Differences in drought sensitivity of photosynthesis between C₄ and C₃ species in the genus *Flaveria* (Asteraceae).

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ABSTRACT

At a global scale, drought is considered the most limiting factor for plants, reducing photosynthesis, growth and yield. There is abundant research exploring the effects of water stress on plants and how it affects the photochemistry in C₃ plants. However, the responses to water stress in C₄ have been much less studied. Due to their carbon concentrating mechanism, C₄ plants exhibit greater assimilation rates and water use efficiency. Despite this advantage, when comparing C₃ and C₄ under water stress conditions, C₄ monocots seem to be more sensitive than C₃ monocots. Since almost no information is available about dicots, the aim of this study was to compare the effects of drought and rewatering on photosynthesis in two C₄ dicot species: Flaveria bidentis and Flaveria trinervia; and one C₃ dicot: Flaveria robusta. Water was withheld in the three species until soil water content reached 30%. F. bidentis showed higher rates of assimilation than F. trinervia and F. robusta under both well-watered and water-stress conditions. The decrease in assimilation was, in proportion, lower in F. bidentis than in F. robusta. Rewatering did not translate into a recovery of any parameter measured in any species, indicating metabolic limitations. The two C₄ exhibited different degrees of tolerance to water stress: F. trinervia was clearly more sensitive, being limited by Rubisco and altering the C₃/C₄ cycle balance, while in F. bidentis the limitation on Rubisco did not alter the coordination, maybe indicating some degree of general downregulation. This finding suggests different ranges of tolerance within the C₄ Flaveria, making it difficult to make comparisons with the C₃.
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INTRODUCTION

Water stress is considered as the main environmental factor limiting photosynthesis, and thus, plant growth and yield worldwide. Water stress causes a reduction in the plant water content, measurable as changes in leaf water potential ($\Psi_{\text{leaf}}$) or leaf relative water content (RWC), which negatively affects photosynthesis. Despite having been extensively reviewed (Lawlor & Cornic 2002; Flexas et al. 2004; Chaves et al. 2009; Lawlor & Tezara 2009; Pinheiro & Chaves 2011), the factors limiting photosynthesis under drought are still in debate. There is a general agreement that mild to moderate water stress alters CO$_2$ diffusion in the leaves through a decrease of stomatal ($g_s$) and mesophyll conductance ($g_m$) (Flexas et al. 2008), which forces plants to operate at lower intercellular CO$_2$ concentration ($C_i$) and hence, reducing photosynthesis. In contrast, the nature of the photosynthesis limitations under more severe water stress is still debated, and although diffusional limitations still persist, metabolic limitations are thought to also play an important role. A number of metabolic causes for decreased photosynthesis in C$_3$ have been proposed (see Lawlor & Tezara 2009 for review), specially reduction of ATP synthesis, RuBP regeneration (Tezara et al. 1999) and reduced Rubisco activity (Flexas et al. 2004; Grassi & Magnani 2005; Galmés et al. 2011), particularly under conditions combining the water stress with high light and temperature, which favour oxidative stress (Flexas et al. 2006; Zhou et al. 2007).

Plants developed different mechanisms to fix Carbon. The majority of them have the so-called C$_3$ pathway that dominates in temperate climate. The C$_4$ pathway is believed to have emerged more recently and is an elaboration of the classical C$_3$ pathway (Sage 2004) the main difference consists in a CO$_2$ concentration mechanism that increases CO$_2$ availability around Rubisco, by the combination of leaf anatomical modifications and metabolic changes. The most common form of anatomical modification is Kranz anatomy, consisting in an anatomical and functional specialization of two photosynthetic cell types: mesophyll (M) and bundle sheath (BS). Mesophyll cells in C$_4$ are reduced in number in comparison to C$_3$, leading to a proportion of M to BS close to 1:1 and allowing close connection between both cell types (Dengler et al. 1994, Dengler & Taylor, 2000). BS cells form a compactly arranged layer surrounding the leaf vasculature and while in C$_3$ they play non-photosynthetic roles (see Leegood (2008) for review), in C$_4$ is where the CO$_2$ carbon reduction through the Calvin-cycle takes place, since M cells do not express Rubisco. The CO$_2$ that enters the M cells is firstly hydrated into bicarbonate (HCO$_3^-$) catalysed by carbonic anhydrase (CA), which reacts with
phosphoenolpyruvate (PEP) through PEP carboxylase (PEPC) to form oxaloacetate. Oxaloacetate can be converted into another 4-carbon acid (malate, aspartate or alanine) and transported to the BS where is decarboxylated, and thus, releasing the CO$_2$ in the BS’s chloroplast. With this process (called the C4 cycle) the concentration of CO$_2$ around Rubisco can be higher than 10-fold the ambient (von Caemmerer & Furbank 1999), reducing photorespiration to minimum and saturating photosynthesis at lower ambient CO$_2$ concentration than C$_3$.

C$_4$ grasses tend to have smaller stomata and/or smaller stomatal density compared to C$_3$ (Taylor et al. 2012) mainly caused by the anatomical modifications implicated in Kranz anatomy (Way 2012). The smaller distance between vascular bundles observed in C$_4$ (resulting in a lower mesophyll to bundle sheath ratio), also limits the proportion of the leaf surface area over which stomata can be distributed, since most stomata are located between vascular bundles (Taylor et al. 2012). In addition, the CO$_2$ concentration mechanism allows C$_4$ plants to maintain a high CO$_2$ assimilation at low $C_i$, in turn, allowing the same rate of photosynthesis to be maintained with a lower stomatal conductance ($g_s$) than C$_3$ plants. This lower $g_s$ at comparable rates of photosynthesis has been extensively reported (Morison & Gifford, 1983; Monson, 1989; Sage, 2004; Taylor et al., 2010). This induces a greater intrinsic water-use efficiency (WUE$_i$) and nitrogen use efficiency (NUE) than C$_3$ species (Long, 1999; Ghannoum, 2011; Taylor et al., 2012; Vogan and Sage, 2011;).

There are few studies comparing the performance of C$_3$ and C$_4$ plants under water stress conditions. In most cases, although C$_4$ showed greater photosynthetic rates and lower $g_s$ than C$_3$ in non-stressed plants, but surprisingly this advantage is lost under water stress, leading to the conclusion that C$_4$ photosynthesis is more severely affected by drought (Ripley et al. 2007; Ibrahim et al. 2008; Ripley et al. 2010; Taylor et al. 2011). This hypothesis still holds when comparing co-occurring C$_3$ and C$_4$ subspecies of Alloteropsis semialata (Ripley et al. 2007) or controlling for phylogeny as in Taylor et al. (2011).

