Large-scale plant growth chamber design for elevated pCO₂ and δ¹³C studies

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RATIONALITY: Throughout at least the next century, CO₂ fertilization and environmental stresses (e.g., nutrient, moisture, insect herbivory) are predicted to affect yields of economically important crop species. Stable isotopes of carbon are used to study plant stresses, which affect yields, but a growth chamber design that can be used to isolate the effects of environmental stresses on crop-sized species through precise maintenance of pCO₂ levels and the δ¹³C values of atmospheric CO₂ (δ¹³CCO₂) is lacking.

METHODS: We designed and built low-cost plant growth chambers for growing staple crop species under precise pCO₂ and δ¹³CCO₂ conditions. Over the course of 14 hours, we assessed for pCO₂ stability at two targeted levels (ambient, ~400 ppm; and 2×, ~800 ppm) and measured the δ¹³CCO₂ value within the two chambers using a stable isotope ratio mass spectrometer. We also compared the temperature and relative humidity conditions within the two growth chambers, and in the ambient, outside air.

RESULTS: Across our experimental period, we achieved δ¹³CCO₂ stability (ambient: -8.05 ± 0.17‰; elevated: -12.99 ± 0.29‰) that showed nearly half the variability of any previously reported values for other chamber designs. The stability of the pCO₂ conditions (ambient: 406 ± 3 ppm; elevated: 793 ± 54 ppm) was comparable with that in previous studies, but our design provided ~8 times more growing space than previous chamber designs. We also measured nearly identical temperature and relative humidity conditions for the two chambers throughout the experiment.

CONCLUSIONS: Our growth chamber design marks a significant improvement in our ability to test for plant stress across a range of future pCO₂ scenarios. Through significant improvement in δ¹³CCO₂ stability and increased chamber size, small changes in carbon isotope fractionation can be used to assess stress in crop species under specific environmental (temperature, relative humidity, pCO₂) conditions. Copyright © 2015 John Wiley & Sons, Ltd.

Over the past 50+ years, hundreds of studies have documented the fertilization effect of elevated pCO₂ on plant growth (see, e.g.,[1–6]). The majority of these studies involved plant growth under pCO₂ levels projected for the next 100 years (i.e., ambient to 800 ppm)[7–8]) using experimental designs ranging from miniature (e.g., 4 L size chambers for bryophytes[9]), to whole-forest scale open-air plots (e.g., Free Air CO₂ Enrichment (FACE) for mature trees).[10] The carbon isotope composition of plant tissue (δ¹³C value) has been used as a proxy for environmental stress (e.g., heat stress,[11] water stress,[12] drought stress,[13] defoliation,[14] and insect herbivory).[15] As an extension of this technique, experiments could be performed to investigate the effect of environmental stress under changing pCO₂ levels. However, such experiments require precise control of the atmospheric conditions under which the plants are grown because both the pCO₂ level[16] and the carbon isotope composition of the atmospheric CO₂ (δ¹³CCO₂ value)[17] have been shown to affect the carbon isotope composition of plant tissue during photosynthesis.

Few studies have successfully varied the pCO₂ level while simultaneously monitoring the δ¹³CCO₂ value within plant growth chambers. Studies that reported sufficiently low δ¹³CCO₂ variability (i.e., <1‰) to assess differences in carbon isotope fractionation among treatments have been limited to small-scale (i.e. <0.8 m²) chamber designs that can accommodate small-stature plants (e.g., herbs and grasses) only.[6,9,16,18,19] Large-scale experiments designed to accommodate shrubs and trees reported relatively large variability in δ¹³CCO₂ values (i.e., ± 0.9 to ± 1.4‰[20] and ± 1.4 to ± 3.3‰[21]). Recent work has intensified interest in the effects that elevated CO₂ has on crop species[22] in order to pursue the importance of environmental stress within this context, the ability to control pCO₂ levels and maintain a constant δ¹³CCO₂ value within larger-sized growth chambers will be crucial. Towards this, we designed a growth chamber of suitable stature for staple crops (i.e., interior volume = 6.6 m³). In order to evaluate the ability of the design to successfully control environmental conditions, we measured a suite of characteristics (i.e., pCO₂, δ¹³CCO₂ relative humidity, and temperature) over a 14-h period (06:00 and 20:00). Here we report on the environmental stability of the chambers at two levels of pCO₂ in order to assess their suitability for plant growth experiments that require simultaneous and precise control over both pCO₂ levels and δ¹³CCO₂ values.
EXPERIMENTAL

