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Photosynthetic Response of an Alpine Plant, *Rhododendron delavayi* Franch, to Water Stress and Recovery: The Role of Mesophyll Conductance

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Rhododendron delavayi Franch is an evergreen shrub or small tree with large scarlet flowers that makes it highly attractive as an ornamental species. The species is native to southwest China and southeast Asia, especially the Himalayan region, showing good adaptability, and tolerance to drought. To understand the water stress coping mechanisms of *R. delavayi*, we analyzed the plant's photosynthetic performance during water stress and recovery. In particular, we looked at the regulation of stomatal (g_s) and mesophyll conductance (g_m), and maximum rate of carboxylation (V_{cmax}). After 4 days of water stress treatment, the net CO_2 assimilation rate (A_N) declined slightly while g_s and g_m were not affected and stomatal limitation (S_L) was therefore negligible. At this stage mesophyll conductance limitation (MC_L) and biochemical limitation (B_L) constituted the main limitation factors. After 8 days of water stress treatment, A_N, g_s, and g_m had decreased notably. At this stage S_L increased markedly and MC_L even more so, while B_L remained relatively constant. After re-watering, the recovery of A_N, g_s, and g_m was rapid, although remaining below the levels of the control plants, while V_{cmax} fully regained control levels after 3 days of re-watering. MC_L remained the main limitation factor irrespective of the degree of photosynthetic recovery. In conclusion, in our experiment MC_L was the main photosynthetic limitation factor of *R. delavayi* under water stress and during the recovery phase, with the regulation of g_m probably being the result of interactions between the environment and leaf anatomical features.

Keywords: mesophyll conductance, photosynthetic limitation, recovery, *Rhododendron delavayi*, stomatal conductance, water stress

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INTRODUCTION

Low water availability is considered as one of the main environmental factors limiting plant growth and productivity worldwide (Chaves et al., 2009). The majority of climate change scenarios predict an increase in drought incidents throughout the world (Lemke et al., 2007). Thus, the strategies of tolerance, adaption, and survival will be of major importance for plants growing under water stress. It has been shown that water stress primarily affects photosynthetic CO_2 assimilation, and

therefore limits plant productivity and growth (Flexas et al., 2006). The response of photosynthesis to water stress has received considerable attention in recent decades, especially the key factors limiting photosynthesis under water stress conditions (Flexas et al., 2002; Lawlor and Cornic, 2002). However, there is an on-going debate about whether the determinant for photosynthesis under water stress conditions is stomatal closure, diffusive resistance, or metabolic uncoupling (Lawlor and Cornic, 2002; Flexas et al., 2009, 2014; Pinheiro and Chaves, 2011; Campos et al., 2014; Chen et al., 2015).

Stomatal closure is generally considered as the initial and main cause of the decrease in photosynthesis under water stress conditions, as diffusion of CO_2 from the atmosphere to the sites of carboxylation in the chloroplast is impaired (Flexas et al., 2009; Rho et al., 2013). However, reduced leaf CO_2 diffusion conductance is not only due to stomatal closure, but also due to the decreased internal conductance of CO_2 diffusion (mesophyll conductance, g_m; Galmés et al., 2007; Zhou et al., 2014; Chen et al., 2015). As a result of the recognition of the importance of g_m, the studies pertaining to g_m have increased exponentially in recent years (Zhou et al., 2007; Flexas et al., 2009; Galle et al., 2009; Niinemets et al., 2009; Rancourt et al., 2015). Some studies suggested that g_m was consistently delayed by a few days compared with g_s (Rancourt et al., 2015), while other studies suggested that g_m can vary at least as fast as g_s, and g_m contributes greatly to the limitation of photosynthesis during water stress and recovery after water stress (Gomes et al., 2008). Recently, the work by Carriquí et al. (2015) suggested that g_s, and g_m were co-responsible for the lower photosynthesis observed in ferns as compared with angiosperms, and that g_m was the most constraining factor for photosynthesis in ferns. These findings support the idea of an important role for g_m in the photosynthetic responses of plants to climatic constraints.

Additionally, photosynthesis also decreases due to metabolic impairments and/or cell damage, especially under severe water stress combined with intensive light and high temperature (Flexas et al., 2006; Zhou et al., 2007). Under these circumstances, down-regulation of photosynthesis increases the production of reactive oxygen species (Takahashi and Murata, 2005) and even leads to photoinhibition (Massacci et al., 2008; Wang et al., 2012; Chastain et al., 2014). Furthermore, the carbon balance of a plant enduring a water stress period depends as much on the rate and degree of photosynthetic recovery (Flexas et al., 2006). The capability for photosynthetic recovery after exposure to water stress conditions is essential to understand the plant response to water stress, and to determine appropriate irrigation in agricultural practices. Many studies have addressed the response of photosynthesis to water stress, but the underlying process of photosynthetic recovery from water stress is poorly understood. In particular, knowledge about the factors limiting photosynthesis under these conditions, and their possible interactions with other environmental conditions are scarce (Flexas et al., 2009; Campos et al., 2014).

