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Differences in instantaneous water use efficiency derived from post-carboxylation fractionation respond to the interaction of CO_2 concentrations and water stress in semi-arid areas

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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. As $\delta^{13}C$ of water-soluble compounds in leaves was depleted from extracellular CO_2 to primary assimilates, no explanation has been offered for ^{13}C fractionation before leaf-exported transportation of photosynthate. Either its variation according to the CO_2 concentration and/or water stress gradients, or their interaction have not yet been identified. 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] × 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. The differences between WUE_{ge} and WUE_{cp} were clearly species-specific, as demonstrated in the interaction of [CO_2] and SWC. Rising [CO_2] coupled with moistened soil generated increasing disparities between WUE_{ge} and WUE_{cp} in *P. orientalis* with an amplitude of 0.0328‰–0.0472‰. Further, the differences between WUE_{ge} and WUE_{cp} of *Q. variabilis* increased as CO_2 concentration increased and water stress alleviated (0.0384‰–0.0466‰). The ^{13}C fractionation in post-photosynthesis was linearly dependent on g_s, and 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

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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 (Farquhar et al., 1982). 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. Several scholars have defined the carbon isotopic fractionations that occur between the leaf and wood cambium 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 sucrose synthesis, ^{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 two sugars before transportation to the twig is associated with the post-carboxylation fractionation generated in leaves. It is also important to consider that the carbon cycling time within plants has an absolute influence on the time integration of photosynthetic carbon discrimination. Several studies have indicated that recently-assimilated carbohydrate that is imprinted with environmental signals is mixed with other carbohydrate pools of different ages during transportation along the basipetal tree axis (Brandes et al., 2006; Richardson et al., 2012). It is necessary to avoid confusion of carbon sources, and further, to determine carbon fractionation within leaves following photosynthetic carboxylation. In addition,

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whether these fractionations are related to environmental variation has not yet been investigated.

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 gas exchange properties on a daily basis (Kodama et al., 2008). However, there is dispute whether the post-carboxylation fractionation process 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 that isotope fractionation responds to different environmental factors, such as elevated [CO_2] and/or soil water gradients, has not yet been observed.

Consequently, we investigated $\delta^{13}C$ 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 growth chambers (FH-230). Our goals are to compare the differences in iWUE derived from ^{13}C-frationation of post-carbonxylation between *P. orientalis* and *Q. variabilis*, and to describe how these differences in iWUE 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* spp. 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 ±0.5℃ from 07:00 to 17:00, and the night-time temperature was 18 ± 0.5℃ 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 a CO_2 concentration of 400 ±50 ppm (during June 2–9, June 12–19, June 21–28, and July 2–9, 2015) and 500 ±50 ppm (during July 11–18, July 22–29, August 4–11, and August 15–22, 2015). The other was adjusted to maintain the CO_2 concentration at 600 ±50 ppm (during June 2–9, June 12–19, June 21–28, and July 2–9, 2015) and 800 ±50 ppm (during July 11–18, July 22–29, August 4–11, and August 15–22, 2015). The CO_2 concentration in the chambers was set to maintain one target level (permitting a standard deviation of ± 50 ppm)

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during cultivation. Thus, we employed a gradient of four CO_2 concentrations in our study (400 ±50 ppm, 500 ±50 ppm, 600 ±50 ppm, and 800 ±50 ppm). Detectors inside the chambers monitored and maintained the CO_2 concentrations continuously at the constant setting.

We designed a device to water the potted plants automatically to 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 chamber could determine whether to water or stop watering the plants. Two drip irrigation devices were installed in both chambers, respectively. After measuring the average Field Capacity (FC) of the pot soil (30.70%), five levels of SWC were maintained before the orthogonal tests, 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.

After establishing the equilibrium circumstances of elevated CO_2 across the soil water gradients, the saplings were ready for investigation. Each orthogonal treatment included three replicates, and each replicate continued for 7 days. Pots were rearranged periodically to minimize non-uniform illumination.

Orthogonal tests: 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).

