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Interactive comment on “Differences in instantaneous water use efficiency derived from post-carboxylation fractionation respond to the interaction of CO_2 concentrations and water stress in semi-arid areas” *by* Na Zhao et al.

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Response to referee’s comments

We thank and greatly appreciate the thoughtful and constructive comments from Professor Ferrio Diaz. We have fully considered your comments in the revision and improved the manuscript (revised manuscript marked in red color).

General comments

In this work, Zhao et al. present an experimental study on the interactive effects of CO2 and water availability on instantaneous water-use efficiency (iWUE) and the carbon isotope composition (d13C) of leaf water-soluble organic matter (LWSOM). Although the study of the interaction between CO2 and drought and its effects on d13C and iWUE is not new (Picon, Ferhi, & Guehl 1997), there is no clear consensus on the interpretation of d13C changes in response to increasing CO2 (Schubert & Jahren 2012). In this context, the comprehensive dataset here presented may contribute to understand the limitations of d13C as a surrogate for iWUE, and to better predict the response of tree species to increasing CO2, particularly in drought-prone environments. This is particularly relevant for the proper interpretation of long-term trends in d13C in relation to changes in water use efficiency, particularly in drought-prone environments, e.g. based on tree-ring records (Duquesnay et al. 1998; Saurer, Siegwolf, & Schweingruber 2004; Voltas et al. 2013), or from herbarium and sub-fossil material (Peñuelas & Azcón-Bieto 1992;Beerling 1996;Köhler et al. 2010). The experiment is well-designed and the data is generally well presented, although some details on the methodology are missing (see technical corrections). However, the manuscript requires some improvements, particularly on the interpretation of results.

Response: Thank you for the careful review and constructive comments. According your helpful suggestions, revisions throughout the whole article have been made and the results have been improved and supplemented with the related contents.

Specific comments

My main concern about the manuscript is that it relies on the assumption that the only source of divergence between gas-exchange iWUE and d13C of recent assimilates could be post-photosynthetic fractionation. Although this is likely to play a role, the authors should consider that what actually defines carbon isotope discrimination (D13C) is the CO2 concentration in the chloroplast (Cc), not in the intercellular space, as used in the simplified equation of the Farquar's model (Evans et al. 1986; Farquhar, Ehleringer, & Hubick 1989). Indeed, the difference between gas-exchange derived values and online measurements of D13C has been widely used to estimate Ci-Cc and mesophyll conductance for CO2 (Le Roux et al. 2001;Warren & Adams 2006;Flexas et al. 2006;Evans et al. 2009;Flexas et al. 2012;Evans & von Caemmerer 2013). In this regard, changes in mesophyll conductance could be partly responsible for the observed variations, as it generally increases in the short term in response to elevated CO2 (Flexas et al. 2007;Flexas et al. 2014), whereas it tends to decrease under drought (Flexas et al. 2004;Ferrio et al. 2012;Hommel et al. 2014;Théroux-Rancourt, Éthier, & Pepin 2014). Hence, the manuscript would be greatly improved by considering both post-photosynthetic fractionation and mesophyll conductance as potential sources of variation. With the data available, the authors may be able to estimate changes in mesophyll conductance, based on the Evans method, which can be adapted to recent assimilates (Pons et al. 2009). Even without alternative estimates for mesophyll conductance, this would provide a useful ground for a deeper discussion.

Response: Thanks for your relevant and helpful comments about our research. The consensus has been reached that the routine of CO2 diffusion into photosynthetic site in plant includes two main procedures, which are CO2 moving from ambient environment surrounding the leaf (Ca) to the sub-stomatic cavities (Ci) through stomata, and from there to the site of carboxylation within the chloroplast stroma (Cc) of leaf mesophyll. The latter diffusion is defined as mesophyll conductance (gm) (Flexas et al., 2008; Evans et al. 2009). Moreover, gm has been identified to coordinate with environmental variables at the faster rate than that of stomatal conductance (Galmés et al., 2007; Tazoe et al., 2011; Flexas et al., 2007). gm as the important factor that could improve water use efficiency under drought pretreatment (Han et al., 2016). There has been a dispute how gm responds to fluctuation of CO2 concentration. Terashima et al. (2006) have confirmed that CO2 permeable aquaporin, located in the plasma membrane and inner envelope of chloroplasts (Uehlein et al. 2008), could regulate the change of gm.