A previous study proposed a method which divides the total limitation into stomatal limitation (S_L), mesophyll conductance limitation (MC_L), and biochemical limitation (B_L; Grassi and Magnani, 2005). Galmés et al. (2007) found that MC_L was the main limiting factor for photosynthesis recovery after re-watering in 10 Mediterranean species. The results of Flexas et al. (2009) suggested that stomatal closure recovered much more slowly than g_m, thus photosynthesis recovery after re-watering was mostly limited by S_L. These findings underline the importance of g_m during water stress conditions, and further suggest an important contribution to the overall adaptation of plants to drought stress conditions. However, current knowledge about the physiological limitations to photosynthesis during short-term water stress and recovery after re-watering is scarce (Flexas et al., 2009, 2014). It would be necessary to apply this method to analyze photosynthetic limitations in plants subjected to water stress, especially in circumstances pertaining to a shorter period of water stress and recovery after re-watering.

Rhododendron is one of the most well-known alpine flowers. Of the approximately 571 *Rhododendron* species in China, 320 species are found within the Yunnan Province of southwestern China (Fang et al., 2005). Most of the above *Rhododendron* species are distributed in alpine areas and commonly are less constrained by water shortage. However, the five consecutive years of spring drought experienced in Southwest China since 2009 have had great adverse effects on the growth and flowering of *Rhododendron*. Furthermore, droughts frequently occur in winter and spring, water supply limitation is gradually becoming one of the dominant limitations for the growth and application of *Rhododendron*. Currently, little is known about the physiological responses during the process of water stress and the recovery after re-watering.

Rhododendron delavayi Franch is an evergreen shrub or small tree with large scarlet flowers that makes it highly attractive as an ornamental species. The species is native to southwest China and southeast Asia, especially the Himalayan region. The leaf of *R. delavayi* is leathery, and the abaxial surface with 1-layered, spongy or somewhat agglutinated, whitish to fawn indumentums (Fang et al., 2005). In our previous study, we found that the stomata of *R. delavayi* exist only on the abaxial surface. When compared with *R. irroratum* and *R. yunnanense* which grow under the same conditions, *R. delavayi* exhibited perfect adaptability and tolerance to dry and high radiation environments, including traits such as with smaller stomata, larger stomatal density, and higher ratio of palisade and spongy tissue (Cai et al., 2014). The aim of the present work was to evaluate the responses of photosynthesis to water stress and recovery, and analyze the main limiting factors of photosynthesis during water stress and recovery, emphasizing the leaf internal diffusive components. Our hypotheses were that: (1) excitation pressure imposed by water stress will cause a general decline of the photosynthetic performance; (2) down-regulation of mesophyll conductance to CO_2 may impose a similar or even greater limitation to photosynthesis than that imposed by stomatal closure during water stress treatment; (3) photosynthetic recovery after re-watering may be mostly limited by mesophyll conductance limitations.

MATERIALS AND METHODS

Plant Materials and Treatments

The experiment was carried out in a greenhouse in Kunming, China (a1t1926 m, E 102°46′, N 25°07′). Five-year-old plants of *R. delavayi* were grown in 9-L plastic pots (one plant per pot) filled with a mixture of red soil and humus (V/V, 1/3). The plants were housed in the experimental greenhouse under natural light and temperature conditions. From budbreak (20 March) to the beginning of the experimental period (25 June), the plants were irrigated three times per week to maintain sufficient water supply. Then, 30 plants were placed in the same greenhouse, and subdivided randomly into two groups: the control and the stressed plants. The control plants were irrigated daily to field capacity, while the treatment plants were not irrigated. Ten days after stopping the irrigation, the leaf stomatal conductance (g_s) decreased to 0.02 mol H_2O $m^{-2}s^{-1}$ (severe water stress), at which point all of the plants were re-watered to field capacity for recovery.

Relative Water Content (RWC)

Plant water status was assessed by relative water content (RWC) on the first whorl of the leaves. In order to determine RWC, four fresh leaves per replication were collected and their fresh weights (FW) were obtained. Next, these leaves were placed in water to float for 24 h at 4°C in the dark to obtain their turgid weights (TW). The leaves were then oven dried at 72°C for 48 h to obtain their dry weights (DW). RWC was determined by the formula: $RWC\,(\%) = (FW - DW)/(TW - DW) \times 100$ (de Souza et al., 2013).

Soil Moisture Content (SMC)

In order to determine soil moisture content (SMC), the soil of four pots per replication were collected in an aluminum box and their fresh weights (FW) determined. The soil sample was then oven dried at 105°C for 48 h to obtain their dry weights (DW). SMC was determined by the formula: $SMC\,(\%) = (FW-DW)/DW \times 100$.

Instantaneous Gas Exchange

Instantaneous gas exchange measurements were tested daily, between 12:00 and 13:00 h local time, using an open gas-exchange system (Li-6400XT; Li-Cor, Inc., Nebraska, USA) equipped with a light source (Li-6400-02B). No measurements were taken on day 6 due to technical problems with the Li-6400. All measurements were made on the young, fully expanded leaves at a saturating photosynthetic photo flux density (PPFD) of 1000 μmol $m^{-2}s^{-1}$, with a CO_2 concentration of 400 μmol mol^{-1} in the leaf cuvette. During the instantaneous measurements, net CO_2 assimilation rate (A_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), transpiration rate (T_r), air temperature (T_{air}), and leaf-to-air vapor pressure deficit (VPD) were recorded automatically by the Li-6400XT.

