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Differences in drought sensitivity of photosynthesis between C_4 and C_3 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 C_3 plants. However, the responses to water stress in C_4 have been much less studied. Due to their carbon concentrating mechanism, C_4 plants exhibit grater assimilation rates and water use efficiency. Despite this advantages, when comparing C_3 and C_4 under water stress conditions, C_4 monocots seem to be more sensitive than C_3 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 C_4 dicot species: *Flaveria bidentis* and *Flaveria trinervia*; and one C_3 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*. Rewatring did not translate into a recovery of any parameter measured in any species, indicating metabolic limitations. The two C_4 exhibited different degree of tolerance to water stress: *F. trinervia* was clearly more sensitive, being limited by Rubisco and altering the C_3/C_4 cycle balance, while in *F. bidentis* the limitation on Rubisco did not alter the coordination, maybe indicating some degree of general downregulation. This findings suggest different ranges of tolerance within the C_4 *Flaveria*, making it difficult make comparisons with the C_3.

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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, measureable as changes in leaf water potential (Ψ_{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)he 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 C4 are reduced in number in comparison to C3, 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 C3 they play non-photosynthetic roles (see Leegood (2008) for review), in C4 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 C_4 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 C_3 and at least five pure C_4, along with some intermediate C_3-C_4 photosynthesis (McKown *et al.* 2005; Sudderth *et al.* 2007). So, given the scarcity of available data about effects of drought on C_4 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_2O m^{-2} s^{-1} (considered a threshold indicating severe water stress; Flexas *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.

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:

$$\mathrm{SWC} = \frac{\mathrm{W} - \mathrm{DW}}{\mathrm{WFC} - \mathrm{DW}} \cdot 100$$

where W is the pot weight, WFC is the pot weight at field capacity and 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:

$$\mathrm{SWC} = 1.747 \cdot \mathrm{SM} + 13.932$$

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: RWC = (Fresh weight – Dry weight) / (Turgid weight – Dry weight). Turgid weight was determined keeping the leafs in

distilled water and in darkness at 4 ºC for 24 h. Dry weight obtained oven-drying the leafs for 48 h at 70 ºC.

Gas exchange measurements

To monitor the process of desiccation, and in parallel to SWC measurements, net CO_2 assimilation (A_N) and stomatal conductance (g_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^2 chamber (using ambient light). The chamber was positioned perpendicular to the light source to uniformly illuminate the leaf (349-375 µmol photons m^{-2} s^{-1}). The chamber conditions consisted in an ambient CO_2 concentration (C_a) of 400 µmol mol^{-1} air, an air flow of 400 µmol min^{-1}, an air temperature of 25 ºC, 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* leafs did not fill de 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_i (A_N-C_i curves), and to different light intensities (A_N-*PPDF*) at low O_2 concentration (<1%) were performed to each plant. A_N-*PPDF* curves at ambient O_2 concentration (21%) were also performed only to the C_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^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 C4 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_N-Ci curves, after waiting 15-30 min to steady-state conditions, C_a was changed stepwise from 400, 350, 300, 200, 100, 50, 400, 400, 500, 600, 750, 1000, 1200 1600 and 2000 µmol mol^{-1}. 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 C_a. Measurements were taken at a saturating light of 2000 µmol photons m^{-2} s^{-1}, an air flow of 400 µmol min^{-1}, 25 ºC 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-photorespiratory 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 Γ* 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 (Γ), which is

mathematically impossible. Instead, Γ* 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 Γ*, do not acclimate to water stress (Galmés *et al.* 2006).

Quantum efficiency of photosystem II (Φ_{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, α is the leaf absorbance and β is the theoretical partition of absorbed *PPDF* between the two photosystems. The product αβ 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*/*J* 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:

$$V_p = \frac{C_m \cdot V_{pmax}}{C_m + K_p}$$

where K_p is the PEPC Michaelis-Menten constant for CO_2 (parameters used are shown in table 1). Φ_{PSII} and J were calculated as previously described.

Statistical analysis

All statistical analysis was performed with R language 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 1. Acronyms, definitions, variables and units used.

