

The effect of vapour pressure deficit on stomatal conductance, sap pH and leaf-specific hydraulic conductance in *Eucalyptus globulus* clones grown under two watering regimes

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• **Background and Aims** Stomatal conductance has long been considered of key interest in the study of plant adaptation to water stress. The expected increase in extreme meteorological events under a climate change scenario may compromise survival in *Eucalyptus globulus* plantations established in south-western Spain. We investigated to what extent changes in stomatal conductance in response to high vapour pressure deficits and water shortage are mediated by hydraulic and chemical signals in greenhouse-grown *E. globulus* clones.

• **Methods** Rooted cuttings were grown in pots and submitted to two watering regimes. Stomatal conductance, shoot water potential, sap pH and hydraulic conductance were measured consecutively in each plant over 4 weeks under vapour pressure deficits ranging 0.42 to 2.25 kPa. Evapotranspiration, growth in leaf area and shoot biomass were also determined.

• **Key Results** There was a significant effect of both clone and watering regime in stomatal conductance and leaf-specific hydraulic conductance, but not in sap pH. Sap pH decreased as water potential and stomatal conductance decreased under increasing vapour pressure deficit. There was no significant relationship between stomatal conductance and leaf-specific hydraulic conductance. Stomata closure precluded shoot water potential from falling below -1.8 MPa. The percentage loss of hydraulic conductance ranged from 40 to 85 %. The highest and lowest leaf-specific hydraulic conductances were measured in clones from the same half-sib families. Water shortage reduced growth and evapotranspiration, decreases in evapotranspiration ranging from 14 to 32 % in the five clones tested.

• **Conclusions** Changes in sap pH seemed to be a response to changes in atmospheric conditions rather than soil water in the species. Stomata closed after a considerable amount of hydraulic conductance was lost, although intraspecific differences in leaf-specific hydraulic conductance suggest the possibility of selection for improved productivity under water-limiting conditions combined with high temperatures in the early stages of growth.

Key words: *Eucalyptus globulus*, stomatal conductance, leaf-specific hydraulic conductance, sap alkalization, acidification, vapour pressure deficit, percentage loss of hydraulic conductance, water potential, water stress.

INTRODUCTION

Eucalyptus globulus is widely used for pulp production around the world and can be considered one of the most important eucalypt species given its high growth rate and pulping properties. *Eucalyptus globulus* plantations established in south-west Spain are subjected to both high temperatures and severe summer drought (Pita *et al.*, 2001). The use of selected clones has improved both growth and survival under such limiting conditions (Supplementary Data Fig. S1). However, some of these clones might fail under extreme meteorological conditions, as shown by the effects of the exceptional drought of 2005.

Stomatal conductance has long been considered of key interest in the study of plant adaptation to drought and high temperatures (Pearce *et al.*, 2005; Grossnickle and Russell, 2010). This is particularly true for *E. globulus* plantations established under

Mediterranean climates, because (1) high vapour pressure deficits (VPDs) may result in water stress even when soil water is abundant, and (2) *E. globulus* was found to reach its highest productivities through lower water-use efficiency in field trials established in south-western Spain (Pita *et al.*, 2001). After analysing the strong dependence of a wide range of photosynthetic parameters on stomatal conductance, Medrano *et al.* (2002) proposed the use of mid-morning, light-saturated stomatal conductance as a reference parameter to reflect the intensity of water stress. Stomata control several trade-offs that determine growth under water-limiting conditions. Minimizing water loss by stomatal closure under drought conditions reduces CO₂ uptake and leaf cooling via transpiration but increases water use efficiency while allowing the plant to avoid low shoot water potentials. Stomata have long been recognized as an efficient

means of controlling the risk of xylem embolism (Jones and Sutherland, 1991), at least under non-extreme soil water deficits (Meinzer et al., 2009). In some species, stomata may close at the incipience of xylem embolism, as in walnut (*Juglans regia* × *nigra*) (Cochard et al., 2002). In other species, stomatal conductance and transpiration are maximized at the expense of a certain degree of embolism (Manzoni et al., 2013).

Both hydraulic and chemical signals participate in the regulation of stomatal conductance. Stomata have been found to respond to, among other things, cavitation-induced changes in stem hydraulic conductance (Ripullone et al., 2007; Hölttä et al., 2012), the abscisic acid (ABA) concentration in the xylem sap (Heilmeier et al., 2007) and xylem sap alkalization (Sobeih et al., 2004; Wan et al., 2004). Although differences between species in hydraulic traits such as vulnerability to cavitation or leaf-specific hydraulic conductance have been widely documented (Fu et al., 2012; Tixier et al., 2014), studies comparing genotypes from a single species are less frequent.

Root-to-shoot signalling is often considered to be important in regulating shoot growth and water use when soil conditions change. Identifying signal molecules and their roles is seen as a potential way to modify crop water use (Dodd, 2005). In contrast, root signalling has been considered less effective for very tall species, in which signal transmission may be too slow for a feed-forward model of short-term stomatal response and thus other factors, such as ABA production or release within the leaves, may be more important (Heilmeier et al., 2007). However, it must be considered that changes in xylem sap may arise from root export of signalling substances and also from changes in sap composition during long-distance transport in the stem (Dodd, 2005).

