

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 °C, 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) matured at control temperature and cultivated in a greenhouse were submitted to heat stress (40 °C, 2 h, 10 days; 23 °C, 10 days) or at a control temperature (23 °C, 20 days); while another 20 SP resulting from ses matured 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 matured at 50 °C 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 °C. 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 during maturation and hardening of somatic plants provoked drought adaptation and epigenetic changes.

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 matured at high temperatures remains to be elucidated.

Pinus 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 matured 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 matured following the protocol described by Montalbán et al. (2010) in Embryo Development Medium (EDM) (Walter et al. 2005) (Fig. 1a–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 seeds were germinated and acclimatized according to Montalbán and Moncaleán

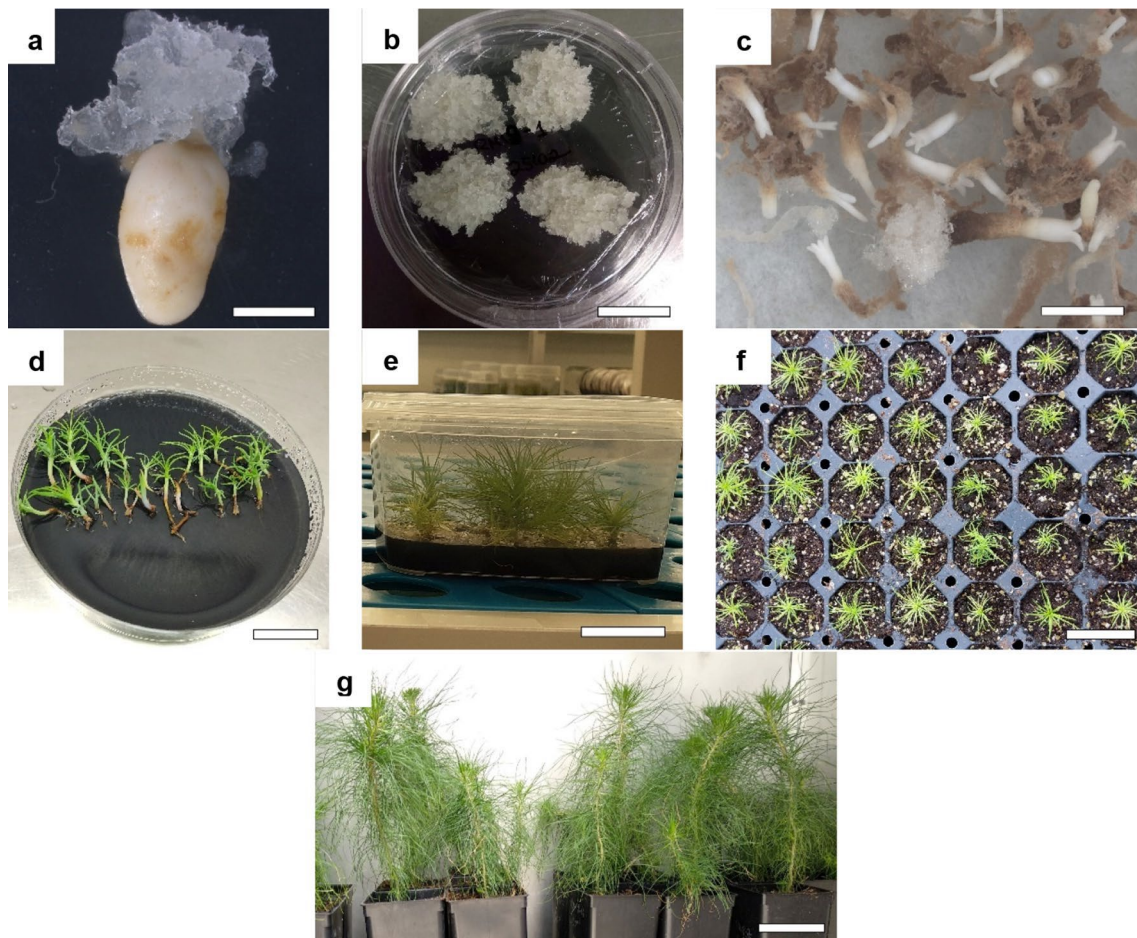


Fig. 1 Somatic embryogenesis in *Pinus radiata* D. Don: **a** Extrusion of embryonal masses (EMs) (bar = 5 mm; 8 weeks), **b** proliferation of EMs (bar = 2 cm; 12 weeks), **c** cotyledonary somatic embryos at the end of maturation stage after heat stress application (bar = 5 mm; 12 weeks), **d** germination of somatic embryos in Petri dishes (bar = 2 cm; 8 weeks), **e** germinated somatic embryos in an Eco-box® (bar = 4 cm; 4 weeks) and **f** acclimatization of plantlets in the greenhouse (bar = 5 cm; 8 weeks). **g** *P. radiata* plants obtained from embryonal masses matured at high temperatures (23 and 50 °C) before the drought experiment in the greenhouse (bar = 9 cm)

