Antecedent Drought Condition Affects Responses of Plant Physiology and Growth to Drought and Post-drought Recovery

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Antecedent environmental conditions may have a substantial impact on plant response to drought and recovery dynamics. Saplings of *Eucalyptus camaldulensis* were exposed to a range of long-term water deficit pre-treatments (antecedent conditions) designed to reduce carbon assimilation to approximately 50 (A50) and 10% (A10) of maximum photosynthesis of well-watered plants (A100). Thereafter, water was withheld from all plants to generate three different levels of water stress before re-watering. Our objective was to assess the role of antecedent water limitations in plant physiology and growth recovery from mild to severe drought stress. Antecedent water limitations led to increased soluble sugar content and depletion of starch in leaves of A50 and A10 trees, but there was no significant change in total non-structural carbohydrate concentration (NSC; soluble sugar and starch), relative to A100 plants. Following re-watering, A50 and A10 trees exhibited faster recovery of physiological processes (e.g., photosynthesis and stomatal conductance) than A100 plants. Nonetheless, trees exposed to the greatest water stress (−5.0 MPa) were slowest to fully recover photosynthesis (Amax) and stomatal conductance (gs). Moreover, post-drought recovery of photosynthesis was primarily limited by gs, but was facilitated by biochemistry (Vcmax and Jmax). During recovery, slow regrowth rates in A50 and A10 trees may result from insufficient carbon reserves as well as impaired hydraulic transport induced by the antecedent water limitations, which was dependent on the intensity of drought stress. Therefore, our findings suggest that antecedent water stress conditions, as well as drought severity, are important determinants of physiological recovery following drought release.

Keywords: *Eucalyptus camaldulensis*, drought, non-structural carbohydrate, photosynthesis, stem growth, post-drought recovery
INTRODUCTION

Understanding the impacts of drought stress on plant physiology and growth is essential for predicting the structure and function of plant communities (O’Brien et al., 2017a; Choat et al., 2018; Brodribb et al., 2020), especially within the context of global climate change in which drought episodes are projected to be more common in the future (Dai, 2013). Such impacts, however, can be complex to assess because the consequence of drought stress is a function of resistance and resilience (Stuart-Haëntjens et al., 2018; Li et al., 2020; Schmitt et al., 2020), and both components can be strongly modified by drought events that plants have previously experienced (i.e., antecedent drought) (Kannenberg et al., 2020). Adjustments in plant morphology and/or physiology triggered by antecedent, non-lethal water deficit can often result in divergent responses during subsequent drought stress, with potential impacts on plant performance during recovery (Kannenberg et al., 2020). This is particularly evident in field-grown trees, for which the growth response to recurring drought differs over time (Xu et al., 2016; O’Brien et al., 2017b; Wu et al., 2018; Anderegg et al., 2020). Although the immediate effects of drought stress on plant physiology have been well documented, knowledge gaps still exist on how drought response and post-drought recovery can be modified by antecedent drought conditions (Ruehr et al., 2019).

Leaves are the primary sites of CO$_2$ and water exchange for the majority of terrestrial plants. How leaves respond to and recover from drought stress can therefore have disproportionately critical effects on plants. Antecedent drought stress can generate multiple adjustments in leaf morphology, physiology, and biochemistry (Gessler et al., 2020). Biological processes related to growth, including cell division and enlargement, are highly sensitive to water stress, and are typically reduced prior to reductions in photosynthesis caused by water deficit (Chapin et al., 1990; Körner, 2015). Consequently, an increase in non-structural carbohydrates (NSCs) is commonly observed in plants subjected to drought stress due to an imbalance in source-sink strength (Sala and Hoch, 2009; Sala et al., 2012; Granda and Camarero, 2017; Piper et al., 2017). Additionally, drought induced variation in tissue NSCs contents may occur in all organs, and the importance of sugar enriched organ to plant performance during water deficit and post-drought recovery can be markedly species-specific (Hagedorn et al., 2016; Joseph et al., 2020; Ouyang et al., 2021). Here, we primarily focus on leaves, the NSCs variation of which has been shown to predominantly regulates plant response to drought stress in some species (Martínez-Vilalta et al., 2016; Signori-Müller et al., 2021), and dominates the variation of whole plant carbohydrates contents in *Eucalyptus* species (Duan et al., 2013). Depending on its specific type (i.e., soluble sugar and starch), NSCs, together with other secondary metabolites derived from their soluble form, can be involved in physiological processes related to drought resistance such as osmotic regulation, or can be stored as carbon reserves for regrowth upon alleviation of drought stress (Sala et al., 2012; Trifilò et al., 2017; Tomasella et al., 2020). Hence, variation in NSCs induced by antecedent drought conditions can have important implications for the fate of plants during subsequent drought and recovery cycles.