Most of the few studies approaching the effects of water stress on C$_4$ species alone or in comparison with C$_3$ have been focused on monocot species. The fact that this class accounts for ≈6300 of the ≈8100 total C4 species (Sage 2016) and includes very important crops (e.g. Zea mays, Sorghum bicolor, Panicum miliaceum, Setaria italic, Saccharum officinarum), confers to monocots a huge interest for research, but leaving a gap of knowledge about the dicots C$_4$ species. Moreover, to isolate differences in water stress tolerance that only come from the different photosynthetic pathway, it is important to studies species phylogenetically closest
as possible. The genus *Flaveria* (Asteraceae) has become model genus for studying the evolution of C4 photosynthesis at physiological and molecular level (Sage 2004). This genus includes in total 23-24 species (McKown *et al.* 2005; The Plant List 2013), with four C3 and at least five pure C4, along with some intermediate C3-C4 photosynthesis (McKown *et al.* 2005; Sudderth *et al.* 2007). So, given the scarcity of available data about effects of drought on C4 dicots (Lal & Edwards 1996; Ward *et al.* 1999) and the need to study phylogenetically close species, the *Flavelia* genus has appeared as an ideal subject for this study.

The aim of this study was to evaluate the effects of water stress and recovery on the photosynthetic parameters of two C4 dicot species (*Flaveria bidentis* and *Flaveria trinervia*), and compare them to phylogenetically closely related C3 (*Flaveria robusta*). The present study was carried under the hypothesis that water stress will cause higher degree of photosynthetic inhibition in C4 dicots than in C3 dicots.

**MATERIALS AND METHODS**

*Plant material, growing conditions and water stress management*

The *Flaveria* species used in this study were *Flaveria bidentis* (L.) Kuntze, *Flaveria trinervia* (Spreng.) C. Mohr and *Flaveria robusta* Rose. All three species were established from seeds provided by Dr Rowan F. Sage at the University of Toronto (Toronto, Ontario, Canada) and the whole experiment was carried out in growing chamber at the University of the Balearic Islands (Mallorca, Spain). Seeds were germinated on Petri plates with filter paper moistened with distilled water. After germination, seedlings were transplanted to seed trays with a soil composed by a 2:1:1 mixture of horticultural substrate (peat), pearlite (granulometry A13) and sand for 40 days, and then transplanted to 3 L pots with the same soil composition. The growing chamber conditions were 12 h/12 h light/dark photoperiod, 21/17 ºC day/night temperature regime and a light intensity of 317±12 µmol photons m⁻² s⁻¹ at the level of the pot. The pots were randomly distributed in the growing chamber to reduce possible effects of non-homogeneity of illumination. Plants were watered every two-three days and fertilized weekly with full-strength Hoagland’s solution until the two treatments were assigned.

When plants were two months old, they were randomly divided in two groups (WW, well-watered plants, and WS, water-stressed plants) and watering was withheld in the water stress
treatment. The effect of water deficit was evaluated every two days by monitoring the water state of the soil concomitantly with instantaneous leaf gas exchange measurements. When soil water content (SWC, see details below) fell below 30%, and \( g_s \) below 0.05 mol H\(_2\)O m\(^{-2}\) s\(^{-1}\) (considered a threshold indicating severe water stress; Flexas \textit{et al.} 2004), full gas exchange characterization (light and CO\(_2\) response curves) was started. Watering was adapted to maintain a constant 30% SWC during the measurements of the water stressed plants. After finishing light and CO\(_2\) response curves on every WS plant, it was watered to field capacity, and light and CO\(_2\) response curves were performed again 24 h after the rewatering (rewatering treatment, RW). The order of measurement of each plant in the whole experiment was randomized.

\textit{Soil and leaf measurements}

Soil water content (SWC) was used monitor the loss of water in the soil as drought progressed. It was calculated as:

\[
\text{SWC} = \frac{W - \text{DW}}{\text{WFC} - \text{DW}} \cdot 100
\]

where \( W \) is the pot weight, \( \text{WFC} \) is the pot weight at field capacity and \( \text{DW} \) the pot dry weight. SWC could not be measured directly during the experiment since it would require drying the pots. Instead, and previous to the experiment, seven 3 L pots with the same soil composition than the experimental pots but without plants were watered to field capacity. After obtaining the WFC and the maximum soil moisture with the probe, the pots were left to slowly dry while weighting them and measuring the soil moisture every day to determine the water lost. Finally, the seven pots were oven-dried for a week at 70ºC to obtain the DW and the relationship between SWC and soil moisture was determined as:

\[
\text{SWC} = 1.747 \cdot \text{SM} + 13.932
\]

where SM is the soil moisture measured with a soil moisture probe (WET Sensor type WET-2, HH2 Moisture Meter, AT Delta-T Devices, Cambridge, UK). The \( r^2 \) of the regression was 0.96 and \( P < 0.0001 \) with a total of 55 measurements. During the experiment, soil moisture was measured immediately after the gas exchange measurements (both instantaneous and curves). Relative water content (RWC) and lead mass area (LMA) were measured in the same leaf than gas exchange measurements (curves). RWC was calculated as: \( \text{RWC} = (\text{Fresh weight} - \text{Dry weight}) / (\text{Turgid weight} - \text{Dry weight}) \). Turgid weight was determined keeping the leafs in
distilled water and in darkness at 4 °C for 24 h. Dry weight obtained oven-drying the leaves for 48 h at 70 °C.

Gas exchange measurements

To monitor the process of desiccation, and in parallel to SWC measurements, net CO\textsubscript{2} assimilation (A\textscript{N}) and stomatal conductance (g\textsubscript{s}) were also measured in each plant. Measurements were taken in the youngest fully-expanded leaf (the same leaf during all the monitoring period) using a gas-exchange system (Li-6400XT, Li-Cor Inc., Nebraska, USA) equipped with an open 6 cm\textsuperscript{2} chamber (using ambient light). The chamber was positioned perpendicular to the light source to uniformly illuminate the leaf (349-375 µmol photons m\textsuperscript{-2}s\textsuperscript{-1}). The chamber conditions consisted in an ambient CO\textsubscript{2} concentration (C\textsubscript{a}) of 400 µmol mol\textsuperscript{-1} air, an air flow of 400 µmol min\textsuperscript{-1}, an air temperature of 25 °C, and a relative humidity of 64.44 ± 0.24 %. After clamping the youngest fully expanded leaf and waiting 30-40 s for gases to stabilize, 4 “logs” were taken every 10 s. The mean of these 4 “logs” was considered the final measurement. Since F. trinervia leaves did not fill the leaf chamber, gas-exchange measurements were corrected by leaf area.