(2019). The seeds were germinated for 8 weeks in LP Quoirin and Lepoivre (1977), modified by Aitken-Christie et al. (1988), with $\frac{1}{2}$ 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 Eco-box® (Eco2box/green filter: a polypropylene vessel with a “breathing” hermetic cover, $125 \times 65 \times 80 \text{ mm}$, Duchefa) (Fig. 1e). The cultures were kept at 23 °C under 16 h photoperiod at $120 \mu\text{mol m}^{-2} \text{ s}^{-1}$ provided by cool white

fluorescent tubes (TFL 58 W/33; Philips, France).

The plantlets obtained were acclimatized in the green-house under controlled conditions at 23 °C, in 43 cm³ pots containing blond peat moss (Pindstrup, Ryomgård, Denmark): vermiculite (8:2, v/v) (Fig. 1f). After 8 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® Top-dress 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.

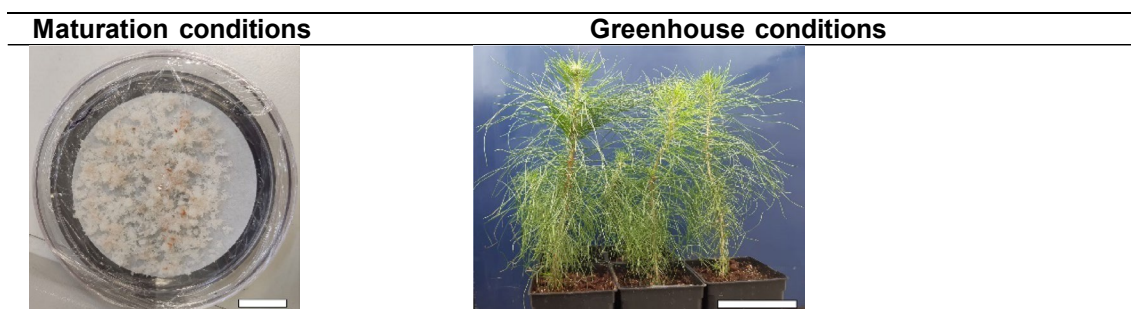
Experiment I: heat experiment

The heat experiment (Fig. 2) comprised ten somatic plants obtained from EMs matured 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 5 days, these somatic plants were exposed to 40 °C for 2 h each day, being kept at 23 °C the remaining 22 h of the day. The next 2 days, the somatic plants were kept at 23 °C, the following 5 days, the somatic plants were again exposed to 40 °C for 2 h each day, being kept at 23 °C for the remaining 22 h of the day. For the remaining 8 days, the somatic plants were kept at 23 °C until completing a total of 20 days. All the plants were watered weekly (Fig. 2).

