The interaction of CO_2 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 CO_2, 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 CO_2 and drought on plant photosynthetic discrimination. It is commonly surveyed that the ^{13}C fractionation derived from the CO_2 diffusion occurred from ambient air to stomatal sub-cavity, and little investigate the ^{13}C fractionation generated from the site of carboxylation to cytoplasm before sugars transportation outward the leaf, which may respond to the environmental conditions (i. e. CO_2 concentration and water stress) and their interactions. Therefore, saplings of species typical 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 CO_2 concentrations [CO_2] $\times$ five soil volumetric water contents (SWC)). The $\delta^{13}C$ of water-soluble compounds extracted from leaves of potted saplings was measured to determine the instantaneous water use efficiency (WUE_{cp}) after cultivation. Instantaneous water use efficiency derived from gas exchange (WUE_{ge}) was integrated to estimate differences in $\delta^{13}C$ signal variation before leaf-exported translocation of primary assimilates. The WUE_{ge} of the two saplings both decreased with increased soil moisture, and increased with elevated [CO_2] at 35%−80% of Field Capacity (FC) by strengthening photosynthetic capacity and reducing transpiration. Differences in instantaneous water use efficiency (iWUE) according to distinct environmental changes differed between the species. The WUE_{ge} of *P. orientalis* was significantly greater than that of *Q. variabilis*, while the opposite results were obtained in a comparison of the WUE_{cp} of the two species. Total ^{13}C fractionation from the site of carboxylation to cytoplasm before sugars transportation (total ^{13}C fractionation) was clearly species-specific, as demonstrated in the interaction of [CO_2] and SWC. Rising [CO_2] coupled with moistened soil generated increasing disparities of $\delta^{13}C$ between the water soluble compounds ($\delta^{13}C_{WSC}$) and estimated by gas-exchange observation ($\delta^{13}C_{obs}$) in *P. orientalis* with an amplitude of 0.0328‰−0.0472‰. Further, the differences between $\delta^{13}C_{WSC}$ and $\delta^{13}C_{obs}$ of *Q. variabilis* increased as CO_2 concentration increased and water stress alleviated (0.0384‰−0.0466‰). Fractionations from mesophyll conductance and post-photosynthesis both contributed to the total ^{13}C fractionation determined by two measurements (1.06%-24.94% and 75.30%-98.9% of total ^{13}C fractionation, respectively). Total ^{13}C fractionations were linearly dependent on g_s, indicating post-carboxylation fractionation was attributed to environmental variation. Thus, cautious descriptions of the magnitude and environmental dependence of apparent post-carboxylation fractionation are worth our attention in photosynthetic fractionation.

Key words: Post-carboxylation fractionation; Carbon isotope fractionation; Elevated CO_2

concentration; Soil volumetric water content; Instantaneous water use efficiency

1 Introduction

Since the onset of the industrial revolution, the 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 also 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 $\delta^{13}C$ 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 physiology and metabolic processes (Gessler et al., 2014; Streit et al., 2013). While the depletion of $\delta^{13}C_{CO_2}$ has been shown in the atmosphere, variations in CO_2 concentration itself also might affect the $\delta^{13}C$ of plant organs that, in turn, respond physiologically to climatic change (Gessler et al., 2014). The carbon discrimination ($^{13}\Delta$) 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_i/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 $\delta^{13}C$ of water-soluble organic matter ($\delta^{13}C_{WSOM}$) of the different plant organs. Meanwhile, several processes during photosynthesis alter the $\delta^{13}C$ of carbon transported within plants considerably. Carbon-fractionation during photosynthetic CO_2 fixation has been described and reviewed well 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, the remobilization or storage of soluble carbohydrates, and starch metabolism fractionations 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 be also considered is the CO_2 concentration in the chloroplast (C_c), not in the intercellular space, as used in the simplified equation of the Farquar's model (Evans et al., 1986; Farquhar et al., 1989) is actually defined as carbon isotope discrimination ($\delta^{13}C$). Indeed, the difference between gas-exchange derived values and online measurements of $\delta^{13}C$ has been widely used to estimate C_i-C_c 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

The simultaneous isotopic analysis of leaves is a recent refinement in isotope studies that allows us to determine the temporal variation in isotopic fractionation (Rinne et al., 2016), and will 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 also can be associated with an assimilation-weighted average of C_i/C_a (and C_c/C_a) photosynthesized over a period ranging from a few hours to 1-2 d (Pons et al., 2009). However, there is 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 the iWUE derived from theses isotopic fractionations responds to different environmental factors, such as elevated [CO_2] and/or soil water gradients, have not yet been observed.

Consequently, we investigated the $\delta^{13}C$ of the fast-turnover carbohydrate pool in leaves from saplings of two trees typical in 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 ^{13}C fractionation from the site of carboxylation to cytoplasm before sugars transportation (total ^{13}C fractionation) of *P. orientalis* and *Q. variabilis*, which were determined from the $\delta^{13}C$ 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 [CO_2] 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 the Chinese Forest Ecosystem Research Network (CFERN, 40°03'45"N, 116°5'45"E) in Beijing, China. This region is populated by warm, temperate, deciduous, broad-leaved trees and mixed tree communities dominated by *Quercus variabilis* Bl. and *Platycladus orientalis* (L.) Franco, respectively. Saplings have similar ground diameters, heights, and growth statuses. The saplings were placed in pots 22 cm in diameter and 22 cm in height. Undisturbed soil samples were collected from the field in the research region, and the sieved soil (with all particles <10 mm removed) was placed in the pots. A single *P. orientalis* sapling was transplanted into each pot. The soil bulk density in the pots was maintained at 1.337–1.447 g cm^{-3}. After one month of rejuvenation, the potted saplings were placed into chambers for cultivation.

The controlled experimental treatments were conducted in growth chambers (FH-230, Taiwan Hipoint Corporation, Kaohsiung City, Taiwan). To imitate the meteorological factors of the growth seasons in the research region, the daytime temperature in the chambers was set to $25 \pm 0.5°C$ from 07:00 to 17:00, and the night-time temperature was $18 \pm 0.5°C$ from 17:00 to 07:00. Relative humidity

was maintained at 60% and 80% during the day 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}. CO_2 concentration was controlled by the central controlling system of the chambers (FH-230). Two growth chambers (A and B) were used in our study. Chamber A was switched in turn to maintain the CO_2 concentration of 400 ppm (during June 2−9, June 12−19, June 21−28, and July 2−9, 2015, C_{400}) and 500 ppm (during July 11−18, July 22−29, August 4−11, and August 15−22, 2015, C_{500}). The other was adjusted to maintain the CO_2 concentration at 600 ppm (during June 2−9, June 12−19, June 21−28, and July 2−9, 2015, C_{600}) and 800 ppm (during July 11−18, July 22−29, August 4−11, and August 15−22, 2015, C_{800}). The target concentrations of CO_2 in the chambers were permitted the standard deviation of ± 50 ppm during cultivation. Thus, the gradient of four CO_2 concentrations in our study (400 ± 50 ppm, 500 ± 50 ppm, 600 ± 50 ppm, and 800 ± 50 ppm) was formed. Detectors inside the chambers monitored and maintained the target concentrations of CO_2.

We designed a device to water the potted plants automatically and avoid heterogeneity caused by interruptions in the watering process (Fig. 1). It consisted of the 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, specific soil water could be set. The soil volumetric water content (SWC) of the pot soil was monitored by the soil moisture sensors. Through the sensors, the irrigation device could determine whether to water or stop watering the plants. Two drip irrigation devices were installed in both chambers, respectively. Since the average Field Capacity (FC) of the pot soil was 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%). Each level of soil water was kept within the specific range thereafter by the irrigation device.

The Orthogonal tests were formed as: elevated CO_2 concentration gradient presented as 400 ppm, 500 ppm, 600 ppm, and 800 ppm, combined with a soil-water gradient 35%−45% of FC, 50%−60% of FC, 60%−70% of FC, and 70%−80% of FC and 100% FC (CK). Undergoing the equilibrium circumstances of elevated CO_2 across the soil water gradients, the saplings were ready for investigation. Each orthogonal treatment continued for 7 days. Pots were rearranged periodically to minimize non-uniform illumination.

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 in the chambers. Two saplings per specie were replicated per treatment ([CO_2] × water stress). 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 (P_n) and transpiration rate (T_r), were measured. Based on the theories proposed by Von Caemmerer and Farquhar (1981), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were calculated by the Li-Cor software. Instantaneous water use efficiency via gas exchange (WUE_{ge}) was calculated as the ratio of P_n to E.

2.3 Plant material collection and leaf water soluble compounds extraction

Recently-expanded, eight sun leaves per sapling were selected and frozen immediately in liquid nitrogen since the gas-exchange measurements accomplished. Two saplings per specie were chosen for

each treatment. A protocol adapted from Gessler et al. (2004) was used to extract the water-soluble compounds (WSCs). All samples were ground to fine powders using mortars and liquid nitrogen. 50 mg of ground leaves and 100 mg PVPP (polyvinylpolypyrrolidone) were weighed, mixed evenly, and incubated in 1 mL double demineralized water for 60 min at 5℃ in a centrifuge tube. Then, the tubes were heated in 100℃ water for 3 min. After they cooled to room temperature, the supernatant was centrifuged at 12000 xg for 5 min and transferred 10 μL supernatant into tin capsule to be dried at 70℃. Folded capsules were then ready for $\delta^{13}C$ analysis of WSOM.

