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Research paper

Solute accumulation and elastic modulus changes in six radiata pine breeds exposed to drought

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Drought is one of the main abiotic factors that determine forest species growth, survival and productivity. For this reason, knowledge of plant drought response and the identification of physiological traits involved in stress tolerance will be of interest to breeding programs. In this work, several *Pinus radiata* D. Don breeds from different geographical origins were evaluated along a water stress period (4 weeks) and subsequent rewatering (1 week), showing different responses among them. Leaf water potential (Ψ_{leaf}) and osmotic potential decreases were accompanied by a variation in the total relative water content (RWC, %). The most tolerant breeds presented the lowest leaf water potential and RWC at turgor loss point, and showed the lowest elastic modulus (ε) values. A high ε value was a characteristic of a less-drought-tolerant plant and was related to membrane alterations (high electrolyte leakage percentages) that could favor cell water loss. Of the group of solutes that contributed to osmotic adjustment, soluble carbohydrates were the most abundant, although stressed plants also increased their content of free amino acids [mainly proline (Pro) and glutamic acid (Glu), and γ-aminobutyric acid (GABA)] and free polyamines. In addition, the most sensitive breeds had a higher GABA/Glu ratio. After rewatering, Pro and GABA were higher in rehydrated plants than in controls.

Keywords: amino acids, drought, elastic modulus, GABA, osmotic adjustment, polyamines, proline, radiata pine, tolerance.

Introduction

In nature, many plants are adversely influenced by several environmental factors that have a negative effect on survival and development (Chen and Jiang 2010). Among these factors, drought has been associated with regional-scale forest mortality worldwide, and predicted climate change is expected to exacerbate these events (McDowell 2011). Clifford et al. (1998) summarized drought tolerance mechanisms as (i) avoidance of damaging plant water deficits; (ii) stress tolerance adaptations that enable plants to continue functioning despite plant water deficit; and (iii) efficiency strategies that enable plants to optimize the utilization of water.

The maintenance of turgor is essential for normal cell activity and survival (Bartlett et al. 2012). Cell turgor loss is a classical indicator of plant water stress, having effects on cellular integrity and whole-plant performance (McDowell 2011). Consequently, the leaf water potential at turgor loss, or bulk turgor loss point (π_{tlp}, MPa), has been used to assess plant drought tolerance (Blackman et al. 2010, Bartlett et al. 2012). Under stress conditions, plants prevent cell turgor loss by promoting water influx as a result of solute accumulation that decreases the osmotic potential (Ψ_s) (Nguyen-Queyrens and Bouchet-Lannat 2003, Chen and Jiang 2010). In this regard, plants synthesize several inorganic and organic solutes such as ions, soluble sugars, free amino acids (AAs) and free polyamines (PAs) that contribute to lowering of Ψ_s (Pérez-López et al. 2010, Silva et al. 2010). Plant species greatly differ with respect to the type of solutes accumulated and their

relative contributions to osmotic adjustment (OA); substantial differences have been reported between species and even cultivars (Bajji et al. 2001, Levy et al. 2006). In many woody species, organic solutes, specifically soluble carbohydrates and AAs, are the principal compounds involved in OA (Clifford et al. 1998, Patakas et al. 2002). Among AAs, proline (Pro) is traditionally considered the most important solute accumulated under drought (Verbruggen and Hermans 2008, Pérez-Pérez et al. 2009). However, some controversies about the role of Pro in drought have been reported, since its synthesis as a stress-induced response and its role as indicator of tolerance (Souza et al. 2004, Silva et al. 2010).

Cell wall elasticity (ε) is also considered an important defense mechanism against water stress (Saito and Terashima 2004, Hessini et al. 2009) and has a critical role in regulating plant–water relations (Saito and Terashima 2004). Indeed, more elastic walls can shrink under osmotic stress to maintain high turgor pressure (Saito and Terashima 2004, Pérez-López et al. 2010), while less elastic walls would permit decreases in leaf water potential (Ψ_{leaf}) with small hydric losses (Kramer and Boyer 1995, Navarro et al. 2007). Both strategies (increases or decreases of ε) have been considered as adaptive changes against drought stress (Martínez et al. 2007, Hessini et al. 2009). For this reason, further analysis of the role of ε is essential to clarify the mechanism that confers water stress tolerance in each species.

Preliminary studies carried out in several *Pinus radiata* D. Don breeds from different geographical and climatological growth areas, including a drought-tolerant species hybrid (*P. radiata* × *Pinus attenuata* Lemmon.), showed different drought responses and recovery capacity (De Diego et al. 2012). A better understanding of the physiological basis of these variations would allow identification and selection of valuable tolerance traits to be included in plant breeding programs. Specifically, variation in pressure potential (Ψ_p) between breeds suggested variations in turgor loss point (π_{tlp}), OA capacity and/or large differences of ε. In the present study, these traits were measured during a drought period and subsequent rewatering and the extent to which they contributed to the different drought responses of *P. radiata* breeds was evaluated. Furthermore, possible differences in the level and type of solutes which contribute to OA were also studied, to determine if they varied between breeds and the value of their potential role in drought tolerance.

Materials and methods

Plant materials and experimental design

Plant materials

Seeds from different geographical and climatological breeds were used in this work:

- O1—*Pinus radiata* var. *radiata* × *P. radiata* var. *binata*: Provided by Proseed (Amberley, North Canterbury, New Zealand) and collected from a seed orchard located in Amberley, New Zealand.
- O2—*Pinus radiata* var. *radiata*: Provided by Servicio de Material Genético of Ministerio de Medio Ambiente (Madrid, Spain) and collected from open-pollinated trees grown in the Basque coastline, Spain.
- O3—*Pinus radiata* var. *radiata*: Provided by Australian Tree Seed Centre (CSIRO Forestry and Forest Products, Clayton South, Australia) and collected from a seed orchard located in Billapaloola, Australia.
- O4—*Pinus radiata* var. *radiata* × *P. attenuate*: Provided by Proseed (Amberley, North Canterbury, New Zealand) and collected from a seed orchard located in Amberley, New Zealand.
- O5—*Pinus radiata* var. *radiata* × *P. radiata* var. *cedrosensis*: Provided by Proseed (Amberley, North Canterbury, New Zealand) and collected from a seed orchard located in Amberley, New Zealand.
- O6—*Pinus radiata* var. *radiata* (GF 17): Provided by Proseed and collected from a control-pollinated trees located in Kaingaroa, New Zealand.

Growth conditions

Seeds were subjected to cold stratification prior to sowing. They were put into a cold chamber at 4 °C in the dark for 3 weeks before being placed in sterilized water to induce germination for 2 days under the same conditions. Finally, seeds were sown in pots of 17 cm Ø with peat : perlite (7 : 3, v/v). The plants were grown in a greenhouse under controlled conditions (T^a = 23 ± 1 °C and relative humidity = 70 ± 5%) for 2 years.

Experimental design

Two-year-old saplings were analyzed during the summer time (from July to September). Ten plants per breed were used. Half were randomly selected for the water stress treatment (D) by withholding water, while the remainder were kept well watered (control plants—W). Water was withheld for 4 weeks and then plants were rewatered for a week. All measurements were performed in apical secondary needles. Ψ_{leaf} (MPa) and Ψ_s (MPa) were determined for each week of the experiment (from T0 to T4) and after rewatering (R-3d and R-7d—3 and 7 days of hydration). Solute accumulation (soluble carbohydrates, AAs and Pas) was quantified at T0, T2 and T4 and at R-7d. The ε was analyzed at T4 and R7.