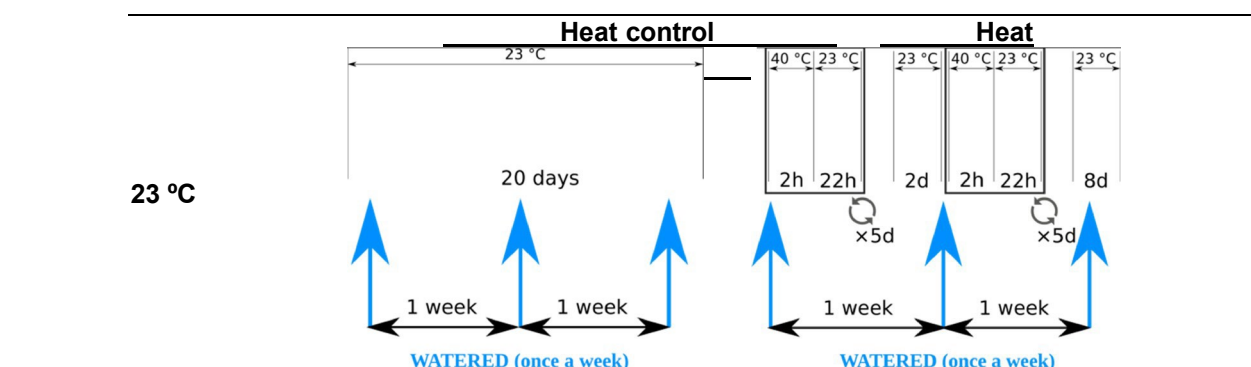
Experiment II: drought experiment

The drought experiment comprised a total of 20 plants. Five plants obtained from maturation at 50 °C and five plants from the same genotypes matured 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 matured 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).

Growth conditions



Experiment I - heat experiment



Experiment II - drought experiment

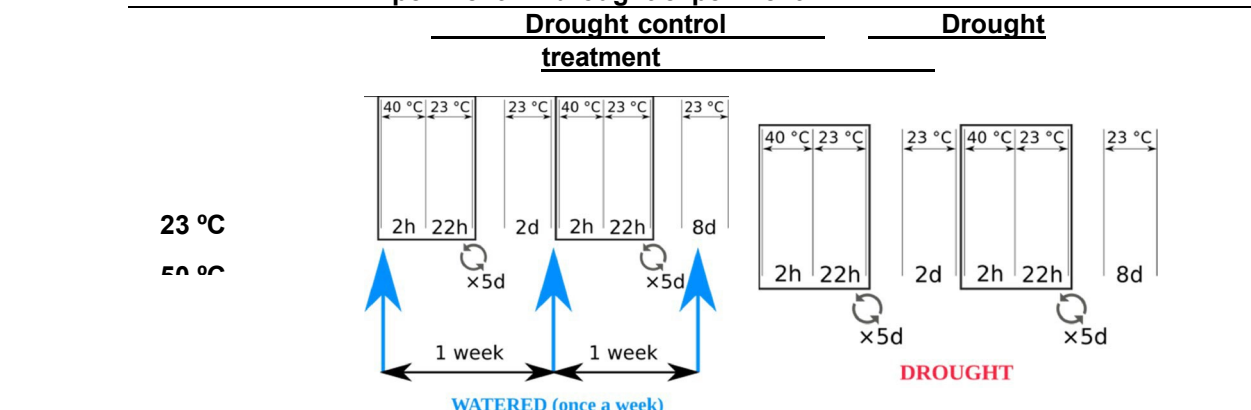


Fig. 2 Experimental design for different in vitro (Maturation conditions) and ex vitro (heat experiment and drought experiment) conditions in the maturation of embryonal masses of *Pinus radiata* D. Don (bar=2 cm) and the growth of somatic plants obtained (bar=9 cm), respectively. *d* days; *h* hours

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:

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

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 24 h (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:

$$E.L.(\%) = \frac{EC_f - EC_i}{EC_t - EC_i} \times 100$$

The response g_s ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) and E ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) 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™ 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 \times 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 $\text{MH}^+ \rightarrow$ fragment ion transitions was recorded (5-mC m/z 242 \rightarrow 126, 5-hmC m/z 258 \rightarrow 142 and C m/z 228 \rightarrow 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-mC 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 matured 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 ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) (10.04 ± 0.22) than those kept at 23 °C (7.32 ± 0.11). For the other physiological parameters (RWC , $E.L.$ and g_s) no statistically significant differences were observed (Table 1).

