The interaction of CO₂ concentrations and water stress in semi-arid areas causes diverging response in instantaneous water use efficiency and carbon isotope composition

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Abstract. In the context of global warming attributable to the increasing levels of CO₂, severe drought can be anticipated in areas with chronic water shortages (semi-arid areas), which necessitates research on the interaction between elevated atmospheric concentrations of CO₂ and drought on plant photosynthetic discrimination. It is commonly surveyed that the ¹³C fractionation derived from the CO₂ diffusion occurred from ambient air to sub-stomatal cavity, and little investigate the ¹³C fractionation generated from the site of carboxylation to cytoplasm before sugars transportation outward the leaf, which may respond to the environmental conditions (i.e. CO₂ concentration and water stress) and their interactions. Therefore, saplings of typical species to a semi-arid area of Northern China that have similar growth status—Platycladus orientalis and Quercus variabilis—were selected and cultivated in growth chambers with orthogonal treatments (four CO₂ concentrations [CO₂] × five soil volumetric water contents (SWC)). The δ¹³C of water-soluble compounds extracted from leaves of saplings was measured to determine the instantaneous water use efficiency (WUEᵢ) after cultivation. Instantaneous water use efficiency derived from gas exchange (WUEₑᵢ) was integrated to estimate differences in δ¹³C signal variation before leaf-exported translocation of primary assimilates. The WUEₑᵢ of the two species both decreased with increased soil moisture, and increased with elevated [CO₂] at 35%–80% of field capacity (FC) by strengthening photosynthetic capacity and reducing transpiration. Differences in instantaneous water use efficiency (WUE) according to distinct environmental changes differed between species. The WUEₑᵢ of P. orientalis was significantly greater than that of Q. variabilis, while the opposite results were obtained in a comparison of WUEᵢ in two species. Total ¹³C fractionation from the site of carboxylation to cytoplasm before sugars transportation (total ¹³C fractionation) was clearly species-specific, as demonstrated in the interaction of [CO₂] and SWC. Rising [CO₂] coupled with moistened soil generated increasing disparities of δ¹³C between the water soluble compounds (δ¹³CWSC) and estimated by gas-exchange observation (δ¹³Cₑᵢ) in P. orientalis with amplitude of 0.0328‰–0.0472‰. Furthermore, differences between δ¹³CWSC and δ¹³Cₑᵢ of Q. variabilis increased as CO₂ concentration and SWC increased (0.0384‰–0.0466‰). The ¹³C fractionations from mesophyll conductance and post-carboxylation both contributed to the total ¹³C fractionation determined by two measurements (1.06%–24.94% and 75.30%–98.9% of total ¹³C fractionation, respectively). Total ¹³C fractionations were linearly dependent on gs, indicating post-carboxylation fractionation was attributed to environmental variation. Thus, clear description of magnitude and environmental dependence of apparent post-carboxylation fractionation is worth our attention in photosynthetic fractionation.

Key words: Post-carboxylation fractionation; Carbon isotope fractionation; Elevated CO₂
1 Introduction

Since the onset of the industrial revolution, atmospheric CO$_2$ concentration has increased at an annual rate of 0.4%, and is expected to increase further to 700 μmol·mol$^{-1}$, together with more frequent periods of low water availability (IPCC, 2014). Increasing atmospheric CO$_2$ concentrations that trigger an ongoing greenhouse effect will not only lead to fluctuations in global patterns of precipitation, but will amplify drought in arid regions, and lead to more frequent occurrences of extreme drought events in humid regions (Lobell et al., 2014). Accompanying the increasing concentration of CO$_2$, the mean δ$^{13}$C of atmospheric CO$_2$ is depleted by 0.02‰–0.03‰ year$^{-1}$ (data available from the CU-INSTAAR/NOAACMDL network for atmospheric CO$_2$; http://www.esrl.noaa.gov/gmd/).

The carbon isotopic composition determined recently could respond more subtly to environmental changes and their influences on diffusion via plant physiological and metabolic processes (Gessler et al., 2014; Streit et al., 2013). While the depletion of δ$^{13}$C$_{CO_2}$ has been shown in the atmosphere, variations in CO$_2$ concentration itself might also affect the δ$^{13}$C of plant organs that, in turn, respond physiologically to climatic change (Gessler et al., 2014). The carbon discrimination (δ$^13D$) of leaves could also provide timely feedback about the availability of soil moisture and the atmospheric vapor pressure deficit (Cernusak et al., 2012). Discrimination against $^{13}$C in leaves relies mainly on environmental factors that affect the ratio of intercellular to ambient CO$_2$ concentration (C/$C_a$) and Rubisco activities, even the mesophyll conductance derived from the difference of CO$_2$ concentrations between intercellular site and chloroplast (Farquhar et al., 1982; Cano et al., 2014). As changes in environmental conditions affect photosynthetic discrimination, they are expected to be recorded differentially in the δ$^{13}$C of water-soluble compounds (δ$^{13}$C$_{WSC}$) of the different plant organs. Meanwhile, several processes during photosynthesis alter the δ$^{13}$C of carbon transported within plants. Carbon-fractionation during photosynthetic CO$_2$ fixation has been described and reviewed elsewhere (Farquhar et al., 1982; Farquhar and Sharkey, 1982).

