RESEARCH ARTICLE

Differential water-use efficiency and growth among *Eucalyptus grandis* hybrids under two different rainfall conditions

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Abstract

Aim of study: To analyze the course of leaf water status, water-use efficiency and growth in *Eucalyptus grandis* and hybrids throughout seasons with different rainfall.

Area of study: The study was conducted in northern Uruguay.

Material and methods: A randomized block trial was established containing E. grandis (ABH17), *E. grandis × Eucalyptus camaldulensis* (GC172), *E. grandis × Eucalyptus tereticornis* (GT529), and *E. grandis × Eucalyptus urophylla* (GU08). Predawn leaf water potential (Ψ_{pd}) and midday leaf water potential (Ψ_{md}) were measured every six weeks from the age of 16 months, throughout two growing seasons. Stomatal conductance (g_s), net photosynthetic rate (A), and leaf-level transpiration (E) were measured once in each growing season, along with leaf carbon isotope discrimination ($\Delta^{13}C$) and tree growth. Stomatal density and distribution were studied.

Main results: ABH17 and GU08 had the lowest daily fluctuation of leaf water potential and showed stronger stomatal regulation; they were hypostomatic, and stomata on the adaxial leaf surfaces remained immature. GC172 and GT529 (Red-Gum hybrids) were amphistomatic and transpired more intensively; they were less efficient in instantaneous and intrinsic water use and grew faster under high soil moisture (inferred from rainfall). Under such conditions, GC172 reached the highest gas-exchange rate due to an increase in tree hydraulic conductance. ABH17 and GU08 were hypostomatic and used water more efficiently because of stronger stomatal regulation.

Research highlights: Red-Gum hybrids evidenced less water use efficiency due to lower stomatal regulation, different stomatal features, and distinct growth patterns as a function of soil moisture (inferred from rainfall).

Keywords: Eucalypt hybrids; stomatal conductance; water-use efficiency; transpiration.

Abbreviations used: Ψ_{pd}: predawn leaf water potential; Ψ_{md}: midday leaf water potential; $\Delta\Psi$: daily fluctuation of leaf water potential ($\Delta\Psi = \Psi_{pd} - \Psi_{md}$); A: net photosynthetic rate, E: leaf transpiration rate, g_s: stomatal conductance, WUE: instantaneous water-use efficiency; WUEi: integrated water-use efficiency; A/E: leaf photosynthesis-to-leaf transpiration ratio; $\Delta^{13}C$: leaf carbon isotope discrimination; K: tree hydraulic conductance; $E/\Delta\Psi$: ratio between leaf transpiration and daily fluctuation of leaf water potential; $\delta^{13}C$: natural abundance of ^{13}C.

Authors' contributions: JG conceived and designed the experiment, performed the analysis, collected the data and wrote the manuscript. SR, LV and GQ collected the data and reviewed the manuscript. PD, JD and GF helped to collect the data. LV supervised the work. Citation by JG, SR, GQ, PD, D, GF and LV.

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Introduction

Eucalyptus afforestation occupies 22 million hectares in more than 90 countries, being one of the most popular woody crops in the world (Dharhi *et al.*, 2018). In Uruguay, eucalypt plantations for hardwood timber or pulpwood supply cover 70% of the Uruguayan afforested area (one million hectares) (Martin, 2018), and

in the last few years, the increase of climate variability (Linderman *et al.*, 2013) led to the selection of promising clones by crossing *Eucalyptus grandis* with *Eucalyptus urophylla* and Red-Gum species (*Eucalyptus camaldulensis* and *Eucalyptus tereticornis*). Since there is a positive correlation between carbon fixation and water loss, breeders face the challenge of selecting genotypes for high plant growth and high water-use efficiency (WUE) (Morris *et al.*, 1998; Dye, 2000; Mokotedi, 2013). However, information regarding several commercial genotypes is still lacking.

To avoid further embolism and xylem dysfunction (Tyree & Ewers, 1991; Brodribb *et al.*, 2003; McDowell *et al.*, 2008; Sperry *et al.*, 2016), many woody species can keep leaf water potential (Ψ) relatively constant through a complex relationship between stomatal conductance (g_s), tree hydraulic conductivity and Ψ (Sperry *et al.*, 1998; Martínez-Vilalta *et al.*, 2004; Lambers *et al.*, 2008). Unlike Red-Gum species (such as *E. camaldulensis* and *E. tereticornis*),*E. grandis* displays stronger stomata control (Kallarackal & Somen, 2008), which means a higher water potential threshold for stomatal closure ($\sim$ -2.0 MPa). On the other hand, Red-Gum species are thought to grow faster and transpire more intensively than other related species (Pohjonen & Pukkala, 1990; Kallarackal & Somen, 1997; Drake *et al.*, 2012) *E. grandis* × E. *urophylla* genotypes show stronger stomata regulation than Red-Gum hybrids and seem to be more sensitive to water stress (Eksteen *et al.*, 2013).

Generally, eucalypts exhibit high transpiration rates to enhance carbon fixation and growth, even under severe drought conditions (Lewis *et al.*, 2011). Such behavior is commonly known as anisohydric and reflects the occurrence of high transpiration rates all year round (Meinzer *et al.*, 2014). On the other hand, isohydric plants close stomata to reduce gas exchange and maintain high Ψ (Mc Dowell *et al.*, 2008). These patterns represent the two ends of the stomatal regulation sensitivity spectrum (Sade & Moshelion, 2014). Gas exchange can be inferred from leaf water potential fluctuation ($\Delta\Psi$), that is, the difference between predawn and midday bulk leaf water potential (Ψ_{pd} and Ψ_{md}, respectively) (Choné *et al.*, 2001; Franks *et al.*, 2007), which varied as a function of soil water content and atmospheric demand (vapor pressure deficit, VPD).

