High Temperature and Water Deficit Cause Epigenetic Changes in Somatic Plants of *Pinus Radiata* D. Don

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Abstract

Current climate changes imply an imminent risk for forest species. In this context, somatic embryogenesis is a valuable tool to study the response of plants to different abiotic stresses. Based on this, we applied a high-temperature regime (50 ºC, 5 min) during the maturation of *Pinus radiata* D. Don embryogenic masses in order to evaluate the development of an epigenetic memory months later. Therefore, somatic plants (SP) resulting from somatic embryos (ses) maturated at control temperature and cultivated in a greenhouse were submitted to heat stress (40 ºC, 2 h, 10 days; 23 ºC, 10 days) or at a control temperature (23 ºC, 20 days); while another 20 SP resulting from ses maturated in the two temperature regimes and cultivated in the greenhouse were submitted to drought stress or weekly irrigated. All plants were evaluated for relative water content, water potential, electrolyte leakage, stomatal conductance, transpiration, methylation (5-mC) and hydroxymethylation (5-hmC) levels. The results showed that the SP obtained from ses maturated at 50 ºC showed an adaptation to drought stress based on water potential and transpiration. Furthermore, SP kept under heat stress in a greenhouse showed lower 5-hmC levels than SP kept at 23 ºC. Furthermore, the 5-hmC and 5-hmC/5-mC ratio showed a significantly negative correlation with changes in water potential; and a significantly negative correlation was observed between the levels of stomatal conductance and 5-mC. We conclude that the manipulation of conditions during the maturation process in somatic embryogenesis modulates the physiological characteristics of the SP obtained.

Key Message

Application of high temperatures in the maturation stage of somatic embryos and the resulting *Pinus radiata* somatic plants provoked a drought adaptation and changes in epigenetic mechanisms.

Introduction

Climate change can cause limitations in the growth of plants due to water and thermal stress (Shahzad et al. 2021). However, plants can develop physiological and biochemical mechanisms that allow them to survive under conditions of abiotic or biotic stress (Krasensky and Jonak 2012; Fox et al. 2018). Currently, in our laboratory, somatic embryogenesis (SE) has been used as model to analyze the hypothesis that abiotic stress applied during the SE process can induce somatic plantlets with different stress tolerance (Castander-Olarieta et al. 2019; Do Nascimento et al. 2020). This is possible because alterations in the chemical and/or physical environmental conditions in the different stages of SE can provoke epigenetic changes such as chromatin organization (Vaissière et al. 2008) and cytosine methylation in the DNA (Alsdurf et al. 2016).

DNA methylation consists in the addition of a methyl group to the fifth carbon of the cytosine ring and is considered to be one of the most important epigenetic mechanisms (Tang et al. 2020; Korotko et al. 2021). The classical epigenetic signature, 5-methylcytosine (5-mC), plays important regulatory functions such as regulation of gene expression (Akhter et al. 2021) and has recently been reported as an

epigenetic marker in response to heat stress, in mammals and plants (Entrambasaguas et al. 2021; Wang et al. 2022). The 5-hydroxymethylcytosine (5-hmC) is another form of cytosine DNA modification and is obtained from the iterative oxidation of 5-mC through the ten-eleven translocation proteins (Wu and Zhang 2017). In mammals and plants, the 5-hmC has been linked to demethylation processes (Richa and Sinha 2014; Shi et al. 2017), but in plants, the biological function of the 5-hmC is still controversial (Kumar et al. 2018). The first discovery of 5-hmC in *Pinus* was reported by our research team in *Pinus radiata* D. Don and more recently noticed in *P. halepensis* Mill., which was associated with an establishment of epigenetic memory (Castander-Olarieta et al. 2020; Pereira et al. 2021).

In previous studies, our research team has reported that high temperatures modulate the different stages of SE in relation to morphology, biochemical and physiological status of embryonal masses (EMs), somatic embryos (ses), and somatic plants of *Pinus* spp. (García-Mendiguren et al. 2016; Castander-Olarieta et al. 2019; Do Nascimento et al. 2020). Castander-Olarieta et al. (2020) reported that the application of heat stress during SE initiation of *P. radiata* provoked the formation of a stable epigenetic memory with a long-term change in the methylation status of the resulting somatic plants. On the other hand, the application of heat stress during SE maturation of *P. radiata* did not affect the number of ses but provoked a significant increase in the stomatal conductance (g_s) and instant leaf transpiration (E) in the resulting somatic plants (Do Nascimento et al. 2020). However, how these mechanisms are also implicated in the epigenome of *ex vitro* somatic plants obtained from ses maturated at high temperatures remains to be elucidated.

P. radiata is a conifer species widely used as a source of wood (Fuentes-Sepúlveda et al. 2020), it has also forestry importance in carbon sequestration in a long term (Mead 2013). However, there is an ecotype-dependent sensitivity to water stress (De Diego et al. 2015). In this sense, the monitoring of the water potential (Ψ_{leaf}), relative water content (*RWC*) and exchange parameters can be used to evaluate stronger changes in plant physiological responses under stress conditions (Neves et al. 2017). Based on physiological parameters, we have reported previously that the somatic plants of *P. radiata* obtained from ses maturated at high temperatures presented better adaptation to drought and heat stress in the greenhouse (Do Nascimento et al. 2020).

Taking into account the abovementioned studies, the objective of this work was to evaluate the influence of high temperatures (50°C after 30 min) applied during the maturation stage of *P. radiata* SE and, consequently, the stress tolerance of somatic plants obtained after the application of heat and water stress in the greenhouse. Moreover, we carried out a biochemical evaluation of these somatic plants in order to study changes that can be attributed to the establishment of an epigenetic memory based on specific modifications of 5-mC and 5-hmC by heat.

Materials And Methods

Plant material

Immature female cones of *P. radiata* were collected in a seed orchard established by Neiker-BRTA in Deba (Spain), and EMs were initiated and proliferated following the protocol described by Montalbán et al. (2012) and maturated following the protocol described by Montalbán et al. (2010) in Embryo Development Medium (EDM) (Walter et al. 2005) (Fig. 1a, b, c, respectively). The cultures in the maturation stage were kept at 23°C for the control treatment during 12 weeks, whereas for the temperature treatment it was applied 50°C for 30 min and then the cultures were kept at control temperature (23°C) for 12 weeks. All cultures were kept in darkness. After this period, the ses were germinated and acclimatized according to Montalbán and Moncaleán (2019). The ses were germinated for 8 weeks in LP Quoirin and Lepoivre (1977), modified by Aitken-Christie et al. (1988), with ½ macronutrients and supplemented with 2 g L^{-1} of activated charcoal and 9 g L^{-1} of Difco Agar granulated (Becton & Dickinson) (Fig. 1d). Then, somatic plantlets were subcultured in the same medium for another month, but in Ecobox® (Eco2box/green filter: a polypropylene vessel with a “breathing” hermetic cover, 125 × 65 × 80 mm, Duchefa) (Fig. 1e). The cultures were kept at 23°C under 16 h photoperiod at 120 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TFL 58 W/33; Philips, France).

The plantlets obtained were acclimatized in the greenhouse under controlled conditions at 23°C, in 43 cm^3 pots containing blond peat moss (Pindstrup, Ryomgård, Denmark): vermiculite (8:2, v/v) (Fig. 1f). After eight weeks the surviving plantlets were transplanted to 2.18 l (90 mm × 270 mm) containing a new substrate of the same composition but adding 3 g L^{-1} Osmocote® Topdress fertilizer (Everris, Geldermalsen, The Netherlands) (Castander-Olarieta et al. 2020).

Growth conditions

Fifteen months old somatic plants (Fig. 1g) growing in the greenhouse and generated under the conditions previously described in the Plant material section were used in the greenhouse experiments. Two environmental stress conditions were applied: the Experiment I - heat experiment and the Experiment II - drought experiment (Fig. 2). Water parameters and gas exchange parameters were analyzed in all plants in both experiments. In addition, a global DNA methylation/hydroxymethylation analysis was performed in all plants.

