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ORIGINAL ARTICLE

Changes in γ-aminobutyric acid concentration, gas exchange, and leaf anatomy in *Eucalyptus* clones under drought stress and rewatering

Marina Shinkai Gentil Otto[1] · Jeane Gisele Francisco[1,2] · Beatriz Teixeira Gonsalez[1] · Lara de Almeida Calvo[1] · Eduardo Moré de Mattos[1,3] · Marcílio de Almeida[1] · Rafael de Andrade Moral[1] · Clarice Garcia Borges Demétrio[1] · José Luiz Stape[1,4] · Ricardo Ferraz de Oliveira[1]

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Abstract Drought stress promotes biochemical and physiological alterations in plant metabolism that limit growth and yield. This study investigated the accumulation of γ-aminobutyric acid (GABA) in plant tissue, the stomatal conductance (gs) and changes in leaf anatomy in *Eucalyptus* following drought stress situation. In this study, eight *Eucalyptus* clones were evaluated under normal water supply (control) and drought stress conditions (stress). For the control treatment, plants were irrigated every day with an automated system until the soil was saturated, and for the stress treatment, drought stress was imposed by non-irrigation of plants, and pots were covered using plastic sheeting to avoid rainfall and humidity. This study has shown that: (1) all clones decreased gs with increasing vapor pressure deficit (*D*) in both treatments. All plastics and drought-tolerant clones (except GG) presented lower stomatal sensitivity to *D* under stress conditions than drought-sensitive clones; (2) GABA concentrations increased fast after drought stress, but we could not find correlation with these changes and resistance to water stress; and (3) all clones increased the number of stomata and reduced leaf thickness after water stress. The finding is that GABA is a fast stress-signaling molecule in *Eucalyptus*, but the response of gs to *D* is a best physiological variable to differentiate drought-tolerant and drought-sensitive *Eucalyptus* clones.

Keywords GABA · Stomatal conductance · Water stress · Recovery

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✉ Marina Shinkai Gentil Otto
msgentil@usp.br

Jeane Gisele Francisco
jeane.quimica@gmail.com

Beatriz Teixeira Gonsalez
beatriz.gonsalez@usp.br

Lara de Almeida Calvo
laracalvo1@gmail.com

Eduardo Moré de Mattos
eduardo@4tree.com.br

Marcílio de Almeida
malmeida@usp.br

Rafael de Andrade Moral
rafael.moral@usp.br

Clarice Garcia Borges Demétrio
clarice.demetrio@usp.br

José Luiz Stape
stape@suzano.com.br

Ricardo Ferraz de Oliveira
rfo@usp.br

[1] Department of Plant Physiology and Biochemistry, University of Sao Paulo, Av. Padua Dias 11, Piracicaba, SP 13418-970, Brazil

[2] Center for Nuclear Energy in Agriculture, University of Sao Paulo, Av. Centenário, 303, Piracicaba 13418-970, Brazil

[3] 4Tree Agroflorestal Company, R. Tiradentes435, Piracicaba, SP 13400-760, Brazil

[4] Suzano Papel e Celulose, Rod. Br 101, km 945,5, s/n, Mucuri, BA 45930-000, Brazil

Introduction

Understanding the interaction between plant physiology and environment allows defining management strategies to alleviate stress conditions in agricultural or forestry species, as well as to select drought-tolerant genotypes in breeding programs. Different physiological responses occur once plants have a perception system composed of a network of molecules with the capability of transferring and processing information about environmental changes (Roshchina 2001). Despite major progress in understanding how water stress affects plant function (Sperry et al. 1998; Breda et al. 2006; Flexas et al. 2009), plant perception of climatic variations remains poorly resolved, and this limits our ability to adequately predict drought tolerance-related variables under stress conditions.

Several studies have identified substances in plants that are also found in the nervous systems of animals, such as histamine (Barger and Dale 1910), acetylcholine (Ewis 1914), dopamine (Buelow and Gisvold 1944), adrenaline (Askar et al. 1972), and serotonin (Bowden et al. 1954). The presence of γ-aminobutyric acid (GABA) in plants was first discovered in 1949 in potato tuber (Steward et al. 1949). GABA is a non-protein amino acid that occurs in high levels in the brain of animals as a neurotransmitter (Steward et al. 1949).

Interest in GABA metabolism in plants has increased following experimental observations that GABA is rapidly produced in response to biotic and abiotic stresses (Kinnersley and Turano 2000; Warren et al. 2011). These situations have been reported in drought-stressed cotton (Hanower and Brzozowska 1975), in beans (Raggi 1994), in turnips (Thompson et al. 1996), in *Eucalyptus* (Warren et al. 2011, 2012a, b), in heat-stressed cowpea cells (Mayer et al. 1990), in cold-stressed soybeans (Wallace et al. 1984), tomato (Snowden et al. 2015), banana (Wang et al. 2014), pear fruit (Yu et al. 2014), and in strawberries subjected to high CO_2 concentration (Deewatthanawong et al. 2010). Furthermore, Bown et al. (2002) demonstrated that when tobacco insect larvae simply walk on soybean plants, a stimulation of GABA synthesis occurs in minutes, indicating that GABA is a stress-signaling molecule.

GABA metabolism has been associated with many physiological responses, including the regulation of cytosolic pH (Carroll et al. 1994; Snedden et al. 1995; Mazzucotelli et al. 2006), nitrogen metabolism (Rolin et al. 2000; Buve et al. 2004), biotic defense (MC Lean et al. 2003; Mac Gregor et al. 2003, Scholz et al. 2015), protection against oxidative stress (Bouché et al. 2003; Fait et al. 2006), osmoregulation (Schawacke et al. 1999; Shelp et al. 1999), signaling (Bouché and Fromm 2004; Michaeli and Fromm 2015), regulation of pollen tube, and root growth (Ramesh et al. 2015). A recent study investigated that the response of the GABA-depleted gad1/2 mutant to drought stress and their findings suggests that GABA accumulation during drought is a stress-specific response and its accumulation induces the regulation of stomatal opening thereby prevents loss of water (Mekonnen et al. 2016).