CO_2 Response Curve and Chlorophyll Fluorescence

The CO_2 response curve (A_N–C_i curves) and chlorophyll fluorescence were measured simultaneously with a combined open gas exchange system and chlorophyll fluorescence system (Li-6400-40; Li-Cor Inc., Nebraska, USA) on four specific sampling days for each treatment: day 4 and day 8 after stopping the irrigation, and then day 1 and day 3 after re-watering.

A_N–C_i curves were constructed as a function of the ambient CO_2 concentration (C_a) ranging from 50 to 2000 μmol mol^{-1}. Light intensity was maintained at 1000 μmol m^{-2} s^{-1}. The flow rate within the chamber was controlled at 500 mmol air min^{-1}, and the block temperature at 25°C. VPD was kept within a variation of 0.5 kPa during the performance of a single curve. At first, C_a in the leaf chamber decreased stepwise from 400 to 50 μmol mol^{-1}. After that C_a was returned to 400 μmol mol^{-1} to restore the original A_N. Then, C_a was increased stepwise until 2000 μmol mol^{-1} to complete the curve. The number of different C_a values used for the response curve was 13, and the time lag between two consecutive measurements at each C_a was restricted to 2–4 min. The A_N-C_i curves were used to estimate the maximum carboxylation rate of Rubisco (V_{cmax}), the maximum electron transport rate (J_{max}) used a utility developed by Sharkey et al. (2007), and was based on an alternative A_N-C_i curve fitting method through a non-rectangular hyperbola version of the model provided by Farquhar et al. (1980).

For the chlorophyll fluorescence measurements, the leaf was irradiated by an actinic radiation of 1000 μmol m^{-2} s^{-1} (90–10% red-blue light) for 15–20 min until stable photosynthesis occurred. Then the steady state fluorescence (F_s) was recorded and subsequently another saturating light pulse around 6000 μmol $m^{-2}s^{-1}$ was applied to determine the maximum fluorescence (F'_m). Actinic light was removed and the leaves were irradiated with far-red light to obtain F_o adapted to light (F'_o). From these values, the photochemical quenching (qP) was calculated as: $qP = (F'_m - F_s)/(F'_m - F'_o)$, the actual photochemical efficiency of photosystem II was calculated as: $(\Phi PSII) = (F'_m - F_s)/F'_m$; Genty et al., 1989), and the electron transport rate was calculated as: $(J_{flu}) = \Phi PSII \times PPFD \times 0.5 \times 0.85$ (Valentini et al., 1995).

After an adaptation period of 30 min in the dark, the minimum fluorescence (F_o) was measured with the light which was sufficiently low to avoid a photochemical reaction. The maximum fluorescence (F_m) was obtained by applying a saturating light pulse of 6000 μmol m^{-2} s^{-1} for 0.8 s. The maximum quantum efficiency of photosystem II (F_v/F_m) was calculated as: $F_V/F_m = (F_m - F_o)/F_m$(Genty et al., 1989).

Determination of Mesophyll Conductance

Mesophyll conductance (g_m) was calculated using the "variable" method as described by Harley et al. (1992):

$$g_m = \frac{A_N}{C_i - \frac{\Gamma^*(J_{flu} + 8(A_N + R_d))}{J_{flu} - 4(A_N + R_d)}}$$

where, A_N and C_i are taken from A_N–C_i curve, and J_{flu} was estimated from chlorophyll fluorescence on the same leaf, and

Γ^* was 37.43 μmol mol^{-1} at 25°C according to Bernacchi et al. (2002). R_d was respiration in the light and determined according to the method of Laisk (1977). g_m values were calculated for measurements of the net assimilation rate at a C_i level of 100–300 μmol mol^{-1}, and the average value of g_m was determined for each leaf. The calculated values for g_m were used to estimate the chloroplast CO_2 concentration (C_c) as: $C_c = C_i - (A_N/g_m)$

Photosynthetic Limitation Analysis

To compare photosynthetic limitations during water stress and recovery, the approach proposed by Grassi and Magnani (2005) was used to partition photosynthetic limitation into three components related to stomatal conductance (S_L), mesophyll conductance (MC_L), and leaf biochemical characteristics (B_L). This requires assuming a reference which had a maximum assimilation rate. In the current study, the maximum assimilation rate, concomitant with g_s, g_m, and V_{cmax}, was generally found under well-watered conditions. Therefore, the control treatment was used as a reference. According to this method, non-stomatal limitations were defined as the sum of the contributions of mesophyll conductance and leaf biochemistry ($NS_L = MC_L + B_L$), while the diffusive limitations were the sum of the contributions of the stomatal and mesophyll conductance ($D_L = S_L + MC_L$).

RESULTS

Experimental Conditions and Plant Water Stress

During the experiment VPD of the control and stressed plants remained mostly above 1.5 kPa. Air temperature (T_{air}) was between 23.9 and 37.9°C (**Figures 1A,B**). The highest VPD and T_{air} corresponded to the most severely stressed plants without irrigation for 10 days. For the three days (3, 7, and 11 days after the onset of the experiment) with clouds or rainfall, T_{air} values of all of the plants were close to 25°C, VPD also decreased regardless of the treatments.