Plant growth chamber design

Two growth chambers (Fig. 1) were built within an outdoor greenhouse at the University of Hawaii Magoon Research Facility; the superstructure of this greenhouse was not glass enclosed, but instead covered with a shade cloth that provided 75% light transmission. The growth chambers were constructed using lengths of Douglas Fir ‘two-by-four’ wood and enclosed in 6 mil, UV-resistant Dura-Film Super 4 polyethylene greenhouse film (International Greenhouse Company, Danville, IL, USA) with a light transmission rating of 91%. The chambers had a footprint 2.4 m wide by 2.4 m long, and increased in height from 0.9 to 1.4 m (front to back) to allow for drainage of rainwater. The roof frames were constructed from Douglas Fir, with cross pieces for support, and positioned on top of the main frames with plastic spacers that provided a 1 cm ventilation gap along the perimeter of the roofline. The roof frames were covered with the same polyethylene greenhouse film as the main frame, with an overhang of 10 cm to provide a wind guard and drip edge. Two sides of the chamber were configured with removable doors to provide access to the interior. Foam weather stripping around the perimeter of the doors minimized atmospheric leakage. The chambers were placed on 0.6 m high nursery benches to provide a solid subflooring, easy access to the interior, and adequate airflow around the full exterior of the chamber.

The chambers featured a flow-through ventilation system where ambient air was supplemented with beverage-grade CO₂ cylinder gas (99.9%; Airgas-Gaspro, Honolulu, HI, USA) and the amount of pure CO₂ gas that entered the intake pipe (A) draws ambient air (B) into the chamber. CO₂ and humidity, and temperature. A fan located within a PVC intake pipe (A) draws ambient air (B) into the chamber. CO₂ from a gas cylinder bleeds into the intake pipe via a stainless steel capillary line (C) in order to elevate the chamber air to the desired pCO₂ level. The air is evenly distributed within the chamber via a perforated PVC manifold (D). Air is exhausted through the top of the chamber through a gap between the main structure and the roof. A 10 cm overhang of polyethylene greenhouse film provides a drip and wind guard (E). Access to the chamber is via removable side panels.

Figure 1. Controlled growth chamber design for maintenance of constant pCO₂ level, δ¹³C_{CO₂} value, relative humidity, and temperature. A fan located within a PVC intake pipe (A) draws ambient air (B) into the chamber. CO₂ from a gas cylinder bleeds into the intake pipe via a stainless steel capillary line (C) in order to elevate the chamber air to the desired pCO₂ level. The air is evenly distributed within the chamber via a perforated PVC manifold (D). Air is exhausted through the top of the chamber through a gap between the main structure and the roof. A 10 cm overhang of polyethylene greenhouse film provides a drip and wind guard (E). Access to the chamber is via removable side panels.

Determination of δ¹³C_{CO₂} values

Between the hours of 06:00 (prior to sunrise) and 20:00 (post sunset), chamber air was sampled every half hour from inside the elevated chamber, and every hour from inside the ambient chamber. Samples were manually collected into 60 mL vials (catalog #26121; Restek Corporation, Bellefonte, PA, USA) and sealed with a Teflon-faced silicone septum. Access to the chamber for sampling was via an 18 cm long opening in the plastic siding of the chamber. The sampling location was 0.6 m from the wall of the chamber where the intake pipe was located. When not in use, the access hole was sealed with a piece of the polyethylene greenhouse film. The positive pressure generated inside the chamber did not allow any external air to enter the growth chamber during sampling. pCO₂ measurements during the experiment were taken at each sampling time at the same location as the δ¹³C_{CO₂} measurements. Prior to this experiment, we tested Monitoring pCO₂, temperature and relative humidity

