (%)	β-Carotene conc. (μg g^{-1})
21	Scrubbed	0	2.0 c	31.9 a	6.6 d	578 abc
	Ethylene	640 ± 44	3.4 ab	29.9 a	11.5 ab	478 bc
	MCP	167 ± 150	2.6 bc	32.1 a	7.7 cd	622 a
6	Scrubbed	0	3.6 ab	35.4 a	10.4 ab	606 ab
	Ethylene	659 ± 100	4.1 a	34.4 a	12.5 a	438 c
	MCP	302 ± 120	2.6 bc	28.9 a	9.8 bc	592 abc

[a] Average headspace ethylene concentration on the day of harvest of three experimental runs.

β-Carotene content was not affected by hypobaria or hypoxia (data not shown).

Plants grown at 25 kPa total pressure had more leaf mineral concentration, in general, than those grown at ambient total pressure (Table 4). Regardless of total pressure, low pO_2 reduced leaf concentration of Ca, Mg, S, Zn, and Mn. The greatest effect of low pO_2 was at ambient total pressure.

3.3. Effects of ethylene on phytochemical content under hypoxia

Under ambient total pressure, ethylene had no significant effect on leaf dry weight under 6 and 21 kPa pO_2 (data not shown). Low pO_2 increased leaf anthocyanin concentration (80%), while not affecting total phenolic concentration compared to ambient pO_2 grown plants (Table 5). Ethylene increased anthocyanin concentration (70%) in 21 kPa pO_2 grown plants but did not significantly affect anthocyanin concentration of 6 kPa pO_2 grown plants. Ethylene treated plants grown at 21 kPa pO_2 had anthocyanin concentration comparable to control plants (ethylene scrubbed) plants grown at 6 kPa pO_2. Ethylene increased the ratio of anthocyanin to total phenolics in 21 kPa pO_2 grown plants but had no affect under 6 kPa pO_2. Ethylene action inhibitor, 1-MCP, had no significant effect on anthocyanin concentration of plants grown at 21 kPa pO_2 but slightly decreased that of plants grown at 6 kPa pO_2. 1-MCP had no effect on total phenolic concentration or free radical scavenging activity (data not shown). Ethylene treatment did not affect chlorophyll concentration (data not shown) but reduced β-carotene concentration.

4. Discussion

Consistent with previous reports (He et al., 2003, 2006, 2007), this research shows that 'Red Sails' lettuce can be grown under hypobaric and/or hypoxic environments. Under ambient total pressure, reducing pO_2 to 12 kPa did not affect plant size, fresh or dry shoot biomass, but further reduction of pO_2 to 6 kPa resulted in ≈25% reduction fresh shoot biomass while not affecting dry shoot biomass. Although the plants were small, the leaves of plants grown at 6 kPa pO_2 appeared to be thicker and more turgid than the leaves of plants grown at 21 or 12 kPa pO_2. He et al. (2007) reported that the leaf area and leaf fresh and dry weights of 'Buttercrunch' lettuce grown at 6 kPa pO_2 were reduced over 30% compared to those grown at 12 or 21 kPa pO_2 and that plants grown at 6 kPa pO_2 had thicker leaves than those grown at 12 or 21 kPa pO_2 as indicated by the lower specific leaf area ($cm^2\ g^{-1}$ fresh weight). In contrast to present observations, Lenz and Antoszewski (1982) reported that green pepper (*Capsicum annuum*) plants grown for 3 weeks under low O_2 (2–5%) environment accumulated more dry matter in leaves and lateral shoots but had fewer flowers and fruits than plants grown in normal air. They attributed the increase in leaf dry matter under low O_2 to the reduction of photorespiration, a process by which plants protect their chloroplast against oxidizing conditions, particularly in C-3 plants such as lettuce and green peppers. Although useful against chloroplast damage, photorespiration results in considerable loss of photosynthates available for biomass production. In controlled environments, such as in greenhouse production, high CO_2 levels are maintained to reduce photorespiration. In the present experiment, CO_2 concentration inside LPPG chambers was maintained at 1000 μmol mol^{-1} (100 Pa) during the photoperiods, 3-fold greater than ambient pCO_2 levels. Therefore, the photorespiration would have been minimal to have an effect on biomass production under our experimental conditions.

In general, under 25 kPa total pressure plants showed a reduction in biomass production and 21 kPa pO_2 resulted in least biomass accumulation. The reduction of biomass production under 25/21 total pressure/pO_2 could at least be partly attributed to a greater photorespiration. Although the CO_2 levels inside LPPG chambers during the photoperiod were maintained at 1000 μmol mol^{-1}, this may not have been high enough to suppress photorespiration under the extremely high levels of O_2 (84%) inside LPPG chambers maintained at 25/21 kPa total pressure/pO_2 thus, limiting the photosynthates available for biomass production.