Water status parameters

Water content

Total relative water content (RWC, %) was determined as

$$RWC = 100 \times (FW - DW) / (TW - DW) \quad (1)$$

where TW is the leaf weight at full turgor, measured after immersion of needles in demineralized water for 24 °C in the dark at room temperature, FW is the leaf fresh weight and DW is the leaf dry weight.

Leaf water potential

The Ψ_{leaf} (MPa) of saplings from all breeds was measured at midday (12:00–14:00) along the drought cycle (from T0 to T4) and after rewatering (R-d3 and R-d7) using a Scholander chamber (Skye SKPM 1400) and the pressure-equilibration technique (Scholander et al. 1965).

Osmotic potential and OA

The Ψ_s (MPa) was determined as described by Pérez-López et al. (2009) with minor modifications. Two needles from each breed and treatment were sampled during the experiment. The material was instantaneously frozen in liquid nitrogen and stored at –80 °C until analysis. Samples were thawed, placed in vials and centrifuged for 20 min to extract the sap. Extracts were equilibrated at 25 °C for 15 min and their osmolarity determined by freezing point osmometry using an Osmomat 030 osmometer (©Gonotec GMBH, Berlin, Germany). Ψ_s was calculated from the van't Hoff equation:

$$\Psi_s = -R \times T \times c_s \quad (2)$$

where R is the gas constant, T is the sample temperature (°K) and c_s is the solute concentration (mol kg^{-1}).

The osmotic potential at full hydration (π_o, MPa), leaf water potential at turgor loss point (π_{tlp}, MPa) and relative water content at turgor loss point (RWC_{tlp}, %) were obtained from the pressure–volume curve according to Bartlett et al. (2012). The maximum OA (MPa) of each breed was estimated as the difference in π_o between irrigated plants and non-irrigated ones at turgor loss point (Table 1).

Osmotic contribution of solutes

The estimated osmotic contribution of solutes ($\Psi_{s,osm}$, MPa) was obtained using the van't Hoff equation (Pérez-López et al. 2010):

$$\Psi_{s,osm} = -0.002479 \times RDW \times c_s \quad (3)$$

where $\Psi_{s,osm}$ indicates the contribution of individual solutes to π_o (Bartlett et al. 2012), c_s is the molar concentration of the solute (mol kg^{-1}), 0.002479 m^3 MPa mol^{-1} is the RT value at 25 °C (solutes are assumed to have ideal behavior; Alarcón et al. 1993), and RDW is the relative DW at saturation, determined using the following equation:

$$RDW = DW / (TW - DW) \quad (4)$$

Table 1. Total relative water content (RWC_{tlp}, %) and osmotic potential at full turgor (π_o, MPa), and osmotic adjustment ($\Delta\pi_o$, MPa) of six *P. radiata* breeds (O1–6) according to pressure–volume curve analysis.

Breed	RWC_{tlp}	π_o	$\Delta\pi_o$
O1	69.59	–1.41	–1.52
O2	63.40	–1.44	–1.84
O3	73.50	–1.30	–1.19
O4	65.94	–1.37	–2.11
O5	63.98	–1.56	–1.86
O6	70.94	–1.44	–1.33

Elastic modulus

The ε (MPa) was calculated from total RWC (Eq. (1)) as described by Bartlett et al.(2012):

$$\varepsilon = \Delta\Psi_p / \Delta RWC \quad (5)$$

where $\Delta\Psi_p$ is the difference of pressure potential (Ψ_p).

The Ψ_p (MPa) was estimated at the same time points as Ψ_{leaf} and was calculated from the following mathematical equation:

$$\Psi_{leaf} = \Psi_p + \Psi_s \quad (6)$$

Solute quantification

Free amino acid and polyamine quantification

Extraction

Free amino acids and PAs were analyzed on two apical needles per sapling every 2 weeks collected at T0, T2 and T4 and at R-d7. Needles were immediately frozen in liquid nitrogen. Samples were maintained at –80 °C until extraction. Free AAs; l-isoleucine (Ile), l-leucine (Leu), l-lysine (Lys), l-methionine (Met), l-phenylalanine (Phe), l-threonine (Thr), l-tryptophan (Trp), l-valine (Val) and l-histidine (His), l-aspartic acid (Asp), l-glutamic acid (Glu), l-asparagine (Asn), l-serine (Ser), l-glutamine (Gln), l-glycine (Gly), l-arginine (Arg), l-alanine (Ala), γ-aminobutyric acid (GABA), l-tyrosine (Tyr), l-proline (Pro), and l-hydroxyproline (OH-Pro) and free PAs; histamine (HA), ethylamine (EA), methylamine (MA), tryptamine (Tryp), β-phenylethylamine (PEA), putrescine (Put), cadaverine (Cad), spermidine (Spd), tiramine (TA) and spermine (Spm) were extracted according to the method described by Calanni et al. (1999). Plant material was pooled and homogenized in liquid nitrogen. Each pooled sample (0.10 g of FW) was placed in a 2 ml vial, and dropped in 1 ml of extraction mixture of ethanol/water (80/20, v/v). Extracts were centrifuged at 2000 *g* for 10 min at 4 °C. Pellets were re-extracted for 10 min in additional 1 ml of the same extraction solution. Supernatants were collected and evaporated to dryness by a stream of compressed air. The pellet was dissolved in 1 ml mobile phase at

initial conditions. Samples were filtered through 13 mm diameter nylon membrane Millex filters (Ø 0.22 μm; ©Millipore, Bedford, MA, USA).

Quantification

Analyses were carried out with an HPLC Model 1100 Agilent (Palo Alto, CA, USA) connected to a fluorescence detector. Free AA and PA derivatization occurred in the loop, mixing 1 μl of borate buffer (pH 10), 2.5 μl of each standard or sample previously filtered, 0.5 μl of *o*-phthaldehyde-2-mercaptoethanol, 0.5 μl of 9-fluorenylmethyl chloroformate and 32 μl of filtered Milli-Q® water. Eight microliters of each mixture was injected onto a GEMINI (NX) C18 column (5 μm, 150 × 0.5 mm, Phenomenex®, Inc., Torrance, CA, USA) with a guard column ZORBAX Eclipse AAA-Pack (Analytical Guard Column 5 μm, 4.6 × 12.5 mm, ©Agilent Technologies, Inc., Palo Alto, CA, USA) installed in an oven Gecko 2000 (Essex, UK) at 40 °C, and eluted at a flow rate of 1.5 ml min^{-1}. Mobile phase A [ammonium formate (20 mM, pH 7.8)] and mobile phase B [acetonitrile/methanol/water (45 : 45 : 10, v/v/v)] were used for the chromatographic separation. The elution consisted of a 42 min linear gradient from 10 to 57% B, followed by another 8 min linear gradient from 57 to 90% B, and finally a 5 min linear gradient from 90 to 100% B. The flow was continuous at a rate of 1.5 ml min^{-1} for 52 min, another continuous flow rate of 0.8 ml min^{-1} for 0.5 min, and a last continuous flow rate of 1.5 ml min^{-1} for 2.5 min. The column was equilibrated with the starting composition of the mobile phase for almost 15 min before each analysis. The fluorescent detector operated at excitation wavelength of 220 nm, and emission wavelengths of 350 nm and 440 nm. Pro and OH-Pro were detected at 350 nm, and the remaining free AAs and free PAs at 440 nm. Standards of known concentrations of each component (free AAs: 10, 25, 50, 100 and 200 mg l^{-1}; free PAs: 5, 12.5, 25 and 50 mg l^{-1}) were also examined under the same conditions. Spectra were obtained using the DataAnalysis program for HPLC-FD (Agilent Technologies, Inc., Palo Alto, CA, USA). Recoveries were determined using internal standards on each emission wavelength (l-sarcosine at 350 nm and α-aminoadipic acid at 440 nm). Recoveries ranged between 85 and 90%. Three biological replicas were quantified per sample.