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. 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. Each leaf was measured three times. Three leaves from each sapling were chosen, and three saplings were measured within one orthogonal treatment. 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 sample preparation

After measuring gas exchange, recently-expanded, sun leaves were removed from the *P. orientalis* and *Quercus variabilis* saplings cultivated in the orthogonal treatments, and frozen immediately in liquid nitrogen. 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 1ml 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 into tin capsules

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Manuscript under review for journal Biogeosciences
Published: 7 September 2016

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):

$$\delta^{13}C = \left(R_{sample}/R_{standard}\right) \times 1000 \quad (1)$$

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

Based on the linear model developed by Farquhar and Sharkey (1982), the isotope discrimination factor, Δ, was calculated as:

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

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:

$$C_i : C_a = (\Delta - a)/(b - a) \quad (3)$$

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 (27‰). Instantaneous water use efficiency (iWUE) is calculated as:

$$\text{iWUE} = P_n : T_r = (C_a - C_i)/1.6\Delta e \quad (4)$$

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:

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

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.

$$WUE_{cp} = \frac{P_n}{T_r} = (1 - \varphi)(C_a - C_i)/1.6\Delta e = C_a(1 - \varphi)\left(1 - \frac{\delta^{13}C_a - \delta^{13}C_p - a}{(b-a)}\right)/1.6\Delta e \quad (6)$$

φ is the ratio between carbohydrates consumed during respiration of the leaves and that of other organs at night (0.3).

3 Results

3.1 Foliar gas exchange measurements

P. orientalis and *Q. variabilis* saplings were exposed to the orthogonal treatments (under gradients of SWC and [CO_2] of 400 ppm, 500 ppm, 600 ppm, and 800 ppm, labeled C_{400}, C_{500}, C_{600}, and C_{800}). When SWC increased, most P_n in *P. orientalis* peaked at 70%–80% of FC, while that of *Q. variabilis* reached higher values of 70%–80% of FC and FC. The uptake capacity of carbon was improved significantly with elevated [CO_2] at any given soil moisture (p<0.05) for *P. orientalis*. We observed

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Manuscript under review for journal Biogeosciences
Published: 7 September 2016

increased P_n of *Q. variabilis* after CO_2 gradient-fumigation, except under C_{400}. Further, greater magnitudes of increments in P_n of *P. orientalis* were found at 50%–70% of FC from C_{400} to C_{800}, which was observed at 35%–45% of FC for *Q. variabilis*. Instantaneous carbon assimilation capacities of *Q. variabilis* among all treatments were stronger than were those of *P. orientalis* (Figs. 2a and 2b, $p<0.01$).

The g_s in *P. orientalis* coincided with P_n as soil moisture increased, and was highest at 70%–80% of FC in C_{400}, C_{500}, and C_{800} (Fig. 2c), while it peaked at FC in C_{600}. When SWC increased from 35%–45% to 50%–60% of FC, g_s in *Q. variabilis* moved up sharply and then increased gradually to its maximum in C_{400}, C_{500}, and C_{800} as soil moisture increased. As the water stress was alleviated (at 70%–80% of FC and 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 ($p<0.01$). g_s in *Q. variabilis* exceeded that in *P. orientalis* under the same treatments ($p<0.01$, Figs. 2c and 2d).

The C_i in *P. orientalis* rose gradually as SWC increased, and peaked at FC at any given [CO_2]. Under the same conditions of cultivation as *P. orientalis*, C_i of *Q. variabilis* reached their maximums at 60%–70% of FC, and declined thereafter with increased SWC. The variation in C_i of the two species was similar and decreased as [CO_2] elevated. C_i of *Q. variabilis* was significantly greater than was that of *P. orientalis* under the same treatment ($p<0.01$, Figs. 2e and 2f).

The T_r of *P. orientalis* and *Q. variabilis* all exhibited single peaks that occurred at 70%–80% of FC in combination with the soil moisture gradient. The T_r of the two saplings in different [CO_2] were compared at each SWC (Figs. 2g and 2h). Except for 35%–60% of FC, the T_r of the two saplings in C_{400} and C_{500} was significantly higher than that in C_{600} and C_{800} ($p<0.01$). With the same [CO_2] and the same SWC, the T_r of *Q. variabilis* was remarkably larger than was that of *P. orientalis* ($p<0.01$).

