Physiological response to drought in radiata pine: phytohormone implication at leaf level

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Pinus radiata D. Don is one of the most abundant species in the north of Spain. Knowledge of drought response mechanisms is essential to guarantee plantation survival under reduced water supply as predicted in the future. Tolerance mechanisms are being studied in breeding programs, because information on such mechanisms can be used for genotype selection. In this paper, we analyze the changes of leaf water potential, hydraulic conductance ($K_{\text{leaf}}$), stomatal conductance and phytohormones under drought in $P.\text{ radiata}$ breeds (O1, O2, O3, O4, O5 and O6) from different climatology areas, hypothesizing that they could show variable drought tolerance. As a primary signal, drought decreased cytokinin (zeatin and zeatin riboside—$Z^+ZR$) levels in needles parallel to $K_{\text{leaf}}$ and gas exchange. When $Z^+ZR$ decreased by 65%, indole-3-acetic acid (IAA) and abscisic acid (ABA) accumulation started as a second signal and increments were higher for IAA than for ABA. When plants decreased by 80%, $Z^+ZR$ and $K_{\text{leaf}}$ doubled their ABA and IAA levels, the photosystem II yield decreased and the electrolyte leakage increased. At the end of the drought period, less tolerant breeds increased IAA over 10-fold compared with controls. External damage also induced jasmonic acid accumulation in all breeds except in O5 ($P.\text{ radiata}$ var. $\text{radiata}$ × var. $\text{cedrosensis}$), which accumulated salicylic acid as a defense mechanism. After rewatering, only the most tolerant plants recovered their $K_{\text{leaf}}$, perhaps due to an IAA decrease and 1-aminocyclopropane-1-carboxylic acid maintenance. From all phytohormones, IAA was the most representative ‘water deficit signal’ in $P.\text{ radiata}$.

Keywords: drought tolerance, leaf level, leaf hydraulic conductance, multivariate analysis, phytohormone, $Pinus\text{ radiata}$, water balance.

Introduction

Drought is one of the main processes that affect plant growth and distribution (Domec and Gartner 2002). According to the accepted prediction (IPCC 2007), climatic change will reduce water availability in some areas, including the Iberian Peninsula (Sánchez et al. 2004, Brunet et al. 2009). Water availability controls productivity because water deficit reduces the growth of forestry species (Duursma et al. 2008). In addition, water limitation decreases post-planting survival due to phenomena, such as hydraulic failure, that can limit water uptake from the soil (McDowell et al. 2008). As a result, plant drought tolerance will be more critical for plantation success and natural forestry regeneration in the future (López et al. 2009). Thus, improving the drought resistance of cultivated species is one of the main objectives of many breeding programs. Nowadays, intensive studies are being carried out to understand the role of drought tolerance mechanisms.

$Pinus$ spp. are frequently used in reforestation programs, and $Pinus\text{ radiata}$ D. Don is one of the most cultivated species in the north of Spain, especially in the Basque Country. Because of its fast growth and wood production, this species has been studied...
to find elite plants with high-quality wood (Ivkovic et al. 2006, Codesido and Fernández-López 2009) and with tolerance to some biotic stresses such as fungal and insect attacks (Grace et al. 2005, Lottmann et al. 2010). With regard to drought, most studies have been based on stress response evaluation (Zou et al. 2000, Mena-Petite et al. 2004, Ortega et al. 2004), but the physiological mechanism underlying drought survival and mortality is poorly understood. The evaluation of breeds from climatologically different areas is essential to know the acclimation mechanisms involved in the different responses to drought. Many ecophysiological parameters, principally water balance and gas exchange, have been studied in conifers (Domisch et al. 2001, Mena-Petite et al. 2003, Brodribb and McAdam 2011). Furthermore, some works have shown that gas exchange parameters and leaf water potential have a strong relationship with plant hydraulic conductance (Sperry 2000, Hubbard et al. 2001, Cochard et al. 2002, Klein et al. 2011). The vulnerability of hydraulic conductance to stress has been recently studied in Pinus edulis Engelm. (West et al. 2007), Pinus halepensis Mill. (Klein et al. 2011), Pinus mugo Turra. (Charra-Vaskou and Mayr 2011), Pinus palustris Mill. (Addigton et al. 2006), Pinus ponderosa L. (Hubbard et al. 2001) and Pinus taeda L. (Domec et al. 2009), but not in P. radiata at intra-species level. Due to the complex relationship among physiological parameters through a drought cycle and subsequent recovery, few studies have evaluated the interconnection among them (Brodribb and Holbrook 2003, Liu et al. 2003). In addition, according to Sack and Holbrook (2006), leaf resistance greatly contributes to whole-plant resistance; for this reason, a deep analysis of plant water status at leaf level could be highly interesting under drought conditions.

Phytohormones are involved in different processes throughout plant growth and development (Skirycz and Inzé 2010, Ross et al. 2011) and are essential for the ability of plants to acclimate to stress by mediating a wide range of adaptive responses (Sanntner and Estelle 2009). The complexity of plant response includes hormone synthesis, transport and signaling pathways, and many interactions among them (Sanntner and Estelle 2009). Thus, growth regulators are being investigated to understand their role in stress situations (Wang et al. 2010, Peleg and Blumwald 2011). In this respect, abscisic acid (ABA) is considered one of the main plant signals in drought stress, mediating several acclimation responses (Duan et al. 2007, Jiang and Hartung 2008). Some studies have shown that ABA is transported to the leaves as a root-to-shoot chemical signal (Pospisilova 2003, Dodd 2005) to induce stomatal closure (Wilkinson and Davies 2002, Schachtman and Goodger 2008), and Ghanem et al. (2008) have recently suggested that ABA has a long-lasting effect on plant hydraulic properties by stimulating leaf growth recovery after watering (Parent et al. 2009). However, other phytohormones are also involved in plant response to stress. In this matter, it has been reported that stomatal function is also regulated by other hormones, such as auxins, cytokinins (Cks), ethylene, jasmonic acid (JA) and salicylic acid (SA) (Acharya and Assmann 2009, Sanntner and Estelle 2009).