Soluble carbohydrate quantification

Carbohydrate analysis was performed in the same extract obtained as described for the free PA and AA quantification. Sucrose, d-glucose and d-fructose content was analyzed using the enzymatic Kit extraction ©Boehringer Mannhein/R-Biopharm (Roche Molecular Biochemicals, Germany).

Statistical analysis

Analysis of variance (ANOVA) was carried out by *proc glm* in the SAS® software package (SAS Institute, Inc., Cary, NC, USA). The Ψ_s was analyzed in five plants per breed and treatment every weeks (Ti); each week (from T0 to T4) and three (R-d3) and seven (R-d7) days after rewatering, according to the following mathematical model:

$$y_{ijkr} = \mu + O_i + T_j + \mathrm{Ti}_k + \mathrm{OT}_{ij} + \mathrm{OTi}_{ik} + \mathrm{TTi}_{jk} + \mathrm{OTTi}_{ijk} + e_{ijkr} \quad (7)$$

where y_{ijkr} is the response variable result of the *r*th plant of the *i*th breed (O1–O6) subjected to *j*th treatment [irrigated (W) or non-irrigated plants (D)] at *k*th time (from T0 to T4 and R7); μ is the experimental mean, O_i the effect of the *i*th breed, T_j the effect of the *j*th treatment, Ti_k the effect of the *k*th time; OT_{ij} is the interaction between the *i*th breed and the *j*th treatment, OTi_{ik} between the *i*th breed and the *k*th time, TTi_{jk} between the *j*th treatment and the *k*th time, OTTi_{ijk} the triple interaction among the *i*th breed, the *j*th treatment and the *k*th time, e_{ijkr} is the random error component.

The changes in ε per breed were measured during drought cycle and after rewatering and were analyzed according to the following mathematical model:

$$y_{ijr} = \mu + O_i + \mathrm{Ti}_j + \mathrm{OTi}_{ij} + e_{ijr} \quad (8)$$

where, y_{ijr} is the response variable result of the *r*th plant of the *i*th breed subjected at *j*th time; μ is the experimental mean, O_i the effect of the *i*th breed, Ti_j the effect of the *j*th time; OTi_{ij} the interaction between the *i*th breed and the *j*th time; e_{ijr} is the random error component.

Multiple comparisons were calculated using the post hoc Tukey's honestly significant difference (HSD) test for balanced data and Tukey–Kramer for unbalanced data. To analyze possible correlations among physiological parameters and their significance *proc reg* was used, and analysis of covariance was carried out by *glm proc* in the SAS® software.

Results

We first considered π_{tlp} and what traits were mainly responsible for its variation in analyzed breeds. Thus, we analyzed the pressure–volume curve of each breed by the evaluation of RWC changes against Ψ_{leaf} and Ψ_s (Figure 1). Needles from O4 and O5 presented the lowest π_{tlp} with values of –2.29 and –2.19 MPa, respectively (Figure 1). On the contrary, O3 and O6 had the less negative values of π_{tlp} (Figure 1). Regarding RWC_{tlp}, O2 and O5 showed the lowest values (63.40 and 63.98%, respectively), and O5 also had the most negative values of π_o (–1.56 MPa) (Table 1). In addition, O4 and O5 presented higher OA capacity, with values of –2.11 and –1.86 MPa, respectively (Table 1).

The Ψ_s variations (Figure 2) were due to the triple interaction among time, treatment and breed according to ANOVA ($P < 0.05$; see Table 1 available as Supplementary Data at *Tree*

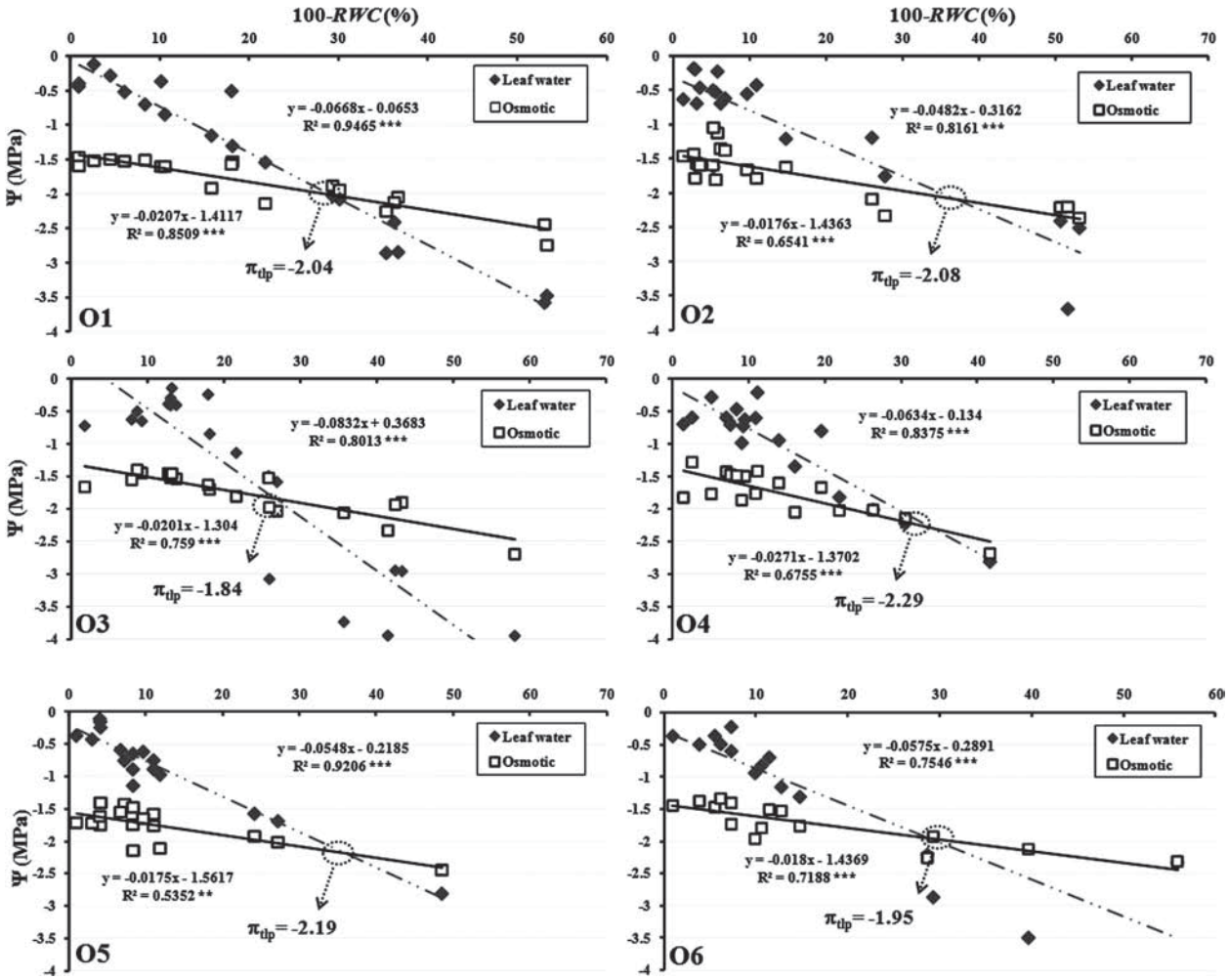

Figure 1. Pressure–volume curve of six *P. radiata* breeds (O1–O6). Leaf water potential (leaf water, MPa) and osmotic potential (osmotic, MPa) *vs* 100-total relative water content (100-RWC, %). Ψ is the potential and π_{tlp} is the water potential at turgor loss point.

