Dear Editor,

We are grateful for the invitation to review our manuscript entitled “A multiplexing system for quantifying oxygen fractionation factors in closed chambers”. We thank the reviewer because he/she helped us to improve the article. We have made the changes suggested by the reviewer in a version provided below. And, a detailed point-by-point response to the reviewers' comments is provided below.

We hope that you will find this revised manuscript suitable for publication,

On the behalf of all co-authors,

Clémence Paul

Point-to-point response

black = reviewer comment / purple = answers / blue = new text / green = unchanged text

Reply to Referee #1

Line 91 -92: A sentence explaining how d18O is used to reconstruct oceanic vs terrestrial productivity would be helpful.

Line 105: Replace “despite our system...” with “although our system...”

Line 108-121: Adding a sentence explaining what overall factors are considered when choosing type of species for experimental setup would be helpful

For these three comments on the introduction, in view of referee 2's proposal, we have decided to repeat the introduction in its entirety (without major modifications). The new introduction is given in the second part of the document “reply to referee #2”.

Line 236: ‘reference’ is highlighted, citation missing

The missing reference (15 W m^{-1}, Technitrace, France) has been added.

Line 292: “Experimental problem: Please describe what the experimental problem was. Since this is a new method and would potentially be adopted by other labs, it would be useful to know what the problem was and how it was addressed.

The sentence explaining the experimental problem: Liquid water entered the instrument due to condensation in the piping connected to the instrument.

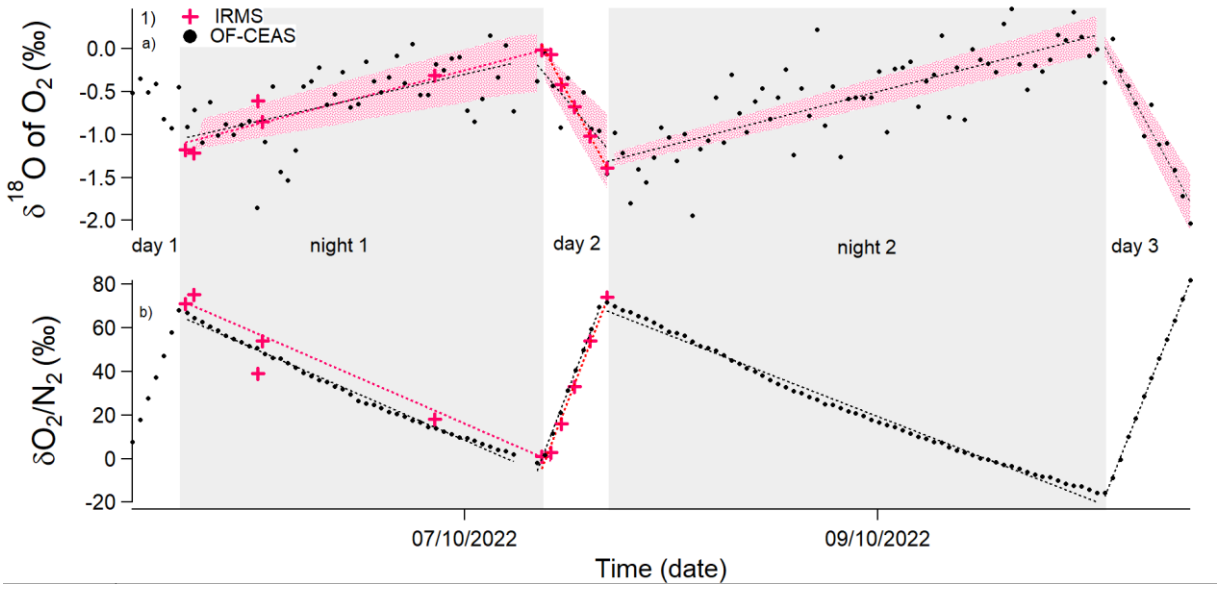
Line 341: Explain why maize was chosen as the preferred option