Parameter	Description	Value / units	References
R_L	Respiration in the light	µmol m^{-2} s^{-1}	
R_m	Mesophyll fraction of R_L	$0.5R_L$ µmol m^{-2} s^{-1}	(von Caemmerer 2000)
g_m	Mesophyll conductance to CO_2 diffusion	mol m^{-2} s^{-1} bar^{-1}	
g_{bs}	Bundle sheath conductance to CO_2 diffusion	mol m^{-2} s^{-1} bar^{-1}	
K_c	Rubisco Michaelis-Menten constant for CO_2	*F. bidentis*: 573.5 µbar *F. trinervia*: 541.2 µbar *F. robusta*: 352.9 µbar	(Perdomo *et al.* 2015) (Perdomo *et al.* 2015) (Zhu *et al.* 1998)
K_o	Rubisco Michaelis-Menten constant for O_2	*F. bidentis*: 491538 µbar *F. trinervia*: 516153 µbar *F. robusta*: 676923 µbar	(Kubien *et al.* 2008) (Kubien *et al.* 2008) (Zhu *et al.* 1998)
K_p	PEPC Michaelis-Menten constant for CO_2	160 µbar	(Boyd, Gandin & Cousins 2015)
O	O_2 concentration in mesophyll cells (either for C_3 or C_4)	210000 µmol mol^{-1}	
α	Fraction of PSII active in Bundle sheath	0.15 (Dimensionless)	
$S_{c/o}$	Rubisco specificity factor	*F. bidentis*: 2092.3 bar bar^{-1} *F. trinervia*: 2040 bar bar^{-1} *F. robusta*: 2667.7 bar bar^{-1}	(Perdomo *et al.* 2015) (Perdomo *et al.* 2015) (Zhu *et al.* 1998)
γ*	Half the reciprocal Rubisco specificity	$0.5/S_{c/o}$	
Γ*	CO_2 compensation point in the absence of mitochondrial respiration	$(0.5O)/ S_{c/o}$ µmol mol^{-1}	

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 where found at any day.