Physiology Online). Stressed plants showed significant differences of Ψ_s compared with each control after 3 weeks under drought, except in O3 which did not show statistical differences until T4 (Figure 2). At this time, O1 showed the lowest Ψ_s value (–2.66 MPa), which was statistically different to the remaining breeds. All stressed plants recovered their Ψ_s control values after 3 days of rewatering (R-3d) (Figure 2).

Drought also induced variations in ε and its changes depended on time and breed according to ANOVA, but not on their interaction (Table S1 available as Supplementary Data at *Tree Physiology* Online). At T4, all stressed plants significantly increased their ε over 1 MPa compared with each control, recovering to the initial levels at R-d7 (Figure 3a). Among breeds, O6 showed the highest ε values at the end of the drought cycle, with non-significant differences with respect to O1, O2 and O3 (Figure 3b). In contrast, O4 and O5 had the lowest ε (3.8 and 2.2 MPa; Figure 3b).

The relationship between ε and Ψ_{leaf} was significant for all saplings except for O1 (Figure 4). In this regard, all breeds increased their ε values in line with Ψ_{leaf} decrease (Figure 4). O3 and O6 showed the greatest curve slopes (from 2.8 to 3.0), whereas O4 and O5 had the smallest ones (from 1.0 to 1.6). On this account, when stressed plants from O3 and O6 reached their π_{tlp}, they presented ε near to 5.8 MPa (Figure 4b), whereas O4 and O5 had ε of 3.9 MPa at their turgor loss point (Figure 4c).

The elastic modulus observed at turgor loss point (ε_{tlp}) of each breed (Figure 4) was correlated with π_{tlp} ($R^2 = 0.63$; $P < 0.1$; Figure 5a). Those breeds with the lowest ε_{tlp}, also showed the most negative π_{tlp} (O4 and O5; Figure 5a). In addition, these breeds also had the smallest RWC_{tlp} values (Figure 5b). In contrast, O3 and O6 presented the highest ε_{tlp} and RWC_{tlp}.

The degree of cell membrane injury induced by water stress may be easily estimated through measurements of electrolyte leakage [EL (%)] from the cells. This technique permits one to quantify damages to cell membranes. For this reason, to evaluate if the increase of ε was related to leaf membrane alterations induced by drought, EL (data re-plotted from De Diego et al.

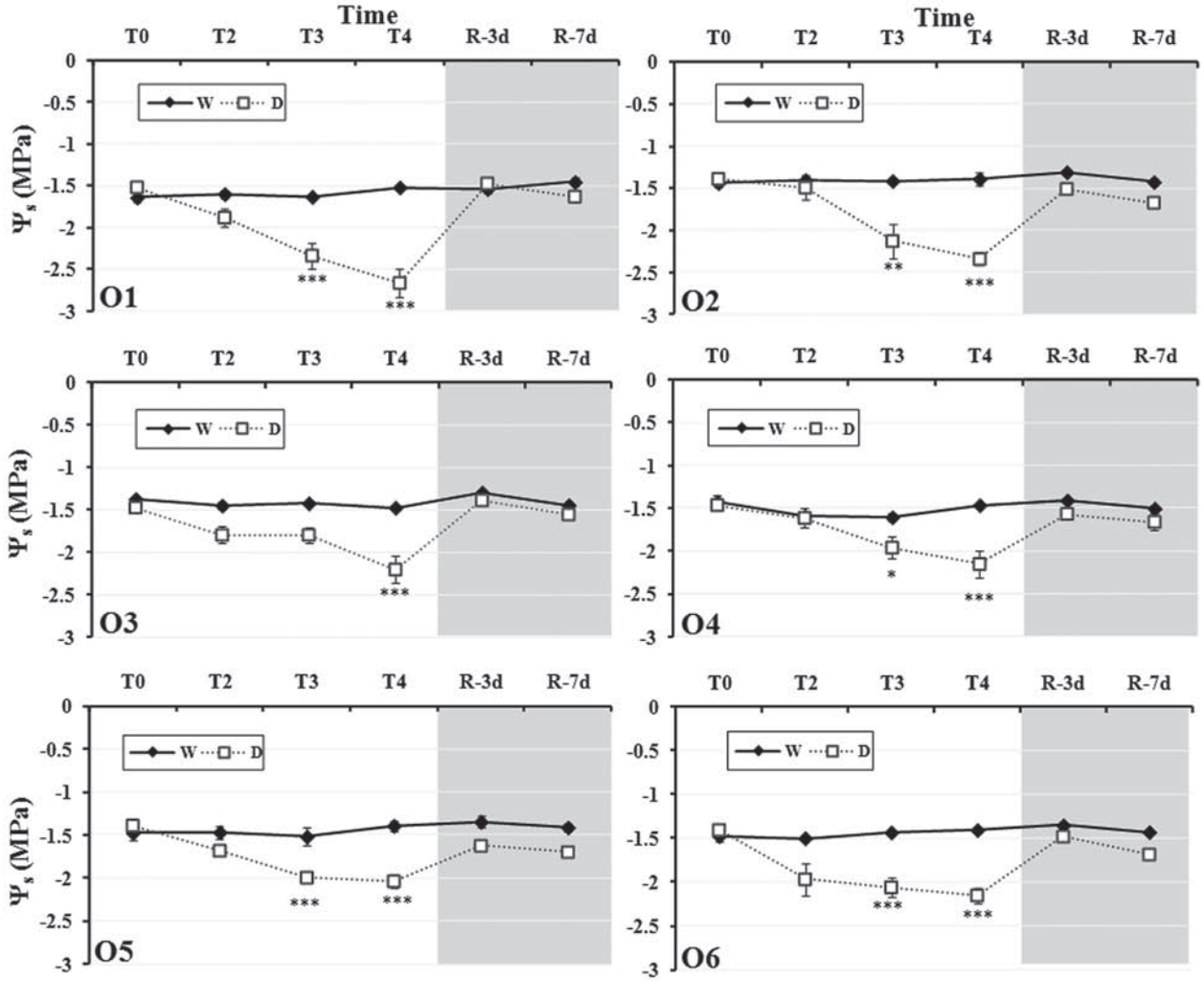

Figure 2. Osmotic potential (Ψ_s, MPa) in six *P. radiata* breeds (O1–O6) exposed to irrigation (W—closed symbols) or no irrigation conditions (D—open symbols) along a drought period of 4 weeks (from T0 to T4) and subsequent recovery after rewatering for a week (shaded area; R-d3 and R-d7 indicate 3 and 7 days of rewatering). M ± SE. Significant differences with regard to each control are represented by asterisks according to Tukey's HSD test after ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

