Answer Letter

Rev #1

This manuscript presents a proof-of-concept study for online monitoring of stem/xylem water isotopic composition in Helianthus annuus using stem vapor in-situ sampling coupled to laser spectroscopy, combined with non-destructive soil water content profiling (SWaP) to infer root water uptake (RWU) dynamics. The manuscript addresses an important topic because stable isotope methods for inferring RWU are increasingly used, while assumptions of instantaneous mixing and negligible internal water storage are often difficult to validate. Extending online isotope monitoring approaches from trees to herbaceous plants is also potentially valuable from a technical point of view.

I have several comments mainly related to uncertainties that could potentially arise from assumptions made during the soil layer-specific estimation of RWU and soil water isotopic composition (δsoil). As detailed below, these uncertainties could potentially have been better constrained by including, for example, a plant-free soil column (blank control) to quantify background soil water dynamics and/or by incorporating direct measurements of soil water isotope composition at different depths and time points throughout the experiment. The lack of such controls or direct measurements makes it difficult to evaluate the robustness of some of the key inferred quantities used in the subsequent analyses.

Dear reviewer, thank you! We greatly appreciate your review of our work. We provide below both our answers to your comments and how we propose to address them in our revised manuscript version.

1. I am concerned about the authors' use of SWaP-derived changes in soil water content to infer layer-specific RWU dynamics. It seems to me that changes in soil water content within a given layer may not necessarily reflect RWU alone, for example, in a soil column without plants, soil water content may still vary over time due to a number of factors including surface evaporation, redistribution/infiltration of water from upper to lower layers following isotope-labeling pulses added to a specific layer, etc.. Such changes could potentially be interpreted as RWU if not explicitly accounted for. In this sense a plant-free control column subjected to the same water additions and environmental conditions (which is lacking in the present study) would be helpful in constraining the uncertainty in SWaP-derived RWU profiles. I suggest that the authors either provide additional evidence that soil water redistribution and evaporation were negligible under their experimental conditions, or explicitly acknowledge this limitation. Ideally, future applications of this approach should benefit from including a blank soil column control to quantify background SWC dynamics unrelated to plant water uptake.

Indeed, this is a very important point and for this experiment we took several precautions to make sure that soil water redistribution between layers did not occur during the time window of the experiment. Before the experiment, we waited for the sunflowers to dry out the soil sufficiently such that a) an injection would lead to a considerable change of the local soil water content (SWC) and b) that the injection of limited size would not be constrained too much by the soil being too dry, or inversely, too wet such that the pulse would diffuse over too much of a region. Based on experience and simulations using Hydrus-1D this would require that the prior SWC is above 0.05 and below 0.12 cm^3 cm^{-3} such that we can be assured that the upper and lower depths of pulse expansion are met. As the plant takes up the added water relatively quickly, this also reduces the risk of the injected water diffusing too far from the point of origin, something that cannot be replicated in a column without a plant. The second step we performed to assure that no water would cross between layers was to adjust the extend of the layer in such a way that we could not

observe an increase of SWC at the boundary of two layers by observing the SWC profiles as they were measured by the SWaP (information is contained within the data series). Here, we also changed the boundary position of the layers by a cm up and down and found no significant changes, indicating that water did not exchange between layers. This can also be seen in Appendix Figure A1 of our original submission (now B1 in the revised version) where the boundary between the layers is indicated and where one can observe that the water of the pulses does not cross these boundaries over time. The lower boundary at about 35 cm depth is not strictly necessary for our analysis but ensures that the effect of the deeper pulse is not diluted by the water at the bottom of the column. The water in the lowest layer does redistribute within that layer as can be observed from Hydrus simulations, without the added pulses:

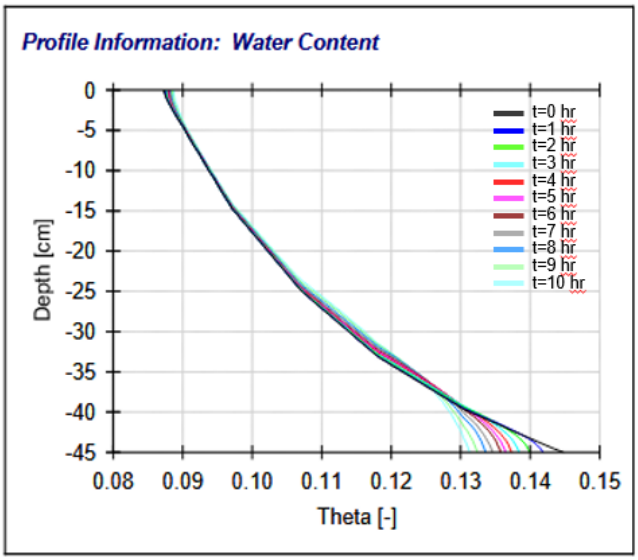


Figure: Evolution of SWC, hourly resolution without root water uptake (bare soil situation)

For SWC below 0.12 cm^3 cm^{-3} redistributive flows are very slow and local and do not cross the second, lower boundary within the timeframe of the experiment. It should be noted too, that the matric potential gradients are in fact quite low, in the order of 10 hPa cm^{-1} so redistributive flows are well contained.