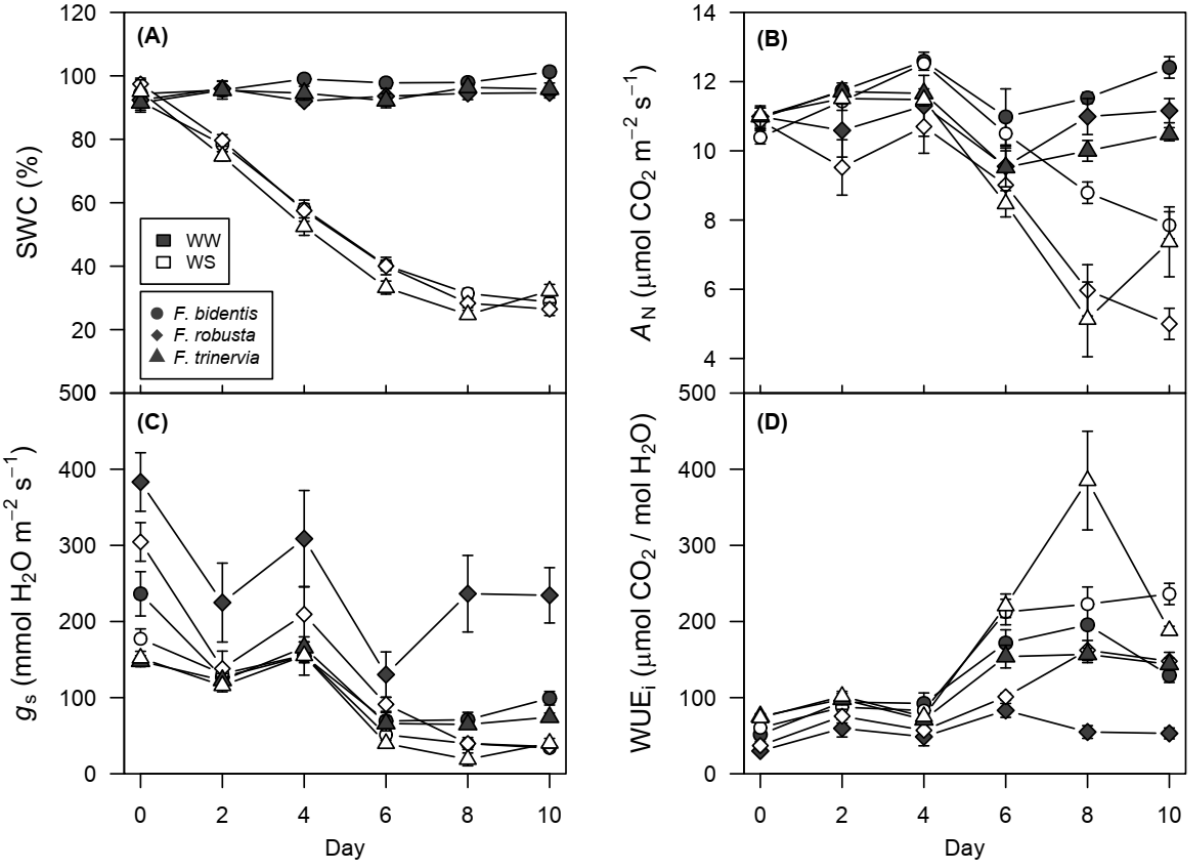

Figure 1. (A) Soil water content (SWC), (B) net CO_2 assimilation (A_N), (C) stomatal conductance (g_s) and (D) intrinsic water use efficiency (WUE_i) along 10 days for *F. bidentis* (C_4; circles), *F. trinervia* (C_4; triangles) and *F. robusta* (C_3; rhombus) under well watered (WW; gray) and water 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 ≈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_2O m^{-2} s^{-1}, while in the two C4 it ranged between 50 and 200 mmol H_2O m^{-2} s^{-1}.