The 13C fractionation of CO2 from air surrounding leaf to sub-stomatal cavity may be simply considered (Eqn. 6), whereas the fractionation induced by mesophyll conductance from sub-stomatic cavities to the site of carboxylation in the chloroplast cannot be neglected (Pons et al., 2009; Cano et al., 2014). As estimating the post-photosynthetic fractionation in leaf, carbon discrimination generated by mesophyll conductance must be subtracted from 13C fractionation from the site of carboxylation to cytoplasm before sugars transportation, estimated from the difference between $\delta\hat{}13$ C_WSC ($\delta\hat{}13$ C of water soluble compounds by carbon isotopic method) and $\delta\hat{}13$ C_model ($\delta\hat{}13$ C modeled from gas exchange measurement), which was closely associated with gm. Consequently, considering your constructive suggestions, gm in our study was determined based on the Evans method, which can be adapted to recent assimilates (Pons et al. 2009). And then we can estimate the variation of gm under SWC $\times$ [CO2] treatments. Related methods, results, discussions and conclusion of gm have been added in the revised manuscript (see Page 6-7, lines 216-261, Page 8-9, lines 319-330 and 347-355, Page 10-11, lines 410-435 and Page 12, lines 473-475). Subsequently, it has been shown that mesophyll conductance and post-carboxylation fractionation both contribute to the 13C fractionation from the site of carboxylation to cytoplasm (the difference between $\delta\hat{}13$ C_WSC and $\delta\hat{}13$ C_obs), which is derived from 13C fractionation following the carboxylation while photosynthate having not been transported to the twigs of plant in our study.

Added citations:

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Flexas, J., Diaz-Espejo, A., Galmés, J., Kaldenhoff, R., Medano, H., and Ribas-Carbo, M.: Rapid variations of mesophyll conductance in response to changes in CO2 concentration around leaves, Plant Cell Environ., 30, 1284–1298, 2007.

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Lanigan, G. J., Betson, N., Griffiths, H., and Seibt, U.: Carbon isotope fractionation during photorespiration and carboxylation in Senecio, Plant Physiol., 148, 2013–2020, 2008.

Pons, T. L., Flexas, J., von Caemmerer, S., Evans, J. R., Genty, B., Ribas-Carbo, M., and Brugnoli, E.: Estimating mesophyll conductance to CO2: methodology, potential errors, and recommendations, J. Exp. Bot., 8, 1–18, 2009.

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Terashima, I., Hanba, Y.T., Tazoe, Y., Vyas, P., and Yano, S.: Irradiance and phenotype: comparative eco-development of sun and shade leaves in relation to photosynthetic CO2 diffusion, J. Exp. Bot., 57, 343–354, 2006.

Uehlein, N., Otto, B., Hanson, D. T., Fischer, M., McDowell, N., and Kaldenhoff, R.: Function of Nicotiana tabacum aquaporins as chloroplast gas pores challenges the concept of membrane CO2 permeability, Plant Cell, 20, 648–657, 2008.

Technical corrections

In its present form, the title may suggest that instantaneous water use efficiency is changing because of post-carboxylation fractionation, which is clearly not the case. Besides, after considering the role of mesophyll conductance, post-carboxylation fractionation should not play such a major role in the title. An alternative might be "The interaction of CO2 concentrations and water stress in semi-arid areas causes diverging response in instantaneous water use efficiency and carbon isotope composition". This leaves open the possibility to discuss both post-photosynthetic fractionation and mesophyll conductance as potential causes for the observed divergence.