Of the numerous physiological processes impacted by water limitation, photosynthesis is extensively studied because of its central role in determining plant fitness and sensitivity to water deficit (Flexas et al., 2006a). Leaf photosynthetic rate is co-determined by stomatal conductance and biochemical components of photosynthesis. During drought stress, leaf stomata will partially or completely close to minimize water loss. Given that CO$_2$ and water molecules share the same pathway at both leaf and cellular levels (e.g., aquaporins), reduced stomatal conductance ($g_s$) will inevitably constrain photosynthesis (Flexas et al., 2008) and is considered to be the major cause for downregulation of photosynthesis during drought stress (Flexas et al., 2004; Grassi and Magnani, 2005; Zait et al., 2019). On the other hand, the biochemical components of photosynthesis, including the capacity for Rubisco carboxylation ($V_{cmax}$) and electron transport ($J_{max}$), are generally less sensitive to dehydration, although a body of literature has shown that both biochemical components can be compromised during drought, and can show similar or even higher drought sensitivity compared to $g_s$ (Zhou et al., 2014). Identifying the key limiting factor for photosynthesis during drought stress and recovery is of utmost importance, especially for modeling vegetation dynamics using ecosystem models. However, acclimation may occur during antecedent drought, thereby generating different physiological responses to subsequent stress conditions (Ruehr et al., 2019; Gessler et al., 2020). Antecedent drought conditions may also alter the relative contribution of each limiting factor during subsequent droughts (Flexas et al., 2009; Menezes-Silva et al., 2017).

The capacity to recover physiological function following the easing of drought stress upon re-watering represents a key dimension of plant drought resilience. However, little is known about the post-drought dynamics and interaction of two fundamental physiological processes, i.e., carbon and water relations (Ruehr et al., 2019). For a given species, the time required for recovery is typically contingent on the severity of drought stress given it determines the extent to which physiological functions are impaired (Flexas et al., 2004; Blackman et al., 2009; Brodribb and Cochard, 2009). With respect to post-drought carbon-relations, it has been shown that stomatal limitation of photosynthesis is prevailing when drought stress is mild or moderate, while biochemical limitation becomes more prominent as drought stress is exacerbated (Flexas et al., 2006a). This is anticipated to result in different recovery dynamics given that reopening of stomata is typically faster than the time it takes to repair photosynthetic machinery after water supply is resumed (Ruehr et al., 2019). Furthermore, rates of photosynthetic recovery from drought are determined by drought severity and its impact on hydraulic function. Recent studies emphasize the pivotal role of water relations in governing plant mortality during drought stress, showing that recovery is no longer possible once the water status has exceeded thresholds of hydraulic impairment due to embolism (Blackman et al., 2009; Brodribb and Cochard, 2009; Choat et al., 2018). Furthermore, plant hydraulics is also intimately coupled with carbon through its impacts on leaf gas
exchange (Brodribb and Holbrook, 2003; Skelton et al., 2017). Thus, assessing the interaction of antecedent drought conditions and drought severity on the recovery dynamics of photosynthesis is crucial for gaining a comprehensive understanding of impacts caused by droughts.

Early experiments studying the influences of drought on plant physiology often subject plants to drought stress differing in either duration (i.e., short or long-term) or severity (i.e., mild to severe), and mainly focused on physiological responses triggered by water limitation (Menezes-Silva et al., 2017). Here, we take a different approach by exposing plants to two drought cycles characterized by different physiological attributes before re-watering and assessing recovery. The first drought was chronic and relatively mild, which was aimed at reducing carbon assimilation and manipulating NSC storage, but not inducing significant impairment in hydraulic function. The second drought was more acute and designed to induce a range of stress levels associated with moderate to severe leaf hydraulic dysfunction. Our aim was to test the potential influences of antecedent drought conditions (i.e., the first drought stress) on the subsequent drought response and eventually the rate of recovery, with particular emphasis on carbon and water relations. We hypothesized that: (1) long-term, mild drought stress will but promote the accumulation of leaf NSC due the hysteresis in ceasing of photosynthesis relative to growth; (2) leaf photosynthesis in plants exposed to antecedent drought conditions will be less affected by subsequent drought than control plants; (3) both stomatal and non-stomatal limitations will restrict the rate of recovery of photosynthesis following re-watering; and (4) the time required for regaining physiological function is dependent on the severity of the drought.

MATERIALS AND METHODS

Plant Material and Experimental Design

_Eucalyptus camaldulensis_, commonly known as river red gum, is one of the most widely distributed tree species across Australia. _E. camaldulensis_ can adapt to diverse climatic conditions, ranging from tropical to temperate ecosystems. At the local scale, the distribution of _E. camaldulensis_ is primarily confined to locations where soil water availability is ample, such as riverbanks or floodplains, where it often appears as the dominant species, providing significant ecological functional services (Butcher et al., 2009; Doody et al., 2015).

Seeds of river red gum (_E. camaldulensis_ subsp. _camaldulensis_) were obtained from the Australian Tree Seed Centre (Canberra, ACT) and germinated in forestry tubes placed in a sunlit poly-tunnel provided by Greening Australia (Richmond, NSW, Australia) for 2 months. On October 20, 2017, 40 similarly sized seedlings (approximately 15 cm tall) were transplanted into 15 × 40 cm (diameter × height) cylindrical pots filled with ca. 9 kg of moderately fertile sandy loam soil. Twenty pots were randomly selected and placed in one of the two adjacent naturally sunlit glasshouse bays located at the Hawkesbury Campus, Western Sydney University, Richmond, NSW Australia. Environmental conditions within both glasshouse bays were controlled, with temperature set to 26/18°C (day/night), which represents the local average air temperature during the growing season, and air CO₂ concentration set to 400 µmol mol⁻¹. This environmental condition was maintained throughout the experimental period. All seedlings were irrigated daily and fed with slow-release fertilizer biweekly (Osmocote, All-Purpose, Scotts, Australia) to ensure the absence of water and nutrient limitations.