In addition, the synthesis of Cks has been related to osmotic adjustment under stress conditions (Pospisilova 2003), and recent evidence suggests that root-synthesized Cks can ameliorate shoot growth inhibition caused by environmental stress (Ghanem et al. 2008). With respect to auxin, indole-3-acetic acid (IAA) is the most studied under several stress conditions (Mahouachi et al. 2007, Albacete et al. 2008, Arbona and Gómez-Cadenas 2008), but relatively little information is available on the changes in auxin content induced by drought (Acharya and Assmann 2009).

Ethylene is considered a primary signal in the regulation of the plant’s immune response (Pieterse et al. 2009). This phytohormone is synthesized through 1-aminocyclopropane-1-carboxylic acid (ACC) by the action of ACC-oxidase (Alexander and Grierson 2002), and its increase plays a critical signaling role in plant response to stress, promoting senescence (Lim et al. 2007, Albacete et al. 2009).

Jasmonic acid and SA are also involved in plant response to stress (Delaney 2007, Howe 2007). Concerning angiosperms, it is well established that JA is implicated in response to biotic stresses such as herbivorous attack and wounding (Liechti and Farmer 2002), pathogenesis defense (Wasternack 2007) and abiotic stresses such as UV irradiation (Demkura et al. 2010), ozone exposure (Rao et al. 2000), flooding (Arbona and Gómez-Cadenas 2008) and drought (Shan and Liang 2010). On the contrary, there is little information about jasmonates and their relation to the defense mechanisms in gymnosperms, particularly in the case of Pinus spp. (Pedranzani et al. 2007). Salicylic acid has also been associated with plant resistance (Delaney et al. 1994, Durner et al. 1997), but the mechanisms of influence are poorly known, especially in conifers (Rajasekaran and Blake 1999).

We hypothesized that needle physiological variations and hormonal levels explain breed-specific drought response and recovery. For this reason, we studied the water balance, gas exchange and phytohormones in different P. radiata breeds subjected to water stress and subsequent recovery. Our goal was to establish the relationships among all these parameters. Besides, the general analysis of those ecophysiological traits could give us a major understanding of the different defense strategies and finally allow us to identify possible markers of drought tolerance in radiata pine.

Material and methods
Plant material and experimental design
Plant material
Seeds from geographically and climatologically different areas were obtained from the following companies (see Supplementary
Table S1 available as Supplementary Data at Tree Physiology Online:

- O1—Pinus radiata var. radiata × Pinus 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 de 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 the Australian Tree Seed Centre (CSIRO Forestry and Forest Products, Clayton South, Australia) and collected from a seed orchard located in Bilalapaloola, Australia.
- O4—Pinus radiata var. radiata × Pinus 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 × Pinus 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 (Amberley, North Canterbury, New Zealand) and collected from control-pollinated trees located in Kaingaroa, New Zealand.

**Growth conditions**

Seeds were subjected to cold stratification prior to sowing. They were stored at 4 °C under dark conditions for 3 weeks, immediately after which they were exposed for 2 days to sterilized water to induce germination under the same conditions. Finally, the seeds were sowed in pots of 17 cm Ø with peat: perlite (7/3, v/v). The plants were grown in a greenhouse under controlled conditions (T = 23 ± 1 °C and RH = 70 ± 5%) for 2 years.

**Experimental design**

The 2-year-old saplings were analyzed during summer (from July to September 2008). Ten plants per breed were used; half of them were randomly selected for water stress treatment by withholding water (water-stressed saplings—D), and the remaining saplings were kept with water supply to field capacity three times a week (controls—W). Drought conditions were maintained for 4 weeks (from T0 to T4), after which the water-stressed saplings were rewatered. Recovery was evaluated 1 week after rewatering (R7). All measurements were taken in apical secondary needles.

**Water status determination**

**Water potential**

The water potential (Ψ, MPa) of saplings from all breeds was measured at predawn (from 5:00 to 6:00 a.m.) (Ψpd) and at midday (leaf water potential, Ψ_leaf) from T0 to T4, and at R3 and R7 using a Scholander chamber (Skye SKPM 1400) and the pressure-equilibration technique (Scholander et al. 1965).

**Turgor pressure**

Plant turgor pressure (Ψ_t, MPa) was estimated at the same time points as Ψpd and Ψ_leaf and was calculated from the following mathematical equation of water potential (Ψ_leaf):

\[
Ψ_leaf = Ψ_π + Ψ_t + Ψ_g
\]

where Ψ_t is osmotic potential calculated by the van’t Hoff equation (Ψ_t = −RTc), R is the gas constant, T is the absolute temperature and c is the solute concentration determined by a cryoscopic osmometer (Osmomat O30). Ψ_g is the gravity potential estimated as Ψ_g = ρ_w gh; ρ_w is the density of water, g is the gravity and h is the height of each sapling.

**Needle hydraulic conductance**

Needle-specific hydraulic conductance (K_leaf, mmol H₂O m⁻² s⁻¹ MPa⁻¹) is derived from Darcy’s law described by Ewers et al. (2000):

\[
E = K_leaf \times (Ψ_s - Ψ_leaf - Ψ_g)
\]

where E is instant leaf transpiration and Ψ_s (soil water potential) is estimated from Ψpd measured at 5:00–6:00 a.m. using a Scholander chamber (Skye SKPM 1400) according to Hubbard et al. (2001); Ψ_g is the gravity potential.