Once plants reached the desired water stress (30% SWC), the response of photosynthesis to varying C\textsubscript{i} (A\textsubscript{N}-C\textsubscript{i} curves), and to different light intensities (A\textsubscript{N}-PPDF) at low O\textsubscript{2} concentration (<1%) were performed to each plant. A\textsubscript{N}-PPDF curves at ambient O\textsubscript{2} concentration (21%) were also performed only to the C\textsubscript{4} species (for specific modelling purposes). For these measurements, the Li-6400 was equipped with a Leaf Chamber Fluorometer 6400-40 with a 2 cm\textsuperscript{2} cuvette. The saturating flash delivered by the red LEDs of the LI-6400-40 system has been reported to be not truly saturating for C\textsubscript{4} plants (Dwyer et al. 2007), reason why fluorescence measurements were taken using the “multiphase flash” option included in the LI-6400XT software for all three species (Loriaux et al., 2013). For the A\textsubscript{N}-Ci curves, after waiting 15-30 min to steady-state conditions, C\textsubscript{a} was changed stepwise from 400, 350, 300, 200, 100, 50, 400, 400, 500, 600, 750, 1000, 1200 1600 and 2000 µmol mol\textsuperscript{-1}. Gas-exchange and fluorescence (F\textsubscript{m}’ and F\textsubscript{s}) measurements were determined at each step after maintaining the leaf for at least 5 min. at the new C\textsubscript{a}. Measurements were taken at a saturating light of 2000 µmol photons m\textsuperscript{-2}s\textsuperscript{-1}, an air flow of 400 µmol min\textsuperscript{-1}, 25 °C of block temperature and 50-70 % of relative humidity.
For the $A_{N}$-PPDF curves at either low or ambient $O_2$, light was lowered from 2500 to 0 µmol photons m$^{-2}$ s$^{-1}$ in 16 steps. Gas-exchange and fluorescence ($F'_m$ and $F_s$) measurements were determined at each step after maintaining the leaf for at least 5 min at the new light intensity. The curves were performed at a $C_a$ of 400 µmol mol$^{-1}$ and the same flow, temperature, relative humidity and steady-state conditions as de $A_{N}$-$C_i$ curves.

Due to the thickness of the leaf raquis, a circle of a putty-like adhesive (Blu-Tack, Bostik) was placed between the leaf and the lower gasket to seal the chamber. $A_{N}$-$C_i$ curves data was corrected for CO$_2$ leakage through the gaskets with the boiled-dead leaf method described in (Flexas et al. 2007), in that case also performed with the putty-like adhesive.

**C$_3$ model calculations**

In the present study, respiration in the light ($R_L$) was calculated from $A_{N}$-PPDF curves in non-photosynthetic conditions according to Yin et al. (2011a). CO$_2$-saturated Rubisco carboxylation rate ($V_{cmax}$), the maximum rate of electron transport ($J_{max}$) and mesophyll conductance ($g_m$) were calculated by curve fitting. As described in von Caemmerer & Evans (1991) or Ethier & Livingston (2004), the equation:

\[
A_c = g_m(C_i - C_c)
\]

solved for $C_c$ can be substituted in the equation for Rubisco-limited CO$_2$ assimilation ($A_c$) or for RuBP-limited CO$_2$ assimilation ($A_j$) from the Farquhar-von Caemmerer-Berry model (Farquhar et al. 1980):

\[
A_c = \frac{(C_c - \Gamma^*)V_{cmax}}{C_c + K_c(1 + O / K_o)} - R_L
\]

\[
A_j = \frac{(C_c - \Gamma^*)J_{max}}{C_c + 2\Gamma^*} - R_L
\]

This results in two quadratic expressions relating $A_N$ to $C_i$ with a non-rectangular hyperbola (see Ethier and Livingston, 2004 for detailed explanation). These equations were used to calculate CO$_2$-saturated Rubisco carboxylation rate ($V_{cmax}$), the maximum rate of electron transport ($J_{max}$) and mesophyll conductance ($g_m$) by curve fitting all at once (Sharkey et al., 2007). The $\Gamma^*$ value used for the calculations could not be any of the ones found in the literature (there are no specific values for $F. robusta$, but some for other C$_3$ Flaveria species) because in all cases these values were higher than the calculated CO$_2$ compensation point ($\Gamma$), which is
mathematically impossible. Instead, $\Gamma^*$ was also fitted along with the other parameters previously mentioned for the CL treatment. The mean value of the six fitted values was then used as the unique value for all three treatments (CL was recalculated with that new value), since it has been demonstrated that $S_{c/o}$ and thus $\Gamma^*$, do not acclimate to water stress (Galmés et al. 2006).

Quantum efficiency of photosystem II ($\Phi_{PSII}$) was calculated as:

$$\Phi_{PSII} = \frac{F_m' - F_s'}{F_m'}$$

where $F_s'$ is the steady-state fluorescence and $F_m'$ is the maximum fluorescence in the light. Electron transport rate ($J$) was calculated as:

$$J = \Phi_{PSII} \cdot PPDF \cdot \alpha \cdot \beta$$

where $PPDF$ is the measuring light intensity, $\alpha$ is the leaf absorbance and $\beta$ is the theoretical partition of absorbed $PPDF$ between the two photosystems. The product $\alpha \beta$ as estimated as a whole following Valentini et al. (1995).