Leaf-level water-use efficiency is frequently estimated by instantaneous and integrated methodologies (WUE and WUE$_i$, respectively). The former is the ratio of leaf photosynthesis to leaf-transpiration rate, while the latter is related to leaf carbon isotope discrimination ($\Delta^{13}C$) during gas diffusion and Rubisco-dependent reactions. In C$_3$ species, such as eucalypts, $\Delta^{13}C$ is negatively correlated with the ratio of transpiration efficiency and whole-plant dry-mass accumulation (Farquhar *et al.*, 1989), and a significant correlation was observed between foliar $\Delta^{13}C$ and

WUE$_i$ in *E. grandis* (Olbrich *et al.*, 1993), *E. globulus* (Osório and Pereira, 1994), *E. camaldulensis* (Akhter *et al.*, 2005) and *E. grandis* × *E. camaldulensis* (Le Roux *et al.*, 1996). However, $\Delta^{13}C$ may change when scaling up to stems, shoots, and whole trees (Olbrich *et al.*, 1993), and so may WUE. Moreover, WUE$_i$ can be inferred from different plant organs, and it can be estimated as the whole-plant WUE (Seib *et al.*, 2008). Although $\Delta^{13}C$ may not be an indicator of WUE, it may be useful as a screening tool (Le Roux *et al.*, 1996). A significant relationship between foliar ^{13}C signature ($\delta^{13}C$) and instantaneous WUE in *E. grandis* was found, which could be used as a proxy for WUE (Casparus *et al.*, 2018). Water-use efficiency can also be studied from wood rings (from the trunk or branch cores), although in many species ring growth may be caused by carbon synthesized in previous growing seasons (Mc Farlane & Adams, 1998; Skomarkova *et al.*, 2006).

Stem biomass can increase without modifying WUE, as previously found by Battie-Laclau *et al.* (2016) in *E. grandis* plantations. Leaf transpiration also depends upon stomatal traits such as density and distribution, both of them varying not only among eucalypt species (David *et al.*, 1997; Morris *et al.*, 1998; Héroult *et al.*, 2013) but also between related hybrids (Eksteen *et al.*, 2013). However, little research has been done in commercial genotypes so far.

The present study was designed to analyze the course of leaf water status and leaf-level WUE in *E. grandis, E. grandis* × *E. camaldulensis, E. grandis* × *E. tereticornis,* and *E. grandis* × *E. urophylla* clones, during two consecutive growing seasons with different rainfall. We hypothesized the following: (a) these genotypes differ in stem growth capacity, transpiration activity ($\Delta\Psi$), stomatal traits and leaf-level water use efficiency (WUE), as inferred either from their instantaneous WUE or leaf carbon isotope discrimination ($\Delta^{13}C$); b) that this behavior is related to different regulation of stomatal opening (g_s) and carbon fixation (A); c) this regulation modifies stem growth and varies between growing seasons as a function of soil water moisture (inferred from effective rainfall).

Materials and methods

Plant material and experiment design

Cuttings from three months old sprouts of each clone were collected in April 2012 and rooted in the clonal nursery of Lumin (formerly Weyerhaeuser Uruguay) with controlled humidity (90 to 95%). They were irrigated from May to August with a Biorend® solution (10 cm^3 L^{-1}) and then transplanted into 3 L plastic pots containing Carolina Soils® substrate, a mix of sphagnum peat

(58%), vermiculite (40%), and trace minerals (2%). They were fertilized three times a week with 18-18-18 (NPK) for two months.

At the age of four months (September 2012), the plants were planted in a field trial in Tacuarembó (Uruguay; 31º 38`15" S, 55º 54' 17" W), where the regional climate is humid subtropical (Cfa, Köppen classification system) with a mean annual rainfall of 1484 mm and an average temperature of 10.3 ºC in July and 22.3 ºC in January. Soils are sandy to loamy (Typic Hapludult, USDA classification system) with a poorly drained B horizon (0.35 to 0.60 m) and Fe-Mn concretions down to 1.50 m deep.

A randomized complete block design with three blocks was used. Within each of the three blocks, ten trees per clone of *E. grandis* (ABH17), *E. grandis × camaldulensis* (GC172), *E grandis × tereticornis* (GT529), and *E. grandis × urophylla* (GU08) were planted in rows, at a spacing of 4.75 m × 5 m. Each row was a plot. Two trees per plot were randomly selected to assess ecophysiological variables. From the age of 16 months (December 2013), Ψ was measured every six weeks throughout two consecutive growing seasons (2013-14 and 2014-15). Diameter at breast height and tree height were measured for all trees of the trial (n=72) at the beginning and end of each growing season. Leaf gas-exchange measurements were performed once a season, as well as foliar sampling for carbon isotope analyses.

Bulk leaf water potential (Ψ_{pd}, Ψ_{md})

Leaf water potential (predawn and midday) was measured with a Schölander pressure chamber (Soil Moisture Equipment®, Santa Barbara, CA) on three current-year branchlets of each selected tree. Predawn leaf water potential (MPa) was recorded before sunrise (4:30 to 06:00 a.m.) on the lower branches. Midday leaf water potential (MPa) was measured from 12:00 to 03:00 p.m. (one hour per block) on the sunlit foliage of the upper tree crown. Average clone Ψ was calculated from three measurements of each selected tree, and the daily fluctuation of Ψ was calculated a $\Delta\Psi = \Psi_{pd} - \Psi_{md}$. We checked for block effect when analyzing the data. Atmospheric demand was used as a covariate (Faustino *et al.*, 2013) when analyzing the date effect.

Gas exchange (*A*, *E*)

Net photosynthetic rate A (μmol m^{-2} s^{-1}), stomatal conductance g_s (mmol m^{-2} s^{-1}), and leaf transpiration rate E (mmol m^{-2} s^{-1}) were measured on three current-year leaves at the top of the crown, between 11:00 a.m. and 03:00 p.m., using a portable gas-exchange device (LiCor 6400,

LiCor®, Lincoln, NE) at a photon flux density of 900.78 ± 1.20 μmol m^{-2} s^{-1} and an airflow rate of 500.16 ± 0.64 mL min^{-1}. For *E. grandis*, this irradiance is saturating (Whitehead and Beadle 2004). Measurements within each block lasted a maximum of one hour and 15 minutes, and we checked for block effect when analyzing the data. Air temperature, CO_2 level, and VPD inside the chamber matched the outside air condition. The purpose was to study gas exchange under water deficits. However, soil moisture (inferred from Ψ_{pd} value) began to decrease in autumn of the second growing season. Measurements were taken on 02/26/2014 (summer) and 05/08/2015 (autumn). The maximum air temperature during measurements was 29.25 ± 1.84 °C in the first growing season and 23.90 ± 0.95 °C in the second. Data were used to calculate instantaneous water-use efficiency (WUE) as the *A/E* ratio, and K as *E/*$\Delta\Psi$ ratio.