The samples of WSCs from leaves were combusted in an elemental analyzer (EuroEA, HEKAtech GmbH, Wegberg, Germany) and analyzed in the mass spectrometer (DELTA^{plus}XP, ThernoFinnigan). Carbon isotope signatures are expressed in δ-notation in parts per thousand, relative to the international Pee Dee Belemnite (PDB):

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \tag{1}$$

where $\delta^{13}C$ is the heavy isotope and R_{sample} and $R_{standard}$ 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 ^{13}C 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 factor, Δ, was calculated as:

$$\Delta = \left(^{13}C_a - {}^{13}C_P\right)/\left(1 + {}^{13}C_P\right) \tag{2}$$

where $^{13}C_a$ is the isotope signature of ambient [CO_2] in the chamber; $^{13}C_P$ is the $^{13}C : {}^{12}C$ of the water-soluble compounds extracted from foliage. The $C_i : C_a$ is determined by:

$$C_i : C_a = (\Delta - a)/(b - a) \tag{3}$$

where C_i is the intercellular CO_2 concentration, and C_a is the ambient CO_2 concentration in the chamber; a is the discrimination dependent on a fraction factor (4‰). b is the discrimination during CO_2 fixation by ribulose 1,5- bisphosphate carboxylase/oxygenase (Rubisco) and internal diffusion (30‰). Instantaneous water use efficiency by gas-exchange measurements (WUE$_{ge}$) is calculated as:

$$WUE_{ge} = P_n : T_r = (C_a - C_i)/1.6\Delta e \tag{4}$$

where P_n is the net carbon assimilation, T_r is the molar rate of transpiration, and 1.6 is the diffusion ratio of stomatal conductance to water vapor to CO_2 in the chamber. Δe is the difference in water vapor pressure between the intracellular in leaves and ambient air, which may be calculated as:

$$\Delta e = e_{lf} - e_{atm} = 0.611 \times e^{17.502T/(240.97+T)} \times (1 - RH) \tag{5}$$

where e_{lf} and e_{atm} represent the extra- and intra-cellular water vapor pressure, respectively. T and RH is temperature and relative humidity on leaf surface. The instantaneous water use efficiency could be determined by the $\delta^{13}C_{WSC}$ of leaves of two species, defined as WUE$_{cp}$:

$$WUE_{cp} = \frac{P_n}{T_r} = (1 - \varphi)(C_a - C_i)/1.6\Delta e = C_a(1 - \varphi)\left[\frac{b - \delta^{13}C_a + (b+1)\delta^{13}C_{WSC}}{(b-a)(1+\delta^{13}C_{WSC})}\right]/1.6\Delta e \tag{6}$$

φ is the ratio between carbohydrates consumed during respiration of the leaves and that of other organs at night (0.3). $\delta^{13}C_{WSC}$ is the carbon isotopic composition of water soluble compounds extracted from leaves.

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 δ^{13}C of water soluble compounds from leaves ($\delta^{13}C_{WSC}$) and the modeled δ^{13}C calculated from gas-exchange ($\delta^{13}C_{model}$). The $\delta^{13}C_{model}$ can be calculated from Δ_{model} from Eqn. (2). The Δ_{model} can be determined by Eqns. (3 and 4) as:

$$\Delta_{model} = (b - a)\left(1 - \frac{1.6\Delta eWUE_{ge}}{C_a}\right) + a \tag{7}$$

$$\delta^{13}C_{model} = \frac{C_a - \Delta_{model}}{1 + \Delta_{model}} \tag{8}$$

Total ^{13}C fractionation $= \delta^{13}C_{WSC} - \delta^{13}C_{model}$ (9)

2.4.2 Methodology of calculating mesophyll conductance

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_c) to that in the ambient environment surrounding plants (C_a). The carbon isotopic discrimination (Δ) could be presented as (Farquhar et al. 1982):

$$\Delta = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + (e_s + a_l)\frac{C_i - C_c}{C_a} + b\frac{C_c}{C_a} - \frac{\frac{eR_D}{k} + f\Gamma_*}{C_a} \tag{10}$$

where $C_a, C_s, C_i,$ 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_b is the fractionation for the CO_2 diffusion at the boundary layer (2.9‰); a is the fractionation occurring CO_2 diffusion in still air (4‰); e_s is the discrimination of CO_2 diffusion when CO_2 enters in solution (1.1‰, at 25 ℃); a_l is the fractionation derived from diffusion in the liquid phase (0.7‰); b is the carboxylation discrimination in C3 plants (27‰); e and f are carbon discrimination derived in dark respiration (R_D) and photorespiration, respectively. k is the carboxylation efficiency, and Γ^* 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 7 could be shown:

$$\Delta = a\frac{C_a - C_i}{C_a} + (e_s + a_l)\frac{C_i - C_c}{C_a} + b\frac{C_c}{C_a} - \frac{\frac{eR_D}{k} + f\Gamma_*}{C_a} \tag{11}$$

There is 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 will influence the calculation for g_m, has 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 also negligible, the Equation 8 could be simplified as:

$$\Delta_i = a + (b - a)\frac{C_i}{C_a} \tag{12}$$

Equation 12 presents the linear relationship between carbon discrimination and C_i/C_a that used normally in carbon isotopic fractionation. That underlined the subsequent comparison between the expected Δ (originated from gas-exchange, Δ_i) and those actually measured (Δ_{obs}), which could evaluate the magnitude of 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

6

245 performing the Δ_{obs} by isotope ratio mass spectrometry and expected Δ_i from C_i/C_a by gas exchange

246 measurements.

247 Then subtract Δ_{obs} of Equation 11 from Δ_i calculated by Equation 12:

248 $$\Delta_i - \Delta_{obs} = (b - e_s - a_l)\frac{C_i - C_c}{C_a} + \frac{\frac{eR_D}{k} + f\Gamma^*}{C_a} \tag{13}$$

249 and the net assimilation rate (A_n) from the first Fick's law is presented by:

250 $$A_n = g_m(C_i - C_c) \tag{14}$$

251 Substitute Equation 14 into Equation 13 we obtain:

252 $$\Delta_i - \Delta_{obs} = (b - e_s - a_l)\frac{A_n}{g_m C_a} + \frac{\frac{eR_D}{k} + f\Gamma^*}{C_a} \tag{15}$$

253 $$g_m = \frac{(b - e_s - a_l)\frac{A_n}{C_a}}{(\Delta_i - \Delta_{obs}) - \frac{eR_D/k + f\Gamma^*}{C_a}} \tag{16}$$

254 In calculation of g_m, the respiratory and photorespiratory terms could be ignored or be given the

255 specific constant values. Here, e and f are assumed to be zero or be cancelled out in the calculation of

256 g_m.

257 Then Equation 16 can be transformed into:

258 $$g_m = \frac{(b - e_s - a_l)\frac{A_n}{C_a}}{\Delta_i - \Delta_{obs}} \tag{17}$$

259 **3 Results**

260 **3.1 Foliar gas exchange measurements**

261 $P.$ $orientalis$ and $Q.$ $variabilis$ saplings were exposed to the orthogonal treatments. When SWC

262 increased, P_n, g_s and T_r in $P.$ $orientalis$ and $Q.$ $variabilis$ peaked at 70%−80% of FC or/and FC (Fig. 2).

263 The C_i in $P.$ $orientalis$ rose as SWC increased, while it peaked at 60%−70% of FC and declined

264 thereafter with increased SWC in $Q.$ $variabilis$. The capacity of carbon uptake and C_i were elevated

265 significantly by elevated [CO_2] at any given SWC for two species ($p<0.05$). Further, greater increasing

266 magnitudes of P_n in $P.$ $orientalis$ were found at 50%−70% of FC from C_{400} to C_{800}, which was at 35%−

267 45% of FC in $Q.$ $variabilis$. As the water stress was alleviated (at 70%−80% of FC and FC), the

268 reduction of g_s in $P.$ $orientalis$ was more pronounced with elevated [CO_2] at a given SWC ($p<0.01$).

269 Nevertheless, g_s of $Q.$ $variabilis$ in C_{400}, $C_{500,}$ and C_{600} was significantly higher than that in C_{800} at

270 50%−80% of FC ($p<0.01$). Coordinated with g_s, T_r of two species in C_{400} and C_{500} was significantly

271 higher than that in C_{600} and C_{800} except for 35%−60% of FC ($p<0.01$, Figs. 2g and 2h). Larger P_n, g_s, C_i

272 and T_r of $Q.$ $variabilis$ was significantly presented than that of $P.$ $orientalis$ ($p<0.01$, Fig. 2).