The objective of this study was to investigate the combined effects of VPD and water shortage on stomatal conductance in *E. globulus* clones. More precisely, we aimed to (1) investigate the extent to which changes in stomatal conductance are mediated by changes in hydraulic conductance and/or xylem sap pH in the species and (2) identify differences between clones in the response to water shortage and VPD. We hypothesized that (1) xylem sap pH may increase with decreasing soil water, (2) xylem sap pH may respond to changes in VPD and (3) hydraulic traits may differ between clones.

To test these hypotheses, a greenhouse experiment was carried out with closely related *E. globulus* genotypes of contrasting drought resistance.

MATERIALS AND METHODS

Plant material and growing conditions

The experiment was carried out in a greenhouse (15–35 °C), from May to the end of June. Maximum photosynthetically active radiation (PAR) was 1600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Air temperature and relative humidity (R_h , %) were recorded with a Lambrecht thermo-hygrometer. Saturation vapour pressure (P_{sat}) was calculated at two-hour intervals from air temperature (Nobel, 2009) and VPD was derived from: $\text{VPD} = P_{\text{sat}}(1 - R_h/100)$. Values of VPD at any other time were calculated from these values by linear interpolation. Daily maximum VPD was typically reached around 1800 h (local time) and ranged from 1.46 to 3.84 kPa.

Seventy *E. globulus* Labill. rooted cuttings grown from scions less than 1 year old were transplanted to 50-litre pots filled with

the same weight of lightly fertilized peat (Kekkilä B6 white 420, Vandaa, Finland) mixed with perlite (1 : 1 v/v). Five extra pots were used to draw the relationship between the volumetric soil water content (H_{vol}) measured with a TRIME TDR system (IMKO GmbH, Ettlingen, Germany) and pot weight (W), covering the range of weight values in the experiment. The relationship between the two variables ($H_{\text{vol}} = 0.0162W - 16.45$, $r^2 = 0.96$, $n = 25$) was used to determine volumetric soil water content from the weight of the potted plants. Plant weight was considered negligible, as it was much lower than the pot weight.

Five clones were selected for the experiment. Clones T and OD are F_1 clones that had been widely used in commercial plantations in south-west Spain. Interestingly, both clones differed in their response to the exceptional drought of 2005 (worst drought since 1947, Aemet, 2005). Clone T was most affected and therefore withdrawn from production from then on. Clone C14 is an F_0 clone that shows an enhanced survival rate but lower growth rates than clones OD and T (Supplementary Data Table S1). Clones PI and SA are F_1 clones that belong to the same half-sib families as OD and T, respectively, and were chosen for this study simply because of their shared affiliation with the others, clone C14 being the common progenitor.

After transplanting, plants were allowed to grow and acclimate for a period of 3 weeks. Plants were watered two to three times a week and fertilized twice with 1 g per plant of soluble Peters fertilizer (N/P/K 20 : 20 : 20) (Scotts International, Heerlen, The Netherlands) during this period. On 31 May [day 0 (d0)], two watering regimes (R1 and R2) were established. In R1, the plants were watered until a weight of 2600 g was reached, while for R2 plants the figure was 2300 g. These values corresponded to 90 and 73 % of the volumetric soil water content at field capacity for R1 and R2, respectively. Plants from the two watering regimes were watered up to these weights throughout the experiment. All plants were watered on Mondays, Wednesdays and Fridays except during the third week of measurements in which R2 plants were not watered on Wednesday to increase the level of water stress.

Measurements

The amount of water lost by evapotranspiration was calculated between irrigations from the weight of each potted plant before and after watering.

Synchronous measurements of stomatal conductance, sap pH, shoot water potential (Ψ) and hydraulic conductance (hereafter ‘physiological parameters’) were carried out plant by plant on d4, d8, d9, d10, d16, d18 d19, d23 and d26. From d4 to d10, six plants per day (three from each watering regime, from the same three clones) were measured and harvested. On d16–d19 the sample size was increased up to ten plants per day (one per clone and watering regime). Eight plants from one single watering regime (one to two per clone) were measured on d23 (R1) and d26 (R2) to establish whether the moment at which the measurements were taken had a significant effect on the physiological parameters.

Stomatal conductance to water vapour and net photosynthetic rate were measured in the youngest fully expanded leaf using a portable gas exchange chamber (Li-Cor 6400XT; Li-Cor, Lincoln, NB, USA). All measurements were made between

