Baartman et al., Isotope discrimination of carbonyl sulfide (^{34}S) and carbon dioxide (^{13}C, ^{18}O) during plant uptake in flow-through chamber experiments

Response to comments by Referee #1

We thank the reviewer for taking the time to read our manuscript and for providing useful suggestions for improvement. The reviewer comments and questions are in black and our responses are in blue below each comment.

The paper deals with an important and exciting topic. The use of COS as a unique tracer of photosynthesis and the rare measurements of the isotopic discrimination, D34S, associated with COS uptake. The paper presents a unique measurement system for gas exchange, COS, and isotopic analysis, and it is well-written.

However, the paper has some rather significant issues that need attention. This is partly so as there seems to be a gap between the impressive analytical measurements and the experimental, plant gas exchange, part . Below are some of the concerns noted as I was reading the paper (i.e., in no special order) that I hope will help to improve the paper.

In general, the motivation is to introduce D34S to "provide useful information on the COS uptake process and help to constrain the COS budget" (upfront in the abstract). However, at the end, the paper does not tell us what we learned in either aspect. **At least some discussion of these aspects is needed, or these should be strongly toned down.**

In fact, the paper goes on to declare another much more modest and specific goal: **To verify the published D34S data obtained in a 'closed system' (Davisson et al.) in their new 'open steady-state system'**. The paper generally confirms the earlier data but in a way that does not provide additional confidence due to the experimental difficulties. In its present form, therefore, it is uncertain whether the paper will advance the field in that respect. **Better focusing on what exactly is the bottom line/take-home message for D34S is needed.**

In the revised manuscript, we will clarify the goal of the manuscript with the reviewer's helpful comments. We agree that measuring plant COS discrimination poses technological and interpretational challenges, but we believe it holds potential to inform us about processes occurring across scales, from the leaf (e.g., test if COS is a unidirectional flux into the leaf) to the globe (e.g., to partition COS oceanic and anthropogenic sources using isotope constrained COS tropospheric mass balances (Davidson *et al.*, 2021)). Additionally, more observational data are required to improve and test mechanistic models of plant COS discrimination. The objective of this work was to design a system to simultaneously measure COS and CO_2 gas exchange and isotope discrimination using a continuous-flow plant chamber. We describe this system, present a first set of measurements in C_3 and C_4 plants, and compare them with the only other available dataset obtained with a closed plant chamber (Davidson *et al.*, 2021; Davidson *et al.*, 2022).

To improve clarity and balance, we will revise the manuscript to better reflect study's objectives and the scope of the results. We will include a perspectives section (see suggestions from Referee #2) with ideas for future research. In a subsequent paper that we are currently writing, we use all available measurements to develop a mechanistic model for COS plant discrimination. In the revised manuscript, we will use some of the information from this accompanying paper to provide context for the measurement results.

On the methodological side, it is not clear how many plants were used as replicates. In the Method, three papyrus cuttings and "a sunflower plant" were noted. In Fig. 3, n=2 is indicated (with SE…). In Fig. 4, no replication is indicated; in Fig. 5, 6, some individual replications are plotted, each with its own SE. While the information is incomplete and

confusing, the impression is that only a few actual replications were made, and there is no clear distinction between the precision (repeating the measurements) and replications. There is also missing information on Blank Testing of the chambers, which seems to be critical in COS experiments. The inlet COS concentration (2-3 ppb) is 4-5 times the atmospheric level) is indicated but not the chamber ambient concentrations (outlet). [BTW, in the Abstract, fluxes are reported in pmo mol-1, which are not flux units.] **More information seems to be required.**

We will include the requested information in the revised document. The data are from two experiments; one with a single sunflower and one with an assemblage of papyrus leaves. At each light level, duplicate samples were collected within the same experiment. We report their averages and standard errors. A few samples were likely affected by contamination and excluded from the analysis.

For the papyrus under dark conditions (PAR=0), the COS uptake was too low to calculate a discrimination value. As a result, these data points are not shown in the discrimination plots but are included in the flux figures. We will clarify the meaning of the duplicates, missing values and the errors in the Methods section and in the caption of Table 3 and relevant figures. Table 3 will also be expanded to include chamber mole fractions and stomatal conductance for each plant and treatment, as suggested in a later comment.

Blank (empty chamber) tests were conducted prior to the plant measurements, though not previously mentioned. We will add a description and the results of these tests in the revised manuscript.

The flux units in the Abstract should have been pmol m^{-2} s^{-1}, this was a typographical error, which we will correct.

The aspects noted above are significant as many of the observations are somewhat unexpected or uncharacteristic, and a range of particular explanations are required, such as non-uniform light level (“low light” in parts), “not optimal behavior”; “stomata not fully open”; increasing Ci with increasing light and increasing A, no response of COS assimilation to light, mostly constant D34S, constant Cm, etc. **In fact, the feeling is that more measurements would help to get more conventional results.**

We agree that more measurements would have strengthened our study, but logistic constraints limited extending the measurements. Furthermore, the isotope system’s capacity further constrained the amount of samples that we could measure (given that it takes a full day to measure only 3 - 4 samples).

We did conduct a follow-up experiment with the goal of expanding the dataset. However, when the experiments were finished, the COS isotope system needed extensive maintenance, after which we were limited in time and personnel. In addition, storage issues may have had an influence on these samples as COS can be unstable during longer storage.