273 **3.2 δ^{13}C of water-soluble compounds in leaves**

274 To observe the photosynthetic traits of the two saplings, the same leaf was frozen immediately and

275 the water-soluble compounds (WSCs) were extracted for all orthogonal treatments. $\delta^{13}C_{WSC}$ (δ^{13}C of

276 water-soluble compounds from leaves) of two species both increased as soil moisture improved (Figs.

277 3a and 3b, $p<0.01$). The average ($\pm$ SD) $\delta^{13}C_{WSC}$ of $P.$ $orientalis$ and $Q.$ $variabilis$ ranged from -27.44 $\pm$

278 0.155‰ to -26.71 $\pm$ 0.133‰, and from -27.96 $\pm$ 0.129‰ to -26.49 $\pm$ 0.236‰, respectively. Similarly

279 with the photosynthetic capacity varying with increased SWC, average $\delta^{13}C_{WSC}$ of two saplings reached

280 their maximums at 70%−80% of FC. Together with the gradual enrichment of [CO_2], average $\delta^{13}C_{WSC}$

281 in two species declined while [CO_2] exceeded 600 ppm ($p<0.01$). Except for C_{400} at 50%−100% of FC,

282 $\delta^{13}C_{WSC}$ of $P.$ $orientalis$ was significantly larger than that of $Q.$ $variabilis$ in any [CO_2] $\times$ SWC

283 treatment ($p<0.01$, Fig. 3).

3.3 Estimations of WUE_{ge} and WUE_{cp}

285 Instantaneous water use efficiency via gas exchange (WUE_{ge}) is calculated as P_n divided by T_r.
286 Figure 4a shows that incremental magnitudes of WUE_{ge} in *P. orientalis* under severe drought (i.e.,
287 35%−45% of FC) were highest at any given $[CO_2]$, ranging from 90.70% to 564.65%. WUE_{ge} in *P.*
288 *orientalis* reduced as SWC increased, while they increased remarkably as $[CO_2]$ elevated. Compared to
289 *P. orientalis*, trends of WUE_{ge} in *Q. variabilis* were promoted slightly at FC in C_{600} or C_{800} as soil
290 moistened (Fig. 4b). The maximum of WUE_{ge} thus occurred at 35%−45% of FC in C_{800} among all
291 orthogonal treatments for *P. orientalis*, as well as that for *Q. variabilis*. Further, elevated $[CO_2]$
292 enhanced the WUE_{ge} of *Q. variabilis* clearly at any SWC except that at 60%−80% of FC. Most saplings
293 of *P. orientalis* had greater WUE_{ge} than did *Q. variabilis* between the same $[CO_2] \times$ SWC treatments
294 ($p<0.05$).

295 The instantaneous water use efficiency could be determined from Eqn. (6) by the $\delta^{13}C_{WSC}$ of leaves
296 of two species, defined as WUE_{cp}. As illustrated in Fig. 5a, WUE_{cp} of *P. orientalis* in C_{600} or C_{800}
297 climbed up as water stress alleviated beyond 50%−60% of FC, while the water threshold was 60%−70%
298 of FC in C_{400} or C_{500}. *Q. variabilis* exhibited no uniform trend of WUE_{cp} with soil wetting (Fig. 5b).
299 Except for C_{400}, WUE_{cp} of *Q. variabilis* decreased abruptly at 50%−60% of FC, and then rose as soil
300 moisture improved in C_{500}, C_{600}, and C_{800}. In contrast to the findings about WUE_{ge} in two species,
301 WUE_{cp} of *Q. variabilis* was more pronounced than that of *P. orientalis* among all orthogonal
302 treatments.

3.4 ^{13}C fractionation from the site of carboxylation to cytoplasm before sugars transportation

304 We evaluated the total ^{13}C fractionation from the site of carboxylation to cytoplasm by gas exchange
305 and $\delta^{13}C$ of water-soluble compounds from leaf measurements (Table 1), which can retrace ^{13}C
306 fractionation before carboxylation transport to the twig. Comparing $\delta^{13}C_{WSC}$ with $\delta^{13}C_{model}$ from
307 Eqns. (4, 7 and 8), total ^{13}C fractionation of *P. orientalis* ranged from 0.0328‰ to 0.0472‰, which
308 was smaller than that of *Q. variabilis* (0.0384‰ to 0.0466‰). The total fractionations of *P. orientalis*
309 were magnified with soil wetting especially that was increased by 21.30%−42.04% at 35%−80% of FC
310 from C_{400} to C_{800}. Fractionation coefficients under C_{400} and C_{500} were amplified as SWC increased until
311 50%−60% of FC in *Q. variabilis*, whereas it was increased at 50%−80% of FC and decreased at FC
312 under C_{600} and C_{800}. Elevated $[CO_2]$ enhanced the average fractionation effect of *P. orientalis*, while
313 those of *Q. variabilis* declined sharply from C_{600} to C_{800}. Total ^{13}C fractionation in *P. orientalis*
314 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

316 According to comparison between online leaf $\delta^{13}C_{WSC}$ and the values of gas exchange measurements,
317 g_m over all treatments was presented in Fig. 6 (Eqns. 10-17). Significant increment trend of g_m was
318 observed with water stress alleviated in *P. orientalis*, ranging from 0.0091−0.0690 mol CO_2 m^{-2} s^{-1}
319 ($p<0.05$), which reached the maximums at FC under a given $[CO_2]$. Yet increases in g_m of *Q. variabilis*
320 with increasing SWC become unremarkable except that under C_{400}. With CO_2 concentration elevated,
321 g_m of two species was increased in different degrees. Comparing with *P. orientalis* under C_{400}, g_m was
322 increased gradiently and reached its maximum under C_{800} at 35%−60% of FC and FC ($p<0.05$),
323 however, that was maximized under C_{600} ($p<0.05$) and slipped down under C_{800} at 60%−80% of FC.
324 The maximum increment magnitude of g_m (8.2%−58.4%) occurred at C_{800} at any given SWC in *Q.*
325 *variabilis*. It is evidently shown that g_m of *Q. variabilis* was larger than that of *P. orientalis* in the same

326 treatment.

3.6 The contribution of post-carboxylation fractionation

328   Here, the difference between Δ_i and Δ_{obs} presented the ^{13}C fractionation derived from mesophyll
329 conductance. So the post-photosynthetic fractionation after carboxylation can be calculated by
330 subtracting the fractionation derived from mesophyll conductance from the total ^{13}C fractionation that
331 is generated from the site of carboxylation to cytoplasm before sugars transportation (Table 1). The
332 fractionation from g_m had less contribution to total ^{13}C fractionation than that from synthesis of sugars
333 belong to post-carboxylation fractionation in any given treatment (Table 1). The contributions of
334 fractionation from g_m with two species were illustrated different variations with soil water increasing,
335 which declined at 50%−80% of FC and rose up at FC in *P. orientalis*, yet it was shown increasing with
336 water stress alleviated at 50%−80% of FC and then decreased at FC in *Q. variabilis*. Nevertheless, the
337 fractionations from synthesis of sugars in leaf and these contributions to total fractionation were all
338 increased as soil moistened with two species. Considering the effects of enriched [CO_2] on
339 fractionations of mesophyll conductance, the fractionation from mesophyll conductance reached its
340 average peak under C_{600} in *P. orientalis*, which occurred under C_{800} with *Q. variabilis*.
341 Post-photosynthetic fractionations were increased along with [CO_2] elevated in *P. orientalis*, which
342 reached those maximums under C_{600} and then slipped down differing in degrees under C_{800}.

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

344   Stoma is the conduit between the plant and atmosphere. Total ^{13}C fractionation after carboxylation
345 may be correlated with resistances derived from stomata and mesophyll cells. Here, we performed
346 linear regressions between g_s/g_m and total ^{13}C fractionation coefficient for *P. orientalis* and *Q. variabilis*,
347 respectively (Fig. 7 and 8). It was apparent that total ^{13}C fractionation coefficient was linearly
348 dependent on the g_s ($p<0.05$), which controls the exchange of CO_2 and H_2O, and responds to
349 environmental variation. Subsequently, it was shown the linear relationships between g_m and the total
350 ^{13}C fractionation coefficient, reflecting variation of CO_2 concentration through the chloroplast was
351 correlated with the carbon discrimination happened after photosynthesis in the leaf.

4 Discussion

4.1 Photosynthetic traits

354   The exchange of CO_2 and water vapor via stomata is modulated in part by the soil or leaf water
355 potential (Robredo et al., 2010). Saplings of *P. orientalis* reached their maximums of P_n and g_s at 70%−
356 80% of FC under any [CO_2]. As SWC exceeded the water threshold, elevated CO_2 caused a greater
357 reduction in g_s, as has been reported for barley and wheat (Wall et al., 2011). Further, Maximal g_s of *Q.*
358 *variabilis* in C_{400}, C_{500}, C_{600}, and C_{800} were generated successively as soil moisture increased, indicating
359 that relative drought can stimulate the stomata which are more sensitive to environmental changes. In
360 addition, C_i of *Q. variabilis* peaked at 60%−70% of FC and followed declines as soil moisture
361 increased (Wall et al., 2006; Wall et al., 2011). This is interpreted as stomata having the tendency to
362 maintain a constant C_i or C_i/C_a when ambient [CO_2] increases, which would determine the CO_2 used
363 directly in chloroplast (Yu et al., 2010). On the basis of theories (Farquhar and Sharkey, 1982) and
364 common experimental technologies (Xu, 1997), this could be explained as the stomatal limitation.
365 However, C_i of *P. orientalis* was increased considerably while SWC exceeded 70%−80% of FC, as
366 found by Mielke et al. (2000). One factor that can account for that is plants close their stomata to

reduce intensive 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, further 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 the soil water irrigation maximum 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 of herbaceous plants.