TABLE 1. Results from the ANCOVA for the variables tested

| | LA (cm ² per plant) | W _p (g per plant) | Ψ (MPa) | Sap pH | g _s (mol m ⁻² s ⁻¹) | LSC (10 ⁻⁵ kg s ⁻¹ m ⁻² MPa ⁻¹) | PLC* |
|-----------------------|--------------------------------|---------------------------------|---------------------------------|--------------------------|---|---|--------------------------|
| <i>P</i> -values | | | | | | | |
| Covariate | <0.0001 | <0.001 | <0.0001 | <0.0001 | <0.0001 | 0.026 | 0.002 |
| Clone | 0.120 | 0.063 | 0.948 | 0.282 | 0.045 | 0.008 | 0.104 |
| Watering | 0.002 | 0.016 | 0.002 | 0.987 | 0.013 | 0.025 | 0.771 |
| Clone × watering | 0.807 | 0.323 | 0.351 | 0.627 | 0.981 | 0.784 | 0.534 |
| Adjusted means ± s.e. | | | | | | | |
| Clone | | | | | | | |
| C14 | 1851 ± 146 ^a | 10.39 ± 0.90 ^a | -1.32 ± 0.05 ^a | 6.6 ± 0.05 ^a | 0.34 ± 0.049^a | 35.2 ± 3.7 ^{ab} | 0.81 ± 0.06 ^a |
| OD | 2050 ± 137 ^a | 13.43 ± 0.91 ^a | -1.35 ± 0.05 ^a | 6.5 ± 0.05 ^a | 0.48 ± 0.050 ^{ab} | 40.2 ± 3.9^a | 0.71 ± 0.06 ^a |
| PI | 1865 ± 141 ^a | 11.49 ± 0.90 ^a | -1.35 ± 0.05 ^a | 6.7 ± 0.05 ^a | 0.40 ± 0.051 ^{ab} | 40.9 ± 3.6^a | 0.76 ± 0.06 ^a |
| SA | 2217 ± 128 ^a | 13.29 ± 0.85 ^a | -1.32 ± 0.05 ^a | 6.6 ± 0.05 ^a | 0.51 ± 0.046^b | 23.8 ± 3.5^b | 0.92 ± 0.05 ^a |
| T | 2270 ± 140 ^a | 13.36 ± 0.94 ^a | -1.30 ± 0.05 ^a | 6.6 ± 0.05 ^a | 0.53 ± 0.052 ^{ab} | 29.9 ± 4.0 ^{ab} | 0.78 ± 0.06 ^a |
| Watering regime | | | | | | | |
| R1 | 2252 ± 87^a | 13.38 ± 0.57^a | -1.26 ± 0.03^a | 6.58 ± 0.03 ^a | 0.51 ± 0.032 ^a | 37.9 ± 2.4^a | 0.77 ± 0.04 ^a |
| R2 | 1849 ± 87^b | 11.4 ± 0.56^b | -1.40 ± 0.03^b | 6.59 ± 0.03 ^a | 0.39 ± 0.032 ^a | 30.1 ± 2.4^b | 0.81 ± 0.04 ^a |

Time was used as covariate to analyse leaf area (LA) and dry biomass (W_p). Vapour pressure deficit was used as covariate for shoot water potential (Ψ), sap pH, stomatal conductance (g_s), leaf specific hydraulic conductance (LSC) and the percentage loss of hydraulic conductance (PLC). Different letters denote significant differences at *P* < 0.05.

*Values were arcsin transformed for the analysis.

1045 and 1315 h (local time), under 300-W metal halide lamps to ensure a PAR above 1000 μmol m⁻² s⁻¹. We later verified that there was no significant relationship between the rate of photosynthesis and PAR values in the range 1000–1600 μmol m⁻² s⁻¹, meaning that light intensity could be considered saturating for all measurements.

Immediately after measuring gas exchange, each plant was taken to the laboratory (less than 5 min from the greenhouse), weighed with its container and cut under water just below the 6th–7th node. Time of harvesting was annotated for each plant. Water potential (Ψ) was determined in the shoot apex using a Scholander-type pressure chamber (Plant Moisture Systems, Santa Barbara, CA, USA). Prior to this, about 3 cm of bark was removed from the cut end of the apical portion of the stem. After recording Ψ, an over-pressure of 0.2–0.4 MPa was applied to the shoot to collect xylem sap. Xylem sap pH was measured immediately afterwards using a microelectrode (Model 5208, CRISON Instruments, Barcelona, Spain) interfaced with a pH meter (CRISON micropH 2002, CRISON Instruments). A similar procedure has been used previously in shoots (Dodd *et al.*, 2003) and leaves (Rodrigues *et al.*, 2008).

At the same time as the water potential and sap pH were measured, the basal portion of the plant was prepared for hydraulic conductance measurements: all leaves were cut off under water and the stem was fixed to a tubing system connected to a low-pressure water reservoir. Hydraulic conductance was determined before (K_i) and after (K_M) removing xylem embolism as explained elsewhere (Pita *et al.*, 2003). The percentage loss of hydraulic conductance (PLC) was calculated from PLC = 100(K_M - K_i)/K_M. When measuring plant leaf area (WinFolia, Regent Instruments, Quebec, Canada), the surface of leaves in the apical portion (L_{ap}) was determined separately from the basal leaves. Leaf-specific hydraulic conductance (LSC) was calculated from LSC = K_i/L_{ap}. We found no significant relationship between LSC and the length of the stem. Therefore, LSC data were not corrected for stem length. Stems and leaves were oven dried at 60 °C and weighed.

Statistical analyses

Analysis of covariance (ANCOVA) was used to analyse the effect of clone and watering regime on all variables. Time (day number) was used as a continuous predictor when analysing differences in growth, whereas VPD was used as a continuous predictor when analysing differences in the physiological parameters.

ANCOVA was also used to verify that there was no significant effect of the time of harvesting on the physiological parameters, using the time of harvesting (hour:minutes) as a continuous predictor, and to compare regression lines. The effect of the clone or watering regime was also tested on a daily basis through one-way ANOVA. Relationships between variables were analysed through simple linear regression.

Percentage data were arcsin transformed prior to analyses. All variables were tested for normality and homogeneity of variance. Differences were considered statistically significant at *P* ≤ 0.05. Tukey's honest significant difference method was used to separate the means. STATISTICA version 6.0 (StatSoft, Tulsa, OK, USA) was used for all tests.

