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Drought tolerance in cork oak is associated with low leaf stomatal and hydraulic conductances

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To investigate the role of seeds origin in drought tolerance, the response to water deprivation of cork oak seedlings differing in climatic conditions at their geographical origin was compared. Gaâfour is the provenance from the driest site and Feija is the provenance from the wettest site. Net photosynthesis (A_n), stomatal conductance (g_s) and leaf water potential were measured during dehydration. A delayed decrease in leaf water potential is observed after water withholding in Gaâfour as compared to Feija leaves. At the onset of dehydration, A_n and g_s were higher in Feija. After withholding watering, Gaâfour leaves were able to maintain a higher A_n and g_s than Feija leaves. Most likely, drought tolerance in Gaâfour leaves is associated to their lower g_s under well-hydrated conditions. The stomatal density (D_s) and specific leaf area (SLA) were not different in well-watered leaves but, leaf hydraulic conductance was lower in Gaâfour leaves when compared to Feija leaves. Our results suggested that lower stomatal and hydraulic conductances of Gaâfour leaves could be involved in bringing about the better resistance to dehydration.

Keywords: Drought, Cork Oak, Photosynthesis, Stomatal Conductance, Hydraulic Conductance

Introduction

Drought is one of the main factors limiting plant growth and production of ecosystems in many parts of the world (Zhao & Running 2010). Water stress significantly alters plant metabolism (Tezara et al. 1999) and profoundly affects agriculture (Reichstein et al. 2002). A better understanding of the mechanisms that allow plants to survive during a prolonged drought would help to select more drought-tolerant genotypes by identifying traits associated with drought resistance (Manavalan et al. 2009). Indeed, acclimation to drought is the result of integration of several events, ranging from the perception and transduction of the stress signal to the regulation of gene expression and metabolic changes (Xiao et al. 2009). Water stress is established in terrestrial plants when the water loss by leaves exceeds that absorbed from the environment by their roots (Schulze et al. 1987). Therefore, relative water content (RWC), water potential (Ψ) and cells turgor are reduced, while concentrations of compatible osmolytes as amino acids and sugars increase, leading to decrease of osmotic potential (Hare et al. 1998). When water limitation is imposed, plants develop a series of responses at the cellular and molecular levels (Chaves et al. 2009). The stomata are gradually closed decreasing the conductance to water vapor diffusion which slows down the transpiration and the rate at which the water deficiency develops (Hsiao 1973).

In addition, carbon photosynthetic assimilation often decreases concomitantly with decreased conductance to CO_2 diffusion (Lawlor & Tezara 2009). There is a controversy over the mechanisms by which photosynthetic activity is diminished under water stress. Stomatal closure is probably the major factor controlling photosynthesis, but the relative role of stomatal limitation on photosynthesis depends on the severity of water deficiency.

The Mediterranean climate is defined by a dry and warm summer. The period of summer drought is increasingly intensified and prolonged due to climatic change effects. Forests are known as highly sensitive to climate change due to the lack of trees adaptive responses to environmental fluctuations. Thereby, forests adaptation strategies should be able to anticipate the expected change. In fact, forests will have to face environmental conditions which are becoming increasingly severe for many decades, even more than a century. Climate changes will have various impacts on forests from different bioclimatic regions, and on the globally distribution of tree species. It seems difficult to predict the impact of climate change without a better understanding of the effect of atmospheric CO_2 rising and drought.

Cork oak (*Quercus suber* L.) is a sclerophyllous, evergreen Mediterranean tree species adapted to long dry summer conditions with little or no precipitation, maximum temperatures reaching 35-40 °C, and irradiance exceeding 2000 µmol m^{-2} s^{-1} at midday. Previous studies show an intraspecific variability between different Tunisian cork oak provenance differing by their ecology in response to summer drought (Ben Fradj 2016), seasonal variations of leaf gas exchange parameters (Nasr et al. 2015, Ben Fradj 2016), and light growth (Rzigui et al. 2015, 2017).

In the present study, we have examined how geographical origin of seeds might affect early responses to drought stress in cork oak seedlings.