Starting on November 28, 2017, 30 plants with similar height and basal diameter (75 cm and 0. 5 cm, respectively) were selected for the experiment. Plants were evenly divided into three groups, so that we could apply three levels of drought in the first round of drought treatment (i.e., antecedent drought condition). Levels of antecedent drought conditions were defined based on the photosynthetic rate measured under saturating light and ambient air CO₂ conditions (_A_\text{max} \text{L}), with _A_\text{max} of stressed plants maintained at approximately 50 (A\text{S}_\text{S}) and 10% (A\text{S}_\text{S}) relative to that of the well-watered plants (A\text{W}_\text{W}). For A\text{S}_\text{S} and A\text{W}_\text{W} plants, drought stress was imposed by withholding irrigation. Leaf _A_\text{max} was monitored daily for each individual until the desired values were achieved. Pots were labeled and weighed immediately. During the period of antecedent drought treatment, pot-specific soil water content was maintained by weighing individual pots daily in the morning and replacing the water loss from the previous day. Leaf gas exchange was measured every 1–3 days during the same period to ensure that the targeted _A_\text{max} could be maintained. It has been shown that complete stomatal closure in _E. camaldulensis_ typically occurs when leaf water potential reaches approximately −1.5 MPa (Zhou et al., 2014), which is less negative than the water potential threshold for the incipient hydraulic dysfunction (see below). Given that the stomata remained open during the phase of antecedent drought treatment, no hydraulic impairment was likely incurred by the intensity of drought stress.

After 48 days of antecedent drought treatment, plants in each drought treatment group were further divided into three sub-groups to implement the second round of drought treatment, so that each sub-group contained 3–4 individuals. The second drought treatment aimed to generate dysfunction in water transport and was therefore applied based on previously determined hydraulic vulnerability curves of leaves. Hence, irrigation water was withheld until midday leaf water potential (Ψ\text{leaf}) reached ca. −3.5, −4.5, or −5.0 MPa for the three drought groups; these values corresponded to the Ψ\text{leaf} at which 12, 50, and 88% loss of leaf hydraulic conductivity (K\text{leaf}) occurred in this species (Blackman et al., unpublished data). For each individual, Ψ\text{leaf} was measured once daily. In the controlled environment of the glasshouse bays, the target Ψ\text{leaf} was typically achieved in 1–3 days. Once the targeted Ψ\text{leaf} was attained, photosynthetic CO₂ response (AC) curves were measured; thereafter, the pot was re-watered to field capacity to alleviate the water stress and allow for recovery. Physiological measurements, including Ψ\text{leaf} and AC curves were repeated on 3, 7 and 14 days following re-watering during the recovery phase to assess the degree of physiological recovery over time.
Gas Exchange Measurements

Leaf gas exchange measurements, including $A_{\text{max}}$, stomatal conductance ($g_s$), and $A_{\text{Ci}}$ curves were measured between 9 am and noon with up to four cross-calibrated open gas exchange systems (LI-6400XT, Li-Cor, Lincoln, NE, United States) equipped with red-blue LED light sources (6400-02B) and an external CO$_2$ injector (6400-01). For each individual plant, one upper canopy, fully expanded leaf was tagged and consistently used for gas exchange measurements throughout the experiment. For spot measurements, gas exchange variables including $A_{\text{max}}$ and $g_s$ were recorded under saturating light (i.e., 1,500 µmol m$^{-2}$ s$^{-1}$). Cuvette CO$_2$ concentration and temperature were set to match the ambient conditions (i.e., 400 µmol mol$^{-1}$ and 26°C, respectively). Leaf $A_{\text{Ci}}$ curves were generated by recording $A_{\text{max}}$ under varying cuvette CO$_2$ concentrations: 400, 300, 200, 100, 400, 600, 800, 1,000, and 1,200 µmol mol$^{-1}$, with other environmental parameters identical to the spot measurements. Before each measurement, leaves were allowed to acclimate in the cuvette for up to 20 min and data were recorded after the readings were stable. During all gas exchange measurements, relative humidity and leaf-to-air water vapor deficit in the cuvette typically varied between 50 and 60% and 1.5–2.0 kPa, respectively.

Leaf Non-structural Carbohydrate Concentrations

Leaf carbohydrate content was assessed at the beginning and the end of the antecedent drought stress phase. Within each antecedent drought treatment group, 2–4 upper canopy
leaves were collected for each plant from 4 randomly selected individuals. The leaf samples were immediately oven-dried at 80°C for 24 h to stop metabolism and then stored in -20°C freezer until measurement. Leaf starch and soluble sugars concentrations were determined following the protocol described by Duan et al. (2013). Concisely, dried materials were grounded to a fine powder in a ball mill, and then weighed for 20 mg. Samples were extracted with 80% aqueous ethanol, then boiled at 95°C for 30 min and centrifuged at 3,000 rpm for 5 min. Supernatant was collected, re-extracted with ethanol and water, and then subjected to the procedures described above. The final supernatant was reserved and evaporated at 40°C using a rotational vacuum concentrator (RVC 2-25 CD, Christ, Germany). Starch content (mg g\(^{-1}\)) was determined from the remaining pellets after extraction and assayed with a total starch assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland). Total soluble sugar content (mg g\(^{-1}\)) was measured from the supernatants by the anthrone method. Total NSCs content (mg g\(^{-1}\)) was calculated as the sum of soluble sugar and starch.