The P_n of the two species increased with elevated [CO_2] in our study, similarly with the results from C_3 woody plants (Kgope et al., 2010). Further, increasing [CO_2] alleviated severe drought and heavy irrigation, which verifies 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 meliorate 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_{cp}

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 the reduction of T_r (Figs. 2a, 2g, 2b and 2h), also were demonstrated by Ainsworth and McGrath (2010). Combining P_n and T_r 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 is that of conifers. Moreover, there has been no consensus on the patterns of iWUE with related soil water states 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 water drying, as presented by Parker and Pallardy (1991), DeLucia and Heckathorn (1989), and Reich et al. (1989). Leakey (2009) also concluded that the WUE of plants in drought could be increased substantially, which was shown more clearly with elevated [CO_2] in this study.

Bögelein et al. (2012) confirmed that WUE_{cp} was more consistent with daily mean WUE_{ge} than was WUE_{phloem}. The WUE_{cp} of two species demonstrated similar variation to those of $\delta^{13}C_{WSC}$, which differentiated with that of WUE_{ge}. Pons et al. (2009) reviewed that Δ in the leaf soluble sugar is coupled tightly with dynamics in the environment integrated over a period ranging from a few hours to 1–2 d. WUE_{cp} of our materials responded synthetically with SWC × [CO_2] gradients over cultivated days whereas WUE_{ge} is characterized by the instantaneous state of plants to conditions. In addition, species-specific $\delta^{13}C_{WSC}$ were observed in the same condition. Consequently, WUE_{cp} and WUE_{ge} have different variable curves according to treatments.

4.3 The influence of mesophyll conductance on the fractionation after carboxylation

The consensus has been reached that the routine of CO_2 diffusion into photosynthetic site in plant includes two main procedures, which are CO_2 moving from ambient environment surrounding the leaf (C_a) to the sub-stomatic cavities (C_i) through stomata, and from there to the site of carboxylation within

10

the chloroplast stroma (C_c) of leaf mesophyll. The latter diffusion is defined as mesophyll conductance (g_m) (Flexas et al., 2008). Moreover, g_m 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). During our 7-day cultivations of water stress $\times$ [CO_2], g_m increased and WUE_{ge} was decreased as soil moistened, which has been verified that g_m as the important factor could improve WUE under drought pretreatment (Han et al., 2016). There has been a dispute how g_m responds to fluctuation of CO_2 concentration. Terashima *et al.* (2006) have confirmed that CO_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, different species has specific-special g_m responding to the gradient of [CO_2]. g_m of *P. orientalis* were significantly reduced by 9.08%-44.42% as [CO_2] rising from 600 to 800 ppm 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 the ^{13}C fractionation following the carboxylation while photosynthate has not been transported to the twigs of plant. The ^{13}C fractionation of CO_2 from air surrounding leaf to sub-stomatal cavity may be simply considered, 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 ^{13}C fractionation from the site of carboxylation to cytoplasm before sugars transportation (the difference between $\delta^{13}C_{WSC}$ and $\delta^{13}C_{model}$), which was closely associated with g_m (Fig 8, $p<0.05$). Similar variations of fractionation derived from g_m were presented with that of g_m under orthogonal tests on Table 1.

4.4 Post-carboxylation fractionation generated before photosynthate leaving leaves

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 already a classic review of the carbon-fractionation in leaves (Farquhar et al., 1989) that covers the significant aspects of photosynthetic carbon isotope discrimination. The post-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 ^{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 sugars transportation as total ^{13}C fractionation. When comparing $\delta^{13}C_{WSC}$ with $\delta^{13}C_{obs}$, 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 ^{13}C fractionation subtracted by fractionation derived from mesophyll conductance, post-photosynthetic fractionation occupied 75.30%-98.9% of total ^{13}C fractionation. Recently, Gessler et al. (2004) reviewed the environmental drivers of variation in photosynthetic carbon isotope discrimination in terrestrial plants. The ^{13}C fractionation of *P. orientalis* were enhanced by soil moistening, consistent with that of *Q. variabilis*, except at FC. The ^{13}C isotope signature of *P. orientalis* was dampened by elevated [CO_2]. Yet, ^{13}C-depletion was weakened in *Q. variabilis* in C_{600}

and C_{800}. Linear regression between g_s and total ^{13}C fractionation coefficient indicated that the post-carboxylation fractionation in leaves depended on the variation of g_s and stomata aperture correlated with environmental change.

5 Conclusions

Through orthogonal treatments of four $[CO_2]$ × five SWC, WUE_{cp} calculated by $\delta^{13}C$ of water-soluble compound and WUE_{ge} derived from simultaneous leaf gas exchange for leaves were estimated to differentiate the $\delta^{13}C$ signal variation before leaf-exported translocation of primary assimilates. The influence of mesophyll conductance on the difference of ^{13}C 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 ^{13}C fractionation from the site of carboxylation to cytoplasm before sugars transportation. In response to the interactive effects of $[CO_2]$ and SWC, WUE_{ge} of the two species of saplings both decreased with soil moistening, and increased with elevated $[CO_2]$ at 35%−80% of FC. We concluded that relative soil drying, coupled with elevated $[CO_2]$, could improve WUE_{ge} by strengthening photosynthetic capacity and reducing transpiration. WUE_{ge} of *P. orientalis* was significantly greater than was that of *Q. variabilis*, while the opposite was the case for WUE_{cp} in two species. Mesophyll conductance and post-photosynthesis were manifested both contributing to the ^{13}C fractionation from the site of carboxylation to cytoplasm before sugars transportation determined by gas exchange and carbon isotopic measurements. Rising $[CO_2]$ and/or soil moistening generated increasing disparities between $\delta^{13}C_{WSC}$ and $\delta^{13}C_{model}$ in *P. orientalis*; nevertheless, the differences between $\delta^{13}C_{WSC}$ and $\delta^{13}C_{model}$ in *Q. variabilis* increased as $[CO_2]$ being less than 600 ppm and/or water stress was alleviated. Total ^{13}C fractionation in leaf was linearly dependent on g_s. With respect to carbon fractionation in post-carboxylation and transportation processes, we cannot neglect that the instantaneous water use efficiency derived from the synthesis of sucrose and starch were influenced inevitably by environmental changes. Thus, cautious descriptions of the magnitude and environmental dependence of apparent post-carboxylation fractionation are worth our attention in photosynthetic fractionation.

References

Adiredjo, A. L., Navaud, O., Lamaze, T., and Grieu, P.: Leaf carbon isotope discrimination as an accurate indicator of water use efficiency in sunflower genotypes subjected to five stable soil water contents, J Agron. Crop Sci., 200, 416–424, 2014.

Ainsworth, E. A. and McGrath, J. M.: Direct effects of rising atmospheric carbon dioxide and ozone on crop yields, Climate Change and Food Security, Springer, 109–130, 2010.

Badeck, F. W., Tcherkez, G., Eacute, N. S. S., Piel, C. E. M., and Ghashghaie, J.: Post-photosynthetic fractionation of stable carbon isotopes between plant organ – a widespread phenomenon, Rapid Commun. Mass S., 19, 1381–1391, 2005.

Bögelein, R., Hassdenteufel, M., Thomas, F. M., and Werner, W.: Comparison of leaf gas exchange and stable isotope signature of water-soluble compounds along canopy gradients of co-occurring Douglas-fir and European beech, Plant Cell Environ., 35, 1245–1257, 2012.

Brandes, E., Kodama, N., Whittaker, K., Weston, C., Rennenberg, H., Keitel, C., Adams, M. A., and Gessler, A.: Short-term variation in the isotopic composition of organic matter allocated from the leaves to the stem of *Pinus sylvestris*: effects of photosynthetic and postphotosynthetic carbon

isotope fractionation, Global Change Biol., 12, 1922–1939, 2006.

Brooks, A. and Farquhar, G. D.: Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light, Planta, 165, 397–406, 1985.

Brugnoli E, Farquhar GD. 2000. Photosynthetic fractionation of carbon isotopes. In: Leegood RC, Sharkey TD, von Caemmerer S. eds. Photosynthesis: physiology and metabolism. Advances in photosynthesis. Dordrecht, The Netherlands: Kluwer Academic Publishers, 399–434.

Cano, F. J., López, R., and Warren, C. R.: Implications of the mesophyll conductance to CO_2 for photosynthesis and water-use efficiency during long-term water stress and recovery in two contrasting Eucalyptus species, Plant Cell Environ., 37, 2470–2490, 2014.

Cernusak, L. A., Ubierna, N., Winter, K., Holtum, J. A. M., Marshall, J. D., and Farquhar, G. D.: Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants, New Phytologist, 200, 950–965, 2013.