Under ambient total pressure, plants grown at 6 kPa pO_2 had greater total carbohydrate concentration than those grown at 21 or 12 kPa pO_2. Low total pressure (25 kPa) in general reduced carbohydrates compared to ambient total pressure. The increase in leaf carbohydrate level in plants grown at 6 kPa pO_2 indicates that more photosynthate was available for metabolic processes in plants grown at 6 kPa pO_2, probably due to greater photosynthesis activity than respiration. Alternatively, the translocation and utilization of photosynthates could have been impaired by low O_2 due to reduced over all metabolism. Lenz and Antoszewski (1982) reported that low O_2 (2–5%) reduced the translocation of photosynthates to storage organs therefore, increasing the soluble carbohydrate in shoot tissue. The increase in leaf carbohydrates under 6 kPa pO_2 could also be at least partially attributed to the restricted root growth as observed by He et al. (2007). Reduced root mass and the metabolic processes in the roots could reduce the translocation of photosynthesis thus, increase the carbohydrate pools in shoot tissue. Priestley et al. (1988) reported that low O_2 did not alter translocation of soluble sugar but reduced the respiratory losses from leaves and the ability of tissue to incorporate soluble sugars into polysaccharides resulting in increased soluble carbohydrate levels in leaf tissue. The increased leaf carbohydrate levels reduced the photosynthesis when plants were exposed to low O_2 levels for longer period of time. However, we did not separately measure the leaf soluble sugar levels in the present experiment.

In addition to nutritionally important compounds such as carbohydrates, proteins and fats, plants contain wide range of functional phytochemicals that act as antioxidants by quenching highly reactive free radicals and protect cellular material such as DNA, proteins and fats from oxidative damage. Plants increase the production of these functional phytochemicals in response biotic and abiotic stress conditions. Our results showed that regardless of the total pressure, stress caused by hypoxia increased the production of stress related phytochemicals, particularly, anthocyanins, and increased free radical scavenging activity. There was no significant difference in anthocyanin or total phenolic concentration between plants grown at 12 and 6 kPa at ambient total pressure indicating that moderate O_2 stress can induce protective compounds. Total anthocyanin content followed a similar increase to that of concentration as the pO_2 decreased indicating the reduced anthocyanin concentration in plants grown at 21 kPa was not due to a dilution effect. Although total phenolics concentration increased, total phenolic content did not follow a similar pattern as pO_2 decreased indicating that the biosynthesis of only certain phenolic compounds were affected by pO_2. The increase of anthocyanin was greater than the increase of total phenolic compounds at low pO_2, suggesting that low pO_2 favored the anthocyanin biosynthetic pathway. Low total pressure reduced anthocyanin concentration and content indicating that reduction of anthocyanins under low pressure is not a result of a dilution effect. Low total pressure also reduced the total phenolic content in plants. Alterations in phytochemical contents under space flight conditions have previously been reported. *B. rapa* L. grown onboard international space station (ISS) had comparable levels of leaf chlorophyll, starch, and soluble carbohydrates than those grown on earth, but ISS grown plants had considerably higher levels (75%) of glucosinolates than earth grown plants (Musgrave et al., 2005).

They also reported that immature seeds grown onboard ISS had more chlorophyll, starch, and soluble sugars but less proteins compared to earth grown plants. These results show that plants grown under suboptimal conditions may have greater free radical scavenging potential than those grown under normal conditions.

In the present experiment, pO_2 had no effect on leaf chlorophyll concentration. Low total pressure reduced leaf chlorophyll concentration compared to ambient pressure grown plants. This was also evident from the pale green appearance of plants grown under low pressure (Fig. 1). In contrast to present results, He et al. (2007) reported that, at ambient total pressure, plants grown under 6 kPa pO_2 had greater leaf chlorophyll levels in 'Buttercrunch' lettuce than those grown at 12 or 21 kPa pO_2. They also reported that the leaf chlorophyll levels were greater in plants grown under 25 kPa than 101 kPa total pressure. The conflicting results under similar experimental conditions could be partly due to the cultivar differences and the differences in the analytical methods used. He et al. (2007) used a SPAD meter to measure leaf greenness and correlated greenness with a previously developed chlorophyll vs. SPAD greenness curve to quantify leaf chlorophyll and expressed results as μg chlorophyll per cm^2 while in the present experiment chlorophyll was extracted using acetone and quantified as μg chlorophyll per gram of freeze dried material. Therefore, the direct comparison of chlorophyll between two experiments is not possible.