Experiment I - heat experiment

The heat experiment (Fig. 2) comprised 10 somatic plants obtained from EMs maturated at 23°C. Five somatic plants were kept at 23°C for 20 days as a control treatment and another five somatic plants with the same genotypes were kept in the following conditions as a heat treatment. During the first five days, these somatic plants were exposed to 40°C for two hours each day, being kept at 23 ºC the remaining 22 hours of the day. The next two days, the somatic plants were kept at 23 ºC, the following five days, the somatic plants were again exposed to 40°C for two hours each day, being kept at 23 ºC for the remaining 22 hours of the day. For the remaining eight days, the somatic plants were kept at 23 ºC until completing a total of 20 days. All the plants were watered weekly (Fig. 2).

Experiment II - drought experiment

The drought experiment comprised a total of 20 plants. Five plants obtained from maturation at 50°C and five plants from the same genotypes maturated at control conditions were subjected to a drought stress treatment by the complete suppression of watering, and the remaining plants (five plants obtained from maturation at 50°C and five plants from the same genotypes maturated at control conditions) were watered weekly (Fig. 2). These last control plants were used to verify if plants coming from different treatments could present varying behaviours in control conditions and thus interfere with the results obtained at drought conditions (Castander-Olarieta et al. 2021). In this experiment, all plants, regardless of the irrigation condition, were submitted to a greenhouse´s temperature at 40°C following the conditions described previously in the heat treatment of Experiment I - heat experiment section (Fig. 2). All plants were watered to maximum retention capacity of the substrate before the start of the experiment, and drought conditions were maintained until at least one plant from drought treatment started to present external symptoms of drought stress such as needle epinasty (20 days) (De Diego et al. 2012).

Water parameters and gas exchange parameters

The water potential (Ψ_{leaf}, MPa) was measured at predawn using a Scholander chamber (Skye SKPM 1400) (Scholander et al. 1965) in one needle per plant following the methodology described by De Diego et al. (2012).

Relative water content (*RWC*, %) was measured in two needles collected from the apical area of each plant following the method described by De Diego et al. (2012). Briefly, the needle fresh weight (FW) was recorded and then samples were immersed in deionized water and maintained overnight in the dark. After 24 h, the excess of water from the surface of the needles was carefully removed by gently pressing them over filter paper, turgid weight (TW) was registered and needles were dried at 60°C for 48 h. After drying, needles were reweighed and dry weight (DW) was recorded. Relative water content was estimated using the following equation:

$$RWC\,(\%)=(FW\text{-}DW)/(TW\text{-}DW)\times 100$$

Electrolyte leakage (*E.L.*, %) to determine leaf membrane damage was measured following the method described by De Diego et al. (2012), using the conductometric method (Bajji et al. 2002). Briefly, two needles per sapling and treatment were collected in both growth conditions, washed, and put in a test tube with 5 mL of deionized water. Electrolytic conductivity was measured using a portable conductivity meter (Cole Parmer Model 19101-10) at the collection date (EC_i) and after 24h (EC_f). Thereafter, samples were autoclaved for 10 min at 121°C and cooled at room temperature to measure the total electrical conductivity (EC_t). The *E.L.* was calculated according to the following equation:

$$E.L.\,(\%)=[(EC_f\text{-}EC_i)/(EC_t\text{-}EC_i)]\times 100$$

The response g_s (mmol H_2O m^{-2} s^{-1}) and E (mmol H_2O m^{-2} s^{-1}) were quantified at midday with a LI-6400XT Portable Photosynthesis System (Li-Cor Biosciences) equipped with the 6400-05 Conifer

Chamber (Li-Cor Biosciences).

DNA extraction and global DNA methylation/hydroxymethylation analysis

Genomic DNA was extracted from needles of the somatic plants generated under the conditions previously described in the Growth conditions section following the methodology described in Castander-Olarieta et al. (2020). Briefly, 100 mg of fresh tissue of needles grounded in liquid nitrogen to a fine powder was employed. All samples were extracted in 800 µL preheated (60°C) buffer containing 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 2% PVP (w/v), 8 mM ascorbic acid and 5 mM DIECA, supplemented with 89 mM β-mercaptoethanol. Samples were incubated at 65°C for 30 min (gently shaking each 10 min), followed by the addition of 500 µL chloroform/isomylalcohol (24:1, v/v). After vortexing and centrifugation at 10,000 rpm for 5 min, the aqueous phase was transferred to a new tube and RNA was digested by the addition of 10 µL of RNase A (50 mg/mL, Sigma-Aldrich) at 37°C for 1 h. Phase separation by chloroform/isoamylalcohol was repeated once and then DNA was precipitated by the addition of 600 µL cold isopropanol (-20°C) and centrifugation at 11,000 rpm for 10 min at 4°C. DNA pellet was washed with 1 mL ethanol 50%, centrifuged at 14,000 rpm for 5 min, and the supernatant discarded. Pellets were air-dried and DNA was resuspended in 50 µL ultra-pure water. Quantification was carried out using a Nanodrop^{TM} 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was hydrolyzed as follows: 10 µL of DNA containing approximately 1 µg DNA were denaturalized at 100°C for 2 min and digested by the addition of 1.13 µL sodium acetate 50 mM and zinc chloride 40 mM solution and 2.5 µL nuclease P1 (2.5 U, Sigma-Aldrich). Samples were incubated at 37°C overnight. Then 2.5 µL Tris buffer (0.5 M, pH 8.3) and 1 µL alkaline phosphatase (0.3 U, Sigma) were added and incubated at 37°C for 2 h and 30 min. After the addition of 40 µL ultra-pure water and precipitation with 200 µL pure ethanol (-20°C) plus centrifugation at 15,000 for 15 min (4°C), supernatants were transferred to low-binding tubes and evaporated using a vacuum-concentrator. Finally, digested free nucleotides were resuspended in 100 µL ultra-pure water. Methylation and hydroxymethylation levels were analyzed on a 1200 Series HPLC system coupled to a 6410 Triple Quad mass spectrometer from Agilent Technologies (Santa Clara, CA, USA). The chromatographic separation was performed on a Zorbax SB-C18 column (2.1 × 100 mm, 3.5 µm, Agilent Technologies). The mobile phase was 11% methanol and 0.1% formic acid in water and 5 µL of samples were injected in the column at a flow rate of 0.1 mL min^{-1}. The electrospray ionization source (ESI) was operated in the positive ion multiple reaction monitoring mode (MRM) set to an ion spray voltage of 3500 V, 40 psi for nebulizer and source temperature at 350°C. The intensity of specific MH+→fragment ion transitions was recorded (5-mC m/z 242→126, 5-hmC m/z 258→142 and C m/z 228→112). Identification of cytosine, 5-mC and 5-hmC were assessed by injection of commercial standards (5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set, Zymo Research, Irvine, CA, USA) under the same LC-ESI-MS/ MS-MRM conditions. The measured percentage of 5-mC and 5-hmC in each experimental sample was calculated from the MRM peak area divided by the combined peak areas for 5 plus 5-hmC plus cytosine (total cytosine pool). In the case of 5-hmC, its percentage in respect to the total modified cytosine pool was also calculated.

Statistical analysis

To assess the effect of the treatments on water and gas exchange parameters and DNA methylation/hydroxymethylation quantification, an analysis of variance (ANOVA) was conducted. The differences in means between treatment combinations were assessed using Tukey's post hoc tests ($p \leq 0.05$) adjusted for multiple comparisons.

The matrix of residuals, obtained from the fitted model in the analysis of variance, was used to calculate Pearson's correlation coefficients between global DNA methylation/hydroxymethylation analysis, Ψ_{leaf}, g_s and E. The significance of the correlation coefficients was verified using a t test. The data obtained in each treatment were also used to build a correlation matrix per treatment.

All the data were analyzed using the R software® (R Core Team, 2021).

Results

Physiological parameters

Experiment I - heat experiment

Greenhouse temperature affected significantly the Ψ_{leaf} and E in plants coming from EMs maturated at 23°C (control temperature) (Table 1). Plants cultured at 23°C had significantly higher negative Ψ_{leaf} (MPa) (-0.40 ± 0.07) than those plants that grew at 40°C (-1.10 ± 0.10). On contrary, the plants kept in the heat stress had a higher E (mmol H_2O m^{-2} s^{-1}) (10.04 ± 0.22) than those kept at 23 ºC (7.32 ± 0.11). For the other physiological parameters (*RWC*, *E.L.* and g_s) no statistically significant differences were observed (Table 1).