**Growth Measurements**

To minimize canopy leaf loss, leaf samples used for \(\Psi_{\text{leaf}}\) determination were measured for leaf area using leaf area meter (LI-3100, Li-Cor, Lincoln, NE, United States). Samples were then oven-dried for at least 72 h to constant mass, and leaf mass per area (LMA, g m\(^{-2}\)) was calculated as the ratio of dry mass to leaf area.

Plant height (H, cm) and basal diameter (D, mm) were measured biweekly throughout the experimental period. Basal diameter was measured using digital calipers at ca. 3 cm above soil level, while plant height was considered as the distance from the soil level to the apex of the main stem. Stem volume was estimated as \(H \times D^2\) (cm\(^3\)), and stem volume index growth rate (VIGR, cm\(^3\) day\(^{-1}\)) was calculated as the increment of stem volume between measurement periods divided by the number of days.

**Data Analysis**

Leaf \(A_C\) curve data were fitted to the FvCB model using \(fitaci\) function of the Planteccphys R Package to estimate the maximum carboxylation rate of Rubisco (\(V_{c\text{max}}, \mu\text{mol m}^{-2} \text{s}^{-1}\)) and electron transport rate (\(J_{\text{max}}, \mu\text{mol m}^{-2} \text{s}^{-1}\)) (Duursma, 2015). The speed of recovery is assessed by calculating the time required for specific physiological variables to regain 50% of its maximum (\(t_{1/2}\), day), according to Brodribb and Cochard (2009). In short, the percentage of recovery relative to its maximum, measured from each individual within the same treatment group, was pooled together and was plotted against the corresponding number of days. The relationship was fitted using a linear regression and \(t_{1/2}\) was then extrapolated from the linear function. The estimation of \(t_{1/2}\) was limited to \(A_{\text{max}}\) and \(g_s\) given that the reduction in other physiological variables never surpassed 50% of their maximum.

Leaf NSCs, including the content of soluble sugar, starch and total NSCs content at the end of antecedent drought treatment, were analyzed using one-way ANOVA to test the difference among treatment groups. Physiological data including the \(A_{\text{max}}\), \(g_s\), \(V_{c\text{max}}\), and \(J_{\text{max}}\) measured during the recovery phase were presented as the percentage relative to their corresponding values measured prior to the implementation of the antecedent drought treatment. All time-series data were analyzed using the one-way repeated measurement ANOVA with the ezANOVA function in the ezANOVA package, with the levels of drought treatment and time of measurement considered as between- and within-subject factors, respectively. In addition, within each measurement time point, one-way ANOVA was used to test the difference among antecedent drought treatment groups. Following all one-way ANOVA, Tukey’s HSD post hoc was applied using the HSD.test function in the agricolae package for comparison among means. All data were tested for normality and homogeneity of variance before statistical analysis was performed. Statistically significant differences were considered when \(p \leq 0.05\). Data process and analysis were performed under R 3.5.3 statistical computing environment (R Core Team, 2013).

**RESULTS**

**Effects of Antecedent Drought Conditions on Leaf Photosynthesis, Carbohydrate Content, and Growth**

Leaf photosynthetic rate under saturating light (\(A_{\text{max}}, \mu\text{mol m}^{-2} \text{s}^{-1}\)) differed significantly across levels of antecedent drought conditions (Figure 1A; \(p < 0.01\)). Consistent with our objective, \(A_{\text{max}}\) in drought-stressed plants (i.e., A\text{50} and A\text{10}) was constantly maintained at the desired levels during the period of antecedent drought treatment. Interaction between time and drought treatment was observed, but only limited to the first day of measurement, with \(A_{\text{max}}\) of A\text{10} being slightly higher (ca. 4.0 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)) than that of A\text{100} and A\text{50}.

Contents of leaf NSCs, including soluble sugar (Figure 1B), starch (Figure 1C), and total NSCs (Figure 1D) did not differ across groups before drought treatments were applied. Differences in these variables were observed after 48 days of water restriction during the antecedent treatment. Soluble sugar of A\text{10} (12.9 ± 0.5 mg g\(^{-1}\)) and A\text{50} (10.9 ± 0.6 mg g\(^{-1}\)) plants were significantly higher than that of A\text{100} plants (8.4 ± 0.4 mg g\(^{-1}\); \(p = 0.02\)). Starch content also differed significantly across groups but exhibited an inverse pattern (\(p = 0.03\)), with starch content being 9.3 ± 0.5 mg g\(^{-1}\), 13.2 ± 0.7 mg g\(^{-1}\) and 14.6 ± 1.7 mg g\(^{-1}\) for A\text{10}, A\text{50}, and A\text{100} plants. Growth rate was strongly impeded by drought treatment (\(p < 0.01\); data not shown). The volume index growth rates (VIGR, cm\(^3\) day\(^{-1}\)) were 5.23 ± 0.31 cm\(^3\) day\(^{-1}\), 0.99 ± 0.11 cm\(^3\) day\(^{-1}\) and 0.39 ± 0.07 cm\(^3\) day\(^{-1}\) for A\text{100}, A\text{50}, and A\text{10} plants, respectively.