Post-photosynthetic fractionation is derived from equilibrium and kinetic isotopic effects, which determines isotopic differences between metabolites and intramolecular reaction positions, defined as “post-photosynthetic” or “post-carboxylation” fractionation (Jäggi et al., 2002; Badeck et al., 2005; Gessler et al., 2008). Post-carboxylation fractionation in plants includes the carbon discriminations that follow carboxylation of ribulose-1, 5-bisphosphate, and internal diffusion (RuBP, 27‰), as well as related transitory starch metabolism (Gessler et al., 2008; Gessler et al., 2014), fractionation in leaves, fractionation-associated phloem transport, remobilization or storage of soluble carbohydrates, and starch metabolism fractionation in sink tissue (tree rings). In the synthesis of soluble sugars, $^{13}$C-depletions of triose phosphates occur during exportation from the cytoplasm, and during production of fructose-1, as does 6-bisphosphate by aldolase in transitory starch synthesis (Rossmann et al., 1991; Gleixner and Schmidt, 1997). Synthesis of sugars before transportation to the twig is associated with the post-carboxylation fractionation generated in leaves. Although these are likely to play a role, what should also be considered is the CO$_2$ concentration in the chloroplast (C$_c$), not in the intercellular space, as used in the simplified equation of the Farquhar’s model (Evans et al., 1986; Farquhar et al., 1989) is actually defined as carbon isotope discrimination (δ$^{13}$C). Indeed, difference between gas-exchange derived values and online measurements of δ$^{13}$C has been widely used to estimate C/$C_a$ and mesophyll conductance for CO$_2$ (Le Roux et al., 2001; Warren and Adams, 2006; Flexas et al., 2006; Evans et al., 2009; Flexas et al., 2012; Evans and von Caemmerer 2013). In this
regard, changes in mesophyll conductance could be partly responsible for the differences from two measurements, as it generally increases in the short term in response to elevated CO₂ (Flexas et al., 2014), whereas it tends to decrease under drought (Hommel et al., 2014; Théroux-Rancourt et al., 2014). Therefore, it is necessary to avoid confusion of carbon isotope discrimination derived from synthesis of soluble sugars or/and mesophyll conductance, and furthermore, whether and what magnitude of these carbon fractionations are related to environmental variation have not yet been investigated.

The simultaneous isotopic analysis of leaves is a recent refinement in isotopic studies that allows us to determine the temporal variation in isotopic fractionation (Rinne et al., 2016), which may help decipher environmental conditions more reliably. Newly assimilated carbohydrates can be extracted, and are defined as the water-soluble compounds (WSCs) in leaves (Brandes et al., 2006; Gessler et al., 2009), which can also be associated with an assimilation-weighted average of C/Ĉ0 (and C/Ĉ0) photosynthesized over a period ranging from a few hours to 1–2 d (Pons et al., 2009). However, there is a dispute whether the fractionation stemmed from post-carboxylation or/and mesophyll resistance may alter the stable signatures of leaf carbon and thence influence instantaneous water use efficiency (iWUE). In addition, the way in which iWUE derived from those isotopic fractionations responds to different environmental factors, such as elevated [CO₂] and/or soil water gradients, has yet to be observed.

Consequently, we investigated the Δ³¹C of fast-turnover carbohydrate pool in leaves from saplings of two typical species to semi-arid areas of China—Platycladus orientalis and Quercus variabilis—together with simultaneous gas exchange measurements in control-environment of growth chambers (FH-230). Our goals are to differentiate the Δ³¹C fractionation from the site of carboxylation to cytoplasm before sugars transportation (total Δ³¹C fractionation) of P. orientalis and Q. variabilis, which were determined from the Δ³¹C of water-soluble compounds and gas-exchange measurements, and then to discuss the potential causes for the observed divergence, estimate the contributions of post-photosynthetic and mesophyll resistance on these differences, and describe how these carbon isotopic fractionations respond to the interactive effects of elevated [CO₂] and water stress.

2 Material and Methods

2.1 Study site and design

Saplings of P. orientalis and Quercus variabilis were selected as experimental material from the Capital Circle forest ecosystem station, a part of Chinese Forest Ecosystem Research Network (CFERN, 40°03’45”N, 116°5’45”E) in Beijing, China. This region is populated by trees of Platycladus orientalis (L.) Franco and Quercus variabilis Bl. Saplings of two species that have similar ground diameters, heights, and growth statuses were selected. One sapling from two species was placed in one pot (22 cm in diameter and 22 cm in height). Undisturbed soil samples were collected from the field, sieved (with all particles >10 mm removed), and placed into the pots. The soil bulk density in each pot was maintained at 1.337–1.447 g·cm⁻³. After the rejuvenation for one month, potted-saplings were placed into chambers for orthogonal cultivation.

The controlled experimental treatments were conducted in growth chambers (FH-230, Taiwan Hipoint Corporation, Kaohsiung City, Taiwan). To imitate the meteorological factors of growth seasons in the research region, the daytime temperature in chambers was set to 25 ± 0.5°C from 07:00 to 17:00, and the night-time temperature was 18 ± 0.5°C from 17:00 to 07:00. Relative humidity was
maintained at 60% and 80% during the daytime and night, respectively. The light system was activated in the daytime and shut down at night. The average daytime light intensity was maintained at 200–240 µmol·m⁻²·s⁻¹. The central controlling system of the chambers (FH-230) can timely monitor and control the CO₂ concentration. Two growth chambers (A and B) were used in our study. Chamber A was switched in turn to maintain the CO₂ concentration of 400 ppm (C₄₀₀) and 500 ppm (C₅₀₀). The other one was adjusted to maintain the CO₂ concentration of 600 ppm (C₆₀₀) and 800 ppm (C₆₀₀). The target concentrations of CO₂ in the chambers were permitted the standard deviation of ± 50 ppm during cultivation. Thus, the gradient of four CO₂ concentrations in our study was formed. Detectors inside the chambers monitored and maintained the target concentrations of CO₂.

We designed a device to irrigate the potted saplings automatically and avoid heterogeneity caused by interruptions in watering process (Fig. 1). It consisted of a water storage tank, holder, controller, soil moisture sensors, and drip irrigation components. Prior to use, the water tank was filled with water, and the soil moisture sensor was inserted to a uniform depth in the soil. After connecting the controller to an AC power supply, target soil volumetric water content (SWC) could be set and monitored by soil moisture sensors. Since timely SWC could be sensed by the sensors, the automatic irrigation device can be regulated to water or stop watering the plants. One drip irrigation device was installed per chamber.

Based on the average field capacity (FC) of potted soil determined (30.70%), five levels of SWC were maintained before the orthogonal cultivations, as follows: 100% FC (or CK) (SWC approximately 27.63%–30.70%), 70%–80% of FC (SWC approximately 21.49%–24.56%), 60%–70% of FC (SWC approximately 18.42%–21.49%), 50%–60% of FC (SWC approximately 15.35%–18.42%), and 35%–45% of FC (SWC approximately 10.74%–13.81%).