^{13}C abundance and carbon isotope discrimination

Nine leaves per tree were collected to obtain the ^{13}C signature (δ ^{13}C), from the same branchlets selected for gas-exchange measurements, and located on the northern (sunny) side of the trees' upper crown. Total carbon concentration and relative abundance of ^{12}C and ^{13}C were measured by mass spectrometry using a Delta Plus® spectrometer (Finnigan MAT, Bremen, Germany). Each sample was analyzed with an elemental Flash EA 112. The standard deviation of total carbon and ^{13}C were calculated from leucine as a reference. The natural abundance of ^{13}C (δ^{13}C, ‰) was expressed in relation to international standard PDB as $[(R_{sample} / R_{standard}) - 1]$ (Craig, 1957), where R is the $^{13}C/^{12}C$ ratio. Data were used to calculate $\Delta^{13}C$ (‰) as $(\delta^{13}C_{air} - \delta^{13}C_{leaf}) / (1 + \delta^{13}C_{leaf}/1000)$, being $\delta^{13}C_{air}$ the atmospheric $\delta^{13}C$ abundance and $\delta^{13}C_{leaf}$ the sample ^{13}C content (Farquar *et al.*, 1989).

Stomatal density and distribution

The observation of these traits was not part of the original work and was considered only for the second growing season. Three one-year-old leaves per tree of similar leaf age were collected to study stomatal density (No. mm^{-2}) and distribution. Three epidermal impressions of adaxial and abaxial leaf surfaces were prepared with nail varnish; dried layers were peeled off with tweezers (modified from D'Ambrogio, 1986). Stomatal density was calculated from images taken with a Dino Eye 2.0 digital camera added to a Nikon E100 light microscope (10X). Five fields (0.15 mm^2) on each leaf surface were analyzed using DinoCapture® software.

Tree growth

Tree height (Ht, m) and diameter at breast height (Dbh, m) of all the trees in a plot were measured at the beginning of each growing season. Data were used to calculate diameter and height relative growth as RG = $(G_n - G_{n-1})/ G_{n-1}$, being G the tree Dbh or Ht, and standing tree volume (V, m³) as V = $Dbh^2 \times \pi /4 \times Ht \times FF$, being FF the form factor An average form factor of 0.5 was used (Da Silva *et al.*, 1999).

Statistical analysis

The effect of the differences and interactions between clone and date on the variances was tested using a two-way analysis of variance (ANOVA). Data were analyzed for homoscedasticity and normality (Shapiro Wilks, $p>0.05$), and when these conditions were met, the ANOVA was performed using InfoStat® software (UNC, Córdoba, Argentina). Mean comparison was performed by Tukey's post hoc test ($p<0.05$). Clone-date interaction was studied for Ψ within each growing season using VPD as a covariate. The proposed model was the following:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \tau_k + (\alpha\tau)_{ik} + \gamma_l + \varepsilon_{ijkl}$$

where y_{ijk} is the response variable, μ is the overall mean, α_i is the effect of the ith clone, $\beta_{j\,is}$ the effect of the jth block, τ_k is the effect of the kth date, $(\alpha\tau)_{ik}$ is the effect of the ith clone and k^{th} date, γ_l is the effect of the covariate, and ε_{ijkl} is the residual error with $\varepsilon_{ij} \sim N(0, \sigma2\varepsilon)$.

Results

Bulk leaf water potential and rainfall

Effective rainfall was higher during the second growing season (2014-15) (Fig. 1), and Ψ_{pd} reflected such variation (Tables 1 and 2). In that season, Ψ_{pd} attained the highest average (-0.21± 0.01 MPa) and no clonal effect was observed. However, differences between clones appeared in the first growing season (the driest one), as *E. grandis* (ABH17) averaged the lowest value (Ψ_{pd} = -0.66 ± 0.03 MPa) ($p<0.0149$).

Unlike Ψ_{pd}, Ψ_{md} did show the clone effect ($p<0.0001$) in both seasons, and Red-Gum hybrids had the lowest average. GT529 showed this behavior throughout both growing seasons (Ψ_{md} = -1.96 ± 0.08 in the first season, and -2.45 ± 0.05 MPa in the second), whereas GC172 showed it during the wettest one (Ψ_{md} = -2.34 ± 0.05 MPa). ABH17 and GU08 behaved similarly, except at the beginning of
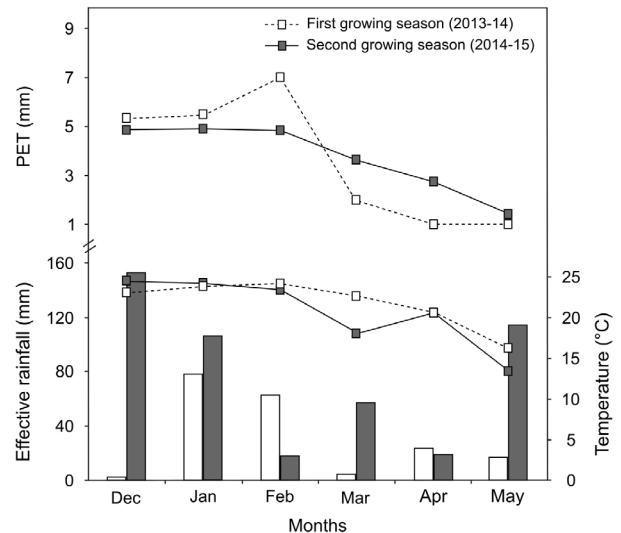
Figure 1. Environmental parameters of water supply and atmospheric demand in the experimental assay; potential evapotranspiration (PET, mm) and monthly average temperature (ºC) (lines), effective rainfall (mm) recorded in the first growing season (2013-14) (white bars) and second growing season (2014-15) (gray bars) at INIA Tacuarembó Station (W 55º 58′ 43"; S 31º 44' 18").

the wettest season (2013-14), when GU08 averaged the minimum value.