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Materials and methods

Plant material and drought experiments

Acorns were collected from two populations originating from contrasting environments in the northwestern provinces of Tunisia (Fig. 1), as described in Rzigui et al. (2017). The first site, the National Park of Feija (36° 30′ 00″ N, 8° 20′ 00″ E), is located in the northern extent of the Kroumirie mountains and is characterized by a cold and humid climate. The average of temperature is 7 °C in January and can drop to 0 °C, which allows for year round snowfall. The second site is located at Gaâfour (36° 32′ 19″ N, 9° 32′ 40″ E) in the southern hills and plains around Siliana, and it is characterized by a semiarid climate with moderate winters and hot dry summers.

Immediately after collection, acorns were planted in the greenhouse of the National Research Institute for Rural Engineering, Waters, and Forestry in Tunis under low light conditions (15% of full sunlight). Seedlings had been grown in 5 L pots containing a mixture of equal parts of soil and compost. During spring 2016, when the two provenances (five year-old seedlings) had similar shoot and leaf sizes, seedlings were used for drought experiments. Drought was imposed by withholding watering. Gas exchange was measured at the onset of experiment and at 3, 7, 14 and 17 days after withholding water. The same plants were used at the five measuring times. All experiments were carried out using fully expanded and developed leaves.

Leaf water potential measurement

Measurements of water potential (ψ_{leaf}, MPa) were made directly after completing the A_n and g_s measurements. Leaves were rapidly pressurized (within 15 s) with nitrogen. We have used a Scholander-type pressure chamber (SKPM 1400®, Skye Instruments Ltd., Powys, UK).

Gas exchange measurements

Gas exchange measurements were carried out on attached intact leaves using a Licor 6400® (Li-Cor, Lincoln, NE, USA). Leaf temperature was maintained at 25 °C, photon flux density at 1200 µmol m^{-2} s^{-1}, ambient CO_2 molar ratio (Ca) at 400 ppm and leaf vapour pressure deficit at around 1 kPa.

Specific leaf area determination

The specific leaf area (SLA) was determined as the ratio of the leaf area (measured using a leaf area meter, Portable Laser, Model CI-202) to leaf dry mass of individual leaves.

Stomatal density determination

Imprints from fully developed Feija and Gaâfour leaves were made by coating the adaxial surface with clear nail varnish. After a few minutes, the varnish was gently peeled off the leaves and then leaf stomatal density, expressed as the number of stomata per unit leaf area, was determined using a light microscope (Leica M205 C, Wetzlar, Germany) at a magnification of 400×. Stomata counting and measuring was conducted on at least three randomized visual areas on each leaf surface.

Leaf hydraulic conductance measurement

Leaf hydraulic conductance on a surface area basis (K_{leaf}, mmol s^{-1} m^{-2} MPa^{-1}) was measured with XYL'EM® apparatus (Bronkhorst, Ruurlo, Netherlands) as described by Cochard et al. (2007). The principle was to measure the water flow (F, mmol s^{-1}) entering the petiole of a cut leaf when exposed to positive pressure (+P, MPa). Upon steady state, K_{leaf} was computed as (eqn. 1):

$$K_{leaf}=\frac{F}{P\cdot LA} \tag{1}$$

where *LA* is the total leaf area (m^2). The XYL'EM was interfaced with a computer to log different data automatically.