Experiment II: drought experiment

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

On the other hand, when plants were water-stressed statistically significant differences for $\Psi_{leaf\text{final}}$, $g_{s\text{final}}$ and the E_{final} were found (Table 1). In this case, plants obtained from EMs matured at 50 °C had a significantly higher $\Psi_{leaf\text{final}}$ (MPa) (-0.97 ± 0.10) than those plants obtained from EMs matured at 23 °C (-1.31 ± 0.08). Moreover, the water-stressed plants had a significant lower in the $\Psi_{leaf\text{final}}$ (MPa) (-1.34 ± 0.07) when compared to irrigated plants (-0.94 ± 0.09). Although statistically significant differences were not observed for $E.L.\text{final}$ and RWC_{final} , low values of $E.L.\text{final}$ ($> 7.40\%$) and high values of RWC_{final} ($> 86.79\%$) were observed in all plants.

A significantly higher $g_{s\text{final}}$ was observed in irrigated plants obtained from EMs exposed to high maturation temperature (50 °C) followed by irrigated plants obtained from EMs matured at control temperature (23 °C), while the water-stressed plants obtained from EMs in both maturation temperatures showed a significantly lower $g_{s\text{final}}$ 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 matured at 50 °C displayed lower E_{final} values than those matured at control conditions (Fig. 3b).

Global DNA methylation/hydroxymethylation analysis

Experiment I: heat experiment

Pinus radiata plants obtained from EMs matured 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 ($5\text{-hmC}/5\text{-mC} \times 100$) (Table 2).

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.

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 (Tables 2, 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 matured 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).

Table I 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 H₂O m⁻² s⁻¹), and instant transpiration (E, mmol H₂O m⁻² s⁻¹) in plants obtained from embryonal masses of *Pinus radiata* D. Don matured 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 H₂O m⁻² s⁻¹), and E (mmol H₂O m⁻² s⁻¹) in plants of *P. radiata* under irrigation or no irrigation conditions (I) in the Experiment II—drought experiment

	Effect	df	F value	p value
Experiment I—heat experiment				
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				
$\Psi_{leaf\text{initial}}$	MT	1	8.03	≤ 0.05*
RWC _{initial} (%)	MT	1	0.19	> 0.05 ^{ns}
E.L. _{initial} (%)	MT	1	4.98	≤ 0.05*
$g_{s\text{initial}}$	MT	1	1.22	> 0.05 ^{ns}
E _{initial}	MT	1	1.86	> 0.05 ^{ns}
After drought experiment				
$\Psi_{leaf\text{final}}$	MT	1	13.86	≤ 0.01**
	I	1	20.25	≤ 0.001***
	MT × I	1	0.03	> 0.05 ^{ns}
RWC _{final} (%)	MT	1	1.26	> 0.05 ^{ns}
	I	1	0.74	> 0.05 ^{ns}
	MT × 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 × I	1	4.25	> 0.05 ^{ns}
$g_{s\text{final}}$	MT	1	0.04	> 0.05 ^{ns}
	I	1	242.63	≤ 0.001***
	MT × I	1	8.21	≤ 0.05*
E _{final}	MT	1	1.81	> 0.05 ^{ns}
	I	1	245.67	≤ 0.001***
	MT × I	1	11.41	≤ 0.01**

