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Long-term acclimation to different stress types: revealing tradeoffs between mesophyll and stomatal conductance

Running Title:

Long-term acclimation of mesophyll and stomatal conductance to different stress types

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Highlight

We have compared the photosynthesis limitation resulting from long-term acclimation to three major a-biotic stresses (drought, salinity and temperature) to understand which limiting-factor are dominant under each type of stress.

Abstract

Ziziphus spina-christi, a thermophilic tree, became more abundant in the Mediterranean, presumably due to increased winter temperatures. In order to predict the plant acclimation to future climate changes, we attempted to understand which factors underlie photosynthetic stress acclimation.

Stress acclimation to three major long-term abiotic stresses (drought, salinity and temperature) was investigated by measuring growth, gas exchange, chlorophyll fluorescence and leaf structure. We developed a stress index that allowed to precisely define stress level, enabling a comparison between stress types. For each stress, photosynthesis-limiting factors were compared: stomatal conductance (g_s), mesophyll conductance (g_m) and maximum capacity for Rubisco carboxylation (V_{cmax}).

Photosynthesis under all stresses was limited mostly by g_s and g_m (80-90%); whereas biochemistry (V_{cmax}) made a minor contribution (10-20%). The relative contribution of g_s and g_m on photosynthetic limitation was influenced by stress type. During acclimation to drought or salinity, photosynthesis was limited by a decline in g_s , while intolerance to low temperatures was driven by decline in g_m . Low mesophyll-imposed limitation was the best predictor of abiotic stress tolerance.

The results demonstrate how warming climate benefits thermophilic species. Moreover, current work gives methodology for stress studies, and defines the main factors underlying the plant response to climate change.

Key words: *Ziziphus spina-christi*; drought stress; mesophyll conductance; photosynthesis limitation; salinity stress; stomatal conductance; temperature

Introduction

An increasing number of studies have shown that climate change affects a wide range of tree species (Saxe *et al.*, 2001; Walther *et al.*, 2002). Climate change can influence habitats in several ways, including increased incidence of extreme temperatures, reduced precipitation and an increase in soil salinity (IPCC, 2014; McDowell *et al.*, 2011). All of these can have a deleterious effect on tree productivity and survival and significantly influence biodiversity (Allen *et al.*, 2010). For this reason, it is important to understand how trees acclimate to environmental changes, particularly drought, increased salinity and increased temperature. Predicting stress-acclimation behaviors is complex, since under natural conditions, trees are exposed to various environmental changes that require them to cope with multiple types of stress simultaneously (Niinemets, 2010). Moreover, stress acclimation is a gradual process that involves structural, physiological and biochemical adjustments (Flexas *et al.*, 2006a; Galmés *et al.*, 2006).

Growth and leaf photosynthetic capacity are the main processes affected by abiotic stress (Chaves *et al.*, 2002). Therefore, studies of photosynthesis under stress conditions can help to explain how different species acclimate and adapt to their environments (Flexas *et al.*, 2014). It is common practice to consider reductions in photosynthesis as a consequence of three limiting factors (Grassi & Magnani, 2005), specifically decreased CO₂ concentrations at the carboxylation site caused by limitations on the diffusion of CO₂ through stomata and mesophyll conductance (stomatal limitation – L_s and mesophyll limitation – L_m) and the capacity for Rubisco carboxylation- V_{cmax} (biochemical limitations – L_b). Though photosynthetic responses to numerous types of environmental stress have been studied extensively, the majority of quantitative limitation analyses have addressed short-term stress responses (Galmés *et al.*, 2007a; Flexas *et al.*, 2009; Galle *et al.*, 2009, 2011; Pérez-López *et al.*, 2012; Chen *et al.*, 2015; Wang *et al.*, 2017). Short-term responses may not reflect

important structural and physiological adjustments that occur under natural conditions (Zhou *et al.*, 2016). A few studies have addressed long-term acclimation to drought (Diaz-Espejo *et al.*, 2007; Perez-Martin *et al.*, 2009; Cano *et al.*, 2014; Tomás *et al.*, 2014) and growth temperatures (Yamori *et al.*, 2006; Warren, 2008; Perdomo *et al.*, 2016) but we know of no previous quantitative limitation analyses reports on long-term acclimation to increased soil salinity. Quantitative limitation analyses have also been used to study the stress responses of trees over their seasonal cycles (Grassi and Magnani, 2005; Limousin *et al.*, 2010; Misson *et al.*, 2010; Egea *et al.*, 2011). To date, there are no clear conclusions as to which is the most dominant limiting factor (L_s , L_m and L_b) for long-term acclimation to stress (Flexas *et al.*, 2008). For this reason, it is important to test each type of stress separately, to facilitate comprehensive comparisons. To the best of our knowledge, there have been no previous comparative studies of quantitative limitations on photosynthesis caused by different stress factors in a single species.

Ziziphus spina-christi (Rhamnaceae) is a thermophilic tree that is able to survive in arid areas (Saied *et al.*, 2008). The species' natural range of distribution extends from Sudan and Ethiopia to North Africa, India and the Eastern Mediterranean. Its fruits are eatable and traditionally the leaves have a medicinal value and the branches are used as valuable firewood. Over the last few decades, *Ziziphus spina-christi* has become more abundant and more widespread in the Eastern Mediterranean region, presumably as a result of higher winter temperatures (BioGIS, 2018). *Ziziphus spina-christi* is basically evergreen, but under conditions of low temperature in the winter and/or severe drought in the summer, the tree is deciduous or semi-deciduous (i.e., facultative deciduous) (Al Yamani *et al.*, 2018). Several questions have been raised regarding changes in the distribution of *Ziziphus spina-christi*: 1. Which environmental parameters define the limits of its growth and photosynthesis? 2. How does the partitioning of photosynthetic limitation (i.e., stomatal, mesophyll, biochemical)

differ under different types of stress? 3. Can we use our knowledge of photosynthetic acclimation to stress to predict shifts in this tree's abundance and natural range of distribution?

To address these questions, under controlled environmental conditions, we thoroughly examined acclimation to three types of environmental stress: drought, salinity and temperature. For each type of stress, we examined four levels of severity. It is important to note that there are various and conflicting definitions of stresses (Levitt, 1980; Lichtenthaler, 1996; Gilbert and Medina, 2016). For this reason, relating photosynthetic limitations directly to the stress severity is a challenge. The types of stress examined in this study were defined in a comparative and precise manner in terms of their impact on the following properties: growth, gas exchange, chlorophyll fluorescence, leaf structure, leaf turgor pressure and leaf hydraulic conductance. Such strict differentiation leads to a better understanding of variations in photosynthesis and to better predictions of plant responses over the course of acclimation to environmental change.

Materials and Methods

Plant material

The study was conducted using the thermophilic tree *Ziziphus spina-christi* (Rhamnaceae). One-year-old saplings were obtained from a public nursery (JNF; Gilat, Israel). All saplings originated from uniform plant material. The saplings were kept in a controlled greenhouse before the stress experiments. They were irrigated with a solution that contained the following concentrations of nutrients: 2.0 ± 0.1 mM Ca, 1.2 ± 0.2 mM Mg, 0.27 ± 0.04 mM NH_4 , 4.6 ± 0.1 mM NO_3 , 0.30 ± 0.02 mM P and 2.3 ± 0.2 mM K.

Stress experiments

Drought experiment

All saplings were grown in 10-L containers filled with highly porous organic planting soil inside a greenhouse (30/22°C day/night, VPD=1.8-2.2 kPa). Full-scale lysimeters were used to measure evapotranspiration (ET), to determine the initial required water supply and to induce the desired levels of drought stress. We later monitored the stress levels via daily measurements of volumetric water content (VWC), carried out using a 5TM soil moisture sensor (Decagon Devices) and measurements of leaf water potential (*LWP*) taken using a pressure bomb (model Ari-Mad, Israel). The period of drought lasted 120 days, in order to let the plants acclimate and grow new leaves. Well-watered plants (WW) received 120% ET. For those plants, pot VWC was kept at 40% and *LWP* was kept between -0.5 and -1 MPa. For the mild-stress treatment (WS1), we applied deficit irrigation of 20% ET, which led to a VWC of $22 \pm 1.7\%$. For the high-stress treatment (WS2), plants received only 5% of the initial ET, which led to a VWC of $10.2 \pm 2.6\%$. Severely stressed plants did not receive any water at all and were evaluated after 10 days when VWC had reached zero and *LWP* exceeded -1.8 MPa.

Salinity experiment

All saplings were grown in 10-L containers filled with Perlite-2. After 60 days, the plants were subjected to four different irrigation-salinity treatments with NaCl concentrations of 0, 30, 60 and 90 mM. Those treatments were continued for 140 days to allow the plants to acclimate and grow new leaves.

To determine stress levels on a leaf basis, leaf fresh weight (FW) was determined. Leaves were dried in a 70°C oven for 7 days and the leaf Na^+ concentration was measured using inductively coupled plasma (ICP; ICP-AES, Arcos, Spectro, Kleve, Germany).

Temperature experiment

This experiment was conducted in an enclosed greenhouse (phytotron). All saplings were grown in 10-L containers filled with highly porous organic planting soil. In order to allow for gradual acclimation, plants were grown for 40 days at 28/22°C (day/night) before being subjected to the different temperature treatments. Those treatments were: 34/28°C, 28/22°C, 22 /16°C and 16/10°C. Greenhouse VPD was kept in the range of 1.6-1.9 kPa in all treatments. We also used a separate growth room kept at 10/5°C in order to induce severe temperature stress. The experiment lasted 150 days to allow the plants to acclimate and grow new leaves.

Gas exchange and chlorophyll a fluorescence measurements

Gas-exchange measurements were conducted with the LiCor-6400 portable photosynthesis measurement system, equipped with a 2-cm² fluorescence leaf chamber (6400-40 Leaf-Chamber Fluorometer; LiCor Inc., Lincoln, NE, USA). All measurements were carried out on leaves that had grown under the specific stress conditions. Measurements were taken using the youngest fully expanded leaves (usually between the 5th and 7th from the apical shoot). Measurements were conducted at a saturated light intensity (1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), with air flow of 300 $\mu\text{mol air s}^{-1}$ at ambient temperature and RH of 60-50%. Block temperature was set to 32 °C in the drought and salinity experiments. Measurements were carried out between 8:00-11:00 (just before mid-day depression) only on clear days. The diffusional leakage was corrected using the empty chamber method according to Rodeghiero *et al.*, (2007). Diffusion coefficients for CO₂ were $K_{\text{CO}_2}=0.24$ for the 2cm² leaf chamber and $K_{\text{CO}_2}=0.31$ for the 6cm² leaf chamber.

CO₂ and light-response curves

After a leaf was clamped to the LiCor-6400 chamber, we allowed 15 min of acclimation to a fixed flux of 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air for non-stressed plants and 50 $\mu\text{mol mol}^{-1}$ for stressed plants. After the leaf had acclimated, we combined the CO₂ response curves for gas-exchange (GE) and chlorophyll a fluorescence (CF). Once the first CO₂ response curves had been calculated, inlet air was replaced with air containing 2% O₂, the leaves were acclimated for 10 min to a fixed 400 $\mu\text{mol mol}^{-1}$ and an additional CO₂ response curve was calculated. Next, light-response curves were calculated in ambient (21% O₂) and low oxygen (2% O₂).

The final step was to construct CO₂ response curves with air containing 21% O₂ at different PPFD levels, according to the Laisk method (Laisk, 1977). For greater accuracy, we used a LiCor-6400XT photosynthesis measurement system with a red-blue light source and a 6-cm² chamber. CO₂ response curves were constructed for several PPFD levels (400, 200, 100 and 50 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) at CO₂ concentrations of 400, 300, 200, 150, 100, 75, 50 and 25 $\mu\text{mol mol}^{-1}$.

