Australian Journal of Crop Science AJCS
AJCS 8(12):1625-1631 (2014) ISSN:1835-2707

A new simple method for labeling field crops with stable isotope tracers

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Abstract

Numerous systems have been used to label plants with ^{13}C, which differ in design and complexity depending upon the desired experimental goals. However, most of these systems have generally been applied to greenhouse grown plants. Here, we report on a relatively simple ^{13}C labeling system designed to label crops such as switchgrass (*Panicum virgatum* L.) grown in the greenhouse or small field plots. The main goals of this study were to validate the system and establish performance benchmarks. We constructed and field tested a simple design plexiglass chamber that was sealed at the soil level with a buried rubber apron. Chamber air was circulated through an infrared gas analyzer to monitor CO_2 levels within the chamber. Provisions were made to control temperatures and minimize condensation inside the chamber during labeling. Control experiments using the empty chamber both under greenhouse and field settings showed that decline in CO_2 levels was only due to plant CO_2 absorption and not due to leakage. Results indicated that the system had generally suitable performance characteristics in both greenhouse and field settings. Isotope enrichment data from our studies revealed that switchgrass fixed $^{13}CO_2$ that was injected into the chamber within 15-20 min labeling periods. The mean and standard deviation of leaf $\delta^{13}C$ values across nine plants enclosed in the chamber was 34 ± 8.9 and 96.1 ± 23.6 for the single and double labeling experiments, respectively. Results indicate that a chamber of this construction type can be effectively used also for labeling other crop plants.

Keywords: $^{13}CO_2$, $^{12}CO_2$, greenhouse grown plants, mixing dynamics, plexi-glass chamber, switchgrass.
Abbreviations: CSTR_continually stirred tank reactors, PMMA_polymethyl methacrylate, NiMH_Nickel – metal hydride battery $Mg(ClO_4)_2$_Magnesium perchlorate

Introduction

The availability of heavy carbon isotopes has provided plant researchers with a useful labeling tool that has yielded valuable insights into carbon partitioning and plant interactions with the environment (Dawson et al., 2002). Tracer studies that have used carbon isotopes employed either pulse or continuous labeling approaches (Kuzyakov et al., 2000), depending upon the specific plant and experimental goals. Pulse labeling has involved exposing plants to labeled CO_2 for a relatively short and specific time during the growth cycle while continuous labeling has involved much longer exposure periods and is significantly more methodologically complex since labeling chamber CO_2 concentration and environmental conditions must be continuously monitored and controlled (Kuzyakov et al., 2000). In both cases, the labeled plants need to be isolated from the atmosphere in some kind of airtight system during the labeling process. Thus, labeling systems share some features of either batch reactors (for pulse labeling systems) or continually stirred tank reactors (CSTR) in the case of continuous labeling systems. These reactor configurations differ in that the CSTR is usually operated under steady state conditions and includes reactant inflow and product outflow streams (Fogler 2005). The similarities to CO_2 labeling systems, though, are not complete: outdoor labeling systems may have one side open to the soil where CO_2 exchange may take place and activities such as respiration and transpiration may alter the internal chamber environment. Nevertheless, like batch and CSTRs, labeling chambers assume rapid mixing and consume a reactant or substrate, and thus reactors provide a conceptual, if idealized, framework for labeling chamber analysis and design. Various systems for labeling plants with $^{13}CO_2$ or $^{14}CO_2$ have been reported. Labeling systems have generally been used in greenhouse or other indoor environments, although plants have also been labeled in field settings. Early labeling approaches used $^{14}CO_2$ because it was readily available and $^{14}CO_2$ measurement techniques were highly sensitive (Bromand et al., 2001; Moore-Kucera et al., 2008). However, $^{14}CO_2$ is radioactive and has been largely replaced with $^{13}CO_2$ for many labeling studies (Moore-Kucera et al., 2008). The design of labeling systems using $^{13}CO_2$ is largely similar to that used in ^{14}C labeling, with the exception that CO_2 concentrations are generally monitored by infra-red gas analyzers (IRGAs). Because $^{12}CO_2$ and $^{13}CO_2$ have different absorbance maxima (2360 and 2270 cm^{-1}, respectively), an infrared gas analyzer (IRGA) designed for $^{12}CO_2$ measurements is only partially sensitive to $^{13}CO_2$ absorbance and thus underestimates actual $^{13}CO_2$ concentrations (Svejcar et al., 1990)). Sources of $^{13}CO_2$ include pressurized gas tanks enriched to a high atom% or using acid to dissolve bicarbonate enriched to a