Despite these limitations, we believe that the current dataset and innovation in methodology provide valuable insights, given the scarcity COS isotope discrimination data. At present, additional measurements are not feasible. The COS isotope system in Utrecht is non-operational and a new system in Bordeaux is still under development.

Fig. 3 presents a nearly complete insensitivity of COS uptake to light level (in sharp contrast to CO2 uptake), and it is explained by the light in-sensitivity of carbonic anhydrase. However, COS should still respond to light for the same CA activity because of its effect on conductance (g). **No information on conductance is given in this paper**.

We will provide the values for stomatal conductance and total conductance in the revised manuscript. We appreciate the reviewer's point - information on these parameters is needed to explain the (lack of) variability with light level of the uptake fluxes.

Briefly, in non-dark conditions (PAR>0), stomatal conductance remained above 0.25 mol m^{-2} s^{-1}, suggesting that stomatal opening was sufficient to maintain COS uptake, even at lower light levels. This is also supported by the relatively small changes observed in Ci^s and Ci/Ca^s, between the highest and the lowest light setting. A more detailed explanation will be included in the revised manuscript.

Details of leaf gas exchange equations are presented, including conductance, internal concentrations, etc. However, all those were developed strictly for the leaf scale, which may not apply here. The photo in the Appendix shows that this was a rather 'dense canopy scale' experiment. The authors note this can explain some of the non-typical observations, but there is no discussion on how to scale from leaf to canopy (or vice versa). The photo clearly indicates no uniformity in conditions and, in turn, in activities. **This scaling gap should be addressed, and if it can be overcome, it should be explained in more detail**. By the way, it seems there are some publications on branch scale measurements, which can be helpful to compare (likely also Yang et al. 2017 or 2018 who tried to scale between leaf to canopy).

We recognize that our gas-exchange approach is relatively simple and involves certain assumptions. We applied a *big leaf* approach, which we consider the most appropriate for our setup, given the use of small plants and a well-ventilated chamber thereby - minimizing boundary layer effects.

We also recognize that likely not all leaves received the same amount of PAR because of shading. However, given the precision at which COS isotope discrimination can be currently determined, it would seem too complex to go beyond a *big leaf* approach in our study. In addition, we did not obtain the gas-exchange data for the stem of the plants, so these could not be included in our calculations.

We will add a section to the Methods in the revised manuscript explaining our choice of the *big leaf* approach and the assumptions involved.

Along these lines, internal concentrations (Ci) are estimated using the leaf-scale equations, and Cm is calculated based on the D34S estimates. **The difference between the Ci and Cm is interesting but not defined or discussed**, except that very different values are reported for Ci/Ca and Cm/Ca.

We calculated the Cm/Ca ratios for COS as the mesophyll cell is the end-point for COS assimilation (the location of CA). We were also interested in whether Cm/Ca values could help explain the limited variation in COS flux across light levels, as. Indeed, we observed little variability in Cm/Ca with changing light. We will expand the discussion on the differences between Ci/Ca and Cm/Ca in the revised manuscript.

Note also that the physiological calculations of conductance, g, based on E and ci, depend on leaf temperature and water vapor saturation assumption. This is tricky in the present study, which uses a dense canopy in a different light and temperature in the chamber. It seems that COS flux, as long as it is based on the assumption of near-zero internal concentrations (i.e., **no compensation point, an issue that is ignored in this paper**), may offer a simpler alternative to total conductance, which could at least be compared (i.e., As=gCa…).

We appreciate the reviewer's thoughtful concerns. The equations by von Caemmerer and Farquhar (1981) do assume saturation of the leaf internal airspaces with water vapor - an assumption that may not hold under high evaporative demands (Cernusak *et al.*, 2018; Cernusak *et al.*, 2024), though such conditions were not present during our measurements.

Our calculations of conductance to COS represent a canopy average and may carry uncertainties related to leaf temperature. However, estimating the conductance under the assumption of zero COS concentration in the mesophyll could introduce even greater uncertainty

As mentioned earlier, we are preparing a companion paper that presents a modelling framework for COS isotope discrimination in plants. This model can account for non-zero internal COS concentrations and emissions, and will allow us to explore their effects on observed isotope discrimination.

Cernusak, L. A., Ubierna, N., Jenkins, M. W., Garrity, S. R., Rahn, T., Powers, H. H., ... & Farquhar, G. D. (2018). Unsaturation of vapour pressure inside leaves of two conifer species. *Scientific reports*, *8*(1), 1-7.

Cernusak, L. A., Wong, S. C., Stuart-Williams, H., Márquez, D. A., Pontarin, N., & Farquhar, G. D. (2024). Unsaturation in the air spaces of leaves and its implications. *Plant, Cell & Environment*, *47*(10), 3685-3698.

The LRU estimates are important. However, it is clearly sensitive to the high ambient COS used as COS uptake can generally be linearly related to Ca-cos, and it also seems that some information on this response may be available in the literature. In this case, **the effect could be estimated to some extent, and an attempt to correct the LRU for comparison with literature values at ambient COS could be made,** and discussed. In fact, a good agreement on the uncorrected LRU does not add confidence, as noted above.

Although the primary goal of our experiments was not to quantify LRU under natural conditions, we appreciate this suggestion. Applying such a correction would rely on a limited dataset (Stimler et al. 2011) and could introduce additional assumptions and uncertainties. Nonetheless, we will acknowledge in the revised manuscript that the LRU values reported may not fully represent natural conditions due to the use of higher-than-ambient COS mole fractions in the chamber air.