RESULTS

Growth and evapotranspiration

Shoot biomass and leaf area increased linearly with time. Water shortage significantly decreased growth (Table 1). Figure 1 shows the time course of average shoot biomass for both watering regimes. Similar results were obtained for leaf area (not shown). There were no significant differences between clones in leaf area or shoot biomass (Table 1).

The mean rate of evapotranspiration measured between irrigations in R1 plants increased from 212 ± 7.5 g day⁻¹ at the beginning of the experiment up to 397 ± 20.8 g day⁻¹ at the end. The total amount of water lost by evapotranspiration increased linearly with time. There were significant differences in

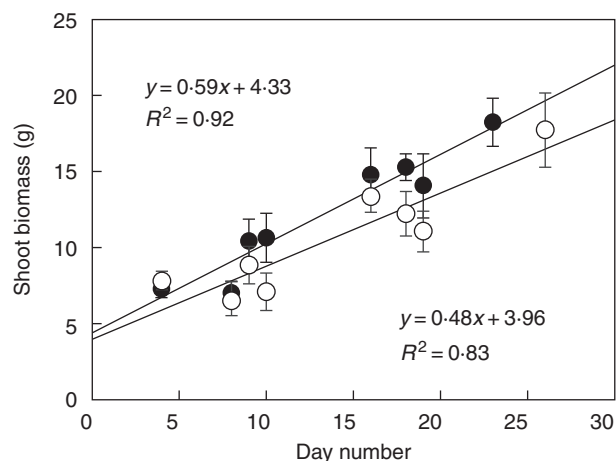


FIG. 1. Linear growth functions fitted by least squares regression to the average shoot biomass ($n=3-10$) of plants grown under two watering regimes, R1 (filled symbols) and R2 (empty symbols) (see text for further details)

TABLE 2. Average rates of evapotranspiration (g d^{-1}) calculated by watering regime as the slope of the relationship between time elapsed from the beginning of the experiment and the total amount of water lost by evapotranspiration

| | Clone | | | | | <i>P</i> |
|-----------------|----------------------|---------------------|---------------------|----------------------|---------------------|----------|
| | C14 | SA | T | OD | PI | |
| Watering regime | | | | | | |
| R1 | 258.86 ^a | 326.02 ^b | 295.59 ^c | 315.15 ^{bc} | 294.36 ^c | 0.006 |
| R2 | 217.12 ^{bc} | 224.1 ^b | 254.95 ^a | 214.36 ^b | 199.83 ^c | <0.001 |

The data were measured in plants from five *E. globulus* clones submitted to two watering regimes ($R^2 > 0.9$ for all the lines). Different letters denote significant differences between clones for each watering regime.

the slope of the regression lines fitted to both watering regimes (ANCOVA, $F=59.69$, $P<0.00001$). We found no significant differences between clones in the rate of evapotranspiration measured at the beginning of the experiment, either in plants from treatment R1 (one-way ANOVA, $F=1.47$, $P=0.23$) or in plants from treatment R2 (one-way ANOVA, $F=0.63$, $P=0.64$). However, we found a significant effect of the clone on the slope of the regression lines fitted to evapotranspiration vs. time. These slopes estimate the average rates of evapotranspiration measured throughout the experiment for each clone–watering regime combination. Under the more favourable watering regime, the highest rate of evapotranspiration corresponded to clone SA and the lowest to clone C14 (Table 2). For treatment R2, the highest evapotranspiration rates were measured in clones SA and T and the lowest in clones OD and PI. Water shortage resulted in decreases in the rate of evapotranspiration that ranged from 14 to 32 % (Table 2).

Physiological parameters

Increasing VPD inside the greenhouse led to a decrease in both Ψ and mid-morning stomatal conductance in plants from both watering regimes for the last days of the study. Stomatal

conductance and Ψ remained above $0.5 \text{ mol m}^{-2} \text{ s}^{-1}$ and -1.4 MPa , respectively, from d4 to d16 and fell below these threshold values from d18 onwards (Fig. 2C, D). Xylem sap pH peaked on d8–d16 and then decreased as stomatal conductance decreased (Fig. 2C, E), whereas PLC remained high and stable throughout the experiment (except for plants from treatment R2 on d4).

No significant effect of the watering regime on xylem sap pH was found on any of the measurement occasions (Fig. 2E). This result was particularly striking for d18 and d19, when R2 plants were submitted to a 4-d drought cycle, while R1 plants were watered every 2 d. Stomatal conductance was significantly higher in R1 plants than in R2 plants on d18 and d19 (Fig. 2C). Despite the differences in soil water (Fig. 2B) and stomatal conductance, xylem sap pH values were almost identical for both watering regimes on both days (Fig. 2E).

Results from the ANCOVA revealed a significant effect of VPD on all variables tested (Table 1). There was a tight coordination in the response of stomatal conductance, xylem sap pH and Ψ to VPD (Fig. 3). Stomata closure precluded Ψ from falling below -1.8 MPa (Fig. 3B, C). Xylem sap pH decreased as VPD increased (Fig. 3A) but there were no significant differences between either clones or watering regimes in xylem sap pH, nor was there a significant clone \times watering regime interaction (Table 1). There was a significant effect of watering regime on stomatal conductance, water potential and LSC. We found significant differences between clones in stomatal conductance and LSC (Table 1). Interestingly, clonal ranking was exactly the same for stomatal conductance and leaf area. The highest stomatal conductance and leaf area values were measured in clones from the same half-sib family (T and SA), and the lowest in the F_0 clone (Table 1).