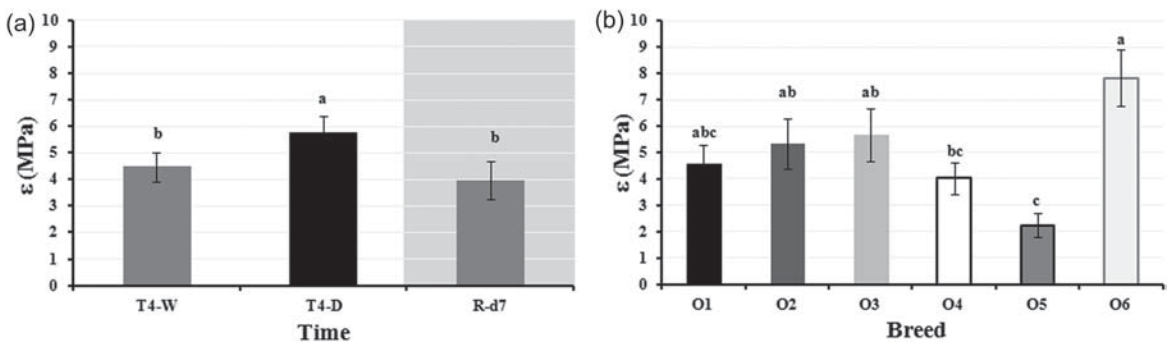

Figure 3. Elastic modulus (ε, MPa) in irrigated (W) and non-irrigated (D) saplings of six *P. radiata* breeds (O1–O6) after 4 weeks under drought conditions (T4) and after rewatering for a week (R-d7 shaded area) (a). ε values of each breed after 4 weeks of drought (b). M ± SE. Different letters mean significant differences according to Tukey's HSD test after ANOVA.

2012) was related to ε_{tlp} (Figure 5c). A strong relationship was found between them and, again, O3 and O6 had the highest EL percentages and ε_{tlp} values and O4 and O5 the lowest ones.

Solute contribution

To determine the relative contribution of organic solutes to OA, some soluble carbohydrates, AAs and PAs were analyzed

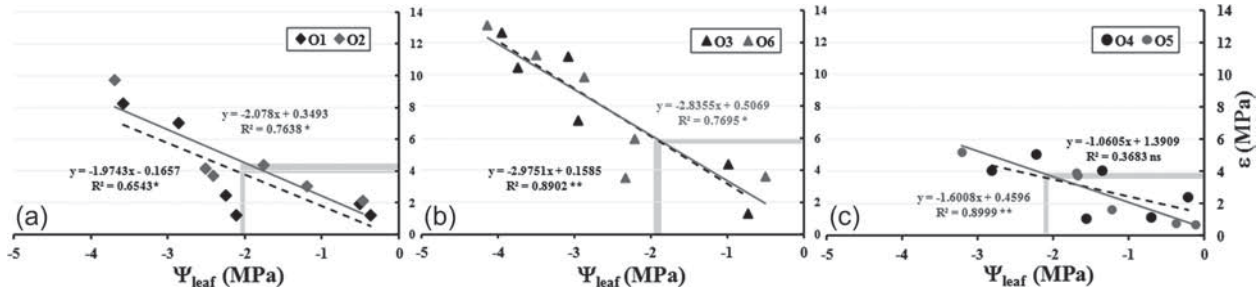

Figure 4. Leaf water potential (Ψ_{leaf}, MPa) vs elastic modulus (ε, MPa) in six *P. radiata* breeds (O1–O6) subjected to drought. Gray line indicates the turgor loss point of each breed. *$P < 0.05$; **$P < 0.01$.

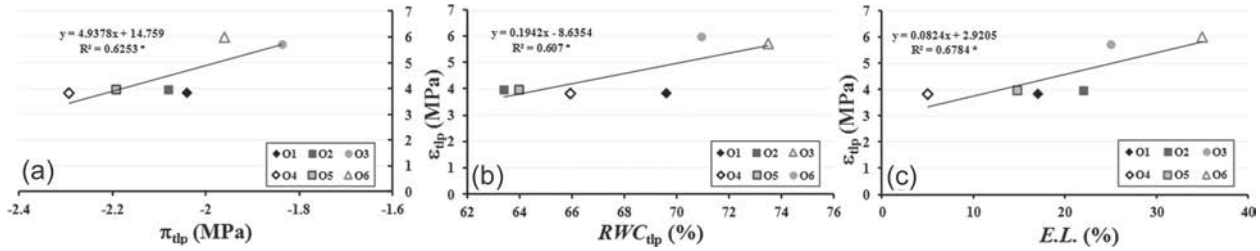

Figure 5. Elastic modulus at turgor loss point (ε_{tlp}, MPa) vs leaf water potential at turgor loss point (π_{tlp}, MPa) in six *P. radiata* breeds (O1–6) (a). ε_{tlp} vs total relative water content at turgor loss point (RWC_{tlp}, %) (b). ε_{tlp} vs electrolyte leakage [EL (%), data partially re-plotted from De Diego et al. (2012)]. •$P < 0.1$; *$P < 0.05$.

(Tables 2 and 3). Soluble carbohydrates were the principal solutes which contributed to OA, varying among breeds from 6.17% (O1) to 21.97% (O4), whereas free AAs and PAs contributed less to OA, with percentages below 3.43 and 0.54%, respectively (Table 3). However, stressed plants increased the levels of free AAs to a greater extent than soluble sugars or free PAs.

Sucrose was the most abundant soluble sugar in all breeds and its levels increased during drought, mainly in O1 (Tables 2 and 3). d-Glucose and d-fructose presented the highest increases in all stressed plants, especially d-glucose in O2 and O3 and d-fructose in O6 (Table 3).

Regarding free AAs, stressed O6 showed the highest increase in osmotic contribution with a value of 3.43% (Table 3). The highest increases after 4 weeks of drought were found in Arg, Asn, Gln, Glu, Gly, Ser, Trp and especially in Pro and GABA (Tables 2 and 3) with increases >800-fold or 400-fold compared with irrigated plants of some of the more susceptible breeds (O3 and O6). Only stressed plants from O5 increased significantly the levels of PAs (Tables 2 and 3), mainly Spd and Spm.

Finally, when the relationship between GABA and Glu was evaluated, breeds varied their GABA/Glu ratio (Figure 6). In this respect, O4 and O5 breeds showed the lowest values.

At R-d7, concentrations of carbohydrates and AAs did not return to basal levels (Figure 7). Whereas O3 plants had the higher ratio of soluble carbohydrates, O1 presented the highest accumulation of total free AAs (Figure 7).