**Relative water content**

Relative water content (RWC (%)) was measured in two needles collected from the apical area of each sapling at the end of the drought period (T4) following the method described by Boyer (1969). RWC was calculated using the following equation:

\[
RWC(%) = (FW - DW) / (TW - DW) \times 100
\]

where FW is the fresh weight at harvesting time, TW is the total weight as total turgor estimated after 24 h of imbibition and DW is the dry weight after 48 h at 60 °C.

**Gas exchange parameters**

Stomatal conductance (gₛ, mmol H₂O m⁻² s⁻¹), instant leaf transpiration (E, mmol H₂O m⁻² s⁻¹) and instant net photosynthesis (A, mmol CO₂ m⁻² s⁻¹) were measured weekly at midday from T0 to T4 and at R7 using an infra-red gas analyzer system (IRGA, CIaras-2 PPS System) equipped with the universal photosynthesis chamber (PLC(U)).

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Fluorescence parameter

Chlorophyll fluorescence was measured in apical needles at room temperature from T0 to T4 and at R7 using a portable fluorometer (Hansatech FMS2). The maximum quantum yield of PSII photochemistry (Fv/Fm) was estimated as described by Rohácek (2002):

\[
Fv / Fm = (Fm - Fo) / Fm
\]

where Fo and Fm are the minimum and the maximum chlorophyll fluorescence yields in the dark-adapted state, respectively.

Electrolyte leakage

To determine leaf membrane damage, measurements of electrolyte leakage [EL (%)] were recorded using the conductometric method (Bajji et al. 2002). Two needles per sapling and treatment were collected, washed and put in a test tube with 5 ml of deionized water. Electrolytic conductivity (EC) was measured using a portable conductivity meter (Cole Parmer Model 19101-10) at the collection date (ECi) and after 24 h (ECf). Thereafter, samples were autoclaved for 10 min at 121 °C and cooled at room temperature to measure the total electrical conductivity (ECT). Electrolyte leakage was evaluated from T0 to T4 and at R7, and was calculated according to the following mathematical model:

\[
EL(\%) = \left( \frac{(ECf - ECi)}{(ECT - ECi)} \right) \times 100
\]

Quantification of parameter decrease

The decrease in percentage of sapling gas exchange parameters and K_{leaf} was estimated as

\[
\text{Decrease (\%)} = \left( \frac{(Xm - Xi)}{Xm} \right) \times 100
\]

where Xi is the parameter value at time i (from T0 to T4) and Xm at T0.

Hormone analysis

Analyses were carried out with an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray interface. Before injection, 100 µl of each fraction was again filtered through Millipore filters (Ø 0.22 µm) and placed into tubes adjusting the volume to 1.5 ml with the extraction solution.

Hormone extraction

Phytohormones were analyzed on two apical needles per sapling. Needles were immediately frozen in liquid nitrogen. Samples were pooled for further analysis and maintained at −80 °C until extraction. The ABA, Cks [zeatin (Z) and zeatin riboside (ZR)], IAA, JA, SA and ACC were extracted and purified according to the method described by Dobrev and Kaminek (2002), and were analyzed as described previously by Albacete et al. (2008). In summary, sapling material (0.5 g FW) was homogenized in liquid nitrogen and dropped in 2.5 ml of cold (−20 °C) extraction solution of methanol/water (80/20, v/v). Extracts were centrifuged at 20,000 g for 15 min at 4 °C, and the pellets were re-extracted for 30 min in an additional 2.5 ml of the same extraction solution. Supernatants were collected and filtered through Sep-Pak Plus C18 (Waters, Milford, MA, USA) to remove interfering lipids and pigments, and evaporated at 40 °C under vacuum. Residues were dissolved in 1 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter nylon membrane Millex filters (Ø 0.22 µm) (Millipore, Bedford, MA, USA) and placed into tubes adjusting the volume to 1.5 ml with the extraction solution.

Hormone quantification

Hormone quantification

Analyses were performed using an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray interface. Before injection, 100 µl of each fraction was again filtered through Millipore filters (Ø 0.22 µm) and placed into tubes adjusting the volume to 1.5 ml with the extraction solution.
Statistical analysis

Multivariate analysis of variance (MANOVA) was carried out by proc glm in the S.A.S.® software package (SAS Institute Inc., Cary, NC, USA) to evaluate the relationship among physiological parameters and to estimate possible different responses in six P. radiata breeds. For the analysis of $\Psi_{\text{psi}}, \Psi_{\text{psr}}, \Psi_{\text{t}}, g_{\text{t}}, A_{\text{g}}, F_{\text{v/Fm}}$ and EL (\%) five saplings per breed and treatment were analyzed according to the following mathematical model:

$$y_{ijr} = \mu + O_i + T_j + T_{ik} + OT_{ij} + OT_{ik} + OT_{jik} + e_{ijr}$$  \hspace{1cm} (7)

where $y_{ijr}$ is the response variable result of the rth sapling of the ith breed (O1–O6) subjected to jth treatment [well-watered (W) or water-stressed sapling (D)] at kth time (from T0 to T4 and at R7); $\mu$ is the experimental mean, $O_i$ the effect of the ith breed, $T_j$ the effect of the jth treatment, $T_{ik}$ the effect of the kth time; $OT_{ij}$ is the interaction between the ith breed and the jth treatment, $OT_{ik}$ between the ith breed and the kth time, $OT_{jik}$ between the jth treatment and the kth time; $e_{ijr}$ is the random error component.