high atom% with $^{13}CO_2$. Large systems have been developed to label whole trees or crowns and measure flux from trunk and soil compartments (Högberg et al., 2008; Plain et al., 2009). Other systems have been developed that include, for example, labeling soybeans (Kouchi et al., 1984), douglas-fir seedlings (Moore-Kucera et al. 2008), grain sorghum (Berg et al., 1991), and field-grown ryegrass and crimson clover (McMahon et al., 2005; Williams et al., 2006; Williams et al., 2007). A relatively sophisticated mobile system was developed for labeling pasture grasses in the field and consisted of 12 labeling chambers and kept $^{13}CO_2$ at a constant concentration (Ostle et al., 2000). This system has been used for studying carbon flow in grasslands (Leake et al., 2006). In addition a mobile flow-through system has also been recently used for a continuous in situ $^{13}CO_2$ pulse-labeling for low vegetation field experiments (Reinsch and Ambus, 2013). Although labeling systems have been used to study carbon partitioning and dynamics in some perennial grasses, there are relatively few studies, to date, focusing on bioenergy crops such as switchgrass (*Panicum virgatum* L.). Switchgrass and other grasses are expected to be developed into major bioenergy crops in the United States (Sanderson et al., 2008; Sarath et al., 2008). However, carbon partitioning and allocation patterns within and among biomass crops are poorly understood, although such information could be useful for breeding purposes or for developing better models of carbon flow in energy crop systems. We have developed a simple $^{13}CO_2$ labeling system to label switchgrass, a model bioenergy crop, in greenhouse or field settings. In this study we evaluated: (1) the basic characteristics of the labeling system, and (2) the system's effectiveness in labeling switchgrass plots using a pulse-chase approach.