3.2 $\delta^{13}C$ of water-soluble compounds in leaves

To observe the photosynthetic traits of the two saplings, the same leaf was frozen immediately and the water-soluble compounds (WSCs) were extracted for all orthogonal treatments. $\delta^{13}C_{WSC}$ ($\delta^{13}C$ of water-soluble compounds from leaves) of *P. orientalis* and *Q. variabilis* saplings cultivated in the four CO_2 concentrations all increased as soil moisture improved (Figs. 3a and 3b, $p<0.01$). The average (±SD) $\delta^{13}C_{WSC}$ values 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. Further, we found that the average $\delta^{13}C_{WSC}$ of the two saplings reached their maximums at 70%–80% of FC in a given [CO_2]. Together with the gradual enrichment of [CO_2], trends of the average $\delta^{13}C_{WSC}$ values of the saplings declined when [CO_2] exceeded 600 ppm ($p<0.01$). Except for C_{400} at 50%–100% of FC, the $\delta^{13}C_{WSC}$ values of *P. orientalis* were significantly larger than were those of *Q. variabilis* for any other [CO_2] × SWC treatments ($p<0.01$).

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

Instantaneous water use efficiency via gas exchange (WUE_{ge}) is calculated as P_n divided by T_r. Figure 4a shows that increased magnitudes of WUE_{ge} of *P. orientalis* under severe drought (i.e., 35%–45% of FC) were highest at any given [CO_2], ranging from 90.70% to 564.65%. As SWC increased, WUE_{ge} reduced along a gradient in C_{400}, C_{500}, C_{600}, and C_{800}, while they increased remarkably as [CO_2] increased. Compared to *P. orientalis*, the trends of WUE_{ge} in *Q. variabilis* were

Biogeosciences Discuss., doi:10.5194/bg-2016-372, 2016
Manuscript under review for journal Biogeosciences
Published: 7 September 2016

promoted slightly at FC in C_{600} or C_{800} as soil water increased (Fig. 4b). The maximum of WUE_{ge} in *P. orientalis* thus occurred at 35%–45% of FC in C_{800} for all orthogonal treatments, and did so in *Q. variabilis* as well. Further, elevated CO_2 concentrations enhanced the WUE_{ge} of *Q. variabilis* clearly at any SWCs except that at 60%–80% of FC. Based on the comparison between the same $[CO_2]$ × SWC treatments, most saplings of *P. orientalis* had greater WUE_{ge} than did *Q. variabilis* (p<0.05).

The $\delta^{13}C$ values of water-soluble compounds from leaves of the two saplings were measured, and the instantaneous water use efficiency could be determined from Eq. (6) for the $\delta^{13}C_{WSC}$ of leaves, defined as WUE_{cp}. As illustrated in Fig. 5a, WUE_{cp} of *P. orientalis* in C_{600} or C_{800} climbed up as water stress was reduced beyond 50%–60% of FC, while the water threshold was 60%–70% of FC in C_{400} or C_{500}. *Q. variabilis* exhibited no uniform trend of WUE_{cp} with soil wetting. Except for C_{400}, WUE_{cp} of *Q. variabilis* decreased abruptly at 50%–60% of FC, and rose as soil moisture improved in C_{500}, C_{600}, and C_{800}. Figure 5b shows the effects of elevated CO_2 on WUE_{cp} in *Q. variabilis*. In contrast to the findings for WUE_{ge} in the two saplings, the WUE_{cp} of *Q. variabilis* was more pronounced than was that of *P. orientalis* for all orthogonal treatments.