Clone-date interaction was significant for Ψ_{md} in both growing seasons ($p<0.0001$), although for Ψ_{pd}, this interaction appeared during the driest season ($p<0.0001$) (Tables 1 and 2). The daily fluctuation of leaf water potential varied among genotypes ($p<0.0001$) and was lowest in the Red-Gum hybrids (Fig. 2). During summertime, it was 30 to 50% higher than that of the other clones.

Gas exchange and instantaneous water-use efficiency

Net photosynthetic rate (*A*) differed among hybrids in both growing seasons ($p<0.0068$ and $p<0.0001$), with the GU08 clone showing the lowest *A* in both study periods (12.80 and 11.91μmol m^{-2} s^{-1}, first and second season respectively). Despite being variable, carbon fixation was similar among the other genotypes (Table 3). Stomatal conductance (g_s) also varied among clones across both growing seasons ($p<0.0001$), when Red-Gum hybrids displayed the highest average and, therefore, transpired more intensively (higher *E*) (Table 3). These results revealed a stronger relationship between *A* and g_s for the ABH17 clone during both growing seasons (r^2 = 0.76 in the first growing season and 0.8 in the second), and for GU08 during the first growing season (r^2 = 0.83) (Fig. 3). Unlike Red-Gum hybrids, these genotypes attained higher carbon fixation under low stomatal conductance (Fig. 3),

Table 1. Mean leaf predawn (Ψ_{pd}) and midday water potential (Ψ_{md}) (MPa) of *Eucalyptus grandis* (ABH17), *E. grandis* × *E. camaldulensis* (GC172), *E. grandis* × *E. tereticornis* (GT529) and *E. grandis* × *E. urophylla* (GU08) clones throughout the first growing season (2013-2014), considering clones as main factor and mid-morning air vapor pressure deficit (VPD) as covariate Means within columns followed by different letters are significantly different (Tukey, $p < 0.05$), p_{VPD} and r_{VPD} for VPD covariance also shown

	Second growing season (2014-15)							
Clone	**Ψ_{pd} (MPa)**				**Ψ_{md} (MPa)**			
	Dec	Feb	Mar	May	Dec	Feb	Mar	May
ABH17	-0.53 a	-0.64 b	-0.58 a	-0.82 a	-2.01 a	-1.66 a	-1.81 a	-0.92 a
GC172	-0.67 a	-0.38 a	-0.49 a	-0.69 a	-2.30 b	-1.59 a	-2.31 b	-0.75 a
GT529	-0.69 a	-0.36 a	-0.46 a	-0.69 a	-2.29 b	-2.30 b	-2.60 b	-0.66 a
GU08	-0.68 a	-0.36 a	-0.48 a	-0.70 a	-1.92 a	-1.48 a	-1.74 a	-0.92 b
p-value	0.1316	<0.0001	0.2098	0.0093	0.0004	<0.0001	<0.0001	0.0005
SE	0.05	0.04	0.04	0.05	0.07	0.09	0.11	0.04
p_{VPD}	0.052				<0.0001			
r_{VPD}	0.06				-0.93			

Means within columns followed by different letters are significantly different (Tukey, $p < 0.05$), p_{VPD} and r_{VPD} for VPD covariance also shown

Table 2. Mean leaf predawn water potential (Ψ_{pd}) and midday water potential (Ψ_{md}) (MPa) of *E. grandis* (ABH17), *E. grandis* × *E. camaldulensis* (GC172), *E. grandis* × *E. tereticornis* (GT529) and *E. grandis* × *E. urophylla* (GU08) clones throughout the second growing season (2014-2015), considering clones as main factor and mid-morning air vapor pressure deficit (VPD) as covariate

	Second growing season (2014-15)							
Clone	**Ψ_{pd} (MPa)**				**Ψ_{md} (MPa)**			
	Dec	Feb	Mar	May	Dec	Feb	Mar	May
ABH17	-0.22 a	-0.19 a	-0.19 a	-0.15 a	-2.33 ab	-2.05 ab	-2.16 ab	-1.94 a
GC172	-0.22 a	-0.20 a	-0.19 a	-0.17 a	-2.42 a	-2.09 ab	-2.17 ab	-2.20 a
GT529	-0.21 a	-0.19 a	-0.22 a	-0.16 a	-2.50 a	-2.36 b	-2.45 b	-2.52 a
GU08	-0.23 a	-0.21 a	-0.21 a	-0.18 a	-2.95 b	-1.72 a	-2.09 a	-2.24 a
p-value	0.8534	0.3835	0.3811	0.5641	<0.0001	0.0009	0.0147	0.1829
SE	0.02	0.01	0.01	0.01	0.06	0.10	0.08	0.10
p_{VPD}	0.04				<0.0001			
r_{VPD}	0.07				0.50			

Means within columns followed by different letters are significantly different (Tukey, $p < 0.05$), p_{VPD} and r_{VPD} for VPD covariance also shown

therefore showing higher instantaneous WUE (*A/E* ratio). In addition, there was no relationship between overall instantaneous WUE and Ψ_{md}, so WUE remained relatively constant for a wide range of Ψ_{md}.

Tree hydraulic conductance (*K*) ($p<0.0001$) varied among clones only during the wettest season. In that period, GC172 had the highest average (2.02 ± 0.14 mmol m^{-2} s^{-1} MPa^{-1}) (Table 4). When comparing *K* between seasons, we found a higher *K* in the less rainy first

season (2.8 ± 0.11 vs. 1.49 ± 0.10 mmol m^{-2} s^{-1} MPa^{-1}) ($p<0.0001$).