Response: We thank referee and greatly appreciate the thoughtful and constructive comments. Following your suggestions, the title was changed as "The interaction of CO2 concentrations and water stress in semi-arid areas causes diverging response in instantaneous water use efficiency and carbon isotope composition" in the revised manuscript, which can more comprehensively discuss both post-photosynthetic fractionation and mesophyll conductance as potential causes for the observed divergence.

In the abstract, lines 11-14: it seems that several concepts are mixed together here, trying to summarize everything in one sentence, but the result is unclear. I would recommend to split the ideas in shorter lines, and to try to go step by step in the argumentation line of the abstract.

Response: Based on your constructive recommendation, we rewrote this part as (starting on Lines 10-13 in the abstract):

"The 13C fractionation may be generated through the transformation from photosynthate to sugars before transporting them outward the leaf. The influence of environmental conditions (i. e. CO2 concentration and water stress) and their interactions on this fractionation have not yet been identified".

The number of replicates (saplings) per treatment is not given in the methods (however it is shown in the figures, n=32). Please add, and also specify the number of leaves measured/sampled per tree, number of gas-exchange measurements per leaf, etc.

Response: Considering your suggestions, we modified and specified the sampling and measuring process in gas-exchange measurements and the extractions of water soluble compound of leaves to read (starting on Page 4, Line 159-161 and on Page 5, Line 168-170, respectively):

"Four replicates were measured with each leaf and four leaves were chosen per tree in the gas-exchange measurement. There were two saplings ready for one orthogonal treatment ([CO2] × water stress)."

"After gas exchange measurements, recently-expanded, eight sun leaves were removed per tree of two species and two cultivated saplings per specie were replicated per treatment, and then were frozen immediately in liquid nitrogen."

In line 263 an attempt to quantify the so-called 'post-carboxylation fractionation' is given, but the methodology used is not described. As it is written, the sentence "When comparing WUEge and WUEcp, the 13C-depletion" is misleading, since it is not WUE calculated by the two methods what is compared here, but observed and modelled d13C. I guess the value results from the difference between observed d13C and modelled d13C calculated from gas-exchange data, i.e. by reverting equations 3 and 4, however this is not explained in the methods.

Response: Thanks for your helpful comments. Consistent with your speculation and considering the effect of mesophyll conductance, the defined ‘post-carboxylation’ or ‘post-photosynthesis’ that can explain part of the 13C fractionation from the site of carboxylation to cytoplasm before sugars transportation that is the difference between observed δ13C of water soluble compounds from leaves and the modeled δ13C calculated from gas-exchange, which in unmodified manuscript was not been explained in the methods, misleading that with the difference between WUEge and WUEcp. Considering with your suggestions, we added the methodology of post-carboxylation in “2.4.1 The 13C fractionation between the sub-stomatic cavities and the ambient environment” that reads (starting on Page 6, Line 209-215):

"Then the 13C fractionation between the sub-stomatic cavities and the ambient environment (total 13C fractionation) can be estimated by the observed δ13C of water soluble compounds from leaves (δ13CWSC) and the modelled δ13C calculated from gas-exchange (δ13Cmodel). The δ13Cmodel can be calculated from Δ_model from Eqn. (2). The Δ_model can be determined by Eqns. (3 and 4) as:

$\Delta\_model=(b-a)(1-(1.6\Delta eWUE\_ge)/C\_a)+a$ (7)

$\delta\hat{}13\ C\_model=(C\_a-\Delta\_model)/(1+\Delta\_model)$ (8)

Total $(_\hat{}13)$C fractionation=$\delta\hat{}13\ C_WSC-\delta\hat{}13\ C_model$ (9)."