**Responses of Leaf Photosynthesis and Water Relations to Subsequent Drought Stress**

All physiological variables, including \(A_{\text{max}}\), light saturated stomatal conductance (\(g_{\text{max}}, \mu\text{mol m}^{-2} \text{s}^{-1}\)), carboxylation rate of Rubisco (\(V_{c\text{max}}, \mu\text{mol m}^{-2} \text{s}^{-1}\)) and electron transport rate (\(J_{\text{max}}, \mu\text{mol m}^{-2} \text{s}^{-1}\)) were significantly decreased by
subsequent drought stress (Figure 2 and Table 1; day 0). In general, the percentage loss of function from maximum values for each physiological parameter, increased with drought severity; however, there was an exception in $V_{\text{cmax}}$ and $J_{\text{max}}$, where the relative values for plants at $-4.5$ MPa were 25.4 and 15.4% higher, respectively, than plants at $-3.5$ MPa. All physiological variables in plants subjected to antecedent drought conditions, and subsequently to variable drought stress, were less affected compared with control plants that were well-watered and did not experience an antecedent drought stress (Figure 2).

**Recovery Dynamics of Leaf Photosynthesis, Water Relations, and Plant Growth After Re-watering**

Recovery in photosynthetic variables was dependent on the degree of antecedent water deficit and drought severity (Figures 2, 3 and Table 1). For $A_{\text{max}}$ and associated components (i.e., $g_s$, $V_{\text{cmax}}$ and $J_{\text{max}}$), $A_{50}$ and $A_{10}$ plants consistently showed faster rates of recovery compared to $A_{100}$ plants; these effects were stronger as the intensity of subsequent drought stress increased. For example, for plants at $-4.5$ MPa, $A_{\text{max}}$, $V_{\text{cmax}}$ and $J_{\text{max}}$ attained nearly or complete recovery on the third day of re-watering, whereas full recovery of these variables in $A_{100}$ plants was much slower; e.g., $A_{\text{max}}$ and $J_{\text{max}}$ did not reach the values measured before the implementation of the antecedent drought treatments even after 14 days of re-watering (Figures 2E,G,H). Notably, for $V_{\text{cmax}}$ and $J_{\text{max}}$, the values during the recovery phase were up to 50% higher than their pre-stress levels, suggesting a compensatory response to the previous drought stress.

Upon re-watering, leaf water potential ($\Psi_{\text{Leaf}}$; MPa) recovered rapidly, reaching pre-stress values within 3 days in most plants, regardless of antecedent and subsequent drought treatment (Figure 4). Across drought treatment, $\Psi_{\text{Leaf}}$ of plants exposed to antecedent drought was generally less negative than that of control plants, although the difference was not significant.

Upon re-watering and release of the drought stress, all plants showed stem regrowth, but with differing rates of growth. For plants dried to $-3.5$ and $-4.5$ MPa, VIGR of $A_{100}$ plants was significantly higher than that of $A_{50}$ and $A_{10}$ plants (Table 2; $p < 0.05$ in both cases), while VIGR did not vary across levels of antecedent drought treatment when exposed to severe water stress ($p = 0.17$).

**The Relationships Between Recovery of Leaf Photosynthesis and Associated Physiological Variables**

Recovery of leaf carbon assimilation was closely related to variables associated with CO$_2$ diffusion and biochemistry.
TABLE 1 | Statistical analysis for the difference in the percentage of recovery in maximum photosynthetic rate ($A_{\text{max}}$), stomatal conductance ($g_{s}$), maximum carboxylation rate of Rubisco ($V_{\text{cmax}}$), maximum electron transport rate ($J_{\text{max}}$), and midday leaf water potential ($\Psi_{\text{leaf}}$) across different drought treatments (i.e., $-3.5$, $-4.5$, and $-5.0$ MPa) during the phase of recovery (i.e., day 0, 3, 7, and 14).