Leaf mineral concentrations were affected by hypoxia and hypobaria (Tables 2 and 4). In general, hypobaria increased leaf mineral concentration while hypoxia decreased it. The reduction in leaf mineral concentrations under hypoxia could be attributed to the poor root growth under low O_2 thus limiting the water and mineral uptake. Root growth was not evaluated in the present experiment. However, He et al. (2007), in a similar experiment with 'Buttercrunch' lettuce, reported that 6 kPa pO_2 resulted in 50–70% reduction in root dry weight compared to 12 or 21 pO_2 suggesting that low O_2 imposes a greater influence on root growth. Although 6 kPa pO_2 does not appear to cause anoxic conditions in shoot tissues it could reduce the O_2 in the root zone to very low levels due to resistance to O_2 movement in the media thus, limiting the metabolic processes in the roots and root growth. In previous studies, it has been shown that hypobaric environments reduced leaf boundary layer resistance and increased the rate of transpiration (Daunicht and Brinkjans, 1996; Goto et al., 1996; Iwabuchi and Furata, 2003). Therefore, the increased leaf mineral concentration under hypobaria could be due to the increased transpiration and water uptake by roots.

It has been well established that plants produce ethylene in response to abiotic stresses. Among many effects, ethylene has been shown to reduce growth rate, reduce yield, decrease chlorophyll and affect anthocyanin production all of which were observed in response to hypoxia (Klassen and Bugbee, 2002). Ethylene has been shown to both promote and inhibit anthocyanin production. Craker and Wettherbee (1973) reported that ethylene had no effect on anthocyanin production in dark but in light, ethylene increased the anthocyanin production. Ethylene has been shown to increase the production of phenylalanine ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway that leads to the production of phenolic compounds and anthocyanins. In our first experiment investigating hypoxia effects, we did not scrub ethylene from the chambers and the headspace ethylene was analyzed on 5th and 10th day of the experiment. Headspace analysis on 5th day into the experiment showed no difference in headspace ethylene levels among treatments but on 10th day, more ethylene had accumulated in the chambers maintained at 12 and 6 kPa pO_2 (≈435 nmol mol^{-1}) than the chambers maintained at 21 kPa pO_2 (≈300 nmol mol^{-1}). Therefore, we hypothesized that hypoxia induced ethylene accumulation which may have played a role in increased anthocyanin production under hypoxia conditions.

In the experiments where we investigated exogenous ethylene effects under hypoxia, plants grown at 6 kPa had more leaf anthocyanin than plants grown at 21 kPa pO_2 (Table 5). Treatment with ethylene (at 1 μL L^{-1}) did not affect fresh leaf biomass or dry leaf biomass. Interestingly, leaf anthocyanin concentration of ethylene treated plants grown at 21 kPa pO_2 showed levels comparable with ethylene scrubbed control plants grown under 6 kPa pO_2 (Table 5). Exogenous ethylene increased the leaf anthocyanin concentration of plants grown at 21 kPa by 70% and about (14%) in plants grown at 6 kPa pO_2 compared to scrubbed plants. Exogenous ethylene had no effect on total phenolic concentration indicating that ethylene preferentially stimulated the production of anthocyanin. On the other hand, ethylene action inhibitor, 1-MCP, had no significant effect on leaf anthocyanin concentration under 21 kPa pO_2 but slightly reduced leaf anthocyanin concentration under 6 kPa pO_2. 1-MCP treated plants showed similar fresh and dry biomass than ethylene treated and scrubbed plants. These observations indicate that ethylene played a role in anthocyanin production but ethylene accumulation was not the sole cause of increased anthocyanin production under hypoxia. It appears that stress caused by hypoxia induced the production of anthocyanin independent of stress induced ethylene.

In summary, our results showed that lettuce can be grown successfully under hypobaria and hypoxia conditions. Hypoxia effects on nutritional and functional phytochemicals were more pronounced than hypobaria effects. Regardless of the total pressure, hypoxia, in general, enhanced leaf anthocyanin levels, enhanced total phenolic compounds, enhanced carbohydrate levels and enhanced free radical scavenging capacity of lettuce thus, enhanced nutritional and functional quality. However, hypoxia generally reduced leaf mineral concentration. Hypobaria enhanced mineral concentration. Hypoxia increased the production of ethylene. Exposure to ethylene enhanced anthocyanin production, particularly under ambient pO_2, but it appears that hypoxia induced the production of anthocyanin independent of ethylene action. Further experiments are needed to better understand the role of hypoxia on key enzymes in the phenolic pathway and the production of anthocyanin. Although hypoxia enhanced the protective compounds and in vitro free radical scavenging capacity, without human intervention studies, if enhanced phytochemicals have significant impact in human body is uncertain. In a recent study, Thomson et al. (2008) reported that consumption of high lycopene tomato resulted in elevated serum lycopene levels but did not reduce the oxidative stress or the inflammation in a healthy adult population.

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