**$C_4$ model calculations**

$R_L$ was calculated according to Yin et al. (2011a). Bundle sheath conductance to CO$_2$ diffusion ($g_{bs}$) was estimated by curve fitting following the $J/I$ method with the excel tool from Bellassio et al. (2015). Having calculated $R_L$ and $g_{bs}$, and with specific in vitro Rubisco parameters ($K_c$, $K_o$, $S_{c/o}$) for *F. bidentis* and *F. trinervia* (Kubien et al. 2008; Perdomo et al. 2015), and other parameters shown in table 1, allowed the calculation of $g_m$, $V_{cmax}$, and CO$_2$-saturated PEPC carboxylation rate ($V_{pmax}$) by fitting modelled values of assimilation ($A_{Nmod}$) to the measured values of enzyme-limited assimilation ($A_N$) from the $A_N$-$C_i$ curves. $A_{Nmod}$ was calculated using the quadratic expression for the enzyme-limited CO$_2$ assimilation rate given in von Caemmerer (2000) (equation 4.21 in von Caemmerer 2000). In addition to the previous parameters mentioned above, which are assumed constant at different CO$_2$ concentrations, two more parameters were still required in the quadratic expression for $A_{Nmod}$: the CO$_2$ concentration in the mesophyll cells ($C_m$) and the PEPC carboxylation rate ($V_p$). These parameters are not constant along the $A_N$-$C_i$ curve and have to be calculated for each value of $A_N$-$C_i$.

$C_m$ can be calculated according to Fick’s first law of diffusion:
\[ C_m = C_i - \frac{A_N}{g_m} \]

\( V_p \) can then be calculated according to von Caemmerer (2000) as:

\[ V_p = \frac{C_m \cdot V_{\text{pmax}}}{C_m + K_p} \]

where \( K_p \) is the PEPC Michaelis-Menten constant for CO\(_2\) (parameters used are shown in table 1). \( \Phi_{\text{PSII}} \) and \( J \) were calculated as previously described.

**Statistical analysis**

All statistical analysis was performed with R [language](https://www.r-project.org) and software environment (R Core Team, 2017). Since WS and RW treatments were established in the same plants, Repeated Measures ANOVA was performed to check for differences between these two treatments and species. However, because in all cases the effect of accounting for treatment as a within factor was negligible, regular two-way ANOVA was performed instead, now also including the CL treatment. If interaction term was not significant it was removed, as well as non-significant factors, reducing the model to one-way ANOVA. In all cases the normality of the model’s residuals and homoscedasticity were checked. If the assumptions were not meet, logarithmic transformation was performed. Statistical differences between means were determined by Tukey-HSD post-hoc tests from “agricolae” package (de Mendiburu, 2017). In the specific cases of SWC and \( g_s \) at ambient CO\(_2\) level, not both assumptions were meet and transformation did not solve it. In these two cases, non-parametric tests (Welch’s ANOVA for non-homoscedastic data and Kruskal-Wallis test for non-normal data respectively) were performed.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value / units</th>
<th>References</th>
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<td>$R_m$</td>
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<td>(von Caemmerer 2000)</td>
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<td>$g_m$</td>
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<td>$\text{mol m}^{-2} s^{-1} \text{bar}^{-1}$</td>
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<td>$g_{bs}$</td>
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<td>$K_c$</td>
<td>Rubisco Michaelis-Menten constant for CO$_2$</td>
<td>$F. \ bidentis$: 573.5 $\mu$bar \n$F. \ trinervia$: 541.2 $\mu$bar \n$F. \ robusta$: 352.9 $\mu$bar</td>
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<td>$K_o$</td>
<td>Rubisco Michaelis-Menten constant for O$_2$</td>
<td>$F. \ bidentis$: 491538 $\mu$bar \n$F. \ trinervia$: 516153 $\mu$bar \n$F. \ robusta$: 676923 $\mu$bar</td>
<td>(Kubien et al. 2008) (Kubien et al. 2008) (Zhu et al. 1998)</td>
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<td>$K_p$</td>
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<td>$O$</td>
<td>O$_2$ concentration in mesophyll cells (either for C$_3$ or C$_4$)</td>
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<td>$\alpha$</td>
<td>Fraction of PSII active in Bundle sheath</td>
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<td>$S_{c/o}$</td>
<td>Rubisco specificity factor</td>
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<td>$\gamma^*$</td>
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<td>$\Gamma^*$</td>
<td>CO$_2$ compensation point in the absence of mitochondrial respiration</td>
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RESULTS

Drought monitoring

After two days since water was withheld, SWC in the WS treatment already differed from the WW treatment (Fig. 1A). In the WW treatment, SWC was maintained along the days at 95.27±0.47 % on average. In all three species, SWC in the WS treatment decreased at the same rate, and no differences among species were found at any day.

Figure 1. (A) Soil water content (SWC), (B) net CO₂ assimilation (Aₜ), (C) stomatal conductance (gₛ) and (D) intrinsic water use efficiency (WUEᵢ) along 10 days for *F. bidentis* (C₄; circles), *F. trinervia* (C₄; triangles) and *F. robusta* (C₃; rhombus) under well watered (WW; gray) and water stress conditions (WS; white). Points represent means ± SE (n = 4-6).
Overall, and not accounting for species, \( A_N \) and \( g_s \) did not differ among treatments until day 8 (\( P > 0.0001 \) for \( A_N \); \( P=0.0033 \) for \( g_s \)), when SWC was between 50\% and 30\%. \( F. \) bidentis tended to have slightly higher rates of CO\(_2\) assimilation than \( F. \) robusta and \( F. \) trinervia in both well-watered and water-stress conditions (Fig. 1B). The difference between \( F. \) bidentis and \( F. \) robusta in WS is especially remarkable at day 10, when \( A_N \) had been reduced by 37\% in the C4 while in the C3 it had been reduced by 55\% at an equal \( \approx 30\% \) of SWC.

As expected from the two different photosynthetic subtypes, \( g_s \) did not follow the same trends. When plotting the relationship between \( A_N \) values from figure 1B and the \( g_s \) from figure 1A (figure 2), for a given rate of CO\(_2\) assimilation both C4 species required lower stomatal conductance than the C3. This is especially clear at well watered conditions, where \( g_s \) in \( F. \) robusta roughly ranged between 200 and 400 mmol H\(_2\)O m\(^{-2}\) s\(^{-1}\), while in the two C4 it ranged between 50 and 200 mmol H\(_2\)O m\(^{-2}\) s\(^{-1}\).