| Traits | Treatment (MPa) | Days of recovery |
|--------|-----------------|-----------------|
|        |                 | **Day 0** | **Day 3** | **Day 7** | **Day 14** |
|        | **SS** | **F-value** | **p** | **SS** | **F-value** | **p** | **SS** | **F-value** | **p** | **SS** | **F-value** | **p** |
| $A_{\text{max}}$ | -3.5 | 1,480.42 | 8.28[$^2,6]$ | 0.02 | 74.60 | 0.36[$^2,6]$ | 0.71 | 213.61 | 1.50[$^2,6]$ | 0.30 | 647.07 | 3.16[$^2,6]$ | 0.12 |
|        | -4.5 | 1,085.27 | 14.16[$^2,7]$ | $<0.01$ | 5,444.10 | 13.00[$^2,7]$ | $<0.01$ | 2,926.70 | 18.34[$^2,7]$ | $<0.01$ | 2,761.74 | 14.25[$^2,7]$ | $<0.01$ |
|        | -5.0 | 2,051.82 | 24.57[$^2,6]$ | $<0.01$ | 2,286.50 | 17.75[$^2,6]$ | $<0.01$ | 660.54 | 3.93[$^2,6]$ | 0.08 | 1,843.29 | 12.21[$^2,6]$ | $<0.01$ |
| $g_{s}$ | -3.5 | 206.62 | 8.49[$^2,6]$ | 0.02 | 590.78 | 7.75[$^2,6]$ | 0.02 | 666.74 | 2.57[$^2,6]$ | 0.16 | 1,051.10 | 2.39[$^2,6]$ | 0.17 |
|        | -4.5 | 28.31 | 4.17[$^2,7]$ | 0.07 | 2,338.60 | 6.56[$^2,7]$ | 0.03 | 4,182.90 | 3.84[$^2,7$] | 0.08 | 11,410.70 | 12.40[$^2,7$] | $<0.01$ |
|        | -5.0 | 156.82 | 17.52[$^2,6]$ | $<0.01$ | 2,154.52 | 11.63[$^2,6$] | $<0.01$ | 1,290.70 | 3.15[$^2,6$] | 0.12 | 3,678.90 | 10.63[$^2,6$] | 0.01 |
| $V_{\text{cmax}}$ | -3.5 | 4,267.40 | 18.86[$^2,6]$ | $<0.01$ | 1,691.20 | 4.88[$^2,6$] | 0.06 | 197.81 | 2.06[$^2,6$] | 0.21 | 215.01 | 3.83[$^2,6$] | 0.32 |
|        | -4.5 | 5,461.40 | 14.96[$^2,7$] | $<0.01$ | 5,689.90 | 11.17[$^2,6$] | $<0.01$ | 1,903.70 | 10.20[$^2,7$] | 0.01 | 5,620.60 | 28.28[$^2,7$] | $<0.001$ |
|        | -5.0 | 2,148.16 | 13.75[$^2,6$] | $<0.01$ | 1,543.22 | 9.49[$^2,6$] | 0.01 | 2,906.22 | 16.11[$^2,6$] | $<0.01$ | 1,064.80 | 4.77[$^2,6$] | 0.06 |
| $J_{\text{max}}$ | -3.5 | 6,181.50 | 19.36[$^2,6$] | $<0.01$ | 7,648.80 | 9.03[$^2,6$] | 0.02 | 599.52 | 4.34[$^2,6$] | 0.07 | 588.69 | 10.66[$^2,6$] | 0.01 |
|        | -4.5 | 1,678.40 | 1,678.40[$^2,6$] | 0.19 | 5,729.80 | 8.66[$^2,7$] | 0.02 | 2,536.37 | 11.80[$^2,7$] | $<0.01$ | 7,610.30 | 19.41[$^2,6$] | $<0.01$ |
|        | -5.0 | 3,821.10 | 31.14[$^2,6$] | $<0.001$ | 2,457.65 | 12.08[$^2,6$] | $<0.01$ | 4,486.60 | 19.31[$^2,6$] | $<0.01$ | 2,183.80 | 10.77[$^2,6$] | 0.01 |
| $\Psi_{\text{leaf}}$ | -0.5 | 0.03 | 3.23[$^2,6$] | 0.11 | 0.63 | 8.58[$^2,6$] | 0.02 | 1.16 | 13.41[$^2,6$] | $<0.01$ | 0.37 | 6.49[$^2,6$] | 0.03 |
|        | -4.5 | 0.01 | 1.36[$^2,6$] | 0.33 | 1.92 | 7.59[$^2,6$] | 0.02 | 2.71 | 36.83[$^2,6$] | $<0.001$ | 0.06 | 1.20[$^2,6$] | 0.37 |
|        | -5.0 | 0.01 | 0.36[$^2,6$] | 0.71 | 0.63 | 9.39[$^2,6$] | 0.01 | 0.68 | 20.40[$^2,6$] | $<0.01$ | 0.07 | 2.45[$^2,6$] | 0.17 |

Presented statistical outcomes including sum of squares (SS), $F$-value, and calculated value of probability ($p$). Subscripted numbers following the $F$ values indicate degrees of freedom for between and within group factors. Post hoc analysis was applied when $p < 0.05$ with the resulting being reported in Figure 2.
FIGURE 3 | Time (day) required for physiological traits (A) maximum photosynthetic rate; (B) stomatal conductance) regain 50% of their maximum value following rewatering across different pretreatment ($A_{100}$, $A_{50}$, and $A_{10}$) and drought treatment levels ($-3.5$, $-4.5$, and $-5.0$ MPa).

FIGURE 4 | Recovery of midday leaf water potential ($\Psi_{\text{leaf}}$) from $-3.5$ MPa (A), $-4.5$ MPa (B), and $-5.0$ MPa (C) during the recovery phase. Colors denote levels of antecedent drought conditions (i.e., $A_{100}$, $A_{50}$, and $A_{10}$). Error bars indicate standard error of mean ($n = 3–4$).

TABLE 2 | Volume index growth rates ($cm^3$ day$^{-1}$; VIGR) of plants across all drought treatments during the recovery phase.  

| Pre-treatment | Drought treatment |
|---------------|-------------------|
|               | $-3.5$ MPa | $-4.5$ MPa | $-5.0$ MPa |
| $A_{100}$     | $6.63 \pm 0.85$  | $5.10 \pm 0.69$  | $3.21 \pm 0.08$  |
| $A_{50}$      | $3.36 \pm 0.47$  | $3.91 \pm 0.42$  | $3.21 \pm 0.38$  |
| $A_{10}$      | $3.24 \pm 0.37$  | $2.97 \pm 0.50$  | $3.58 \pm 0.24$  |

Data are shown as mean ± standard deviation of mean ($n = 3–4$). Superscripts indicate significant difference at $p \leq 0.05$ level within each drought treatment group.