→ We added in the text of section 2.4.3 (Data analysis) the following sentences (L178-182):

> *"The boundaries between layers were chosen based on the absence of observable cross-over of water between layers based on the sequential SWaP data. Shifting the position of the boundaries by 1cm up or down had no significant effect on the observed SWC decay rates of the adjoining layers, indicating minimal to no water flow occurred between layers (see also Appendix Fig. B1 for a graphical explanation)."*

We are not claiming that our method is perfectly accurate but that it is associated with a low and systematic error.

We would like to finally point out that a blank column without a plant would not appropriately reflect soil water flow occurring in a planted column: root water uptake rapidly lowers soil hydraulic conductivity by decreasing soil water content at the labeling pulse locations, therefore significantly minimizes soil water flow. In other words, cross-boundary water transport is minimized in a planted columns as compared to in a bare soil one.

2. Related to the above concern, I also feel that the reported whole-column RWU rates shown in Fig. 2b appear relatively low compared with what might be expected for a sunflower plant at the

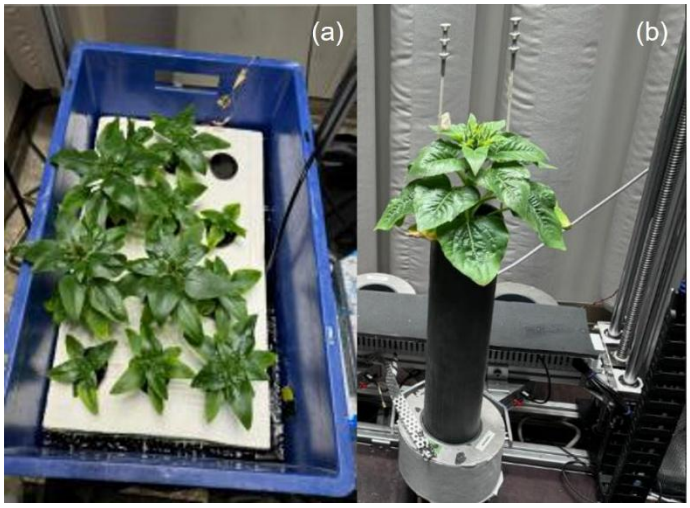
flowering stage. For example, Fig. 2b suggests a peak whole-plant uptake rate (blue line) of only approximately 1.5–1.75 ml per 10 min under high light intensity (~1200 µmol m^{-2} s^{-1}). Assuming a representative leaf-level transpiration rate of approximately 3 mmol H_2O m^{-2} s^{-1} for sunflower (which could potentially be even higher under such conditions, given that sunflower is a highly transpirational species), this uptake rate would correspond to only around 500 cm^2 total transpiring leaf area. This seems relatively small for a flowering sunflower plant, although actual leaf area was not reported.

The reviewer addresses a valid issue, but the sunflower cultivar we used here is a very small (<50 cm) growing cultivar, the "Dwarf yellow spray", and under our conditions was no higher than 30 to 40 cm. Whereas for this experiment we do not have the leaf area, the typical leaf area of these sunflowers at this growth stage is in the order of 500 to 1000 cm^2. The calculation of the reviewer is perfectly in line with our measurements, which is also expected from the published performance of the SWaP.

→ We added 1) the cultivar name in the text, sections 2.3 ("Calibration experiment") and 2.4.1 ("Experimental design and environmental conditions"):

> "*To test both hypotheses, sunflower plants of the 'Yellow Spray' cultivar, a small (<50 cm height) sunflower variety, were grown…*" (L124)
>
> "*Two 'Yellow Spray' sunflower plants…*" (L141)

→ We added 2) in a new Appendix A one picture of the sunflowers grown in hydroponics for the calibration experiment and one picture of Plant #1 in its column about a week before the case study was conducted. This should highlight the small size of the plants.