During the experiment period, RWC in the control plants had some fluctuation, with an average of 82.8%. After 6 days of water stress, RWC in the stressed plants decreased slightly. After 9 days of water stress, RWC in the stressed plants decreased to 72.1%, and there was a significant difference between the control and stressed plants. After re-watering, RWC in the water stressed plants increased to values similar to those in the control plants (**Figure 1C**).

During the experiment period, SMC of the control plants was between 134.1 and 147.8%, while SMC of the stressed plants decreased significantly, from 147.8% at the beginning of the treatment to 25.3% after 9 days of water stress (**Figure 1D**).

Photosynthetic Responses to Water Stress and Recovery

A_N of the control plants oscillated during the experiment, from 15.2 μmol CO_2 $m^{-2}s^{-1}$ by day 4 to 10.5 μmol CO_2 $m^{-2}s^{-1}$ by day 7. Water stress caused a slight reduction in A_N from day 1 to day 7, and A_N of the stressed plants was more than 80% of the control plants. However, water stress resulted in a significant reduction in A_N by day 8, and the value of A_N rapidly decreased to 1.68 μmol CO_2 $m^{-2}s^{-1}$ by day 9, only 14% of the value of the control plants. After 1 d of re-watering, A_N was restored to 80% of the control value, but no further recovery of A_N was observed afterwards (**Figure 2A**).

Stomatal conductance (g_s) showed a similar variation to A_N. In the control plants, g_s were maintained above 0.20 mol CO_2 $m^{-2}s^{-1}$, with an average value of 0.22 mol CO_2 $m^{-2}s^{-1}$. The stressed plants had similar g_s values to the control plants from day 1 to day 4. Water stress caused a gradual decline in g_s from day 5 to day 8, and g_s values of the stressed plants were approximately 0.10 mol CO_2 $m^{-2}s^{-1}$ by day 8. However, g_s quickly declined to 0.02 mol CO_2 $m^{-2}s^{-1}$ by day 9. Like A_N, the recovery of g_s after re-watering was very quick, restored to 70% of the control value in the first day, and restored to 86% of the control plants after 3 days of re-watering (**Figure 2B**).

As a consequence of the decreased g_s during water stress treatment, C_i and T_r were also depressed (**Figures 2C,D**). It is worth noting that the change in C_i did not simply follow that in g_s. During the last 2 days of water stress treatment, C_i increased quickly and exceeded the control plants. After re-watering, C_i decreased and maintained lower values than that observed in the control plants.

When compared with the control plants, V_{cmax} and J_{max} in the stressed plants were reduced by 22 and 14% by day 4, respectively. However, V_{cmax} and J_{max} did not decrease further after 8 days of water stress treatment. After re-watering, V_{cmax} and J_{max} increased to the levels equivalent to that in the control plants, and even higher than the control plants after 3 days of re-watering (**Table 1**).

Mesophyll conductance (g_m) did not decline by day 4, but suddenly decreased to 53% of the control plants by day 8. However, g_m totally recovered during the first day of re-watering. As a consequence of the decreased g_m during water stress treatment, C_c was also depressed. The depression was more remarkable by day 8. After re-watering, due to the rapid recovery of g_m, C_c increased and was higher than the control plants during the first day after re-watering (**Table 1**).

Change in Chlorophyll Fluorescence During Water Stress and Recovery

The chlorophyll fluorescence parameters are shown in **Figure 3**. The values for F_v/F_m were kept above 0.82 throughout the experimental period, and were not significantly different between the control plants and stressed plants (**Figure 3A**). By day 4, the values for ΦPSII, qP, and J_{flu} of the stressed plants significantly declined, and further declined by day 8. After re-watering, the recovery of ΦPSII, qP, and J_{flu} was slight on the first day, and these recovered progressively to the levels seen in the control plants within 3 days (**Figures 3B–D**).

Photosynthetic Limitations During Water Stress and Recovery

After 4 days of water stress treatment, S_L was negligible, while MC_L and B_L imposed some limitations to photosynthesis. With