Leaf water relations

After the GE and CF measurements, the measured leaf was cut and inserted into a pressure bomb, in order to measure its water potential (Ψ_w). The same leaf was then immediately transferred into liquid nitrogen. Samples were also put into 0.5-ml Eppendorf tubes and centrifuged to extract the tissue sap. The osmotic potential (Ψ_s) of the extracted sap was determined using a vapor pressure osmometer (Model 5600, ELITech Group, Puteaux, France). Leaf turgor pressure (Ψ_p) was later calculated using the water potential equation:

$$\Psi_p = \Psi_w - \Psi_s.$$

To estimate leaf hydraulic conductance, we measured midday stem water potentials (Ψ_{stem}) by covering leaves, approximately 3 h prior to cutting, with a bag that was

impermeable to water vapor. Leaf hydraulic conductance (K_{leaf}) was estimated using the evaporative flux method (Sack *et al.*, 2002), that is, $K_{leaf} = E / (\Psi_{stem} - \Psi_{leaf})$ where E is the transpiration rate measured earlier with the LiCor-6400.

Analysis of leaf starch and soluble sugars

One hundred mg of fine powder were extracted three times in hot 80% ethanol. Total soluble sugar content was determined using the Anthrone reagent method (Dubois *et al.*, 1956). To determine starch content, the dried residue was analyzed following amyloglucosidase digestion.

Estimation of mesophyll conductance and theoretical considerations

To estimate mesophyll conductance (g_m), we used the variable J (Harley *et al.*, 1992) and curve-fitting methods (Ethier and Livingston, 2004). These two methods rely on different assumptions, but showed good agreement (Fig. S2). g_m estimation by the variable J method is expressed as:

$$g_m = \frac{A_N}{C_i - C_c}$$

where A_N is the net photosynthesis rate and C_i is the CO_2 concentration inside leaf intercellular airspaces (both determined from GE measurements). C_c is the CO_2 concentration in the chloroplast stroma, estimated as:

$$C_c = \frac{\Gamma^* * (J + 8 * (A_N + R_d))}{J - 4 * (A_N + R_d)}$$

where J is electron-transport rate calculated based on chlorophyll fluorescence, calibrated by the GE data (as described below). R_d is the non-photorespiratory respiration in light and Γ^* is the apparent photo-compensation point estimated according to the Laisk method (Laisk 1977). Laisk CO_2 curves were fitted curvilinearly (Tholen *et al.*, 2012) and y and x values of

the average of four CO₂ response intersections were taken as R_d and C_i^* . According to von Caemmerer *et al.*, (1994), Γ^* is dependent on g_m and R_d , which are related to one another as follows:

$$\Gamma^* = C_i^* + R_d/g_m.$$

However, since no g_m value was available prior to the measurements, conversion was not completed and C_i^* was taken as a good representation of Γ^* (Qiu *et al.*, 2017). To calculate the rate of electron transport (J), we constructed CO₂ and light-response curves in the absence of photorespiration (2% O₂ in air).

The actual quantum efficiency of photosystem II (ΦPS_2) was calculated according to Genty *et al.* (1989):

$$\Phi PS_2 = \frac{(F'_m - F_s)}{F'_m}$$

where F'_m is the maximum fluorescence after a light-saturating pulse of ~8000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and F_s is the steady-state fluorescence under continuous light exposure.

The electron transport rate (J_f) was calculated as:

$$J_f = \Phi PS_2 * PPFD * (\alpha * \beta)$$

where α is the coefficient of leaf absorbance and β is the fraction of absorbed quanta that reach PS₂.

The quantum yield of CO₂ fixation (ΦCO_2) was calculated from the GE measurements as: $\Phi CO_2 = (A_N + R_d)/PPFD$.

Agreement between GE and CF was predicted on the assumption that under non-(photo)respiratory conditions, there is a linear relationship between ΦPS_2 and ΦCO_2 , since CO₂ fixation is the only sink for electrons (Valentini *et al.*, 1995). The theoretical model and

experimental observations were limited to the linear region of $\Phi CO_2 < 0.05$, and $\Phi PS_2 < 0.5$.

The slope of the linear regression (k) and the y-axis intercept (b) were used to recalculate the ($\alpha^*\beta$) for the electron-transport rate (J_{cal}) based on CF measured under ambient oxygen conditions ($O_2 = 21\%$) as:

$$J_{cal} = (4 * (\Phi PS_2 - b)/k) * PPFD.$$

Considerations for the estimation of g_m under stress conditions

We estimated g_m for stressed plants by allowing the plants to acclimate to low concentrations of CO_2 (Centritto *et al.*, 2003). This method reduces stomatal limitation and maintains sufficient C_i to avoid the negative effect of (photo)respiration on g_m (see Tholen *et al.*, 2012; Th  roux-Rancourt *et al.*, 2014). In addition, in severely stressed plants, the epidermis transfers significantly more water vapor than CO_2 , which may bias the estimation of C_i (Boyer *et al.*, 1997). Taking all of this into consideration, we measured cuticular conductance (g_{cut}) for severely stressed leaves by covering the abaxial epidermis with paraffin. Cuticular conductance (g_{cut}) was found to be $0.005 \pm 0.001 \text{ mol m}^{-2} \text{ s}^{-1}$. Measurements of fluorescence quantum yield at several locations on stressed leaves revealed that clear stomatal patchiness could be observed only when stomatal conductance of water vapor (g_{sw}) was less than $0.05 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (or $g_{sc} > 0.0312 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$). Therefore, in order to avoid any negative effects of stomatal patchiness and dominant cuticular conductance on the estimation of C_i , we decided not to perform any measurements on plants with g_{sw} lower than $0.05 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (i.e., g_{cut} that was less than 10% of g_{sw}). Since the dependence of g_m on C_i is sensitive to measurement error (Pons *et al.*, 2009; Gilbert *et al.*, 2011), g_m values were considered reliable only when dC_c/dA_N values were within the criterial range of $10 < dC_c/dA_N < 50$, as described in detail by Harley *et al.* (1992).

Quantitative limitations of photosynthesis

The A_N/C_i curves were converted into A_N/C_c curves and analyzed using the fitting tool developed by Sharkey *et al.* (2007). The maximum carboxylation rate (V_{cmax}) and rate of photosynthetic electron transport (J_{max}) were computed. Parameters were adjusted to 25°C to enable comparisons (Bernacchi *et al.*, 2001). We later used the A_N/C_c curves to calculate the absolute limitations on net photosynthesis, as described by Grassi and Magnani (2005). In brief and as described in detail by Farquhar *et al.* (1980), light-saturated photosynthesis can be expressed as:

$$A_c = \frac{V_{cmax}(C_c - \Gamma^*)}{C_c + K_c(1 + \frac{O}{K_o})} + R_d$$

where A_c is the rate of photosynthesis during the Rubisco carboxylation-limited stage, O is the O_2 partial pressure at the site of carboxylation and K_c and K_o are the Michaelis-Menten constants of Rubisco for carboxylation and oxygenation were taken from Bernacchi *et al.*, 2002. The rate of photosynthesis can be limited by substance availability (C_c) caused by diffusional restrictions (g_s and g_m) or by biochemical factors (changes in V_{cmax}). Therefore, total photosynthetic limitations are divided into three components: stomatal limitation (L_s), mesophyll limitation (L_m) and biochemical limitation (L_b). Changes in light-saturated photosynthesis ($\Delta A_c/A_c$) can be expressed in parallel terms of relative change in g_{sc} ($g_{sw}/1.6$),

$$g_m \text{ and } V_{cmax}: \frac{\Delta A_c}{A_c} = L_s + L_m + L_b = l_s * \frac{\Delta g_{sc}}{g_{sc}} + l_m * \frac{\Delta g_m}{g_m} + l_b * \frac{\Delta V_{cmax}}{V_{cmax}}$$

$$l_s = g_t * \frac{\partial A_N / \partial C_c}{g_s + (g_t + \partial A_N / \partial C_c)}$$

$$l_m = g_t * \frac{\partial A_N / \partial C_c}{g_m + (g_t + \partial A_N / \partial C_c)}$$

$$l_b = g_t * \frac{1}{g_t + \partial A_N / \partial C_c}$$

where g_t is the total CO_2 leaf conductance, calculated as:

275 $g_t = 1/(1/g_m + 1/g_{sc})$

276 and $\partial A_N/\partial C_c$ is the first derivative of A_N/C_c curves calculated as:

277 $\partial A_N/\partial C_c = V_{cmax} * (\Gamma^* + K_m)/(C_c + K_m)^2$

278 where K_m is the effective Michaelis–Menten constant for CO₂. Total limitations on the rate of
279 photosynthesis were calculated based on the maximum reference photosynthesis (A_{max}):

280 $\Delta A_N/A_N = (A_{max} - A_N)/A_N$.

281 **Leaf structural analyses**

282 *Leaf mass per unit area (LMA)*

283 Leaf area was measured using an area meter (model LI-3100, Li-Cor, Lincoln, NE, USA).

284 Leaves were dried in a 70°C oven for 7 days, and then dry weight was measured.

285 *Growth measurements*

286 Measurements of the elongation of the main stem were carried out by attaching plastic
287 markers on the apical meristem at a period of every 5 days.

288 *Histology*

289 The central area of the uppermost fully expanded leaf was measured. Samples were
290 immediately fixed in FAA (5:5:90, formalin: acetic acid:70% ethanol), dehydrated in a
291 graded alcohol series and embedded in paraffin wax (Paraplast Plus, Leica). Cross-sections
292 were cut 12-µm thick using a rotary microtome (Leica, Germany) and stained in Toluidine
293 Blue O. Samples were viewed and micro-graphed using an inverted microscope imaging
294 system (EVOSTM XL Core, Fisher Scientific).

295 Image analysis was done using ImageJ software (Rasband, W.S., ImageJ, U. S.

296 National Institute of Health, Bethesda, MD, USA, <http://imagej.nih.gov/ij/>, 1997–2015).

Leaf thickness was measured at three different locations on the cross section. The mesophyll surface area exposed to intercellular airspace per unit leaf area (S_{mes}/S) was calculated according to Syvertsen *et al.* (1995) as $S_m = (L_m/W) * F$, where L_{mes} is the total length of mesophyll cells facing the intercellular air space, W is the section width and F is the cell-shape curvature correction factor for ellipsoids (Evans *et al.* 1994). The fraction of the intercellular air space (%IAS or f_{IAS}) was calculated as $f_{IAS} = 1 - \Sigma S_s / t_{mes} * W$, where ΣS_s is the sum of the cross-sectional areas of the mesophyll cells and t_{mes} is the thickness of the mesophyll between two epidermal layers. Stomatal density was measured from epidermal peels, as described by Shtein *et al.* (2011).

Stress index

The stress index was calculated as a percentage of the difference in physiological variables between the optimal reference (o) and the stress value (s) using the following equation:

$$SI = \frac{1}{4} \left[\frac{\psi_{Po} - \psi_{Ps}}{\psi_{Po}} + \frac{K_{leaf\ o} - K_{leaf\ s}}{K_{leaf\ o}} + \frac{F_v/F_{m_o} - F_v/F_{m_s}}{F_v/F_{m_o}} + \frac{SC_o - SC_s}{SC_o} \right]$$

where ψ_P is the leaf turgor pressure and K_{leaf} is the leaf hydraulic conductance. ψ_P and K_{leaf} are both indicative of leaf water status. F_v/F_m is the dark-adapted chlorophyll fluorescence, indicating the potential efficiency of PS_2 . SC is the soluble carbohydrate content, which is indicative of the leaf's metabolic state.