3.4 Post-carboxylation fractionation generated before photosynthate leaves the leaves

We evaluated the differences between instantaneous water use efficiency via leaf gas exchange and $\delta^{13}C$ of water-soluble compounds (Table 1), which can retrace ^{13}C fractionation before carboxylation transport to the twig. When comparing WUE_{ge} and WUE_{cp}, the ^{13}C-depletion of *P. orientalis* ranged from 0.0328‰ to 0.0472‰, which was smaller than that of *Q. variabilis* (0.0384‰ to 0.0466‰). The fractionation effects of *P. orientalis* were magnified with increased soil moisture, and particularly at 35%–80% of FC from C_{400} to C_{800}, the magnitudes of the increments reached 21.30%–42.04%. Under C_{400} and C_{500} in *Q. variabilis*, the coefficients were amplified, as SWC increased until 50%–60% of FC, then remained constant. With respect to C_{600} and C_{800}, the coefficients of *Q. variabilis* were amplified at 50%–80% of FC, and decreased at FC. The average coefficients of *P. orientalis* increased gradually as $[CO_2]$ rose, while those of *Q. variabilis* declined sharply from C_{600} to C_{800}. Coefficients of *P. orientalis* increased at a faster rate than did those of *Q. variabilis* with increased soil moisture.

Stoma are the conduit between the plant and atmosphere. Post-carboxylation fractionation may be correlated with the degree to which the stomata are open and the resistance they exert. Here, we performed linear regressions between g_s and the ^{13}C fractionation coefficient for *P. orientalis* and *Q. variabilis* (Fig. 6). It was apparent that the ^{13}C fractionation coefficient was linearly dependent on the g_s (p<0.05), which controls the exchange of CO_2 and H_2O, and responds to environmental variation.

4 Discussion

4.1 Photosynthetic traits

The exchange of CO_2 and water vapor via stomata is modulated in part by the soil or leaf water potential (Robredo et al., 2010). Saplings of *P. orientalis* reached their maximums of P_n and g_s at 70%–80% of FC under any $[CO_2]$. As SWC exceeded the water threshold, elevated CO_2 caused a greater reduction in g_s, as has been reported for barley and wheat (Wall et al., 2011). Nonetheless, maximal values of g_s in *Q. variabilis* in C_{400}, C_{500}, C_{600}, and C_{800} were generated successively as soil moisture increased, indicating that drought stimulated the stomata, which are more sensitive to

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environmental changes. In addition, C_i of *Q. variabilis* peaked at 60%–70% of FC, following declines as soil moisture increased (Wall et al., 2006; Wall et al., 2011). The g_s of *P. orientalis* and *Q. variabilis* decreased with elevated [CO_2], which was evidenced by FACE and non-FACE experiments (Ainsworth and Rogers, 2007; Wall et al., 2011). This is interpreted as stomata having the tendency to maintain a constant C_i or C_i/C_a when ambient [CO_2] increases, which would determine the CO_2 used directly in chloroplast (Yu et al., 2010). On the basis of theories proposed by Farquhar and Sharkey (1982) and common experimental technologies (Xu, 1997), this could be explained as a stomatal limitation. However, C_i of *P. orientalis* was observed to increase considerably when SWC exceeded 70%–80% of FC in all CO_2 treatments (Mielke et al., 2000). One factor that can account for this is that 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 that 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). When SWC exceeds the threshold (70%–80% of FC), further elevations in [CO_2] may cause nonstomatal limitation, 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 and ecological characteristics of perennial *Leymus chinensis*. They demonstrated that there was an evident threshold in the gradient at which a plant could manage its structure and function to adjust to environmental conditions. 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 saplings increased with increased [CO_2] in our study, as was found previously for C_3 in woody plants (Kgope et al., 2010). Further, increasing [CO_2] appeared to alleviate soil water stress at 35%–45% or 50%–60% of FC, which proves that photosynthetic inhibition produced by water stress (or excess) may be moderated by increased [CO_2] (Robredo et al., 2007; Robredo et al., 2010). These results are consistent with a number of studies in which elevated CO_2 was expected to ameliorate 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 $\delta^{13}C$ of water-soluble compounds