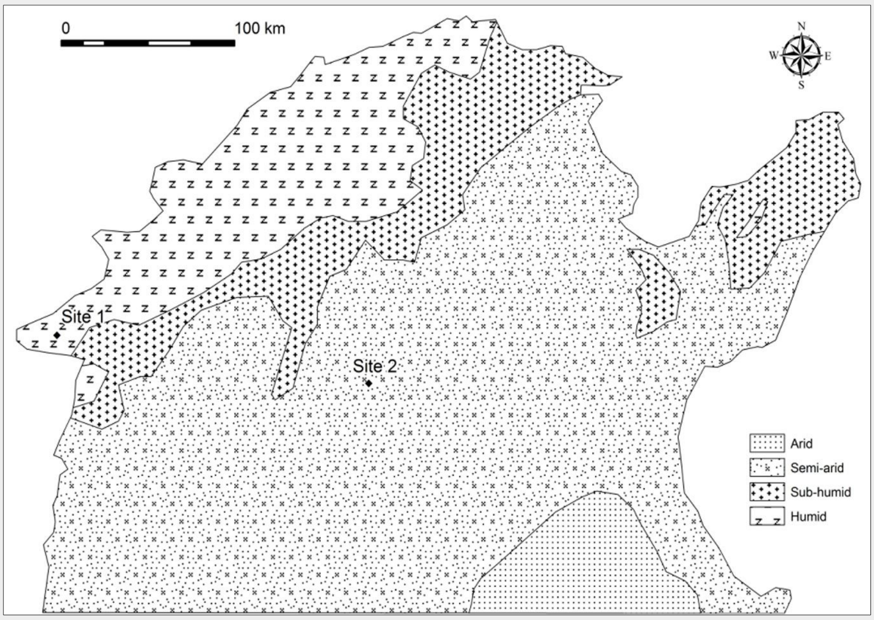

Fig. 1 - Seed source locations (site 1: Feija and site 2: Gaâfour).

Chlorophyll fluorescence measurements

In vivo chlorophyll *a* fluorescence emissions in 30-min dark-adapted leaves were measured with a portable modulated chlorophyll fluorometer (OS-30p+®, Opti-Sciences, Hudson, NH, USA) at predawn and midday. After adaptation to the darkness, the modulated fluorometer allows the accurate measurement of minimum fluorescence (F_o) using a weak, modulated light that is too low to induce photosynthesis. In this state, photosystem II is maximally oxidized. A subsequent saturating flash of white light (3500 µmols) reduces all available PSII reaction centers, and the maximum fluorescence (F_m) during the saturating light radiation is recorded. The maximum photochemical efficiency of PSII was calculated as the ratio of the light-induced variable and maximum fluorescence of chlorophyll, F_v/F_m (eqn. 2):

$$F_v/F_m=(F_m-F_o)/F_m \tag{2}$$

Photosynthetic pigments

Leaf tissue of known area (measured using a leaf area meter, Portable Laser, Model CI-202) and fresh weight was incubated in 80% acetone until all of the chlorophyll was visibly extracted. Spectrophotometer readings of the extracts were obtained at 750, 663, 645 and 453 nm to determine the chlorophyll *a* and *b* and total carotenoid contents using the equations of Arnon (1949). Chlorophyll and carotenoid concentrations were estimated on the basis of fresh weight and leaf area.

Statistical analyses

All experiments were repeated independently at least for three times and mean values and the standard errors are shown. Significant statistical differences at the level 5% were calculated with the Student's *t*-test using the software Sigma Plot® (Systat Software Inc., San Jose, CA, USA). Two-way analysis of variance was also conducted to test the interaction effect (provenance × time) on net photosynthesis rate (A_n) and stomatal conductance (g_s).

Results

It was possible to apply the dehydration treatment to the two provenances at a similar developmental stage. At the onset of the drought, Gaâfour and Feija well-watered plants (-0.5 and -0.7 MPa of ψ_{leaf}) had the same foliar evaporative surfaces (Mannai 2016). Specific leaf area (SLA) and the total chlorophyll concentration on the basis of leaf area were similar in both provenances (Tab. 1).

On the other hand, stomatal conductance (g_s) and net CO_2 uptake (A_n) were lower in Gaâfour leaves (Tab. 1). Stomatal density was similar in both provenances (Tab. 1), while leaf hydraulic conductance (K_{leaf}) was higher in Feija leaves (Tab. 1).

After withholding watering, Gaâfour plants maintained a higher leaf water potential than Feija plants (Fig. 2). After 14 days of drought, leaf water potential was reduced by 76% and 64% in Feija and Gaâfour seedlings, respectively.