Statistical analysis

We used five to seven trees as biological repeats for each treatment and control. We performed three technical repeats for each biological repeat. Analysis of variance (ANOVA) was used (JMP 14 software, SAS Institute Inc., Cary, NC, USA) to identify any statistical differences between treatments. The Tukey-Kramer post hoc test was used to compare the

treatments found to be significantly different from one another. Data-fitting was carried out using MATLAB software (MathWorks Inc., Natick, MA, USA).

Results

Precise definition of stress

This study focused on three different types of abiotic stress (drought, temperature and salinity) and examined four levels of severity for each type of stress. In order to physiologically define each type of stress and set stress thresholds, measurements of main stem elongation were carried out for all of the stress treatments. The moderate stress threshold was defined as a 50% decline compare to the average of the control growth rate. In the drought-stress experiment, a 50% decline in the growth rate was observed as growth of 0.6 ± 0.06 cm per day (Fig. 1A), which was within the leaf water potential (*LWP*) range of $-1 > LWP > -1.4$ MPa. The lowest *LWP* values that allowed for any growth were between -1.4 and -1.8 MPa. Under those conditions, the growth rate was 0.34 ± 0.03 cm per day, which represented a 72% reduction relative to the control. *LWP* values below -1.8 MPa caused a cessation of growth and were, therefore, defined as severe water stress.

Under salinity stress, a 50% reduction in the growth rate was defined as growth of 0.7 ± 0.05 cm per day, which was observed at a NaCl concentration of 90 mM (Fig. 1B). Therefore, severe salt stress was not reached. For temperature stress, a 50% reduction was defined as growth of 0.77 ± 0.1 cm per day in the 22/16°C treatment, which corresponded to leaf temperatures below 25°C (Fig. 1C). In the 16/10°C temperature treatment, the growth rate was 0.12 ± 0.03 cm per day, which represented a 92% reduction relative to the control.

In order to define GE under various stress conditions, we analyzed the different types of stress in terms of their effects on leaf properties during the acclimation process: leaf water

potential for the drought experiment, leaf Na^+ concentration for the salinity experiment and leaf temperature for the temperature experiment. As shown in Figure 2, the net photosynthetic rate (A_N) declined hyperbolically ($R^2 = 0.79$, $P < 0.0001$) with the decrease in LWP (Fig. 2A), declined nearly exponentially with the increase in leaf Na^+ ($R^2 = 0.66$, $P < 0.0001$) (Fig. 2C) and decrease in leaf temperature ($R^2 = 0.79$, $P < 0.0001$) (Fig. 2E). The maximum capacity for Rubisco carboxylation (V_{cmax}) exhibited threshold-like behavior in its response to drought and dropped only when LWP exceeded -1.5 MPa (Fig. 2B). However, it decreased exponentially with the increase in leaf Na^+ ($R^2 = 0.55$, $P = 0.0004$) (Fig. 2D) and with the reduction in leaf temperature ($R^2 = 0.78$, $P < 0.0001$) (Fig. 2F).

Mesophyll to stomatal conductance ratio ($g_m:g_s$) as a driver of intrinsic water-use efficiency

Under favorable conditions, in all experiments, stomatal conductance to CO_2 (g_{sc}) was ~ 10 -50% higher than g_m (Fig. 3). g_{sc} and g_m were not coordinated during acclimation to drought and salinity. In the drought experiment, while g_{sc} decreased exponentially with the decline in LWP ($R^2 = 0.79$), g_m was nearly constant ($\sim 0.16 \text{ mol m}^{-2} \text{ s}^{-1}$) under moderate water stress ($-0.5 > LWP > -1.5$ MPa,) and dropped significantly when LWP exceeded -1.5 MPa (Fig. 3A).

Variation in g_m was relatively high within the range of $-1.4 > LWP > -1.8$ MPa, to the extent that in some plants it was reduced to nearly zero ($\sim 0.05 \text{ mol m}^{-2} \text{ s}^{-1}$); whereas in other plants, it remained high (~ 0.15 - $0.2 \text{ mol m}^{-2} \text{ s}^{-1}$). g_m reached zero under severe water stress ($LWP < -1.8$ MPa). A greater decline in g_{sc} relative to g_m had a strong impact on intrinsic water-use efficiency ($iWUE$), that is, the ratio of A_N to g_{sw} (Fig. 3B). There was a moderate increase in $iWUE$ between LWP values of -0.5 to -1.5 MPa, from ~ 40 up to $\sim 130 \mu\text{mol mol}^{-1}$, but $iWUE$ fell back to its initial lower values when LWP was less than -1.5 MPa.

In the salinity experiment (Fig. 3C), g_{sc} decreased exponentially as leaf Na^+ increased ($R^2 = 0.67$, $P < 0.0001$), while g_m decreased moderately and linearly ($R^2 = 0.57$, $P < 0.0001$). g_{sc} was greater than g_m (~ 0.18 vs. $0.15 \text{ mol m}^{-2} \text{ s}^{-1}$) at low leaf Na^+ levels ($< 50 \text{ mmol kg}^{-1}$). When Na^+ levels were raised to above 130 mol kg^{-1} , g_m was larger than g_{sc} (~ 0.05 vs. $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$). As in the case of drought, when there was a sharp decline in g_{sc} relative to g_m , within the range of $0 < \text{leaf Na}^+ < 200 \text{ mmol kg}^{-1}$, we observed an increase in $iWUE$ from 60 to $120 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ (Fig. 3D).

In the temperature experiment (Fig. 3E), g_{sc} and g_m both decreased exponentially (g_{sc} : $R^2 = 0.81$, $P < 0.0001$, g_m : $R^2 = 0.94$, $P < 0.0001$) over the course of acclimation to decrease in leaf temperatures. However, g_m decreased more sharply and g_m values were lower than g_{sc} values across all of the examined leaf temperatures, which led to low ($\sim 40\text{--}50 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) and relatively constant $iWUE$ across the entire range of examined leaf temperatures (Fig. 3F).

Limitation of photosynthesis during long-term acclimation to stress

As shown in Figure 4, across all of the examined types of stress, the net photosynthesis rate (A_N) was limited mostly by factors related to the diffusion of CO_2 : stomatal limitation and mesophyll limitation (L_s and L_m). In spite of the obvious decline in V_{cmax} , biochemical limitation (L_b) of the rate of photosynthesis remained relatively low for all types of stress ($\sim 5\%$ drought $\sim 15\%$ salinity $\sim 20\%$ temperature). In the drought experiment (Fig. 4A), the total photosynthesis limitation (L_t) increased exponentially ($R^2 = 0.72$, $P < 0.0001$) as LWP decreased. L_s was hyperbolically correlated with the ongoing decrease in LWP ($R^2 = 0.51$, $P < 0.0001$). L_m was relatively constant ($\sim 15\%$) under well-watered and moderate drought conditions ($-0.5 > LWP > -1.5 \text{ MPa}$) and increased sharply to $\sim 75\%$ when LWP was above -1.5 MPa ($R^2 = 0.74$, $P < 0.0001$). In the salinity experiment (Fig. 4B), L_t increased hyperbolically ($R^2 = 0.79$, $P < 0.0001$), reaching a plateau ($\sim 70\%$) at a leaf Na^+ concentration

of $\sim 200 \text{ mmol kg}^{-1}$. L_s was the most dominant limiting factor along all of the examined levels of leaf Na^+ ; it increased hyperbolically ($R^2 = 0.54$, $P=0.0005$) and plateaued ($\sim 40\%$) when leaf Na^+ was $\sim 200 \text{ mmol kg}^{-1}$. L_m exhibited more moderate behavior, as it was $\sim 10\%$ at low leaf Na^+ ($< 200 \text{ mmol kg}^{-1}$), and increased inversely to L_s ($R^2 = 0.41$, $P=0.0043$), up to $\sim 30\text{-}40\%$, only when leaf Na^+ exceeded 200 mmol kg^{-1} .

In the temperature experiment (Fig. 4C), all types of limitation increased hyperbolically (L_t : $R^2 = 0.89$, $P<0.0001$; L_s : $R^2 = 0.6$, $P<0.0001$; L_m : $R^2 = 0.72$, $P<0.0001$; L_b : $R^2 = 0.36$, $P=0.0032$) during acclimation to decreased leaf temperatures. Unlike acclimation to drought or salinity, acclimation to low temperature ($30^\circ\text{C} \rightarrow 10^\circ\text{C}$) was dominated by L_m ($\sim 30\text{-}50\%$), while L_s and L_b ($\sim 10\text{-}20\%$) remained significantly lower.

In order to precisely quantify the stress levels, we created a stress index (SI) that expresses a general index for the decrease of leaf physiological performance (excluding GE parameters; Fig. 5). SI is composed of the decrease in K_{leaf} , Ψ_p , dark-adapted chlorophyll fluorescence (F_v/F_m) and the amount of soluble sugar in the leaf (SC , see Fig. S1). Higher SI values indicate stress susceptibility; whereas lower values indicate advanced stress tolerance. SI is correlated with relative growth so that a 50% reduction in growth is equivalent to a SI of 0.4 (Fig. 5A). A SI of less than 0.5 allows for continued growth, as well as the improved $iWUE$ (Fig. 5C) accompanied with increase in the $g_m:g_s$ ratio (Fig. 5B). In addition, it can be seen that SI is significantly correlated with the overall limitation of photosynthesis (L_t) for all treatments combined ($R^2 = 0.89$, $P < 0.0001$) (Fig. 6). Plotting L_t components (L_s , L_m and L_b) versus SI revealed that SI is significantly correlated with L_m ($R^2 = 0.91$, $P < 0.0001$), has a weaker correlation with L_s ($R^2 = 0.24$, $P=0.1$) and is not correlated with L_b ($R^2 = 0.03$, $P=0.25$).

Correlation of g_m with leaf-water-relations parameters in the different under various stresses

Plotting g_m versus K_{leaf} shows different patterns for each stress (Fig 7A). In the case of drought stress, g_m and K_{leaf} were not coordinated. A sharp decline in K_{leaf} from 9 ± 1.57 to $1.8 \pm 0.5 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ did not affect g_m . No correlation was found between K_{leaf} and g_m in the salinity experiment. However, in the temperature experiment, g_m and K_{leaf} were strongly correlated ($R^2 = 0.97$, $P = 0.001$). The relationship between g_m and Ψ_p was not correlated with any of the examined types of stress (Fig. 7B). However, we did find a threshold of $\Psi_p < 0.4 \text{ MPa}$ for the drop in g_m that was observed in the drought-stress experiment.

Stress, leaf structural traits and g_m

Examination of structural and anatomical leaf characteristics revealed differences between treatments in the drought and temperature experiments (Table 1). In contrast, no significant structural or anatomical changes were found in the salinity experiment. Unlike many other forest trees characterized by a classic dorsoventral mesophyll arrangement, *Ziziphus spina-christi* has a unique mesophyll arrangement that does not include any clear spongy mesophyll (Fig. S3). Acclimation to drought and temperature were each characterized by an increase in leaf mass per area (LMA) and leaf width (L_t), accompanied by a decrease in the leaf intercellular spaces ($\%IAS$). Analysis of leaf cross-sections revealed a significantly larger mesophyll surface area exposed to the intercellular air space (S_m) and thicker leaves in the low-temperature treatment ($16/10^\circ\text{C}$).