The limitation of plant growth imposed by water stress is mainly due to a reduction in photosynthesis. For this reason, photosynthetic response to drought has been subject of studies and debate for decades (Lawlor and Cornig 2002). There are many studies from gas exchange to water stress that show that stomatal control is an important mechanism for plant survival under stress conditions, and different species may exhibit different stomatal sensitivity to vapor pressure deficit (D) (Oren et al. 1999; Mediavilla and Escudeiro 2004; Addington et al. 2004; Huaman 2010; Mokotedi 2013; Eksteen et al. 2013; Ocheltree et al. 2013). Species and individuals that exhibit high gs at a low D tend to have greater stomatal sensitivity to an increasing D (Oren et al. 1999; Addington et al. 2004; Maherali et al. 2006).

The effects of water stress on photosynthesis have been intensively studied, but the recovery of photosynthesis following drought has been studied less intensively. Even though there are many studies showing different concentrations of GABA in response to drought and differences in the response of stomatal conductance in water-stress situations, there are few studies that examine these physiological variables under water-stress conditions simultaneously. To understand how plants are affected by water status, we need to examine simultaneously the multiple effects of water stress on stomatal changes, internal conductance, and leaf metabolites (Warren et al. 2011).

Differences in morphological and anatomical parameters of leaves are important to understanding the mechanisms related to plants living in water-stressed conditions. Stomatal control and stomatal density are important factors determining water relationships and their relationship to photosynthesis and GABA accumulation is poorly understood. This is important, since one way for plants to adjust to environmental conditions is to modify their leaf anatomy (Cutler et al. 2011). Such modification includes changes in palisade layers, spongy layers, and intercellular space in plants living in water shortage conditions (Chartzoulakis et al. 2002).

Eucalyptus is an important species that accounts for 8% of planted forests in the world (FAO 2011). With the expansion of *Eucalyptus* planted in regions under water stress, it becomes necessary to increase our understanding of physiological and anatomical processes triggered by plants under water-stress conditions. Development of varieties exhibiting increased drought tolerance for any plant species would result in a more stable yield under stress conditions (Eksteen et al. 2013). However, breeding, specifically for drought tolerance, is still time-consuming and expensive (Pidgeon et al. 2006). The distinction between drought-tolerant clones and drought-sensitive clones is particularly relevant to assisting *Eucalyptus* breeding programs for selecting clones that are more productive under water-stress situations.

We hypothesized that GABA is an early indicator of water-stress situation and that drought-tolerant and sensitive *Eucalyptus* clones differ in physiological and anatomical parameters following water-stress situation. To provide an integrated picture of how plants responds to drought stress and recovery, this study examines responses of physiology variables (water potential, stomatal control in response to water pressure deficit-*D*), GABA concentration, and leaf anatomy in eight *Eucalyptus* clones from dry and humid regions of Brazil.

Materials and methods

Plant material and experimental design

Cuttings of eight *Eucalyptus* clones from different geographical and climatological breeds were evaluated in this study. Clones planted in humid regions were considered drought-sensitive to water stress, clones planted in dry regions were considered drought-tolerant, and clones planted in both (dry and humid regions) were considered plastics (Table 1). These clones were planted in 320 L pots containing 66% soil, 17% sand, and 17% peat in February 2013. Two plants were planted in each pot.

The study was carried out in Piracicaba, São Paulo, Brazil (22°42′30″S, 47°38′00″W). Piracicaba has an average annual temperature of 21.6 °C, average precipitation of 1230 mm $year^{-1}$, and average potential evapotranspiration of 1042 mm $year^{-1}$. The site's climate is classified as Cfa (humid temperate) under the Koppen Classification (Alvares et al. 2013) and consists of three winter months (June, July, and August), a dry season during the winter and a wet season during the summer. Total rainfall for the study period (October 2013–January 2014) was 684 mm, and the monthly average temperature was 25 °C.

It was adopted a completely randomized experimental design, in a factorial scheme 2 × 8: two treatments (drought stress—stress; and normal water supply—control) and eight *Eucalyptus* clones, with six replicates, totaling 96 experimental units. For the normal water supply treatment, plants were irrigated every day with an automated system until the soil was saturated. Plants of both treatments received similar water supply in the first 9 months. Drought stress treatment began on November 12th when plants were 9 months and 10–15 m tall. Water stress was imposed by non-irrigation of the plants, and the pots were covered using plastic sheeting to avoid rainfall and humidity. Water was withheld in three cycles: two water-stress days (cycle 1), four water-stress days (cycle 2), and two water-stress days (cycle 3). Between cycle 1 and cycle 2, water-stressed plants were rehydrated for 2 weeks, so they could completely recover.

Leaf water potential

Leaf water potential (Ψ_{leaf}) was determined during drought stress days (T1 and T2; T15–T18; and T41 and T42) and after recovery watering (R1d, R2d, and R3d; R22d, R28d, and R30d). We measured four fully expanded leaves for each treatment, located in the middle of the tree crown, at midday (11–12 am) and predawn (4–5 am) during the drought cycle using a Scholander chamber (1965).

GABA analysis

Extraction and quantification

Leaf samples were collected on the same days that we measured water potential. All leaves were collected on the same time and between noon and 1 pm on sunlit days when water stress is most severe and to control possible diurnal variations in GABA concentration. We punched three leaves of four plants per treatment (12 repetitions) from the middle part of the crown, and they were immediately frozen in liquid N and subsequently stored at −80 °C.

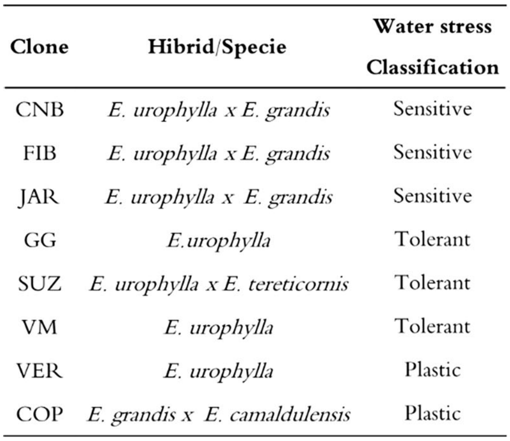
GABA concentration was extracted using mixture of methanol, chloroform, and water (12:5:3, v:v) according to the method described by De diego et al. (2012).