Withholding watering caused a slower decrease of g_s in Gaâfour leaves (Fig. 3). As a result, g_s was higher in Gaâfour leaves than in those of Feija after 10 days. The trend followed by A_n during drought treatment was similar to g_s (Fig. 3). There is a statistically significant interaction (Tab. 2) between provenance and measuring time on both g_s ($p < 0.001$) and on A_n ($p < 0.05$). The relation between A_n and g_s was the same for Feija and Gaâfour leaves (Fig. 4). However, both g_s and A_n were lower in Gaâfour at ψ_{leaf} ranging between -0.5 and -2.5MPa, as compared to Feija seedlings (Fig. 4).

The maximum photochemical efficiency of PSII (given by F_v/F_m in dark-adapted leaves) remained constant at approximately 0.8 during drought (measured at 0, 7 and 17 days after withholding watering)

Tab. 1 - Physiological properties of well-watered Feija and Gaâfour seedlings at the onset of experiment: net photosynthesis (A_n, μmol m^{-2} s^{-1}); stomatal conductance (g_s, mmol m^{-2} s^{-1}); leaf hydraulic conductance (K_{leaf}); stomatal density (D_s, pores mm^{-2}); and total chlorophyll (mg m^{-2}). Significant differences ($P < 0.05$) between WT and CMSII leaves are indicated by different letters.

Parameters	Feija	Gaâfour
A_n (μmol m^{-2} s^{-1})	12.9 ± 0.5 ^{a}	10.4 ± 0.7 ^{b}
g_s (mol m^{-2} s^{-1})	0.3 ± 0.04 ^{a}	0.2 ± 0.01 ^{b}
K_{leaf} (mmol s^{-1} m^{-2} MPa^{-1})	7.1 ± 1.2 ^{a}	4.9 ± 0.5 ^{b}
D_s (pores mm^{-2})	282.6 ± 24 ^{a}	285.0 ± 20 ^{a}
Total chlorophyll (mg m^{-2})	395.7 ± 36 ^{a}	362.7 ± 39 ^{a}
SLA (cm^2 g^{-1})	126.0 ± 15 ^{a}	125.0 ± 9 ^{a}

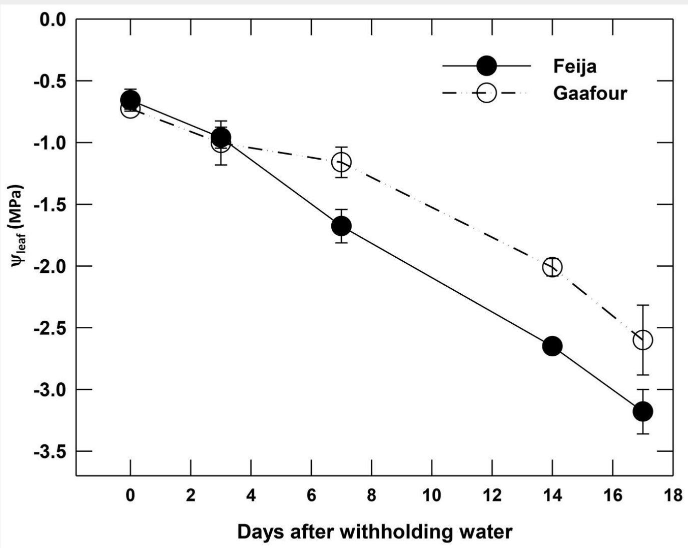

Fig. 2 - Leaf water potential (ψ_{leaf}, MPa) as a function of time after withholding watering. Data are mean ± SE of four independent measurements.