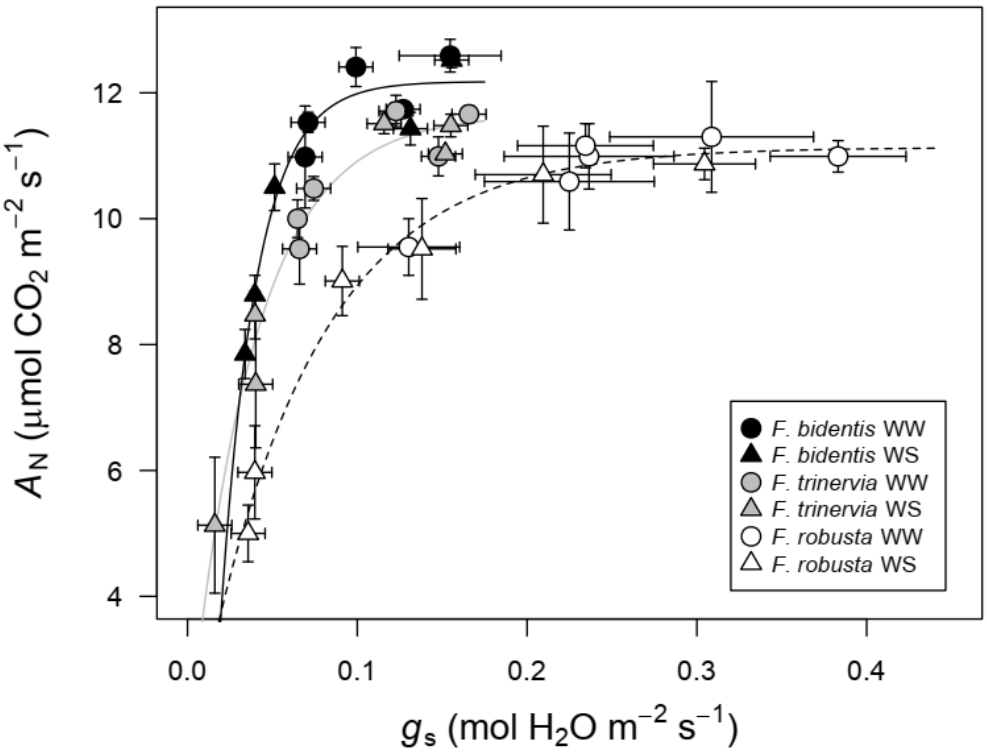

Figure 2. Relationship between net CO_2 assimilation (A_N) and stomatal conductance (g_s) of *F. bidentis* (C_4; black), *F. trinervia* (C_4; grey) and *F. robusta* (C_3; white) under well watered (WW; circles) and water stress conditions (WS; triangles). Points represent means ± SE (n = 5-6). Measurements were taken at an atmospheric CO_2 concentration of 400 µmol mol^{-1}, light intensity of 346 µmol photons m^{-2} s^{-1} and 25ºC.

Between days 4 and 6 there was a drop in A_N, and especially in g_s 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 g_s, but both *F. bidentis* and *F. trinervia* had its g_s reduced by half from days 6 to 10. That general reduction in g_s but not in A_N caused an improvement on intrinsic water-use efficiency (WUE_i; Fig. 1D). As expected, during days 0 to 4 *F. bidentis* and *F. trinervia* showed higher WUE_i than *F. robusta* although there were no differences between treatments. However, from day 6, the two C_4 improved their WUE_i in WW plants and to a higher extent in WS plants. *F. robusta* increased its WUE_i 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 A_N and g_s. 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 A_N-C_i and A_N-*PPDF* curves were performed.

Response to WS and RW for common C_3-C_4 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 CO_2 and common for C_4 and C_3 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 CO_2 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 (≈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 C4 are compared, only V_{cmax} had a differential decrease between the two species ($P = 0.046$), decreasing to a greater extent in *F. trinervia*.

Table 2. Soil water content (SWC), relative water content (RWC), respiration in the light (R_L), photosynthetic rate (A_N), stomatal conductance (g_s), electron transport rate (J), CO_2-saturated Rubisco carboxylation rate (V_{cmax}), mesophyll conductance (g_m), bundle-sheath conductance (g_{bs}) and CO_2-saturated PEPC carboxylation rate (V_{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).

Species	Treatment	SWC %	RWC %	R_L µmol CO_2 m^{-2} s^{-1}	A_N µmol CO_2 m^{-2} s^{-1}	g_s mol H_2O m^{-2} s^{-1}
F. bidentis (C4)	CL	111.85 ± 2.84 a	86.94 ± 2.28 a	2.22 ± 0.21 ab	33.26 ± 1.94 a	0.23 ± 0.02 a
	WS	24.75 ± 1.27 c	89.41 ± 1.44 a	1.77 ± 0.41 abc	21.78 ± 0.61 bc	0.16 ± 0.04 b
	RW	90.49 ± 5.42 b	83.24 ± 0 a	2.13 ± 0.16 ab	25.11 ± 1.32 b	0.16 ± 0.02 b
F. trinervia (C4)	CL	111.02 ± 1.72 a	86.15 ± 1.97 a	1.51 ± 0.16 bc	22.65 ± 1.45 b	0.18 ± 0.05 a
	WS	28.66 ± 1.19 c	83.09 ± 1.1 a	1.14 ± 0.07 c	13.35 ± 2.72 d	0.1 ± 0.04 b
	RW	94.28 ± 5.28 b	83.48 ± 1.51 a	1.52 ± 0.1 bc	14.55 ± 2.3 cd	0.17 ± 0.03 b
F. robusta (C3)	CL	113.6 ± 1.06 a	84.46 ± 0.98 a	2.43 ± 0.12 a	24.08 ± 2.39 b	0.28 ± 0.04 a
	WS	25.57 ± 1.67 c	81.79 ± 4.74 a	1.81 ± 0.29 abc	13.17 ± 0.98 d	0.12 ± 0.01 b
	RW	101.6 ± 6.47 b	85.02 ± 3.46 a	2.26 ± 0.23 ab	12.72 ± 1.4 d	0.15 ± 0.04 bb
Species	Treatment	J µmol e^- m^{-2} s^{-1}	V_{cmax} µmol CO_2 m^{-2} s^{-1}	g_m µmol CO_2 m^{-2} s^{-1}	g_{bs} mmol CO_2 m^{-2} s^{-1}	V_{pmax} µmol CO_2 m^{-2} s^{-1}
F. bidentis (C4)	CL	230.86 ± 14.72 ab	38.48 ± 1.74 c	1.99 ± 0.01 a	1.67 ± 0.39 a	222.4 ± 74.24 a
	WS	147.62 ± 6.06 c	29.08 ± 0.74 c	2 ± 0 a	2.28 ± 0.59 a	171.18 ± 34.81 a
	RW	172.29 ± 8.41 c	27.87 ± 1.45 c	2 ± 0 a	1.48 ± 0.38 a	142.04 ± 31.31 a
F. trinervia (C4)	CL	145.04 ± 6.97 c	26.57 ± 1.55 c	2 ± 0 a	1.84 ± 0.85 a	101.47 ± 7.31 a
	WS	83.54 ± 12.69 d	15.58 ± 2.53 d	1.8 ± 0.2 a	2.47 ± 0.75 a	169.56 ± 52.43 a
	RW	88.81 ± 10.67 d	16.42 ± 2.48 d	2 ± 0 a	1.78 ± 0.43 a	130.69 ± 14.29 a
F. robusta (C3)	CL	240.69 ± 12.49 a	135.5 ± 10.83 a	0.26 ± 0.04 b	—	—
	WS	182.57 ± 16.92 bc	84.07 ± 12.83 b	0.22 ± 0.07 b	—	—
	RW	177.09 ± 10.12 bc	83.31 ± 7.47 b	0.23 ± 0.08 b	—	—