While undergoing 20 groups of orthogonal treatments for [CO₂] × SWC, the saplings were ready for sampling. Due to one chamber only containing five plant-pots (per species) and one pot one SWC level under one CO₂ concentration, two saplings per specie in one orthogonal treatment were replicated for two periods, respectively. Each period per orthogonal treatment continued for 7 days. Pots were rearranged periodically to minimize non-uniform illumination. All orthogonal tests were formed as: elevated CO₂ concentration gradient for C₄₀₀ (during June 2–9, June 12–19, June 21–28, and July 2–9, 2015, C₄₀₀), C₅₀₀ (during July 11–18, July 22–29, August 4–11, and August 15–22, 2015, C₅₀₀), C₆₀₀ (during June 2–9, June 12–19, June 21–28, and July 2–9, 2015, C₆₀₀), and C₆₀₀ (during July 11–18, July 22–29, August 4–11, and August 15–22, 2015, C₆₀₀), combined with a soil-water gradient for 35%–45% of FC, 50%–60% of FC, 60%–70% of FC, and 70%–80% of FC and 100% FC (CK).

2.2 Foliar gas exchange measurement

Fully expanded primary annual leaves of the saplings were measured with a portable infrared gas photosynthesis system (LI-6400, Li-Cor, Lincoln, US) before and after the 7-day cultivation. Two saplings per specie were replicated per treatment (SWC× [CO₂]). For each sapling, four leaves were chosen and then four measurements were conducted on each leaf. The main photosynthetic parameters, such as net photosynthetic rate (Pₙ) and transpiration rate (Tᵣ), were measured. Based on the theories proposed by Von Caemmerer and Farquhar (1981), stomatal conductance (gₛ) and intercellular CO₂ concentration (Cᵢ) were calculated by the Li-Cor software. Instantaneous water use efficiency via gas exchange (WUEₑₑ) was calculated as the ratio of Pₙ to Tᵣ.

2.3 Plant material collection and leaf water soluble compounds extraction

Recently-expanded, eight sun leaves per sapling were selected and homogenized in liquid nitrogen since the gas-exchange measurements accomplished. For the extraction of the water-soluble
compounds (WSCs) from the leaves (Gessler et al., 2004), 50 mg of ground leaves and 100 mg of
PVPP (polyvinylpyrrolidone) were mixed and incubated in 1 mL double demineralized water for
60 min at 5°C in a centrifuge tube. Each leaf was replicated two times. Two saplings per specie were
chosen for each orthogonal treatment. The tubes containing above mixture were heated in 100°C
water for 3 min. Waiting for cooling to the room temperature, the supernatant of the mixture was
centrifuged (12000 × g for 5 min, g represents one gravity) and transferred 10 μL supernatant into tin
capsule to be dried at 70°C. Folded capsules were then ready for δ13C analysis of WSCs. The samples
of WSCs from leaves were combusted in an elemental analyzer (EuroEA, HEKAtech GmbH,
Wegberg, Germany) and analyzed with a mass spectrometer (DELTAplus-XP, ThermoFinnigan).
Carbon isotope signatures are expressed in δ-notations in parts per thousand, relative to the
international Pee Dee Belemnite (PDB):
\[
\delta^{13}C = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000
\]
(1)
where \(\delta^{13}C\) is the heavy isotope and \(R_{\text{sample}}\) and \(R_{\text{standard}}\) refer to the isotope ratio between the particular
substance and the corresponding standard, respectively. The precision of the repeated measurements
was 0.1 ‰.

2.4 Isotopic calculation
2.4.1 13C fractionation from the site of carboxylation to cytoplasm before sugars transportation
Based on the linear model developed by Farquhar and Sharkey (1982), the isotope discrimination, \(\Delta\),
is calculated as:
\[
\Delta = \left( \delta^{13}C_a - \delta^{13}C_{WSC} \right) / \left( 1 + \delta^{13}C_{WSC} \right)
\]
(2)
where \(\delta^{13}C_a\) is the isotope signature of ambient \([CO_2]\) in chambers; \(\delta^{13}C_{WSC}\) is the carbon isotopic
composition of water soluble compounds extracted from leaves. The \(C_i; C_a\) is determined by:
\[
C_i; C_a = (\Delta - a) / (b - a)
\]
(3)
where \(C_i\) is the intercellular CO2 concentration, and \(C_a\) is the ambient CO2 concentration in chambers;
\(\alpha\) is the fractionation occurring CO2 diffusion in still air (4 ‰) and \(b\) refers to the discrimination during
CO2 fixation by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and internal diffusion
(30 ‰). Instantaneous water use efficiency by gas-exchange measurements (WUEge) is calculated as:
\[
\text{WUE}_g = \frac{P_n}{T_r} = \frac{(C_a - C_i) / 1.6\Delta \varepsilon}{(1 - \rho)}
\]
(4)
where 1.6 is the diffusion ratio of stomatal conductance to water vapor to CO2 in chambers and \(\Delta\varepsilon\) is
the difference between \(e_{if}\) and \(e_{atm}\) that represent the extra- and intra-cellular water vapor pressure,
respectively:
\[
\Delta\varepsilon = e_{if} - e_{atm} = 0.611 \times \left( e^{17.52/240.97 + T} \right) \times (1 - \text{RH})
\]
(5)
where \(T\) and RH are the temperature and relative humidity on leaf surface, respectively. Combining
Eqns. (2, 3 and 4), the instantaneous water use efficiency could be determined by the \(\delta^{13}C_{WSC}\) of leaves,
defined as WUEcp:
\[
\text{WUE}_{cp} = \frac{P_n}{T_r} = \left( 1 - \varphi \right) \frac{(C_a - C_i) / 1.6\Delta \varepsilon}{C_a (1 - \varphi)} \left[ \frac{b - \delta^{13}C_a + (b+1)\delta^{13}C_{WSC}}{b - a + (b+1)\delta^{13}C_{WSC}} \right] / 1.6\Delta \varepsilon
\]
(6)
where \(\varphi\) is the respiratory ratio of leaf carbohydrates to other organs at night (0.3).
Then the $^{13}$C fractionation from the site of carboxylation to cytoplasm before sugars transportation (total $^{13}$C fractionation) can be estimated by the observed $^{13}$C of water soluble compounds from leaves ($\delta^{13}$C$_{WSC}$) and the modeled $^{13}$C calculated from gas-exchange ($\delta^{13}$C$_{model}$). The $\delta^{13}$C$_{model}$ is calculated from $\Delta_{model}$ from Eqn. (2). The $\Delta_{model}$ can be determined by Eqs. (3 and 4) as:

$$
\Delta_{model} = (b - a) \left( 1 - \frac{1.6 \delta_{WUEge}}{c_a} \right) + a
$$