Table 1

Analysis of variance (ANOVA) to assess the effect of different temperature conditions (TC, 23°C or 40°C) in the greenhouse on the: water potential (Ψ_{leaf}, MPa), relative water content (*RWC*, %), electrolyte leakage (*E.L.*, %), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), and instant transpiration (*E*, mmol H_2O m^{-2} s^{-1}) in plants obtained from embryonal masses of *Pinus radiata* D. Don maturated at 23°C in the Experiment I - heat experiment. And ANOVA to assess the effect of different maturation temperatures (MT, 23°C or 50°C) on the: Ψ_{leaf} (MPa), *RWC* (%), *E.L.* (%), g_s (mmol H_2O m^{-2} s^{-1}), and *E* (mmol H_2O m^{-2} s^{-1}) in plants of *P. radiata* under irrigation or no irrigation conditions (I) in the Experiment II - drought experiment

Experiment I - heat experiment				
	Effect	**df**	**F-value**	**p-value**
Ψ_{leaf}	TC	1	33.09	≤0.001 ***
RWC (%)	TC	1	0.47	>0.05 ns
E.L. (%)	TC	1	2.65	>0.05 ns
g_s	TC	1	2.57	>0.05 ns
E	TC	1	96.64	≤0.001 ***
Experiment II - drought experiment				
Before drought experiment				
	Effect	**df**	**F-value**	**p-value**
$\Psi_{leafinitial}$	MT	1	8.03	≤0.05 *
$RWC_{initial}$ *(%)*	MT	1	0.19	>0.05 ns
$E.L._{initial}$ *(%)*	MT	1	4.98	≤0.05 *
$g_{sinitial}$	MT	1	1.22	>0.05 ns
$E_{initial}$	MT	1	1.86	>0.05 ns
After drought experiment				
$\Psi_{leaffinal}$	MT	1	13.86	≤0.01 **
	I	1	20.25	≤0.001 ***
	MT x I	1	0.03	>0.05 ns
RWC_{final} *(%)*	MT	1	1.26	>0.05 ns

*; **; ***: Significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively; ns: Non-significant at $p \leq 0.05$; df: degrees of freedom

	Experiment I - heat experiment			
	I	1	0.74	>0.05 ^{ns}
	MT x I	1	0.30	>0.05 ^{ns}
$E.L._{final}$ (%)	MT	1	2.85	>0.05 ^{ns}
	I	1	4.93	>0.05 ^{ns}
	MT x I	1	4.25	>0.05 ^{ns}
g_{sfinal}	MT	1	0.04	>0.05 ^{ns}
	I	1	242.63	≤0.001 ***
	MT x I	1	8.21	≤0.05 *
E_{final}	MT	1	1.81	>0.05 ^{ns}
	I	1	245.67	≤0.001 ***
	MT x I	1	11.41	≤0.01 **

*; **; ***: Significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively; ^{ns}: Non-significant at $p \leq 0.05$; df: degrees of freedom

Experiment II - drought experiment

Different maturation temperatures affected significantly the $\Psi_{leaf initial}$ and the $E.L._{initial}$, but did not affect the $RWC_{initial}$, $g_{sinitial}$, and the $E_{initial}$ in plants of *P. radiata* before drought experiment (Table 1). The plants obtained from EMs maturated at 23°C had a significantly higher negative $\Psi_{leaf initial}$ (MPa) (-0.71 ± 0.12) than those maturated at a temperature of 50°C (-1.10 ± 0.97). For the $E.L._{initial}$ (%), a significantly low value was observed in somatic plants obtained from EMs maturated at 23°C (5.77 ± 1.01) than those plants obtained from EMs maturated at 50°C (10.80 ± 2.02).

On the other hand, when plants were water-stressed statistically significant differences for $\Psi_{leaf final}$, g_{sfinal} and the E_{final} were found (Table 1). In this case, plants obtained from EMs maturated at 50°C had a significantly higher $\Psi_{leaf final}$ (MPa) (-0.97 ± 0.10) than those plants obtained from EMs maturated at 23°C (-1.31 ± 0.08). Moreover, the water-stressed plants had a significant lower in the $\Psi_{leaf final}$ (MPa) (-1.34 ± 0.07) when compared to irrigated plants (-0.94 ± 0.09). Although statistically significant differences were not observed for $E.L._{final}$ and RWC_{final}, low values of $E.L._{final}$ (> 740 %) and high values of RWC_{final} (> 8679 %) were observed in all plants.

A significantly higher g_{sfinal} was observed in irrigated plants obtained from EMs exposed to high maturation temperature (50°C) followed by irrigated plants obtained from EMs maturated at control temperature (23°C), while the water-stressed plants obtained from EMs in both maturation temperatures showed a significantly lower g_{sfinal} than the others (Fig. 3a).

As shown in Fig. 3b, the plants under irrigation conditions maintained similar E_{final} values regardless of the maturation temperature, however, when the plants were water-stressed, the plants coming from EMs maturated at 50°C displayed lower E_{final} values than those maturated at control conditions (Fig. 3b).

Global DNA methylation/hydroxymethylation analysis

Experiment I – heat experiment

P. radiata plants obtained from EMs maturated at 23°C and grown under different temperatures in the greenhouse did not show statistically significant differences in 5-mC levels (Table 2). However, statistically significant differences were found for 5-hmC and the percentage of hydroxylated cytosine forms with respect to the total amount of modified cytosine bases (5-hmC/5-mC × 100) (Table 2).

Table 2

Analysis of variance (ANOVA) to assess the effect of different temperature conditions (TC, 23°C or 40°C) in the greenhouse on the methylation and hydroxymethylation rates of needles of plants obtained from embryonal masses of *Pinus radiata* D. Don maturated at 23°C in the Experiment I – heat experiment. And ANOVA for the effect of different maturation temperatures (MT, 23°C or 50°C) on the methylation and hydroxymethylation rates of needles of *P. radiata* plants grown under irrigation or no irrigation conditions (I)

Experiment I – heat experiment							
Effect	**df**	**5-mC %**		**5-hmC %**		**5-hmC/5-mC × 100**	
		F-value	*p*-value	F-value	*p*-value	F-value	*p*-value
TC	1	0.25	>0.05 ^{ns}	5.40	≤0.05 *	6.29	≤0.05 *
Experiment II - drought experiment							
Effect	**df**	**5-mC %**		**5-hmC %**		**5-hmC/5-mC × 100**	
		F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
MT	1	2.65	>0.05 ^{ns}	2.57	>0.05 ^{ns}	2.44	>0.05 ^{ns}
I	1	0.11	>0.05 ^{ns}	0.05	>0.05 ^{ns}	0.08	>0.05 ^{ns}
MT x I	1	0.01	>0.05 ^{ns}	0.02	>0.05 ^{ns}	0.01	>0.05 ^{ns}

*: Significant differences at $p \leq 0.05$; ^{ns}: Non-significant at $p \leq 0.05$; df: degrees of freedom

Although non-significant differences were detected between the different temperature conditions in the greenhouse for the 5-mC, 5-mC levels higher than 37% in both treatments were observed (Table 3). Nevertheless, the change of growth conditions provoked a significant lower in the 5-hmC and in the 5-hmC/5-mC ratio when plants were exposed to heat stress (40°C) in the greenhouse (Table 3), indicating that DNA methylation of *P. radiata* plants was lower with heat stress in the growth conditions assayed.

Table 3
Methylation and hydroxymethylation rates of needles of *Pinus radiata* D. Don plants obtained from embryonal masses maturated at 23°C and grown in different temperature conditions (23°C or 40°C) in the greenhouse in the Experiment I – heat experiment. And methylation and hydroxymethylation rates of needles of *P. radiata* plants obtained from embryonal masses maturated at different maturation temperatures (MT, 23°C or 50°C) and grown under irrigation (UI) or no irrigation (NI) conditions in the Experiment II - drought experiment

Experiment I – heat experiment			
Treatment (°C)	**5-mC %**	**5-hmC %**	**5-hmC/5-mC × 100**
23	38.40 ± 0.23 [a]	0.0129 ± 0.0006 [a]	0.0335 ± 0.0014 [a]
40	37.98 ± 0.75 [a]	0.0089 ± 0.0015 [b]	0.0233 ± 0.0035 [b]
Experiment II - drought experiment			
Treatment	**5-mC %**	**5-hmC %**	**5-hmC/5-mC × 100**
23°C -UI	37.98 ± 0.75	0.0089 ± 0.0015	0.0233 ± 0.0035
50°C -UI	38.77 ± 0.39	0.0114 ± 0.0014	0.0292 ± 0.0034
23°C -NI	37.83 ± 0.34	0.0094 ± 0.0018	0.0247 ± 0.0047
50°C -NI	38.60 ± 0.31	0.0115 ± 0.0007	0.0298 ± 0.0020
Data are presented as mean values ± SE. Significant differences among treatments at $p<0.05$ are indicated by different letters in the column			

Experiment II - drought experiment

Regardless of the maturation temperature, when *P. radiata* plants were exposed to irrigation or no irrigation conditions, no statistically significant differences were observed for global DNA methylation/hydroxymethylation analysis (Table 2, Table 3).