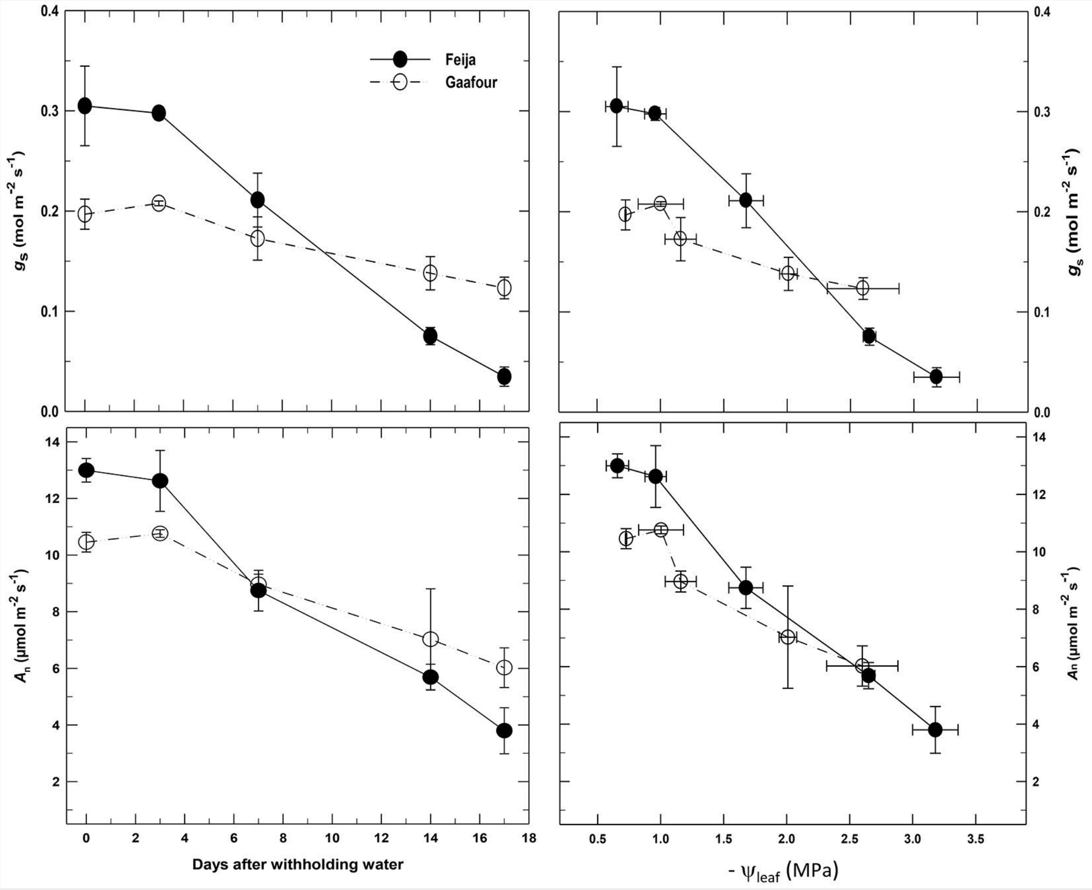

Fig. 3 - Stomatal conductance (g_s) and net CO_2 uptake (A_n) as a function of time after withholding watering and leaf water potential. Conditions: leaf temperature 25 °C; vapour pressure deficit (VPD) 1 ± 0.2 kPa; photon flux density (PFD) 1200 μmol m^{-2} s^{-1}. Data are mean ± SE of four independent measurements.

Tab. 2 - Summary of two-way analysis of variance (*F*-values) for differences in net photosynthesis rate (A_n) and stomatal conductance (g_s) between measuring times and provenances. (ns): non-significant; (**): p < 0.01; (*): p <0.05.

Factors	A_n	g_s
Provenance	0.0731^{ns}	28.710^{**}
Measuring time	38.194^{**}	70.675^{**}
Provenance × Measuring time	4.241^{*}	37.981^{**}