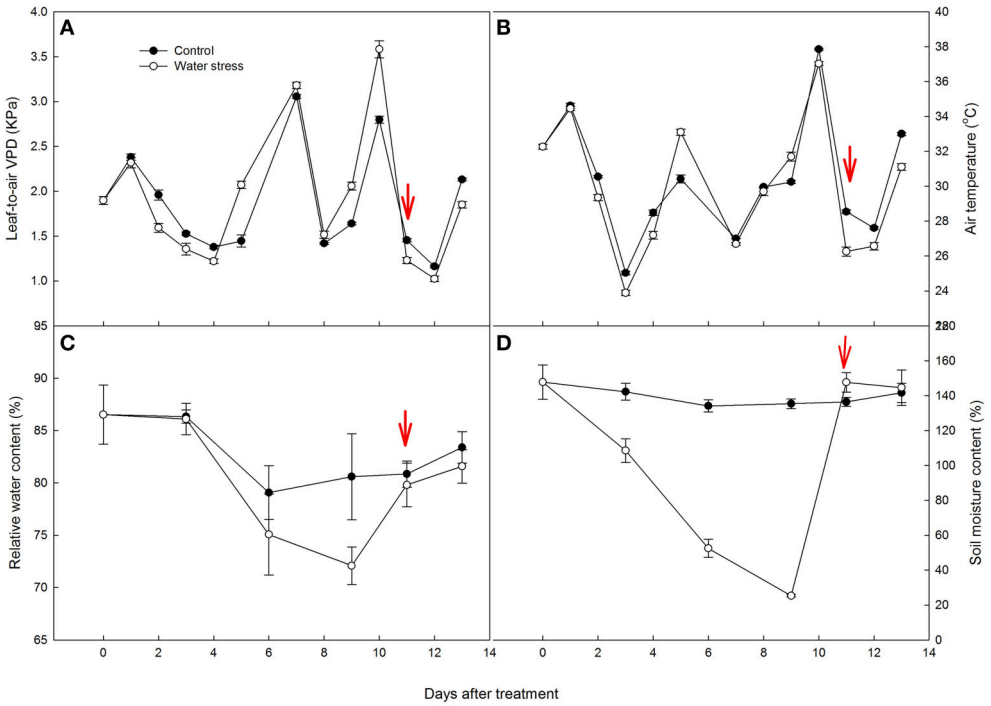

FIGURE 1 | Variations of (A) leaf-to-air vapor pressure deficit (VPD), (B) air temperature (T_{air}), (C) leaf relative water content (RWC), and (D) soil moisture content (SMC) during the experiment. The values represent means ± SE ($n = 10$ for **A,B**, $n = 4$ for **C,D**). Day 0 corresponds to the first day of water stress and the arrow indicates the start of re-watering.

the further imposition of water stress treatment, S_L and MC_L increased rapidly, while B_L did not increase by day 8. It is worth noting that MC_L was more than two times the level observed for S_L. After 1 d of re-watering, S_L and MC_L decreased rapidly, while B_L did not change. After 3 days of re-watering, S_L and B_L further declined, while MC_L remained stable, and thus MC_L was the main limitation to photosynthesis during recovery (**Table 2**).

DISCUSSION

Photosynthetic Responses to Water Stress and Recovery

The water stress-induced decline of A_N, g_s, and g_m was always slower than the recovery after re-watering. After 4 days of water stress treatment, A_N declined slightly, and there was almost no change in the values for g_s and g_m. As the duration of the water stress treatment increased, A_N, g_s, and g_m decreased markedly by day 8. After 9 days of water stress, the values of A_N was close to zero, g_s reached 0.02 mol CO_2 $m^{-2}s^{-1}$. There was no further reduction in the next continued 2 days drought. After re-watering for 1 day, no plant died and all of them showed some recovery. Results suggested that g_s can use as a reference parameter to justify the levels of water stress of *R. delavayi*, i.e., severe water stress (g_s near 0.02 mol CO_2 $m^{-2}s^{-1}$). Flexas et al. (2009) also use g_s to define the levels of water stress of *Vitis* hybrid Richter-110, i.e., moderate water stress (g_s near 0.15 mol CO_2 $m^{-2}s^{-1}$) and severe water stress (g_s near 0.05 mol CO_2 $m^{-2}s^{-1}$).

Once water stress was established and maintained, g_s was more stable than g_m. An intriguing behavior of g_m was a total recovery after irrigation for 1 day, while g_s and A_N were restored to above 70% of the control values. Throughout the periods of water stress and recovery, A_N and g_s of followed the same course, indicating a strong co-regulation of these parameters, as shown in many studies (Chaves et al., 2002; Lawlor and Cornic, 2002). However, with the further recovery of g_s and A_N, the values for g_m were not restored further, and even slightly decreased after re-watering for 3 days, indicating an independent regulation for g_s and g_m. Previous studies suggested that g_s appears to be more independent of environmental conditions except for VPD (Pou et al., 2008; Hanson et al., 2013), and the opening and closing of stomata is regulated by the integration of environmental signals and endogenous hormonal stimuli (Daszkowska-Golec and Szarejko, 2013). However, the response of g_m to water stress strongly depends on the water channels aquaporins (Daszkowska-Golec and Szarejko, 2013), and the impact of additional environmental factors, especially light condition (Zhou et al., 2007). Regulatory mechanisms such as the phosphorylation of aquaporins can be light dependent (Tournaire-Roux et al., 2003). The results of Galle et al. (2009) further showed that g_m declines considerably and recovers slightly under high light conditions; while under low light conditions it does not decrease under water stress. In the present study, the first day of re-watering was cloudy with rain, and T_{air} was close to 25°C with a decreasing VPD. Considered together with the present results, it seems that the adaptation of g_m to