When data from all of the stress treatments were pooled, none of the examined structural or anatomical traits exhibited any correlation with g_m (Fig. 8). When the data sets collected for the different experiments were examined separately, we found a tight negative

correlation between g_m and LMA in the drought experiment ($R^2 = 0.52$, $P = 0.26$) and in the temperature experiment ($R^2 = 0.17$, $P = 0.037$) (Fig. 8A). g_m was correlated with L_t only in the drought experiment ($R^2 = 0.79$, $P < 0.01$) (Fig. 8B). Among the pooled data, no relationship was observed between g_m and S_m (Fig. 8C). A correlation between g_m and the %IAS was observed only in the temperature experiment ($R^2 = 0.34$, $P < 0.01$) (Fig. 8D).

Discussion

Our method of precisely defining stress allowed us to compare three important types of abiotic stress and investigate the physiological acclimation of our species of interest to different levels of those types of stress (Figs. 1,5,6). For all of our experiments, we examined the youngest leaves, which developed under the applied stress conditions. In this manner, we were able to avoid the well-known negative effects of leaf senescence on photosynthesis-limiting factors (Niinemets *et al.*, 2005). As expected, growth and photosynthesis gradually declined during the course of acclimation to each of the different types of stress (Figs. 1,2). Quantitative-limitations analysis demonstrated that for all types of stress, photosynthesis is mostly limited by the diffusion of CO_2 into the chloroplasts, through g_{sc} and g_m (~90% for drought and salinity, ~80% temperature); whereas biochemistry has only a minor effect (~10% drought and salinity, ~20% temperature; Fig. 4). These findings are consistent with earlier reports on trees' acclimation to drought and salinity (Bongi and Loreto, 1989; Grassi and Magnani, 2005; Galmés *et al.*, 2007a; Perez-Martin *et al.*, 2009; Keenan *et al.*, 2010; Limousin *et al.*, 2010; Egea *et al.*, 2011; Cano *et al.*, 2013, 2014). As for growth temperature, information about long-term acclimation to higher temperatures is currently available for only one tree species (*Eucalyptus regnans*; Warren, 2008b). However, that report did not involve a quantitative analysis of photosynthetic limitations that included L_b .

Though g_{sc} and g_m were the main limiting factors for all of the examined types of stress, their relative contributions to the diffusive limitation differed between the types of stress (Figs. 3,4). The limitation of photosynthesis during acclimation to drought and salinity was controlled mostly by stomata (L_s), whereas mesophyll limitation (L_m) was important only under progressive stress ($LWP > 1.5$ MPa or leaf $Na^+ > 200$ mmol kg⁻¹). However, the limitation of photosynthesis during acclimation to lower growth temperatures (34→10°C) was significantly controlled by the mesophyll (~30-50%), while the stomata and biochemical limitation accounted for less than 20% of the observed reduction in the rate of photosynthesis.

It is well established that in forest trees a reduction in g_s is the earliest response to drought and salinity, as well as the most perceptible photosynthesis limitation (Ziska *et al.*, 1990; Wilson *et al.*, 2000; Maseyk *et al.*, 2008; Limousin *et al.*, 2010). Stomatal closure is the most well-described characteristic of leaf stress avoidance (Raschke, 1975; Levitt, 1980). Unlike g_s , which is actively regulated, g_m does not have any known regulatory mechanism (Parkhurst, 1994; Tholen *et al.*, 2012; Barbour, 2017). Mesophyll cells are protected from damage caused by dehydration as they are partially hydraulically buffered from the transpiration stream (Buckley *et al.*, 2017). Most studies indicate that g_m drops at a later stage of drought stress, but the initial influence and magnitude varies greatly among species (Galmés *et al.*, 2007a; Cano *et al.*, 2014; Thérroux-Rancourt *et al.*, 2014; Zhou *et al.*, 2015). Stress acclimation of g_m and its role in the limitation of photosynthesis are relatively difficult to predict. Our proposed stress index may explain trees' vast investment in mesophyll protection. As the stress index (SI) increases, there is a linear decrease in the growth rate (Fig. 5A). In parallel, $iWUE$ and $g_m:g_s$ increase until SI reaches 0.4 (Fig. 5 B,C). This is still the avoidance stage, in which the leaf is effectively acclimated to its new non-optimal conditions. Later on (when SI exceeds 0.4), the ratio of g_m to g_s is impaired, which may represent the

breakdown of the g_m protective barriers (i.e., stomatal regulation and hydraulic compartmentalization). We suggest that g_m drops only at the stage at which stress prevails (i.e., when the stomatal protective mechanism is less effective). Moreover, the integrative relationship for photosynthesis limitation accounts for all types of stress, revealing a strong correlation with SI only for L_m (Fig. 6). This also emphasizes how g_m is a key factor in tolerance and acclimation to non-optimal conditions.

It has been suggested that the exact point at which g_m begins to decrease is a determining factor for drought resistance across different species and genotypes (Cano *et al.*, 2014; Th  roux-Rancourt *et al.*, 2014, 2015; Barbour & Kaiser, 2016). Our results are in line with those previous findings and further suggest that a stable low L_m may be a strong indicator of stress tolerance in acclimation to salinity and low temperature.

Our results also showed that improved $iWUE$ under intensified drought and salinity stresses was made possible by a greater decline in g_s , rather than g_m (a high $g_m:g_s$ ratio; Fig. 3 A,B,C,D). On the other hand, relatively low $iWUE$ at low temperatures was accompanied by a low $g_m:g_s$ ratio, mostly due to the opposite effect: a stronger influence of g_m relative to g_s ; Fig. 3E,F). It was recently suggested that trees originating in a xeric environment have higher $g_m:g_s$ ratios than mesic trees (Stpaul *et al.*, 2012; Cano *et al.*, 2014; Zhou *et al.*, 2014; Peguero-Pina *et al.*, 2017). It has also been proposed that species from warmer climates exhibit a steep g_m -temperature response (leading to low $g_m:g_s$); whereas the g_m levels of species from cooler climates are slightly change in response to changes in temperature (von Caemmerer & Evans, 2015). Our results are congruent with these two hypotheses.

Under stress conditions, leaves exhibited structural rearrangements, including an increase in LMA and reduced IAS (Table 1), which may have a negative influence on g_m (Poorter *et al.*, 2009; Tom  s *et al.*, 2013; Muir *et al.*, 2014; Onoda *et al.*, 2017). However,

increases in LMA were accompanied by increases in S_m , which is a major positive determinant of g_m under optimal conditions (von Caemmerer and Evans, 1991; Peguero-Pina *et al.*, 2017). In *Ziziphus*, it appears that mesophyll anatomy alone does not explain g_m acclimation to drought, salinity or temperature stress (Fig. 8).

The fact that g_m , similarly to g_s , can change rapidly within seconds or minutes in response to environmental or internal stimuli, indicating that leaf anatomy is not the only determining factor (Flexas *et al.*, 2007). There is some evidence that CO₂ and water can both pass through aquaporins (Terashima and Ono, 2002; Flexas *et al.*, 2006b). Thus, g_m and K_{leaf} are expected to be positively correlated under optimal conditions (Flexas *et al.*, 2013). However, it is still unclear whether this correlation holds under stress conditions (Loucos *et al.*, 2017).

In our case, a correlation between g_m and K_{leaf} was observed only in the temperature experiment (Fig. 7A). This finding strengthens the argument that shared pathways for CO₂ and water lead to coordination between g_m and K_{leaf} and that that process is temperature dependent (Bernacchi *et al.*, 2002). Although we observed a sharp drop in K_{leaf} in the drought experiment, under those conditions, g_m decreased only slightly. This suggests that mesophyll cell-to-cell CO₂ pathways are not influenced by moderate drought and that only severe drought will cause a coupled decline of both K_{leaf} and g_m . Even so, the relationship between g_m and K_{leaf} may vary among species and may also be related to anatomical properties of the mesophyll (Xiong *et al.*, 2016, 2017). Further study of these relationships under different stress conditions is needed.

We also demonstrated that g_m was not correlated with Ψ_p for any of the examined types of stress (Fig. 7B). g_m appears to respond to drought at Ψ_p levels lower than 0.4 MPa. Still, threshold-like responses and mechanisms remain obscure. We suggest that g_m becomes

severally impaired only when the mesophyll becomes flaccid. Flaccid mesophyll tissues may go through transformative changes involving cell collapse and reduced IAS (Levitt and Ben Zaken, 1975). This would reduce leaf internal effective CO₂ diffusion pathways and explain low g_m (von Caemmerer and Evans, 1991). Other possible explanations are based on the idea that deactivation of aquaporins occurs only under conditions of dramatic turgor loss (Miyazawa *et al.*, 2008). However, there is currently no known mechanism for aquaporin-turgor sensing in the mesophyll cells and further research is needed to explain this phenomenon.

For salinity stress, the point at which g_m dropped can be explained in other ways. Plants responses to salt stress are generally accepted to have two components: an osmotic component and an ionic component (Munns & Tester, 2008). The primary osmotic stress characterized by a reduction in both the water potential and the osmotic potential results in a rapid decrease in g_s . Following that rapid effect, under prolonged salt stress, ion imbalance and toxic levels of Na⁺ and Cl⁻ inhibit enzyme activity and protein synthesis (Chaves *et al.*, 2009; Shapira *et al.*, 2009). Yet, it is still unclear whether the reduction in g_m can be attributed to the osmotic effect or to the ionic toxicity (Delfine *et al.*, 1999; Centritto *et al.*, 2003; Chen *et al.*, 2015; Razzaghi *et al.*, 2015). In our case, the lowest LWP measured for the extreme salinity treatment (90 mM, leaf Na⁺ > 300 mmol kg⁻¹) was -1.1 MPa and, at that level, the leaves remained turgid (Fig. 7B). This indicates that the leaves were osmotically acclimated and that the reduction in g_m was triggered mostly by leaf accumulation of Na⁺ and Cl⁻ (i.e., ionic factors).

Conclusions

In this work, we have compared for the first time the photosynthesis limitation resulting from acclimation to three different types of stress. Such a comparison can lead to a better

understanding of which aspects of photosynthesis limitation are dominant under each type of stress. It may also help to predict how this species adapts to its environment and to future climate change. Our results clarify that *Z. spina-christi* is unable to withstand low temperatures due to an extremely sensitive g_m . On the other hand, this species exhibits high tolerance to drought and salinity characterized by a low g_s accompanied by a stable and constant g_m . With ongoing climate changes, the Mediterranean basin is likely to experience more frequent and intense drought conditions, accompanied by higher winter temperatures (Alpert *et al.*, 2008). These trends may give *Z. spina-christi* an ecological advantage that may lead to its becoming more widely distributed across this region in future decades. Consequently, this tree may serve as an indicator species for climate change, which is pushing the limits of the distribution ranges of many species.

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575 **Author Contributions:** YZ – planning and performing the experiments, data analysis,
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577

578 **References**

579 **Allen CD, Macalady AK, Chenchouni H, *et al.*** 2010. A global overview of drought and
580 heat-induced tree mortality reveals emerging climate change risks for forests. *Forest Ecology*
581 *and Management* **259**, 660–684.

582 **Alpert P, Krichak SO, Shafir H, Haim D, Osetinsky I.** 2008. Climatic trends to extremes
583 employing regional modeling and statistical interpretation over the E. Mediterranean. *Global*
584 *and Planetary Change* **63**, 163–170.

585 **Barbour MM.** 2017. Understanding regulation of leaf internal carbon and water transport
586 using online stable isotope techniques. *New Phytologist* **213**, 83–88.

587 **Barbour MM, Kaiser BN.** 2016. The response of mesophyll conductance to nitrogen and
588 water availability differs between wheat genotypes. *Plant Science* **251**, 119–127.