(7)

$$
\delta^{13}C_{model} = \frac{C_a - \Delta_{model}}{1 + \Delta_{model}}
$$

(8)

Total $^{13}$C fractionation = $\delta^{13}$C$_{WSC}$ - $\delta^{13}$C$_{model}$

(9)

2.4.2 Methodology of calculating mesophyll conductance and estimating contribution of post-carboxylation fractionation

Actually, the carbon isotope discrimination is generated from the relative contribution of diffusion and carboxylation, reflected by the ratio of CO$_2$ concentration at the site of carboxylation ($C_i$) to that in the ambient environment surrounding plants ($C_a$). The carbon isotopic discrimination ($\Delta$) could be presented as (Farquhar et al. 1982):

$$
\Delta = a_e \frac{C_a - C_i}{c_a} + a \frac{C_a - C_i}{c_a} + (e_s + a_i) \frac{C_i - C_e}{c_a} + b \frac{C_e}{c_a} - \frac{\epsilon_{RDO+} f \Gamma}{c_a}
$$

(10)

Where $C_a$, $C_i$, $C_e$, and $C_c$ indicate the CO$_2$ concentrations in the ambient environment, at the boundary layer of leaf, in the intercellular air spaces before entrancing into solution, and at the sites of carboxylation, respectively; $a_e$ is the fractionation for the CO$_2$ diffusion at the boundary layer (2.9‰); $e_s$ is the discrimination of CO$_2$ diffusion when CO$_2$ enters in solution (1.1‰, at 25 ℃); $a_i$ is the fractionation derived from diffusion in the liquid phase (0.7‰); $e$ and $f$ are carbon discrimination derived in dark respiration ($R_D$) and photorespiration, respectively; $k$ is the carboxylation efficiency, and $\Gamma^*$ is the CO$_2$ compensation point in the absence of dark respiration (Brooks and Farquhar, 1985).

When the gas in the cuvette could be well stirred during measurements of carbon isotopic discrimination and gas exchange, the diffusion in the boundary layer could be neglected and Equation 10 could be shown:

$$
\Delta = a_e \frac{C_a - C_i}{c_a} + (e_s + a_i) \frac{C_i - C_e}{c_a} + b \frac{C_e}{c_a} - \frac{\epsilon_{RDO+} f \Gamma}{c_a}
$$

(11)

There was no agreement about the value of $e$, although recent measurements estimated it as 0-4‰.

Value of $f$ has been estimated ranging at 8-12‰ (Gillon and Griffiths, 1997; Igamberdiev et al., 2004; Lanigan et al., 2008). As the most direct factor, the value of $b$ would influence the calculation for $g_{sv}$, had been thought to be close to 30‰ in higher plants (Guy et al., 1993).

The difference of CO$_2$ concentration between the substomatal cavities and the chloroplast is omitted while diffusion discrimination related with dark-respiration and photorespiration is negligible, Equation 11 could be simplified as:

$$
\Delta_t = a + (b - a) \frac{C_i}{c_a}
$$

(12)

Equation 12 presents the linear relationship between carbon discrimination and $C_i/C_a$ that is used normally in carbon isotopic fractionation. That underlines the subsequent comparison between the
expected \( \Delta \) (originated from gas-exchange, \( \Delta_i \), and those actually measured \( \Delta_{\text{obs}} \)), that is the \( ^{13}\text{C} \) fractionation from mesophyll conductance, could evaluate the differences of CO\(_2\) concentration between the intercellular air and the sites of carboxylation that generated by mesophyll resistance. Consequently, \( g_m \) can be estimated by performing the \( \Delta_{\text{obs}} \) by isotope ratio mass spectrometry and expected \( \Delta_i \) from \( C_i/C_a \) by gas exchange measurements.

Then the \( ^{13}\text{C} \) fractionation from mesophyll conductance is calculated by subtracting \( \Delta_{\text{obs}} \) of Equation 11 from \( \Delta_i \) (Equation 12):

\[
\Delta_i - \Delta_{\text{obs}} = (b - e_x - a_i) \frac{C_i - C_c}{C_a} + \frac{eP_a + f^m}{c_a} \tag{13}
\]

and the \( P_n \) from the first Fick’s law is presented by:

\[
P_n = g_m (C_i - C_c) \tag{14}
\]

Substitute Equation 14 into Equation 13 we obtain:

\[
\Delta_i - \Delta_{\text{obs}} = (b - e_x - a_i) \frac{P_n}{g_m C_a} + \frac{eP_a + f^m}{c_a} \tag{15}
\]

\[
g_m = \frac{(b - e_x - a_i) P_n}{(\Delta_i - \Delta_{\text{obs}}) \frac{eP_a + f^m}{c_a}} \tag{16}
\]

In calculation of \( g_m \), the respiratory and photorespiratory terms could be ignored or be given the specific constant values. Here, \( e \) and \( f \) are assumed to be zero or be cancelled out in the calculation of \( g_m \).