For the quantification, analyses were carried out on an LC–ESI–MS/MS system, a Liquid Agilent (Wilmington, DE, USA) Chromatograph 1200. The chromatographic separations were carried out using a Thermo Scientific Hypersil GOLD C18 column (100 mm × 2.1 mm, 3 µm particle size). Table 2 shows the chromatographic parameters used for GABA detection.

The mobile phases were (A) 0.1% formic acid in Milli-$Q^{®}$water (Millipore; Bedford, USA) and (B) 0.1% formic acid in acetonitrile. The elution was in isocratic mode with the proportion of A:B being 20:80, v/v. The flow remained constant at 0.40 mL min^{-1}, the column temperature was fixed at 30 °C, and the injection volume was 5 µL.

We used a Quadruple Triple 6430 mass spectrometer as a detector. The ESI parameters in the positive ionization mode were: gas flow of 10 L/min, gas nebulizer at 50 psi, gas temperature at 350 °C, and capillary voltage of 4000 V. Nitrogen 99.99% was used as a nebulizer and 99.9999% as a collision gas. We used the Agilent Mass

Table 1 Origin of the eight *Eucalyptus* clones (map modified from Alvares et al. 2013)



Clone	Hibrid/Specie	Water stress Classification
CNB	*E. urophylla x E. grandis*	Sensitive
FIB	*E. urophylla x E. grandis*	Sensitive
JAR	*E. urophylla x E. grandis*	Sensitive
GG	*E.urophylla*	Tolerant
SUZ	*E. urophylla x E. tereticornis*	Tolerant
VM	*E. urophylla*	Tolerant
VER	*E. urophylla*	Plastic
COP	*E. grandis x E. camaldulensis*	Plastic

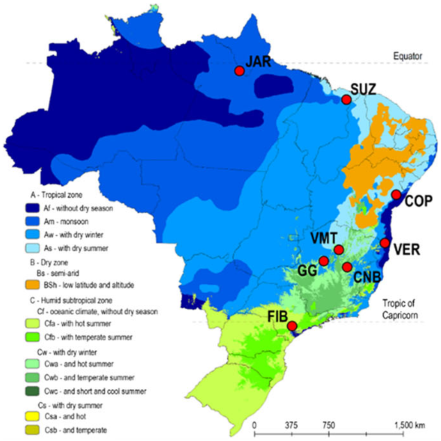


Table 2 Detection and chromatographic parameters

Transitions	Fragmentation energy (V)	Collision energy (V)
104.1–> 87.2[a]	45	4
104.1–> 69.2[b]	45	12

[a] Quantifier

[b] Qualifier

Hunter software for data acquisition, and we used the MRM mode for detection in the MS/MS.

Leaf gas-exchange measurements

We evaluated gas-exchange variables using the LI-6400 system (Li-Cor, Lincoln, NE, USA) on every day that we measured the water potential and GABA concentration. Measurements were performed after 10–15 min of stabilization in a light saturation of 1500 μmol m^{-2} s^{-1}, ambient humidity greater than 50%, and CO_2 concentration of 400 ppm. We measured nine repetitions per treatment on fully expanded leaves located in the middle of the tree crown. To observe variations between stomatal conductance and *D*, measurements were taken in the morning (8–12 am) and afternoon (2–5 pm).

Stomatal anatomy

Samples of fully expanded leaves, chosen at random from the middle third portion of shoots, were collected from each clone on two dates: (1) before water-stress days (T1) and (2) after all water-stress cycles (T42). To capture the water-stress effect, newly emergent leaves were tagged in the upper third of the crown after cycle 1 and collected after three water-stress cycles when they were fully expanded (Fig. 1).

Leaf spring samples of two sides were taken on three leaves for each clone by the superglue technique adapted from Gulcan and Misirli (1990). This sampling was performed on the middle portion of the leaf because previous studies showed that the highest stomatal frequency is found near the leaf tip, the lowest frequency near the leaf base, and an intermediate frequency in the middle (Salisbury 1927; Miranda et al. 1981).

Stomatal frequency was studied for 3072 fields [8 clones × 3 leaves × 2 leaf sides (abaxial and adaxial) × 4 slides × 4 fields × 2 treatments × 2 periods] chosen at random in the middle of the blade. Stomatal quantity was measured under an optical microscope, and the stomata were manually counted on digital images for each field. Approximately 27,195 stomata were counted in total. From these measurements, mean stomatal frequency was determined for each leaf surface for each replicate. Stomatal frequency was calculated as the number of stomata/mm^2 (n. mm^{-2}).

Statistical methodology

Generalized additive models (Wood 2006) were fitted to the stomatal conductance data including different smoothing functions with a cubic spline basis over *D* for each treatment (different linear predictors). Sub-models were also fitted, i.e., parallel and coincident linear predictors and tested using likelihood-ratio tests. Then, the same models were fitted to these data split for each clone. Generalized additive models were also fitted to the GABA data including a smoothing function with a cubic spline basis over water potential.

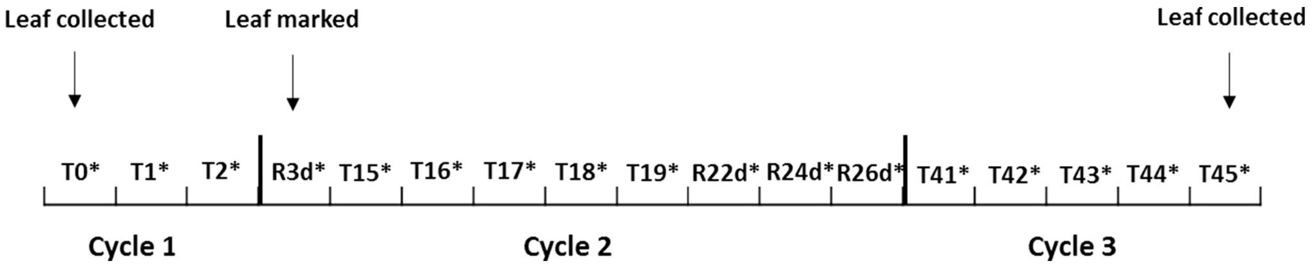


Fig. 1 Water-stress cycles. T1 and T2, T15–T19, and T41 and T42 are days with water stress, and R3d and R22d–R26d are watering days. The *asterisks* represent days with stomatal conductance and water potential measurements, and *arrows* indicate days when leaves were collected and marked for anatomical evaluations

For comparison of GABA concentration under water stress and after rehydrating, multiple comparisons were performed using Tukey's test ($p = 0.05$).