Correlation analysis between global 5-mC%, 5-hmc%, and physiological parameters

Experiment I - heat experiment

Different conditions in the greenhouse affected the correlation between physiological parameters and the global 5-mC, 5-hmC profiles. In this sense, when the control plants were exposed to different temperatures in the greenhouse, significantly positive correlations between 5-mC and Ψ_{leaf}, and 5-mC and E were observed in the plants growing at 23°C in the greenhouse (Fig. 4a). However, when the plants obtained from EMs maturated at 23°C grew at 40°C in the greenhouse significantly negative correlations between the 5-hmC and Ψ_{leaf} and 5-hmC/5-mC ratio and Ψ_{leaf} were observed (Fig. 4b).

Experiment II - drought experiment

When correlation analysis between global 5-mC, 5-hmC, the 5-hmC/5-mC ratio and physiological parameters were analyzed (Fig. 5) for drought experiment, it was observed that somatic plants of *P. radiata* that had high levels of 5-hmC (Fig. 6b) and 5-hmC/5-mC ratio (Fig. 6c) showed low $\Psi_{leaf\text{final}}$ with a significantly negative correlation between them. On the other hand, the somatic plants that lost more water through the epidermis were also the somatic plants with the low 5-mC contents, with a significantly negative correlation between g_s and 5-mC contents (Fig. 6d), as well as, a marginally significant negative correlation between E and 5-mC contents was observed (Fig. 6g). For the other correlation analyses, a significant correlation was not observed (Fig. 6a, e, f, h, i).

When we analyzed the values of different variables in each treatment for drought experiment, the positive correlation between 5-hmC and 5-mC was predominant in all treatments (Fig. 4c, d and e), except in the somatic plants obtained from EMs maturated at 50°C under drought conditions (Fig. 4f). Furthermore, those plants obtained from maturation temperature at 23°C and maintained in irrigation conditions had a significantly negative correlation between the $\Psi_{leaf\text{final}}$ and 5-hmC contents, as well as, a significantly negative correlation with the 5-hmC/5-mC ratio was observed (Fig. 4c). However, when the plants obtained from this temperature of maturation (23°C) were maintained under drought stress, a significantly negative correlation was observed between the $g_{s\text{final}}$ with 5-hmC contents and $g_{s\text{final}}$ with the 5-hmC/5-mC ratio (Fig. 4d). The same significantly negative correlation was observed between the E_{final} with 5-hmC contents and E_{final} with 5-hmC/5-mC ratio (Fig. 4d). On the other hand, the plants obtained from EMs maturated at 50°C and maintained in irrigation conditions had a marginally negative correlation between $g_{s\text{final}}$ and 5-mC contents (p = 0.057) (Fig. 4e). However, the plants obtained from EMs maturated at 50°C and maintained in drought stress did not show a significant correlation between the physiological parameters ($\Psi_{leaf\text{final}}$, $g_{s\text{final}}$ and E_{final}) and the global 5-mC, 5-hmC and the 5-hmC/5-mC ratio (Fig. 4f).

Discussion

High temperature is one of the main forms of abiotic stress in nature (Bita and Gerats 2013). Although is often aggravated by additional abiotic stresses such as drought, it is important to study the independent action and biological consequences of high temperature to alleviate the effects of combined abiotic stress in plants (Bita and Gerats 2013; Lamaoui et al. 2018; Imran et al. 2021). In this work, when one-year and three-month-old *P. radiata* somatic plants were grown at 40°C in the greenhouse, their Ψ_{leaf} was significantly lower than the Ψ_{leaf} of the plants growing at 23°C. In contrast, we reported in a previous study (Do Nascimento et al. 2020) that the Ψ_{leaf} of six-month-old *P. radiata* somatic plants growing at 23°C was significantly lower (-0.3 MPa) than in those growing at 40°C (-0.26 MPa) in the greenhouse, indicating that the Ψ_{leaf} is not constant under the same growing conditions, but it varies with the age of the *P. radiata* somatic plants as reported in other conifers (Rosner et al. 2019).

Drought triggers an instructional mechanism that guides the epigenetic machinery, causing plants to respond more quickly and effectively to a subsequent drought event (Colaneri and Jones 2013; Akhter et

al. 2021). The degree of resistance to drought and, consequently, changes in physiological parameters depend on several factors such as phenological stages and exposure time (Ozturk et al. 2021). Our results in the drought experiment showed that *P. radiata* somatic plants derived from EMs maturated at high maturation temperature (50°C, 30 min) showed higher $\Psi_{leaf\text{final}}$ and a significant lower E_{final} when exposed to drought conditions, indicating that these somatic plants showed a much higher degree of regulation of water loss under drought conditions than those obtained from EMs maturated at 23°C. In line with these findings, similar reductions in E and Ψ_{leaf} of somatic plants obtained from EMs of *P. radiata* initiated at high temperatures (40°C, 4 h; 50°C, 30 min; 60°C, 5 min) and maintained under water stress were observed by Castander-Olarieta et al. (2021). In this way, in a short time, the regulation of water loss in isohydric plants, such as *P. radiata*, allows plants to limit the transpiration losses and, thus, keep Ψ_{leaf} within tolerable limits (Martínez-Vilalta and Garcia-Forner 2017), avoiding complete stomatal closure without severe restrictions on carbon assimilation and tissue preservation against dehydration (Meinzer et al. 2014). Furthermore, a reduction in E in *Fragaria x ananassa* Duch. plants under saline stress was related to a state of pre-adaptation to saline and/or drought stress, preserving tissues from dehydration and a more effective adjustment to the hyperosmotic environment (Orsini et al. 2012).

The abovementioned results confirmed the hypothesis that stresses applied at different stages of SE can induce memory in the ses that is maintained at later stages of plant development (Do Nascimento et al. 2020; Castander-Olarieta et al. 2021; Pérez-Oliver et al. 2021). Similarly, two-year-old *P. pinaster* Ait. plants derived from EMs induced at high initiation temperature (50°C, 3 h) showed an improvement in defense mechanisms against drought stress with a better osmotic adjustment and higher chlorophyll, soluble sugars and starch contents when exposed to high temperatures (45°C, for three hours per 12 days) in the greenhouse (Pérez-Oliver et al. 2021). Furthermore, in our study, the plants coming from EMs maturated at 50°C compared with those plants coming from EMs maturated at 23°C had a higher $g_{s\text{final}}$ under irrigation conditions. This high g_s has been reported to be beneficial for pine growth (Urban et al. 2017). In *P. tabulaeformis* Carr. inoculated with ectomycorrhizal fungi, although the increase in g_s could increase the loss of water, it also permitted that the seedlings, that grew under drought could be carried out more photosynthesis by means of increasing CO_2 absorption (Augé et al. 2015; Gehring et al. 2017; Wang et al. 2021a).