Results

Static and dynamic characteristics

An image of the plant labeling chamber in a late June field setting is shown in Fig 1B. Static characteristics of the system are shown for both the greenhouse (Fig 2A) and field (Fig 2B) experiments. In both cases, the CO_2 signal was stable over time after CO_2 addition, although slight CO_2 leakage was detected in some experiments. Mean CO_2 loss 11 minutes after CO_2 addition in the greenhouse stability trials was 0.6% ± 0.5%, and the largest CO_2 loss occurred when the largest CO_2 gradient was imposed across system boundaries. Stability characteristics in a field setting (Fig 2B) were largely similar, although more care was taken to ensure that a good seal was present with the chamber bottom edge and ground. In one case, a 1.6% increase in CO_2 concentration was observed, possibly due to instrumental drift. These data indicated that loss of CO_2 would not present a problem during labeling of plants, and even extended exposure (>40 minutes) of enclosed plants to labeled CO_2 was possible. This was an important criterion to verify, since uptake of CO_2 by plants within an enclosed chamber would be influenced by environmental conditions, including incident light, humidity, and temperature. Dynamic response of the empty chamber in greenhouse experiments to a CO_2 pulse was assessed by graphical inspection of the data and analyzing the CO_2 standard deviation of three successive time points across the data set. These values were used to define a window where data variability was abnormally high. For this analysis, high data variability was defined as a standard deviation that fell above the 75 th percentile of all standard deviation values that were calculated across successive time points in the data set. Dynamic response results from greenhouse experiments are shown in Table 1. The empty chamber had the lowest mean stabilization time when compared to chamber configurations that used potted switchgrass plants. However, differences among the mean stabilization times were not statistically significant due to the larger variability in the stabilization time when plants were present in the chamber. Several experiments were conducted to analyze CO_2 depletion characteristics using a non-isotope enriched CO_2 source in order to observe chamber performance when filled with switchgrass. Experiments were done in both greenhouse and field settings and typical CO_2 depletion curves are shown in Fig 3. In most cases, CO_2 concentration within the chamber decreased to at or below initial CO_2 levels within 20 minutes. However, the total time needed was dependent upon several variables including the amount of CO_2 initially injected into the chamber, total number of plants, time of day, light intensity, and plant physiological status. The mean CO_2 depletion rates across the tested experimental conditions are reported in Table 2. Statistically significant ($p = 0.047$) differences were observed between the two summer depletion rates as well as the six-plant Kanlow rate and the four plant Summer rate. The greenhouse results showed that the system was sensitive to the total number of plants in the chamber, which further supported plant CO_2 absorption, rather than chamber leakage, as the primary mechanism behind the observed declines in CO_2 concentration. Results from the $^{13}CO_2$ labeling experiments are shown as a contour plot in Fig 4. Each plot was generated using isotope enrichment data that was obtained by harvesting two tillers from each of the nine individual plants that were enclosed within the chamber during the labeling periods. The data showed that relatively large amounts of $^{13}CO_2$ were fixed over the 15-20 minute labeling periods. Furthermore, there was a clear increase in the amount of label taken up by switchgrass in the double labeling experiment. The mean and standard deviation of the $\delta^{13}CO_2$ values across nine plants was 34.00 ± 8.85 and 96.06 ± 23.56 for the single and double-labeling experiments, respectively.

Discussion

While designing a relatively simple $^{13}CO_2$ labeling system for field grown crop plants such as switchgrass, the prime concern that needs to be assessed is the mixing dynamics within the chamber. Although it was expected that the presence of plants in the chamber would slow overall mixing and result in longer stabilization, results obtained in this study showed that the differences were not statistically significant ($p = 0.31$) when compared to the empty box. The relatively large error terms associated with the stabilization time estimates may have been partly due to the limits in temporal resolution that were achievable with the LI-6200 that was used to monitor and log chamber CO_2 concentration. The chamber design allowed it to be successfully used for CO_2 depletion and labeling studies on moderately-sized crop plants, such as switchgrass plants that were generally in the V2-E0 growth stages (Moore et al., 1991). Greenhouse experiments tended to show a more constant (linear) decline in CO_2 concentration while field experiments revealed nonlinearity in the CO_2 depletion profile. This may have been due to the more complex dynamics that occurred in the field: in addition to plant uptake, the CO_2 pulse could also enter the soil matrix which would have contributed to the observed depletion characteristics. It is known that in the $^{13}CO_2$ pulse labeling experiments, CO_2 not only enters the soil via plant CO_2 uptake but also through diffusion processes

Table 1. Dynamic response to a CO_2 pulse in greenhouse experiments.

Configuration	Stabilization time (min.)*
Empty box	1.04[a] ± 0.26
P. virgatum cv. Summer	1.83[a] ± 0.83
P. virgatum cv. Kanlow	1.36[a] ± 0.73

*Error values represent the standard deviation. Values with the same letter are not statistically different.

Fig 1. Performance evaluation of the constructed plexiglass chamber without and with enclosed plants. A (Top). Labeling chamber dimensions were 1.0×1.0×0.0016 m. The main components of the system were (A) CO_2 sampling ports, (B) air circulation fans, (C) CO_2 injection port, (D) Li-6200. Figure 1B (Bottom). Labeling chamber in a typical field setting over a switchgrass plot.