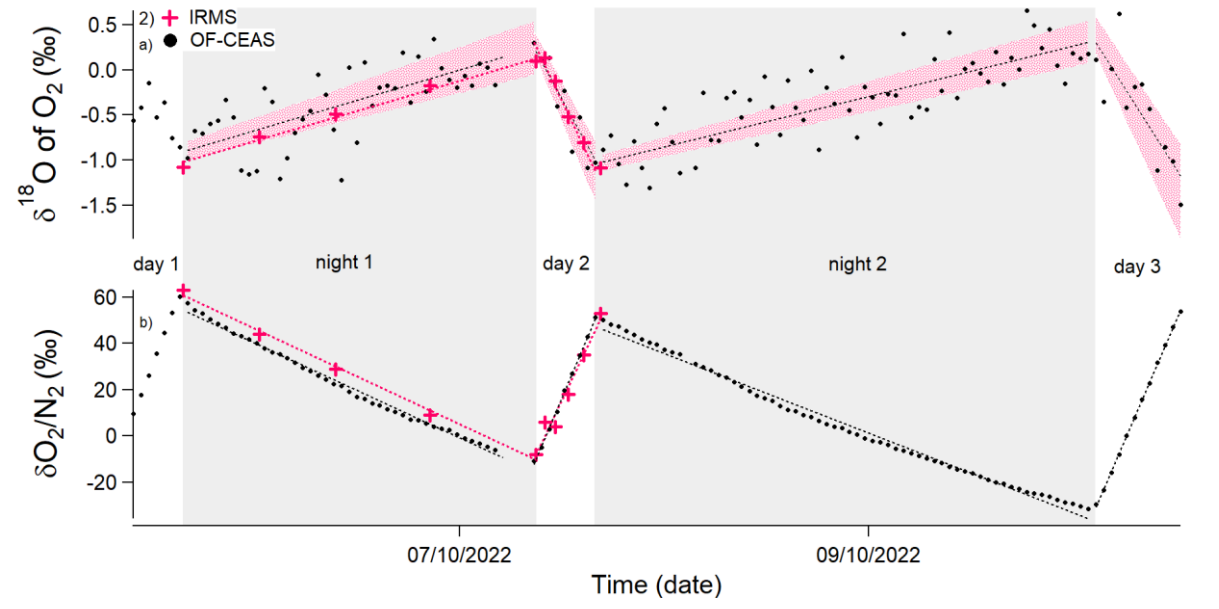
The dark periods were imposed to be longer than the light periods because the production rate of oxygen during photosynthesis was much stronger than the consumption rate of oxygen by respiration. Maize was chosen as the preferred option, as it is a C4 model plant and enables photosynthetic fluxes

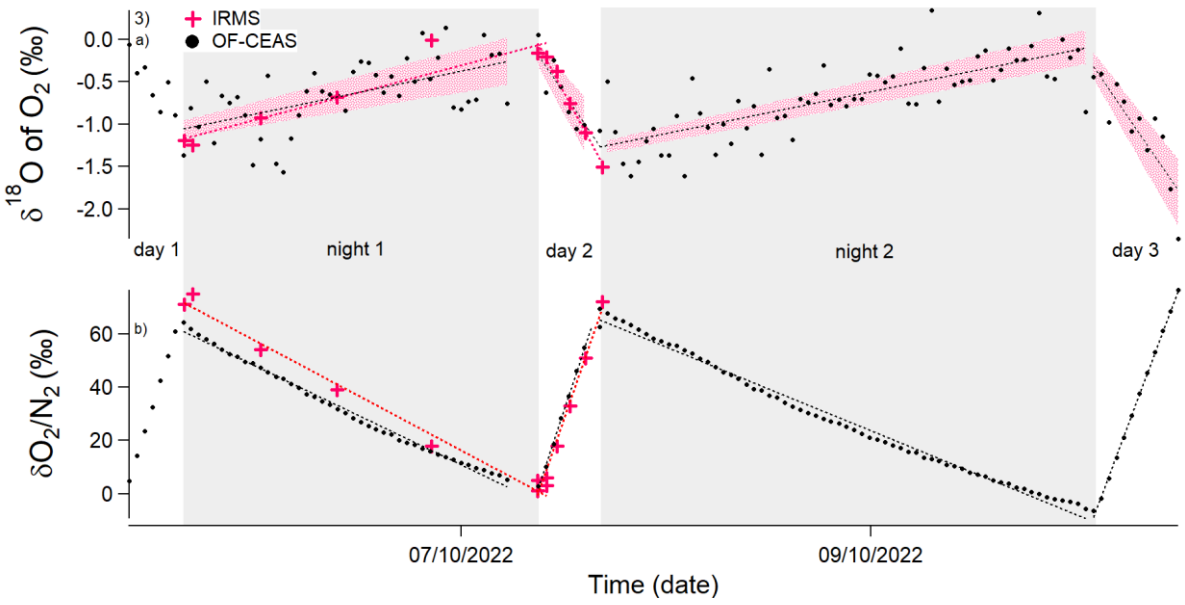
to be clearly differentiated from respiratory fluxes (no photorespiration for C4 plants), so that biological fractionation factors can be calculated easily.