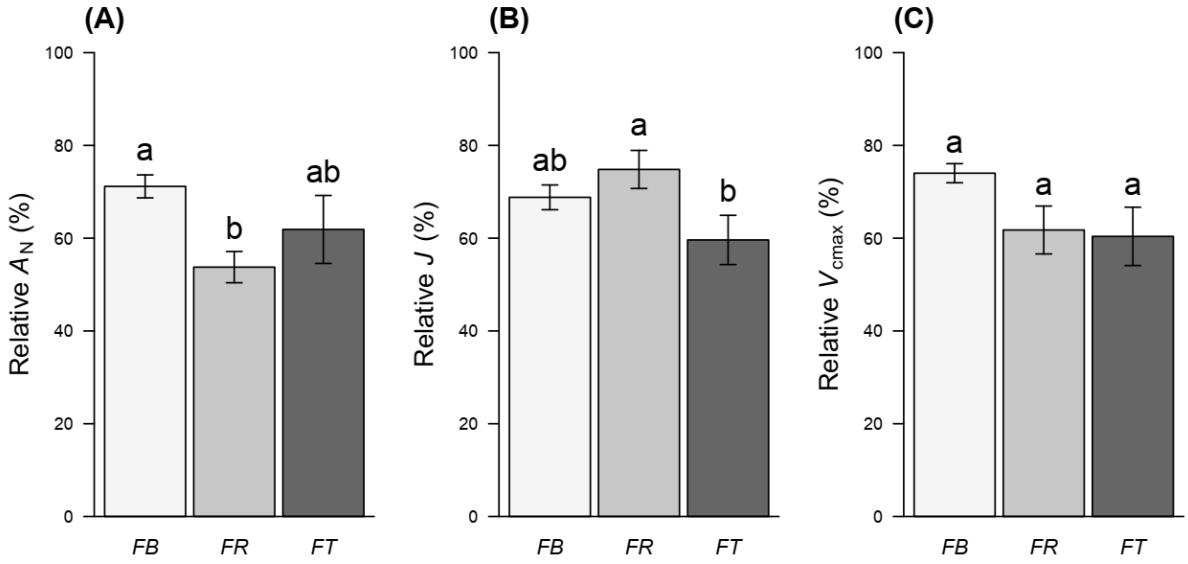

Figure 3. WS values of net CO_2 assimilation (A_N; A), electron transport rate (J; B) and CO_2-saturated Rubisco carboxylation rate (V_{cmax}; C) relativized to their corresponding WW values. *FB = F. bidentis* (C_4); *FR = F. robusta* (C_3); *FT = F. trineriva* (C_4). Bars are means with SE (n = 9-11). Different letters indicate statistically different responses between species at $P < 0.05$ (Tukey's HSD *post hoc* test).