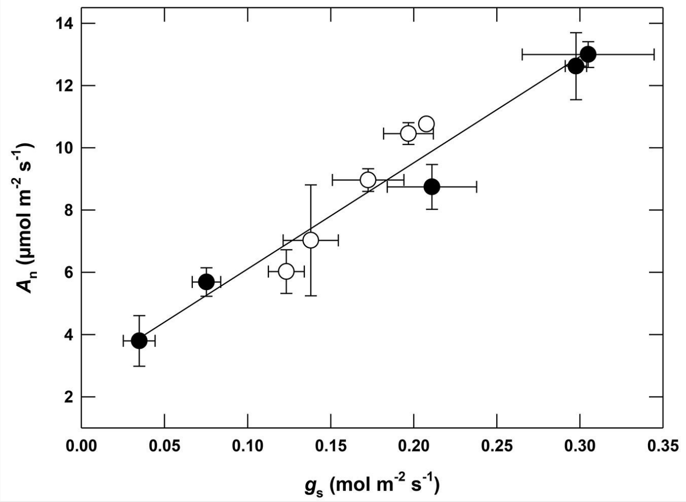

Fig. 4 - Relation between A_n and g_s during drought. Closed symbols: Feija; open symbols: Gaâfour.

in both Feija and Gaâfour leaves (data not shown).

Discussion

Intraspecific differences in response to the drought

This study showed that intraspecific variability within a species changes the tolerance for water stress. Indeed, the leaf water potential of Gaâfour leaves exhibited a slower decrease than in Feija after a water withholding. In previous studies (Valladares & Sánchez-Gómez 2006, Galmés et al. 2007, Vaz et al. 2010), this variability in response to drought has been investigated mainly between Mediterranean species (interspecific variability). In these species, photosynthesis has been shown to be essentially limited by stomatal closure (Vaz et al. 2010) and more specifically by the concentration of CO_2 at the catalytic sites of Rubisco (Galmés et al. 2007). The interpopulation variability of the response to environmental constraints, including drought, has also been studied in many species. In cork oak, some studies have investigated intra-specific variability as a source of adaptation to different regional climatic conditions. For example, Ramirez-Valiente et al. (2010, 2014) analyzed phenotypic plasticity and local adaptation of foliar ecophysiological parameters in European populations subjected to different levels of water availability. In Tunisia, Staudt et al. (2008) examined the release of volatile organic compounds during drought in Tunisian cork oak populations from contrasting climatic conditions. More recently, Rzigui et al. (2015, 2017) used seedlings from Feija and Gaâfour provenances to show the intra-specific variability of cork oak in response to high light. Several studies examined the intraspecific variability in the Tunisian cork oak forest, mainly concerning the seasonal variability of gas exchange (Nasr et al. 2015, Ben Fradj 2016). These studies have also shown that the response of photosynthetic capacity to summer drought was different among trees of three populations of cork oak belonging to different habitats. Results of the present study showed that plants from two different regions with different ecological conditions do not have the same response to dehydration by stopping watering. The analysis of light response of different populations of *Quercus coccifera* from different localities, led to the conclusion that there is an ecotypical differentiation rather than a phenotypic plasticity (Blaguer et al. 2001). In this sense, Ramírez-Valiente et al. (2014) showed that the variability of the present cork oak distribution is correlated with a variability of morphological and biochemical properties (SLA, nitrogen content, water use efficiency) of leaves. Current results indicate a variation in the plasticity of the provenances of *Quercus suber* in response to watering arrest. Indeed, two response types were distinguished in the studied provenances. For Feija provenance, a very rapid and simultaneous decrease of A_n and g_s was registered. In contrast, this decrease was slower in seedlings of the Gaâfour provenance. Recently, Ramírez-Valiente et al. (2015) have found that climatic origins of eleven *Quercus virginiana* populations induced variations in photoprotective pigments (anthocyanin and xantophyll cycle pigments) in response to temperature and drought.

The drought tolerance is associated to lower stomatal conductance (g_s) under well-hydrated conditions

The delayed water loss in Gaâfour compared to Feija seedlings was not the result of a smaller transpiring leaf surface, since the total leaf surface of the plants at the beginning of the experiment were similar in both provenances (Mannai 2016). Water loss is delayed in Gaâfour leaves due to their lower stomatal conductance (g_s) at the onset of the drought treatment and during the first days of the drought period (Fig. 2). It is obvious that the higher levels of stomatal conductance and net photosynthesis after 10 days of water withholding in Gaâfour leaves was related to their capacity to maintain their water potential and not to a differential response to a lack of water, because g_s and A_n were lower in Gaâfour than in Feija leaves (Fig. 2) within the range -0.5 to -2.5 MPa of Ψ_{leaf}. Furthermore, Gaâfour and Feija seedlings were similarly affected by drought as indicated by identical relations between A_n and g_s (Fig. 4). Thus, when compared to Feija, the plants of Gaâfour provenance were able to maintain a higher photosynthetic activity during the first days of dehydration conditions.