Temperature and relative humidity were measured and logged using a HOBO U12-012 data logger (Onset Computer Corp., Bourne, MA, USA) and pCO₂ levels were measured using a WMA-4 CO₂ Analyzer (PP Systems, Amesbury, MA, USA). Data were downloaded from the data logger using the HOBOware Lite software, and then exported to Microsoft Excel for processing. We compared the environmental conditions within the chambers to that recorded by a weather station maintained just outside the greenhouse where the chambers were located. The weather station logged temperature, relative humidity, and solar radiation levels every 30 min.
whether the air was well mixed within the chambers by elevating the $p$CO$_2$ levels to ~800 ppm and measuring $p$CO$_2$ at eight different locations within the chamber. The readings were all within 5 ppm, which indicated that the air was well distributed within the chamber.

The $\delta^{13}$CCO$_2$ value of the chamber air was measured using the direct injection method described in Schubert and Jahren.[16] Briefly, sample aliquots were drawn from the sample vials using an SGE gas-tight syringe (model #008962; SGE Analytical Science) and injected into a modified Eurovector EA3000 automated combustion system (Eurovector SpA, Milan, Italy). Water was removed using a magnesium perchlorate trap, the CO$_2$ was frozen into a loop cooled with liquid nitrogen, and atmospheric N$_2$ and O$_2$ were sent to waste. Any nitrogen oxides in the sample were reduced to N$_2$ gas by passing the sample over a reduced copper layer held at 650°C. The purified CO$_2$ within a flow of helium, then continued to an Isoprime stable isotope ratio mass spectrometer (Micromass UK Ltd, Manchester, UK) for $\delta^{13}$C analysis. The $\delta^{13}$CCO$_2$ value of each sample was normalized to the Vienna Pee Dee Belemnite (VPDB) scale using two internal reference gases calibrated using CO$_2$ gas generated from NBS-19 calcium carbonate ($\delta^{13}$C consensus value = 1.95‰) and LSVEC lithium carbonate ($\delta^{13}$C consensus value = $-46.6$‰)[23] via reaction with 100% H$_3$PO$_4$.[24] The precision for reference injections and sample injections was better than 0.2‰ (1σ).

Cylinder CO$_2$ was sampled for $\delta^{13}$CCO$_2$ analysis using a 250-cm$^3$ metal vessel. The vessel was purged with the cylinder CO$_2$ for 1 min, and then both ends were closed. The gas was transferred to the dual micro-inlet of the Isoprime and measured against pure aliquots of CO$_2$ gas distributed within the chamber. All measurements were within 5 ppm, which indicated that the air was well mixed within the chamber.

RESULTS AND DISCUSSION

Environmental conditions

The temperature, relative humidity, and solar radiation levels within the two chambers are shown in Fig. 2 and Table 1. The differences between the average daytime (08:00 to 18:30) temperature and relative humidity between the two chambers were very small: 0.03°C and 0.78%, respectively, which fell within the precision specified for each gauge (0.36°C and 3.5%). The daytime temperature within the chambers averaged 3.9°C higher than open-air conditions reported by the greenhouse weather station. The largest difference occurred at midday when the chambers reached 8.4°C warmer than air outside the shaded greenhouse. Because the chambers were a semi-enclosed system, they were expected to operate at higher temperature than open-air experiments. Relative humidity is inversely proportional to temperature, which explains why the relative humidity was 7.9% lower, on average, within the chambers than that of the outside air where temperatures were lower. We calculated solar radiation levels within the chambers as being 68% of the open air values recorded at the greenhouse weather station by accounting for the amount of light transmission provided by the greenhouse shade cloth (75%) and greenhouse film (91%).