(Reinsch and Ambus, 2013). The lower depletion rates observed in greenhouse experiments may also been partly due to differences in plant age and plant biomass amounts used in its and field settings. Additionally, the minimal variation observed between technical replicates in the greenhouse trials, may likely due to the better environmental control. Greenhouse experiments did not reveal any difference in the gross depletion rates between the two switchgrass cultivars, although the experiments were not explicitly designed to test this hypothesis. Larger differences were observed between the switchgrass plots that were used in the field studies: the mean CO_2 depletion rate using ^{12}C enriched CO_2 was almost three-fold higher than the apparent rate on the plots where $^{13}CO_2$ was applied. However, this higher rate was likely a response to mechanical trimming of plants that had occurred over 24 hours earlier. Plants have evolved sophisticated signaling and response networks to mechanical wounding and herbivore attacks, and the importance of jasmonate in the wound response has been established (León et al., 2001; Schilmiller et al., 2005; Wu et al., 2010). Although wounding has been shown to down-regulate many of the genes involved in photosynthesis as plants divert energy from growth to defense (Bilgin et al., 2010), there are also compensatory mechanisms employed as plants adjust to tissue loss. For instance, photosynthesis was shown to increase in *Agropyron desertorum* tussocks after partial defoliation (Nowak et al., 1984; Gold et al., 1990) and gas exchange was shown to increase within 24 hours after clipping (Gold et al., 1989). In alfalfa and *Eucalyptus globulus* photosynthesis also increased after partial defoliation (Baysdorfer et al., 1985; Turnbull et al., 2007), and the effect has been noted to occur in numerous other species (Turnbull et al., 2007). Therefore, the increase in observed CO_2 depletion rates on the clipped switchgrass plants is consistent with compensatory mechanisms increasing CO_2 uptake after clipping. Assessing these results in the context of other labeling systems is difficult as distributional aspects of labeling systems are seldom reported, and it can be difficult to determine the relative importance of label distribution effects versus biological variation. When analyzing observed ^{13}C incorporation, though, it can plausibly be assumed that biological variation should have a random character. In one system where 14 labeling pulses were applied over 45 days, the $\delta^{13}C$ values for 16 individual grain sorghum plants averaged 293.60 ± 56.48 and 172.08 ± 26.06 for leaf and stem tissues, respectively (Berg et al., 1991). Labeled soybean plants showed apparent between-plant differences of approximately 3 – 43% when comparing assimilated $^{13}CO_2$ values (Kouchi et al., 1984). Field pea and canola were labeled in a controlled environment chamber over multiple 1.5 hour intervals with $^{13}CO_2$ using a system capable of labeling four plants simultaneously (Sangster et al., 2010). The reported mean $\delta^{13}C$ enrichment values for field pea stems (132.7 ± 21.7), leaves (138.0 ± 24.7), and canola stems (95.9 ± 13.7) and leaves (162.8 ± 19.4) again demonstrated significant plant-to-plant variability (Sangster et al., 2010). Our results thus appeared to fall within the range of values that were obtained by other labeling systems, and suggested that field labeling of small plots of bioenergy crops such as switchgrass was feasible with the relatively short (~20 minute) exposure times used in our system. Short exposure times are critical for field applications since environmental conditions are highly variable. Adding features to control the internal chamber environment, such as dehumidification or air temperature control, adds to the cost, power requirements, and most importantly, the weight and portability of the system in the field. The results also indicated that two labeling events were capable of yielding plants that were highly enriched with ^{13}C, which was an important goal for our field labeling studies. However, as indicated in Figure 4, a gradient existed within the chamber in both labeling experiments. In both experiments, values of assimilated $^{13}CO_2$ were higher on the side of the chamber with the CO_2 injection port and were lower as distance from this port increased. This finding highlighted the importance of two design considerations. First, the gradient was likely caused by inadequate air mixing due to the size of the plants that were used in this pilot study. At the time of labeling (late June), switchgrass in the field plots was already sufficiently tall to reach the top of the chamber, thus challenging its designed capability. The size of the plants likely resulted in poor mixing dynamics, which resulted in areas of the chamber that were not well-stirred. In essence, the flow regime within the box may have been

Table 2. CO_2 depletion rates.