In conifers, low *E.L.* values associated with high *RWC* values were related to a greater capacity of plants to survive in drought conditions (Mantova et al. 2021). In this work, under irrigation, the plants obtained from maturation at 50°C showed a significantly higher $E.L._{\text{initial}}$ (%) (10.80 ± 2.02) than those maturated at 23°C (5.77 ± 1.01), but these differences were not observed when plants were submitted to drought. These values of *E.L.* were considered low when compared with other works with *P. radiata* and other conifers subjected to biotic and abiotic stress (De Diego et al. 2012). For example, in *P. radiata* plants subjected to biotic stress conditions (infection by *Trichoderma viride* and *Fusarium circinatum*), there was an increase in *E.L.* (approximately 26%) characterizing damage to cell membranes in the stressed plants when compared to control plants (Amaral et al. 2019). Also, an increase of *E.L.* (> 40%) in *Pseudotsuga menziesii* Mirb. Franco plants submitted to drought was reported (Mantova et al. 2021). In addition, the

RWC contents were similar in all treatments showing higher than 86%. Likewise, in *P. pinaster* somatic plants obtained from ses embryos maturated at different temperatures (18, 23, or 28°C) and kept under heat stress (45°C for 3 h/day for 10 days) the *RWC* was not significantly affected regardless of its origin (Sales et al. 2022). The maintenance of *RWC* values in *Pinus* needles is necessary for normal physiological processes, but contrary to our results, in two-year-old *P. sylvestris* needles obtained from mature trees, the *RWC* values decreased by about 27% with high-temperature stress (55° C, 10 min) (Gette et al. 2020).

Our results showed that different stress conditions in *P. radiata* plants did not affect significantly the percentage of 5-mC independently of the maturation temperature or growth condition in the greenhouse. A percentage higher than 37% of 5-mC in all treatments was found. Similar to this, high values of 5-mC were observed in needles of *in vitro* somatic plants of *P. halepensis* Mill. (> 40%) initiated at different temperatures (Pereira et al. 2021) and in needles of one-year-old somatic plants of *P. radiata* (> 37%) obtained from different initiation temperatures (Castander-Olarieta et al. 2020). However, in this last case, the authors reported statistically significant differences between initiation treatments with a significant decrease in the percentage of 5-mC at the highest temperature (60°C, 5 min). Also, contrary to our results, Entrambasaguas et al. (2021) reported that plants of *Posidonia oceanica* L. and *Cymodocea nodosa* (Ucria) Aschers. from different ecotypes showed different epigenetic responses with a modification of the global levels of methylation improving their response to an increase in temperature.

In this work, the percentage of 5-hmC and the 5-hmC/5-mC ratio were affected by the greenhouse temperature in somatic plants from maturation at 23°C with a significant decrease when the greenhouse temperature was 40°C. These results are in line with previous findings by Castander-Olarieta et al. (2020) who reported that the percentage of 5-hmC and the 5-hmC/5-mC ratio varied with different temperatures, but in their case, this variation was due to the initiation temperature of the EMs. On contrary to our results, in *Brassica napus* L. an increase in 5-hmC in response to heat stress was reported (Golubov and Kovalchuk 2017).

We observed that changes in the DNA methylation/hydroxymethylation contents were significantly correlated with changes in the physiological parameters. This fact supports the idea that physiological responses during abiotic stress can be influenced by methylation/hydroxymethylation changes, as previously suggested Castander-Olarieta et al. (2020). In this work, within each treatment, the $\Psi_{leaf\,\mathrm{final}}$ significantly correlated with methylated and hydroxymethylated DNA, being water and thermal dependent; since it is an essential component of any biological system (Singh et al. 2020). Furthermore, the presence or absence of irrigation exerted changes in the state of correlation between these variables in agreement with Colaneri and Jones (2013). However, they reported that drought stress, caused for the addition of polyethylene glycol in the culture medium, imposed on *Arabidopsis* seedlings triggered changes in DNA methylation with hypermethylation of protein-coding genes related to stress responses. Methylation has also been associated with aquaporins, which are proteins that regulate the movement of water across cell membranes, but the methylation does not interfere with the intrinsic permeability of aquaporin to water in *Arabidopsis* (Santoni et al. 2006).

On the other hand, the correlation between g_s and E with 5-mC, 5-hmC, the 5-hmC/5-mC ratio changed in the control somatic plants kept under heat and drought stress. Similar to our results, in *Populus nigra* L. plants grown in medium culture supplemented with 100 - 1000 μM of 5-Azacytidine, which had a significantly negative correlation with DNA methylation level, showed changes in with g_s and E indicating that the phenotypic changes were related to the methylation changes in the regenerated plants (Zhong et al. 2021). Contrariwise, Auler et al. (2021) reported a simultaneous decrease in physiological parameters (g_s and photosynthesis), with 5-mC during the application of recurrent water stress in the reproductive stage of *Oryza sativa* L. plants. In addition, they reported that proteome and the transcripts associated with the guard cells showed a positive correlation between the highest accumulation of proteins and genes with the highest percentages of 5-mC in the vegetative stage and the vegetative and at the reproductive stages. Furthermore, an increase in the DNA methylation associated with phenotypic changes, such as higher antioxidant activity in *Hibiscus cannabinus L.* seedlings was related to chromium tolerance mechanisms, and these changes then affected the expression of specific genes involved in the chromium stress response (Tang et al. 2021). In this sense, several works reported that DNA methylation modulates the expression of various genes and consequently phenotypic changes in response to stressful environmental conditions (He et al. 2021; Tang et al. 2021; Wang et al. 2021b; Su et al. 2022). Thus, in the future, it would be interesting to combine the results of our experiments with genetic analysis to understand how the methylation of genes responsible for responses to water and heat stress affects the physiological parameters in these conditions.

Conclusions

Plants obtained from EMs submitted to a maturation temperature of 50°C (30 min) presented better adaptation to drought stress based on the $\Psi_{leaf\text{final}}$ and E_{final}. Somatic plants kept at heat stress (40 ºC, 10 days) in the greenhouse had lower 5-hmC levels than plants kept at 23 ºC. Furthermore, 5-hmC and 5-hmC/5-mC ratio presented a significantly negative correlation with the changes in the Ψ_{leaf} and a significantly negative correlation between g_s and 5-mC contents was observed.

Declarations

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References

1. Aitken-Christie J, Singh AP, Davies H (1988) Multiplication of meristematic tissue: a new tissue culture system for radiata pine. In: Genetic manipulation of woody plants. Springer, pp 413–432
2. Akhter Z, Bi Z, Ali K, et al (2021) In response to abiotic stress, DNA methylation confers epigenetic changes in plants. Plants 10(6):1096. https://doi.org/10.3390/plants10061096
3. Alsdurf J, Anderson C, Siemens DH (2016) Epigenetics of drought-induced trans-generational plasticity: Consequences for range limit development. AoB Plants 8:146. https://doi.org/10.1093/aobpla/plv146
4. Amaral J, Pinto G, Flores-Pacheco JA, et al (2019) Effect of *Trichoderma viride* pre-inoculation in pine species with different levels of susceptibility to *Fusarium circinatum*: physiological and hormonal responses. Plant Pathol 68:1645–1653. https://doi.org/10.1111/ppa.13080
5. Augé RM, Toler HD, Saxton AM (2015) Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. Mycorrhiza 25:13–24. https://doi.org/10.1007/s00572-014-0585-4
6. Auler PA, Nogueira do Amaral M, Bolacel Braga EJ, Maserti B (2021) Drought stress memory in rice guard cells: Proteome changes and genomic stability of DNA. Plant Physiol Biochem 169:49–62. https://doi.org/10.1016/j.plaphy.2021.10.028
7. Bajji M, Kinet JM, Lutts S (2002) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul 36:61–70. https://doi.org/10.1023/A:1014732714549
8. Bita CE, Gerats T (2013) Plant tolerance to high temperature in a changing environment: Scientific fundamentals and production of heat stress-tolerant crops. Front Plant Sci 4:1–18. https://doi.org/10.3389/fpls.2013.00273
9. Castander-Olarieta A, Moncaleán P, Pereira C, et al (2021) Cytokinins are involved in drought tolerance of *Pinus radiata* plants originating from embryonal masses induced at high temperatures. Tree Physiol 41:912–926. https://doi.org/10.1093/treephys/tpaa055
10. Castander-Olarieta A, Montalbán IA, De Medeiros Oliveira E, et al (2019) Effect of thermal stress on tissue ultrastructure and metabolite profiles during initiation of radiata pine somatic embryogenesis. Front Plant Sci 9:1–16. https://doi.org/10.3389/fpls.2018.02004
11. Castander-Olarieta A, Pereira C, Sales E, et al (2020) Induction of radiata pine somatic embryogenesis at high temperatures provokes a long-term decrease in DNA methylation/hydroxymethylation and differential expression of stress-related genes. Plants 9:1–16. https://doi.org/10.3390/plants9121762