Carbon isotope discrimination and water-use efficiency

Red-Gum hybrids showed higher $\Delta^{13}C$ ($p<0.0001$), suggesting less efficiency in leaf-level intrinsic water use (*A/E* integrated over time). In contrast, ABH17 and
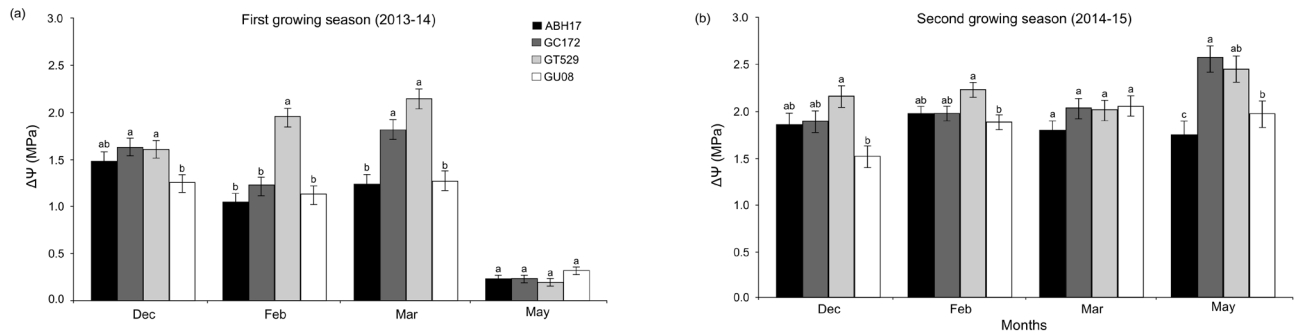
Figure 2. Mean leaf water potential gradient (ΔΨw, MPa) of *E. grandis* (ABH17), *E. grandis × E. camaldulensis* (GC172), *E. grandis × E. tetericornis* (GT529) and *E. grandis × E. urophylla* (GU08) clones during the first (a) and second (b) growing season; bars topped with different letters differ statistically within the same date (Tukey, $p < 0.05$).

GU08 showed the opposite behavior, as inferred either from their higher instantaneous WUE or their lower foliar $\Delta^{13}C$ (Table 3).

Stomatal density and distribution

Stomatal density showed no clonal difference and ranged between 428 ± 82 and 340 ± 56 stomata mm^{-2} (Fig. 4). The stomatal distribution pattern did vary among clones, since Red-Gum hybrids were amphistomatous, whereas ABH17 and GU08 were hypostomatic and stomata on their upper epidermis remained entirely immature (Fig. 5).

Tree growth

Clonal difference ($p < 0.0001$) in diametric relative growth was observed in the second year of the study

(*i.e.*, during the wettest growing season). By then, the *E. grandis × camaldulensis* (GC172) clone had the highest diametric relative increment (0.23 ± 0.01), whereas *E. grandis × urophylla* (GU08) showed the lowest one (0.07 ± 0.01). On the other hand, differences in height growth appeared during the driest year of the study (the first growing season), but they did not affect volumetric tree growth (all clones averaged $0.065 \pm 5.8 \times 10^{-3}$ m^3; data not shown). However, differences in stem volume were observed at the end of the study, when GU08 averaged the lowest value (Tables 5 and 6).

Discussion

Leaf water potential and transpiration

Leaf bulk water potential and transpiration reflected the differences in soil moisture (inferred from the effective

Table 3. Mean net photosynthetic rate (A, μmolCO$_2$ m^{-2} s^{-1}), stomatal conductance (g_s, molH$_2$O m^{-2} s^{-1}), transpiration rate (E, molH$_2$O m^{-2} s^{-1}), instantaneous water-use efficiency (μmolCO$_2$ m^{-2} s^{-1} / molH$_2$O m^{-2} s^{-1}) and foliar carbon isotope discrimination ($\Delta^{13}C$, ‰) of *E. grandis* (ABH17), *E. grandis × E. camaldulensis* (GC172), *E. grandis × E. tereticornis* (GT529) and *E. grandis × E. urophylla* (GU08) clones

Clone	n	First growing season (2013-14)					Second growing season (2014-15)				
		A	g_s	E	WUE	$\Delta^{13}C$	A	g_s	E	WUE	$\Delta^{13}C$
ABH17	10	17.49 a	0.13 b	3.51 bc	4.98 a	21.77 b	13.89 ab	0.11 c	2.20 c	6.31 a	20.93 b
GC172	17	17.18 ab	0.18 a	4.13 ab	4.14 ab	23.42 a	15.94 a	0.30 a	4.85 a	3.28 c	22.25 a
GT529	17	18.28 a	0.17 a	4.67 a	3.91 b	23.30 a	15.27 a	0.18 b	3.40 b	4.49 b	21.88 a
GU08	14	12.80 b	0.09 b	2.84 c	4.51 ab	20.32 c	11.91 b	0.10 c	2.05 c	5.81 a	20.47 b
p-value		0.0068	<0.0001	<0.0001	0.02	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
SE		1.07	0.01	0.24	0.22	0.16	0.60	0.01	0.13	0.22	0.16

Means within columns followed by different letters are significantly different (Tukey, $p < 0.05$), standard error of the mean (SE) also shown
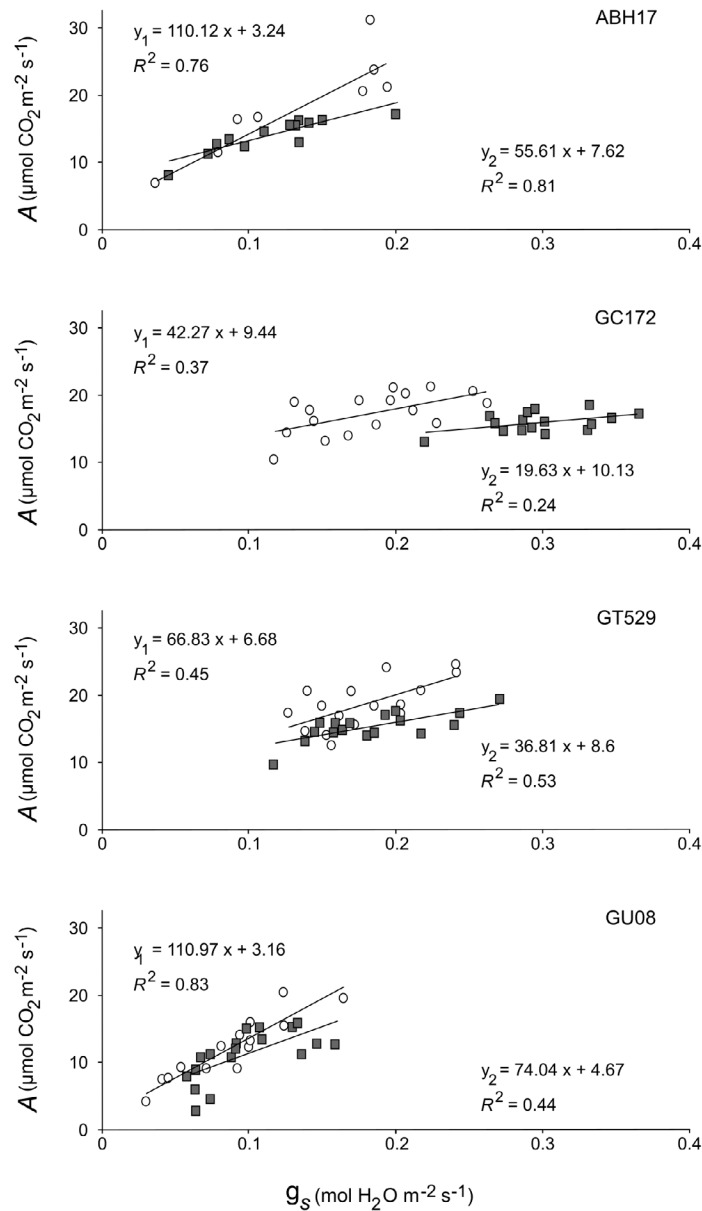
Figure 3. Relationship between net photosynthetic rate (*A*) and stomatal conductance (*g_s*) of (a) *E. grandis* (ABH17), (b) *E. grandis* × *E. camaldulensis* (GC172), (c) *E. grandis* × *E. tetereticornis* (GT529) and (d) *E. grandis* × *E. urophylla* (GU08) clones in the first and second growing season (circles and squares, respectively); regression equations for both growing seasons also shown (y_1 and y_2, first and second respectively)