Figures: Adding shaded error bars/ or some sort of way to indicate uncertainty in regression slopes would be visually helpful.

We have calculated the uncertainties in the regression slopes and added them on the graph as uncertainty envelops.







New caption:

Fig.5 Evolution of the different isotopic ratios of the soil and maize experiment due to dark respiration and photosynthesis (starting 05/10/22 and ending 10/10/22) in closed chambers over 5 days. Grey rectangles correspond to dark periods and white rectangles to light periods. (1) corresponds to chamber 1, (2) chamber 2, (3) chamber 3. (a) $\delta^{18}O$ of O_2 variations. (b) $\delta O_2/N_2$ variations. Black points: optical spectrometer's data (OF-CEAS). Red stars: data obtained by IRMS. Red dashed line: linear regression of optical spectrometer data for one period (dark or light). Black dashed line: linear regression of IRMS data for one period (dark or light). Pink envelopes represent uncertainty envelops associated with linear regression slopes and intercept of optical spectrometer data for $\delta^{18}O$ of O_2. Note that the first period of light is not considered because the system is not stable at that stage.

Reply to Referee #2

A scientific article on an innovative but non-exclusive topic deserves a more extensive literature search. This would also make the introduction of the paper more robust.

Thank you for your interesting comment. We add information about Dole effect to have a more detailed and comprehensive introduction. Here's the new introduction.

Oxygen, the most abundant chemical element on Earth, is present in all the geological layers, both internally and externally. In the surface layers of the Earth (atmosphere, biosphere, ocean), it is produced from water through the well-known biological process of photosynthesis. Consumption of O_2 is mainly due to respiration. The photosynthesis and respiration fluxes are responsible for the seasonal variations of dioxygen concentration in the atmosphere (Keeling and Shertz, 1992) and play a role in the longer-term evolution of O_2 (Stolper et al., 2016). Oxygen consists of three stable isotopes: ^{16}O, ^{17}O and ^{18}O. By measuring the ratios of these isotopes, we can document the physicochemical and

biological processes involved in the oxygen cycle. We use the $\delta^{18}O$ notation to express the isotopic signal of oxygen compared to a reference isotopic ratio (Eq. 1):

$$\delta^{18}O_{calibrated} = \left[\frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample}}{\left(\frac{^{18}O}{^{16}O}\right)_{standard}} - 1 \right] \times 1000 \quad (1)$$

Oxygen isotopes do not have the same thermodynamic properties. Thus, during phase changes, fractionation occurs which is measured by the fractionation factor α (Eq. 2):

$$^{18}\alpha = \frac{^{18}R_{product}}{^{18}R_{substrate}} \quad (2)$$

where ^{18}R is the ratio of the concentration $^{18}R = \frac{n(^{18}O)}{n(^{16}O)}$ and n the number of moles of O_2 containing ^{18}O or ^{16}O.

The isotopic discrimination is related to the isotopic fractionation factor through:

$$^{18}\varepsilon = {}^{18}\alpha - 1 \quad (3)$$

The isotopic composition of dioxygen in the atmosphere, $\delta^{18}O$ of O_2 in air, is often noted $\delta^{18}O_{atm}$. This signal, measured in the air bubbles in ice cores, can be used for ice core dating, related to the past variations of the hydrological cycle of water in the low latitudes and the relative proportion of oceanic vs terrestrial productivity. First, using the analyses of isotopic composition of dioxygen in the air trapped in ice cores, Bender et al. (1994) demonstrated that $\delta^{18}O_{atm}$ varies synchronously with precession, a discovery that has been instrumental in using this proxy for dating ice cores (Petit et al., 1999; Dreyfus et al., 2007). This influence of precession on $\delta^{18}O_{atm}$ is possibly due to changes in the low-latitude hydrological cycle driven by precession (Bender et al., 1994; Severinghaus et al., 2009; Landais et al., 2010; Seltzer et al., 2017). Such variations in low-latitude hydrological cycle influence the $\delta^{18}O$ of meteoric water which is then transmitted to the $\delta^{18}O_{atm}$ through terrestrial photosynthesis. Supporting this, over the past 650,000 years, $\delta^{18}O_{atm}$ has shown a strong correlation with $\delta^{18}O_{calcite}$ variations in East Asian speleothems (Wang et al., 2008; Cheng et al., 2016), which are largely controlled by shifts in the low-latitude water cycle, particularly monsoonal activity.