Response to WS and RW for C_4 measured variables

No differences were found in g_{bs} or V_{pmax} neither for species nor treatment. Other exclusive parameters from the C_4 model and the Rubisco carboxylation rate (V_c) are also presented in figure 4. No differences were found in PEPC carboxylation rate (V_p; Fig. 4A). In contrast, differences in V_c (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. trinervia* by 34.87% on average. The ratio V_p/V_c was not altered by WS in *F. bidentis* but it increased twofold in *F. trinervia* (Fig. 4C). Leakiness (ϕ), theratio between the Leak rate (rate of CO_2 leaking out of the BS back to the M) and V_p followed the same trend as V_p/V_c: it did not change in *F. bidentis* but it also doubled in *F. trinervia* (Fig. 4D).

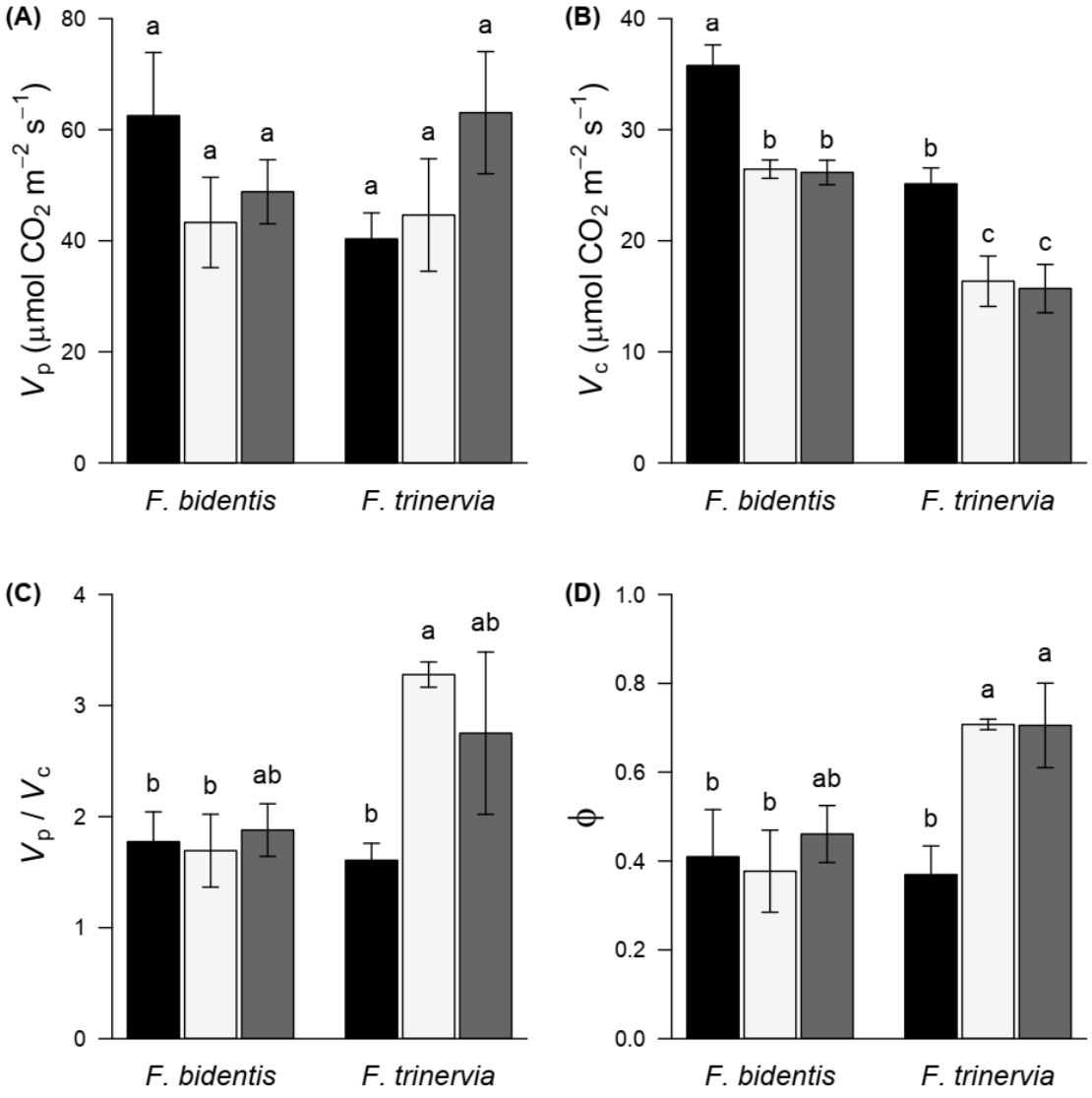