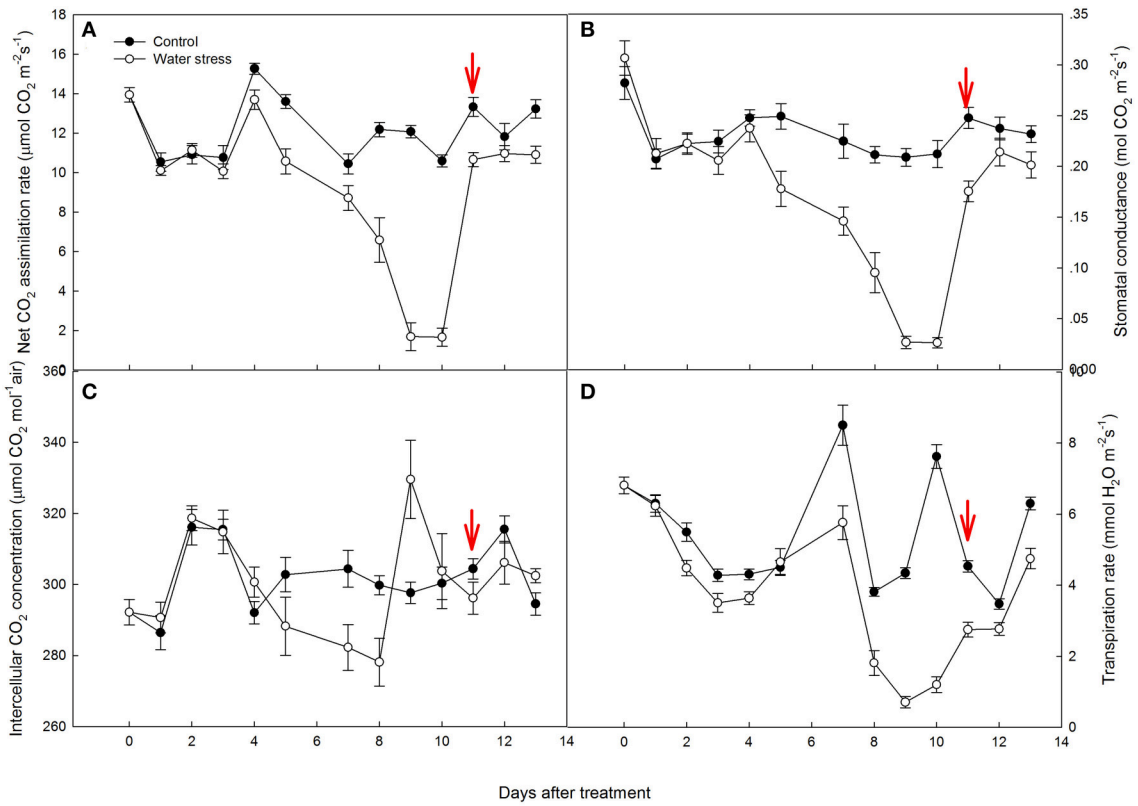

FIGURE 2 | Variations of (A) net CO_2 assimilation rate (A_N), (B) stomatal conductance (g_s), (C) intercellular CO_2 concentration (C_i), and (D) transpiration rate (T_r) during the experiment. Values represent means ± SE (n =10). Day 0 corresponds to the first day of water stress and the arrow indicates the start of re-watering.

water stress and its rapid recovery after rewatering is related to additional factors, such as light intensity and temperature, and suggests the necessity to better understand the regulation of mesophyll conductance, which conceivably depends on the environmental conditions.

In plants under water stress treatment, stomatal closure results in rapid decrease of g_s and A_N (Campos et al., 2014). However, non-stomatal factors were also important for the regulation of photosynthetic capacity, as reflected by both the reduction of V_{cmax} and J_{max}. The decrease in V_{cmax} is mostly due to the reduced activity of fructose-1,6-biphosphate phosphatase, as well as the decreased activity of Rubisco (Maroco et al., 2002; Bota et al., 2004). However, recent transcriptomic analysis in plants subjected to water stress showed that some genes related to Rubisco activase, Calvin cycle enzymes, and PSI and PSII, are conversely up-regulated during the acclimation to water stress (Cramer et al., 2007; Song et al., 2014). Proteomic analysis also confirmed that some photosynthetic proteins such as notably Rubisco and sedoheptulose 1,5-bisphosphatase, and mitochondrial glycine decarboxylase complex (GDC) protein were up-regulated (Vincent et al., 2007; Zhang et al., 2010). It has been verified that irrigated and water-stressed plants often show similar values for V_{cmax} and J_{max} (de Souza et al., 2005), and V_{cmax} remained almost unchanged both under water stress and recovery (Galle et al., 2009). In the present study, these two effects were observed. Firstly, the reduction of A_N, g_s, and g_m were accompanied by reductions in V_{cmax} and J_{max} under water stress conditions (**Table 1**), but the magnitudes of the reduction of V_{cmax} and J_{max} were much smaller than for A_N, g_s, and g_m. Secondly, A_N, g_s, and g_m remained below the levels of the control plants after 3 days of re-watering, but V_{cmax} and J_{max} were totally restored and even surpassed the control value. In accordance with the reports by Tang et al. (2013) and Galle et al. (2011), the increased V_{cmax} in response to the decreased C_c and g_m improved the photosynthetic capacity, and contributed to the maintenance of photosynthesis under water stress treatment, most notably, during recovery.

Indeed, chlorophyll fluorescence analysis also supported this conclusion. After 4 days of water stress treatment, decreases in J_{flu} and ΦPSII were observed, and indicated a decline in the quantum yield of the electron transport in PSII (de Souza et al., 2013), and limited the synthesis of ATP and the regeneration of RuBP (Lin et al., 2009), which caused the low activation of Rubisco (V_{cmax}). However, F_v/F_m was maintained between 0.82 and 0.85 throughout the experiment, indicating that PSII was quite resistant to water stress treatment. In addition, qP, J_{flu}, and ΦPSII were fully recovered after 3 days of re-watering. The above results suggest that water stress inevitably damaged the

TABLE 1 | Net CO_2 assimilation rate (A_N), stomatal conductance (g_s), Rubisco maximum carboxylation rate (V_{cmax}), maximum electron transport rate (J_{max}), mesophyll conductance (g_m) and chloroplast CO_2 concentration (C_c) under water stress and re-watering.