Discussion

Drought induced an osmotic response in *P. radiata* breeds from various geographic areas, with variations in needle Ψ_s and Ψ_{leaf} to regulate water loss (Figure 1). These variations implicate different π_{tlp}, π_o and RWC_{tlp} for each breed, O5 and O4 showing the lowest values (Figure 1 and Table 1). Most existing literature about pressure–volume curve traits deals with how plants respond to water stress (Aranda et al. 1996, Lenz et al. 2006). In this regard, it has been observed that the π_{tlp} is recognized as the highest-level trait that quantifies leaf and plant drought tolerance most directly, because a more negative π_{tlp} extends the range of Ψ_{leaf} at which the leaf remains turgid and maintains function (Lenz et al. 2006, Bartlett et al. 2012). On this account, whereas the most tolerant breeds, O4 and O5 (De Diego et al. 2012), reached their turgor loss point (π_{tlp}), at –2.29 and –2.19 MPa, O3 and O6 (the most sensitive ones) showed their π_{tlp} in values of –1.84 and –1.95 MPa, respectively (Table 1). RWC_{tlp} also varied among breeds, and again O4 and O5 showed the lowest values. These results pointed out that RWC_{tlp} and mainly π_{tlp} are more reliable indicators of drought tolerance in *P. radiata* than π_o. In this sense, Bartlett et al. (2012) observed that π_{tlp} and π_o were the best indicators of species drought tolerance whereas RWC_{tlp} did not differ between species, perhaps due to a variable intraspecific response.

Elastic modulus, ε, increased after drought and recovered the initial values after rewatering (Figure 3a). During water

Table 2. Soluble carbohydrate (mg g^{-1} FW), free AAs and free PAs (μg g^{-1} FW) in six *P. radiata* breeds (O1–O6) under irrigated and non-irrigated conditions for 4 weeks.

Solutes	Breeds											
	O1		O2		O3		O4		O5		O6	
	W	D	W	D	W	D	W	D	W	D	W	D
Carbohydrates (mg g^{-1} FW)	**3.30 ± 0.08**	**5.30 ± 0.02**	**2.88 ± 0.07**	**6.44 ± 0.28**	**3.98 ± 0.39**	**4.96 ± 0.75**	**14.83 ± 0.36**	**9.46 ± 1.30**	**2.09 ± 0.08**	**3.48 ± 0.44**	**2.78 ± 0.16**	**4.44 ± 0.17**
Sucrose	1.25 ± 0.09	2.86 ± 0.21	2.17 ± 0.02	2.76 ± 0.64	3.36 ± 0.73	3.68 ± 0.87	8.37 ± 1.11	5.92 ± 1.24	1.12 ± 0.08	1.28 ± 0.13	1.92 ± 0.01	0.84 ± 0.18
D-Glucose	1.01 ± 0.06	1.18 ± 0.02	**0.51 ± 0.15**	**2.69 ± 0.90**	**0.15 ± 0.00**	**0.72 ± 0.05**	5.09 ± 0.57	1.99 ± 0.05	0.57 ± 0.02	1.35 ± 0.08	**0.58 ± 0.10**	**1.84 ± 0.06**
D-Fructose	1.04 ± 0.05	1.26 ± 0.17	**0.20 ± 0.06**	**1.00 ± 0.03**	**0.14 ± 0.08**	**0.57 ± 0.07**	3.38 ± 0.47	1.55 ± 0.11	0.40 ± 0.02	0.85 ± 0.23	**0.28 ± 0.06**	**1.76 ± 0.07**
Free AAs (μg g^{-1} FW)	**198.4 ± 22.0**	**1430.0 ± 55.2**	**314.1 ± 14.5**	**1101.4 ± 5.63**	**182.3 ± 7.19**	**950.8 ± 36.1**	**335.1 ± 15.3**	**662.4 ± 12.9**	**232.9 ± 17.3**	**679.2 ± 27.1**	**289.7 ± 67.3**	**1826.2 ± 69.5**
Asp	8.25 ± 1.04	4.80 ± 0.18	23.40 ± 0.52	8.24 ± 0.02	6.01 ± 0.22	6.70 ± 0.15	7.02 ± 0.08	20.15 ± 0.80	9.60 ± 0.03	<0.01	10.20 ± 0.49	11.73 ± 1.08
Glu	**48.35 ± 0.84**	**138.2 ± 0.08**	**96.39 ± 1.27**	**218.8 ± 0.69**	**36.46 ± 1.75**	**142.8 ± 1.25**	**86.08 ± 1.38**	**95.65 ± 0.15**	**55.89 ± 1.11**	**75.26 ± 0.62**	**68.10 ± 0.71**	**198.5 ± 0.88**
Asn	<0.01	8.61 ± 0.07	<0.01	17.46 ± 0.01	<0.01	12.86 ± 0.12	<0.01	8.55 ± 0.04	<0.01	<0.01	<0.01	36.88 ± 0.99
Ser	2.18 ± 0.11	33.86 ± 0.60	8.39 ± 0.09	35.66 ± 0.01	1.80 ± 0.66	31.56 ± 0.24	14.38 ± 0.76	21.51 ± 1.37	6.17 ± 0.55	13.51 ± 0.10	6.92 ± 0.21	48.90 ± 0.08
Gln + His	22.12 ± 1.82	205.9 ± 4.95	59.20 ± 0.32	245.48 ± 0.52	31.06 ± 2.19	153.4 ± 3.74	72.43 ± 3.46	138.4 ± 2.67	37.68 ± 1.97	330.6 ± 2.75	69.09 ± 3.25	592.5 ± 39.92
Gly	<0.01	12.52 ± 0.38	<0.01	4.67 ± 0.09	<0.01	2.53 ± 0.14	6.49 ± 0.67	<0.01	<0.01	0.14 ± 0.09	<0.01	4.13 ± 0.20
Thr	<0.01	6.32 ± 0.14	<0.01	5.71 ± 0.01	<0.01	3.18 ± 0.08	<0.01	1.92 ± 0.12	<0.01	<0.01	<0.01	<0.01
Arg	0.15 ± 0.10	84.94 ± 0.52	6.62 ± 0.17	123.7 ± 0.10	8.46 ± 0.66	76.28 ± 1.40	19.31 ± 0.79	50.75 ± 0.59	3.28 ± 0.41	78.27 ± 0.32	8.35 ± 4.04	122.7 ± 3.23
Ala	0.16 ± 0.10	182.6 ± 8.22	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	29.00 ± 6.48	76.25 ± 37.10	14.38 ± 14.38
GABA	**0.17 ± 0.16**	**78.19 ± 1.15**	**< 0.01**	**133.51 ± 0.95**	**< 0.01**	**70.98 ± 3.32**	**< 0.01**	**6.06 ± 1.18**	**< 0.01**	**18.57 ± 0.98**	**< 0.01**	**130.9 ± 1.24**
Tyr	<0.01	<0.01	<0.01	<0.01	<0.01	16.55 ± 0.35	<0.01	0.76 ± 0.06	<0.01	<0.01	<0.01	48.49 ± 0.37
OH-Pro	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Val + Met	5.64 ± 0.74	0.62 ± 0.06	20.51 ± 1.66	6.31 ± 0.02	8.80 ± 0.76	11.15 ± 0.47	30.55 ± 0.93	6.45 ± 0.57	8.01 ± 0.69	8.01 ± 0.28	8.87 ± 0.04	29.00 ± 0.69
Trp	<0.01	60.56 ± 1.79	0.80 ± 0.42	42.69 ± 0.15	<0.01	38.61 ± 1.24	<0.01	17.86 ± 0.87	3.43 ± 0.67	38.61 ± 8.84	8.85 ± 0.31	44.16 ± 0.68
Phe	19.26 ± 0.00	19.26 ± 0.01	19.26 ± 0.01	19.26 ± 0.01	19.26 ± 0.01	17.94 ± 1.32	19.26 ± 0.01	19.26 ± 0.01	19.26 ± 0.01	24.19 ± 0.00	19.26 ± 0.00	9.36 ± 0.00
Pro	**11.35 ± 3.37**	**538.3 ± 2.11**	**6.54 ± 5.14**	**174.9 ± 0.34**	**0.44 ± 0.44**	**299.6 ± 21.10**	**4.98 ± 0.37**	**205.3 ± 3.62**	**5.37 ± 1.97**	**34.36 ± 2.23**	**2.61 ± 2.61**	**442.3 ± 3.43**
Ile	<0.01	0.02 ± 0.00	<0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	<0.01	<0.01	<0.01
MA + Leu	12.63 ± 6.62	4.33 ± 4.33	8.04 ± 4.81	4.43 ± 2.21	<0.01	0.12 ± 0.12	6.48 ± 6.48	<0.01	12.51 ± 9.14	24.31 ± 4.35	11.21 ± 8.20	10.37 ± 1.30
Lys	68.15 ± 1.02	51.06 ± 0.63	64.97 ± 0.04	60.57 ± 0.50	70.00 ± 0.50	66.54 ± 1.21	68.09 ± 0.34	69.81 ± 0.83	71.70 ± 0.72	4.34 ± 0.12	<0.01	81.86 ± 1.05
Free PAs (μg g^{-1} FW)	**65.26 ± 0.28**	**65.16 ± 1.95**	**38.81 ± 1.01**	**43.35 ± 0.83**	**41.47 ± 3.58**	**38.37 ± 0.48**	**56.37 ± 2.57**	**54.23 ± 0.50**	**36.48 ± 1.26**	**217.6 ± 1.73**	**122.4 ± 2.20**	**124.1 ± 8.15**
EA	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	51.34 ± 1.24	75.47 ± 1.75	3.97 ± 0.05
Tryp	19.31 ± 0.00	19.31 ± 0.01	18.40 ± 0.52	19.31 ± 0.01	19.31 ± 0.01	19.31 ± 0.01	19.31 ± 0.01	19.31 ± 0.02	19.31 ± 0.02	5.18 ± 0.00	19.31 ± 0.02	5.18 ± 0.00
PEA	22.84 ± 0.19	<0.01	<0.01	<0.01	<0.01	<0.01	20.68 ± 0.40	19.81 ± 0.26	<0.01	17.92 ± 0.01	<0.01	36.07 ± 0.58
Put	**5.74 ± 0.08**	**2.92 ± 0.01**	**4.24 ± 0.21**	**3.35 ± 0.09**	**8.98 ± 0.05**	**7.09 ± 0.03**	**5.54 ± 0.10**	**6.35 ± 0.24**	**6.47 ± 0.30**	**14.70 ± 0.10**	**6.21 ± 0.04**	**14.02 ± 0.08**
Cad	8.77 ± 0.00	8.77 ± 0.01	8.77 ± 0.01	8.51 ± 0.25	8.77 ± 0.01	8.77 ± 0.01	8.77 ± 0.01	8.77 ± 0.02	8.77 ± 0.02	11.41 ± 0.00	8.77 ± 0.02	11.41 ± 0.00
Spd + TA	<0.01	<0.01	<0.01	0.08 ± 0.00	2.25 ± 2.25	<0.01	2.08 ± 1.00	<0.01	<0.01	**69.68 ± 0.33**	6.43 ± 0.03	33.45 ±3.26
HA	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	1.16 ± 0.07
Spm	**8.60 ± 0.00**	**34.16 ± 7.41**	**7.41 ± 0.28**	**12.10 ± 0.40**	**2.16 ± 1.27**	**3.20 ± 0.46**	**< 0.01**	**< 0.01**	**1.93 ± 0.96**	**47.41 ± 0.06**	**6.25 ± 0.39**	**11.40 ± 3.89**