When data were pooled together, a curvi-linear relationship was found between the percentage recovery of $A_{\text{max}}$ and $g_s$. These two variables were linearly related up to 70% recovery of $A_{\text{max}}$, with a slope of 1.38. In addition, recovery of $A_{\text{max}}$ was also linearly related to $V'_{\text{cmax}}$ ($r^2 = 0.91, p < 0.001$) and $J_{\text{max}}$ ($r^2 = 0.77, p < 0.001$), with the slope of the linear regression being 0.94 and 0.97, respectively.

DISCUSSION

We applied different levels of drought stress and measured plant carbohydrates, gas exchange, plant water status and growth, to investigate the influences of antecedent drought conditions on the response of plants during subsequent drought stress and recovery dynamics. Although photosynthesis, growth and the form of leaf carbohydrate varied across different levels of antecedent drought treatment, total NSC content did not vary. During subsequent drought stress, plants that experienced antecedent drought conditions (i.e., $A_{50}$ and $A_{10}$) were less affected compared with the control (i.e., $A_{100}$) in terms of carbon assimilation, maximum rate of Rubisco carboxylation and...
E. camaldulensis resumption in of leaf carbon assimilation to ensuing water limitation and found that antecedent drought conditions altered the response exposed to the more severe drought treatment. Overall, we compared to controls observed only when plants had been stress, with faster physiological recovery in antecedent plants drought conditions and the severity of subsequent drought Importantly, time to recovery was dependent on both antecedent was controlled by both stomatal and non-stomatal limitation. 

During Sustained Drought Variation of Leaf Carbohydrate Content

Greater reductions in growth relative to photosynthesis will theoretically lead to carbohydrate accumulation (Chapin et al., 1990; Sala et al., 2012; Piper et al., 2017). However, experimental evidence regarding the impact of drought on leaf carbohydrate content is inconsistent (Klein et al., 2014; Martínez-Vilalta et al., 2016; Chuste et al., 2020; He et al., 2020). For example, leaves of Eucalyptus saligna seedlings exposed to drought exhibited higher NSC content compared to well-watered controls (Ayub et al., 2011), whereas an opposite pattern was observed in leaves of Eucalyptus globulus and Eucalyptus smithii (Duan et al., 2013; Mitchell et al., 2013). In the present study, growth nearly ceased while leaf photosynthesis continued during the phase of antecedent drought treatment, yet total NSC content remained stable across treatment groups (Figure 1), similar to the findings reported by Klein et al. (2014) in branches of Pinus halepensis subjected to drought. The lack of change in NSC content during drought may emerge due to several reasons. Firstly, the total NSC content can vary markedly among organs (He et al., 2020); therefore, the NSC content of a single organ does not comprehensively reflect drought-induced changes in whole plant carbon reserve, although it can be driven by one organ (e.g., Duan et al., 2013). Secondly, the carbohydrate content may be influenced by the duration of drought exposure. For example, initially accumulated carbohydrate due to growth cessation may be depleted due to increased rates of other carbon-consuming metabolic processes such as respiration or synthesis of compatible osmolytes, as drought stress proceeds (Flexas et al., 2005; Tomasella et al., 2020).

Although total NSC content was not affected by drought, the relative contribution of soluble sugar and starch content to NSC was consistent with the findings of earlier studies (He et al., 2020). It has been shown that the depolymerization of starch into soluble sugar during drought stress will facilitate osmotic regulation, thereby lowering the risk of hydraulic impairment by increasing water acquisition (Trifilò et al., 2017; Tomasella et al., 2020). The concomitant increase in soluble sugar and decrease in starch, therefore reflects the shift in the functionality of carbohydrate from growth to maintenance, in which starch primarily acts as carbon reserve while soluble sugar performs immediate metabolic functions (Martínez-Vilalta et al., 2016).

Antecedent Drought Condition Promotes Drought Resistance of Photosynthesis

Our second hypothesis was partially supported by the observation that leaf $A_{\text{max}}$ was generally higher in plants exposed to antecedent drought conditions, and then exposed to subsequent drought. The smaller reduction in $A_{\text{max}}$ was attributed to the biochemical components of photosynthesis rather than gas exchange, as $g_s$ did not differ across levels of drought stress in plants exposed to antecedent drought (Figure 2). On the other hand, biochemical components of photosynthesis can also acclimate to drought. With respect to Rubisco carboxylation, it has been reported that water limitation, although uncommon, can increase the catalytic efficiency of Rubisco by increasing either the amount of enzyme or activation.
state (Galmés et al., 2013; Menezes-Silva et al., 2017). Likewise, in some species, an increased electron transport rate has been found in leaves exposed to drought (Kitao et al., 2003), although the functional significance of the adjustment is unclear, given that photosynthesis is rarely limited by RuBP regeneration under drought stress.