For the phytohormone and ACC analysis, three pools per breed and treatment were measured during the drought period (T0, T2 and T4) and subsequent rewatering (R7), and the relation between irrigated (W) and non-irrigated saplings (D) was statistically analyzed according to the following mathematical model:

$$y_{ip} = \mu + O_i + T_{ik} + OT_{ik} + e_{ip}$$ \hspace{1cm} (8)

where $y_{ip}$ is the response variable result of the rth relation (D/W) of the ith breed (O1–O6) subjected to kth time (from T0 to T4 and at R7); $\mu$ is the experimental mean, $O_i$ the effect of the ith breed, $T_{ik}$ is the effect of the kth time, $OT_{ik}$ is the interaction between the ith breed and kth time and $e_{ip}$ is the random error component.

The characteristic vectors of associated matrix from MANOVA were also calculated to identify the most representative phytohormone involved in P. radiata response to drought. Vectors were obtained from the product of the square matrix sum of the model and the experimental error. The characteristic root was the square root of an eigenvalue and the percentage accounted for the variability in the effect of breed relation (D/W) along the experiment.

Multiple comparisons were calculated using post hoc Tukey’s test to determine the different significance levels among the factors and their possible interactions. Furthermore, to analyze possible correlations between hormones and physiological parameters proc reg was used. Analysis of covariance (ANCOVA) was carried out by glm proc using the S.A.S.® software package.

Results

Drought induced varied intensities of response in P. radiata plants of different breeds, and the differences were generally due to the triple interaction among time (from T0 to T4 and at R7), treatment (irrigation or no irrigation conditions) and breed (O1–O6), according to MANOVA (see Supplementary Table S2 available as Supplementary Data at Tree Physiology Online).

All saplings showed decreased $\Psi_{\text{leaf}}$ at T4. In this respect, O3 and O6 reached $\Psi_{\text{leaf}}$ below $-3\text{ MPa}$ and lost their turgor ($\Psi_{\text{t}} < 0\text{ MPa}$) (Table 1). On the contrary, stressed saplings from O4 and O5 maintained their turgor and showed $\Psi_{\text{leaf}}$ values around $-1.8\text{ MPa}$. A strong correlation emerged between $\Psi_{\text{leaf}}$, $\Psi_{\text{t}}$, and Fv/Fm. Only O1 showed lower Fv/Fm values than its controls (Table 1).

At T4, all stressed saplings showed similar $K_{\text{leaf}}$, $g_{\text{t}}$ and $A_N$ decreases (over 90\%) (Table 2). At R7, only O4 and O5 recovered their $K_{\text{leaf}}$ values and showed the highest $g_{\text{t}}$ and $A_N$ levels.

The most evident external symptoms of P. radiata saplings subjected to drought were needle epinasty and apical curvature (Figure 1). These symptoms showed high variability among breeds. At T2, 40\% of the plants from O1, O3 and O6, and only 20\% of the saplings from O2 showed external symptoms (data not shown). On the other hand, O4 and O5 presented normal appearance. At T4, all saplings from O6 showed symptoms, whereas only 40 and 20\% from O4 and O5, respectively, showed symptoms. At R7, all plants recovered normal appearance except for 20\% of saplings from O6 (Table 2).

Phytohormonal profiling

Drought induced changes in the endogenous hormone levels of P. radiata apical needles. Phytohormonal changes were highly significant ($P < 0.001$) due to the effect of the double interaction between time and breed, according to MANOVA (see Supplementary Table S3 available as Supplementary Data at Tree Physiology Online). At T2, ABA accumulation was only significant in O6 (Figure 2a). At T4, O1, O2, O3 and O5 significantly increased their ABA ratios. At R7, O5 recovered its ABA initial values (Figure 2a). Finally, ABA accumulation was still increasing in water-stressed saplings from O3.

Indole-3-acetic acid was the least abundant hormone in P. radiata needles, with levels around 15–20 ng g$^{-1}$ FW at the beginning of the drought, whereas the other phytohormones showed values 15- or 30-fold higher [from 250 to 600 ng g$^{-1}$ FW].
Table 1. Leaf water potential ($\Psi_{\text{leaf}}$) and turgor pressure ($\Psi_t$) (MPa), and the maximum quantum yield of PSII photochemistry (Fv/Fm) in six $P.\ radiata$ breeds (O1–O6) exposed to irrigation (W—well watered) or no irrigation conditions (D—water-stressed saplings) for 4 weeks (T4) and a subsequent rewatering for 1 week (R7). M ± SE. Significant differences with regard to each control are represented by asterisks according to Tukey’s HSD test after MANOVA.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Treatment</th>
<th>$\Psi_{\text{leaf}}$ (MPa)</th>
<th>$\Psi_t$ (MPa)</th>
<th>Fv/Fm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T4</td>
<td>R7</td>
<td>T4</td>
</tr>
<tr>
<td>O1</td>
<td>W</td>
<td>$-0.50 \pm 0.09$</td>
<td>$-0.49 \pm 0.09$</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>$-2.86 \pm 0.03^{***}$</td>
<td>$-0.39 \pm 0.14^{**}$</td>
<td>$0.20 \pm 0.41^{***}$</td>
</tr>
<tr>
<td>O2</td>
<td>W</td>
<td>$-0.49 \pm 0.12$</td>
<td>$-0.33 \pm 0.08$</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>$-2.31 \pm 0.48^{***}$</td>
<td>$-0.51 \pm 0.11^{**}$</td>
<td>$-0.02 \pm 0.37^{**}$</td>
</tr>
<tr>
<td>O3</td>
<td>W</td>
<td>$-0.47 \pm 0.05$</td>
<td>$-0.42 \pm 0.14$</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>$-3.53 \pm 0.21^{***}$</td>
<td>$-0.41 \pm 0.11^{**}$</td>
<td>$-1.34 \pm 0.13^{***}$</td>
</tr>
<tr>
<td>O4</td>
<td>W</td>
<td>$-0.57 \pm 0.09$</td>
<td>$-0.66 \pm 0.12$</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>$-1.83 \pm 0.32^{***}$</td>
<td>$-0.45 \pm 0.12^{**}$</td>
<td>$0.31 \pm 0.20^{**}$</td>
</tr>
<tr>
<td>O5</td>
<td>W</td>
<td>$-0.59 \pm 0.09$</td>
<td>$-0.56 \pm 0.09$</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>$-1.87 \pm 0.35^{***}$</td>
<td>$-0.23 \pm 0.07^{**}$</td>
<td>$0.13 \pm 0.23^{**}$</td>
</tr>
<tr>
<td>O6</td>
<td>W</td>
<td>$-0.51 \pm 0.08$</td>
<td>$-0.36 \pm 0.10$</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>$-3.01 \pm 0.36^{***}$</td>
<td>$-0.69 \pm 0.05^{**}$</td>
<td>$-0.58 \pm 0.35^{**}$</td>
</tr>
</tbody>
</table>