	A_N (μmol CO_2 $m^{-2}s^{-1}$)	g_s (mol CO_2 $m^{-2}s^{-1}$)	V_{cmax} (μmol CO_2 $m^{-2}s^{-1}$)	J_{max} (μmol e^{-1} $m^{-2}s^{-1}$)	g_m (mol CO_2 $m^{-2}s^{-1}$)	C_c (μmol CO_2 mol^{-1} air)
TREATMENTS AFTER WITHOUT IRRIGATION						
Control	15.26 ± 0.28^{a}	0.25 ± 0.01^{a}	33.71 ± 1.18^{a}	144.59 ± 5.83^{ab}	0.074 ± 0.013^{a}	121.43 ± 8.78^{a}
Stress (4)	13.69 ± 0.49^{b}	0.24 ± 0.01^{a}	26.34 ± 1.11^{bc}	124.92 ± 5.29^{c}	0.069 ± 0.003^{ab}	122.35 ± 15.86^{a}
Control	12.18 ± 0.36^{bc}	0.21 ± 0.01^{b}	32.62 ± 1.52^{a}	148.93 ± 1.88^{ab}	0.070 ± 0.012^{a}	120.35 ± 10.63^{a}
Stress (8)	6.59 ± 1.13^{d}	0.09 ± 0.02^{d}	24.74 ± 0.95^{c}	123.07 ± 3.48^{c}	0.037 ± 0.012^{b}	106.42 ± 1.37^{a}
TREATMENTS AFTER RE-WATERING						
Control	13.33 ± 0.49^{b}	0.25 ± 0.01^{a}	33.08 ± 3.64^{a}	153.75 ± 5.51^{a}	0.057 ± 0.014^{ab}	125.49 ± 6.92^{a}
Recovery (1)	10.66 ± 0.36^{c}	0.18 ± 0.01^{c}	27.23 ± 0.84^{bc}	118.87 ± 2.14^{c}	0.060 ± 0.013^{ab}	137.20 ± 10.41^{a}
Control	13.22 ± 0.46^{b}	0.23 ± 0.01^{ab}	30.21 ± 1.09^{abc}	128.51 ± 5.72^{c}	0.087 ± 0.019^{a}	122.09 ± 12.21^{a}
Recovery (3)	10.90 ± 0.43^{c}	0.20 ± 0.01^{bc}	30.40 ± 2.64^{ab}	135.67 ± 10.06^{bc}	0.054 ± 0.004^{ab}	103.03 ± 7.52^{a}

Data are means ± SE. Numbers in brackets on the first part show days of water stress whereas in the second part indicate days of re-watering. Different letters in the same column indicate statistical difference ($p < 0.05$).

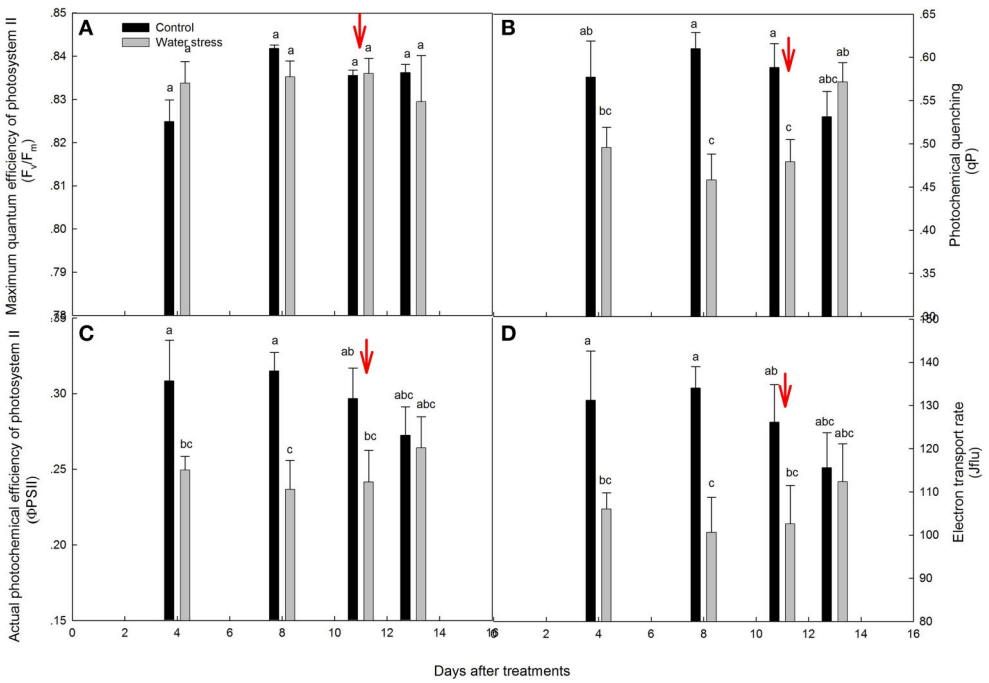

FIGURE 3 | Variations of (A) maximum quantum efficiency of photosystem II (F_v/F_m), (B) photochemical quenching (qP), (C) actual photochemical efficiency of photosystem II (ΦPSII), and (D) electron transport rate (J_{flu}) during the experiment. Values represent means ± SE ($n = 4$). Day 0 corresponds to the first day of water stress and the arrow indicates the start of re-watering.

light reactions, with possible damage to PSII functionality, but the occurrence of damage did not seem to be irreversible. The same trend has already been shown in some species, particularly in those showing increased paraheliotropism in response to water stress (Pastenes et al., 2005; Wang et al., 2012).