12. Colaneri AC, Jones AM (2013) Genome-wide quantitative identification of DNA differentially methylated sites in *Arabidopsis* seedlings growing at different water potential. PLoS One 8:e59878. https://doi.org/10.1371/journal.pone.0059878
13. De Diego N, Pérez-Alfocea F, Cantero E, et al (2012) Physiological response to drought in radiata pine: Phytohormone implication at leaf level. Tree Physiol 32:435–449. https://doi.org/10.1093/treephys/tps029
14. De Diego N, Saiz-Fernández I, Rodríguez JL, et al (2015) Metabolites and hormones are involved in the intraspecific variability of drought hardening in radiata pine. J Plant Physiol 188:64–71. https://doi.org/10.1016/j.jplph.2015.08.006
15. Do Nascimento AMM, Barroso PA, Do Nascimento NFF, et al (2020) *Pinus* spp. somatic embryo conversion under high temperature: Effect on the morphological and physiological characteristics of plantlets. Forests 11:1–14. https://doi.org/10.3390/f11111181
16. Entrambasaguas L, Ruocco M, Verhoeven KJF, et al (2021) Gene body DNA methylation in seagrasses: inter-and intraspecific differences and interaction with transcriptome plasticity under heat stress. Sci Rep 11:1–15. https://doi.org/10.1038/s41598-021-93606-w
17. Fox H, Doron-Faigenboim A, Kelly G, et al (2018) Transcriptome analysis of *Pinus halepensis* under drought stress and during recovery. Tree Physiol 38:423–441. https://doi.org/10.1093/treephys/tpx137
18. Fuentes-Sepúlveda R, García-Herrera C, Vasco DA, et al (2020) Thermal characterization of *Pinus radiata* wood vacuum-impregnated with octadecane. Energies 13:. https://doi.org/10.3390/en13040942
19. García-Mendiguren O, Montalbán IA, Correia S, et al (2016) Different environmental conditions at initiation of radiata pine somatic embryogenesis determine the protein profile of somatic embryos. Plant Biotechnol 33:143–152. https://doi.org/10.5511/plantbiotechnology.16.0520a
20. Gehring CA, Swaty RL, Deckert RJ (2017) Mycorrhizas, Drought, and Host-Plant Mortality. In Mycorrhizal Mediation of Soil. Elsevier Inc. pp. 279-298
21. Gette IG, Pakharkova N V., Kosov I V., Bezkorovaynaya IN (2020) Influence of high-temperature convective flow on viability of Scots pine needles (*Pinus sylvestris* L.). J For Res 31:1489–1497. https://doi.org/10.1007/s11676-019-00990-1
22. Golubov A, Kovalchuk I (2017) Analysis of DNA hydroxymethylation using colorimetric assay. In: Plant Epigenetics. Springer, pp 89–97
23. He S, Zhang Y, Wang J, et al (2021) H3K4me2, H4K5ac and DNA methylation function in short- and long-term heat stress responses through affecting the expression of the stress-related genes in *G. hirsutum*. Environ Exp Bot 194:104699. https://doi.org/10.1016/j.envexpbot.2021.104699
24. Imran QM, Falak N, Hussain A, et al (2021) Abiotic stress in plants, stress perception to molecular response and role of biotechnological tools in stress resistance. Agronomy 11:. https://doi.org/10.3390/agronomy11081579

25. Korotko U, Chwiałkowska K, Sańko-Sawczenko I, Kwasniewski M (2021) Dna demethylation in response to heat stress in *Arabidopsis thaliana*. Int J Mol Sci 22:1–20. https://doi.org/10.3390/ijms22041555
26. Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J Exp Bot 63:1593–1608. https://doi.org/10.1093/jxb/err460
27. Kumar S, Chinnusamy V, Mohapatra T (2018) Epigenetics of modified DNA bases: 5-Methylcytosine and beyond. Front Genet 9:1–14. https://doi.org/10.3389/fgene.2018.00640
28. Lamaoui M, Jemo M, Datla R, Bekkaoui F (2018) Heat and drought stresses in crops and approaches for their mitigation. Front Chem 6:1–14. https://doi.org/10.3389/fchem.2018.00026
29. Mantova M, Menezes-Silva PE, Badel E, et al (2021) The interplay of hydraulic failure and cell vitality explains tree capacity to recover from drought. Physiol Plant 172:247–257. https://doi.org/10.1111/ppl.13331
30. Martínez-Vilalta J, Garcia-Forner N (2017) Water potential regulation, stomatal behaviour and hydraulic transport under drought: deconstructing the iso/anisohydric concept. Plant Cell Environ 40:962–976. https://doi.org/10.1111/pce.12846
31. Mead DJ (2013) Sustainable management of *Pinus radiata* plantations. FAO Forestry Paper No. 170. Rome, FAO
32. Meinzer FC, Woodruff DR, Marias DE, et al (2014) Dynamics of leaf water relations components in co-occurring iso- and anisohydric conifer species. Plant Cell Environ 37:2577–2586. https://doi.org/10.1111/pce.12327
33. Montalbán IA, De Diego N, Moncaleán P (2012) Enhancing initiation and proliferation in radiata pine (*Pinus radiata* D. Don) somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments. Acta Physiol Plant 34:451–460
34. Montalbán IA, De Diego N, Moncaleán P (2010) Bottlenecks in *Pinus radiata* somatic embryogenesis: improving maturation and germination. Trees 24:1061–1071
35. Montalbán IA, Moncaleán P (2019) Rooting of *Pinus radiata* somatic embryos: factors involved in the success of the process. J For Res 30:65–71
36. Neves DM, Almeida LADH, Santana-Vieira DDS, et al (2017) Recurrent water deficit causes epigenetic and hormonal changes in *Citrus* plants. Sci Rep 7:1–11. https://doi.org/10.1038/s41598-017-14161-x
37. Orsini F, Alnayef M, Bona S, et al (2012) Low stomatal density and reduced transpiration facilitate strawberry adaptation to salinity. Environ Exp Bot 81:1–10. https://doi.org/10.1016/j.envexpbot.2012.02.005
38. Ozturk M, Turkyilmaz Unal B, García-Caparrós P, et al (2021) Osmoregulation and its actions during the drought stress in plants. Physiol Plant 172:1321–1335. https://doi.org/10.1111/ppl.13297
39. Pereira C, Castander-Olarieta A, Sales E, et al (2021) Heat stress in *Pinus halepensis* somatic embryogenesis induction: Effect in DNA methylation and differential expression of stress-related

genes. Plants 10:. https://doi.org/10.3390/plants10112333

40. Pérez-Oliver MA, Haro JG, Pavlović I, et al (2021) Priming maritime pine megagametophytes during somatic embryogenesis improved plant adaptation to heat stress. Plants 10:. https://doi.org/10.3390/plants10030446
41. Quoirin M, Lepoivre P (1977) Etude de milieux adaptes aux cultures *in vitro* de *Prunus*. Acta Hortic 78:437–442
42. R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing.
43. Richa R, Sinha RP (2014) Hydroxymethylation of DNA: an epigenetic marker. EXCLI J 13:592–610
44. Rosner S, Heinze B, Savi T, Dalla-Salda G (2019) Prediction of hydraulic conductivity loss from relative water loss: new insights into water storage of tree stems and branches. Physiol Plant 165:843–854. https://doi.org/10.1111/ppl.12790
45. Sales E, Cañizares E, Pereira C, et al (2022) Changing temperature conditions during somatic embryo maturation result in *Pinus pinaster* plants with altered response to heat stress. Int J Mol Sci 23(3): 1318. https://doi.org/10.3390/ijms23031318
46. Santoni V, Verdoucq L, Sommerer N, et al (2006) Methylation of aquaporins in plant plasma membrane. Biochem J 400:189–197. https://doi.org/10.1042/BJ20060569
47. Scholander PF, Bradstreet ED, Hemmingsen EA, Hammel HT (1965) Sap pressure in vascular plants: negative hydrostatic pressure can be measured in plants. Science (80-) 148:339–346
48. Shahzad A, Ullah S, Dar AA, et al (2021) Nexus on climate change: agriculture and possible solution to cope future climate change stresses. Environ Sci Pollut Res 28:14211–14232. https://doi.org/10.1007/s11356-021-12649-8
49. Shi D-Q, Ali I, Tang J, Yang W-C (2017) New insights into 5hmC DNA modification: generation, distribution and function. Front Genet 8:100. https://doi.org/10.3389/fgene.2017.00100
50. Singh RK, Deshmukh R, Muthamilarasan M, et al (2020) Versatile roles of aquaporin in physiological processes and stress tolerance in plants. Plant Physiol Biochem 149:178–189. https://doi.org/10.1016/j.plaphy.2020.02.009
51. Su T, Fu L, Kuang L, et al (2022) Transcriptome-wide m6A methylation profile reveals regulatory networks in roots of barley under cadmium stress. J Hazard Mater 423:127140. https://doi.org/10.1016/j.jhazmat.2021.127140
52. Tang D, Gallusci P, Lang Z (2020) Fruit development and epigenetic modifications. New Phytol 228:839–844. https://doi.org/10.1111/nph.16724
53. Tang M, Yue J, Huang Z, et al (2021) Physiological and DNA methylation analysis provides epigenetic insights into chromium tolerance in kenaf. Environ Exp Bot 194:104684. https://doi.org/10.1016/j.envexpbot.2021.104684
54. Urban J, Ingwers M, McGuire MA, Teskey RO (2017) Stomatal conductance increases with rising temperature. Plant Signal Behav 12:. https://doi.org/10.1080/15592324.2017.1356534