> *"Appendix A. (a) Picture of the hydroponics setup for growing the sunflower plants to be used for the calibration experiment. (b) Picture of Plant #1 in its column about a week before the case study was conducted."*

3. Lines 199-209 describe how delta_soil water for each soil layer was estimated. According to this description, layer-specific detla_soil was calculated as a volume-weighted mixture of added labeled water and antecedent soil water. I have two concerns regarding this procedure:

> 1) without direct isotope measurement of soil water, how was delta_ant determined for each individual layer? Was a pre-established, depth-dependent isotope distribution model used to estimate delta_ant? or was delta_ant assumed identical among different layers (e.g., equivalent to the irrigation water isotopic composition)?

As the reviewer points out, Eq. (4) is basically a simple volume-weighed mixing model to calculate δ_{soil} at time step (t) in one layer from the value at time step (t-1), namely δ_{ant} and that of the added, labelled water (δ_{add}). As the latter is known, Eq. (4) only needs to be initialized, which we explain in L203-208 of the original manuscript:

> *"Finally, values for the initial soil water isotopic composition, i.e., prior to the first addition of isotopically enriched water in the upper soil layer on DoE 1 16:00-17:00, was determined – since it was not measured in situ during the experiment – and was assumed to be the same for both soil columns (plant #1 and plant #2). More precisely, it was computed to fall onto an evaporation line with a slope of 4‰ /‰ (Rothfuss et al., 2015) and passing through the isotopic composition value of local tap water (δ^2H=–50.4‰ and $\delta^{18}O$=–7.4‰) in a dual isotope (δ^2H vs. $\delta^{18}O$) plot."*

The obtained soil water δ^2H ($\delta^{18}O$) initial values in the top, middle, and bottom layers of both soil columns (in which plant #1 and #2 are grown) were -47.7, -50.4, and -50.4 ‰ (-6.7, -7.4, and -7.4 ‰) and are reported in Appendix E.

→ We now provide these numbers L212-218 of our revised manuscript. In addition, we now name the initial isotopic composition of soil prior labeling, δ_{ini}, to avoid confusion with δ_{ant}:

> "*Finally, values for the initial soil water isotopic composition (δ_{ini}), i.e., prior to the first addition of isotopically enriched water in the upper soil layer on DoE 1 16:00-17:00, was determined – since it was not measured in situ during the experiment – and was assumed to be the same for both soil columns (plant #1 and plant #2). More precisely, δ_{ini} was computed to fall onto an evaporation line with a slope of 4‰ /‰ (Rothfuss et al., 2015) and passing through the isotopic composition value of local tap water (δ^2H=–50.4‰ and $\delta^{18}O$=–7.4‰) in a dual isotope (δ^2H vs. $\delta^{18}O$) plot. The calculated soil water δ^2H_{ini} ($\delta^{18}O_{ini}$) in the top, middle, and bottom layers of both soil columns (in which plant #1 and #2 were grown) were –47.7, –50.4, and –50.4 ‰ (–6.7, –7.4, and –7.4 ‰).*"

In addition, we added to the legend of Appendix F, Table 1, the following sentence (L707-709):

> "*The soil water stable isotopic compositions values simulated initially (δ_{ini}), that is, prior the first labeling pulse on DoE 1, 15:10-15:59, are reported in the first line of Table F1.*"

2) Eq. 4 also gives the impression that, once isotopically labeled water was added, the calculated delta_soil for each layer remained effectively constant thereafter. This means that temporal variation in delta_soil was neglected. This may need further justification. More generally I think that at least some attempts to make measurements of soil water isotope compositions at spatiotemporal scales would be beneficial than to just rely entirely on estimated values based on ideal assumptions.

Yes, that is correct, δ_{soil} is affected by water addition only, that is, remains constant within each layer until the next water addition. We acknowledged in our manuscript that this is a simplification (see L403-404), yet we believe it to be valid at the layer scale, since we made sure that there was 1) no (convective) water transport across the defined layers (please refer to our answer to your previous comment) and 2) no or negligible diffusion of the labeling pulse across layers during the time window of the experiments. We also believe

that there was no significant evaporation and therefore changes in δ_{soil} in the top layer during the experiments.

→ We agree that this needs further justification and added L210-212:

> "*We acknowledge that, in between labeling pulses, the isotopic composition of soil may locally change as it is directly affected by soil water redistribution (and by RWU indirectly). However, at the layer scale* $\{\delta_{soil}\}_{i=1:3}$ *remains constant since there is no water transport across soil layers.*"

Taking destructive measurements for determination of δ_{soil} in such conditions (small size of the column) would have been very challenging without affecting the plants themselves. The best option would be to monitor non-destructively the changes in δ_{soil} (e.g., by following the method of, e.g., Rothfuss et al. 2013). This would require to significantly modify the setup. We hope to perform such a follow-up experiment soon.