DeLucia, E. H. and Heckathorn, S. A.: The effect of soil drought on water-use efficiency in a contrasting Great Basin desert and Sierran montane species, Plant Cell Environ., 12, 935–940, 1989.

Epron, D., Nouvellon, Y., and Ryan, M. G.: Introduction to the invited issue on carbon allocation of trees and forests, Tree physiol., 32, 639–643, 2012.

Evans, J. R., Kaldenhoff, R., Genty, B., and Terashima, I.: Resistances along the CO_2 diffusion pathway inside leaves, J. Exp. Bot., 60, 2235–2248, 2009.

Evans, J. R., Sharkey, T. D., Berry, J. A., and Farquhar, G. D.: Carbon isotope discrimination measured concurrently with gas-exchange to investigate CO_2 diffusion in leaves of higher-plants, Funct. Plant Biol., 13, 281–292, 1986.

Evans, J. R. and von Caemmerer, S.: Temperature response of carbon isotope discrimination and mesophyll conductance in tobacco, Plant Cell Environ., 36, 745–756, 2013.

Farquhar, G. D., Ehleringer, J. R., and Hubick, K. T.: Carbon isotope discrimination and photosynthesis, Ann. Rev. Plant Physiol., 40, 503–537, 1989.

Farquhar, G. D., O'Leary, M. H., and Berry, J. A.: On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves, Funct. Plant Biol., 9, 121–137, 1982.

Farquhar, G. D. and Sharkey, T. D.: Stomatal conductance and photosynthesis, Ann. Rev. Plant Physiol., 33, 317–345, 1982.

Flexas, J., Barbour, M. M., Brendel, O., Cabrera, H. M., Carriquí M., Díaz-Espejo, A., Douthe, C., Dreyer, E., Ferrio, J. P., Gago, J., Gallé, A., Galmés, J., Kodama, N., Medrano, H., Niinemets, Ü., Peguero-Pina, J. J., Pou, A., Ribas-Carbó, M., Tomás, M., Tosens, T., and Warren, C. R.: Mesophyll diffusion conductance to CO_2: An unappreciated central player in photosynthesis, Plant Science, 193–194, 70–84, 2012.

Flexas, J., Carriquí M., Coopman, R. E., Gago, J., Galmés, J., Martorell, S., Morales, F., and Diaz-Espejo, A.: Stomatal and mesophyll conductances to CO_2 in different plant groups: Underrated factors for predicting leaf photosynthesis responses to climate change? Plant Science, 226, 41–48, 2014.

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 CO_2 concentration around leaves, Plant Cell Environ., 30, 1284–1298, 2007.

Flexas, J., Ribas-Carbó, M., Diaz-Espejo, A., Galmés, J., and Medrano, H.: Mesophyll conductance to CO_2: current knowledge and future prospects, Plant Cell Environ., 31, 602–621, 2008.

Flexas, J., Ribas-Carbó, M., Hanson, D.T., Bota, J., Otto, B., Cifre, J., McDowell, N., Medrano, H., and Kaldenhoff, R.: Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 *in vivo*, Plant J., 48, 427–439, 2006.

Galmés, J., Medrano, H., and Flexas, J.: Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms, New Phytol., 175, 81–93. 2007.

Gessler, A., Brandes, E., Buchmann, N., Helle, G., Rennenberg, H., and Barnard, R. L.: Tracing carbon and oxygen isotope signals from newly assimilated sugars in the leaves to the tree-ring archive, Plant Cell Environ., 32, 780–795, 2009.

Gessler, A., Ferrio, J. P., Hommel, R., Treydte, K., Werner, R. A., and Monson, R. K.: Stable isotopes in tree rings: towards a mechanistic understanding of isotope fractionation and mixing processes from the leaves to the wood, Tree Physiol., 34, 796–818, 2014.

Gessler, A., Rennenberg, H., and Keitel, C.: Stable isotope composition of organic compounds transported in the phloem of European beech-evaluation of different methods of phloem sap collection and assessment of gradients in carbon isotope composition during leaf-to-stem transport, Plant Biology, 6, 721–729, 2004.

Gessler, A., Tcherkez, G., Peuke, A. D., Ghashghaie, J., and Farquhar, G. D.: Experimental evidence for diel variations of the carbon isotope composition in leaf, stem and phloem sap organic matter in *Ricinus communis*, Plant Cell Environ., 31, 941–953, 2008.

Gillon, J. S., Griffiths, H.: The influence of (photo)respiration on carbon isotope discrimination in plants. Plant Cell Environ., 20, 1217–1230, 1997.

Gleixner, G. and Schmidt, H.: Carbon isotope effects on the fructose-1, 6-bisphosphate aldolase reaction, origin for non-statistical ^{13}C distributions in carbohydrates, J. Biol. Chem., 272, 5382–5387, 1997.

Guy, R. D., Fogel, M. L., and Berry, J. A.: Photosynthetic fractionation of the stable isotopes of oxygen and carbon, Plant Physiol., 101, 37–47, 1993.

Han, J. M., Meng, H. F., Wang, S. Y., Jiang, C. D., Liu, F., Zhang, W. F., and Zhang, Y. L.: Variability of mesophyll conductance and its relationship with water use efficiency in cotton leaves under drought pretreatment, J. Plant Physiol., 194, 61–71, 2016.

Hommel, R., Siegwolf, R., Saurer, M., Farquhar, G. D., Kayler, Z., Ferrio, J. P., and Gessler, A.: Drought response of mesophyll conductance in forest understory species-impacts on water-use efficiency and interactions with leaf water movement, Physiol. Plantarum, 152, 98–114, 2014.

Igamberdiev, A. U., Mikkelsen, T. N., Ambus, P., Bauwe, H., and Lea, P. J.: Photorespiration contributes to stomatal regulation and carbon isotope fractionation: a study with barley, potato and Arabidopsis plants deficient in glycine decarboxylase, Photosynth. Res., 81, 139–152, 2004.

IPCC: Summary for policymakers, in: Climate Change 2014, Mitigation of Climate Change, contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, edited by: Edenhofer, O., Pichs-Madruga, R., Sokona, Y., Farahani, E., Kadner, S., Seyboth, K., Adler, A., Baum, I., Brunner, S., Eickemeier, P., Kriemann, B., Savolainen, J., Schlomer, S., von Stechow, C., Zwickel, T., and Minx, J. C., Cambridge University Press, Cambridge, UK and New York, NY, USA, 1–30, 2014.

Jäggi, M., Saurer, M., Fuhrer, J., and Siegwolf, R.: The relationship between the stable carbon isotope composition of needle bulk material, starch, and tree rings in *Picea abies*, Oecologia, 131, 325–

332, 2002.

Kadam, N. N., Xiao, G., Melgar, R. J., Bahuguna, R. N., Quinones, C., Tamilselvan, A., Prasad, P. V. V., and Jagadish, K. S. V.: Chapter three-agronomic and physiological responses to high temperature, drought, and elevated CO_2 interactions in cereals, Adv. Agron., 127, 111–156, 2014.

Kgope, B. S., Bond, W. J., and Midgley, G. F.: Growth responses of African savanna trees implicate atmospheric [CO_2] as a driver of past and current changes in savanna tree cover, Austral Ecol., 35, 451–463, 2010.

Kirkham, M. B.: Elevated carbon dioxide: impacts on soil and plant water relations, CRC Press, London, New York, 2016.

Kodama, N., Barnard, R. L., Salmon, Y., Weston, C., Ferrio, J. P., Holst, J., Werner, R. A., Saurer, M., Rennenberg, H., and Buchmann, N.: Temporal dynamics of the carbon isotope composition in a *Pinus sylvestris* stand: from newly assimilated organic carbon to respired carbon dioxide, Oecologia, 156, 737–750, 2008.

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.

Le Roux, X., Bariac, T., Sinoquet H., Genty, B., Piel, C., Mariotti, A., Girardin, C., and Richard, P.: Spatial distribution of leaf water-use efficiency and carbon isotope discrimination within an isolated tree crown, Plant Cell Environ., 24, 1021–1032, 2001.

Leakey, A. D.: Rising atmospheric carbon dioxide concentration and the future of C4 crops for food and fuel, Proceedings of the Royal Society of London B: Biological Sciences, 276, 1517–2008, 2009.

Leakey, A. D., Ainsworth, E. A., Bernacchi, C. J., Rogers, A., Long, S. P., and Ort, D. R.: Elevated CO_2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE, J. Exp. Bot., 60, 2859–2876, 2009.

Lobell, D. B., Roberts, M. J., Schlenker, W., Braun, N., Little, B. B., Rejesus, R. M., and Hammer, G. L.: Greater sensitivity to drought accompanies maize yield increase in the US Midwest, Science, 344, 516–519, 2014.

Medlyn, B. E., Barton, C. V. M., Broadmeadow, M. S. J., Ceulemans, R., Angelis, P. D., Forstreuter, M., Freeman, M., Jackson, S. B., Kellomäki, S., and Laitat, E.: Stomatal conductance of forest species after long-term exposure to elevated CO_2 concentration: a synthesis, New Phytol., 149, 247–264, 2001.