Differences between clones in LSC were highly significant. However, if K_M instead of K_i was used to calculate LSC_{\max} , no significant differences were found between clones (ANCOVA, $F=1.04$, $P=0.40$) or watering regimes (ANCOVA, $F=1.22$, $P=0.27$). Therefore, differences in LSC appear to be related to the loss of hydraulic conductance rather than to anatomical traits such as vessel size. In accordance with these results, there was a strong and negative relationship between LSC and PLC ($r^2=0.58$, $P<0.0001$). The lowest PLC values were measured in the same clones in which the highest LSC values were measured (OD and PI) and the highest PLC and lowest LSC were measured in clone SA (Table 1).

Qualitative analysis of stomatal conductance showed that both the lowest median and the lowest 75th percentile were measured in clone C14 (Fig. 4D). The highest stomatal conductance for the 25th percentile was measured in clone T (Fig. 4D). The latter result shows that under the most stressful conditions plants from clone T did not close stomata as efficiently as the others. The lowest pH value for the 75th percentile was measured in clone T, for which a pH higher than 6.6 was measured only in 25 % of the plants (Fig. 4C). Median PLC values were between 70 and 80 % for all the clones (Fig. 4B). The lowest LSC values for the 25th percentile were measured in clones T and SA, whereas the highest LSC median was measured in clone OD (Fig. 4A). Interestingly, LSC values measured in clones OD and PI were significantly different from the remaining clones (Table 1). These clones belong to the same half-sib family.

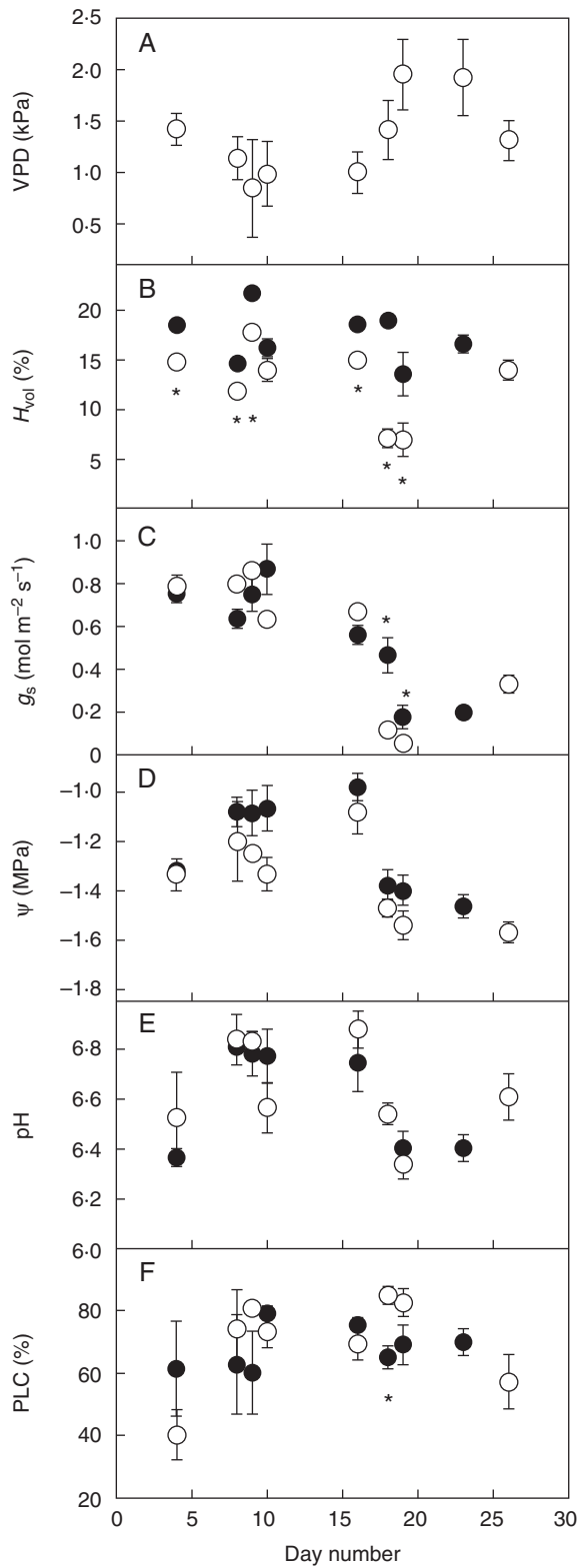


FIG. 2. Daily mean \pm s.e. values of (A) average air vapour pressure deficit inside the greenhouse at the time of measuring, (B) mean volumetric soil water content for the plants measured each day, (C) light-saturated mid-morning stomatal conductance, (D) shoot water potential, (E) xylem sap pH and (F) percentage loss of stem hydraulic conductance. Data are means of 3–10 observations for two watering regimes, R1 (filled symbols) or R2 (empty symbols). An asterisk denotes significant differences between watering regimes.