589 **Bernacchi CJ, Portis AR, Nakano H, Caemmerer S Von, Long SP.** 2002. Temperature
590 Response of Mesophyll Conductance . Implications for the Determination of Rubisco
591 Enzyme Kinetics and for Limitations to Photosynthesis in Vivo. *Plant Physiology* **130**, 1992–
592 1998.

593 **Bernacchi CJ, Singaas EL, Pimentel C, Portis AR, Long SP.** 2001. Improved temperature
594 response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and*
595 *Environment* **24**, 253–259.

596 **BioGIS.** 2018. Israel Biodiversity Information System. [<http://www.biogis.huji.ac.il>].

597 **Bongi G, Loreto F.** 1989. Gas-Exchange Properties of Salt-Stressed Olive (*Olea-Europea* L)
598 Leaves. *Plant Physiology* **90**, 1408–1416.

599 **Boyer JS, Wong SC, Farquhar GD.** 1997. CO₂ and Water Vapor Exchange across Leaf
600 Cuticle (Epidermis) at Various Water Potentials. *Plant Physiology* **114**, 185–191.

601 **Buckley TN, John GP, Scoffoni C, Sack L.** 2017. The Sites of Evaporation within Leaves.
602 *Plant Physiology* **173**, 1763–1782.

603 **von Caemmerer S, Evans JR.** 1991. Determination of the average partial pressure of CO₂
604 in chloroplasts from leaves of several C₃ plants. *Australian Journal of Plant Physiology* **18**,
605 287–305.

606 **von Caemmerer S, Evans JR.** 2015. Temperature responses of mesophyll conductance
607 differ greatly between species. *Plant, Cell and Environment* **38**, 629–637.

608 **von Caemmerer S, Evans JR, Hudson GS, Andrews TJ.** 1994. The kinetics of ribulose-
609 1,5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of
610 photosynthesis in leaves of transgenic tobacco. *Planta* **195**, 88–97.

611 **Cano FJ, López R, Warren CR.** 2014. Implications of the mesophyll conductance to CO₂
612 for photosynthesis and water-use efficiency during long-term water stress and recovery in
613 two contrasting Eucalyptus species. *Plant Cell and Environment* **37**, 2470–2490.

614 **Cano FJ, Sánchez-gómez D, Rodríguez-calcerrada J, Warren CR, Gil L, Aranda I.**
615 2013. Effects of drought on mesophyll conductance and photosynthetic limitations at
616 different tree canopy layers. *Plant Cell and Environment* **36**, 1961–1980.

617 **Centritto M, Loreto F, Chantzoulakis K.** 2003. The use of low [CO₂] to estimate
618 diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive
619 saplings. *Plant Cell and Environment* **26**, 585–594.

620 **Chaves MM, Flexas J, Pinheiro C.** 2009. Photosynthesis under drought and salt stress :
621 regulation mechanisms from whole plant to cell. *Annals of Botany* **103**, 551–560.

622 **Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CPP, Osório ML, Carvalho**
623 **I, Faria T, Pinheiro C.** 2002. How plants cope with water stress in the field. Photosynthesis
624 and growth. *Annals of Botany* **89**, 907–916.

625 **Chen TW, Kahlen K, Stützel H.** 2015. Disentangling the contributions of osmotic and ionic
626 effects of salinity on stomatal, mesophyll, biochemical and light limitations to
627 photosynthesis. *Plant, Cell and Environment* **38**, 1528–1542.

628 **Delfine S, Alvino A, Villani MC, et al.** 1999. Restrictions to Carbon Dioxide Conductance
629 and Photosynthesis in Spinach Leaves Recovering from Salt Stress. *Plant Physiology* **119**,
630 1101–1106.

631 **Díaz-Espejo A, Nicolás E, Fernández JE.** 2007. Seasonal evolution of diffusional
632 limitations and photosynthetic capacity in olive under drought. *Plant, Cell and Environment*
633 **30**, 922–933.

634 **Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F.** 1956. Colorimetric Method for
635 Determination of Sugars and Related Substances. *Analytical Chemistry* **28**, 350–356.

636 **Egea G, González-Real MM, Baille A, Nortes PA, Díaz-Espejo A.** 2011. Disentangling the
637 contributions of ontogeny and water stress to photosynthetic limitations in almond trees.
638 *Plant, Cell and Environment* **34**, 962–979.

639 **Ethier GJ, Livingston NJ.** 2004. On the need to incorporate sensitivity to CO₂ transfer
640 conductance into the Farquhar–von Caemmerer–Berry leaf photosynthesis model. *Plant Cell*
641 and Environment **27**, 137–153.

642 **Farquhar GD, von Caemmerer S, Berry JA.** 1980. A biochemical model of photosynthetic
643 CO₂ assimilation in leaves of C₃ species. *Planta* **149**, 78–90.

644 **Flexas J, Barón M, Bota J, et al.** 2009. Photosynthesis limitations during water stress

acclimation and recovery in the drought-adapted Vitis hybrid Richter-110 (V. berlandieri×V. rupestris). Journal of Experimental Botany **60**, 2361–2377.

Flexas J, Bota J, Galmes J, Medrano Hilito, Ribas-carbo M. 2006a. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiologia Plantarum **127**, 343–352.

Flexas J, Carriquí M, Coopman RE, Gago J, Galmés J, Martorell S, Morales F, Diaz-Espejo A. 2014. Stomatal and mesophyll conductances to CO₂ in different plant groups: Underrated factors for predicting leaf photosynthesis responses to climate change? Plant Science **226**, 41–48.

Flexas J, Diaz-Espejo A, Galmés J, Kaldenhoff R, Medrano H, Ribas-Carbo M. 2007. Rapid variations of mesophyll conductance in response to changes in CO₂ concentration around leaves. Plant, Cell and Environment **30**, 1284–1298.

Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H. 2008. Mesophyll conductance to CO₂: Current knowledge and future prospects. Plant, Cell and Environment **31**, 602–621.

Flexas J, Ribas-Carbó M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R. 2006b. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO₂ in vivo. Plant Journal **48**, 427–439.

Flexas J, Scoffoni C, Gago J, Sack L. 2013. Leaf mesophyll conductance and leaf hydraulic conductance: An introduction to their measurement and coordination. Journal of Experimental Botany **64**, 3965–3981.

Galle A, Florez-Sarasa I, Aououad H El, Flexas J. 2011. The Mediterranean evergreen Quercus ilex and the semi-deciduous Cistus albidus differ in their leaf gas exchange

668 regulation and acclimation to repeated drought and re-watering cycles. *Journal of*
669 *Experimental Botany* **62**, 5207–5216.

670 **Galle A, Florez-Sarasa I, Tomas M, Pou A, Medrano H, Ribas-Carbo M, Flexas J.** 2009.
671 The role of mesophyll conductance during water stress and recovery in tobacco (*Nicotiana*
672 *sylvestris*): Acclimation or limitation? *Journal of Experimental Botany* **60**, 2379–2390.

673 **Galmés J, Medrano H, Flexas J.** 2006. Acclimation of Rubisco specificity factor to drought
674 in tobacco: Discrepancies between in vitro and in vivo estimations. *Journal of Experimental*
675 *Botany* **57**, 3659–3667.

676 **Galmés J, Medrano H, Flexas J.** 2007a. Photosynthetic limitations in response to water
677 stress and recovery in Mediterranean plants with different growth forms. *New Phytologist*
678 **175**, 81–93.

679 **Galmés J, Pou A, Alsina MM, Tomàs M, Medrano H, Flexas J.** 2007b. Aquaporin
680 expression in response to different water stress intensities and recovery in Richter-110 (*Vitis*
681 *sp.*): Relationship with ecophysiological status. *Planta* **226**, 671–681.

682 **Genty B, Briantais JM, Baker NR.** 1989. The relationship between the quantum yield of
683 photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et*
684 *Biophysica Acta - General Subjects* **990**, 87–92.

685 **Gilbert ME, Medina V.** 2016. Drought Adaptation Mechanisms Should Guide Experimental
686 Design. *Trends in Plant Science* **21**, 639–647.

687 **Grassi G, Magnani F.** 2005. Stomatal , mesophyll conductance and biochemical limitations
688 to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. *Plant, Cell*
689 *and Environment* **28**, 834–849.

690 **Harley PC, Loreto F, Marco G Di, Sharkey TD.** 1992. Theoretical Considerations when

691 Estimating the Mesophyll Conductance to CO₂ Flux by Analysis of the Response of
692 Photosynthesis to CO₂. *Plant Physiology* **98**, 1429–1436.

693 **IPCC**. 2014. *Wong, Poh Poh Losada, Iñigo J. Gattuso, Jean-Pierre Hinkel, Jochen Khattabi,*
694 *Abdellatif McInnes, Kathleen L. Saito, Yoshiki Sallenger, Asbury Coastal systems and low-*
695 *lying areas. In: Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Glo.*
696 Cambridge United Kingdom and New York, NY, USA: Cambridge University Press.

697 **Keenan T, Sabate S, Gracia C**. 2010. The importance of mesophyll conductance in
698 regulating forest ecosystem productivity during drought periods. *Global Change Biology* **16**,
699 1019–1034.

700 **Laisk AK**. 1977. *Kinetics of Photosynthesis and Photorespiration in C₃ plants*. Nauka,
701 Moscow.

702 **Levitt J**. 1980. *Responses of Plant to Environmental Stress: Water, Radiation, Salt and*
703 *Other Stresses*. New York: Academic Press, 365.

704 **Levitt J, Ben Zaken R**. 1975. Effects of Small Water Stresses on Cell Turgor and
705 Intercellular Space. *Physiologia Plantarum* **34**, 273–279.

706 **Lichtenthaler HK**. 1996. Vegetation Stress : an Introduction to the Stress Concept in Plants.
707 *Journal of Plant Physiology* **148**, 4–14.

708 **Limousin JM, Misson L, Lavoie AV, Martin NK, Rambal S**. 2010. Do photosynthetic
709 limitations of evergreen *Quercus ilex* leaves change with long-term increased drought
710 severity? *Plant, Cell and Environment* **33**, 863–875.

711 **Loucos KE, Simonin KA, Barbour MM**. 2017. Leaf hydraulic conductance and mesophyll
712 conductance are not closely related within a single species. *Plant Cell and Environment* **40**,
713 203–215.

- 714 **Maseyk KS, Lin T, Rotenberg E, Grünzweig JM, Schwartz A, Yakir D.** 2008.
715 Physiology-phenology interactions in a productive semi-arid pine forest. *New Phytologist*
716 **178**, 603–616.
- 717 **McDowell NG, Beerling DJ, Breshears DD, Fisher RA, Raffa KF, Stitt M.** 2011. The
718 interdependence of mechanisms underlying climate-driven vegetation mortality. *Trends in*
719 *Ecology and Evolution* **26**, 523–532.
- 720 **Misson L, Limousin JM, Rodriguez R, Letts MG.** 2010. Leaf physiological responses to
721 extreme droughts in Mediterranean *Quercus ilex* forest. *Plant, Cell and Environment* **33**,
722 1898–1910.
- 723 **Miyazawa S, Yoshimura S, Shinzaki Y, Maeshima M, Miyake C.** 2008. Deactivation of
724 aquaporins decreases internal conductance to CO₂ diffusion in tobacco leaves grown under
725 long-term drought. *Functional Plant Biology* **35**, 553–564.
- 726 **Muir CD, Hangarter RP, Moyle LC, Davis PA.** 2014. Morphological and anatomical
727 determinants of mesophyll conductance in wild relatives of tomato (*Solanum* sect .
728 *Lycopersicon* , sect . *Lycopersicoides* ; Solanaceae). *Plant Cell and Environment* **37**, 1415–
729 1426.
- 730 **Niinemets Ü.** 2010. Responses of forest trees to single and multiple environmental stresses
731 from seedlings to mature plants: Past stress history, stress interactions, tolerance and
732 acclimation. *Forest Ecology and Management* **260**, 1623–1639.
- 733 **Niinemets Ü, Cescatti A, Rodeghiero M, Tosens T.** 2005. Leaf internal diffusion
734 conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen
735 broad-leaved species. *Plant, Cell and Environment* **28**, 1552–1566.
- 736 **Onoda Y, Wright IJ, Evans JR, Hikosaka K, Kitajima K, Poorter H, Tosens T, Westoby**

737 **M.** 2017. Physiological and structural tradeoffs underlying the leaf economics spectrum. *New*
738 *Phytologist* **214**, 1447–1463.