55. Vaissière T, Sawan C, Herceg Z (2008) Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res - Rev Mutat Res 659:40–48. https://doi.org/10.1016/j.mrrev.2008.02.004
56. Walter C, Find JI, Grace LJ (2005) Somatic embryogenesis and genetic transformation in *Pinus radiata*. In: Protocol for somatic embryogenesis in woody plants. Springer, pp 11–24
57. Wang J, Zhang H, Gao J, et al (2021a) Effects of ectomycorrhizal fungi (*Suillus variegatus*) on the growth, hydraulic function, and non-structural carbohydrates of *Pinus tabulaeformis* under drought stress. BMC Plant Biol 21:1–13. https://doi.org/10.1186/s12870-021-02945-3
58. Wang Q, Wang W, Sun B, Zhang A (2022) Genomic DNA hydroxymethylation reveals potential role in identification of lung injury in coal-burning arsenicosis populations. Environ Res 204:112053. https://doi.org/https://doi.org/10.1016/j.envres.2021.112053
59. Wang S, Yan W, Yang X, et al (2021b) Comparative methylome reveals regulatory roles of DNA methylation in melon resistance to *Podosphaera xanthii*. Plant Sci 309:110954. https://doi.org/10.1016/j.plantsci.2021.110954
60. Wu X, Zhang Y (2017) TET-mediated active DNA demethylation: mechanism, function and beyond. Nat Rev Genet 18:517–534. https://doi.org/10.1038/nrg.2017.33
61. Zhong S, Gao Y, Lu J, et al (2021) Production of an epigenetic mutant population of *Populus nigra*: DNA methylation and phenotype analyses. J Plant Biochem Biotechnol 30:354–363. https://doi.org/10.1007/s13562-020-00600-w

Figures

Figure 1

Somatic embryogenesis in *Pinus radiata* D. Don: **a** Extrusion of embryonal masses (EMs) (bar = 5 mm; eight weeks), **b** proliferation of EMs (bar = 2 cm; 12 weeks), **c** cotyledonary somatic embryos at the end of maturation stage after heat stress application (bar = 5 mm; 12 weeks), **d** germination of somatic embryos in Petri dishes (bar = 2 cm; eight weeks), **e** germinated somatic embryos in an Ecobox® (bar = 4 cm; four weeks) and; **f** acclimatization of plantlets in the greenhouse (bar = 5 cm; eight weeks). **g** *P. radiata* plants obtained from embryonal masses maturated at high temperatures (23 and 50 °C) before the drought experiment in the greenhouse (bar = 9 cm)

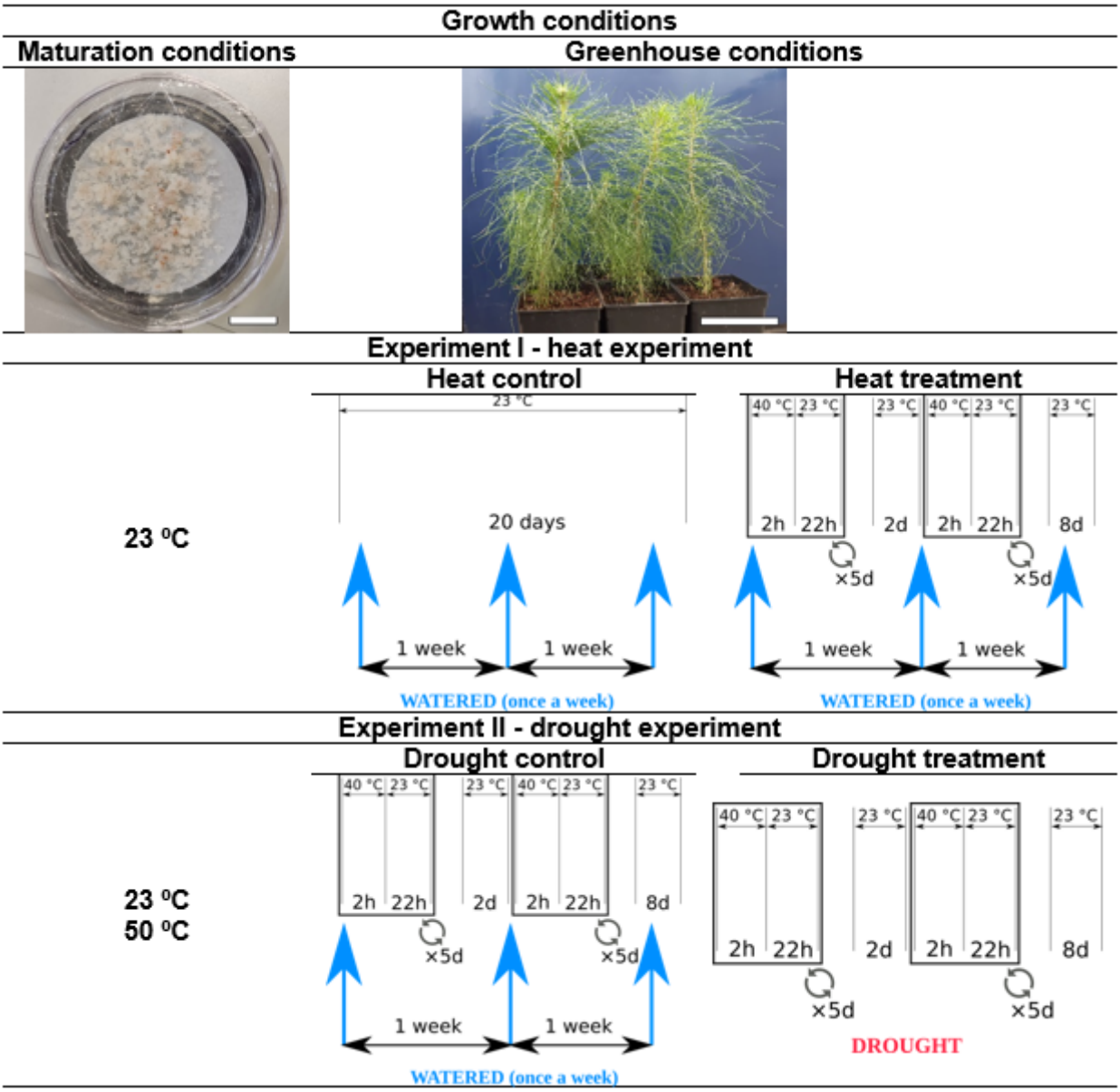

Figure 2

Experimental design for different *in vitro* (Maturation conditions) and *ex vitro* (heat experiment and drought experiment) conditions in the maturation of embryonal masses of *Pinus radiata* D. Don (bar = 2 cm) and the growth of somatic plants obtained (bar = 9 cm), respectively. d: days; h: hours