Then Equation 16 can be transformed into:

\[
g_m = \frac{(b - e_x - a_i) P_n}{\Delta_i - \Delta_{\text{obs}}} \tag{17}
\]

Therefore, the contribution of post-carboxylation fractionation could be estimated by:

\[
\text{Contribution of post – carboxylation fractionation} = \frac{(\text{Total } ^{13}\text{C} \text{ fractionation – fractionation from mesophyll conductance})}{\text{Total } ^{13}\text{C} \text{ fractionation}} \times 100 \% \tag{18}
\]

3 Results

3.1 Foliar gas exchange measurements

Saplings of \( P. \) orientalis and \( Q. \) variabilis were exposed to the orthogonal treatments. When SWC increased, \( P_n \), \( g_s \), and \( T_i \) in \( P. \) orientalis and \( Q. \) variabilis peaked at 70%–80% of FC or/and 100% FC (Fig. 2). The \( C_i \) in \( P. \) orientalis rose as SWC increased, while it peaked at 60%–70% of FC and declined thereafter with increased SWC in \( Q. \) variabilis. The capacity of carbon uptake and \( C_i \) were improved significantly by elevated [CO\(_2\)] at any given SWC for two species (\( p<0.5 \)). Furthermore, greater increments of \( P_n \) in \( P. \) orientalis were found at 50%–70% of FC from \( C_{400} \) to \( C_{800} \), which was at 35%–45% of FC in \( Q. \) variabilis. As the water stress was alleviated (at 70%–80% of FC and 100% FC), the reduction of \( g_s \) in \( P. \) orientalis was more pronounced with elevated [CO\(_2\)] at a given SWC (\( p<0.01 \)). Nevertheless, \( g_s \) of \( Q. \) variabilis in \( C_{400} \), \( C_{500} \), and \( C_{600} \) was significantly higher than that in \( C_{800} \) at 50%–80% of FC (\( p<0.01 \)). Coordinated with \( g_s \), \( T_i \) of two species in \( C_{400} \) and \( C_{500} \) was significantly
higher than that in $C_{600}$ and $C_{400}$ except for 35%–60% of FC ($p<0.01$, Figs. 2g and 2h). Larger $P_n$, $g_s$, $C_i$ and $T_c$ of *Q. variabilis* was significantly presented than that of *P. orientalis* ($p<0.01$, Fig. 2).

### 3.2 δ¹³C of water-soluble compounds in leaves

After the observations of the photosynthetic traits in two species, the same leaf was frozen immediately and the water-soluble compounds (WSCs) were extracted for all orthogonal treatments. The carbon isotope composition of WSCs (δ¹³C_WSC) of two species both increased as soil moistened (Figs. 3a and 3b, $p<0.01$). The average (± SD) δ¹³C_WSC of *P. orientalis* and *Q. variabilis* ranged from -27.44 ± 0.155‰ to -26.71 ± 0.133‰, and from -27.96 ± 0.129‰ to -26.49 ± 0.236‰, respectively. Similarly with the photosynthetic capacity varying with increased SWC, average δ¹³C_WSC of two species reached their maxima at 70%–80% of FC. Together with the gradual enrichment of [CO₂], average δ¹³C_WSC in two species declined while [CO₂] exceeded 600 ppm ($p<0.01$). Except for $C_{400}$ at 50%–100% of FC, δ¹³C_WSC of *P. orientalis* was significantly larger than that of *Q. variabilis* in any [CO₂] × SWC treatment ($p<0.01$, Fig. 3).

### 3.3 Estimations of WUEge and WUEsp

Figure 4a showed that increments of WUEge in *P. orientalis* under severe drought (i.e., 35%–45% of FC) were highest at any given [CO₂], ranging from 90.70% to 564.65%. The WUEge in *P. orientalis* decreased as SWC increased, while they increased as [CO₂] increased. Differing from variation in WUEge of *P. orientalis* with soil moistened, WUEge in *Q. variabilis* were improved slightly at 100% FC in $C_{600}$ or $C_{800}$ (Fig. 4b). The maximum of WUEge thus occurred at 35%–45% of FC in $C_{800}$ among all orthogonal treatments for *P. orientalis*; this was also observed in *Q. variabilis*. Furthermore, elevated [CO₂] enhanced the WUEge of *Q. variabilis* clearly at any SWC except that at 60%–80% of FC. Thirty-two saplings of *P. orientalis* had greater WUEge than did *Q. variabilis* between the same [CO₂] × SWC treatments ($p<0.5$).

The instantaneous water use efficiency could be determined from Eqn. (6) by the δ¹³C_WSC of leaves of two species, defined as WUEsp. As illustrated in Fig. 5a, WUEsp of *P. orientalis* in $C_{600}$ or $C_{800}$ climbed up as water stress alleviated beyond 50%–60% of FC, as well as that in $C_{400}$ or $C_{500}$ while SWC exceeding 60%–70% of FC. *Q. variabilis* exhibited no uniform trend of WUEsp with soil wetting (Fig. 5b). Except for $C_{400}$, WUEsp of *Q. variabilis* decreased abruptly at 50%–60% of FC, and then rose as soil moisture improved in $C_{500}$, $C_{600}$, and $C_{800}$. In contrast to the results of WUEge in two species, WUEsp of *Q. variabilis* was more pronounced than that of *P. orientalis* among all orthogonal treatments.

### 3.4 ¹³C fractionation from the site of carboxylation to cytoplasm before sugars transportation

We evaluated the total ¹³C fractionation from the site of carboxylation to cytoplasm by gas exchange measurements and δ¹³C of water-soluble compounds from leaf (Table 1), which can retrace ¹³C fractionation before carboxylation to the twig. Comparing δ¹³C_WSC with δ¹³C_model from Eqs. (4, 7–9), 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 reached 35%–80% of FC from $C_{400}$ to $C_{600}$ (increased by 21.30%–42.04%). The total fractionation under $C_{600}$ and $C_{800}$ 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 100% FC under $C_{600}$ and $C_{800}$. Elevated [CO₂] enhanced the average total fractionation of *P. orientalis*, while those of *Q. variabilis* declined sharply from $C_{600}$ to $C_{800}$. Total ¹³C fractionation in *P. orientalis* increased faster than did those of *Q. variabilis* with increased soil moisture.
3.5 $g_m$ imposed on the interaction of CO$_2$ concentration and water stress

According to comparison between online leaf $\delta^{13}$C$_{WSC}$ and the values of gas exchange measurements, $g_m$ over all treatments was presented in Fig. 6 (Eqns. 10–17). Significant increment trend of $g_m$ was observed with water stress alleviated in *P. orientalis*, ranging from 0.0091–0.0690 mol·CO$_2$·m$^{-2}$·s$^{-1}$ ($p<0.5$), which reached the maximum at 100% FC under a given [CO$_2$]. Yet increases in $g_m$ of *Q. variabilis* with increasing SWC become unremarkable except that under C$_{600}$. With CO$_2$ concentration elevated, $g_m$ of two species was increased in different degrees. Comparing with *P. orientalis* under C$_{400}$, $g_m$ was increased gradiently and reached its maximum under C$_{600}$ at 35%–60% of FC and 100% FC ($p<0.5$), however, that was maximized under C$_{600}$ ($p<0.5$) and slipped down under C$_{800}$ at 60%–80% of FC. The maximum increment of $g_m$ (8.2%–58.4%) occurred at C$_{800}$ at any given SWC in *Q. variabilis*. It is evidently shown that $g_m$ of *Q. variabilis* was larger than that of *P. orientalis* in the same treatment.