rainfall). During the wettest growing season, Ψ_{pd} showed a three-fold increase in comparison to the first seasons, and all genotypes averaged a similar value. In addition, $\Delta\Psi$ was 1.88 times larger, which suggests a higher leaf-level water loss. Red-Gum hybrids (GC172 and GT529) transpired more intensively in both growing seasons, with an increase of 67% in their $\Delta\Psi$ under well-watered conditions (2014-15).

Stomatal opening can be quite sensitive to atmospheric demand in some eucalypt species (White *et al.*, 2000). However, soil moisture modifies the relationship between g_s and VPD (Leuning *et al.*, 1991). Under well-watered conditions, as in the second growing season, the magnitude of the increase in water loss (*E*) depends on the sensitivity of decreasing g_s with increased VPD. We observed an increase of *E* in Red-Gum hybrids during the summer (high $\Delta\Psi$), and higher g_s when measuring instantaneous gas exchange (measured once a season). Therefore, midday leaf water potential decreased more steeply because of transpiration (Tables 1 and 2), which reveals a lower stomatal sensitivity in these clones. *E. camaldulensis* and *E. tereticornis* seem to display higher

Table 4. Mean (± SE) tree hydraulic conductance (K, mmol m^{-2} s^{-1} MPa^{-1}) of *E. grandis* (ABH17), *E. grandis* × *E. camaldulensis* (GC172), *E. grandis* × *E. tereticornis* (GT529) and *E. grandis* × *E. urophylla* (GU08) clones

	Tree hydraulic conductance (K)	
	First growing season	**Second growing season**
	(February 2104)	**(May 2015)**
ABH17	3.10 ± 0.32 a	1.19 ± 0.16 b
GC172	3.24 ± 0.24 a	2.02 ± 0.14 a
GT529	2.47 ± 0.22 a	1.53 ± 0.16 ab
GU08	2.66 ± 0.24 a	1.14 ± 0.15 b
p-value	0.094	0.0002

Means within columns followed by different letters are significantly different (Tukey, $p<0.05$)

Table 5. Mean (± SE) tree height (m) and height relative growth (RG, unitless) of *E. grandis* (ABH17), *E. grandis* × *E. camaldulensis* (GC172), *E. grandis* × *E. tereticornis* (GT529) and *E. grandis* × *E. urophylla* (GU08) clones

	Tree height (H, m)			Height relative growth	
Clone	**Dec 2013**	**Dec 2014**	**Dec 2015**	**Dec 2013-Dec 2014**	**Dec2014-Dec 2015**
ABH17	6.33 ± 0.18 a	10.52 ± 0.6 a	13.54 ± 0.53 a	0.40 ± 0.02 b	0.29 ± 0.53 a
GC172	5.80 ± ab	9.49 ± 0.22 ab	12.73 ± 0.33 ab	0.39 ± 0.01 b	0.35 ± 0.003 a
GT529	5.59 ± b	9.86 ± 0.30 ab	13.6 ± 0.47 a	0.43 ± 0.01 ab	0.38 ± 0.004 a
GU08	4.73 ± 0.13 c	8.84 ± 0.26 b	11.47 ± 0.47 b	0.46 ± 0.01 a	0.31 ± 0.04 a
p-value	<0.0001	0.0033	0.0168	0.0004	0.4197

Means within columns followed by different letters are significantly different (Tukey, $p<0.05$)

Table 6. Mean (± SE) diameter at breast height (Dbh, m), diametric relative growth (RG, unitless) and stem volume (V, m^3) of *E. grandis* (ABH17), *E. grandis* × *E. camaldulensis* (GC172), *E. grandis* × *E. tereticornis* (GT529) and *E. grandis* × *E. urophylla* (GU08) clones

	Diameter at breast height			Diametric relative growth		Stem volume
	Dec 2013	**Dec 2014**	**Dec 2015**	**2013-14**	**2014-15**	**Dec 2105**
ABH17	0.07 ± 3.1* a	0.13 ± 4.2* a	0.16 ± 4.7* b	0.48 ± 0.01 a	0.17 ± 0.02 b	0.13 ± 0.01 a
GC172	0.07 ± 2.0* a	0.14 ± 2.6* a	0.17 ± 3.0* a	0.49 ± 0.01 a	0.23 ± 0.01 a	0.14 ± 0.01 a
GT529	0.06 ± 2.0* b	0.12 ± 3.5* b	0.14 ± 4.2* b	0.50 ± 0.01 a	0.20 ± 0.01 ab	0.11 ± 0.01 ab
GU08	0.07 ± 3.1* a	0.14 ± 3.1* a	0.14 ± 4.2* b	0.49 ± 0.01 a	0.07 ± 0.01 c	0.09 ± 0.01 b
p-value	0.0130	0.0012	<0.0001	0.7397	<0.0001	0.0013

* Dbh × 10^{-3}
Means within columns followed by different letters are significantly different (Tukey, $p< 0.05$)

g_s at saturating irradiance (Whitehead & Beadle, 2004), similarly to GC172 and GT529 in the present work.