Experimental setup	Mean CO_2 depletion rate (ppm CO_2 min^{-1})*
Greenhouse	
cv. Summer (9 plants)	$18.3^a \pm 3.0$
cv. Summer (4 plants)	$8.0^b \pm 2.5$
cv. Kanlow (6 plants)	$17.6^a \pm 1.8$
cv. Kanlow (3 plants)†	10.5
Field	
Micro-plot (cv. Summer)	71.8 ± 16.1
$^{13}CO_2$ labeling	24.1 ± 13.0

*Error represents the standard deviation, $n \geq 3$. Means with the same letter are not statistically different. †$n = 2$ for this experiment

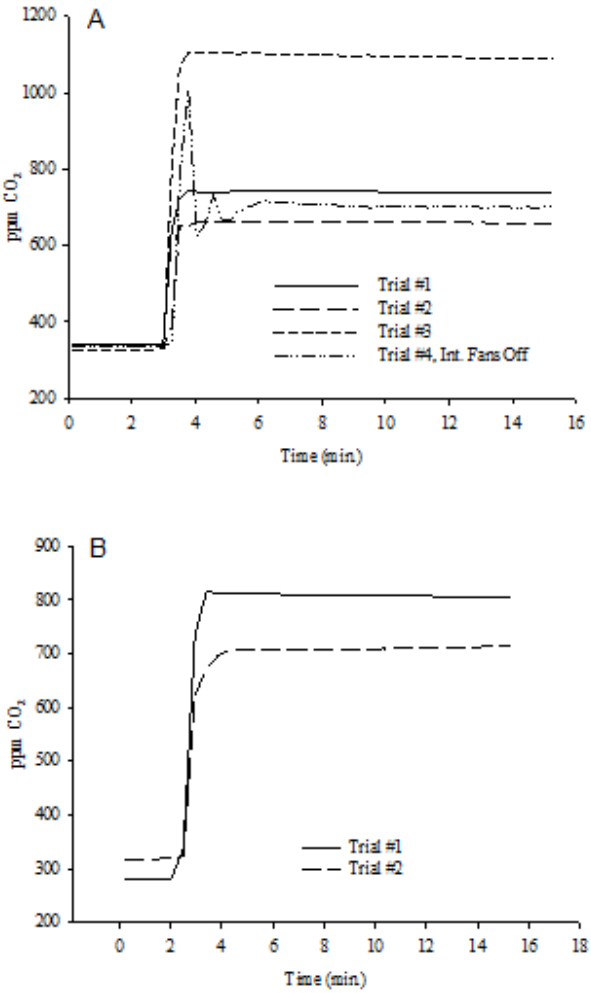

Fig 2. Stability characteristics of the empty chamber. (A) In the greenhouse and (B) in the field. CO_2 signal from the empty box was mostly stable over time after CO_2 addition under both conditions.

laminar at certain points, characterized by a low Reynolds number near the surfaces of some switchgrass tillers and leaves. Thus, variations in the leaf boundary layer and CO_2 mass transfer limitations may have contributed to the observed variability in the labeling results. This problem can be solved through several means. In actual field labeling studies, the labeling will occur earlier in the growing season when the plants are much shorter (less than 0.5 meters) and thus mixing dynamics of the box will be substantially improved with less plant height and total biomass. Additional fans can also be added to improve mixing capabilities and the box can be oriented so that opposite sides of the plot are close to the CO_2 injection port when double-labeling the plots, which will help to minimize variation across the plot. Alternatively, additional CO_2 injection ports can be added so that the labeled CO_2 enters through multiple ports which will enhance distribution throughout the chamber. The second consideration involves the importance of testing static and dynamic characteristics as well as distributional aspects of CO_2 labeling systems that are designed for parallel labeling of multiple plants. Although this requires added expense and effort, our results indicated that this is an important step that leads to design insights and is useful for system validation and design focused on minimizing variation between individual plants.