3.6 The contribution of post-carboxylation fractionation

Here, the difference between $\Delta_i$ and $\Delta_{obs}$ presented the $^{13}$C fractionation derived from mesophyll conductance. So the post-photosynthetic fractionation after carboxylation can be calculated by subtracting the fractionation derived from mesophyll conductance from the total $^{13}$C fractionation that is generated from the site of carboxylation to cytoplasm before sugars transportation (Table 1). The fractionation from $g_m$ had less contribution on total $^{13}$C fractionation than that from synthesis of sugars belonging to post-carboxylation fractionation in any given treatment (Table 1). The contributions of fractionation from $g_m$ in two species were illustrated different variations with soil water increasing, which declined at 50%–80% of FC and rose up at 100% FC in *P. orientalis*, yet it was shown increasing with water stress alleviated at 50%–80% of FC and then decreased at 100% FC in *Q. variabilis*. Nevertheless, the fractionations from synthesis of sugars in leaf and these contributions to total fractionation were all increased as soil moistened in two species. Considering the effects of enriched [CO$_2$] on $g_m$, fractionation from $g_m$ reached its average peak under C$_{600}$ in *P. orientalis*, which occurred under C$_{800}$ with *Q. variabilis*. Post-carboxylation fractionations were increased along with [CO$_2$] increased in *P. orientalis*, which reached those maxima under C$_{600}$ and then slipped down under C$_{800}$ differing in degrees.

3.7 Relationship between $g_s$, $g_m$ and total $^{13}$C fractionation

Total $^{13}$C fractionation after carboxylation may be correlated with the resistances derived from stomata and mesophyll cells. Here, we performed linear regressions between $g_s$/$g_m$ and total $^{13}$C fractionation for *P. orientalis* and *Q. variabilis*, respectively (Fig. 7 and 8). It was apparent that total $^{13}$C fractionation was linearly dependent on the $g_s$ ($p<0.01$) that controls the exchange of CO$_2$ and H$_2$O, and responds to environmental variation. Subsequently, the linear relationships between $g_m$ and total $^{13}$C fractionation were shown ($p<0.01$), which reflected the variation of CO$_2$ concentration through the chloroplast was correlated with carbon discrimination happened after photosynthesis in the leaf.

4 Discussion

4.1 Photosynthetic traits

The exchange of CO$_2$ and water vapor via stomata is modulated in part by the soil/leaf water potential (Robredo et al., 2010). Saplings of *P. orientalis* reached their maxima of $P_n$ and $g_s$ at 70%–80% of FC irrespective of [CO$_2$] treatments. As SWC exceeded this water threshold, elevated CO$_2$ would cause a greater reduction in $g_s$, as has been reported for barley and wheat (Wall et al., 2011). The
decrease of $g_s$ responding to elevated [CO$_2$] could be mitigated by the coupling effects of soil wetting. In addition, $C_i$ of *Q. variabilis* peaked at 60%–70% of FC and followed declines as soil moisture increased (Wall et al., 2006; Wall et al., 2011). This is interpreted as stomata having the tendency to maintain a constant $C_i$ or $C_d/C_a$ when ambient [CO$_2$] increased, which would determine the CO$_2$ used directly in chloroplast (Yu et al., 2010). On the basis of theories (Farquhar and Sharkey, 1982) and common experimental technologies (Xu, 1997), this could be explained as the stomatal limitation. However, $C_i$ of *P. orientalis* was increased considerably while SWC exceeded 70%–80% of FC, as found by Mielke et al. (2000). One factor that can account for that is plants close their stomata to reduce the loss of water during the synthesis of organic matter, simultaneously decreasing the availability of CO$_2$ and generating respiration of organic matter (Robredo et al., 2007). Another explanation is the limited root volume in potted experiments may not be able to absorb sufficient water to support full growth of shoots (Leakey et al., 2009; Wall et al., 2011). In our study, the coupling of increasing [CO$_2$] may cause nonstomatal limitation as SWC exceeding the threshold (70%–80% of FC), i.e., accumulation of nonstructural carbohydrates in leaf tissue that induces mesophyll-based and/or biochemical-based transient inhibition of photosynthetic capacity (Farquhar and Sharkey, 1982). Xu and Zhou (2011) developed a five-level SWC gradient to examine the effect of water on the physiological characteristics of perennial *Leymus chinensis*, demonstrating that there was a clear irrigation maximum of SWC below which the plant could manage itself to adjust changing environment. Miranda Apodaca et al. (2015) also concluded that, in suitable water conditions, elevated CO$_2$ augmented CO$_2$ assimilation in herbaceous plants.

The $P_n$ of two species increased with elevated [CO$_2$] in our study, similarly with the results from $C_3$ woody plants (Kgope et al., 2010). Furthermore, increasing [CO$_2$] alleviated severe drought and heavy irrigation, which suggests that photosynthetic inhibition produced by water stress or excess may be mediated by increased [CO$_2$] (Robredo et al., 2007; Robredo et al., 2010) and mitiorate the adverse effects of drought stress by decreasing plant transpiration (Kirkham, 2016; Kadam et al., 2014; Miranda Apodaca et al., 2015; Tausz Posch et al., 2013).