ns, non-significant; *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2. Decrease (%) of needle hydraulic conductance ($K_{\text{leaf}}$), stomatal conductance ($g_s$), photosynthesis ($A_N$) and percentage of saplings with external symptoms (Symptoms, %) evaluated as epinastic needles and apical curvature in six $P.\ radiata$ breeds (O1–O6) after 4 weeks under drought conditions (T4), and the recovery capacity (%) related to each control after rewatering for 1 week (R7).

<table>
<thead>
<tr>
<th>Breed</th>
<th>T4</th>
<th>R7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Decrease (%)</td>
<td>Symptoms (%)</td>
</tr>
<tr>
<td></td>
<td>$g_s$</td>
<td>$A_N$</td>
</tr>
<tr>
<td>O1</td>
<td>98.06</td>
<td>95.80</td>
</tr>
<tr>
<td>O2</td>
<td>91.72</td>
<td>88.16</td>
</tr>
<tr>
<td>O3</td>
<td>90.39</td>
<td>94.96</td>
</tr>
<tr>
<td>O4</td>
<td>90.57</td>
<td>91.43</td>
</tr>
<tr>
<td>O5</td>
<td>94.75</td>
<td>100</td>
</tr>
<tr>
<td>O6</td>
<td>99.52</td>
<td>96.96</td>
</tr>
</tbody>
</table>

Figure 1. Apical part of 2-year-old plants of $P.\ radiata$. Well-watered saplings (a). Saplings with needle epinasty and apical curvature after 4 weeks under drought conditions (from T0 to T4) (b).
However, IAA explained most of the model variance (66%) (see Supplementary Table S4 available as Supplementary Data at Tree Physiology Online). The highest IAA increment was observed at T4 in all saplings analyzed (Figure 2b), except in O4, which did not show statistically significant differences during the experiment. The highest increments were observed in O1, the levels of which increased 27-fold their control values [from 16.8 to 456.0 ng g\(^{-1}\) FW (data not shown)], whereas these were only doubled in O5 [from 5.1 to 13.6 ng g\(^{-1}\) FW (data not shown)]. At R7, O5 recovered its initial values, while IAA was still accumulating in O1, O2 and O3 (Figure 2b).

All saplings contained between 100- and 1000-fold higher values (ng g\(^{-1}\) FW) of Z than ZR (data not shown). Therefore, Z and ZR were evaluated together. According to characteristic vectors, Z + ZR explained a model variance of 10.44%, being the third most representative hormone of the experimental model (see Supplementary Table S4 available as Supplementary Data at Tree Physiology Online). Zeatin + ZR changes were evident since the beginning of the drought period, so all breeds except O5 showed significantly decreased D/W at T2 (Figure 2c). At T4, all stressed saplings except those from O6 maintained their Z + ZR levels below the initial amounts. At R7, O1 and O6 recovered their initial Z + ZR relation (Figure 2c).

The ACC ratio of O5 did not vary during the experiment (Figure 2d). At T4, only O1 and O6 significantly dropped their ACC levels. At R7, O1 recovered its ACC initial levels whereas O3 increased them (Figure 2d).

Drought also induced changes in endogenous JA levels (Figure 2e). O5 did not change its JA ratio during the water stress period. At T2, JA levels were significantly decreased in O2 and O6, and maintained in O3. At T4, the ratios in these three breeds showed strong increases. O1 and O4 significantly accumulated JA throughout the drought period, showing the highest JA ratios at T4 (Figure 2e).

Finally, SA was the second most representative phytohormone, explaining a model variance of 16.64% (see Supplementary Table S4 available as Supplementary Data at Tree Physiology Online). At T2, all stressed plants, except O4 and O5, showed significantly decreased SA levels compared with T0 (Figure 2f). At T4, O4, O5 and O6 showed significantly increased SA content. At R7, only stressed saplings from O3 and O4 recovered their SA values, whereas O1 and O6 presented higher ratios than those observed in T0 (Figure 2f).

Correlation analysis
At T2, a strong correlation was detected among Z + ZR variations, \(\Psi_{\text{leaf}}\), \(K_{\text{sat}}\) and \(g_c\) (Figure 3). A 50% decrease in Z + ZR
content (D/W = 1.20) was observed when the \( \Psi_{\text{leaf}} \) of plants dropped below \(-0.7 \) MPa (Figure 3a), and this decrease was related to a reduction of 50 and 35% in \( K_{\text{leaf}} \) and \( g_s \) respectively (Figure 3b and c). When stressed saplings reached \( \Psi_{\text{leaf}} \) of \(-1.4 \) MPa, both Cks content and \( K_{\text{leaf}} \) were reduced by 80%, with \( g_s \) presenting a decrement of 55%.