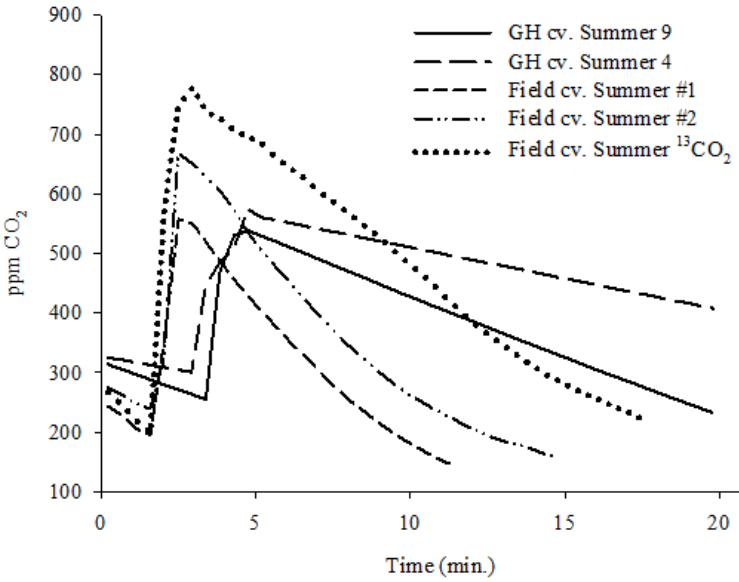

Fig 3. Representative data showing CO_2 concentration changes in the chamber from the tested experimental conditions. The terms "Summer 9" and "Summer 4" refer to chamber configurations with nine and four plants, respectively. The dotted line represents data from one of the $^{13}CO_2$ labeling experiments.

Materials and Methods

Plant material

Stands of switchgrass cv Summer and cv Kanlow were established in the fields of the University of Nebraska-Agricultural Research Division near Mead, NE in 2009, using seedlings raised in the greenhouse and transplanted to the fields. Each plot (1 m61.2 m) contained 12 closely-spaced plants to mimic sward densities. Plants were maintained in the field following the procedures and recommendations as described in Vogel and Mitchell, 2008. Greenhouse experiments were performed using potted switchgrass cv. Summer and cv. Kanlow plants that had been previously growing in the greenhouse. Greenhouse conditions were 80-84°F day / 70-74°F night with a 15 h / 9 h day/night lighting cycle.

Chamber construction and instrumentation

The plant labeling chamber was constructed by H & H Plastics, Lincoln, NE and used five sheets of clear polymethyl methacrylate (PMMA) measuring 1.0×1.0×0.0016 m to form a 1.0 m^3 cube that was open on the bottom for placement over plants (Figure 1A). The sheets were connected by chemically welding the vertical and top edges to 0.0254 x 0.0254 x 1 m rods of PMMA, and the chamber bottom was reinforced by chemically welding PMMA rods to the interior bottom edge. Strips of butyl rubber measuring 0.33 x 1 m were attached to the chamber exterior 6 cm above the bottom edges to provide better air sealing on uneven surfaces. On two opposing sides, two 16 cm metal handles were attached per side, centered 23 cm from the vertical and bottom edges of the side. On the other two opposing sides, two 80 mm diameter fans were attached to the chamber interior to provide air circulation. Placement of each fan was 12 cm from the right vertical edge and 31 cm from the chamber bottom and both fans were raised 10 cm from the PMMA surface by stainless steel rods. Each fan was powered by 8 D-cell NiMH batteries (Tenergy, Inc.,