Mielke, M. S., Oliva, M. A., de Barros, N. F., Penchel, R. M., Martinez, C. A., Da Fonseca, S., and de Almeida, A. C.: Leaf gas exchange in a clonal eucalypt plantation as related to soil moisture, leaf water potential and microclimate variables, Trees, 14, 263–270, 2000.

Miranda Apodaca, J., Pérez López, U., Lacuesta, M., Mena Petite, A., and Muñoz Rueda, A.: The type of competition modulates the ecophysiological response of grassland species to elevated CO_2 and drought, Plant Biolog, 17, 298–310, 2015.

Parker, W. C. and Pallardy, S. G.: Gas exchange during a soil drying cycle in seedlings of four black walnut (*Juglans nigra* l.) Families, Tree physiol., 9, 339–348, 1991.

Pons, T. L., Flexas, J., von Caemmerer, S., Evans, J. R., Genty, B., Ribas-Carbo, M., and Brugnoli, E.: Estimating mesophyll conductance to CO_2: methodology, potential errors, and recommendations, J. Exp. Bot., 8, 1–18, 2009.

Reich, P. B., Walters, M. B., and Tabone, T. J.: Response of *Ulmus americana* seedlings to varying nitrogen and water status. 2 Water and nitrogen use efficiency in photosynthesis, Tree Physiol., 5,

627   173–184, 1989.

628   Rinne, K. T., Saurer, M., Kirdyanov, A. V., Bryukhanova, M. V., Prokushkin, A. S., Churakova
629   Sidorova, O. V., and Siegwolf, R. T.: Examining the response of larch needle carbohydrates to
630   climate using compound-specific δ^{13}C and concentration analyses, EGU General Assembly
631   Conference, 1814949R, 2016.

632   Robredo, A., Pérez-López, U., de la Maza, H. S., González-Moro, B., Lacuesta, M., Mena-Petit,e A.,
633   and Muñoz-Rueda, A.: Elevated CO_2 alleviates the impact of drought on barley improving water
634   status by lowering stomatal conductance and delaying its effects on photosynthesis, Environ. Exp.
635   Bot., 59, 252–263, 2007.

636   Robredo, A., Pérez-López, U., Lacuesta, M., Mena-Petite, A., and Muñoz-Rueda, A.: Influence of
637   water stress on photosynthetic characteristics in barley plants under ambient and elevated CO_2
638   concentrations, Biologia. Plantarum, 54, 285–292, 2010.

639   Rossmann, A., Butzenlechner, M., and Schmidt, H.: Evidence for a nonstatistical carbon isotope
640   distribution in natural glucose, Plant Physiol., 96, 609–614, 1991.

641   Streit, K., Rinne, K. T., Hagedorn, F., Dawes, M. A., Saurer, M., Hoch, G., Werner, R. A., Buchmann,
642   N., and Siegwolf, R. T. W.: Tracing fresh assimilates through *Larix decidua* exposed to elevated
643   CO_2 and soil warming at the alpine treeline using compound-specific stable isotope analysis, New
644   Phytol., 197, 838–849, 2013.

645   Tausz Posch, S., Norton, R. M., Seneweera, S., Fitzgerald, G. J., and Tausz, M.: Will intra-specific
646   differences in transpiration efficiency in wheat be maintained in a high CO_2 world? A FACE study,
647   Physiol. Plantarum, 148, 232–245, 2013.

648   Tazoe, Y., von Caemmerer, S., Estavillo, G. M., and Evans, J. R.: Using tunable diode laser
649   spectroscopy to measure carbon isotope discrimination and mesophyll conductance to CO_2
650   diffusion dynamically at different CO_2 concentrations, Plant Cell Environ., 34, 580–591, 2011.

651   Terashima, I., Hanba, Y.T., Tazoe, Y., Vyas, P., and Yano, S.: Irradiance and phenotype: comparative
652   eco-development of sun and shade leaves in relation to photosynthetic CO_2 diffusion, J. Exp. Bot.,
653   57, 343–354, 2006.

654   Théroux-Rancourt, G., Éthier, G., and Pepin, S.: Threshold response of mesophyll CO_2 conductance to
655   leaf hydraulics in highly transpiring hybrid poplar clones exposed to soil drying, J. Exp. Bot., 65,
656   741-753, 2014.

657   Uehlein, N., Otto, B., Hanson, D. T., Fischer, M., McDowell, N., and Kaldenhoff, R.: Function of
658   Nicotiana tabacum aquaporins as chloroplast gas pores challenges the concept of membrane CO_2
659   permeability, Plant Cell, 20, 648–657, 2008.

660   Von Caemmerer, S. V. and Farquhar, G. D.: Some relationships between the biochemistry of
661   photosynthesis and the gas exchange of leaves, Planta, 153, 376–387, 1981.

662   Wall, G. W., Garcia, R. L., Kimball, B. A., Hunsaker, D. J., Pinter, P. J., Long, S. P., Osborne, C. P.,
663   Hendrix, D. L., Wechsung, F., and Wechsung, G.: Interactive effects of elevated carbon dioxide
664   and drought on wheat, Agron. J., 98, 354–381, 2006.

665   Wall, G. W., Garcia, R. L., Wechsung, F., and Kimball, B. A.: Elevated atmospheric CO_2 and drought
666   effects on leaf gas exchange properties of barley, Agr. Ecosyst. Environ., 144, 390–404, 2011.

667   Warren, C. R. and Adams, M. A.: Internal conductance does not scale with photosynthetic capacity:
668   implications for carbon isotope discrimination and the economics of water and nitrogen use in
669   photosynthesis, Plant Cell Environ., 29, 192–201, 2006.

670   Xu, D. Q.: Some problems in stomatal limitation analysis of photosynthesis, Plant Physiol. J., 33, 241–

244, 1997.

Xu, Z. and Zhou, G.: Responses of photosynthetic capacity to soil moisture gradient in perennial rhizome grass and perennial bunchgrass, BMC Plant Boil., 11, 21, 2011.

Yang, B., Pallardy, S. G., Meyers, T. P., GU, L. H., Hanson, P. J., Wullschleger, S. D., Heuer, M., Hosman, K. P., Riggs, J. S., and Sluss D. W.: Environmental controls on water use efficiency during severe drought in an Ozark Forest in Missouri, USA, Global Change Biol., 16, 2252–2271, 2010.

Yu, G., Wang, Q., and Mi, N.: Ecophysiology of plant photosynthesis, transpiration, and water use, Science Press, Beijing, China, 2010.

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.

Figure

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 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.
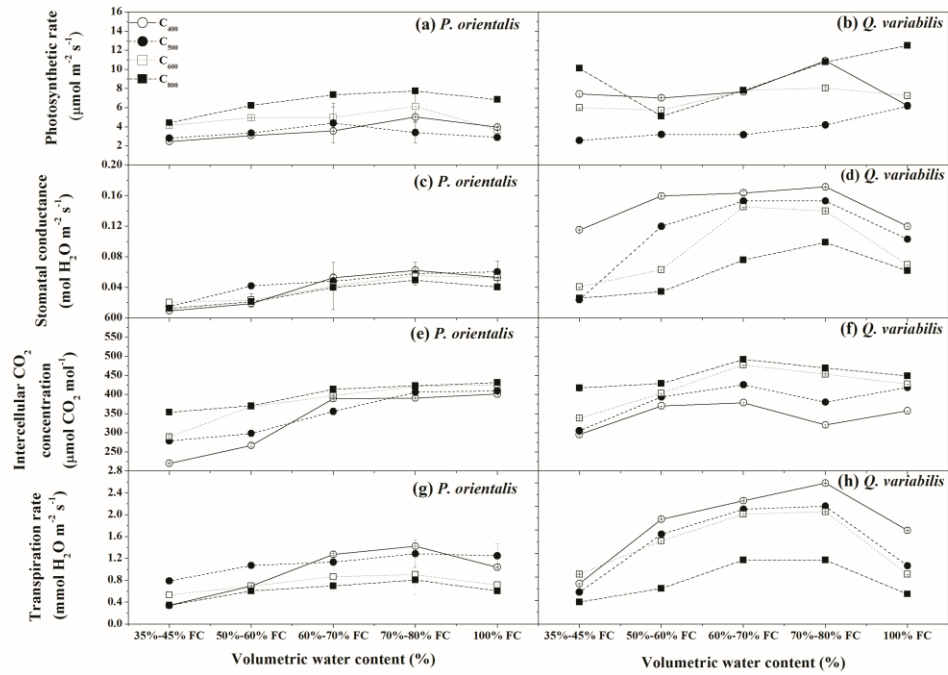
Figure 2. Photosynthetic parameters of *P. orientalis* and *Q. variabilis* saplings in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm and 800 ppm across five soil volumetric water contents. The net photosynthetic rates (P_n, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol H$_2$O m^{-2} s^{-1}), intercellular CO_2 concentration (C_i, μmol CO_2 mol^{-1}), and transpiration rates (T_r, mmol H$_2$O m^{-2} s^{-1}) are shown in Figs. 2a and 2b, 2c and 2d, 2e and 2g, and 2g and 2h, respectively. Means ±SDs, n = 32.