739 **Parkhurst DF.** 1994. Tansley Review No . 65. Diffusion of CO₂ and other gases inside
740 leaves. *New Phytologist* **126**, 449–479.

741 **Peguero-Pina JJ, Sisó S, Flexas J, Galmés J, García-Nogales A, Niinemets Ü, Sancho-**
742 **Knapik D, Saz MÁ, Gil-Pelegrín E.** 2017. Cell-level anatomical characteristics explain high
743 mesophyll conductance and photosynthetic capacity in sclerophyllous Mediterranean oaks.
744 *New Phytologist* **214**, 585–596.

745 **Perdomo JA, Carmo-Silva E, Hermida-Carrera C, Flexas J, Galmés J.** 2016.
746 Acclimation of Biochemical and Diffusive Components of Photosynthesis in Rice, Wheat,
747 and Maize to Heat and Water Deficit: Implications for Modeling Photosynthesis. *Frontiers in*
748 *Plant Science* **7**, 1–16.

749 **Pérez-López U, Robredo A, Lacuesta M, Mena-Petite A, Muñoz-Rueda A.** 2012.
750 Elevated CO₂ reduces stomatal and metabolic limitations on photosynthesis caused by
751 salinity in *Hordeum vulgare*. *Photosynthesis research* **111**, 269–283.

752 **Perez-Martin A, Flexas J, Ribas-Carbo M, Bota J, Tomas M, Infante JM, Diaz-Espejo**
753 **A.** 2009. Interactive effects of soil water deficit and air vapour pressure deficit on mesophyll
754 conductance to CO₂ in *Vitis vinifera* and *Olea europaea*. *Journal of Experimental Botany* **60**,
755 2391–2405.

756 **Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R, Niinemets U, Poorter L, Wright**
757 **IJ, Villar R.** 2009. Causes and consequences of variation in leaf mass per area (LMA):a
758 meta-analysis. *New Phytologist* **182**, 565–588.

759 **Qiu C, Ethier G, Pepin S, et al.** 2017. Persistent negative temperature response of

760 mesophyll conductance in red raspberry (*Rubus idaeus* L .) leaves under both high and low
761 vapour pressure deficits : a role for abscisic acid ? *Plant Cell and Environment* **40**, 1940–
762 1959.

763 **Raschke K.** 1975. Stomatal Action. *Annual Review of Plant Physiology* **26**, 309–340.

764 **Razzaghi F, Jacobsen SE, Jensen CR, Andersen MN.** 2015. Ionic and photosynthetic
765 homeostasis in quinoa challenged by salinity and drought - Mechanisms of tolerance.
766 *Functional Plant Biology* **42**, 136–148.

767 **Rodeghiero M, Niinemets ÜLO, Cescatti A.** 2007. Major diffusion leaks of clamp-on leaf
768 cuvettes still unaccounted : how erroneous are the estimates of Farquhar et al . model
769 parameters ? *Plant Cell and Environment* **30**, 1006–1022.

770 **Sack L, Melcher PJ, Zwieniecki MA, Holbrook NM.** 2002. The hydraulic conductance of
771 the angiosperm leaf lamina: a comparison of three measurement methods. *Journal of*
772 *experimental botany* **53**, 2177–2184.

773 **Saied AS, Gebauer J, Hammer K, Buerkert A.** 2008. *Ziziphus spina-christi* (L.) Willd.: A
774 multipurpose fruit tree. *Genetic Resources and Crop Evolution* **55**, 929–937.

775 **Saxe H, Cannell MGR, Johnsen Ø, Ryan MG, Vourlitis G.** 2001. Tree and forest
776 functioning in response to global warming. *New Phytologist* **149**, 369–400.

777 **Shapira O, Khadka S, Israeli Y, Shani U, Schwartz A.** 2009. Functional anatomy controls
778 ion distribution in banana leaves: Significance of Na⁺ seclusion at the leaf margins. *Plant,*
779 *Cell and Environment* **32**, 476–485.

780 **Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL.** 2007. Fitting photosynthetic
781 carbon dioxide response curves for C₃ leaves. *Plant, Cell and Environment* **30**, 1035–1040.

782 **Shtein I, Meir S, Riov J, Philosoph-Hadas S.** 2011. Interconnection of seasonal

783 temperature, vascular traits, leaf anatomy and hydraulic performance in cut *Dodonaea* ‘Dana’
784 branches. *Postharvest Biology and Technology* **61**, 184–192.

785 **Stpaul NKM, Limousin JM, Rodríguez-Calcerrada J, Ruffault J, Rambal S, Letts**
786 **MG, Misson L.** 2012. Photosynthetic sensitivity to drought varies among populations of
787 *Quercus ilex* along a rainfall gradient. *Functional Plant Biology* **39**, 25–37.

788 **Syvertsen J, Lloyd J, Meconchie C, Kriedtmann PE, Farquhar GD.** 1995. On the
789 relationship between leaf anatomy and CO₂ diffusion through the mesophyll of
790 hypostomatous leaves. *Plant Cell and Environment* **18**, 149–157.

791 **Terashima I, Ono K.** 2002. Effects of HgCl₂ on CO₂ Dependence of Leaf Photosynthesis :
792 Evidence Indicating Involvement of Aquaporins in CO₂ Diffusion across the Plasma
793 Membrane. *Plant and Cell Physiology* **43**, 70–78.

794 **Théroux-Rancourt G, Éthier G, Pepin S.** 2014. Threshold response of mesophyll CO₂
795 conductance to leaf hydraulics in highly transpiring hybrid poplar clones exposed to soil
796 drying. *Journal of experimental botany* **65**, 741–753.

797 **Théroux-Rancourt G, Éthier G, Pepin S.** 2015. Greater efficiency of water use in poplar
798 clones having a delayed response of mesophyll conductance to drought. *Tree Physiology* **35**,
799 172–184.

800 **Tholen D, Éthier G, Genty B, Pepin S, Zhu X.** 2012. Variable mesophyll conductance
801 revisited : theoretical background and experimental implications. *Plant Cell and Environment*
802 **35**, 2087–2103.

803 **Tomás M, Flexas J, Copolovici L, Galmés J, Hallik L, Medrano H, Ribas-Carbó M,**
804 **Tosens T, Vislap V, Niinemets Ü.** 2013. Importance of leaf anatomy in determining
805 mesophyll diffusion conductance to CO₂ across species: Quantitative limitations and scaling

up by models. *Journal of Experimental Botany* **64**, 2269–2281.

Tomás M, Medrano H, Brugnoli E, Escalona JM, Martorell S, Pou A, Ribas-Carbó M, Flexas J. 2014. Variability of mesophyll conductance in grapevine cultivars under water stress conditions in relation to leaf anatomy and water use efficiency. *Australian Journal of Grape and Wine Research* **20**, 272–280.

Valentini R, Epron D, Deangelis P, Matteucci G, Dreyer E. 1995. In-Situ Estimation of Net Co₂ Assimilation, Photosynthetic Electron Flow and Photorespiration in Turkey Oak (Q-Cerris L) Leaves - Diurnal Cycles under Different Levels of Water-Supply. *Plant Cell and Environment* **18**, 631–640.

Walther G-R, Post E, Convey P, Menzel A, Parmesan C, Beebee TJC, Fromentin J, Hoegh-Guldberg O, Bairlein F. 2002. ecological responses to recent climate change. *Nature* **416**, 389–395.

Wang X, Wang W, Huang J, Peng S, Xiong D. 2017. Diffusional conductance to CO₂ is the key limitation to photosynthesis in salt-stressed leaves of rice (*Oryza sativa*). *Physiologia Plantarum*.

Warren CR. 2008. Does growth temperature affect the temperature responses of photosynthesis and internal conductance to CO₂? A test with *Eucalyptus regnans*. *Tree Physiology* **28**, 11–19.

Wilson KB, Baldocchi DD, Hanson PJ. 2000. Quantifying stomatal and non-stomatal limitations to carbon assimilation resulting from leaf aging and drought in mature deciduous tree species. *Tree Physiology* **20**, 787–797.

Xiong D, Douthe C, Flexas J. 2017. Differential coordination of stomatal conductance, mesophyll conductance and leaf hydraulic conductance in response to changing light across

species. Plant Cell and Environment.

Xiong D, Flexas J, Yu T, Peng S, Huang J. 2016. Leaf anatomy mediates coordination of leaf hydraulic conductance and mesophyll conductance to CO₂ in *Oryza*. New Phytologist **213**, 572–583.

Al Yamani W, Green S, Pangilinan R, Dixon S, Shahid SA, Kemp P, Clothier B. 2018. Water use of Al Ghaf (*Prosopis cineraria*) and Al Sidr (*Ziziphus spina-christi*) forests irrigated with saline groundwater in the hyper-arid deserts of Abu Dhabi. Agricultural Water Management **203**, 105–114.

Yamori W, Noguchi K, Hanba YT, Terashima I. 2006. Effects of internal conductance on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. Plant and Cell Physiology **47**, 1069–1080.

Zhou SX, Medlyn BE, Prentice IC. 2016. Long-term water stress leads to acclimation of drought sensitivity of photosynthetic capacity in xeric but not riparian *Eucalyptus* species. Annals of Botany **117**, 133–144.

Zhou S, Medlyn B, Sabaté S, Sperlich D, Prentice IC, Sabate S, Sperlich D, Prentice IC. 2014. Short-term water stress impacts on stomatal, mesophyll and biochemical limitations to photosynthesis differ consistently among tree species from contrasting climates. Tree Physiology **34**, 1035–1046.

Zhou L, Wang S, Chi Y, Li Q, Huang K, Yu Q. 2015. Responses of photosynthetic parameters to drought in subtropical forest ecosystem of China. Scientific Reports **5**, 1–11.

Ziska LH, Seemann JR, Dejong TM. 1990. Salinity Induced Limitations on Photosynthesis in *Prunus salicina*, a Deciduous Tree Species. Plant Physiology **93**, 864–70.

Table 1 Leaf structural and anatomical characteristics under long-term drought, salinity and temperature stress. For each treatment, the following information is presented: leaf mass/area (*LMA*), stomatal density (*SD*), leaf width (*L_t*), mesophyll surface area exposed to intercellular airspaces (*S_m*) and fraction of intercellular air space (*IAS*). Different letters denote a statistically significant difference between treatment means for each stress factor.