Figure 4. (A) PEPC carboxylation rate (V_p), (B) Rubisco carboxylation rate (V_c), (C) ratio V_p/V_c and (D) Leakiness (ϕ, ratio V_p/L) of the two C_4 species *F. bidentis* and *F. trinervia* under well-watered (WW; black bars) and water-stress conditions (WS; white bars) and after 24h since rewatering 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} C_{bs}

is 14.57% smaller than the WW). For *F. trinervia* (Fig. 5B), a theoretical C_i = 200 μmol 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.

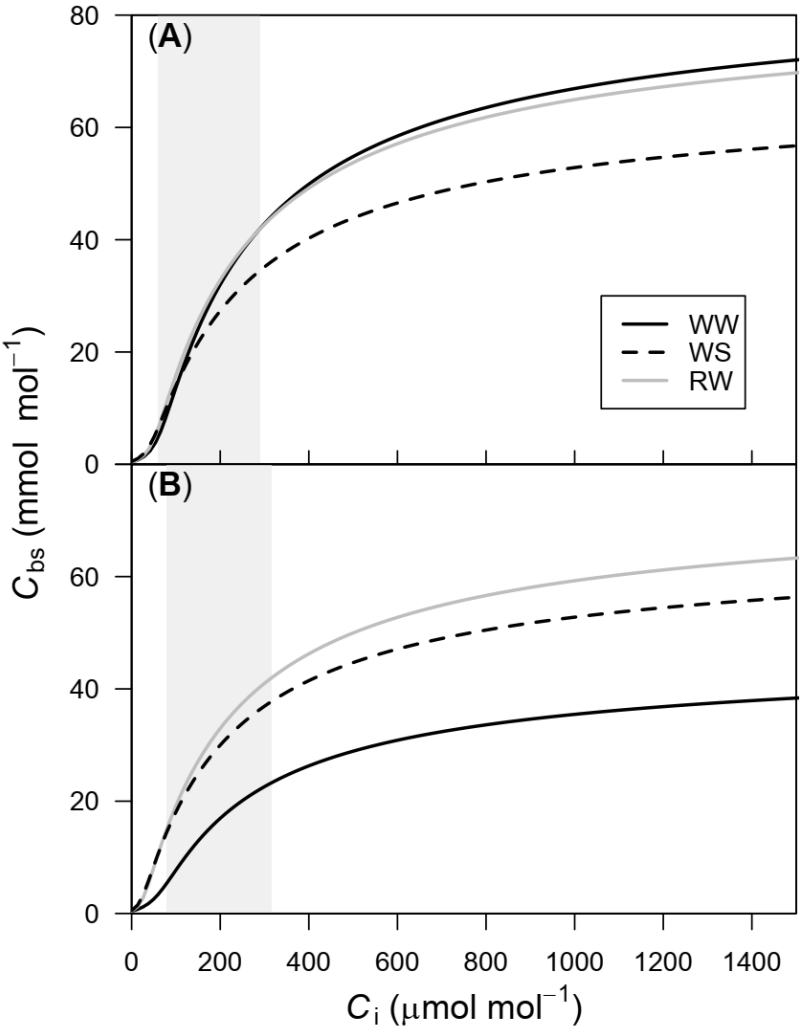

Figure 5. Modelled response of C_{bs} to increasing C_i with the C_4 model from von Caemmerer (2000) in well-watered (WW; black continuous line) and water-stress conditions (WS; black dashed line) and after 24h from rewatering (RW; continuous gray line) in *F. bidentis* (A) and *F. trinervia* (B). The 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 μmol mol^{-1})