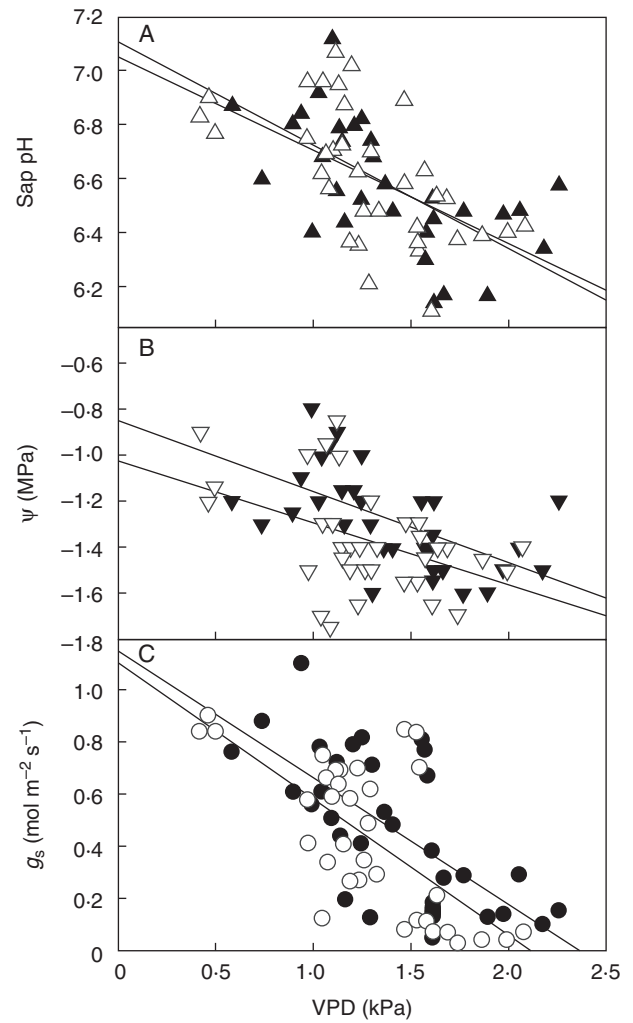


FIG. 3. Relationships between air vapour pressure deficit inside the greenhouse at the time of measuring and (A) sap pH, (B) shoot water potential and (C) stomatal conductance. Every point within each graph corresponds to data measured on one single plant. Filled symbols correspond to plants under the more favourable watering regime (R1) and empty symbols to the less favourable R2 watering. Regression lines were fitted to both watering regimes.

For all clones and treatments combined, there was a highly significant relationship between stomatal conductance and values of Ψ ($r^2=0.34$, $P<0.0001$) or sap pH ($r^2=0.21$, $P=0.0004$) and no relationship with either PLC ($r^2=0.04$, $P=0.08$) or LSC ($r^2=0.007$, $P=0.51$). Stomatal conductance decreased significantly as Ψ became more negative (Fig. 5). The highest values of sap pH were measured under concurrent comparatively high stomatal conductance ($g_s > 0.4 \text{ mol m}^{-2} \text{ s}^{-1}$) and high Ψ (Fig. 5).

DISCUSSION

Stomatal conductance and hydraulic conductance of the stem

High values of stomatal conductance, such as those measured in the present study (Fig. 3), have been associated with low water-use efficiency in fast-growing pioneer species

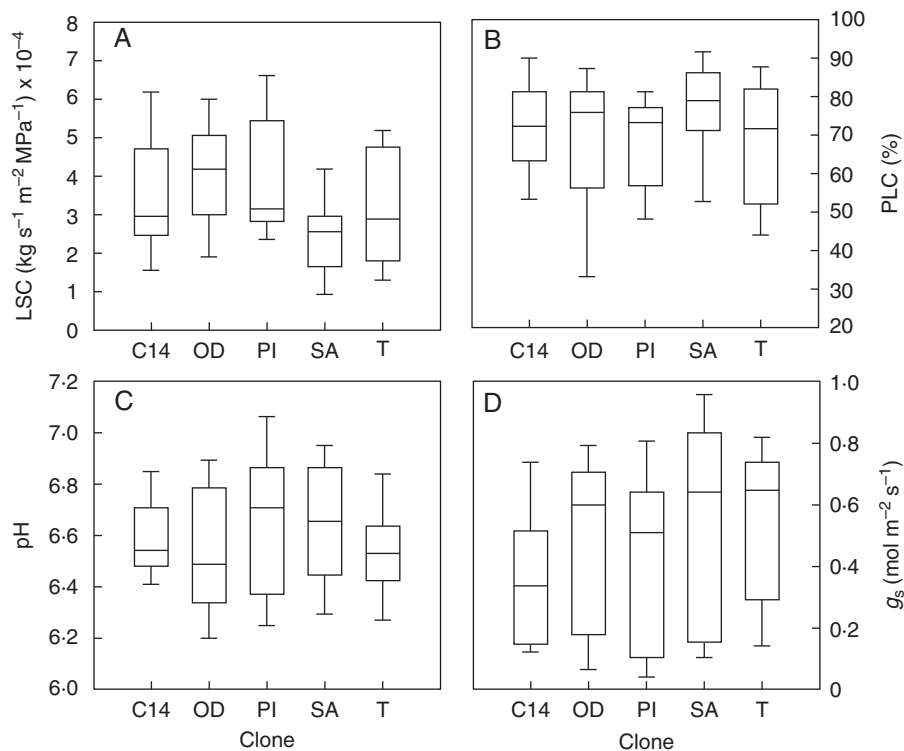


FIG. 4. Box and whisker plots of (A) leaf specific hydraulic conductance, (B) the percentage loss of hydraulic conductance, (C) xylem sap pH and (D) stomatal conductance for the five clones tested. The boundaries of the box represent the 25th and 75th percentiles, the mid-line within the box indicates the median, and whisker caps show the 10th and 90th percentiles.