A highly significant correlation was observed among IAA accumulation, \( \Psi_{\text{leaf}} \) and \( \Psi_t \) (Figure 4). Stressed saplings started to accumulate IAA in the needles when the \( \Psi_{\text{leaf}} \) of plants decreased to \(-1 \) MPa and their \( \Psi_t \) to 0.66 MPa (Figure 4a). When stressed saplings reached the turgor loss point (TLP), their IAA levels increased to fourfold the initial values. \( K_{\text{leaf}} \), gas exchange, \( Fv/Fm \) and EL showed a higher correlation with IAA than ABA (data not shown). In this respect, stressed saplings started to accumulate IAA when \( A_n \) was reduced to 45% (D/W = 0.55) in relation to their control levels (Figure 4b). Indole-3-acetic acid increments were accompanied by an \( Fv/Fm \) reduction and a high solute loss [membrane damage, EL (\%)] during the drought cycle (Figure 4c and d). At T4, only O1 showed a different EL tendency due to a higher IAA increase (Figure 4d).

The relationship between phytohormones and RWC (\%) was also evaluated. A high correlation was observed between ABA and IAA levels and RWC (\%) (Figure 5). RWC (\%) induced IAA accumulation in all saplings [increasing 12-fold the initial values at T4 but only tripling their ABA levels (Figure 5a and b)], corroborating a higher influence for IAA than ABA in \( P. \ radiata \) saplings subjected to drought.

External symptoms were highly correlated to IAA, JA and SA content (Figure 6a and b). When 50% of the saplings of all

Figure 3. Correlation among leaf water potential (\( \Psi_{\text{leaf}} \)) (a), leaf hydraulic conductance (\( K_{\text{leaf}} \)) (b) and stomatal conductance (\( g_s \)) (c) vs. Cks (\( Z + Z R \)) ratio content (D—water-stressed saplings/W—well-watered saplings) in six \( P. \ radiata \) breeds (O1–O6) from T0 to T2. Gray dashed lines represent 50 and 80% of \( Z + Z R \) decrease. \( R^2 \) is Pearson's correlation: *\( P < 0.05 \); ***\( P < 0.001 \).

Figure 4. Correlations among leaf water potential (\( \Psi_{\text{leaf}} \)) and turgor pressure (\( \Psi_t \)) (MPa) vs. IAA (ng g\(^{-1}\) FW) in six \( P. \ radiata \) breeds during one drought cycle of 4 weeks (from T0 to T4) (a). Instant net photosynthesis (\( A_n \)) (b), the maximum quantum yield of PSII (\( Fv/Fm \)) (c) and electrolyte leakage [EL (\%)] (d) vs. IAA ratio content (D—water-stressed saplings/W—well-watered saplings). TLP, turgor loss point. Circles indicate the regression. \( R^2 \) is Pearson's correlation: ***\( P < 0.001 \).
breeds presented with needle epinasty and apical curvature, their IAA levels were double (Figure 6a). Besides, an IAA accumulation of 30-fold the control values was reached when 100% of saplings showed external symptoms. The accumulation of JA was related to high percentages of saplings with symptoms, whereas an SA increase was associated with the lowest ones. Although a strong correlation was observed between JA and external symptoms \( (P < 0.01) \), the JA/ACC relation showed the strongest correlation \( (P < 0.001) \) (Figure 6b and c).

Recovery capacity
At R7, all saplings presented recovery capacity, but the intensity varied among breeds. All parameters were evaluated by principal component (PC) analysis, with PC-1 explaining a total of 44% of model variance (Figure 7). A strong positive correlation between O4 and O5 was noticed. They also showed a direct correlation with high values of \( A_{NT} \), \( E \), fluorescence parameters and \( K_{f_{leaf}} \), and high values of \( Z + ZR/IAA \) and ACC/IAA. On the contrary, O4 and O5 were negatively related to IAA, SA and ACC levels, suggesting a strong implication of these hormones in sapling water status. This trend was reversed in O3. According to PC-2 (24% of model variance), the O1 recovery was inversely correlated to \( Z + ZR \) content, jasmonate levels and phytohormone relations such as JA/ACC and JA/SA (Figure 7).

Discussion

Drought and physiological changes
This study provided experimental evidence of the relationship between phytohormonal levels and parameters such as water balance, gas exchange and fluorescence in \( P. \) radiata saplings during a water deficit period and subsequent recovery. Different responses were observed in the evaluated breeds. The \( \Psi_{pd} \) of all saplings significantly decreased at T2, reaching values of \(-2 \) MPa at T4 (data not shown). In this regard, Hubbard et al. (2001) indicated that \( \Psi_{pd} \) was a good indicator of soil water status, pointing out that all saplings were subjected to the same soil moisture. When \( \Psi_{leaf} \) was analyzed, we observed that the \( \Psi_{leaf} \) of O1, O2, O3 and O6 reached below \(-2 \) MPa (TLP). On the other hand, O4 and O5 showed \( \Psi_{leaf} \) of \(-1.8 \) MPa (Table 1) and did not lose their turgor (see Supplementary Figure S1 available as Supplementary Data at Tree Physiology Online). According to these results, Mena-Petite et al. (2001) observed that when \( P. \) radiata seedlings reached water potentials lower than \(-2.5 \) MPa, they were subjected to severe water stress. In addition, our results showed that gas exchange parameters and \( K_{f_{leaf}} \) decreased in line with \( \Psi_{leaf} \), as noticed in previous studies (Ewers et al. 2000, Brodribb and Cochard 2009). It was remarkable that, at T4, O4 and O5 showed decreased gas exchange and \( K_{f_{leaf}} \) values in the same way as did the other breeds, but maintained \( \Psi_{leaf} \) over the TLP. This stronger gas exchange and \( K_{f_{leaf}} \) regulation, together with an efficient osmotic adjustment (data not shown), is characteristic of isohydric species (McDowell et al. 2008, Quero et al. 2011).