Analysis of variance models were fitted to the continuous variables associated with leaf anatomy, including the effects of clone, treatment, and the interaction between clone and treatment in the linear predictor. Multiple comparisons were made using Tukey's test ($p = 0.05$). All analyses were carried out using the statistical software R (R Core Team 2014).

Results

Plant water status

During the control treatment, the Ψ_{pd} average was −0.2 MPa, with few variations during the entire period, and ranged from −0.06 to −1.2 MPa, suggesting that water availability for control treatment was adequate for all clones during the study period. The lowest Ψ_{pd} in this treatment occurred on November 29th (T16) and 30th (T17), which were the hottest days during the entire period, with 29 and 28.7 °C as average temperatures, respectively. Measured values of Ψ_{pd} during good water supply were in agreement with the values reported by Mielke et al. (1998) for *Eucalyptus grandis* plantation in Espirito Santo, Brazil, and Dye (1996) in South Africa.

Changes in water potential are illustrated in Fig. 2. Values of Ψ_{pd} during stress treatment ranged from −0.6 to −2.7 MPa, with the minimum values occurring on day T41 when the maximum temperature reached 34 °C at noon, DPV was 3.2 kPa, and relative humidity was 41%.

During stress days, the values of Ψ_{pd} for all clones were around −1.5 MPa, with the lowest value of Ψ_{pd} occurring during days with maximum water stress for each cycle, i.e., T2 (cycle 1), T18 (cycle 2), and T44 (cycle 3). On those days, the predawn leaf water potential ranged from −2.3 to −3.6 MPa, and 1 day after rehydration, all clones recovered and increased Ψ_{pd}, to values of −0.05 to −0.6 MPa. For the midday water potential (Ψ_{md}), water-stressed plants exhibited −1.8 MPa (ranging from −0.7 to −3 MPa), and exhibited −0.4 to −2 MPa after rewatering.

In the first cycle, all clones increased Ψ_{pd} and Ψ_{md} 1 day after rehydration, matching the values of Ψ_{pd} and Ψ_{md} of the control treatment samples. The recovery of Ψ_{pd} in the second cycle also occurred 1 day after rewatering, but Ψ_{md} increased 2 days after rewatering, showing that water stress in the second cycle caused more damage to all clones, which needed 2 days to recover Ψ_{md} completely.

Responses of gas exchange to water stress

Stomatal conductance decreased with increased vapor pressure deficit during both treatments. One of the most significant environmental variables controlling gs is the *D*, which is a stomatal response that prevents excessive dehydration and hydraulic failure (Schulze et al. 1982; Mott and Parkhurst 1991; Oren et al. 1999).

Increased stomatal sensitivity to *D* is an indication of isohydric behavior, i.e., stomata limit transpiration once *D* is increased to prevent the leaf water potential from decreasing to levels that endanger the hydraulic system integrity (Gharun et al. 2015).

Moreover, the likelihood-ratio test between the different linear prediction model vs the parallel linear predictor model was significant ($\chi^2_{1.6} = 21.27, p < 0.0001$), the stomatal conductance decreased with increasing *D* for both treatments (stress and control), but the equation for relationships was different comparing both treatments (Fig. 3).

During the stress treatment, the relationship between stomatal conductance and *D* was lower than during control treatment, showing that, in this case, in addition to *D*, water restriction in the soil also contributed to stomatal closure. Plants in more drought-prone environments generally show lower minimum stomatal conductance (Christman et al. 2008).

Comparing all clones, we can define different allometric equations for relationships between gs and *D* for each clone (Table 3). For instance, drought-sensitive clones (CNB, FIB, and JAR) yielded parallel curves between stress and