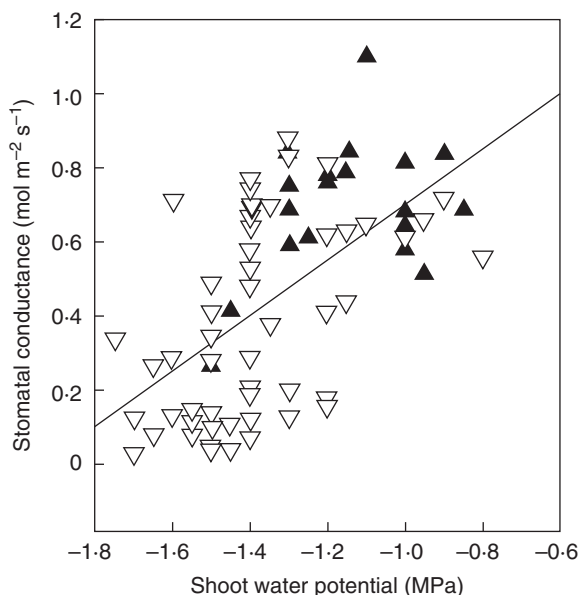


FIG. 5. Relationship between stomatal conductance and shoot water potential. Each pair of values was measured consecutively on a single plant. Filled symbols correspond to data measured in plants with a xylem sap pH higher than 6.77, which was the average 75th percentile for pH data. Data from all clones and treatments are pooled together. See text for further details.

(Pearce *et al.*, 2005) and may be advantageous to compete for available soil water. The adaptive advantage of comparatively high stomatal conductance for eucalypt plantations will depend on the ability to keep a tight control of stomata, to avoid a catastrophic xylem failure. Previous studies have reported high values of native embolism in this (Pita *et al.*, 2003) and other woody species. For example, a PLC as high as 76.7 % was measured in less than 2-year-old twigs from field-grown *Eucalyptus crebra* and *Eucalyptus xanthoclada* (Rice *et al.*, 2004). It has been suggested that the formation of embolism may have some positive side effects, such as increasing the hydraulic capacitance (Vergeynst *et al.*, 2015). Although the common occurrence of xylem cavitation remains controversial (Cochard and Delzon, 2013), the differences in stomatal regulation between coexisting ferns and angiosperms reported by Brodribb and Holbrook (2004) suggest that the evolution of a more specialized stomatal physiology may allow gas exchange to be maximized by forcing the xylem to achieve its highest flow rate, in a riskier but more successful water-use strategy (Sperry, 2004). Tolerance of a certain degree of embolism may depend, although not exclusively, on the ability of some species to refill embolized vessels (Trifilò *et al.*, 2014). The suggestion that the hydraulic conductance of the stem is overbuilt (Sterck *et al.*, 2011) must be taken into consideration. In addition, growth of xylem tissue may allow a significant recovery of lost hydraulic function in some tree species (Urli *et al.*, 2013). Furthermore, tree species may lose all their leaves when reaching a PLC higher than 80 %, but still be capable of resprouting

after watering (Urli *et al.*, 2013). Therefore, not only the loss of hydraulic conductance but also the amount of remaining hydraulic conductance must be considered when analysing the vulnerability to cavitation (Hacke *et al.*, 2015) or stem hydraulic constraints to gas exchange. LSC is a measure of the hydraulic sufficiency of the stem to supply water to the leaves (Tyree and Zimmermann, 2002). Decreases in LSC under drought conditions have been previously reported in other tree species (Domec *et al.*, 2009; Sellin *et al.*, 2014) and are considered a plastic response to water stress that may compromise water use, and therefore growth, even after the water supply is restored (Eamus *et al.*, 2000). The effect of decreasing LSC on growth was inconclusive in our study, as both LSC and growth decreased in R2 plants in relation to R1 plants but the significant effect of the clone on LSC values did not result in significant differences in growth between clones.

We found no significant relationship between values of stomatal conductance and either PLC or LSC. Moreover, values of stomatal conductance as high as $0.81 \text{ mol m}^{-2} \text{ s}^{-1}$ were measured in plants with a PLC close to 80 % in the present study (Fig. 2). These results suggest that loss of hydraulic conductance had little effect on limiting stomatal conductance under our experimental conditions. Similarly, PLC values as high as 50–60 % were found to be compatible with a relatively high stomatal conductance in *Ceratonia siliqua*, *Laurus nobilis* and *Olea europea* trees (Trifilò *et al.*, 2014). By contrast, stomata closure precluded shoot water potential from dropping below -1.8 MPa (Fig. 3) while average PLC remained below 85 % throughout the experiment (Fig. 2F). Interestingly, the PLC corresponding to -1.8 MPa ranged from 67 to 82 % in field-grown *E. globulus* clones, as calculated from the vulnerability curves constructed by Pammenter and Vander Willigen (1998). This range of values is quite similar to ours, despite differences in the age of the plants and techniques used to dehydrate the shoots in the two studies. These results suggest that stomata closure was not acting to prevent the onset of cavitation, but rather was an attempt to prevent stem hydraulic conductance from decreasing any further. In accordance with these results, Urli *et al.* (2013) found that the embolism threshold leading to irreversible drought damage was close to 88 % in five angiosperm tree species. As four of these species reached water potentials close to their minimum recoverable potential under drought conditions, they concluded that safety margins calculated for an 88 % loss of hydraulic conductance would be much more meaningful in angiosperms than the P_{50} safety margin traditionally used.