"3.4 13C fractionation from the site of carboxylation to cytoplasm before sugars transportation" has been modified as (starting on Page 8, Line 307-318):

"We evaluated the total 13C fractionation from the site of carboxylation to cytoplasm by gas exchange and δ13C of water-soluble compounds from leaf measurements (Table 1), which can retrace 13C fractionation before carboxylation transport to the twig. Com-

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paring $\delta\hat{}13$ C_WSC with $\delta\hat{}13$ C_model from Eqns. (4, 7 and 8), total 13C fractionation of P. orientalis ranged from 0.0328‰ to 0.0472‰ which was smaller than that of Q. variabilis (0.0384‰ to 0.0466‰. The total fractionations of P. orientalis were magnified with soil wetting especially that was increased by 21.30%–42.04% at 35%–80% of FC from C400 to C800. Fractionation coefficients under C400 and C500 were amplified as SWC increased until 50%–60% of FC in Q. variabilis, whereas it was increased at 50%–80% of FC and decreased at FC under C600 and C800. Elevated [CO2] enhanced the average fractionation effect of P. orientalis, while those of Q. variabilis declined sharply from C600 to C800. Total 13C fractionation in P. orientalis increased faster than did those of Q. variabilis with increased soil moisture."

"4.4 Post-carboxylation fractionation generated before photosynthate leaving leaves" was been improved as (starting on Page 11, Line 450-453):

"When comparing $\delta\hat{}13$ C_WSC with $\delta\hat{}13$ C_obs, total fractionations of P. orientalis ranged from 0.0328‰ to 0.0472‰ less than that of Q. variabilis (from 0.0384‰ to 0.0466‰. Then total 13C fractionation subtracted by fractionation derived from mesophyll conductance, post-photosynthetic fractionation occupied 75.30%-98.9% of total 13C fractionation."

The conclusion of this manuscript need to be modified as (starting on Page 12, Line 465-468 and 475-479):

"The influence of mesophyll conductance on the difference of 13C fractionation between the sub-stomatic cavities and the ambient environment need to be considered, while testing the hypothesis that the post-carboxylation will contribute to the 13C fractionation from the site of carboxylation to cytoplasm before sugars transportation."

"Rising [CO2] and/or soil moistening generated increasing disparities between $\delta\hat{}13$ C_WSC and $\delta\hat{}13$ C_model in P. orientalis; nevertheless, the differences between $\delta\hat{}13$ C_WSC and $\delta\hat{}13$ C_model in Q. variabilis increased as [CO2] being less than 600 ppm and/or water stress was alleviated. Total 13C fractionation in leaf was linearly dependent on gs."

Text in the legends of Figs. 2-5 could be larger. Since each panel is associated to one single species, they could be simplified by including the name of the species elsewhere in the figure, and using the symbols only for the CO2 levels. The symbols for a given CO2 level could be the same in all panels, regardless of the species (in this way, one legend would be enough for all the panels).

Response: Thanks for your constructive comments. Considering your suggestions, the legends of Figs. 2-5 were simplified in the revised manuscript. The symbols for CO2 concentration of 400 ppm, 500 ppm, 600 ppm and 800 ppm were uniformly presented as C400, C500, C600 and C800 in sequence. One legend was shown in all panels of one Figure shown in Figs. 2-5 of revised manuscript.

In Figure 6 I would use the symbols to indicate CO2 levels, as in the rest of figures. This would be useful to see whether the positive association between "fractionation" and gs is linked with CO2 or water availability.

Response: Thank you for suggestions about the graphic settings. According your consideration, we have redrawn the images of Figs. 7 and 8 in the revised manuscript, which could obviously illustrate the relationships between gs/gm and total 13C fractionation. The legends of Figs. 7 and 8 were simplified. The symbols for CO2 concentration of 400 ppm, 500 ppm, 600 ppm and 800 ppm were uniformly presented as C400, C500, C600 and C800 in sequence.

Please also note the supplement to this comment:
http://www.biogeosciences-discuss.net/bg-2016-372/bg-2016-372-AC1-supplement.zip

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