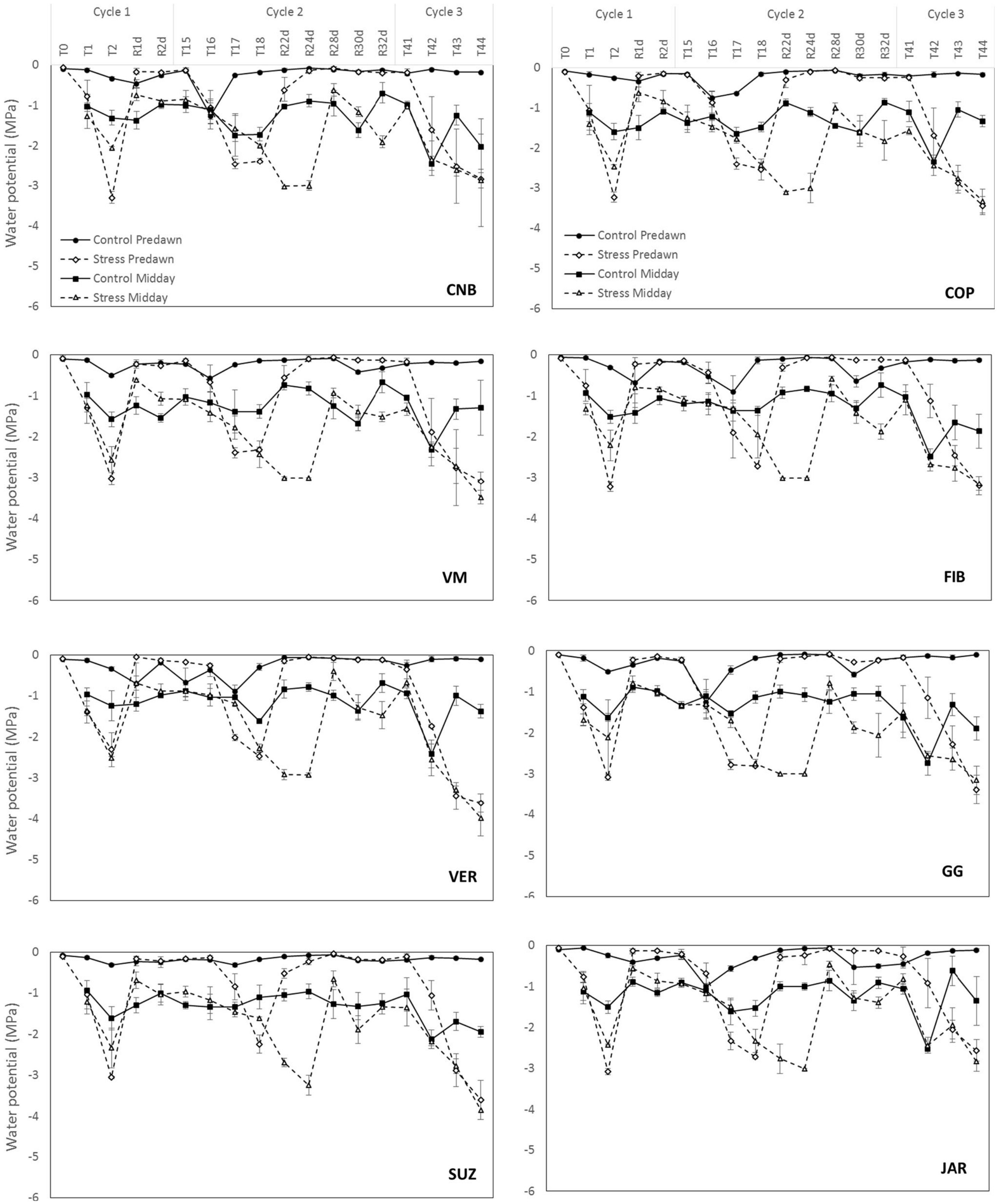


Fig. 2 Leaf water potential in eight *Eucalyptus* clones exposed to water stress during a drought period (T0–T45) with subsequent recovery after rewatering (R1d–R32d) and control treatment

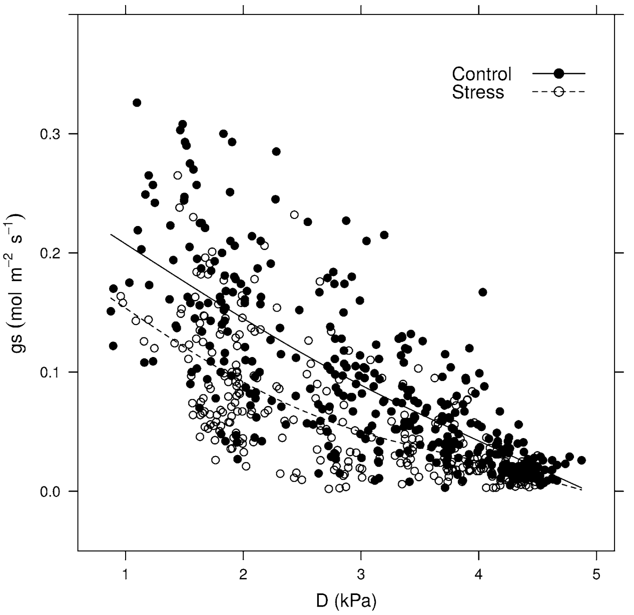


Fig. 3 Relationship between stomatal conductance (gs) and *D* in plants subjected to stress (*white circles*) and control (*black circles*) treatments

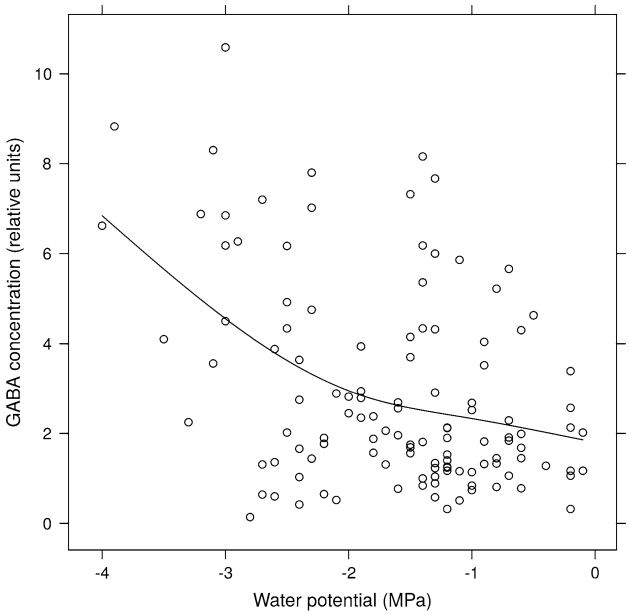


Fig. 4 Relationship between GABA concentration and midday water potential

Table 3 Likelihood-ratio tests for the generalized additive models fitted to the gs data for each *Eucalyptus* clone

Clone	Test	
	Different vs. parallel	Parallel vs. coincident
CNB	$\chi^2_{2.7} = 1.49, p = 0.6275$	$\chi^2_{1.1} = 14.03, p = 0.0002$
COP	$\chi^2_{1.5} = 14.81, p = 0.0003$	–
FIB	$\chi^2_{1.3} = 1.30, p = 0.1586$	$\chi^2_{1.1} = 17.02, p < 0.0001$
GG	$\chi^2_{3.0} = 5.47, p = 0.1427$	$\chi^2_{1.0} = 0.95, p = 0.3170$
JAR	$\chi^2_{1.2} = 2.69, p = 0.1310$	$\chi^2_{1.3} = 11.75, p = 0.0010$
SUZ	$\chi^2_{1.4} = 18.75, p < 0.0001$	–
VER	$\chi^2_{1.8} = 10.49, p = 0.0041$	–
VM	$\chi^2_{2.5} = 11.76, p = 0.0050$	–

control treatments. This means that the stomatal sensitivity to *D* was the same between treatments, but during the stress treatment, stomata were more closed than during the control treatment, a clear effect of water stress. Even though these clones revealed parallel curves, they had different behaviors when compared. For example, FIB exhibited a continuous curve in both treatments, but CNB and JAR clones exhibited curves with a lower slope after approximately 3.0 kPa of *D* in both treatments (Table 3).