Stable isotope ratios of plant tissues have been applied widely to evaluate the ecophysiological processes that interact with environmental variation, especially those that control plant-atmosphere exchanges of mass circulation and energy flow (McCarroll and Loader, 2004; Poussart et al., 2004; Rinne et al., 2010). Based on the relationship between photosynthetic carbon isotope fractionation ($\Delta^{13}C$ or Δ) and the ratio between internal leaf and ambient CO_2 concentration (C_i/C_a: Eq. (2) and Eq. (3), Farquhar et al. 1982), the $\delta^{13}C$ of plant tissue could characterize effects of environmental interaction on internal reactions and processes of photosynthesis (Gessler et al., 2014). Further, the leaf carbon isotope ratios are an excellent surrogate for direct measurement of iWUE (Eq. (4) shows the determination of iWUE by $\delta^{13}C$), which was fractionated over CO_2 diffusion into leaf via stomata and carboxylation in chloroplast. The $\delta^{13}C$ of water-soluble compounds ($\delta^{13}C_{WSC}$) extracted from saplings' leaves has been measured to examine the physiological and metabolic responses to current environmental variation (Streit et al., 2013). The authors found that the average $\delta^{13}C_{WSC}$ of *P. orientalis* and *Q. variabilis* were correlated positively with the increment of soil moisture during

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35%–80% of FC, which was demonstrated by Adiredjo et al. (2014) as well. Thus, $^{13}C_{WSC}$ may respond according to the availability of soil moisture, which was reviewed by Cernusak et al. (2012). Once it exceeds 70%–80% of FC, the average $\delta^{13}C_{WSC}$ values of the two saplings with soil moistening were consistent with the trends of g_s. Elevated CO_2 concentrations affected physiological performance profoundly (Gimeno et al., 2015), especially by increasing the CO_2 supply to the chloroplasts and reducing stomatal conductance, which would have influenced $\delta^{13}C_{WSC}$ indirectly in this study.

4.3 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. 1a, 1g, 1b and 1h), also were demonstrated by Ainsworth and McGrath (2010). Combining the P_n and T_r of *P. orientalis* and *Q. variabilis* in the same treatment, the lower WUE_{ge} in *Q. variabilis* is achieved generally by the plant's 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, with respect to the patterns of iWUE, there has been no consensus on soil water states at the leaf level, although some have discussed this topic (Yang et al., 2010). As SWC decreased, the WUE_{ge} of *P. orientalis* and *Q. variabilis* was enhanced, as presented by Parker and Pallardy (1991), DeLucia and Heckathorn (1989), and Reich et al. (1989). Leakey (2009) also concluded that the WUE of stressed plants 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 *P. orientalis* and *Q. variabilis* demonstrated similar variations to those of $\delta^{13}C_{WSC}$, and water stress was alleviated when combined with elevated $[CO_2]$, which differentiated the trends in WUE_{ge}. The assumption has been made that $\delta^{13}C_{WSC}$ is coupled tightly with dynamics in the environment several days before the water-soluble compounds is extracted. As observed, the WUE_{cp} of *P. orientalis* and *Q. variabilis* responded synthetically with increasing SWC across different $[CO_2]$ gradients over the course of several days. Consequently, WUE_{cp} and WUE_{ge} have different variable curves according to treatments. In addition, there were characteristic species-specific responses of $\delta^{13}C_{WSC}$ under the same environmental conditions.

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 transportation route of photosynthate production, from leaf to wood formation, consists of post-assimilation fractionations/processes, referred to as "post-photosynthetic" or "post-carboxylation" fractionation (Jäggi et al., 2002; Gessler et al., 2008). 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-

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carboxylation fractionation generated in leaves. Hence, we hypothesized that ^{13}C fractionation might exist. The WUE_{ge} of the plant responds to instantaneous carbon assimilation and transpiration. When we finished the leaf gas exchange measurements, the leaf samples were collected immediately to determine the $\delta^{13}C$ of water-soluble compounds (WUE_{cp}). Presumably, the ^{13}C fractionation generated in the synthetic processes of sucrose and starch was approximately equal to the $\delta^{13}C$ difference between WUE_{ge} and WUE_{cp}. When comparing WUE_{ge} *and* WUE_{cp}, the ^{13}C-depletion of *P. orientalis* ranged from 0.0328‰ to 0.0472‰, less than that of *Q. variabilis* (from 0.0384‰ to 0.0466‰). Recently, Gessler et al. (2004) reviewed the environmental drivers of variation in photosynthetic carbon isotope discrimination in terrestrial plants. The ^{13}C fractionation effects 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 the ^{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. 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 the two species. Rising $[CO_2]$ and/or soil moistening generated increasing disparities between WUE_{ge} and WUE_{cp} in *P. orientalis*; nevertheless, the differences between WUE_{ge} and WUE_{cp} in *Q. variabilis* increased as $[CO_2]$ increased and/or water stress was alleviated. The ^{13}C fractionation also was linearly dependent on g_s. With respect to post-photosynthesis fractionation in post-carboxylation and transportation processes, we cannot neglect the fact 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.