**Figure 2.** Relationship between net CO\(_2\) assimilation (\( A_N \)) and stomatal conductance (\( g_s \)) of \( F. \) bidentis (C4; black), \( F. \) trinervia (C4; grey) and \( F. \) robusta (C3; white) under well watered (WW; circles) and water stress conditions (WS; triangles). Points represent means ± SE (n = 5-6). Measurements were taken at an atmospheric CO\(_2\) concentration of 400 \( \mu \)mol mol\(^{-1}\), light intensity of 346 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) and 25ºC.
Between days 4 and 6 there was a drop in AN, and especially in gs, that affected all species and both treatments. During the following days, photosynthesis raised again to previous values, but not the stomatal conductance or at least not in the same extent (Fig. 1C). F. robusta regained part of its previous gs, but both F. bidentis and F. trinervia had its gs reduced by half from days 6 to 10. That general reduction in gs but not in AN caused an improvement on intrinsic water-use efficiency (WUEi; Fig. 1D). As expected, during days 0 to 4 F. bidentis and F. trinervia showed higher WUEi than F. robusta although there were no differences between treatments. However, from day 6, the two C4 improved their WUEi in WW plants and to a higher extent in WS plants. F. robusta increased its WUEi at days 8 to 10 in WS, but remained essentially unaltered for the ten days in well watered conditions.

At day 10, SWC had fallen to ≈30% and the effects of water scarcity were evident in AN and gs. Photosynthesis in F. robusta had been reduced by half, and clear signs of leaf turgor loss were observable. At that point water stress was considered established and AN-Ci and AN-PPDF curves were performed.

**Response to WS and RW for common C3-C4 measured variables**

There was a general decrease in almost all photosynthetic parameters in all three species, with no recovery in any of the measured parameters after 24h since rewatering (except for SWC). Table 2 summarizes the main parameters derived from gas exchange at ambient CO2 and common for C4 and C3 species, together with SWC, RWC and LMA. WS treatment was well established with no differences between species, and SWC being 24.75 ± 1.27% for F. bidentis, 28.66 ± 1.19% for F. trinervia and 25.57 ± 1.67% for F. robusta. After rewatering, SWC increased in all three cases to 90-100%. RWC however, did not show any difference between treatments. Water scarcity did not altered LMA, although it was different for each species (P < 0.0001): 55.85 ± 2.71, 46.44 ± 2.12 and 35.31 ± 1.27 for F. robusta, F. bidentis and F. trinervia respectively.

Stomatal conductance at ambient CO2 concentration and saturating light was the same for all three species (P = 0.36), which contrasts with previous results with instantaneous measurements at growing light, but was affected by water stress (P = 0.004), being reduced by 43.23% in average for all three species.
Mesophyll conductance on the contrary, was not affected by water stress, but differed greatly between the two C$_4$ and _F. robusta_, although some issues related to its calculation for the C$_4$ are addressed in discussion.

Net CO$_2$ assimilation, electron transport rate and CO$_2$-saturated Rubisco carboxylation rate were highly affected by drought in all three species and in a similar degree (no interaction effect between species and treatment). _F. bidentis_ exhibited higher photosynthetic rates than _F. trinervia_ and _F. robusta_ in WW conditions ($\approx$40% higher). Under WS, photosynthesis was reduced from 33.26 ± 1.94 to 21.78 ± 0.61 µmol CO$_2$ m$^{-2}$ s$^{-1}$ (34.52% less) in _F. bidentis_, from 22.65 ± 1.45 to 13.35 ± 2.72 (41.06% less) in _F. trinervia_ and from 24.08 ± 2.39 to 13.17 ± 0.98 (45.35% less) in _F. robusta_.

_{F. bidentis_ and _F. robusta_ presented similar rates of $J$ in WW: 230.89 ± 14.17 and 240.69 ± 12.49 µmol e$^{-}$ m$^{-2}$ s$^{-1}$ respectively while _F. trinervia_ presented considerably lower rates. In WS, ETR was reduced in a very similar proportion as $A_N$ for the two C$_4$: 36.05% in _F. bidentis_ and 42.4% in _F. trinervia_, whereas in _F. robusta_ the decrease was approximately half the decrease in $A_N$ (24.15%). In the case of $V_{cmax}$, the C$_4$ presented much lower rates than the C$_3$ (3.5 to 5-fold lower). The 24% decrease in _F. bidentis_ with WS was not significantly different from values at WW, in contrast with the 41.36% and 37.96% decrease observed in _F. trinervia_ and _F. robusta_ respectively.

In figure 3 the relativized values of $A_N$, $J$ and $V_{cmax}$ for the water-stressed plants to their mean WW values are presented. Since the RW treatment was never different from WS, the factor treatment was removed from the ANOVA model, increasing the number of observations and thus, the power of the model. The relative decrease of $A_N$ and $J$ differed between species ($P = 0.04$ for $A_N$; $P = 0.046$ for $J$) but not the decrease in $V_{cmax}$ ($P = 0.098$). In _F. robusta_, $A_N$ decreased to a 53.7 ± 3.36% of non-stressed values, which is more than the decrease in _F. bidentis_ (71.16 ± 2.47%; Fig. 3A). In the case of $J$, the decrease was more important in _F. trinervia_ (59.61 ± 5.31%) than in _F. robusta_ (74.81 ± 4.1%; Fig. 3B). If just the two C$_4$ are compared, only $V_{cmax}$ had a differential decrease between the two species ($P = 0.046$), decreasing to a greater extent in _F. trinervia_.

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Table 2. Soil water content (SWC), relative water content (RWC), respiration in the light ($R_L$), photosynthetic rate ($A$), stomatal conductance ($g_s$), electron transport rate ($J$), CO$_2$-saturated Rubisco carboxylation rate ($V_{\text{cmax}}$), mesophyll conductance ($g_m$), bundle-sheath conductance ($g_b$) and CO$_2$-saturated PEPC carboxylation rate ($V_{\text{pmax}}$) of *Flaveria bidentis* (C$_4$), *Flaveria trinervia* (C$_4$) and *Flaveria robusta* (C$_3$) under well-watered (WW) and water-stress conditions (WS), and after rewatering (RW). Values are means ± SE (n = 3-6). Different letters indicate statistically different responses between species and treatments at $P < 0.05$ (Tukey’s HSD post hoc test).