Drought-tolerant clones (SUZ, VM, except GG) and plastic clones (VER and COP) exhibited concurrent curves; hence, stress and control treatments exhibited different behaviors. In the control treatment, SUZ and VM exhibited higher gs values until ~2.0 kPa, after which they exhibited a reduced curve slope. In the stress treatment, the curve slope was lower than in the control treatment and was uniform for variations of *D*. GG was the only clone that exhibited coinciding curves; therefore, this clone showed no difference in its gs and *D* relationship when comparing both treatments (Table 3).

In contrast to SUZ and VM, plastic clones (COP and VER) in the control treatment had a lower slope at the beginning of the curve and a higher slope after 2.5 kPa of *D*. In the stress treatment, stomatal conductance was lower compared with the control treatment, and the curve slope was lower and uniform for all values of *D*, similar to SUZ and VM.

GABA concentration

Water-stressed plants exhibited increased GABA concentration during days with the lowest midday leaf water potential (Fig. 4).

Clones exhibited more GABA on the second stress day (T2) of cycle 1, except COP and GG, which showed a higher concentration on the first day (T1). During cycle 2, all clones exhibited a higher concentration on the fourth stress day. During cycle 3, all clones exhibited concentrated GABA on the second stress day (T42), except JAR, which showed a lower GABA concentration (0.07) (Fig. 5).

Water stress decreased photosynthesis and water potential; in addition, GABA levels were quantitatively

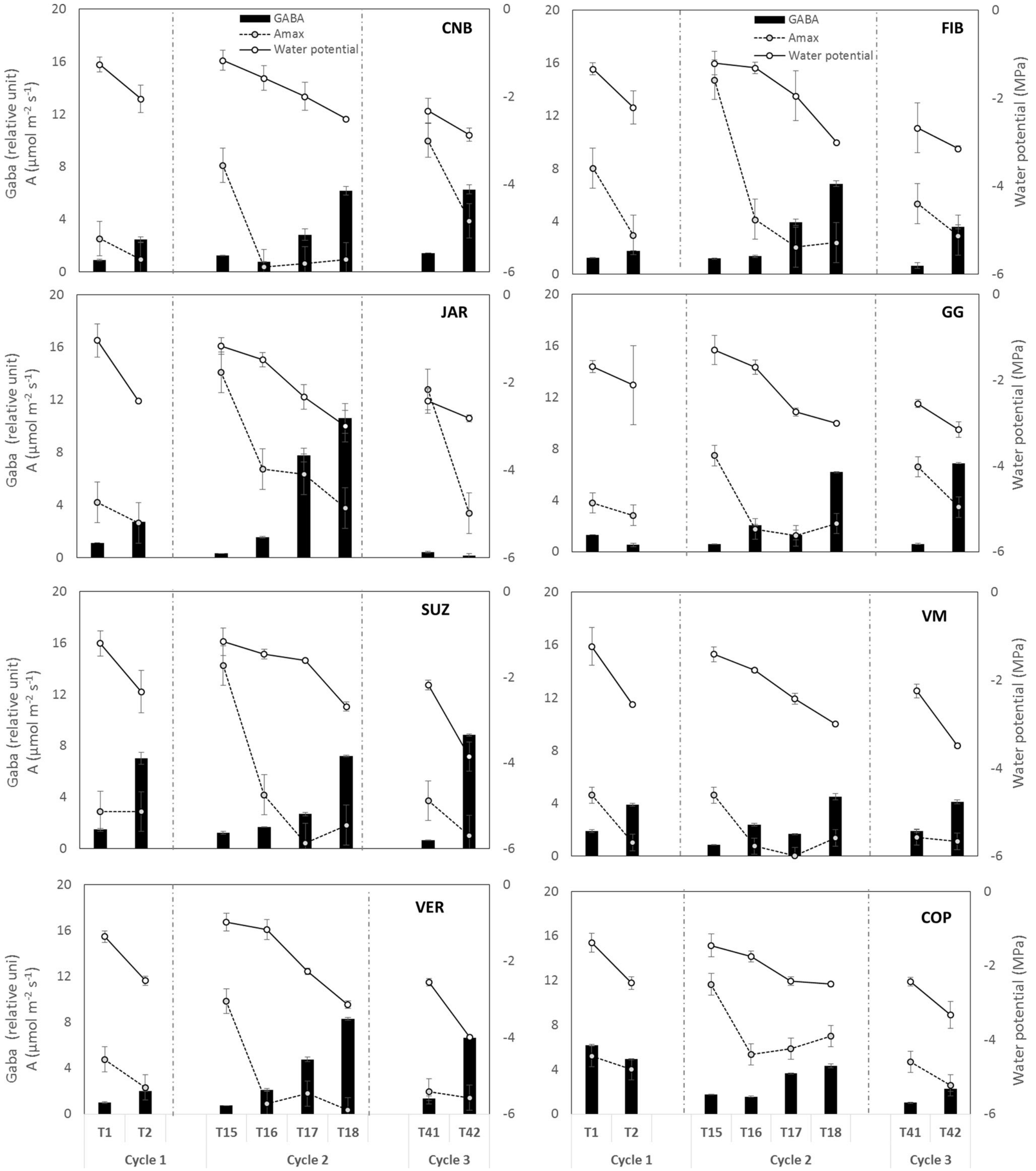


Fig. 5 Variations in water potential, photosynthesis (**a**) and the GABA relationship in three cycles of water-stressed days for eight *Eucalyptus* clones. *Error bars* indicate standard deviation for $n = 4$

significant and changed by a factor of 0.1–10.6 after water stress of *Eucalyptus* clones (Fig. 5).