The interpretation of $\delta^{18}O_{atm}$ as an indicator for reconstructing oceanic and terrestrial productivity relies on the definition of the Dole effect (DE) calculated as the difference between $\delta^{18}O_{atm}$ and $\delta^{18}O_{sw}$ (sw referring to sea water). The present Dole effect has a value which is estimated to 24 ‰ (Bender et al., 1994; Hoffmann et al., 2004; Luz and Barkan, 2011).

Bender et al. (1994), Malaizé et al. (1999) and Hoffmann et al. (2004) proposed that changes in the Dole effect are driven by the relative contribution of terrestrial and oceanic productivity. This

conclusion arises from the fact that the terrestrial Dole effect defined as the enrichment of atmospheric O_2 $\delta^{18}O$ relative to $\delta^{18}O_{sw}$ due to terrestrial biosphere fluxes is estimated to be several permil higher than the oceanic Dole effect, which results from oceanic biosphere fluxes. This conclusion is based on of the available determinations of O_2 fractionation coefficients associated with biological processes, including terrestrial and oceanic respiration and photosynthesis (Guy et al., 1993; Angert and Luz, 2001; Hendricks et al., 2004; Helman et al., 2005)). In contrast, Luz and Barkan (2011) used updated estimates of O_2 fractionation coefficients and arrived at a different conclusion: both the terrestrial and oceanic Dole effects are approximately 24 ‰. It is thus of primary importance to determine robust values of fractionation coefficients of O_2 during biological processes.

Previous studies conducted over previous decades at the cell or organism level (Guy et al., 1993; Angert et al., 2001; Helman et al., 2005; Eisenstadt et al., 2010; Stolper et al., 2018) have already revealed variations in oxygen fractionation among different biological species and methods employed. Guy et al. (1993) conducted investigations on spinach thylakoids, cyanobacteria (*Anacystis nidulans*) and diatoms (*Phaeodactylum tricornutum*), and estimated a respiratory discrimination of oxygen by about 21 ‰. Kroopnick and Craig (1972) measured this effect on plankton incubated in natural seawater and obtained a similar value. Luz and Barkan (2002) found a respiratory fractionation of 21.6 ‰ on incubation experiments with natural plankton in Lake Kinneret. Finally, the global average oceanic respiratory fractionation value given by Luz and Barkan (2011) is 19.7 ‰ on samples from the Celtic Sea, Southern Ocean, North Atlantic and Red Sea. For terrestrial respiration, using a compilation of values from previous experiments, Bender et al. (1994) gave a global respiratory fractionation value of 18 ‰. Angert et al. (2001) focused on soil samples and gave a soil respiratory fractionation (roots and micro-organisms) of around 12 ‰. This lower value is the result of the role of roots in limiting oxygen diffusion in the consumption site.

Guy et al. (1993) showed that photosynthesis does not fractionate oxygen between the water consumed and the dioxygen produced by the organism. However, Eisenstadt et al. (2010) found later a discrimination up to 6 ‰ for oceanic photosynthesis on a study on oceanic phytoplankton, whereas Paul et al. (2023) found a discrimination of 3.7 ± 1.3 ‰ for terrestrial photosynthesis with an experiment performed at the scale of a terrarium with *Festuca arundinacea.* Such different contributions lead to different interpretation of past variations in $\delta^{18}O_{atm}$ or Dole effect.

The variety of values found for the different studies can be attributed to the different set-up used, different environment or different species. To determine robust values of fractionation coefficients, it is necessary to proceed in a systematic way and use the same set-up for a large variety of plants and environments which is the goal of the set-up detailed in this study.

Finally, note that isotopic composition of O_2 can be used to quantify global biosphere productivity (Bender et al., 1994; Luz et al., 1999; Severinghaus et al., 2009; Brandon et al., 2020; Yang et al., 2022). Such reconstruction relies on the observation that biological productivity processes (respiration and photosynthesis) fractionate oxygen in a mass dependent manner (i.e. there is a consistent relationship between changes in $\delta^{17}O$ and $\delta^{18}O$, approximately equal to 0.5), while dioxygen originating from exchanges with the stratosphere has an isotopic composition affected by mass independent fractionation (hence a relationship between changes in $\delta^{17}O$ and $\delta^{18}O$ significantly different from 0.5 i.e. between 1 and 2). The relative proportion of biosphere productivity vs stratospheric exchange fluxes sets the value of the relationship between $\delta^{17}O$ vs $\delta^{18}O$ in the troposphere, which is often

described as $\Delta^{17}O = \ln(1 + \delta^{17}O) - 0.516 \times \ln(1 + \delta^{18}O)$ (Luz et al., 1999). In parallel, the same parameter $\Delta^{17}O$ measured in the air dissolved in the ocean permits to constrain the gross biosphere productivity when combined with the concentration of O_2 measured as the ratio O_2/Ar (Luz et al., 2000).