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>SWC</th>
<th>RWC</th>
<th>$R_L$</th>
<th>$A$</th>
<th>$g_s$</th>
<th>$g_m$</th>
<th>$g_b$</th>
<th>$V_{\text{cmax}}$</th>
<th>$V_{\text{pmax}}$</th>
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<tbody>
<tr>
<td><em>F. bidentis</em></td>
<td>CL</td>
<td>111.85 ± 2.84 a</td>
<td>86.94 ± 2.28 a</td>
<td>2.22 ± 0.21 ab</td>
<td>33.26 ± 1.94 a</td>
<td>0.23 ± 0.02 a</td>
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<td></td>
<td>WS</td>
<td>24.75 ± 1.27 c</td>
<td>89.41 ± 1.44 a</td>
<td>1.77 ± 0.41 abc</td>
<td>21.78 ± 0.61 bc</td>
<td>0.16 ± 0.04 b</td>
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<td>RW</td>
<td>90.49 ± 5.42 b</td>
<td>83.24 ± 0 a</td>
<td>2.13 ± 0.16 ab</td>
<td>25.11 ± 1.32 b</td>
<td>0.16 ± 0.02 b</td>
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<td><em>F. trinervia</em></td>
<td>CL</td>
<td>111.02 ± 1.72 a</td>
<td>86.15 ± 1.97 a</td>
<td>1.51 ± 0.16 bc</td>
<td>22.65 ± 1.45 b</td>
<td>0.18 ± 0.05 a</td>
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<td>WS</td>
<td>28.66 ± 1.19 c</td>
<td>83.09 ± 1.1 a</td>
<td>1.14 ± 0.07 c</td>
<td>13.35 ± 2.72 d</td>
<td>0.1 ± 0.04 b</td>
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<td>RW</td>
<td>94.28 ± 5.28 b</td>
<td>83.48 ± 1.51 a</td>
<td>1.52 ± 0.1 bc</td>
<td>14.55 ± 2.3 cd</td>
<td>0.17 ± 0.03 b</td>
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<td><em>F. robusta</em></td>
<td>CL</td>
<td>113.6 ± 1.06 a</td>
<td>84.46 ± 0.98 a</td>
<td>2.43 ± 0.12 a</td>
<td>24.08 ± 2.39 b</td>
<td>0.28 ± 0.04 a</td>
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<td>25.57 ± 1.67 c</td>
<td>81.79 ± 4.74 a</td>
<td>1.8 ± 0.29 abc</td>
<td>13.17 ± 0.98 d</td>
<td>0.12 ± 0.01 b</td>
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<td>RW</td>
<td>101.6 ± 6.47 b</td>
<td>85.02 ± 3.46 a</td>
<td>2.26 ± 0.23 ab</td>
<td>12.72 ± 1.4 d</td>
<td>0.15 ± 0.04 bb</td>
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<td><em>F. bidentis</em></td>
<td>CL</td>
<td>230.86 ± 14.72 ab</td>
<td>38.48 ± 1.74 c</td>
<td>1.99 ± 0.01 a</td>
<td>1.67 ± 0.39 a</td>
<td>222.4 ± 74.24 a</td>
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<td>WS</td>
<td>147.62 ± 6.06 c</td>
<td>29.08 ± 0.74 c</td>
<td>2 ± 0 a</td>
<td>2.28 ± 0.59 a</td>
<td>171.18 ± 34.81 a</td>
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<td>RW</td>
<td>172.29 ± 8.41 c</td>
<td>27.87 ± 1.45 c</td>
<td>2 ± 0 a</td>
<td>1.48 ± 0.38 a</td>
<td>142.04 ± 31.31 a</td>
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<td><em>F. trinervia</em></td>
<td>CL</td>
<td>145.04 ± 6.97 c</td>
<td>26.57 ± 1.55 c</td>
<td>2 ± 0 a</td>
<td>1.84 ± 0.85 a</td>
<td>101.47 ± 7.31 a</td>
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<td>WS</td>
<td>83.54 ± 12.69 d</td>
<td>15.58 ± 2.53 d</td>
<td>1.8 ± 0.2 a</td>
<td>2.47 ± 0.75 a</td>
<td>169.56 ± 52.43 a</td>
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<td>RW</td>
<td>88.81 ± 10.67 d</td>
<td>16.42 ± 2.48 d</td>
<td>2 ± 0 a</td>
<td>1.78 ± 0.43 a</td>
<td>130.69 ± 14.29 a</td>
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<td><em>F. robusta</em></td>
<td>CL</td>
<td>240.69 ± 12.49 a</td>
<td>135.5 ± 10.83 a</td>
<td>0.26 ± 0.04 b</td>
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<td>WS</td>
<td>182.57 ± 16.92 bc</td>
<td>84.07 ± 12.83 b</td>
<td>0.22 ± 0.07 b</td>
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<td>RW</td>
<td>177.09 ± 10.12 bc</td>
<td>83.31 ± 7.47 b</td>
<td>0.23 ± 0.08 b</td>
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Figure 3. WS values of net CO₂ assimilation (Aₐ; A), electron transport rate (J; B) and CO₂-saturated Rubisco carboxylation rate (Vₖₐ₅ₐₓ; C) relativized to their corresponding WW values. FB = F. bidentis (C₄); FR = F. robusta (C₃); FT = F. trineriva (C₄). Bars are means with SE (n = 9-11). Different letters indicate statistically different responses between species at P < 0.05 (Tukey’s HSD post hoc test).

Response to WS and RW for C₄ measured variables

No differences were found in gₙₐ₅ₐₓ or Vₚₖₐ₅ₐₓ neither for species nor treatment. Other exclusive parameters from the C₄ model and the Rubisco carboxylation rate (Vₖₐ₅ₐₓ) are also presented in figure 4. No differences were found in PEPC carboxylation rate (Vₚ; Fig. 4A). In contrast, differences in Vₖₐ₅ₐₓ (Fig. 4B) where highly significant for both main factors (P < 0.0001 for both Species and Treatment), with F. bidentis having it reduced by 26.08% and F. trineriva by 34.87% on average. The ratio Vₚ/Vₖₐ₅ₐₓ was not altered by WS in F. bidentis but it increased twofold in F. trineriva (Fig. 4C). Leakiness (ϕ), the ratio between the Leak rate (rate of CO₂ leaking out of the BS back to the M) and Vₚ followed the same trend as Vₚ/Vₖₐ₅ₐₓ: it did not change in F. bidentis but it also doubled in F. trineriva (Fig. 4D).
Figure 4. (A) PEPC carboxylation rate ($V_p$), (B) Rubisco carboxylation rate ($V_c$), (C) ratio $V_p/V_c$ and (D) Leaksiness ($\phi$, ratio $V_p/L$) of the two C$_4$ species *F. bidentis* and *F. trinervia* under well-watered (WW; black bars) and water-stress conditions (WS; white bars) and after 24h since rewatering the WS plants to full capacity (RW; gray). Bars are means with SE (n = 3-6). Different letters indicate statistically different responses between species and treatments at $P < 0.05$ (Tukey’s HSD post hoc test).