	Treatment	LMA (g m ⁻²)	SD (n mm ⁻²)	L _t (μm)	S _m (m ² m ⁻²)	IAS (%)
Drought	WW	77.5±2.4 a	197.9±18 a	115.7±5.9 a	9.3±0.2 a	25.2±1.2 a
	WS1	99.7±3.5 b	200.0±18 a	116.2±18.4 a	8.9±0.1 a	15.3±1.7 b
	WS2	124.5±3.5 c	196.0±31 a	146.4±15.2 a	10.2±0.2 b	14.9±1.0 b
	<i>P</i> value	0.0003	n.s	n.s	0.014	0.0065
Salinity (mM)	0	108.7±3.7 a	229.3±13.0 a	134.0±6.0 a	8.8±0.4 a	23.8±0.4 a
	30	91.5±11.6 a	227.5±25.5 a	122.5±11.4 a	9.8±1.6 a	21.5±0.8 a
	60	120.6±11.4 a	219.0±32.6 a	142.4±7.6 a	10.4±0.5 a	16.7±1.1 a
	90	106.7±3.4 a	196.9±19.4 a	145.4±14.4 a	10.6±1.1 a	21.0±1.2 a
	<i>P</i> value	n.s	n.s	n.s	n.s	n.s
Temperature (°C day/night)	34/28	77.8±4.3 a	319.4±26.0 a	177.4±5.7 ab	8.4±0.7 a	21.5±2.2 a
	28/22	75.1±4.5 a	274.8±26.9 ab	174.4±5.9 ab	7.3±0.9 a	21.7±2.3 a
	22/16	87.7±4.0 ab	252.2±24.4 ab	160.6±5.4 b	6.9±0.9 a	12.8±2.0 b
	16/10	93.9±5.5 b	197.7±32.8 b	196.8±7.2 a	11.3±0.2 b	14.6±2.8 ab
	<i>P</i> value	0.0239	0.0667	<.0001	0.0007	0.0361

Figure Legends

Fig. 1 Physiological definition of the types of stress examined, as demonstrated in terms of changes in the growth rate of the main stem and aspects of the leaf stress response. **(a)** The drought experiment included four different irrigation treatments defined by ranges of leaf water potential, **(b)** the salinity experiments were defined in terms of different leaf Na^+ concentrations and **(c)** the temperature experiment was based on the use of different leaf temperatures. The gray line represents a 50% reduction in the growth rate for each type of stress.

Fig. 2: Effects of different types of environmental stress on the rate of photosynthesis (A_N) and the maximum capacity for Rubisco carboxylation (V_{cmax}). Leaf-level parameters were quantified differently following long-term stress acclimation: **(a)** drought stress was quantified in terms of leaf water potential, **(b)** salinity stress was quantified in terms of leaf Na^+ concentration and **(c)** temperature stress as quantified in terms of leaf temperature. Continuous lines represent best-fit adjustments.

Fig. 3 Mesophyll and stomatal CO_2 conductance (g_m and g_s , respectively) and the change in the leaf-level intrinsic water-use efficiency ($i\text{WUE}$) following long-term stress acclimation. Mesophyll conductance and stomatal conductance trends are distinguished by the leaf-level response: **(a)** drought stress as quantified in terms of leaf water potential, **(b)** salinity stress as quantified in terms of leaf Na^+ concentration and **(c)** temperature stress in terms of leaf temperature. Continuous lines represent the best-fit adjustment for stomatal conductance (blue circles) and broken lines represent the best-fit for mesophyll conductance (red triangles).

Fig. 4 Effects of different environmental stress factors on the overall limitation of photosynthesis (L_t) and its components: stomatal limitation (L_s), mesophyll limitation (L_m)

and the biochemical limitation component (L_b). The leaf-level response is expressed differently for each type of stress. **(a)** Drought stress is expressed in terms of leaf water potential, **(b)** salinity is expressed in terms of leaf Na^+ concentration and **(c)** temperature is expressed in terms of leaf temperature. The continuous line represents the best-fit adjustment for the total limitation (gray diamonds), the line made up of dashes represent the best fit for stomatal limitation (blue circles) and the line made up of dots and dashes represents the best fit for mesophyll limitation (red triangles). The dotted green line represents biochemical limitation of photosynthesis (green stars).

Fig. 5 The relationships between **(a)** relative growth, **(b)** relative intrinsic water-use efficiency and **(c)** relative change in the $g_m:g_s$ ratio and the leaf stress index for all of the stress treatments: drought (red circles), salinity (green triangles) and temperature (blue squares). The continuous line represents the best fit for all types of stress combined.

Fig. 6 The relationships between the total limitation of photosynthesis (L_t – gray diamonds) and its components: stomatal limitation (L_s – blue circles), mesophyll limitation (L_m – red triangles) and biochemical limitation (L_b – green stars), and the stress index for all of the stress treatments combined. The continuous line represents the best fit for L_t , the dashed line represent the best fit for L_s , the line made up of dots and dashes represents the best fit for L_m and the dotted line represents the best fit for L_b .

Fig. 7 Relationships between mesophyll conductance and **(a)** the estimated leaf hydraulic conductance (K_{leaf}) and **(b)** the calculated leaf turgor pressure (Ψ_p). The different types of stress are represented as follows: drought (red circles), salinity (green triangles) and temperature (blue squares). The broken blue line represents the best-fit adjustment for the g_m/K_{leaf} correlation in the temperature experiment.

Fig. 8 Relationships between g_m and leaf structural and anatomical traits under different types of environmental stress: drought (red circles), salinity (green triangles) and temperature (blue squares). **(a)** Leaf mass per area, **(b)** leaf thickness, **(c)** mesophyll surface area exposed to the intercellular airspace (S_m) and **(d)** percentage of leaf intercellular airspace (IAS).

Supplementary Data

Table S1 Parameters derived from the analysis of the A_N/C_c and A/Q curves for the different types of environmental stress (drought, salinity and temperature). Laisk-method estimation of the mitochondrial respiration in the light (R_d) and the apparent intercellular CO_2 photo-compensation point (C_i^*). Valentini method estimation of the total electron-transport rate (J_t) calculated using the, electron-transport rate derived for carboxylation (J_c), electron-transport rate derived for oxygenation (J_o), $J_c:J_o$ ratio and CO_2 emitted in photorespiration (F). Different letters denote a statistically significant difference between the treatments means for each type of stress.

	Treatment	"Laisk method "			"Valentini method "			
		R_d ($\mu mol CO_2 m^{-2} s^{-1}$)	C_i^* ($\mu mol mol^{-1}$)	J_t ($\mu mol e^- m^{-2} s^{-1}$)	J_c ($\mu mol e^- m^{-2} s^{-1}$)	J_o ($\mu mol e^- m^{-2} s^{-1}$)	$J_c:J_o$	F ($\mu mol CO_2 m^{-2} s^{-1}$)
Drought	WW (n = 7)	1.0 ± 0.08 a	38.4 ± 2.0 a	155.1 ± 5.9 a	114.0 ± 3.0 a	41.1 ± 3.0 a	3.0 ± 0.12 a	5.1 ± 0.4 a
	MWS1 (n = 7)	0.82 ± 0.08 b	36 ± 1.3 a	132.0 ± 7.9 b	84.7 ± 4.1 b	47.3 ± 4.3 a	1.9 ± 0.16 b	5.9 ± 0.5 a
	MWS2 (n = 7)	0.7 ± 0.08 b	36.4 ± 1.5 a	99.4 ± 6.6 c	54.8 ± 3.4 c	44.6 ± 3.6 a	1.3 ± 0.13 c	5.5 ± 0.4 a
	SWS (n = 7)	0.6 ± 0.1 b	39.0 ± 1.5 a	81.7 ± 5.7 d	34.6 ± 3.0 d	47.1 ± 3.1 a	0.75 ± 0.1 d	5.8 ± 0.3 a
	P value	0.0012	n.s	< 0.0001	< 0.0001	n.s	< 0.0001	n.s
Salinity	0 mM (n = 6)	0.95 ± 0.1 a	34.1 ± 1.5 a	159.7 ± 6.6 a	107.4 ± 4.6 a	52.3 ± 2.7 a	2.0 ± 0.08 a	6.5 ± 0.3 a
	30 mM (n = 5)	0.93 ± 0.04 a	34.0 ± 1.9 a	148.9 ± 8.0 a	98.2 ± 5.6 a	49.7 ± 3.3 a	2.0 ± 0.1 a	6.2 ± 0.4 a
	60 mM (n = 6)	0.84 ± 0.05 a	36.9 ± 1.4 a	105.7 ± 7.0 b	63.9 ± 4.7 b	42.2 ± 2.8 a	1.5 ± 0.09 b	5.2 ± 0.36 a
	90 mM (n = 6)	0.83 ± 0.04 a	32.1 ± 1.3 a	111.5 ± 7.4 b	59.9 ± 5.2 b	51.7 ± 3.0 a	1.1 ± 0.09 b	6.4 ± 0.38 a
	P value	n.s	n.s	< 0.0001	< 0.0001	n.s	< 0.0001	n.s
Temperature	34°C (n = 6)	1.2 ± 0.13 a	41.8 ± 2.9 a	178.8 ± 9.1 a	122.0 ± 5.0 a	56.8 ± 4.0 a	2.1 ± 0.2 a	7.1 ± 0.5 a
	28°C (n = 6)	0.9 ± 0.1 b	39.0 ± 2.2 a	140.3 ± 8.3 b	104.1 ± 4.7 a	36.1 ± 3.7 b	2.8 ± 0.18 ab	4.5 ± 0.4 b
	22°C (n = 6)	0.6 ± 0.1 c	29.9 ± 2.2 b	103.1 ± 7.7 c	70.6 ± 4.7 b	32.4 ± 3.7 b	2.2 ± 0.18 ab	4.0 ± 0.4 b
	16°C (n = 6)	0.4 ± 0.12 c	28.6 ± 2.0 b	54.5 ± 7.7 d	33.9 ± 5.0 c	20.5 ± 4.0 bc	1.8 ± 0.19 bc	2.5 ± 0.5 bc
	10°C (n = 5)			26.7 ± 9.1 d	14.0 ± 5.5 c	12.7 ± 4.4 c	1.1 ± .20 c	1.5 ± 0.5 c
	P value	< 0.0001	0.001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Fig. S1 Changes in soluble carbohydrate (SC) and starch content in the (a) drought, (b) salinity and (c) temperature treatments.

Fig. S2 Correlation of mesophyll conductance (g_m) estimated using the methods described by Harley *et al.* (1992) and Ethier and Livingston (2004) for the drought (red circles), salinity (green triangles) and temperature treatments (blue squares).

Fig. S3 Representative samples of leaf anatomical cross-sections for the control and extreme treatment for each type of stress, showing differences in mesophyll shape and density.