df degrees of freedom

*, **, *** Significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively

^{ns}Non-significant at $p \leq 0.05$

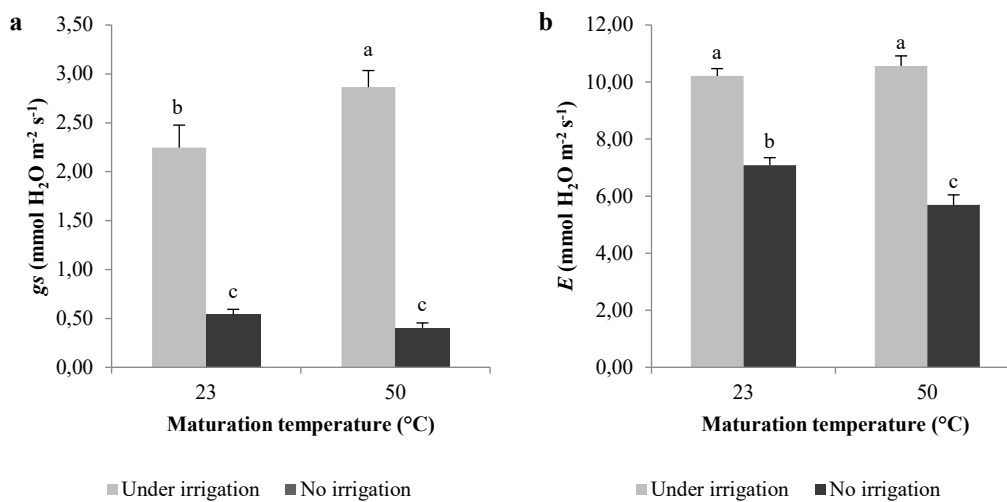


Fig. 3 Physiological parameters in plants under irrigation or no irrigation conditions obtained from embryonal masses of *Pinus radiata* D. Don submitted to different maturation temperatures (23 °C or 50 °C). **a** Stomatal conductance (g_s , $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$), and **b** instant transpiration (E , $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$). Bars indicate standard errors. Different letters show significant differences according to the Tukey's post hoc test ($p \leq 0.05$)

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 matured 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)

Effect	df	5-mC %		5-hmC %		5-hmC/5-mC × 100	
		F value	p value	F value	p value	F value	p value
Experiment I—heat experiment							
TC	1	0.25	> 0.05 ^{ns}	5.40	≤ 0.05*	6.29	≤ 0.05*
Experiment II—drought experiment							
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 × I	1	0.01	> 0.05 ^{ns}	0.02	> 0.05 ^{ns}	0.01	> 0.05 ^{ns}

Table 3 Methylation and hydroxymethylation rates of needles of *Pinus radiata* D. Don plants obtained from embryonal masses matured 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 matured 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 ± 0.23a	0.0129 ± 0.0006a	0.0335 ± 0.0014a
40	37.98 ± 0.75a	0.0089 ± 0.0015b	0.0233 ± 0.0035b
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

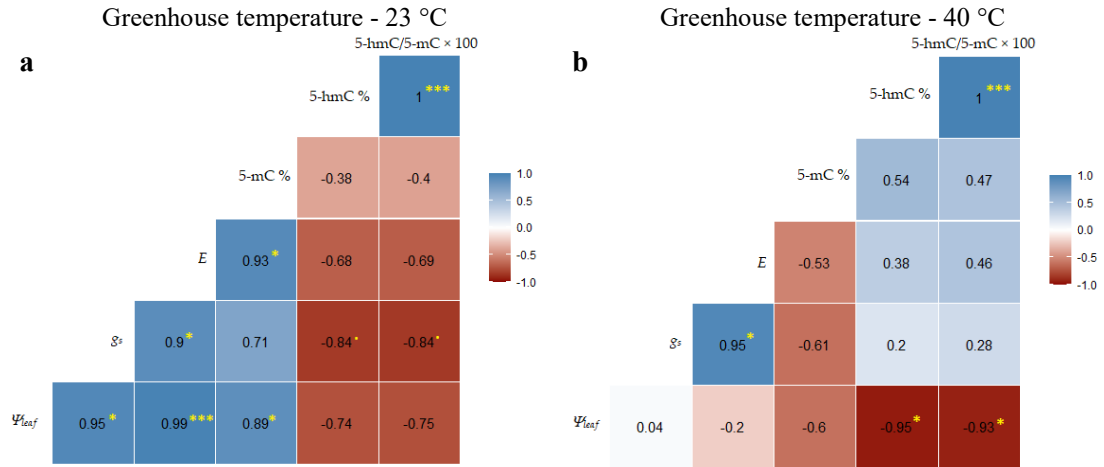
Data are presented as mean values ± SE. Significant differences among treatments at $p < 0.05$ are indicated by different letters in the column