The most important values are in bold.

Table 3. Solute content (D–water stressed/W–well watered plants) and each contribution to π_o (%) in six *P. radiata* breeds (O1–6) subjected to drought conditions for 4 weeks.

Solute	Breeds											
	O1		O2		O3		O4		O5		O6	
	D/W	%	D/W	%	D/W	%	D/W	%	D/W	%	D/W	%
Carbohydrates (mg g^{-1} FW)	1.61	6.17	2.24	8.52	1.36	7.09	**0.65**	**21.97**	**1.67**	**8.65**	1.60	8.30
Sucrose	2.34	3.33	1.26	3.65	1.08	5.25	0.88	**13.76**	1.14	**3.18**	0.44	1.57
D-Fructose	1.20	1.47	6.69	1.32	2.50	0.81	0.47	3.59	2.20	2.12	**7.15**	**3.31**
D-Glucose	1.18	1.38	5.18	3.55	4.87	1.02	0.39	4.61	2.36	3.35	**3.40**	**3.45**
Free AAs (mg g^{-1} FW)	7.22	1.66	3.50	1.46	5.21	1.36	1.98	1.54	2.91	1.69	**6.30**	**3.43**
Arg	73.86	0.10	18.69	0.16	9.02	0.11	2.63	0.12	23.86	0.19	**14.70**	**0.23**
Asn	8.61	0.01	17.45	0.02	12.86	0.02	2.09	0.02	1.00	0.00	**36.87**	**0.07**
Glu	2.86	0.16	2.27	0.29	3.94	0.20	1.12	0.22	1.35	0.19	**2.92**	**0.37**
Gln + His	9.31	0.08	4.14	0.14	4.94	0.10	0.52	0.05	8.80	0.82	8.58	0.66
Gly	12.50	0.01	4.67	0.01	2.53	0.00	6.49	0.01	1.14	0.00	4.12	0.01
Ser	15.55	0.04	4.25	0.05	17.52	0.05	0.66	0.03	2.18	0.03	**7.06**	**0.09**
GABA	**468.40**	**0.09**	133.50	0.02	**71.00**	**0.10**	6.10	0.01	18.60	0.05	**130.90**	**0.09**
Pro	**47.50**	**0.63**	26.70	0.23	868.90	0.43	41.40	0.48	6.40	0.08	**169.20**	**0.83**
Free PAs (mg g^{-1} FW)	1.00	0.07	1.12	0.06	0.93	0.05	0.96	0.13	**5.97**	**0.54**	1.01	0.23

The most important values are in bold.

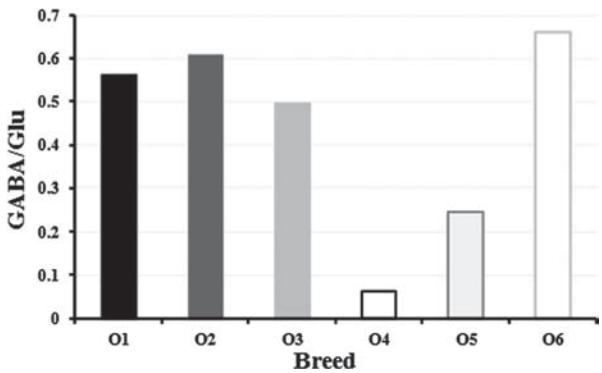

Figure 6. GABA/Glu ratio in six *P. radiata* breeds (O1–6) subjected to 4 weeks of drought (T4).