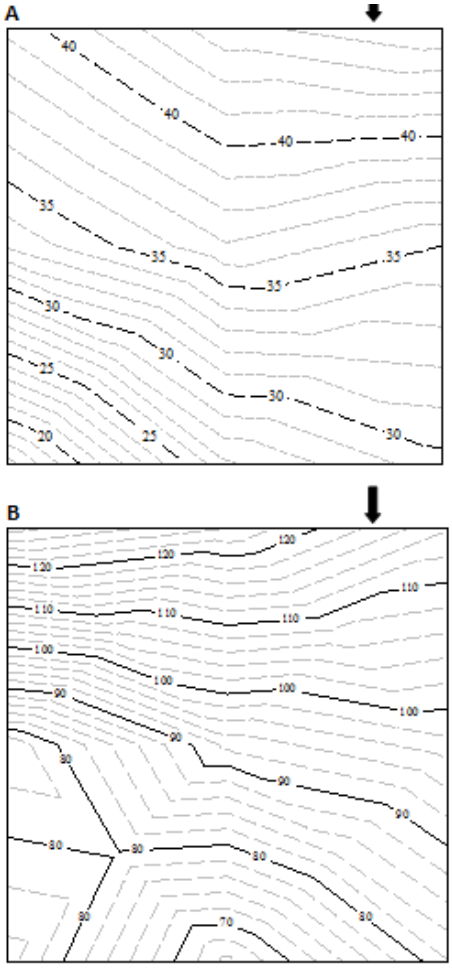

Fig 4. Distribution of $^{13}CO_2$ label across plants in the labeling chamber. Shown are the contour plots of $^{13}CO_2$ levels within the plant tissue 24 hours after labeling in (A) the single-labeling experiment and (B) the double-labeling experiment. Arrow indicates location of CO_2 injection port.

Freemont, CA) that were housed in externally mounted battery holders and power was controlled by a switch mounted to the exterior of the box. An air sampling port was located 14 cm from the left vertical edge and 14 cm from the bottom edge on the same chamber wall where one of the circulation fans was mounted. An air return port was located 4.3 cm directly below the air sampling port. Both ports consisted of a valved, ¼ inch diameter quick-disconnect panel-mount hose barb body and fitting assembly (Colder Products Company, #S-06360-55 and #S-06360-80, Cole Parmer, Inc.). To avoid sampling air from the return port, 20 cm of 3/8 in. diameter PVC tubing was attached to the interior hose barb of the air sampling port. A CO_2 injection port was located on the chamber wall opposite the sampling ports, 13 cm from the left vertical edge and 82 cm from the bottom edge. The injection used the same panel mounted house barb assembly that was used for the air sampling and return ports. A LI-6200 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE) was used to monitor CO_2 concentration as well as air temperature and relative humidity during box experiments (Figure 1B). Upon completion of an experiment, data was downloaded from the instrument intro Microsoft Excel 2007 using the LI-6200 serial port and WinWedge Standard (TAL Technologies, Inc., Philadelphia, PA). The $Mg(ClO_4)_2$ desiccant was replaced frequently during experimental runs.

Greenhouse experiments

All greenhouse experiments were conducted at the University of Nebraska Beadle Center for Biotechnology. The CO_2 source for these experiments was a pressurized tank of research grade CO_2 (Matheson Tri-Gas Inc., Lincoln, NE). For each switchgrass cultivar, two different numbers of plants were used: for cv. Summer, experiments were conducted using nine and four plants, and for cv. Kanlow, experiments used six and three plants. In order to determine basic CO_2 depletion characteristics, plants were placed in a suitable arrangement and the chamber was lowered over them. Data logging was then initiated on the LI-6200 and CO_2 was injected into the box after three minutes of initial data collection. The concentration of CO_2 inside the chamber was continuously monitored using the IRGA for the duration of each experiment which usually lasted for about 20-30 minutes. Experiments were terminated when the CO_2 concentration had fallen to at least 20% below ambient. After each experiment, the box was immediately lifted off of the plants and a fan was used to equilibrate the box interior with ambient air.

Field experiments and tissue processing

Field experiments were conducted on research plots located at the University of Nebraska Agricultural Research and Development Center near Mead, NE. The field was established in summer 2009. Each plot consisted of twelve space-planted switchgrass plants of the same cultivar or experimental strain arranged in a 4 × 3 grid (to simulate sward density). For the $^{13}CO_2$ labeling experiments, two plots containing cv. Summer plants were chosen. Data collection was initiated on the LI-6200 and after collecting data for about one minute, the gas regulator was opened for approximately 15 seconds to allow 99 atom % $^{13}CO_2$ (Sigma-Aldrich Co. St. Louis, MO) to enter the chamber. CO_2 concentration inside the chamber was continuously monitored via the LI-6200.