The design for a growth experiment can affect the environmental conditions greatly. FACE experiments were designed to most closely preserve natural environmental conditions, while closed environmental growth chambers have been designed to regulate each environmental parameter (i.e., temperature, light levels, relative humidity, and $p$CO$_2$ levels) precisely. Open top chambers are an intermediate design that mimics the natural environment more closely, although not exactly, with temperatures typically higher, relative humidity lower, and light intensity decreased compared with the surrounding environment.[25] The flow-through chambers described in this paper altered the temperature, relative humidity, and light levels in predictable ways, while preserving the diurnal patterns of the natural system (Fig. 2). The observation that the environmental conditions inside and outside the chambers differed is not problematic as long as: (1) the conditions within the chambers are conducive to growing the types of
species selected for an experiment, and (2) that the environmental parameters (e.g. light, relative humidity, temperature) are consistent between chambers. As with all chamber experiments, we suggest taking additional precautions to eliminate both inter- and intra-chamber effects by rotating the plants both within a chamber, and between chambers, on a regular basis.

**pCO₂ and δ¹³CO₂ values**

The pCO₂ and δ¹³CO₂ results are displayed in Fig. 3 and Table 1. The average pCO₂ level over the course of daylight hours was 406 ± 3 ppm (1σ) and 793 ± 54 ppm (1σ) for the ambient and elevated chambers, respectively. This compares favorably with environmental growth cabinets, greenhouse growth rooms that utilize an automatic CO₂ injection, and flow-through designs that were able to maintain stability of pCO₂ within ±8% of the average values (Table 2).²⁵,²⁶,²⁹ FACE studies defined an acceptable level of pCO₂ stability as maintaining pCO₂ levels within 10% of the mean for 90% of the time.²¹,²⁶,³¹ We maintained pCO₂ within 10% of the mean for 100% of the readings for the ambient chamber and for 91% of the readings for the elevated chamber.

The average daytime δ¹³CO₂ value in the ambient chamber was −8.05‰ (1σ = 0.17‰; SE = 0.06‰; n = 10) and −12.99‰ (1σ = 0.29‰; SE = 0.05‰; n = 21) in the elevated chamber. While previous studies reported δ¹³CO₂ values of environmental air for both ambient and elevated treatments, only half (n = 6) reported uncertainties for these values (Table 2). The standard deviations (and standard errors) of the average δ¹³CO₂ values within our chambers were lower than those of all previous designs.

The higher variability in pCO₂ levels and δ¹³CO₂ values for the elevated versus the ambient chamber can be attributed to several factors. Environmental temperature fluctuations can affect the flow characteristics of the CO₂ cylinder gas delivery system via thermal expansion and contraction of the gas regulator diaphragms, micro-valves, and metal transfer lines. A change in temperature can also affect the resistance and conductivity of the electrical components of the chamber intake fans, which can contribute to variations in intake flow. In addition, a change in temperature can change the number of moles of ambient air molecules flowing into the chamber (following the ideal gas law PV = nRT). Since the pCO₂ level of the elevated chamber is the result of two CO₂ sources mixing: (1) supplemental CO₂ from a cylinder and (2) ambient air, changes in amounts of either of these entering the chamber, due to one or more of the above causes, can affect the resulting pCO₂ level within the elevated chamber. The supplemental CO₂ has a different δ¹³CO₂ value from the ambient air (−18.47‰ versus −8.05‰); therefore, changes in the ratio of CO₂ sources can also affect the δ¹³CO₂ value of the elevated chamber air. As a result, we suggest shielding the CO₂ delivery system from direct sunlight and providing adequate airflow around the components to minimize temperature fluctuations within the system.