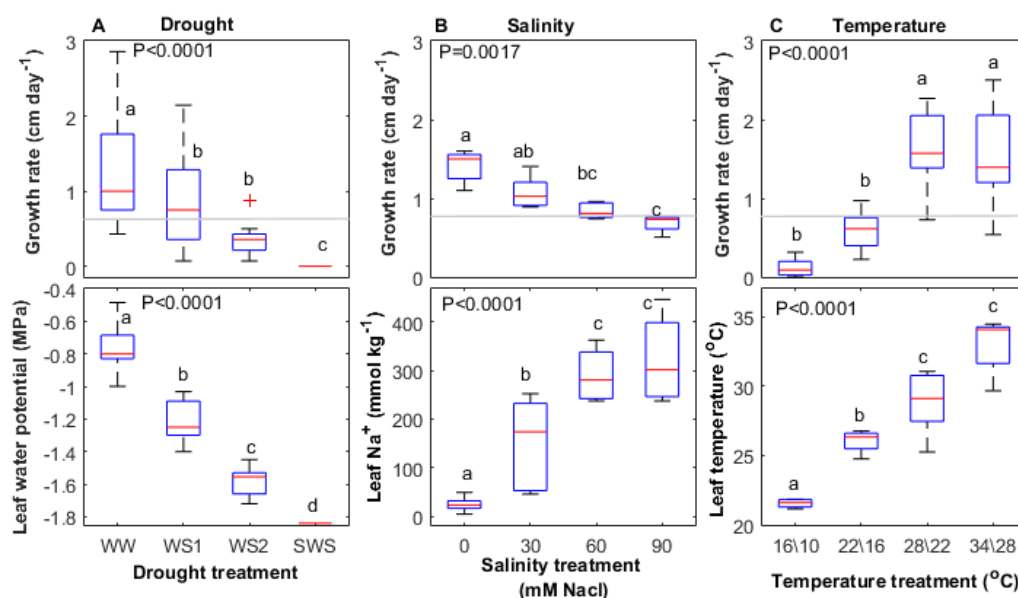


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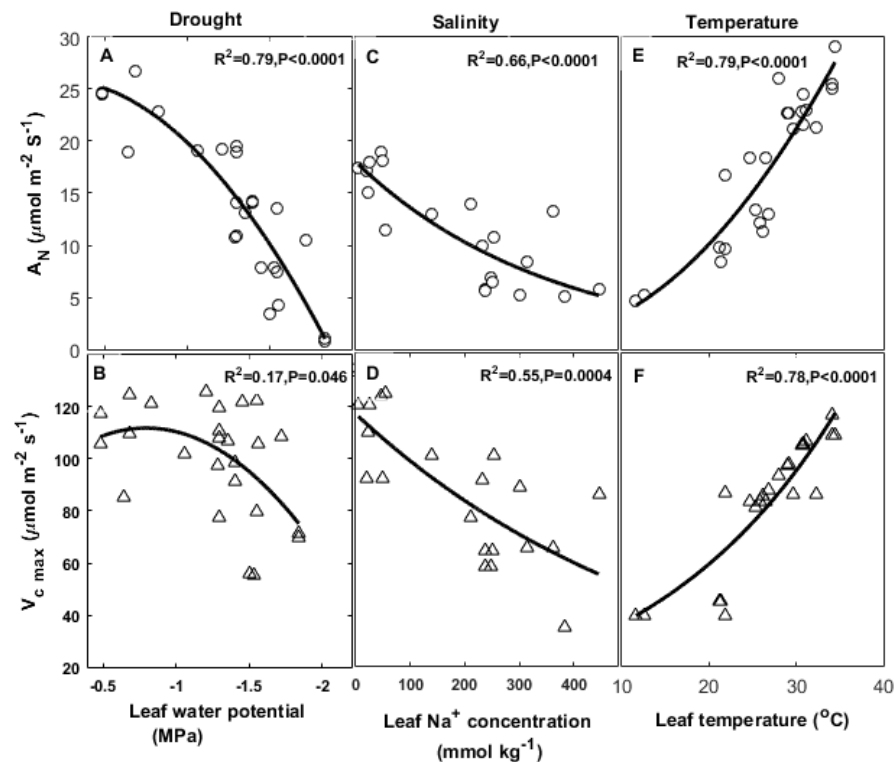


Fig. 2: Effects of different types of environmental stress on the rate of photosynthesis (A_N) and the maximum capacity for Rubisco carboxylation ($V_{c \text{ max}}$). Leaf-level parameters were quantified differently following long-term stress acclimation: **(a)** drought stress was quantified in terms of leaf water potential, **(b)** salinity stress was quantified in terms of leaf Na^+ concentration and **(c)** temperature stress as quantified in terms of leaf temperature. Continuous lines represent best-fit adjustments.

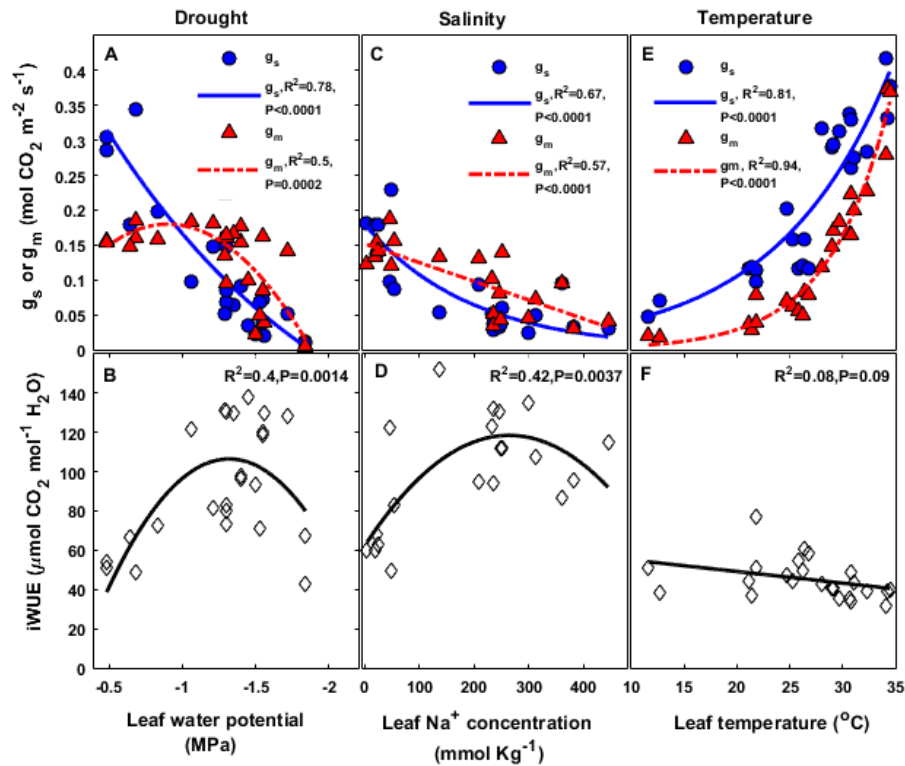


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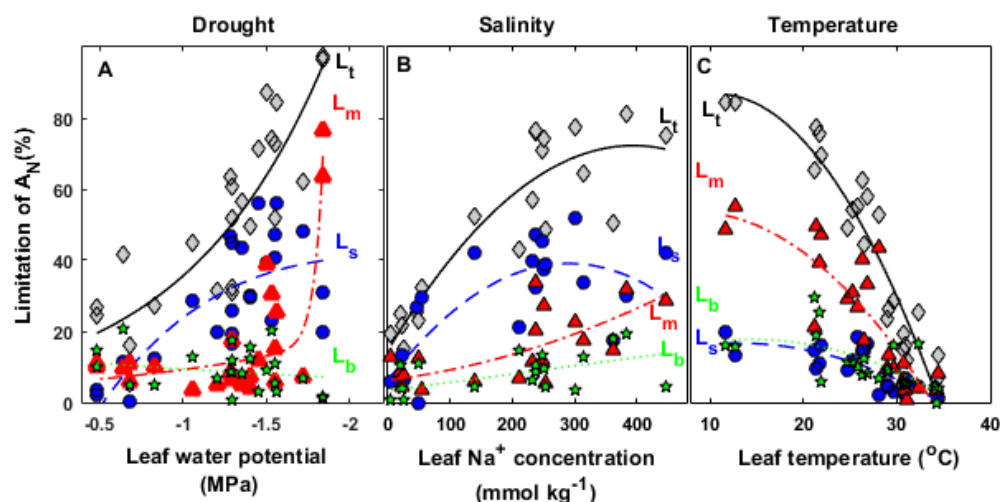


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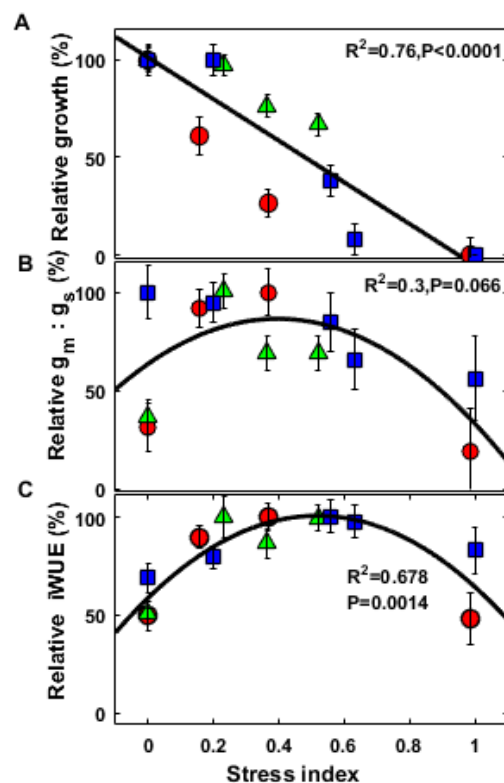


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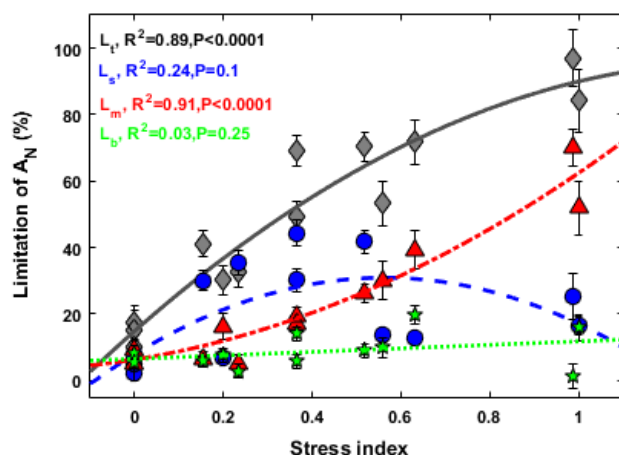


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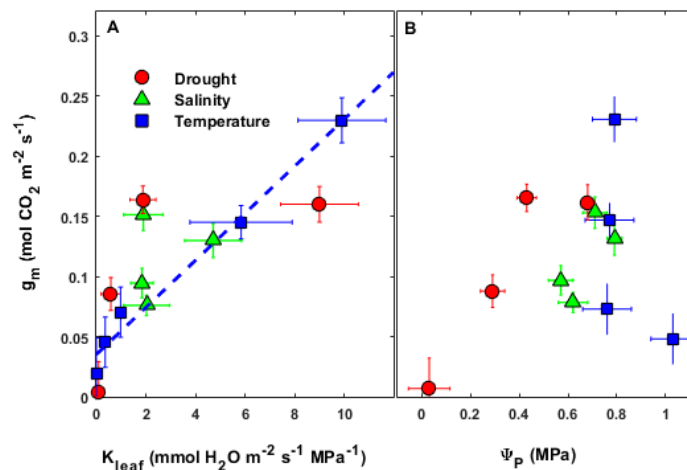


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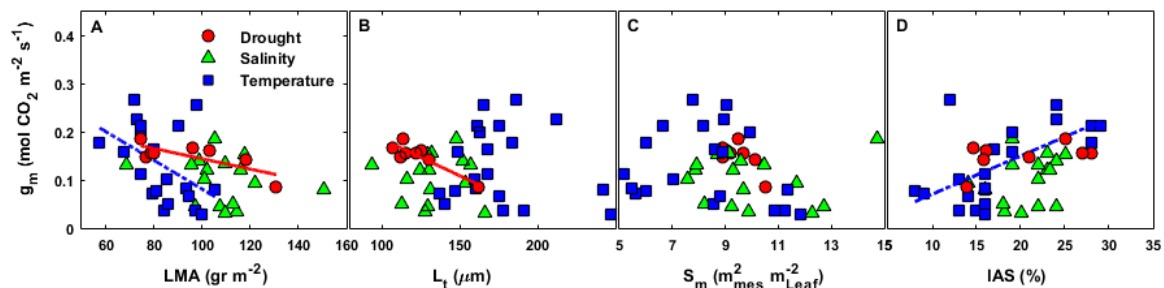


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