Figure 5. Correlations of ABA (a) and IAA (b) (ng g\(^{-1}\) FW) vs. RWC (%) in six \( P. \) radiata breeds (O1–O6) during one drought cycle of 4 weeks (from T0 to T4). W and D are irrigated and non-irrigated plants, respectively. \( R^2 \) is Pearson’s correlation: ***\( P < 0.001 \).

Figure 6. Correlations of external symptoms (%) with IAA (a), JA and SA (b), and JA/ACC (c) ratio content (D—water-stressed saplings/W—well-watered saplings) in six \( P. \) radiata breeds (O1–O6) during a drought period of 4 weeks (from T0 to T4). \( R^2 \) is Pearson’s correlation: *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \).
Concerning phytohormonal signaling, a strong decrease of Z+ZR content in *P. radiata* needles was observed at T2, and this decrement was closely bound to \( \Psi \) leaf, K leaf and gs variations (Figure 3). This assumption corroborated the previously described important role of Cks in regulating the early response to drought (Granda et al. 2011). In this respect, as Goodger et al. (2005) suggest, chemical signals are produced before hydraulic signals, and represent an ‘early warning’ of soil water status. Thus, it was observed that minimal \( \Psi \) leaf variations produce significant changes in the Ck concentration of all plant tissues, although the effect is stronger at leaf level as previously described in *Medicago sativa* L. (Goicoechea et al. 1995) and *Vitis vinifera* L. (Stoll et al. 2000). Furthermore, Cks are considered ABA antagonists in processes such as stomatal aperture control during the early response (Haisel et al. 2008, Peleg and Blumwald 2011). The low Z+ZR content and the subsequent high ABA accumulation could be mainly responsible for the practically negligible \( g_s \) noticed at T4.

The role of ABA as a stress signal has been discussed in depth in several studies (Hartung et al. 2002, Pospisilova 2003, Dodd 2005, Sánchez-Díaz et al. 2008), specifically in relation to stomatal closure regulation (Li et al. 2000, Bauerle et al. 2006, Acharya and Assmann 2009, ). Under drought conditions, all saplings presented needle ABA accumulation, although the highest levels were detected at T4 (Figure 2a). The initial low ABA accumulation observed at the beginning of the drought could be because the ABA presented in leaves was re-cycled from xylem to phloem (Dodd 2005) and/or transported to other plant areas (Jeschke et al. 1997, Sauter et al. 2001). Other authors point out that the needle ABA levels are controlled by a dynamic equilibrium between ABA biosynthesis and catabolism (Ren et al. 2007). Moreover, ABA accumulation requires an activated and accelerated production of ABA precursors (Ren et al. 2007), a process that could delay the ABA increment in the leaves. The highest ABA values were observed in stressed O5 (537 ng g\(^{-1}\) FW) (Figure 5a), which maintained the turgor (Table 1), practically losing K leaf and gas exchange capacity at T4 (Table 2). Furthermore, stressed O5 showed the highest recovery capacity of K leaf at R7. In this respect, ABA accumulation is assumed to be a water defense mechanism through the increment of some antioxidant enzyme activity to prevent cell damage (Jiang and Zhang 2002), and it could explain the high stress tolerance and the lowest damage percentage observed in O5 (Table 2). Moreover, O5 presented the highest ABA values with respect to RWC (%) (Figure 5a), this being a possible cause of its faster stomata closure despite a less negative \( \Psi \) leaf.

All breeds except O5 accumulated JA at T4 (Figure 2d). Stressed O5 did not have significant JA variations, did not lose turgor (Table 1) and presented the lowest number of plants with external symptoms (20%) (Table 2). These results confirmed that JA levels were rapidly and transiently increased by plant cell changes under biotic and abiotic stresses (Creelman and Mullet 1997, Wasternack 2007), including cell turgor reduction (Schaller and Stintzi 2009). This behavior might be due to jasmonate synthesis being normally stimulated by physical damage as a consequence of stress effect (Gould et al. 2009). Thus, stressed plants that showed high stress signs [low Fv/Fm and high EL (%)] (O1, O3 and O6) showed increased JA and JA/ACC levels (Figure 6b and c). EL is considered a good indicator of cell membrane status (Campos et al. 2003). Thus, high EL (%) indicates loss of membrane integrity and stability under water stress situations (Lauriano et al. 2000). For all these assumptions and according to our results, JA seems to be a possible indicator of damage response in *P. radiata*.

Salicylic acid is also considered a signal molecule that modulates plant response to stress (Senaratna et al. 2000,
Delaney 2007). Various physiological and biochemical effects of SA in plants, including ion uptake, membrane permeability, mitochondrial respiration and photosynthesis, have been well documented (Barksosky and Einhellig 1993, Delaney et al. 1994, Wang et al. 2010). Besides, SA regulates plant growth, triggers local resistance and activates systemic acquired resistance response (Delaney 2007). O5 showed the highest SA accumulation (Figure 2f), maintained its Fv/Fm values (Table 1) and practically did not show apparent external damage (Table 2). The remaining breeds presented a high JA accumulation and low SA changes. In this regard, it has been previously reported that there is antagonistic behavior between JA and SA in plant response to drought (Felton and Korth 2000, Kunkel and Brooks 2002, Howe 2007). This negative crosstalk seems to provide plants with defense response mechanisms (Turner et al. 2002). This possible mechanism is specific to the stress situation and minimizes the expression of inappropriate defense genes (Kunkel and Brooks 2002, Howe 2007).