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547 **Author contribution**

548 Na Zhao and Yabing He collected field samples, and performed the experiment. Na Zhao engaged
549 in data analysis and writing this paper. Ping Meng proposed the suggestions on the theory and
550 practice of experiment. Xinxiao Yu revised the paper and contributed to edit the manuscript.
551
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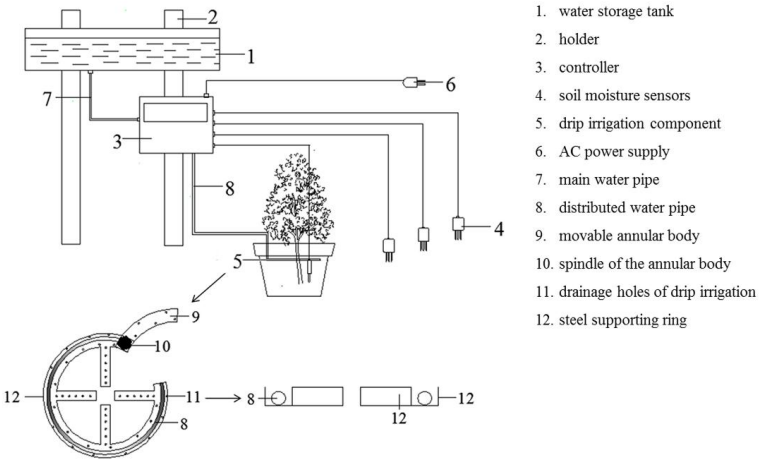

Figure 1. Structural diagram of the device for automatic drip irrigation

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.

Biogeosciences Discuss., doi:10.5194/bg-2016-372, 2016
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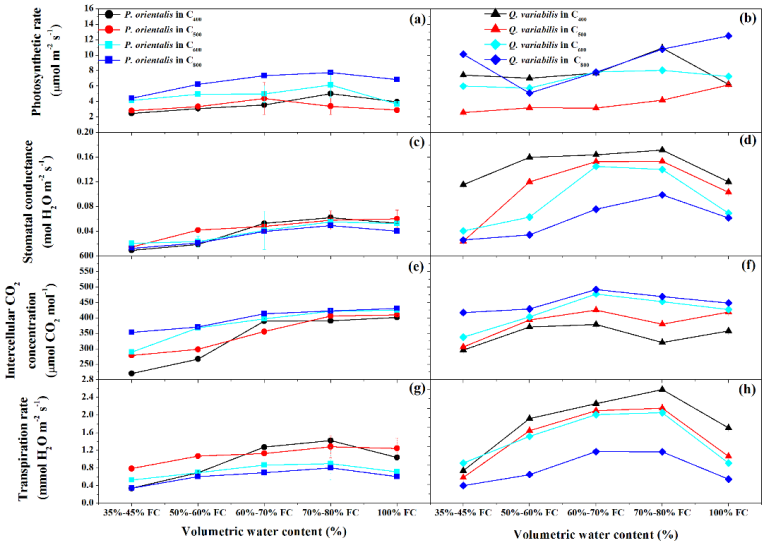

Figure 2. Photosynthetic parameters of *P. orientalis* and *Q. variabilis* saplings in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm and 800 ppm across five soil volumetric water contents. The net photosynthetic rates (P_n, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (C_i, μmol CO_2 mol^{-1}), and transpiration rates (T_r, mmol H_2O 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.