**Recovery Is Co-limited by Stomatal and Non-stomatal Limitation**

The decreased $g_s$, $V_{cmax}$, and $J_{max}$ relative to their pre-stress values, in conjunction with the correlations between the percentage recovery of $A_{max}$ and these components, indicate stomatal and non-stomatal limitation of photosynthesis during both drought stress and post-drought recovery phase (Figure 2). Noticeably, after the resumption of irrigation, although the recovery of $A_{max}$ was associated with recovery of both $g_s$ and biochemical components, the slope of the $A_{max} - g_s$ relationship was much steeper than the slope of the $A_{max} - V_{cmax}$ or $A_{max} - J_{max}$ relationship, up to ca. 70% of full recovery (Figure 5); hence, the initial phase and major fraction of the full recovery of $A_{max}$ was primarily driven by the restoration of $g_s$. It has been suggested that drought induced down-regulation of leaf photosynthesis is mainly attributed to diffusive limitation rather than biochemical limitation (Flexas et al., 2004, 2006b). However, down-regulation of photosynthesis due to decreased $V_{cmax}$ caused by inactivation of Rubisco and RuBP content have been reported in a number of species (Parry et al., 2002; Carmo-Silva et al., 2010; Galmés et al., 2013). It is likely that the occurrence of biochemical limitation depends on the strength of water limitation. For instance, decreased $V_{cmax}$ or $J_{max}$ only emerged when drought stress was severe and $g_s$ approached very low values, approximately 20% of the control (Flexas et al., 2004), suggesting that decreased biochemical efficiency of photosynthesis may have limited the recovery of photosynthesis as the highest $g_s$ accounted for ca. 14% of the pre-stressed value even for plants subjected to the mildest drought stress (i.e., −3.5 MPa). Nonetheless, it could be argued that decreased biochemical function could be an artifact due to drought-induced reduction in mesophyll conductance ($g_m$). Indeed, $V_{cmax}$ remains unaffected by water stress when the resistance of $g_m$ to CO$_2$ diffusion is accounted for when calculating Rubisco carboxylation rate from response curves (Flexas et al., 2006a, 2008). However, by accounting for $g_m$, Zhou et al. (2014) found that both diffusive and biochemical limitation can contribute to down-regulation of photosynthesis during drought in a wide range of species, including *E. camaldulensis*; e.g., $V_{cmax}$ can decrease by 50% at −2.0 MPa, regardless of the variation in the value of $g_m$ under drought. Still, further work is clearly needed to confirm our observation by assessing the potential effects of $g_m$ on the measurement of biochemical components of photosynthesis, using plants subjected to the same drought treatment.

**Recovery From Drought Depends on Antecedent Condition and Severity**

Shorter $T_{1/2}$ for $A_{max}$ and $g_s$ was found in seedlings exposed to antecedent drought conditions, which apparently generated drought-induced acclimation of photosynthesis, although the difference in $T_{1/2}$ for $A_{max}$ at −3.5 MPa was less pronounced between well-watered control and plants exposed to antecedent drought treatment. Notably, $A_{10}$ and $A_{90}$ plants exhibited similar $T_{1/2}$ for both $A_{max}$ and $g_s$ at less negative water potentials (i.e., −3.5 and −4.5 MPa), while the time for recovery diverged at −5.0 MPa. Hence, increasing the level of antecedent drought conditions would not consistently confer rapid recovery, especially when subsequent drought stress was severe (Figure 4), suggesting an interaction between the intensity of consecutive drought conditions. Overall, trees exposed to more severe drought stress typically need longer $T_{1/2}$. This finding is consistent with early studies showing that the time required for recovery is dependent on the severity of drought stress (Blackman et al., 2009; Brodribb and Cochard, 2009). Given that the subsequent drought stress was designed to reduce $K_{Leaf}$, a mechanistic explanation bridging the time to recovery and drought severity may be the following: that the greater the impairment of $K_{Leaf}$, the longer it would take $A_{max}$ and $g_s$ to fully recover, thereby delaying whole plant recovery. Of note, two implicit assumptions behind this interpretation are: (a) recovery of leaf photosynthesis is driven by the alleviation of stomatal limitation; and (b) the recovery dynamics of $g_s$ are primarily governed by the recovery in $K_{Leaf}$. As has been discussed above, stomatal limitation played a major role in limiting the recovery of photosynthesis during the recovery phase. On the other hand, it is known that the stomatal openness highly depends on leaf water status, which is a function of $K_{Leaf}$ at a given stem-to-leaf water potential gradient. In support, the variation of $K_{Leaf}$ has been found to synchronize with that of $g_s$ at diurnal scales (Brodribb and Holbrook, 2004) and the restoration of leaf gas exchange has been facilitated by the recovery of $K_{Leaf}$ following drought stress (Galmés et al., 2007; Skelton et al., 2017). In the current study, the recovery of $g_s$ was slower as drought stress and in turn hydraulic impairments intensified, which is consistent with the established theory linking plant hydraulics and gas exchange.

**CONCLUSION**

Our findings demonstrate that antecedent drought conditions may modify leaf biochemistry and physiology, which can be translated into different responses upon subsequent drought stress and recovery dynamics, following the alleviation of water limitation. Antecedent drought triggered an increase in soluble sugar content, which may have facilitated the recovery of water status by osmotic regulation and the maintenance of hydraulic integrity by lowering the vulnerability to embolism (De Baerdemaecker et al., 2017). The recovery of carbon assimilation was ultimately limited by decreased gas exchange capacity because of hydraulic impairment, which depended on the severity of the subsequent drought. Growth following re-watering was limited by the lack of carbon reserves, thus highlighting the importance of plant hydraulics and carbohydrates in regulating plant drought resistance and resilience. Overall, the response of plants to drought stress appears to be a function of the antecedent conditions and the subsequent drought, which should be considered in combination when assessing the response of plants to drought.
DATA AVAILABILITY STATEMENT
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS
DT and CB conceived and designed the research and revised the manuscript. JW and JB conducted the experiments. XL analyzed the data and wrote the early draft of the manuscript. All authors read and approved the manuscript.

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