There was a significant difference in the GABA concentration of all clones on water-stress days, but there was no relationship between these differences and tolerance to water stress (Table 4). This lack of correlation between GABA and tolerance to water stress does not support the hypothesis that GABA is associated with tolerance. In this

Table 4 GABA concentration of eight *Eucalyptus* clones during water-stress days and after rewatering

Clone	GABA (relative units)	
	Stress (%)	Rewatering (%)
GG	2.05aA	1.36aA
CNB	2.53aA	1.12aB
VER	2.93abA	1.66aA
COP	3.72abcA	1.18aB
JAR	4.19abcA	1.52aB
SUZ	4.39bcA	1.85aB
VM	4.94cA	2.58aB
FIB	6.37dA	0.95aB
P<	0.3305	
DMS	2.2514	
CV (%)	0.5213	

* Lowercase letters indicate differences in the column and capital letters indicate differences in the row ($p = 0.05$)

study, we determined GABA content in plant leaves; however, the clones studied exhibited different leaf quantities in their canopy and exhibited a specific dynamic leaf area index; therefore, the total amount of GABA in the crown may vary for each clone, which was not included in this work.

In all clones except GG and VER, water stress led to reversible changes in GABA levels. After rehydrating, GABA was rapidly reduced (Table 4); this occurrence was also observed by Warren et al. (2011). Shelp et al. (2012) suggested that GABA may, in turn, be used to rapidly generate succinate and energy via the tricarboxylic acid cycle upon stress removal.

Anatomical changes after water stress

There was a significant reduction of leaf thickness (LT) and an increase of abaxial stomatal density (AbD) in all clones after water stress (Table 5). All clones increased their number of stomata after water stress (Table 5; Fig. 6). The stress treatment had ~573.5 n. mm^{-2}, and the control treatment had 491.6 n. mm^{-2}. In addition, there was also an increase in the adaxial stomatal density of COP and SUZ clones with an average of 112.7 in the control treatment, compared to 159.6 n. mm^{-2} in the water-stress treatment (Table 5).

For all clones, there was no significant change in other anatomical variables after water stress. Although the GG clone exhibited a thicker abaxial and adaxial epidermis compared with the other clones—a characteristic that is considered a part of the plants' defense against desiccation (James and Bell 1995)—there was no significant difference between the stress and control treatments.

Discussion

Our investigations of the behavior of Ψ_{pd} and Ψ_{md} are an evidence that *Eucalyptus* is a species that exhibits a high resilience to water stress, since the plants recovered 1–2 days after water stress. Warren et al. (2012a, b) observed complete recovery of Ψ_{pd} 2 days after a severe water stress in two species of *Eucalyptus*. In another study comparing five species of *Eucalyptus*, recovery of Ψ_{pd} occurred 4 days after rewatering (Warren et al. 2011).

We also observed that tolerant and plastic clones exhibit different stomatal sensitivity to *D*, a signal that these clones have greater ability to adapt to water-stressed situations. The relationship between gs and *D* provides a convenient tool for describing *Eucalyptus* clone sensitivity under water-stress conditions (Whotehead and Beadle 2004; Gharun et al. 2015).

GABA levels increased by a factor of 0.1–10.6 after water stress of the *Eucalyptus* clones evaluated, confirming previous research that indicates that GABA is a fast stress-signaling molecule. Similar to our findings, in response to drought stress, GABA levels in five species of *Eucalyptus* and two of Acacia leaves increased 5–16-fold (Warren et al. 2011). High and rapid GABA accumulation was also reported in the leaves of bean (Raggi 1994), turnip (Thompson et al. 1996), sesame (Bor et al. 2009), and *Pinus* under drought stress (De Diego et al. 2013).

Studies have reported that the relationship between GABA concentration and stress is able to directly protect or promote other benefits in leaves, thereby increasing stress tolerance (Li et al. 2017). Schaberg et al. (2011) associated the increase in GABA concentration with increased tolerance to cold stress in red spruce trees (*Picea rubens*). Moreover, a study of *Arabidopsis thaliana* suggests that GABA helps plants to survive stress (Bouché and Fromm 2004), while Hatmi et al. (2015) found higher GABA concentration in a drought-resistant grapevine genotype than in a sensitive variety. However, there are studies that show that the role of GABA under stress conditions is more related to stress perception than to protection, because growth was limited under stress conditions when GABA levels increased (Bor et al. 2009).

Analysis of anatomical changes after water stress showed that there was a significant reduction of leaf thickness (LT) and an increase of abaxial stomatal density in all clones after water stress. Many abiotic stresses (such as drought and high temperatures) cause water reduction in cells and consequent reduction of leaf thickness (Bussotti et al. 2002). Chartzoulakis et al. (2002) observed an LT reduction after water stress in two avocado cultivars, which was attributed to the reduction of mesophyll cell size. Cell size is related to cell wall elasticity; in general, bulk

Table 5 Anatomical measurements in cross sections of fully expanded leaves of eight *Eucalyptus* clones under control and water-stress conditions