Biogeosciences Discuss., doi:10.5194/bg-2016-372, 2016
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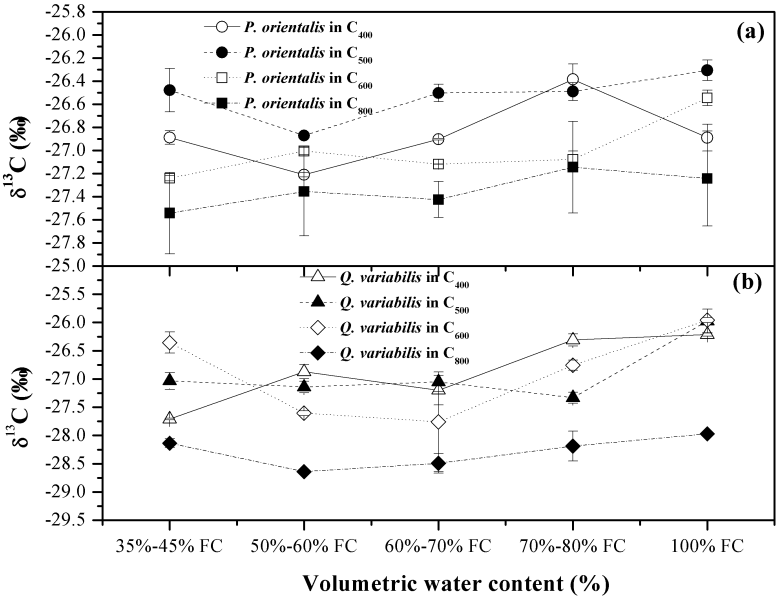

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598 **Figure 3.** $\delta^{13}C$ of water-soluble compounds extracted from leaves of *P. orientalis* and *Q. variabilis*
599 cultivated in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm and 800 ppm across five soil
600 volumetric water contents are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

Biogeosciences Discuss., doi:10.5194/bg-2016-372, 2016
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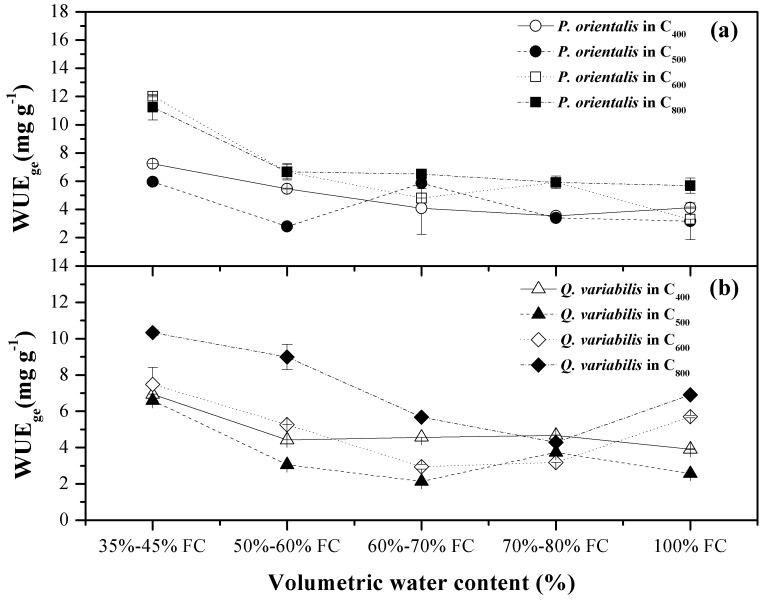

Figure 4. Instantaneous water use efficiency through gas exchange (WUE_{ge}) in leaves of *P. orientalis* and *Q. variabilis* cultivated in CO_2 concentrations of 400 ppm, 500 ppm, 600 ppm and 800 ppm across five soil volumetric water contents are shown in Figs. 4a and 4b. Means ± SDs, n = 32.