Hydraulic conductance is lower in Gaâfour plants under well hydrated conditions

As suggested by previous studies (Woodward & Bazzaz 1988, Hetherington & Woodward 2003, Zhenzhu & Guangsheng 2008), the low g_s in Gaâfour may be a consequence of low leaf stomatal density (D_s). However, contrary to expectation, D_s was similar in both provenances (Tab. 1), and the difference of g_s between well watered plants recorded in this study is probably a consequence of a difference in stomatal aperture level. This is in accordance with Marenco et al. (2017) which shown that, in six forest tree species of central Amazonia, g_s did not increase with increasing D_s, and concluded that the aperture of the stomatal pores was below its maximum width. The opening and closing of stomata are strongly and finely regulated by many signaling factors (Lind et al. 2015), the most important are the abscisic acid (ABA) and hydraulic factors (Comstock 2002), which were acting in interaction. ABA interacts with so many processes, outside and inside the guard cell, that it is difficult to assess its importance by just measuring total leaf ABA content (Wilkinson & Davies 2002).

In the case of this study, leaf hydraulic conductance (K_{leaf}) was lower in Gaâfour plants, and it could be critical in setting the maximum possible g_s. Indeed, it has been shown that K_{leaf} is strongly related to gs, indicating that there is an internal leaf-level regulation of liquid and vapour conductances (Brodribb et al. 2005). In leaf hydraulic system, the stomata are placed in series with the xylem. Since stomata control both CO_2 and water vapor exchanges between the leaf and its environment, it is expected that the values of hydraulic conductance will be related to those of g_s and the assimilation of CO_2 (Cornic 2007). It could be that the lowest g_s in Gaâfour leaves result from its lower K_{leaf}.

Inhibition of A_n depends on g_s during dehydration

The decrease of A_n and g_s as a function of the dehydration is faster in Feija than Gaâfour plants. For all $\Psi_{leaf} \geq -2$ MPa, A_n and g_s are higher in Feija as compared to Gaâfour plants. However, this difference disappears with Ψ_{leaf} below -2MPa. The relationship between A_n and g_s is similar in both provenances, showing that the decrease in g_s is the main factor of photosynthesis inhibition during dehydration as previously reported (Cornic & Briantais 1991, Cornic & Fresneau 2002, Flexas & Medrano 2002). This does not eliminate the possibility of non-stomatal limitation of photosynthesis. However, water stress may reduce leaf net photosynthetic assimilation (A_n) by both stomatal and metabolic limitations (Farquhar & Sharkey 1982). In addition, many studies have reported that stomatal effects are major under moderate stresses, but biochemical limitations are quantitatively important during leaf ageing or during severe drought (Grassi & Magnani 2005, Gallé et al. 2007). The maximum photochemical efficiency of PSII (given by F_v/F_m in dark-adapted leaves) in both Feija and Gaâfour leaves remained constant during the drought experiment. Cork oak, a widely distributed forest tree species in the Mediterranean basin, is able to maintain maximum photochemical efficiency of PSII during periods of drought (Faria et al. 1998). Additionally, Ghouil et al. (2003) demonstrated its tolerance to high temperatures.

In conclusion, previous studies (Aranda et al. 2005, Ramírez-Valiente et al. 2014) showed a large provenance-level differentiation in cork oak, with provenance from dry places exhibiting the higher tolerance. A distinct difference was observed in response to drought stress between both studied provenances (Gaâfour and Feija). The presented results show that Gaâfour seedlings exhibited lower stomatal and hydraulic conductances and a better tolerance to water deprivation. These physiological changes are likely to be the consequence of divergences between populations with respect to phenotypic plasticity.

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