### 4.2 Differences between WUE$_{ge}$ and WUE$_{wp}$

The increments of WUE$_{ge}$ in *P. orientalis* and *Q. variabilis* that resulted from the combination of an increase in $P_n$ and decrease in $g_s$, followed by a reduction in $T_i$ (Figs. 2a, 2g, 2b and 2h), were also demonstrated by Ainsworth and McGrath (2010). Combining $P_n$ and $T_i$ of two species in the same treatment, lower WUE$_{ge}$ in *Q. variabilis* is obtained due to its physiological and morphological traits, such as larger leaf area, rapid growth, and higher stomatal conductance than that of *P. orientalis* (Adiredjo et al., 2014). Medlyn et al. (2001) reported that the stomatal conductance of broadleaved species is more sensitive to elevated CO$_2$ concentrations than in conifers. Moreover, there has been no consensus on the patterns of WUE with related SWC at the leaf level, although some have discussed this topic (Yang et al., 2010). The WUE$_{ge}$ of *P. orientalis* and *Q. variabilis* was enhanced with soil drying, as presented by Parker and Pallardy (1991), DeLucia and Heckathorn (1989), Reich et al. (1989), and Leakey (2009).

Bögelein et al. (2012) confirmed that WUE$_{wp}$ was more consistent with daily mean WUE$_{ge}$ than WUE$_{phloem}$. The WUE$_{wp}$ of two species demonstrated similar variation to those $\delta^{13}$C$_{WC}$, which differentiated with that of WUE$_{ge}$. Pons et al. (2009) reviewed that $\Delta$ leaf soluble sugar is coupled with environmental dynamics over a period ranging from a few hours to 1–2 d. The WUE$_{wp}$ of our materials could respond to [CO$_2$] × SWC treatments over cultivated conditions, whereas WUE$_{ge}$ is characterized as the instantaneous physiology of plants to conditions. In addition, species-specific
\[\delta^{13}C_WSC\] were observed in the same environmental treatment. Consequently, WUE\text{sp} and WUE\text{ge} have different variable curves according to different treatments.

### 4.3 The influence of mesophyll conductance on the fractionation after carboxylation

The consensus has been reached that the routine of CO\textsubscript{2} diffusion into photosynthetic site includes two main procedures, which are CO\textsubscript{2} moving from ambient air surrounding the leaf (\(C_a\)) to the sub-stomatal cavities (\(C_s\)) through stomata, and from there to the site of carboxylation within the chloroplast stroma (\(C_p\)) of leaf mesophyll. The latter procedure of diffusion is defined as mesophyll conductance (\(g_m\)) (Flexas et al., 2008). Moreover, \(g_m\) has been identified to coordinate with environmental factors more faster than stomatal conductance (Uehlein et al., 2007; Tazoe et al., 2011; Flexas et al., 2007). During our 7-day cultivations of SWC \(\times [\text{CO}_2]\), \(g_m\) was increased and WUE\text{ge} was decreased as soil moistened, which has been verified that \(g_m\) as an important factor, could improve WUE under drought pretreatment (Han et al., 2016). There has been a dispute how \(g_m\) responds to the fluctuation of CO\textsubscript{2} concentration. Terashima et al. (2006) have confirmed that CO\textsubscript{2} permeable aquaporin, located in the plasma membrane and inner envelope of chloroplasts (Uehlein et al. 2008), could regulate the change of \(g_m\). In our study, \(g_m\) is specific-special to the gradient of [CO\textsubscript{2}]. The \(g_m\) of \(P.\) orientalis was significantly decreased by 9.08%-44.42% from \(C_{600}\) to \(C_{800}\) at 60%-80% of FC, being similar to the results obtained by Flexas et al. (2007). Although larger \(g_m\) of \(Q.\) variabilis under \(C_{800}\) was observed, it made almost no difference.

Furthermore, \(g_m\) contributed to total \(^{13}\)C fractionation that followed the carboxylation while photosynthate has not been transported to the twigs of sapling. The \(^{13}\)C fractionation of CO\textsubscript{2} from the air surrounding leaf to sub-stomatal cavity may be simply considered, whereas the fractionation induced by mesophyll conductance from sub-stomatal cavities to the site of carboxylation in the chloroplast cannot be neglected (Pons et al., 2009; Cano et al., 2014). As estimating the post-carboxylation fractionation, carbon isotope fractionation derived from \(g_m\) must be subtracted from the total \(^{13}\)C fractionation (the difference between \(\delta^{13}C_WSC\) and \(\delta^{13}C_{\text{model}}\)), which was closely associated with \(g_m\) (Fig. 8, \(p=0.01\) or \(p<0.01\)). Similar variations of \(^{13}\)C fractionations derived from \(g_m\) were presented with that of \(g_m\) under orthogonal treatments on Table 1.

### 4.4 Post-carboxylation fractionation generated before photosynthetic leaf

Photosynthesis, a biochemical and physiological process (Badeck et al., 2005), is characterized by discrimination against \(^{13}\)C, which leaves an isotopic signature in the photosynthetic apparatus. There is a classic review of the carbon-fractionation in leaves that covers the significant aspects of photosynthetic carbon isotope discrimination (Farquhar et al., 1989). The post-carboxylation/photosynthetic fractionation associated with the metabolic pathways of non-structural carbohydrates (NSC; defined here as soluble sugars + starch) within leaves, and fractionation during translocation, storage, and remobilization prior to tree ring formation remain unclear (Epron et al., 2012; Gessler et al., 2014; Rinne et al., 2016). The synthetic processes of sucrose and starch before transportation to the twig are within the domain of post-carboxylation fractionation generated in leaves. Hence, we hypothesized that the \(^{13}\)C fractionation might exist. When we finished the leaf gas-exchange measurements, the leaf samples were collected immediately to determine the \(\delta^{13}C\) of water-soluble compounds (\(\delta^{13}C_WSC\)). Presumably, the \(^{13}\)C fractionation generated in the synthetic processes of sucrose and starch was approximately contained within the \(^{13}\)C fractionation from the site of carboxylation to cytoplasm before sugar transportation as total \(^{13}\)C fractionation.