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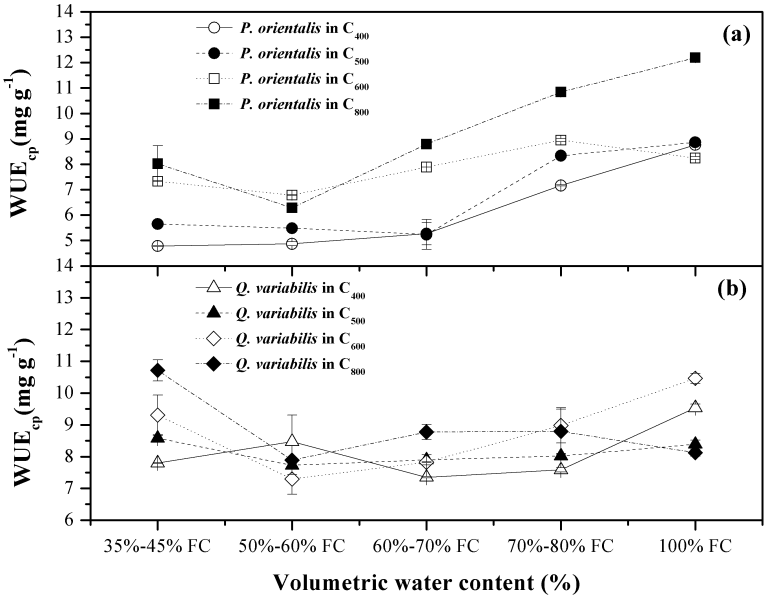

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607 **Figure 5.** Instantaneous water use efficiency through $\delta^{13}C$ of water-soluble compounds (WUE_{cp}) in
608 leaves of *P. orientalis* and *Q. variabilis* cultivated in CO_2 concentrations of 400 ppm, 500 ppm, 600
609 ppm, and 800 ppm across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

Biogeosciences Discuss., doi:10.5194/bg-2016-372, 2016
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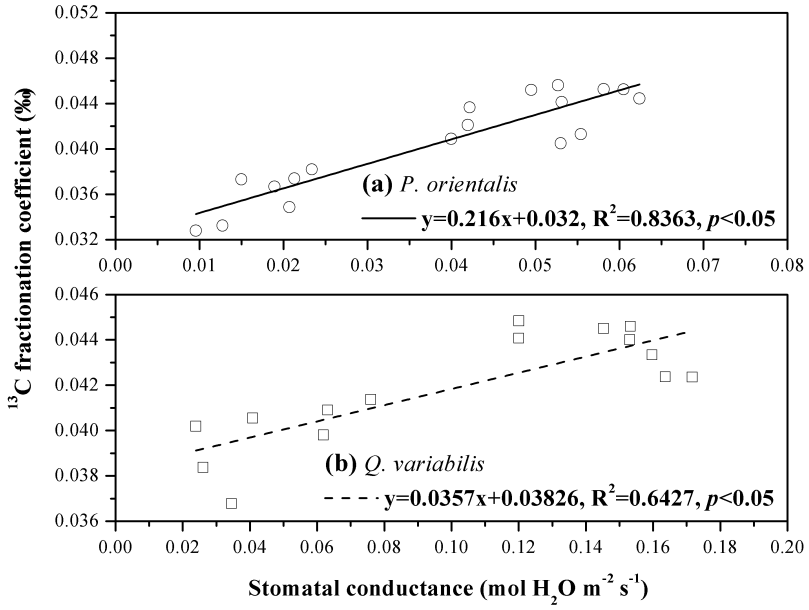

Figure 6. Regression between stomatal conductance and ^{13}C fractionation coefficient of *P. orientalis* and *Q. variabilis* under four CO_2 concentrations × five soil volumetric water contents are established in Figs. 6a and 6b. p=0.05, n = 32.

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635 **Table 1.** ^{13}C fractionation coefficients of *P. orientalis* and *Q. variabilis* under four CO_2
636 concentrations × five soil volumetric water contents.

^{13}C fractionation coefficients (‰)	CO_2 concentration (ppm)				
	SWC	400	500	600	800
Platycladus orientalis	35%–45% *FC*	0.0328	0.0373	0.0349	0.0332
	50%–60% *FC*	0.0367	0.0437	0.0382	0.0374
	60%–70% *FC*	0.0405	0.0366	0.0421	0.0409
	70%–80% *FC*	0.0444	0.0453	0.0413	0.0452
	100% *FC*	0.0441	0.0453	0.0456	0.0472
Quercus variabilis	35%–45% *FC*	0.0388	0.0402	0.0406	0.0384
	50%–60% *FC*	0.0433	0.0448	0.0409	0.0368
	60%–70% *FC*	0.0424	0.0440	0.0445	0.0414
	70%–80% *FC*	0.0424	0.0446	0.0482	0.0457
	100% *FC*	0.0441	0.0466	0.0466	0.0398

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