Although Red-Gum hybrids had the highest E (and $\Delta\Psi$) during the summer, GC172 was more sensitive to soil water content and displayed stronger regulation of stomata opening (Table 3). In addition, gas exchange results for this clone revealed a 40% reduction in g_s during that period. Stomatal conductance in *E. camaldulensis* decreases steeply as a function of soil water status (White, 2000), the same behaviour recorded for the GC hybrid. However, transpiration also depends on K (Tyree & Ewers, 1991; Bond & Kavanagh, 1999). Our
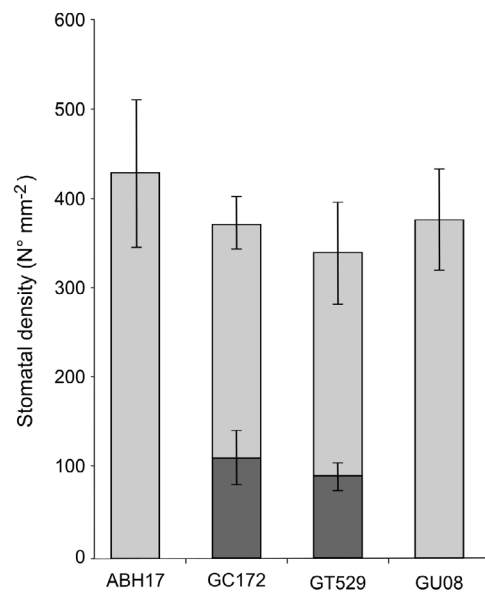
Figure 4. Stomatal density on adaxial and abaxial leaf surfaces (dark and gray bars, respectively) of *E. grandis* (ABH17), *E. grandis × E. camaldulensis* (GC172), *E. grandis × E. tetereti-cornis* (GT529) and *E. grandis × E. urophylla* (GU08) clones; error bars indicate the standard deviation of the mean.

results suggest that all genotypes increased their K under low soil moisture (first growing season) to sustain gas exchange and, consequently, carbon gain. In well-watered conditions, GC172 had the highest K, which confirms the findings of White (2000) in *E. camaldulensis*. In addition, GC172 displayed a lower control of stomatal opening under high soil moisture (second growing season) (Table 3), mainly due to an increase in K (Table 4) to deal with rising xylem tension (lower Ψ_{md}). Under such conditions, this species is known to produce extensive adventitious roots, to display stem hypertrophy (increased stem diameter), and to increase root porosity to sustain higher g_s and K, which modify tree growth (Argus *et al.*, 2015). Some of these features could explain the behavior of the GC clone in the wettest season, mainly if stem growth is considered (Tables 5 and 6).

In recent work, we studied the clonal variation of hydraulic traits and wood anatomy of one-and-a-half-year specimens of GC172, GT529, and ABH17, grown without water limitations under greenhouse conditions. We found that GC172 had the highest leaf-specific hydraulic conductivity without embolism (k_L max = 7.53 ± 0.53 kg m^{-1} MPa^{-1} s^{-1}) and wider xylem vessels (0.04 ± 0,001 mm). This clone showed the highest $\Delta\Psi$ and was also the most vulnerable to cavitation (higher percentage loss of hydraulic conductivity; unpublished data). These results are consistent with the high transpiration of Red-Gum hybrids in the present study, and the higher hydraulic conductance of GC172 under well-watered conditions.

Water-use efficiency and growth

The analysis of instantaneous WUE (A/E) and $\Delta^{13}C$ led to similar conclusions. Red-Gum hybrids were the least efficient in intrinsic leaf-level water use, most likely due to lower stomatal regulation over time (long term A/E ratio) (Table 3, Fig. 3). This outcome confirms the information provided by leaf water potential and suggests that Red-Gum hybrids adjust $\Delta\Psi$ to sustain carbon gain (Le Roux *et al.*, 1996). Conversely, ABH17 and GU08 seemed to be more VPD sensitive and would therefore be more suitable for withstanding drought conditions by reducing leaf gas exchange. These clones were highly efficient in carbon fixation (high A), although GU08 was more water-use efficient in during the driest period (as inferred by $\Delta^{13}C$ data) (Table 3). This behavior could have led to higher WUE by decoupling stomatal conductance and carbon gain (A), as previously reported for humid-zone eucalypt species (Héroult *et al.*, 2013).