The role of IAA during water stress

Most plants subjected to abiotic stresses have shown changes in their IAA levels (Albacete et al. 2008, Kong et al. 2008). In our study, at leaf level IAA was the most influential phytohormone under drought (see Supplementary Table S4 available as Supplementary Data at Tree Physiology Online), showing increments in P. radiata saplings subjected to stress (Figure 2b) and high correlations with other physiological processes such as Ψleaf and Aleaf (Figure 4a and b). Indole-3-acetic acid changes were frequently due to crosstalk with other hormones (Chandler 2009), regulating stomata closure processes (Pospisilova 2003), reactive oxygen species activation (Tognetti et al. 2012) and/or ethylene synthesis (Hansen and Grossmann 2000, Merritt et al. 2001). For example, it is well known that auxins interact with Cks in the control of many central developmental processes (Tanaka et al. 2006, Zhao 2008). The interconnection between Cks and auxins is still being studied due to the difficulty in resolving which is the cause or the effect of the other synthesis (Nordström et al. 2004). In our study, an evident reduction of Z + ZR (65%) was first observed in needles under drought earlier than the increment of ABA and IAA levels. Indole-3-acetic acid accumulation was well correlated to the RWC (%) decrease (Figure 5b) and the induction of epinasty symptoms (Figures 1 and 6), previously observed in some angiosperms (Keller and Van Volkenburgh 1997, Kawano et al. 2003) and even in conifers (Blake et al. 1980). In this matter, some studies have demonstrated that leaf curvature is a defense strategy that slows down the damage under stress conditions (Abreu and Munné-Bosch 2008). In V. vinifera it has been reported that shoots with downward orientation accumulated IAA in the apex and induced Kleaf reduction; this fact did not occur in upward-oriented shoots (Lovisolo et al. 2002). In P. radiata saplings the apical curvature could be induced by IAA accumulation in the apical needles (Figure 6a), but the accumulation might be a consequence of Kleaf reduction and not its possible cause. In addition, IAA accumulation presented a strong negative relationship with Fv/Fm and a positive correlation with EL (%) and the presence of symptoms, which pointed to high IAA accumulation as an indicator of severe water deficit. Moreover, and in agreement with Abreu and Munné-Bosch (2008), IAA could be considered a defense mechanism signal to reduce greater damage in the PSII (Figure 4c and d). Finally, some studies have observed that IAA can influence ethylene biosynthesis and vice versa (Swarup et al. 2002, Santner and Estelle 2009). On this account, Tsuchisaka and Theologis (2004) found 1-aminocyclopropane-1-carboxylate synthase to be regulated by auxin presence. Thus, O1 and O6 also showed the highest decrements of ACC levels due to a possible conversion to ethylene at T4 (Figure 2d). Else et al. (1995) observed petiole epinasty on plants under stress stimulated by conversion of ACC to ethylene (Dodd 2005, Ghanem et al. 2008). Thus, the needle epinasty in our study could be due to the additive effect of IAA and ethylene accumulation.

Recovery capacity

Breed recovery capacity was also analyzed. All saplings recovered Ψleaf and Ψt at R7 (Table 1). However, only O4 and O5 recovered their Kleaf levels (Table 2). In this matter, Blackman et al. (2009) found a shift in the relationship between E and Kleaf. In addition, they observed that possible candidates for the inhibition of gas exchange recovery were the damage to leaf photosynthesis apparatus or/and ABA accumulation. Based on these results, we observed that O3 showed the lowest gas exchange parameters and Kleaf recovery, and was still accumulating ABA at R7 (Table 2, Figure 2a).

According to PC analysis, the recovery capacity of the highest drought tolerance breeds (O4 and O5) was correlated to the lowest IAA values (Figure 7), so only these plants recovered Kleaf (Table 2). In this sense, although Salleo et al. (1996) observed that high IAA concentrations were necessary for Kleaf recovery, they could also induce a great number of small xylem vessels that delay the hydraulic conductance restoration (Lovisolo et al. 2002). This assumption could be one reason for the low percentage of Kleaf observed in stressed plants from O1, O2, O3 and O6 at R7 (Table 2). So they did not recover the IAA initial ratios (Figure 2b). Besides, O4 and O5 were also positively correlated to Z + ZR/IAA and ACC/IAA relations, which can induce the stomatal aperture due to the Ck increase and IAA decrease (Pospisilova 2003, Acharya and Assmann 2009).

To summarize, our study provides new insights into the role of major phytohormones (ABA, Z, IAA, JA and SA) and ACC in P. radiata needles during drought periods, the recovery
capacity after rewatering and their relationships with some physiological traits commonly associated with stress ($\Psi_{\text{leaf}}$, $K_{\text{leaf}}$ and $g_s$) (Figure 8). Although ABA has been traditionally considered the principal water stress indicator, our results show IAA to be the most important phytohormone (66% of model variance) and the most representative ‘water deficit signal’. The main indicator was $K_{\text{leaf}}$, which dropped in line with $Z + ZR$ content that acted as a first drought signal. When $Z + ZR$ content decreased by over 65%, plants started to accumulate ABA and IAA at leaf level. Indole-3-acetic acid changes presented a decreased by over 65%, plants started to accumulate ABA and tent that acted as a first drought signal. When Z main indicator was ance) and the most representative ‘water deficit signal’. The to be the most important phytohormone (66% of model vari - ered the principal water stress indicator, our results show IAA and stress, this being interpreted as a ‘warning alarm’ (Figure 8). reveals that saplings have been subjected to severe water due to the water stress situation. The accumulation of JA ‘water stress tolerance’. Finally, SA accumulation was shown to be a mechanism of ‘water stress tolerance’.

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

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Conflict of interest
None declared.

References