When comparing \(\delta^{13}C_WSC\) with \(\delta^{13}C_{\text{obs}}\), total \(^{13}\)C fractionation of \(P.\) orientalis ranged from 0.0328‰ to
0.0472‰, less than that of Q. variabilis (from 0.0384‰ to 0.0466‰). The post-carboxylation fractionation contributed 75.30%–98.9% on total 13C fractionation, which was determined by subtracting the fractionation of mesophyll conductance from total 13C fractionation. Recently, Gessler et al. (2004) reviewed the environmental drivers of variation in photosynthetic carbon isotope discrimination in terrestrial plants. Total 13C fractionation of P. orientalis was enhanced by soil moistening, consistent with that of Q. variabilis, except at 100% FC. The 13C isotope signature of P. orientalis was dampened by elevated [CO2]. Yet, 13C-depletion was weakened in Q. variabilis at C500 and C600. Linear regressions between gs and total 13C fractionation indicated that the post-carboxylation fractionation in leaves depended on the variation of gs and stomata aperture correlated with environmental change.

5 Conclusions

Through orthogonal treatments of four [CO2]s × five SWCs, WUEp calculated by δ13C of water-soluble compound and WUEg derived from simultaneous leaf gas exchange were estimated to differentiate the δ13C signal variation before leaf-exported translocation of primary assimilates. The influence of mesophyll conductance on the difference of 13C fractionation between the sub-stomatal cavities and the ambient environment need to be considered, while testing the hypothesis that the post-carboxylation will contribute on the 13C fractionation from the site of carboxylation to cytoplasm before sugars transportation. In response to the interactive effects of [CO2] and SWC, WUEg of two species both decreased with soil moistening, and increased with elevated [CO2] at 35%–80% of FC. We concluded that relative soil drying, coupled with elevated [CO2], could improve WUEg by strengthening photosynthetic capacity and reducing transpiration. WUEg of P. orientalis was significantly greater than that of Q. variabilis, while the opposite was the case for WUEg in two species. Mesophyll conductance and post-carboxylation were manifested both contributing on the 13C fractionation from the site of carboxylation to cytoplasm before sugars transportation determined by gas-exchange and carbon isotopic measurements. Rising [CO2] and/or soil moistening generated increasing disparities between δ13CWSC and δ13Ccmodel in P. orientalis; nevertheless, the differences between δ13CWSC and δ13Ccmodel in Q. variabilis increased as [CO2] being less than 600 ppm and/or water stress alleviated. Total 13C fractionation in leaf was linearly dependent on gs. With respect to carbon isotope fractionation in post-carboxylation and transportation processes, we cannot neglect that the 13C fractionation derived from the synthesis of sucrose and starch were influenced inevitably by environmental changes. Thus, clear description of the magnitude and environmental dependence of apparent post-carboxylation fractionation are worth our attention in photosynthetic fractionation.

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Parker, W. C. and Pallardy, S. G.: Gas exchange during a soil drying cycle in seedlings of four black


Author contribution

Na Zhao and Yabing He collected field samples, and performed the experiment. Na Zhao engaged in data analysis and writing this paper. Ping Meng proposed the suggestions on the theory and practice of experiment. Xinxiao Yu revised the paper and contributed to edit the manuscript.

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Figure 1. Structural diagram of the device for automatic drip irrigation

Arabic numerals indicate the individual parts of the automatic drip irrigation device (No. 1–7). The lower-left corner of this figure presents the detailed schematic for the drip irrigation components (No. 8–12). The lower-right corner of this figure shows the schematic for the drip irrigation component in profile.

1. water storage tank
2. holder
3. controller
4. soil moisture sensors
5. drip irrigation component
6. AC power supply
7. main water pipe
8. distributed water pipe
9. movable annular body
10. spindle of the annular body
11. drainage holes of drip irrigation
12. steel supporting ring
Figure 2. Net photosynthetic rates ($P_n$, µmol m$^{-2}$ s$^{-1}$, a and b), stomatal conductance ($g_s$, mol H$_2$O m$^{-2}$ s$^{-1}$, c and d), intercellular CO$_2$ concentration ($C_i$, µmol CO$_2$ mol$^{-1}$, e and f), and transpiration rates ($T_r$, mmol H$_2$O m$^{-2}$ s$^{-1}$, g and h) of *P. orientalis* and *Q. variabilis* for four CO$_2$ concentrations × five soil volumetric water contents. Means ± SDs, n = 32.
Figure 3. Carbon isotope composition of water-soluble compounds (δ\(^{13}\)C\(_{WSC}\)) extracted from leaves of *P. orientalis* (a) and *Q. variabilis* (b) for four CO\(_2\) concentrations × five soil volumetric water contents. Means ± SDs, n = 32.
**Figure 4.** Instantaneous water use efficiency through gas exchange measurements ($WUE_{ge}$) for leaves of *P. orientalis* (a) and *Q. variabilis* (b) for four CO$_2$ concentrations × five soil volumetric water contents. Means ± SDs, n = 32.
**Figure 5.** Instantaneous water use efficiency estimated by $\delta^{13}$C of water-soluble compounds (WUE$_{cp}$) from leaves of *P. orientalis* (a) and *Q. variabilis* (b) for four CO$_2$ concentrations × five soil volumetric water contents. Means ± SDs, $n = 32$. 

[WUE vs Volumetric water content graph]
Figure 6. Mesophyll conductance of *P. orientalis* (a) and *Q. variabilis* (b) for four CO₂ concentrations × five soil volumetric water contents. Means ± SDs, n = 32.
Figure 7. Regression between stomatal conductance and total $^{13}$C fractionation of *P. orientalis* (a) and *Q. variabilis* (b) for four CO$_2$ concentrations x five soil volumetric water contents ($p=0.01$, $n=32$).
Figure 8. Regression between mesophyll conductance and total $^{13}$C fractionation of *P. orientalis* (a) and *Q. variabilis* (b) for four CO$_2$ concentrations $\times$ five soil volumetric water contents ($p=0.01$, $n=32$).
Table 1. Carbon-13 isotope fractionation of *P. orientalis* and *Q. variabilis* for four CO₂ concentrations × five soil volumetric water contents.

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<tr>
<th>Species</th>
<th>SWC (of FC)</th>
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<th>CO₂ concentration (ppm)</th>
<th>13C fractionation (‰)</th>
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<td>400 500 600 800</td>
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<td>100%</td>
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