We observed that δ¹³CO₂ and pCO₂ measurements made before sunrise and after sunset within the ambient chamber followed the same trends reported in the literature for ambient air that contained a source of nighttime respired CO₂ from roots and microbial decomposition.²⁵,³²,³³,³⁵ We used the Keeling plot method to determine the δ¹³CO₂ value of this contribution by plotting ambient chamber δ¹³CO₂ values (06:00 to 20:00) against 1/pCO₂. The least squares fit line through these points resulted in the equation: δ¹³CO₂ = 5624/pCO₂ − 21.9 (R² = 0.78), where the intercept represented the δ¹³CO₂ value of mean local surface emissions.²⁵,³⁶ This

**Table 1. Daytime environmental conditions (08:00 to 18:30)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>Relative humidity (%)</th>
<th>pCO₂ (ppm)</th>
<th>δ¹³CO₂ value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient</td>
<td>27.4 (21.1–32.8)</td>
<td>57.6 (48.6–78.8)</td>
<td>406 ± 3</td>
<td>−8.05 ± 0.17</td>
</tr>
<tr>
<td>Elevated</td>
<td>27.4 (20.0–33.0)</td>
<td>56.8 (45.2–82.0)</td>
<td>793 ± 54</td>
<td>−12.99 ± 0.29</td>
</tr>
<tr>
<td>Open Air</td>
<td>23.5 (21.1–24.6)</td>
<td>65.1 (59.3–80.6)</td>
<td>N.M.</td>
<td>N.M.</td>
</tr>
<tr>
<td>N.M. = not measured.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Average values; ranges in parentheses.

*Average values ± 1σ

*Figure 3. pCO₂ (a) and δ¹³CO₂ (b) measurements of ambient (circles) and elevated (squares) chamber air throughout the 14-h experiment. Shaded areas indicate nighttime, non-shaded areas indicate daytime. Uncertainty associated with pCO₂ measurements was ± 10 ppm, and ± 0.2‰ for δ¹³CO₂ measurements. Dashed lines indicate averages for the daylight hours (08:00 to 18:30).
Table 2. Comparison of published plant growth studies that vary pCO₂ levels and record δ¹³C CO₂ levels of the air used for photosynthesis

<table>
<thead>
<tr>
<th>Type</th>
<th>Size</th>
<th>Plants studied</th>
<th>pCO₂ levels (ppm)</th>
<th>δ¹³C CO₂ values uncertainty</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Air CO₂ Enrichment (FACE), open air plots</td>
<td>25 m dia. plots</td>
<td>Triticum aestivum</td>
<td>360, 550 ±10%</td>
<td>±1.4% and ±3.3% (1σ)</td>
<td>Leavitt et al. [21]</td>
</tr>
<tr>
<td>Glass greenhouse room</td>
<td>not reported</td>
<td>Sequoia sempervirens, Metasequoia glyptostroboides, Taxodium distichum</td>
<td>400, 800 ±5%</td>
<td>not reported</td>
<td>Llorens et al. [29]</td>
</tr>
<tr>
<td>Growth room w/CO₂ injection</td>
<td>not reported</td>
<td>Quercus petraea</td>
<td>300,700</td>
<td>not reported</td>
<td>Tu et al. [40]</td>
</tr>
<tr>
<td>Growth Cabinet</td>
<td>not reported</td>
<td>Aristida glabra, Bouteloua curtipendula, Eragrostis lemanniana</td>
<td>370,690</td>
<td>±0.3% and ±0.6%</td>
<td>Fravolini et al. [41]</td>
</tr>
<tr>
<td>Open top chambers</td>
<td>3 m dia. Sorghum bicolor, Glycine max</td>
<td>358 to 732 ±&lt;0.6%</td>
<td>±0.9% and ±1.4 %</td>
<td>Torbert et al. [20]</td>
<td></td>
</tr>
<tr>
<td>Closed chamber w/CO₂ injection</td>
<td>0.72 m³</td>
<td>Sorghum bicolor</td>
<td>350, 700 ±7%</td>
<td>±0.22 % and ±0.24% (stand error)</td>
<td>Watling et al. [27]</td>
</tr>
<tr>
<td>Closed chamber w/CO₂ injection</td>
<td>0.72 m³</td>
<td>Arabidopsis thaliana</td>
<td>380 to 3000</td>
<td>not reported</td>
<td>Lomax et al. [42]</td>
</tr>
<tr>
<td>Flow through</td>
<td>0.004 m³</td>
<td>Lumularia cruciata, Marchantia polymorpha, Funaria hygrometrica, Leptobryum pyriforme Phaseolus vulgaris and Sinapis alba</td>
<td>375 to 6000</td>
<td>not reported</td>
<td>Fletcher et al. [9]</td>
</tr>
<tr>
<td>Flow through</td>
<td>0.004 m³</td>
<td>Phaseolus vulgaris and Sinapis alba</td>
<td>300, 360, 450</td>
<td>~ ±1 %</td>
<td>Beering et al. [43]</td>
</tr>
<tr>
<td>Flow through</td>
<td>0.125 m³</td>
<td>Puccinellia nuttalliana</td>
<td>350, 1300</td>
<td>not reported</td>
<td>Guy and Reid [18]</td>
</tr>
<tr>
<td>Flow through</td>
<td>0.50 m³</td>
<td>Arabidopsis thaliana, Raphanus sativus</td>
<td>370-4200 ±3-8%</td>
<td>±0.54% (1σ)</td>
<td>Schubert and Jahren [6,16]</td>
</tr>
<tr>
<td>Flow through</td>
<td>2.43 m³</td>
<td>17 herb and grass species</td>
<td>350, 525, 700 ±3%</td>
<td>not reported</td>
<td>Beering and Woodward [26]</td>
</tr>
</tbody>
</table>