Xylem sap pH

Water shortage reduced shoot growth, evapotranspiration, water potential, stomatal conductance and LSC, but had no significant effect on sap pH. Under our experimental conditions, the values for xylem sap pH ranged from 6.1 to 7.0. These values are similar to those reported for *Populus deltoides* (Aubrey *et al.*, 2011) and *Populus nigra* (Secchi and Zwieniecki, 2012) but are higher than those measured in stems of field-grown *E. globulus* by Cerasoli *et al.* (2009). Despite this wide range of values, we found no significant variation in xylem sap pH between watering regimes. This result suggests that sap alkalization did not act as a mechanism of root to shoot signalling of

soil water deficit under our experimental conditions. This is particularly interesting given the shortness of the plants used, as the effect of path length on signal transmission is negligible in seedlings compared with tall trees.

Sap alkalization can be triggered by environmental conditions that stimulate transpiration, such as VPD (Chaves and Oliveira, 2004). In a recent study, Aubrey *et al.* (2011) observed that xylem sap pH derived from stems and twigs of *Populus deltoides* L. increased when VPD was lowest, and concluded that sap pH may increase under environmental conditions that result in low transpiration rates. We found a negative correlation between sap pH and VPD (Fig. 3), but measured the highest values of sap pH in plants that showed no strong stomatal limitations to transpiration (Fig. 5).

Whereas xylem sap alkalization has been reported as a common effect of several kinds of stress, the response pattern of stomatal conductance to elevated xylem pH remains unclear. Despite the decrease in stomatal conductance observed in response to modification of xylem sap pH by alkaline foliar sprays, Sharp and Davies (2009) only found concurrent sap alkalization and stomatal closure in four out of 22 perennial species receiving mild and severe soil water deficits. By contrast, Secchi and Zwieniecki (2012) observed that severe water stress resulted in a sudden drop of xylem sap pH in *Populus nigra* and found that sap pH decreased in embolized vessels undergoing osmotically driven refilling. In accordance with this, we measured a decrease of about 0.4 pH units from d16 to d19 (Fig. 2). The lowest values of stomatal conductance were measured on d18–d23. It has been suggested that stomata closure may favour embolism repair (Tombesi *et al.*, 2015). Interestingly, PLC values measured on d26 in R2 plants were lower than those measured in plants from the more favourable R1 watering regime 3 d before (Fig. 2). Changes in sap pH due to different factors may add to those arising in response to soil drying either antagonistically or synergistically (Sharp and Davies, 2009). The lowering of sap pH under osmotically driven refilling may contribute to explain why sap alkalization is considered a common response to soil drying in herbaceous species but remains elusive in woody species (Sharp and Davies, 2009), as woody species (and particularly tree species) cannot rely on root pressure as a mechanism of embolism repair.

Clonal effects

The lowest evapotranspiration rate was measured under the more favourable watering regime in the F_0 clone (C14). Stomatal conductance was also lowest in this clone (Table 1). Therefore, F_1 clones were able to profit from extra soil water to a greater extent than the F_0 clone. In a previous study we obtained a strong positive correlation between carbon isotope discrimination and the average diameter at breast height in *E. globulus* plantations established in south-western Spain (Pita *et al.*, 2001). This result highlights the relevance of reaching high values of stomatal conductance to achieve enhanced growth under a dry Mediterranean climate. In the present study we found a tight relationship between leaf area and stomatal conductance that further supports these findings and is in accordance with the poor growth shown by clone C14 if compared with T or OD in the field (Supplementary Data Table S1). The

highest rate of evapotranspiration was measured in clone T under the less favourable R2 treatment. Accordingly, values of stomatal conductance measured under the most stressful conditions were higher in clone T than in the remaining clones (Fig. 4). These results could contribute to explain why clone T was severely affected by the exceptional drought of 2005, particularly if we consider that the lowest values of LSC were measured in clones T and SA (Table 1). In a previous study, Vilagrosa *et al.* (2003) suggested the existence of an LSC threshold for early leaf shedding. This is a common response to drought in *E. globulus* plantations established in south-west Spain (Supplementary Fig. S2). It is also a costly response in terms of growth and productivity. Early leaf shedding can also be considered the last line of plant defence against the effects of drought. Clones with a low LSC therefore seem less capable of achieving reasonable growth and survival under Mediterranean climates. This seems particularly relevant under a climate change scenario with a predicted increase in extreme meteorological events.

In conclusion, xylem sap alkalization did not show a clear relationship with soil water status, contrary to our first hypothesis, but sap pH decreased significantly as VPD increased, in agreement with our second hypothesis. Stomata closed after a considerable amount of hydraulic conductance was lost, although the clone effect for LSC was significant, suggesting the possibility of selection for improved productivity under water-limiting conditions combined with high temperatures in the early stages of growth.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following: Table S1: survival and growth at age 3 years in two field trials established in south-west Spain prior to the exceptional drought of 2005. Figure S1: photo showing the enhanced growth of clone OD under the most water-limiting conditions. Figure S2: hemispherical photos taken in June and September at the same eucalypt plantation established in south-west Spain, showing the effect of early leaf shedding.

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