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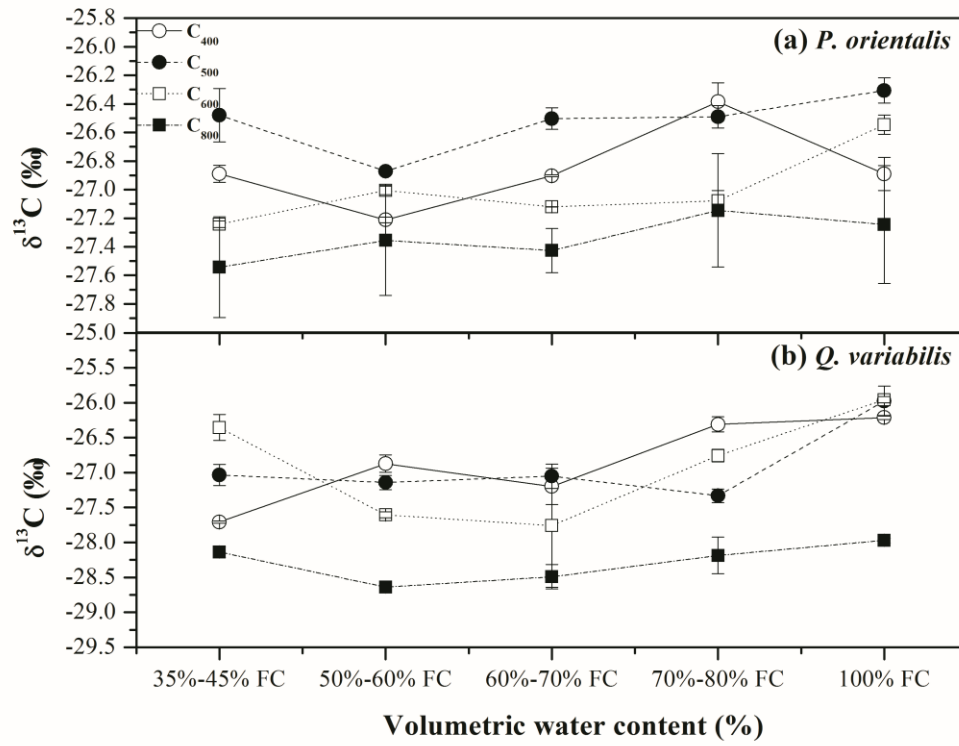**Figure 3.** $\delta^{13}C$ of water-soluble compounds extracted from leaves of *P. orientalis* and *Q. variabilis*
cultivated in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm and 800 ppm across five soil
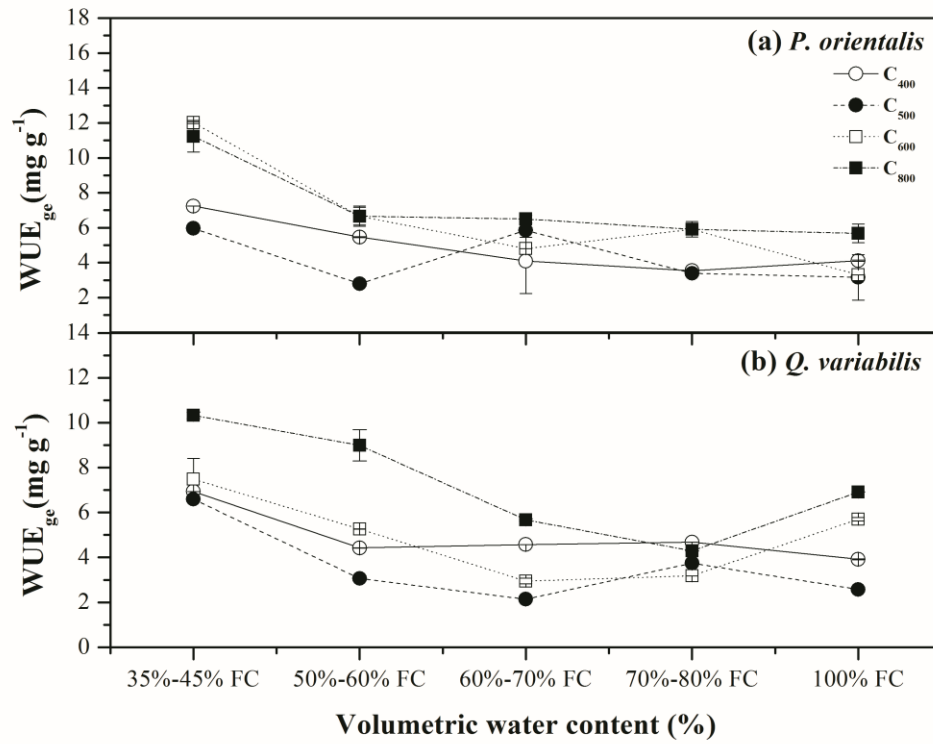volumetric water contents are shown in Figs. 3a and 3b. Means ±SDs, n = 32.

Figure 4. Instantaneous water use efficiency through gas exchange (WUE_{ge}) in leaves of *P. orientalis* and *Q. variabilis* cultivated in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm and 800 ppm across five soil volumetric water contents are shown in Figs. 4a and 4b. Means ± SDs, n = 32.
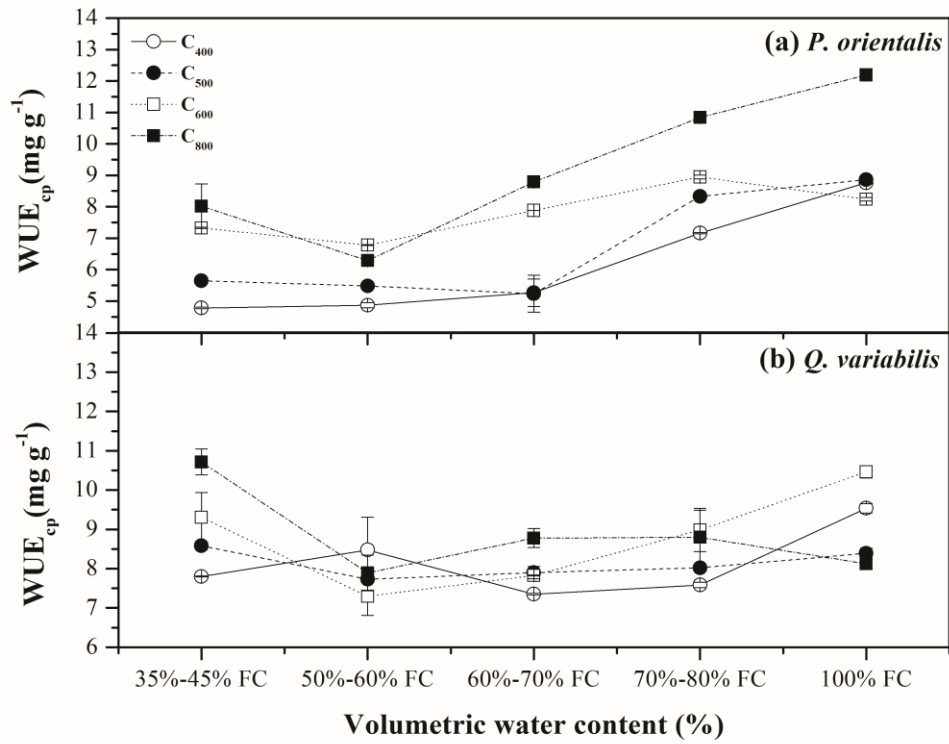
Figure 5. Instantaneous water use efficiency through $\delta^{13}C$ of water-soluble compounds (WUE_{cp}) in leaves of *P. orientalis* and *Q. variabilis* cultivated in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm, and 800 ppm across five soil volumetric water contents are shown in Figs. 5a and 5b. Means ± SDs, n = 32.