To our knowledge, hybrids with *E. urophylla* appear to be highly sensitive to VPD, which implies a reduction of carbon gain under high VPD. However, WUE is not a constant characteristic of a given genotype, and it varies according to a combination of site conditions, weather and tree age. The frequency and duration of soil water deficits are crucial in determining WUE, which is sensitive to annual variations in rainfall amount as well as to rainfall distribution throughout the year (Dye, 2000). Under soil water deficits, *E. urophylla* is known to show higher WUE (lower $\Delta^{13}C$) due to stable stomatal
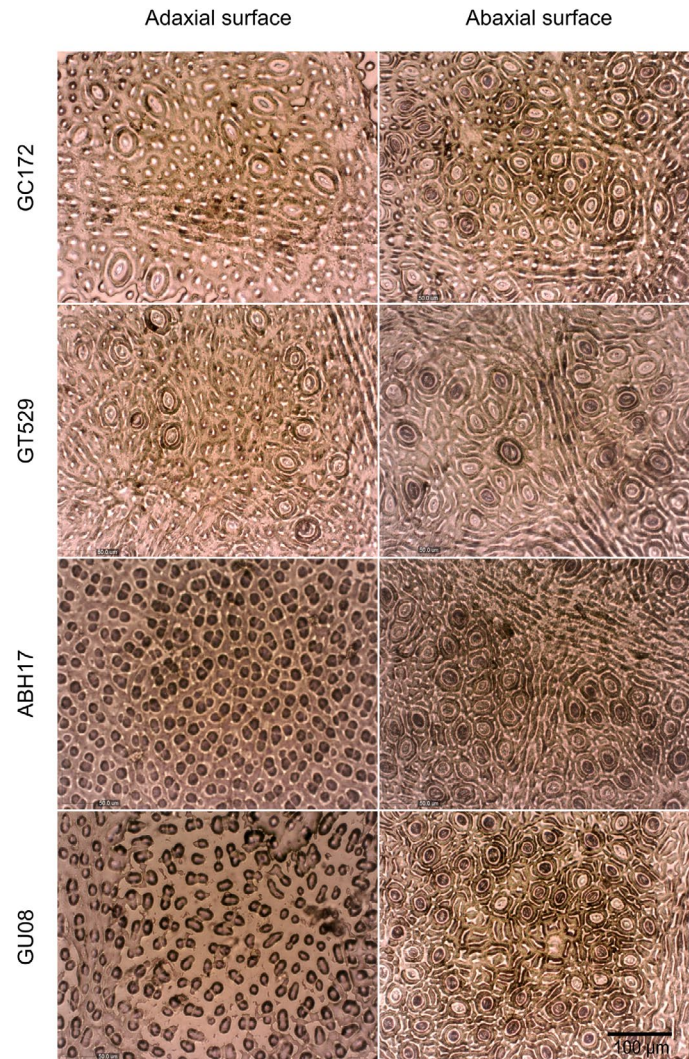
Adaxial surface   Abaxial surface

Figure 5. Photomicrographs of adaxial and abaxial leaf epidermis (columns) of *E. grandis* (ABH17), *E. grandis* × *E. camaldulensis* (GC172), *E. grandis* × *E. tereticornis* (GT529) and *E. grandis* × *E. urophylla* (GU08) clones (files); images (10X) were obtained on 0.15-mm2 fields.

conductance as leaf water potential decreases (Zhang *et al.*, 2016). In our work, this mechanism was displayed by GU08 throughout the less rainy season (2013-14), as inferred from WUE$_i$ (Table 3).

All genotypes had similar diametric relative growth and volumetric stem growth during the first year of the study (the less rainy one). During the wettest year, the GC clone attained the highest diametric relative growth (0.23 ± 0.01), whereas GU averaged the lowest (0.07 ± 0.01). The latter also showed the lowest stem volume at the end of the study (Table 6) and was the most efficient in water use (lower $\Delta^{13}C$) during the driest growing season. This behavior is consistent with the so-called "hare strategy," which means that growth increases rapidly with soil water availability, as observed for diameter growth in 2014-15 (Table 6). Drew *et al.* (2009) reported this pattern for some GU clones in South Africa. These authors found *E. grandis* × *camaldulensis* hybrids to keep growing even under severe drought conditions, referred to as the "tortoise" growing pattern. In our study, this hybrid grew faster than other clones in the driest season (Table 6), mainly due to a higher gas exchange (as inferred by $\Delta\Psi$, WUE, and WUE$_i$), which promoted carbon fixation.

Stomatal traits and water status

Transpiration was influenced by stomatal density and distribution. ABH17 and GU08 had hipostomatous leaves and showed stronger stomatal control that reduced gas exchange. In GU clones, Eksteen *et al.* (2013) reported that stomatal sensitivity to drought could be a consequence of both stomatal closure and the absence of stomata on

adaxial leaf surfaces. These traits are highly variable among closely related clones, and we did not study stomata development or their functionality. Leaves were sampled at the end of the second growing season on the same branchlets used for gas-exchange measurements, therefore reflecting the effect of environmental conditions on stomatal anatomy during this time (the preceding days or even weeks).

On the other hand, Red-Gum hybrids transpired more intensively and were amphistomatic. *E. grandis × camaldulensis* hybrid seems to have higher K under well-watered conditions to sustain carbon gain, which is consistent with gas-exchange results. Conversely, ABH17 and GU08 enhanced their net carbon fixation. Eksteen *et al.* (2013) observed that GC hybrids had higher growth than GU clones after 12 months of chronic water stress. This behavior was related to a higher gas exchange and different stomata density and distribution.

A noteworthy observation was that stomata on adaxial surfaces of ABH17 and GU08 remained entirely immature, similarly to some tropical herbs (Kagan & Sachs, 1991). England & Attiwill (2011) studied stomata development in *Eucalyptus regnans* and reported that they evolved progressively from margin to midrib in juvenile leaves. However, in the current study, stomata remained immature on the whole adaxial surface of adult leaves. This pattern could have reduced leaf gas exchange and it probably prevented leaf water potential from falling (*i.e.*, from being more negative). Hydraulic isolation in amphistomatic leaves can induce surface-specific stomatal closure in *E. globulus* (Richardson *et al.*, 2017), and this could explain why Red-Gum hybrids attained the highest gas exchange in our work, even in the driest growing season. Nevertheless, further research is needed to study leaf hydraulics and the functionality of such immature stomata.

Conclusions

We found differences in gas-exchange regulation, water status, and growth among genotypes as a function of soil water moisture (inferred from rainfall) in two growing seasons. Red-Gum hybrids (*E. grandis × E. camaldulensis*, GC172, and *E. grandis × E. tereticornis*, GT529) transpired more intensively (higher daily $\Delta\Psi$), and were therefore less water-use efficient as confirmed either by instantaneous (A/E) or intrinsic ($\Delta^{13}C$) WUE. These clones were amphistomatic and displayed lower stomatal control over time (higher $\Delta^{13}C$). The *E. grandis × E. camaldulensis* hybrid showed the highest gas exchange under well-watered conditions (wettest growing season), mainly due to an increase in tree hydraulic conductance (K). This behavior granted this hybrid constant g_s, therefore promoting carbon gain and stem growth. Conversely, *E.*

grandis (ABH17) and *E. grandis × E. urophylla* (GU08) genotypes were more water-use efficient over time because of stronger stomatal regulation that increased the A/E ratio. Therefore, they were more VPD sensitive across both growing seasons (and therefore in different soil water moisture conditions). These clones were hypostomatic, and stomata on their adaxial leaf epidermis remained entirely immature.

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