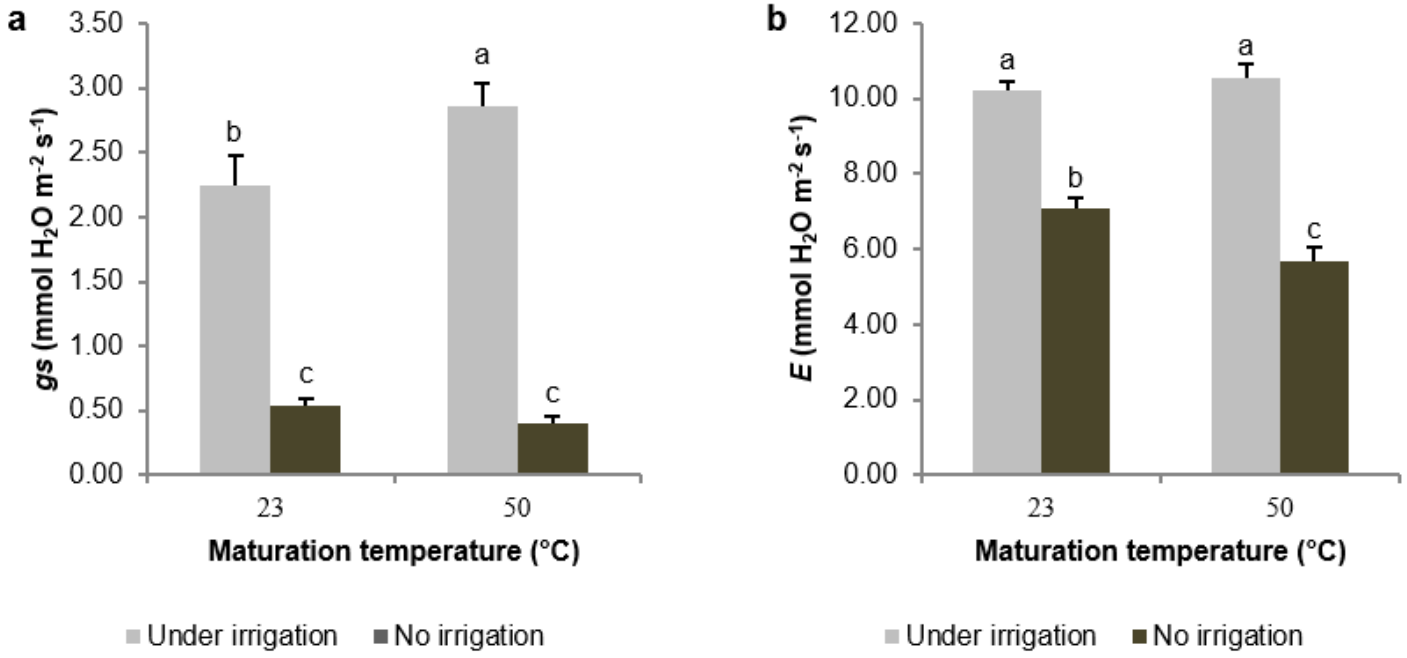

Figure 3

Physiological parameters in plants under irrigation or no irrigation conditions obtained from embryonal masses of *Pinus radiata* D. Don submitted to different maturation temperatures (23 °C or 50 °C). **a** stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}); and **b** instant transpiration (E, mmol H_2O m^{-2} s^{-1}). Bars indicate standard errors. Different letters show significant differences according to the Tukey's post hoc test ($p \leq 0.05$)

Figure 4

Pearson's correlation network of global 5-methylcytosine (5-mC, %), 5-hydroxymethylcytosine (5-hmC, %), the 5-hmC/5-mC ratio (5-hmC/5-mC, %) and physiological parameters in plants of *Pinus radiata* D. Don; red lines represent negative correlation with $R < -0.57$ and green lines represent correlation with $R > 0.0$ according to Pearson's correlation coefficient (R). Y_{leaf} water potential (MPa); g_s stomatal conductance (mmol H_2O m^{-2} s^{-1}); E instant transpiration (mmol H_2O m^{-2} s^{-1})

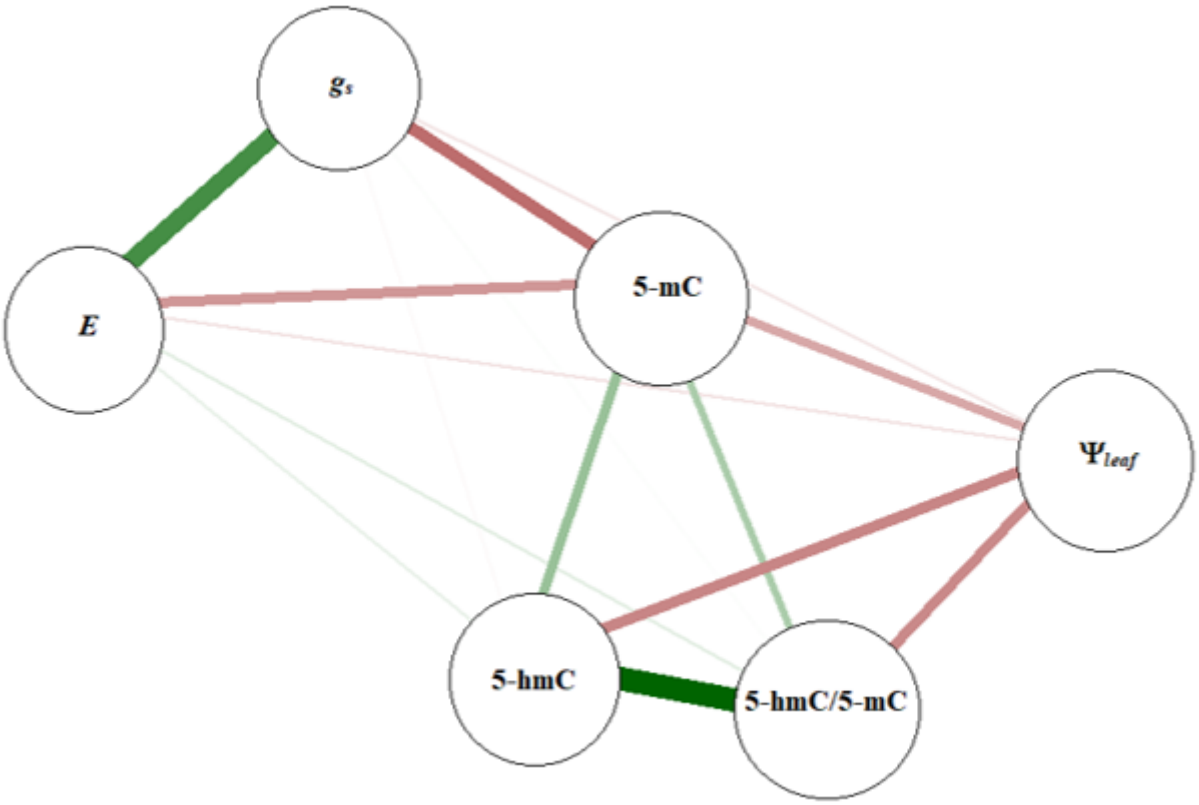

Figure 5

Pearson's correlation network of global 5-methylcytosine (5-mC, %), 5-hydroxymethylcytosine (5-hmC, %), the 5-hmC/5-mC ratio (5-hmC/5-mC, %) and physiological parameters in plants of *Pinus radiata* D. Don; red lines represent negative correlation with $R < -0.57$ and green lines represent correlation with $R > 0.0$ according to Pearson's correlation coefficient (R). Y_{leaf} water potential (MPa); g_s stomatal conductance (mmol H_2O m^{-2} s^{-1}); E instant transpiration (mmol H_2O m^{-2} s^{-1})

Figure 6

Pearson correlation pair between relationships between water potential (Y_{leaf}, MPa) and **a** 5-methylcytosine (5-mC, %), **b** 5-hydroxymethylcytosine (5-hmC, %), and **c** the 5-hmC/5-mC ratio (5-hmC/5-mC x 100, %). Person correlations between stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) and **d** 5-mC, **e** 5-hmC and **f** the 5-hmC/5-mC x 100. Finally, Pearson correlations between instant transpiration (E, mmol H_2O m^{-2} s^{-1}) and **g** 5-mC, **h** 5-hmC and **i** the 5-hmC/5-mC x 100. *; **: Significant correlations at $p \leq 0.05$ or $p \leq 0.01$, respectively, according to a t test