→ We agree that this needs further justification and incorporated the present answer to the discussion section 4.2 (L415-417):

> "*Finally,* δ_{xyl_calc} *is also sensitive to the initial conditions in soil water isotopic composition prior the first labeling (DoE 1 at 16:00, see Appendix E),* δ_{ini}*, which was not measured in situ but simulated as well. As destructive sampling was not a viable option due to the limited size of the columns, a possible alternative would be to monitor* δ_{soil} *in situ using the methods of Rothfuss et al. (2013) or Volkmann and Weiler (2014), albeit at the cost of a substantial modification of our setup.*"

The following reference was added:

> "Volkmann, T. H. M., and Weiler, M.: Continual in situ monitoring of pore water stable isotopes in the subsurface, Hydrol. Earth Syst. Sc., 18(5), 1819-1833, https://www.doi.org/10.5194/hess-18-1819-2014, 2014."

Rev #2

General comments

The authors present a novel and highly relevant methodological approach for ecohydrology and plant physiology. The authors successfully demonstrate minimally invasive monitoring of stem water isotopic composition in an herbaceous plant (sunflower) using a laser spectrometer coupled with a sampling tube. The manuscript is generally well structured and the figures are clear.

Despite these limitations, the work represents a promising step towards real-time isotope monitoring in herbaceous plants, but the current level of replication, calibration verification, and modelling detail is insufficient for full acceptance. A revision that addresses the above points would substantially improve the manuscript.

Dear Reviewer, thank you very much for your comments! Please find below our answers and details on how we propose to address your comments in the revised version of our manuscript.

Specific comments

1. The case study relies on only two sunflower plants (plant #1 and plant #2). The observed differences between the two plants are interpreted in terms of experimental timing, but without

statistical replication it is impossible to know whether these differences reflect genuine biological variability or are simply artefacts of the low n.

Thank you for this very important point! Our intention was not to draw definitive conclusions on the physiology and water transit time of sunflower plants, rather to present a proof of concept and a first application of the simple isotope monitoring method. We do not think that our estimate of capacitance should be representative of the tested cultivar: we just highlight its importance. There is naturally no wish to overinterpret the differences between the two plants investigated - there are indeed more possibilities, including biology.

→ We mentioned the low n in the introduction paragraph of the Material and Method sections (L82-87):

> "*The second experiment (called 'case study' afterwards) was conducted on two potted sunflowers plants using soil columns under controlled conditions to (i) investigate their response to irrigation pulses that differed in their soil location and timing and (ii) assess the importance of their water flow capacitance. In this experiment, the Soil Water Profiler, SWaP (van Dusschoten et al., 2020) was used to provide independent, quantitative estimates of root water uptake profiles. Because of the absence of experimental replicates and repetitions, our intention was not to draw definitive conclusions on the physiology and water transit times that would be representative of the investigated sunflower cultivar.*"

2. The calibration was performed in hydroponic solution, yet the case study uses soil-grown plants. The authors acknowledge that the calibration might need to be re-determined for soil conditions, but they do not test whether the hydroponic-derived calibration functions are valid under the soil condition.

The calibration functions obtained in hydroponic solutions would not be transferable to the case of a soil-grown plant only if a sunflower behaves differently from an isotope standpoint, i.e., discriminates differently against heavier stable isotopes during uptake from a soil than from pure liquid water. We however do not think it is case.

→ We therefore modified that section (now L391-395) and formulated it in a positive manner, that is:

> "*Finally, we are confident that the calibration functions are specific to the plant species, rather than being influenced by the environment in which the plant is grown. Therefore, the obtained calibration functions should be transferable to soil-grown plants. Nevertheless, the calibration experiment could be extended to include sunflowers grown in soil columns for verification and validation.*"

On a side note, calibrating in soil poses other challenges, e.g., prevention from soil evaporation to conserve the δ_{soil} value while growing the plant in aerobic conditions.

3. The method is described as "minimally invasive", but the authors provide no comparison with other non-destructive alternatives. The extent to which the drilling perturbs the plant is not quantified.

There are currently no non-destructive alternatives to our method. This can only be done by sampling the stem destructively and by extracting its water in the laboratory using one of the available techniques, e.g., the cryogenic vacuum distillation, centrifugation, direct vapor equilibration (Ceperley et al., 2024). Also, the extent to which the drilling affected the plant was

observed with MRI pictures and discussed in the text (please see Figure 5 and text 450-455, Appendix Fa and b – now Figure 5, L463-468, Appendix Ga and b).