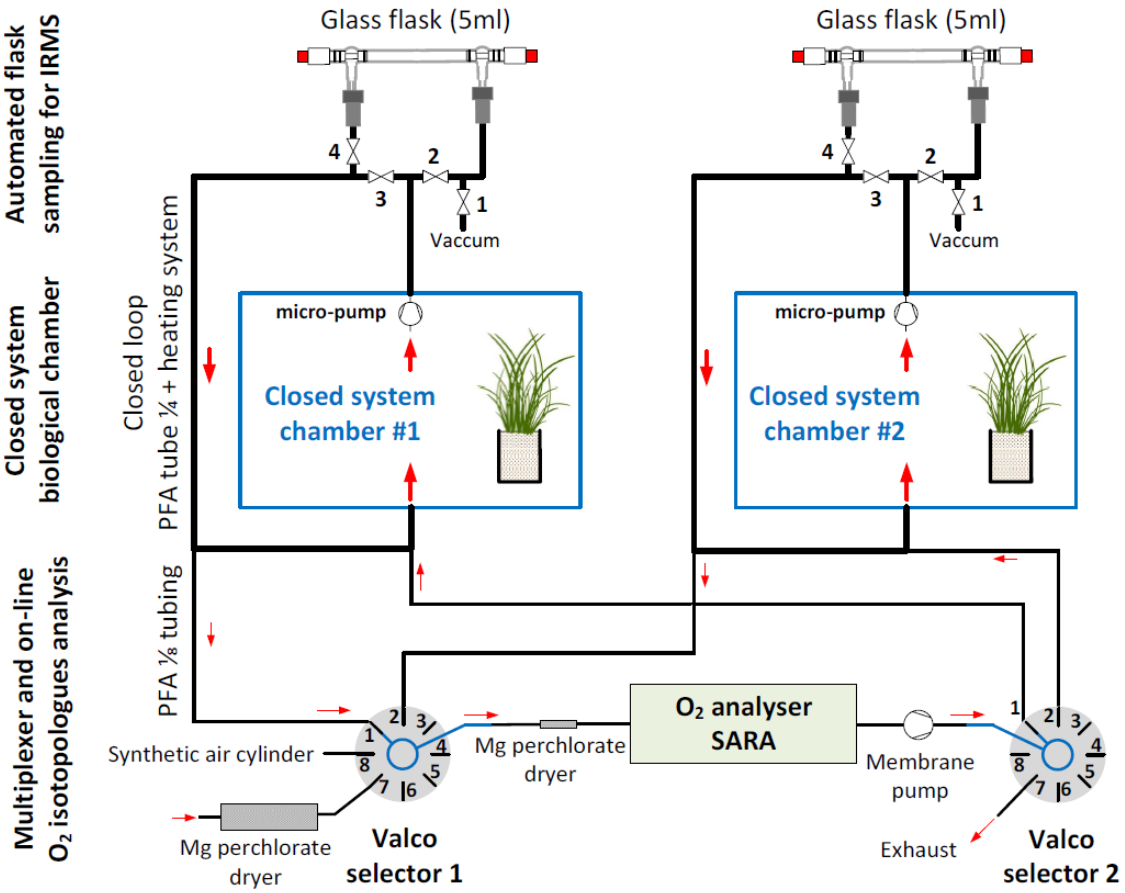
Although our system can in theory enable determination of the triple isotopic composition of O_2 (through IRMS, Isotopic Ratio Mass Spectrometry), we will focus on $\delta^{18}O$ of O_2 in the present study. We thus concentrate on fractionation coefficients needed to interpret $\delta^{18}O_{atm}$ records only.