Labeling experiments were terminated when chamber CO_2 concentration had dropped at least 100 ppm below ambient. Tissue was harvested 48 hours after initial labeling by cutting two tillers from each of the 12 plants in the labeled plots, oven-dried at 50 ºC for 48 hours and ground using a Wiley mill equipped with a 1-mm mesh. In order to obtain finely ground samples suitable for isotope analysis, a sub-sample of the ground material was then ground to a smaller particle size using a roller mill designed to eliminate risks of sample cross contamination (Arnold et al., 2004). $\delta^{13}C$, $\delta^{15}N$, and C and N concentrations were determined using an elemental analyzer (Carlo Erba EA-1108, CE Elantech, Lakewood, NJ) interfaced with an isotope ratio mass spectrometer (Delta Plus, Thermo Electron Corp., Waltham, MA) operating in continuous flow mode. Carbon isotope ratios are presented in δ notation:

$$\delta = [(R_{SAMPLE} - R_{STD}) / R_{STD}] \times 10^3$$

where R_{SAMPLE} is the $^{13}C/^{12}C$ ratio of the sample and R_{STD} is the $^{13}C/^{12}C$ ratio of the V-PDB standard (Coplen 1996). For experiments that used $^{12}CO_2$, the same general procedures were followed with the exception that plant tissue was not harvested for isotope analysis. Control experiments were conducted using bare ground where no vegetation was present.

Data analysis

Data analysis was conducted using PROC UNIVARIATE and PROC GLM in SAS for Windows 9.2 (SAS Institute Inc., Cary, NC) and SigmaPlot 11.2 (Systat Software, Inc., San Jose, CA). The statistical model used for determining differences in mean depletion rates as well as stabilization times was a single factor linear model. Multiple comparison procedures used Tukey's HSD and controlled the family error rate at a=0.05. Replicate tests in the greenhouse were conducted by randomly selecting plants from a larger greenhouse population and placing them in the chamber. Field replicates for $^{13}CO_2$ consisted of three labeling events using two different plots (dual labeling occurred on the same plot). The field data on $^{12}CO_2$ depletion represents the mean of three individual plots. In order to calculate CO_2 depletion rates, a five-minute span of CO_2 data was used after CO_2 was injected into the chamber and the concentration had stabilized. A line was fit to this data using linear least squares and the slope of the line was used as the mean CO_2 depletion rate.

Conclusions

The main purpose of this study was to design and validate a simple, easy to handle chamber under field conditions for labeling bioenergy crop plants such as switchgrass with $^{13}CO_2$. We have provided here the details of such a set up that was tested both under the greenhouse and field conditions, with or without plants. Results from both depletion and $^{13}CO_2$ labeling studies revealed that the developed system was almost leak free under both conditions. We were able to label the enclosed plants in the chamber twice in quick succession with relative ease under field conditions. However, the density of plants and their size at the time of labeling have to be given careful consideration in order to have a good mixing dynamics of the label in the chamber. We suggest adding a few more fans in strategic positions to aid in achieving a good mixing dynamics and perhaps have two ports to inject the $^{13}CO_2$.

Acknowledgements

The authors wish to thank Professor Thomas Boutton for performing carbon isotope ratio analyses, Prof. Timothy Arkebauer for the LI6200 IRGA system and Nathan Palmer for excellent technical assistance. This work was supported by the Office of Science (BER), U. S. Department of Energy Grant Number DE-AI02-09ER64829, and by the USDA-ARS CRIS project 5440-21000-028-00D. The U.S. Department of Agriculture is an equal opportunity/affirmative action employer and all agency services are available without discrimination. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply endorsement by the U.S. Department of Agriculture. It does not constitute endorsement over other products and organizations not mentioned.

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