Figure 6. Variations in mesophyll conductance of *P. orientalis* and *Q. variabilis* cultivated in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm, and 800 ppm across five soil volumetric water contents are shown in Figs. 6a and 6b. Means ±SDs, n = 32.
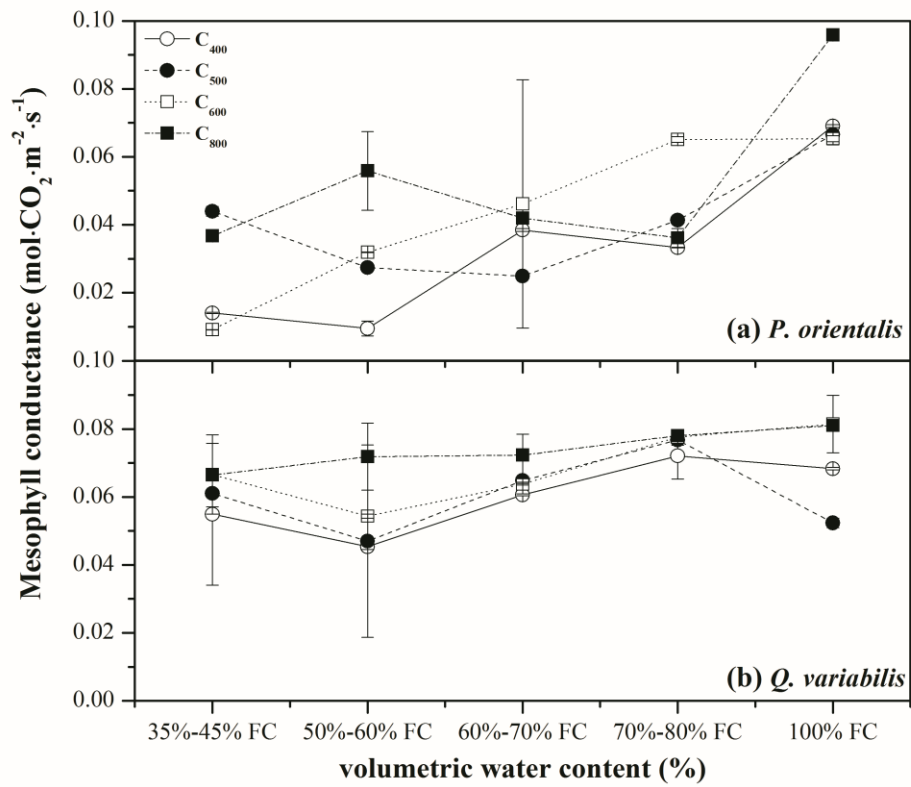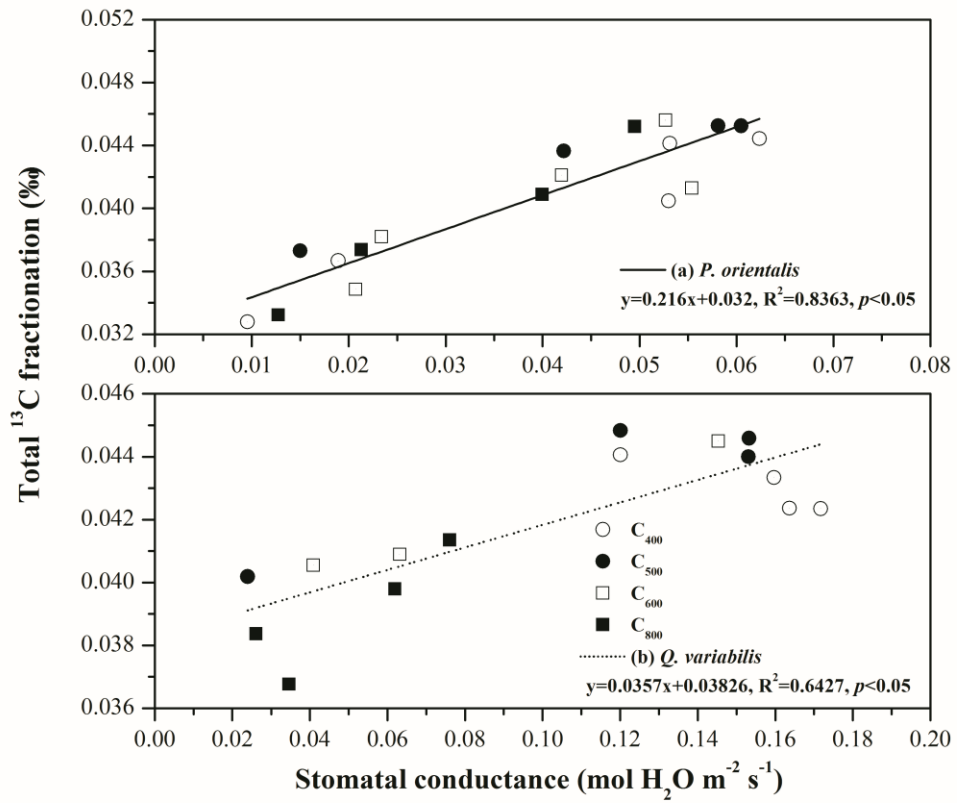
Figure 7. Regression between stomatal conductance and total ^{13}C fractionation of *P. orientalis* and *Q. variabilis* under four CO_2 concentrations $\times$ five soil volumetric water contents are established in Figs. 7a and 7b. *p*=0.05, n = 32.
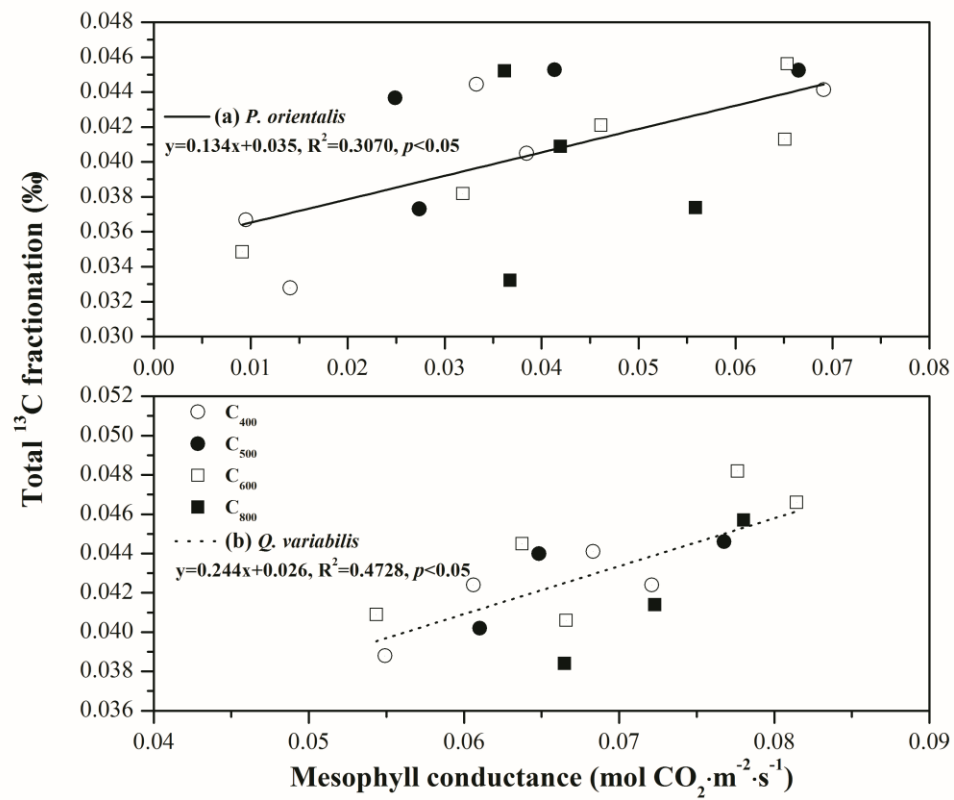
Figure 8. Regression between mesophyll conductance and total ^{13}C fractionation of *P. orientalis* and *Q. variabilis* under four CO_2 concentrations × five soil volumetric water contents are established in Figs. 8a and 8b. *p*=0.05, n = 32.

Table 1. ^{13}C fractionation of *P. orientalis* and *Q. variabilis* under four CO_2 concentrations $\times$ five soil volumetric water contents.

Species	SWC (of FC)		CO₂ concentration (ppm)													
		Total ^{13}C fractionation (‰)	400	500	600	800	^{13}C fractionation (‰)	400	500	600	800	^{13}C fractionation (‰)	400	500	600	800
P. orientalis	35%–45%		0.0328	0.0373	0.0349	0.0332		0.0081	0.0030	0.0034	0.0072		0.0247	0.0343	0.0315	0.0260
	50%–60%		0.0367	0.0437	0.0382	0.0374		0.0018	0.0058	0.0094	0.0004		0.0349	0.0379	0.0288	0.0370
	60%–70%	Total ^{13}C fractionation (‰)	0.0405	0.0366	0.0421	0.0409		0.0018	0.0050	0.0026	0.0007		0.0387	0.0316	0.0395	0.0402
	70%–80%		0.0444	0.0453	0.0413	0.0452		0.0044	0.0052	0.0103	0.0013		0.0400	0.0401	0.0310	0.0439
	100%		0.0441	0.0453	0.0456	0.0472	Mesophyll conductance	0.0057	0.0040	0.0025	0.0039	Post-photosynthesis	0.0384	0.0413	0.0431	0.0433
Q. variabilis	35%–45%		0.0388	0.0402	0.0406	0.0384		0.0007	0.0025	0.0006	0.0091		0.0381	0.0377	0.0400	0.0293
	50%–60%		0.0433	0.0448	0.0409	0.0368		0.0061	0.0084	0.0023	0.0018		0.0372	0.0364	0.0386	0.0350
	60%–70%		0.0424	0.0440	0.0445	0.0414		0.0066	0.0086	0.0078	0.0041		0.0358	0.0354	0.0367	0.0373
	70%–80%		0.0424	0.0446	0.0482	0.0457		0.0034	0.0016	0.0074	0.0028		0.0390	0.0430	0.0408	0.0429
	100%		0.0441	0.0466	0.0466	0.0398		0.0027	0.0076	0.0022	0.0125		0.0414	0.0390	0.0444	0.0273