DISCUSSION

C_4 modeling

Due to its complexity, the C_4 model for leaf CO_2 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 C_4 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", g_m was calculated by curve fitting together with V_{cmax} and V_{pmax}. The curve fitting procedure requires maximum and minimum values to be set. g_m upper bound was set to 2 μmol CO_2 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 g_m is not considered to be limiting for photosynthesis. With the new methods developed recently (Barbour et al. 2016; Ubierna et al. 2017), g_m seems to range between 0.75 and 1.78 μmol CO_2 m^{-2} s^{-1}, and still, very unlikely to be an important limitation for photosynthesis (Ubierna *et al.* 2013).

Bundle-sheath conductance to CO_2 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_{PSII}, needed to estimate J_{ATP}, represents in C_4 leafs an unknown contribution from mesophyll versus bundle-sheath chloroplasts (Kromdijk *et al.* 2014). The estimates of g_{bs} 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 g_{bs} 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_{bs} 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 C_4 are more sensitive than C_3 (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 C_3 than in C_4 (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 C_4 *F. bidentis*. The C_4 *F. trinervia*, on the contrary, showed more signs of wilding 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_{cmax} of water-stressed plants relative to their well-watered values shown in figure 3, the C_3 *F. robusta* suffered a more important reduction in A_N than *F. bidentis*, although not in J and V_{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 C_3 plants are more known than for C_4. For C_3, 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_4 plants will be discussed below.

C_4 vs C_4

A good coordination between the C_4 and C_3 cycles within the leaf are considered crucial for a good functioning of the C_4 plants. An imbalance between the two cycles would translate on a reduced efficiency and energy waste (Pengelly *et al.* 2012). Using antisense RNA targeted to different enzyme involved in the C_4 photosynthesis to reduce its activity, it is possible to simulate possible cases of the C_4/C_3 balance due to ambient factors. Furbank *et al.* (1996) created transformants of *F. bidentis* with reduced Rubisco concentration (up to 85%) and observed reductions in net CO_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 *et al.* (2012) also transformed *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_2 assimilation to decrease by half and also a decrease in V_p, C_{bs} and thus leak rate and leakiness. However, Rubisco activity did not change. They concluded that under this scenario a reduction in C_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 *et al.* (2008b) concluded that under drought conditions photorespiration not only remained slow but decreased with severe water stress in two C_4 grasses, indicating metabolic inhibition at Rubisco level. In another study, Carmo-Silva *et al.* (2008a) observed that PEPC and the three C_4 acid decarboxylases were not affected by water deficit to an extent to limit photosynthesis. Later on, Carmo-Silva *et al.* (2010) reported a decline in the quantity of RuBP in leafs as water deficit increased. These and other evidences (Ripley *et al.* 2007; Ghannoum 2009) all point to the C_3 enzymes and not the C_4 as the main cause of the observed decline in photosynthesis observed in C_4 plants under water stress.

In the present study, at ≈30% of SWC *F. trinervia* showed clear signs of water stress, with important wilting and reductions of 41.06, 42.4 and 41.36% of A_N, J and V_{cmax} respectively.

The cause of these reductions can be speculated from data in figure 4 and the large reduction in V_{cmax}: the C_4 cycle activity in the mesophyll (reflected by V_p and V_{pmax}) did not seem affected by water stress whereas the C_3 cycle in the BS did (reflected by V_c and V_{cmax}). 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_{cmax} as a percentage of the WW-V_{cmax} of each C_4 species, *F. bidentis*'s V_{cmax} 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.

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