Experiment II: drought experiment

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 $\Psi_{leaf\ final}$ 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–e), except in the somatic plants obtained from EMs matured 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 $\Psi_{leaf\ final}$ 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 $g_{s\ final}$ with 5-hmC contents and $g_{s\ final}$ 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 matured at 50 °C and maintained in irrigation conditions had a marginally negative correlation between $g_{s\ final}$ and 5-mC contents ($p = 0.057$) (Fig. 4e). However, the plants obtained from EMs matured at 50 °C and maintained in drought stress did not show a significant correlation between the physiological parameters ($\Psi_{leaf\ final}$, $g_{s\ final}$ and $E_{\ final}$) and the global 5-mC, 5-hmC and the 5-hmC/5-mC ratio (Fig. 4f).

Experiment I - heat experiment



Experiment II - drought experiment

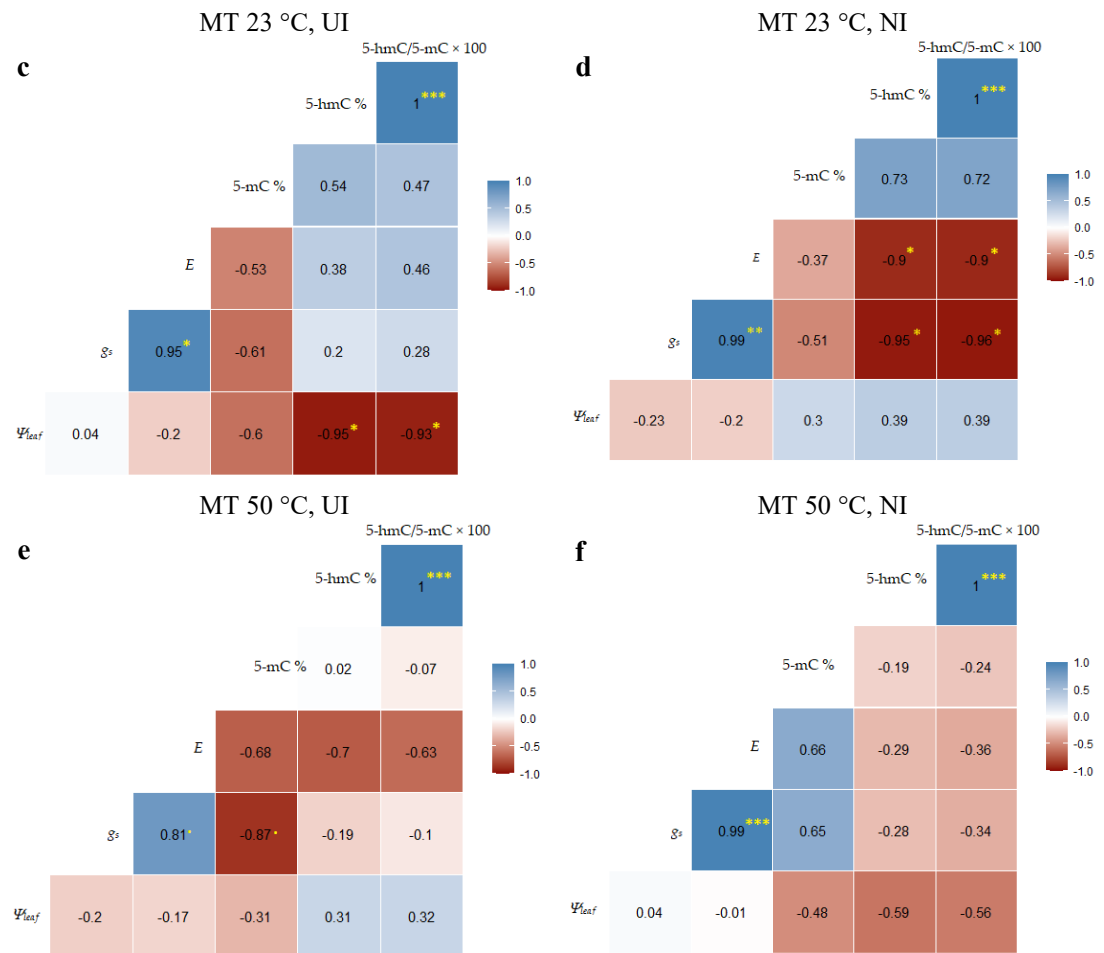


Fig. 4 Heat map of Pearson's correlation coefficients pair between global 5-methylcytosine (5-mC, %), 5-hydroxymethylcytosine (5-hmC, %), the 5-hmC/5-mC ratio (5-hmC/5-mC × 100, %), water potential (Ψ_{leaf} , MPa), stomatal conductance (g_s , mmol H₂O m⁻² s⁻¹) and instant transpiration (E , mmol H₂O m⁻² s⁻¹) in *Pinus radiata* D. Don plants obtained from embryonal masses matured at 23 °C and kept at **a** 23 °C and **b** 40 °C, or heat map of Pearson's correlation coefficients pair between global 5-mC (%), 5-hmC (%), the 5-hmC/5-mC × 100 (%), Ψ_{leaf} (MPa), g_s (mmol H₂O m⁻² s⁻¹) E (mmol H₂O m⁻² s⁻¹) in *P. radiata* plants obtained from different maturation temperatures (MT) and under irrigation (UI) or no irrigation (NI) conditions in the greenhouse. **c** MT 23 °C, UI; **d** MT 23 °C, NI; **e** MT 50 °C, UI; and **f** MT 50 °C, NI. *, **, ***: Significant differences at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively, according to the t test

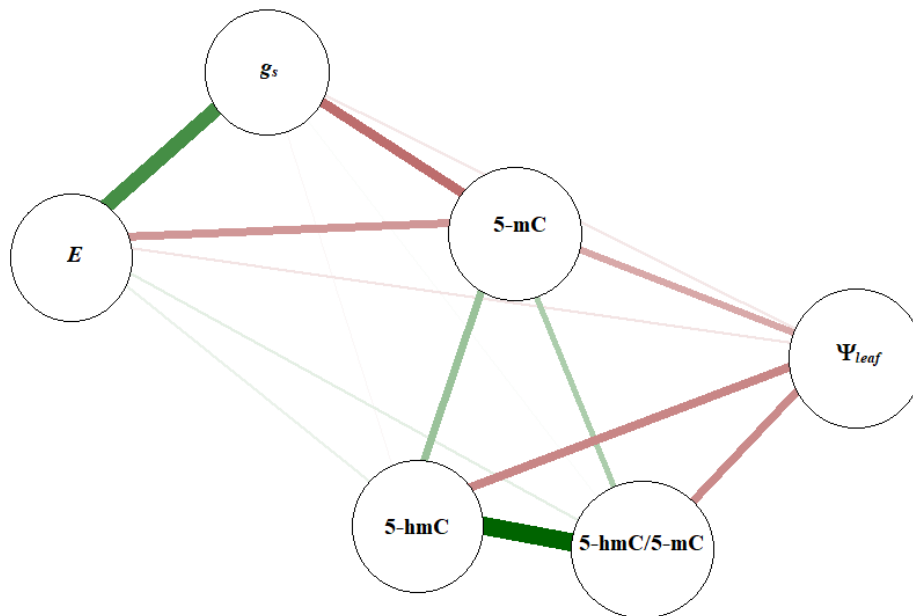


Fig. 5 Pearson's correlation network of global 5-methylcytosine (5-mC, %), 5-hydroxymethylcytosine (5-hmC, %), the 5-hmC/5-mC ratio (5-hmC/5-mC, %) and physiological parameters in plants of *Pinus radiata* D. Don; red lines represent negative correlation with $R < -0.57$ and green lines represent correlation with $R > 0.0$ according to Pearson's correlation coefficient (R). Ψ_{leaf} water potential (MPa); g_s stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$); E instant transpiration ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$). (Color figure online)