Clone	Abaxial epiderm thickness (10^{-2} μm)	Adaxial epiderm thickness (10^{-2} μm)	Palisade parenchyma (10^{-2} μm)	Spongy parenchyma (10^{-1} μm)	Homogeneous parenchyma (10^{-1} μm)	Leaf thickness (10^{-1} μm)	Abaxial stomatal density (10^{-1} n mm^{-2})	Adaxial stomatal density (10^{-1} n mm^{-2})
CNB	1.57ab	1.38a	6.55a	1.32ab	ND	2.27a	678.68a	ND
COP	1.47b	1.30a	ND	ND	2.04a	2.32a	413.77d	169.17a
FIB	1.63ab	1.25a	6.92a	1.33ab	ND	2.31a	560.43abcd	ND
GG	1.85a	1.45a	7.13a	1.21b	ND	2.25a	462.09cd	ND
JAR	1.43b	1.18a	6.58a	1.30ab	ND	2.22a	482.51bcd	ND
SUZ	1.45b	1.28a	ND	ND	1.91a	2.19a	443.72d	103.15b
VER	1.55ab	1.30a	6.82a	1.40ab	ND	2.36a	613.86ab	ND
VM	1.57ab	1.30a	6.28a	1.47a	ND	2.39a	605.18abc	ND
	$p = 0.0105$	$p = 0.0096$	$p = 0.3934$	$p = 0.0568$	$p = 0.1210$	$p = 0.3357$	$p < 0.0001$	$p = 0.0276$
Treatment								
Control	1.60a	1.32a	6.89a	1.37a	2.00a	2.34a	491.57b	112.71a
Stress	1.53a	1.30a	6.54a	1.30a	1.96a	2.24b	573.49a	159.61a
	$p = 0.1898$	$p = 0.5125$	$p = 0.1525$	$p = 0.1269$	$p = 0.6169$	$p = 0.0313$	$p = 0.0011$	$p = 0.0927$

Means followed by the same letter do not differ statistically ($p = 0.05$); *ND* not detected

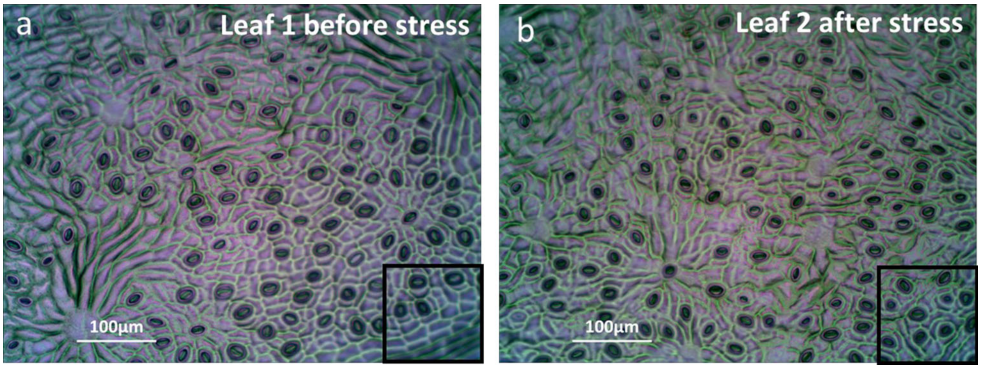


Fig. 6 Stomatal density on *Eucalyptus* leaves. Images of abaxial epidermis of leaf 1 before water stress (**a**) and leaf 2 after water stress (**b**)

modulus of elasticity increases with cell size, and thus small cells can withstand lower pressure better than large cells (Steudle et al. 1977). This is confirmed by our results, in which mesophyll thickness decreased in stressed plants, indicating a reduction in cell size. This strategy can be considered as a drought adaptation mechanism (Cutler et al. 1977; Steudle et al. 1977).

The higher number of stomata in leaves is very common in xerophytes plants; this strategy is called “get-it-while-you-can” and may increase their photosynthetic rates (Moore et al. 1998). Having stomata on both sides, like COP and SUZ, may increase the supply of carbon dioxide to the mesophyll cell area (Mott et al. 1982, Parkhurst 1994; Parkhurst and Mott 1990).

Higher stomatal density and smaller stomata size are forms of adaptation to drought, because these traits enable plants to regulate water transport and transpiration more effectively (Fahn and Cutler 1992; Dickison 2000; Ennajeh et al. 2010). In addition to stomatal density, stomatal behavior is very important in controlling different gas-exchange parameters. For instance, opening and closing stomata and the stomatal orientation on leaf surfaces may prove vital (Nejad et al. 2006).

Modification of stomatal density in response to drought varies between plant species and depends on the severity of water deficit. For example, a drought-induced reduction in stomata numbers was observed in *Eucalyptus camaldulensis* × *tereticornis* (Nautiyal et al. 1994), almonds (Camposeo et al. 2011), olives (Bosabalidis and Kofidis 2002), apples (Slack 1974; Elias 1995), and umbu trees (Silva et al. 2009). In contrast, increased stomatal density was observed in grass (Xu and Zhou 2008), Acacia (Craven et al. 2010), olives (Ennajeh et al. 2010), and *Eucalyptus* (Eksteen et al. 2013). Species that have unchanged stomatal characteristics in response to drought are reported for groundnuts (Clifford et al. 1995), grapes (Barbagallo et al. 1996), and olives (Gucci et al. 2002).

In summary, water stress affected multiple aspects of *Eucalyptus* physiology, such as water potential, stomatal control in response to water pressure deficit-*D*, and leaf anatomy. The concentration of GABA changed after drought and this occurrence allows identify the beginning and the end of critical periods of water stress. However, there was no correlation of GABA concentration and tolerance to water stress in the clones evaluated. The finding is that GABA is a fast stress-signaling molecule in *Eucalyptus*, but the response of gs to *D* is a best physiological variable to differentiate drought-tolerant and drought-sensitive *Eucalyptus* clones.

Conclusions

In conclusion, this study has shown that:

1. All clones decreased gs with increasing *D* in both treatments. All plastics and drought-tolerant clones (except GG) presented lower stomatal sensitivity to *D* under stress conditions than drought-sensitive clones.
2. GABA concentrations increased fast after drought stress, but we could not find correlation with these changes and resistance to water stress.
3. All clones increased the number of stomata and reduced leaf thickness after water stress.
4. Evaluating the response of gs to *D* is more promising than evaluating GABA concentration to differentiate drought-tolerant and drought-sensitive *Eucalyptus* clones.

Author contribution statement MSGO: literature search, study design, data collection (leaf water potential, leaf gas-exchange measurements, and stomatal anatomy), data interpretation, and writing; JGF: GABA analysis (extraction and quantification); BTG and EMM: study design, data collection (leaf water potential and leaf gas-exchange measurements); LAC and MA: study design, data collection (Stomatal anatomy), and data interpretation; RAM and CGBD: data analysis and data interpretation; and JLS and RFO: contributed to the idea of this work.

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