δ¹³C CO₂ uncertainty included for studies that reported it.

δ¹³C CO₂ uncertainty is included for studies that reported it. Uncertainty was reported as 1σ, standard error, or not indicated.
could not be directly measured during the experiment without affecting the chamber pCO2).

Using Eqn. (4), we calculated the average daytime δ13C = –13.14% (σ = 0.44%; n = 10), which was not statistically different (p = 0.33) from the average δ13C CO2 value measured via the direct injection method (–12.99%; σ = 0.29%; n = 21). This verified that the composition of the elevated chamber air was a simple two-end-member mixing system that consisted of ambient CO2 and supplemental cylinder CO2.

These chambers, along with the majority of controlled pCO2 experiments, utilized local ambient air supplemented with CO2 cylinder gas to achieve elevated pCO2 levels. In urban and industrial settings, anthropogenic CO2 emissions can alter localized/daytime ambient pCO2 levels and δ13C CO2 values by as much as 550 ppm and 11%, respectively.[33,37–39] It is therefore important to choose a location for the chambers where local ambient conditions are stable for the length of the growth experiment.

We expect plant stress growth experiments to last longer than 14 h (of the order of weeks to months), and the data presented here suggests that the chamber conditions will remain sufficiently stable over these longer time periods. We believe that temperature changes are of most concern to the stability of the pCO2 levels and δ13C CO2 values within the elevated chamber, and we have shown that across a wide range of temperatures (i.e., 20 to 33°C), stability was maintained. For changes in large-scale external environmental conditions (e.g., atmospheric pressure), maintenance of the elevated pCO2 level would involve a simple adjustment of the supplemental CO2 flow rate.

CONCLUSIONS

We have described low-cost growth chambers capable of maintaining nearly identical environmental conditions and precise δ13C CO2 control under both ambient and elevated pCO2 levels. Previous chamber designs aimed at using the δ13C value of plant tissue as an investigative tool reported greater δ13C CO2 variability and were limited in interior chamber volume (maximum of 0.72 m³). The chamber design that we tested here provided at least 8 times more interior volume (6.6 m³) while limiting δ13C CO2 variability to nearly half that of all previous designs. The large interior volume allows for growth experiments of economically important crop-sized species, such as sweet potato, soybean, and tomato. Because the δ13C CO2 value[12] and pCO2 level[16] both affect the measured carbon isotope fractionation in plant tissues, the unmatched stability in atmospheric conditions (i.e., δ13C CO2 and pCO2) of this design will enable researchers to detect differences in isotope discrimination from environmental stresses that would not otherwise be possible.

Acknowledgements

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