Photosynthetic Limitations During Water Stress and Recovery

The photosynthetic limitation analysis showed that S_L was negligible, and that MC_L and B_L were the main limitation factors after 4 days of water stress treatment (**Table 2**). This indicates that 4 days of water stress treatment did not affect the opening of the stomata, the internal transfer of CO_2, or the photosynthetic rate. With the continuing water stress treatment, A_N, g_s and g_m declined rapidly and significantly by day 8. At the same time, S_L and MC_L increased markedly, and the increase of MC_L was far greater than that of S_L, and thus MC_L became the main important limitation factor under water stress conditions. It is worth noting that g_m of *R. delavayi* was remarkably smaller than g_s, even in the control plants, although the differences became smaller as

TABLE 2 | Photosynthetic limitation of photosynthesis under water stress and re-watering.

Treatments	Limitations (%)					
	Stomatal limitation (S_L)	Mesophyll conductance limitation (MC_L)	Biochemical limitation (B_L)	Diffusive limitation (D_L)	Non-stomatal limitation (NS_L)	Total limitation (T_L)
Stress (4)	0.5	6.7	10.7	7.4	17.4	17.9
Stress (8)	11.9	27.9	8.0	39.8	35.9	47.8
Recovery (1)	4.0	11.1	8.8	15.1	19.9	23.9
Recovery (3)	2.5	16.9	3.6	19.4	20.5	23.0

Numbers in brackets show days of water stress or re-watering.

the water stress intensified. This phenomenon has been widely described in woody plants, especially for sclerophyllous species (Hanba et al., 2002; Warren and Dreyer, 2006; Galmés et al., 2007), although not in all cases. Previous studies indicate that leaf anatomical characteristics have an important role in driving the photosynthesis and the potential g_m (Tosens et al., 2012; Tomás et al., 2013). In particular, CO_2 diffusion through the mesophyll tissues is significantly limited by the cell wall thickness and the chloroplast envelope. Besides, the chloroplast surface area exposed to intercellular air spaces per leaf area have been proposed as major determinants of differences in g_m between species (Terashima et al., 2011; Tomás et al., 2013). Our previous study found that leaf anatomical characteristics of *R. delavayi* may have effects on g_m and CO_2 transfer, including traits such as smaller stomata, higher stomatal density, and a higher ratio of palisade to spongy mesophyll tissues (Cai et al., 2014). Also, other leaf anatomical characteristics might have contributed to the regulation of g_m, emphasizing the need for further investigations.

After re-watering for 1 day, g_m was almost complete recovery. g_s showed some recovery and not fully restored to the control level after 3 days of re-watering. The recovery extent largely depended on the species, from almost null (e.g., *Pistacia lentiscus*) to almost complete (e.g., *Limonium magallufianum*) after re-watering for 1 day (Galmés et al., 2007). The limitation analysis performed for recovery data revealed that the recovery of photosynthesis was most affected by MC_L rather than by S_L and B_L (**Table 2**). This result contrasts with the results of Ennahli and Earl (2005), who showed in cotton that, after severe water stress, recovery 24 h after re-watering was mostly caused by biochemical limitations, while stomatal and mesophyll limitations were almost totally absent. However, our results are in agreement with the results of Galmés et al. (2007), who showed that g_m was the most important factor limiting photosynthesis recovery after severe water stress treatment, regardless of the plant growth form and leaf anatomy. To our knowledge, there are few reports showing that limited recovery of g_m is the most important factor limiting photosynthesis recovery after a severe water stress. Our findings strongly reinforced the important role of g_m in response of photosynthesis in *R. delavayi* plants under water stress and recovery, and indicate the necessity of better understanding the regulation of g_m, which likely depend on the metabolism related to environmental conditions and leaf anatomy.

In conclusion, 4 days of water stress had little effect on A_N, g_s, and g_m of *R. delavayi*. After 8 days of water stress treatment, a marked stomatal closure and a decrease in A_N and g_m were observed. After re-watering, g_m recovered faster than A_N and g_s did. Photosynthetic limitation revealed that down-regulation of g_m was the main limitation factor both under water stress and recovery.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01089

Table S1 | Leaf relative water content (RWC) of *R. delavayi*.

Figure S1 | The leaf adaxial surface of *R. delavayi*.

Figure S2 | The leaf abaxial surface of *R. delavayi*.

Figure S3 | Recovery of the *R. delavayi* during the experiment was repeated in 2015, the red circles indicate survival plants after re-watering. Re-watering was done when the stomatal conductance reached 0.02 mol CO_2 $m^{-2}s^{-1}$ and the drought continued for 4 days.

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