In this study, we present an automated setup which can be used to perform numerous systematic studies of the fractionation factor of oxygen during biological processes. Similar to the study of Paul et al. (2023), we used closed growth chambers to quantify oxygen fractionation factors associated with respiration and photosynthesis of *Festuca arundinacea*. The novelty is that we worked with up to three closed chambers simultaneously in an automated way which allows an exploration of numerous different plant species and climatic conditions. Moreover, the isotopic analyses are now performed with an optical spectrometer (Optical-Feedback Cavity-Enhanced Absorption Spectroscopy, i.e. OF-CEAS technique) in addition to IRMS. This spectrometer allows studying the concentration and the isotopic composition of O_2 in the different chambers in a continuous way.

This manuscript is organized as follows. First, we will present new developments on closed biological chambers compared to the study of Paul et al. (2023) as well as the multiplexing system integrating continuous measurements of elemental and isotopic composition of O_2. Then, we will present the results of a biological experiment where photosynthesis and respiration took place. Finally, we will provide estimate of fractionation factors through two analytical techniques: optical spectrometry and IRMS.

Figure 2 has too small, unreadable lettering. I suggest replacing it.

Here a best version of the schema.



In general, I always find it confusing to merge discussions with conclusions. In any case, I suggest adding in the conclusions your group's future perspectives on this very interesting research.

We separate conclusions and perspectives like this:

4- Discussion

The value of isotopic discrimination, ${}^{18}\varepsilon_{dark\_respi}$, associated with maize growing on soil agreed with the literature. Guy et al. (1989) found a value equal to - 17 and - 19 ‰ for ${}^{18}\varepsilon_{dark\_respi}$ for Phaeodactylum tricornutum and terrestrial plants. Helman et al. (2005) found a value of ${}^{18}\varepsilon_{dark\_respi}$ equal to -17.1 ‰ for bacteria from the Lake Kinneret and a value of - 19.4 ‰ for *Synechocystis*. Paul et al. (2023), found, for *Festuca arundinacea* a value equal to - 19.1 ± 2.4 ‰.

Our value of ${}^{18}\varepsilon_{photosynthesis}$ for maize is also close to the value determined by Paul et al. (2023): + 3.7 ± 1.3 ‰ for *Festuca arundinacea* species. In both cases we observe a positive value which contradicts the value classically used of 0 ‰ from Guy et al. (1993). Our value hence confirms the existence of an apparent isotopic discrimination for terrestrial photosynthesis. This leads to an increase of the $\delta^{18}O$ of O_2 value associated with terrestrial biosphere compared to the latest study of Luz and

Barkan (2011). As a consequence, it is still an open question to know of $\delta^{18}O_{atm}$ or Dole effect variations should be interpreted solely as a change in the low latitude atmospheric water cycle or if the relative change in the marine vs terrestrial biological productivity also plays a role. Future studies should hence use a set-up similar to ours to systematically study the O_2 fractionation coefficients associated with biological processes.

5- Conclusions and perspectives

We have developed and presented a new automated multiplexing system that facilitates the study of gas exchange between plants and the atmosphere. This system offers several key advantages. First, it allows continuous measurements of the isotopic and elemental composition of dioxygen in the biological chamber, removing the need for manual sampling. Second, it provides near-real-time monitoring of $\delta^{18}O$ of O_2 and O_2 concentration during experiments, enabling adjustments to environmental conditions, such as dark and light cycles, in real time. Finally, it supports the convenient replication of experiments, enabling systematic studies across a wide range of environmental conditions, plant species, and soil types.

In the application of this system to maize, the fractionation factors for dark respiration ($^{18}\varepsilon_{dark\_respi}$: - 17 ± 2 ‰) and photosynthesis ($^{18}\varepsilon_{photosynthesis}$: + 6.7 ± 3.3 ‰) are consistent with literature values, though the relatively large uncertainties highlight some current limitations, including suboptimal performance of the optical spectrometry and excessive calibration time. Stability tests of the calibration gases indicated that less frequent calibrations (e.g., measuring both gases twice daily and one calibration gas every 15 – 20 minutes) would be sufficient to ensure accuracy.

Our automated system has significant potential for broader applications. First, its open-code design and use of relatively low-cost sensors (excluding the optical spectrometry analyzer) make it easily adaptable to other biological experiments. Second, coupling this system with other optical spectrometers, such as Picarro or Los Gatos Research (LGR) trace gas instruments, could enable the quantification of trace gas exchanges, including N_2O and CH_4 (and their isotopologues), between the plant/soil system and the atmosphere.

Future studies should focus on upgrading the instrumentation to enhance performance and reduce uncertainties in isotopic fractionation measurements. Additionally, optimizing calibration frequency will improve experimental efficiency and reliability. This system paves the way for more comprehensive and systematic investigations into gas exchange processes under diverse conditions.