From all Species x Treatment combinations the average values of $V_{cmax}$, $V_{pmax}$, $g_{bs}$, $g_m$, $R_L$ and the parameters from Table 1 were used to model the CO$_2$ concentration in the Bundle-sheath ($C_{bs}$) at increasing $C_i$ (Fig. 5). In figure 5A, *F. bidentis* the model predicts $C_{bs}$ from WW and RW tretments to be almost identical while in WS it is smaller (at $C_i$ = 200 µmol mol$^{-1}$).
is 14.57% smaller than the WW). For *F. trinervia* (Fig. 5B), a theoretical $C_i = 200 \mu\text{mol mol}^{-1}$ would imply 16.95 mmol mol$^{-1}$ of CO$_2$ in the BS at WW conditions, but it would be increased by 76.73% and 94.26% in WS and RW respectively.

**Figure 5.** Modelled response of $C_{bs}$ to increasing $C_i$ with the C$_4$ model from von Caemmerer (2000) in well-watered (WW; black continuous line) and water-stress conditions (WS; black dashed line) and after 24h from rewatering (RW; continuous gray line) in *F. bidentis* (A) and *F. trinervia* (B). The parameters used for modelling are the mean values of $V_{cmax}$, $V_{pmax}$, $R_L$, $g_{bs}$ and $g_m$ presented in table 2 and the ones described in table 1. The shaded area represents the measured range of $C_i$ of each species at atmospheric CO$_2$ (400 $\mu\text{mol mol}^{-1}$).
DISCUSSION

C₄ modeling

Due to its complexity, the C₄ model for leaf CO₂ assimilation (von Caemmerer and Furbank, 1999) requires a large number of parameters for which a precise calculation or measurement is not easy or even impossible. Because of that, in most research papers found in the literature, the majority of these parameters are assumed. In recent years, however, a number of articles have thrown some light on methods to calculate some of the key parameters of the C₄ model such as mesophyll conductance (Barbour et al. 2016; Ubierna et al. 2017), bundle-sheath conductance (Ubierna et al. 2011, 2013; Yin et al. 2011b; Bellasio & Griffiths 2014) or leakiness (Kromdijk et al. 2010, 2014).

As explained in “material and methods”, gₘ was calculated by curve fitting together with Vₖₘₐₓ and Vₚₖₐₓ. The curve fitting procedure requires maximum and minimum values to be set. gₘ upper bound was set to 2 µmol CO₂ m⁻² s⁻¹. In almost all cases, the fitting procedure took that value as the best. This values has been traditionally used for C4 modelling, since gₘ is not considered to be limiting for photosynthesis. With the new methods developed recently (Barbour et al. 2016; Ubierna et al. 2017), gₘ seems to range between 0.75 and 1.78 µmol CO₂ m⁻² s⁻¹, and still, very unlikely to be an important limitation for photosynthesis (Ubierna et al. 2013).

Bundle-sheath conductance to CO₂ was calculated with the “J/J” method proposed by Bellasio & Griffiths (2014). The method consists of fitting the chlorophyll fluorescence estimated J (J_ATP) to the theoretical total electron transport rate J (J_MOD). This method does not require isotopic discrimination data but only gas exchange and chlorophyll fluorescence which makes it easier to use. However, it carries some issues, mainly because Φ_PSIΙ, needed to estimate J_ATP, represents in C₄ leafs an unknown contribution from mesophyll versus bundle-sheath chloroplasts (Kromdijk et al. 2014). The estimates of gₙₛ obtained with this method ranged from 0.5 to 6.1 mmol m⁻² s⁻¹ bar⁻¹ with averages for species of 1.8 ± 0.26 mmol m⁻² s⁻¹ bar⁻¹ for F. bidentis and 2.16 ± 0.38 mmol m⁻² s⁻¹ bar⁻¹ for F. trinervia. This values fall within the range of gₙₛ measurements found in the literature in recent years, which range from 0.18 to 10 mmol m⁻² s⁻¹ bar⁻¹, although measured with different methods (Kromdijk et al. 2010; Yin et al. 2011b; Sun et al. 2012; Bellasio & Griffiths 2014; Retta et al. 2016). The majority of estimations found
are from *Zea mays*, and the only dicot species found was *Amaranthus edulis*, with $g_{\text{tr}}$ from 5.6 to 10 mmol m$^{-2}$ s$^{-1}$ bar$^{-1}$ (Kiirats et al. 2002).

$C_3$ vs $C_4$

There is very limited data comparing C4 and C3 under drought. Most papers conclude that C4 are more sensitive than C3 (Ripley et al. 2007, 2010; Ibrahim et al. 2008; Taylor et al. 2010) mainly due to higher metabolic limitations. Others however, have reported higher sensitivity in C3 than in C4 (Alfonso & Brüggemann 2012), or no real differences (Ward et al. 1999).

Overall, the C3 species *F. robusta* seems to be less resistant to rapid and short drought conditions than the C4 *F. bidentis*. The C4 *F. trinervia*, on the contrary, showed more signs of wilting but its photosynthetic machinery remained relatively functional, not being possible to consider it neither more nor less sensitive to water stress than *F. bidentis* and *F. robusta*. Comparing the decrease in $A_N$, $J$ and $V_{\text{cmax}}$ of water-stressed plants relative to their well-watered values shown in figure 3, the C3 *F. robusta* suffered a more important reduction in $A_N$ than *F. bidentis*, although not in $J$ and $V_{\text{cmax}}$.

According to bibliography, under mild to moderate stress, plants tend to recover within 1 or 2 days (Flexas et al. 1999; Chaves et al. 2009). If the stress is more severe, a two-stage process has been described to explain recovery (Pinheiro & Chaves 2011): in the first stage (first hours or days upon rewatering) the plant rehydrates and re-opens stomata; and in the second stage (lasts days) the plant re-synthetizes photosynthetic proteins. That second stage implies biochemical limitations and metabolic impairment that occurs only under severe stress (Flexas et al. 2004; Grassi & Magnani 2005).

In the present experiment rewatering did not translate into a recovery in any of the parameters measured in any of the three species (RW means tended to be higher than WS but not statistically different), indicating that all three species were suffering biochemical limitations. If that is the case, 24h was a short time to measure recovery since the plants would probably be in the first stage described above and no recovery in the photochemistry would be expected.