4. The assumption that the stem water reservoir is well mixed and that the exchange fraction is constant during the day is a gross simplification. The authors can present a sensitivity analysis.

Thank you. It is not entirely clear which section this comment refers to. In case it refers to Eq. (5) (replacement model), it is correct that we cannot proof our assumption, but this is not the purpose for providing this equation. It serves only as a rough estimate of how large the fraction of water is that exchanges with the stationary water within the stem. It serves as an indicator of order of magnitude only.

Also, we do think that we tested the sensitivity of the replacement model to the exchange fraction parameter in L424-429 (section 4.3) of the original manuscript (now L434-438):

> "*If f_{ex}=1 is set in the simple replacement model (Eq. 5), that is, all water flowing in the xylem is isotopically exchanging with water from the (non-conducting) stem tissues, stem water would reach a constant δ_{stem} in roughly 2 h. On the other hand, if f_{ex}=1/6, it would take 10 h for stem water to be replaced nearly fully with the xylem water, which would be roughly in line with our observation for plant #1.*"

5. The statement that the method "failed" when the tubing was inserted too deeply or too high above the soil is important. The authors should provide quantitative thresholds (e.g., maximum insertion depth, minimum stem diameter) that would allow other researchers to avoid these failures.

Thank you for this important point! Our method did not "fail", rather "[f]ailure to [insert the tubing (i) only a couple of millimeters inside the stem and (ii) close to the root crown] resulted in invalid data during the experiments". The installation of the tubing inside the stem always worked and always yielded an isotope response. It could be observed quite fast (<1 min) from the raw readings if the tubing was inserted correctly (water vapor mixing ratio values equal to the saturated values at the temperature of the stem).

→ Statement was rephrased as (L458-462):

> "*The installation of the tubing inside the stem always worked and yielded an isotope response. The method showed to be sensitive to the insertion depth of the PTFE sampling tubing inside the stem and to the installation height above the soil surface. To work, the tubing had to be inserted (i) only a couple of millimeters inside the stem and (ii) close to the root crown. Not doing so resulted in invalid data during the experiments, e.g., the WVMR was either too low or too high – the latter pointing to the presence of liquid water inside the sample tubing because of a direct contact with the wet (pith) tissue.*"

6. "assuming no transport of water occurred across soil layers" (L199) this assumption is not justified. The authors can give former references or explanations on why lateral or vertical redistribution can be neglected

This statement in line 199 should be seen more like a standard, conservative phrasing, because we did verify that this is substantially correct. As commented to Reviewer 1, the layers were carefully selected. In fact, we tried several boundary positions over a range of a few cm from within the SWaP data. Up to 1 cm up or down showed no significant changes in our results, giving more than sufficient confidence that no relevant cross-layer exchange occurred between the upper and middle soil layers.

→ We rephrased the sentence L199 of our original manuscript by (now L205):

> "*Based on the SWaP data we can safely assume that no transport of water occurred across soil layers, δ_{soil} was calculated*"

Technical Corrections

The reference list contains several formatting inconsistencies (e.g., missing journal names, incomplete page ranges). Check the journal guidelines for references.

References are reported in accordance with the journal guidelines (https://www.hydrology-and-earth-system-sciences.net/submission.html#references). We did find two articles (Ceperley et al., 2024; Fabiani et al., 2022) for which an article number was missing (no page ranges). Thank you! Corrections were made:

> "*Ceperley, N., Gimeno, T. E., Jacobs, S. R., Beyer, M., Dubbert, M., Fischer, B., Geris, J., Holko, L., Kuebert, A., Le Gall, S., Lehmann, M. M., Llorens, P., Millar, C., Penna, D., Prieto, I., Radolinski, J., Scandellari, F., Stockinger, M., Stumpp, C., Tetzlaff, D., van Meerveld, I., Werner, C., Yildiz, O., Zuecco, G., Barbeta, A., Orlowski, N., and Rothfuss, Y.: Toward a common methodological framework for the sampling, extraction, and isotopic analysis of water in the Critical Zone to study vegetation water use, Wiley Interdisciplinary Reviews-Water, 11(4), e1727, https://www.doi.org/10.1002/wat2.1727, 2024."*

> "*Fabiani, G., Penna, D., Barbeta, A., and Klaus, J.: Sapwood and heartwood are not isolated compartments: Consequences for isotope ecohydrology, Ecohydrology, 15(8), e2478, https://www.doi.org/10.1002/eco.2478, 2022."*