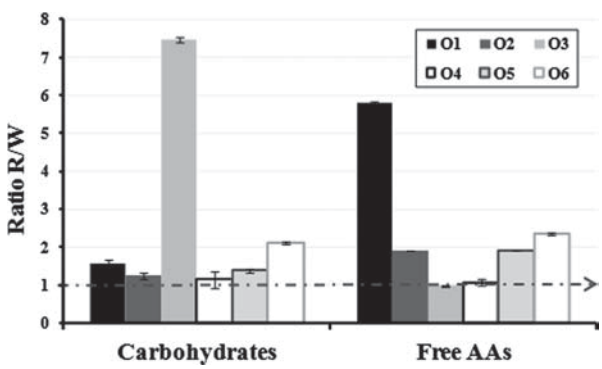

Figure 7. Soluble carbohydrates and free amino acid (AAs) ratio content (rewatered plants/well-watered plants) in six *P. radiata* breeds (O1–6) subjected to a drought cycle of 4 weeks (T4) and subsequent rewatering for a week (R-d7). M ± SE. Discontinuous gray line indicates *R/W* ratio = 1.

stress, all stressed saplings increased their ε values in line with the Ψ_{leaf} decrease, O4 and O5 being those breeds with a lower ε_{tlp} and the most negative π_{tlp} (Figures 4c and 5a). In gymnosperms, both increases and decreases of ε have been related to drought tolerance in different species (Marshall and Dumbroft 1999, Major and Johnson 2001), but recent studies of several woody plants point out that a coordinated reduction of π_o and increase of ε allow plants to maintain their RWC and achieve drought tolerance (Bartlett et al. 2012). In accordance with this assumption, O5 could delay its water loss either by the ε increase or the π_o reduction, presenting the lowest one (–1.56 MPa) (Table 1). Thus, concerning our results, we concluded that an increase of cell rigidity conferred temporary drought tolerance in *P. radiata* plants, but the higher ε values are from the plants which get their π_{tlp} faster (O1 and O3 breeds, De Diego et al. 2012), possibly due to high levels of EL (De Diego et al. 2012, Figure 5c) that could point out cell membrane alteration. Thus, variations in EL may be used as a 'predictive' criterion of putative water stress resistance in some whole plants and low EL variation as a drought tolerance quality (Bajji et al. 2001). According to this assumption, although O5 reached EL percentages of 15% under stress conditions, it presented the lowest increase because the controls also showed high values (6.56 ± 1.15%, data not shown). For this reason, the EL variation could be also a good indicator of *P. radiata* drought tolerance. Furthermore, under water stress conditions π_{tlp} and ε were more correlated than to π_o. Although Bartlett et al. (2012) did not find this relationship between π_{tlp} and ε in a wide range of species, they observed that at low ε values, these traits could be strongly correlated, as it occurred in *P. radiata* breeds ($\varepsilon < 8$ MPa). Finally, it was remarkable that plants recovered their ε values after rewatering, as also was observed in *Quercus* spp. (Saito and Terashima 2004) and *Pseudotsuga menziesii* (Mirb.) Franco (Joly and Zaerr 1987) when the water supply was restored.

Solute accumulation

Soluble carbohydrates were the main solutes that contributed to OA (%): sucrose in O4 and to a lesser extent in O5, and hexoses in O6 (Tables 2 and 3). According to this assumption, recent studies reported an interaction of plant hydraulics with carbon metabolism, and observed that carbohydrate content increases early in the drought, for maintaining the cellular survival by the implication in respiratory metabolism and OA (McDowell 2011). Less membrane damage (low EL variations) has been correlated with an increased capacity to accumulate sugars at the leaf level during water stress (Bajji et al. 2001). In fact, it was hypothesized that sugars, particularly non-reducing disaccharides such as sucrose, interact with cellular membranes to increase the stability of the lipid layers (Nilsen and Orcutt 1996), justifying the high OA contribution observed in O4 and O5. In contrast, hexose sugars increased mainly in O6 stressed saplings, and this accumulation might result from starch hydrolysis (Clifford et al. 1998, Meinzer et al. 2002).

Free PAs and free AAs moderately contributed to OA (Table 3) but drought induced significant changes in some of them (Tables 2 and 3). Hasegawa et al. (2000) suggested that osmotic contributions of certain metabolites to stress tolerance may not describe their function completely and the pathway leading to a particular solute may be more important than accumulation per se. Concerning these results, Glu and Pro, and to less extent GABA, increased in all *P. radiata* plants subjected to drought (Tables 2 and 3), corroborating a close relationship with plant drought response. These solutes were mainly accumulated in the most stressed breeds, which also presented higher GABA/Glu ratio than the most tolerant ones (O4 and O5) (Figure 6). Stress-induced Pro or GABA accumulation has been correlated to stress-tolerance (Bouché and Fromm 2004, Xiong et al. 2011), as these compounds may act as protective molecules and favour the transport of other organic compatible solutes implicated in OA (Rentsch et al. 1996, Schwacke et al. 1999). Besides, GABA production is highly linked to the glutamate content because it is the first step of the pathway that converts Glu to succinate via GABA (Shelp et al. 1999). In this sense, the high GABA/Glu ratio observed in all breeds except for O4 and O5 could be due to the GABA shunt being associated with carbon flux into the tricarboxylic acid cycle to provide carbon skeletons which maintain normal cellular metabolism when carbon availability is reduced (photosynthesis decrease; De Diego et al. 2012).

Other amino acids such as Asn, Arg, Gln, Gly and Ser increased mainly in the more susceptible breeds. The accumulation of these free AAs could be associated to leaf senescence induced by drought and/or their role in specific metabolic process (Araújo et al. 2011). On the contrary, free polyamines such as Put and Spm increased in O5, one of the most tolerant breeds (Tables 2 and 3). Some studies have demonstrated that they play versatile roles in stress responses (Takahashi and Kakehi 2010), including the control of ion channel and receptor activities in membranes (Liu et al. 2000, Shabala et al. 2007) and the protection of DNA from free radical attack (Ha et al. 1998).

After rewatering stressed plants recovered their initial Ψ_s and ε values, but the levels of soluble carbohydrates and AAs were higher than in controls (Figure 7). In this respect, the most tolerant breeds presented the lowest carbohydrate and AA accumulation but they were still accumulating Pro and GABA after rewatering (data not shown). The low contribution of AAs to OA (Table 3) and the total recovery of Ψ_π suggests that these metabolites, especially Pro and GABA, could act as long-term carbon and nitrogen reserves, allowing plants to quickly re-activate growth after stress (Silveira et al. 2003), or even improve their tolerance to further stress.

To summarize, this study corroborates that π_{tlp} is the main indicator of *P. radiata* drought tolerance within species. Their values were due to shifts in π_o with coordinated adjustments in ε that regulate the total RWC. An increase of ε has also a role in drought tolerance until plants reach their π_{tlp}, after that plants present a higher water loss by possible cell damage and/or metabolism disruption. Finally, either soluble carbohydrates or AAs and PAs don't contribute to a high extent to OA but they could provide additional information of plant status against stress. In addition, due to the elevated levels of solutes such as GABA, Pro and Glu, in further studies we will evaluate their possible role in plants of stress conditioning and its implications in the carbon/nitrogen metabolism interaction during drought stress.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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