The causes of metabolic limitations in C3 plants are more known than for C4. For C3, the limitations have been attributed to alterations in Rubisco content and activity, decreased ATP
synthesis and RuBP regeneration, decreased chlorophyll content and lower photochemical efficiency (see Lawlor & Cornic 2002; Ribas-carbo et al. 2006; Lawlor & Tezara 2009 for review). The nature of the metabolic limitation on photochemistry in C\textsubscript{4} plants will be discussed below.

\textit{C\textsubscript{4} vs C\textsubscript{3}}

A good coordination between the C\textsubscript{4} and C\textsubscript{3} cycles within the leaf are considered crucial for a good functioning of the C\textsubscript{4} plants. An imbalance between the two cycles would translate on a reduced efficiency and energy waste (Pengelly \textit{et al.} 2012). Using antisense RNA targeted to different enzyme involved in the C\textsubscript{4} photosynthesis to reduce its activity, it is possible to simulate possible cases of the C\textsubscript{4}/C\textsubscript{3} balance due to ambient factors. Furbank \textit{et al.} (1996) created transformants of \textit{F. bidentis} with reduced Rubisco concentration (up to 85\%) and observed reductions in net CO\textsubscript{2} assimilation proportional to the reduction in Rubisco activity but not in activities of the C4 cycle enzymes such as PEP carboxylase or NADP-malic enzyme. Pengelly \textit{et al.} (2012) also transformed \textit{F. bidentis} with antisense RNA but targeting the NADP-malic enzyme reducing its activity by 34-75\% relative to wild type. That did not cause an effect on growth but caused net CO\textsubscript{2} assimilation to decrease by half and also a decrease in \textit{V_{p}}, \textit{C_{bs}} and thus leak rate and leakiness. However, Rubisco activity did not change. They concluded that under this scenario a reduction in C\textsubscript{4} cycle regeneration rate was more likely to be the cause of the reduced photosynthetic rate and that NADP-ME activity can be reduced by half without affecting assimilation rate.

In addition, Carmo-Silva \textit{et al.} (2008b) concluded that under drought conditions photorespiration not only remained slow but decreased with severe water stress in two C\textsubscript{4} grasses, indicating metabolic inhibition at Rubisco level. In another study, Carmo-Silva \textit{et al.} (2008a) observed that PEPC and the three C\textsubscript{4} acid decarboxylases were not affected by water deficit to an extent to limit photosynthesis. Later on, Carmo-Silva \textit{et al.} (2010) reported a decline in the quantity of RuBP in leaves as water deficit increased. These and other evidences (Ripley \textit{et al.} 2007; Ghannoum 2009) all point to the C\textsubscript{3} enzymes and not the C\textsubscript{4} as the main cause of the observed decline in photosynthesis observed in C\textsubscript{4} plants under water stress.

In the present study, at \textapprox 30\% of SWC \textit{F. trinervia} showed clear signs of water stress, with important wilting and reductions of 41.06, 42.4 and 41.36\% of \textit{A_{N}}, \textit{J} and \textit{V_{cmax}} respectively.
The cause of these reductions can be speculated from data in figure 4 and the large reduction in $V_{c_{\text{max}}}$: the C$_4$ cycle activity in the mesophyll (reflected by $V_p$ and $V_{p_{\text{max}}}$) did not seem affected by water stress whereas the C$_3$ cycle in the BS did (reflected by $V_c$ and $V_{c_{\text{max}}}$). That disruption between the two cycles caused an increased $V_p/V_c$ ratio in relation to WW conditions (from 1.61 ± 0.15 to 3.28 ± 0.11), meaning that much more CO$_2$ was being pumped into the BS than the CO$_2$ that could be fixed in the Calvin cycle. Since NADP-ME was not likely to be limiting (Pengelly et al. 2012), an increased $V_p/V_c$ ratio would explain the modelled increase of $C_{bs}$ above non-stressed levels (Fig. 5B) and thus the increased estimated leakiness (Fig. 4D).

Leakiness estimations in this experiment (from 0.1 to 0.64 in WW plants) are larger than other estimations found in literature, that range roughly between 0.14 and 0.45 (Cousins et al. 2006; Tazoe et al. 2008; Kromdijk et al. 2010; Pengelly et al. 2010, 2012; Sun et al. 2012; Ubierna et al. 2013; Gong et al. 2017). Very few information is available about leakiness under water stress conditions, although it is described to increase with water deficit (Saliendra et al. 1996; Williams et al. 2001). Saliendra et al. (1996) found that in sugarcane it increased from control values of 0.3 to 0.34-0.38 in water-stressed plants, and in Williams et al. (2001) from 0.27-0.34 in control to up to 0.42 in water-stress Sorgum bicolor. No values higher than 0.6 have been measured although the C$_4$ model predicts such values at very high $C_{bs}$, as would be the case of this experiment.

*F. bidentis* showed a reduction in $A_N$ and $J$ in the same proportion as *F. trinervia* with water stress (reduced to 71.16 and 68.8% of WW values respectively). However, when comparing the WS-$V_{c_{\text{max}}}$ as a percentage of the WW-$V_{c_{\text{max}}}$ of each C$_4$ species, *F. bidentis*’s $V_{c_{\text{max}}}$ was reduced in a lower proportion than *F. trinervia* (a 26% vs a 39.6% reduction). According to the results, that slight reduction in the C$_3$ cycle in *F. bidentis* did not alter the coordination C$_4$/C$_3$ (no change in $V_p/V_c$ ratio between WW and WS treatments) which would cause no change in $C_{bs}$ in respect to WW conditions at ambient $C_i$ (60-100 µmol mol$^{-1}$ in WS *F. bidentis* plants) and thus, maintaining leakiness as in WW plants. Note that figure 5A predicts essentially the same $C_{bs}$ in WW and WS when $C_i$ is below 100 µmol mol$^{-1}$.

**CONCLUSIONS**

From this results, it seems that *F. bidentis* is more drought resistant than *F. robusta* and *F. trinervia* at equal SWC. However, *F. robusta* and *F. trinervia* showed similar sensitivity to
water stress. All three species suffered mainly metabolic limitations, evidenced by the lack or recovery. The case of *F. trinervia* is in total agreement with the previous research cited above. In contrast, the reduction in assimilation of *F. bidentis* could not be explained by a disruption in the C$_4$/C$_3$ coordination from the data available. It is assumed that a certain degree of regulation exists coordinating the two cycles, and although the nature of the controlling mechanisms is still unclear (Pengelly *et al.* 2012), a certain degree of general downregulation might have happened to adequate to a reduction of the C$_3$ fixation.
BIBLIOGRAPHY