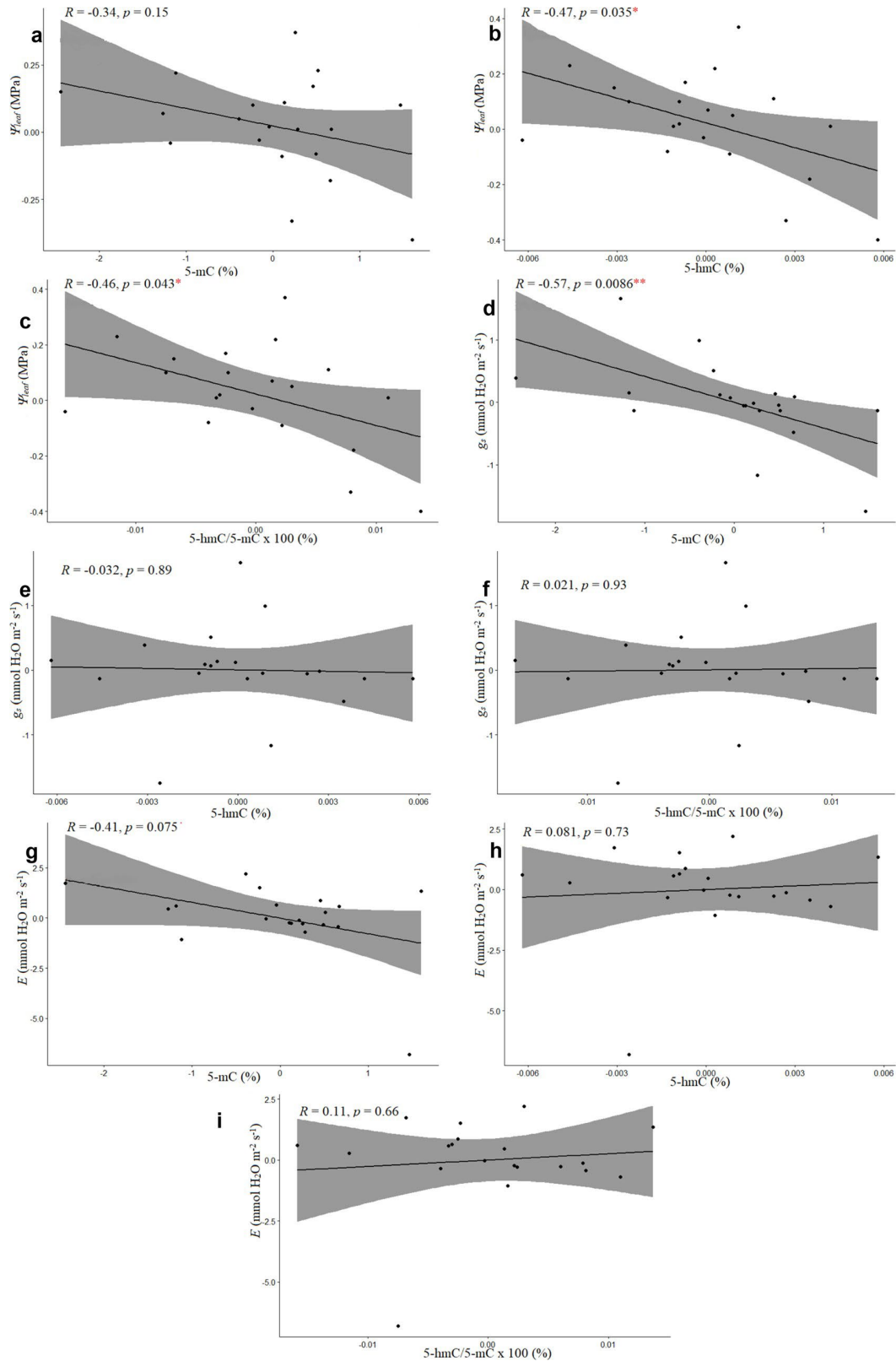


Fig. 6 Pearson correlation pair between relationships between water potential (ψ_{leaf} , MPa) and **a** 5-methylcytosine (5-mC, %), **b** 5-hydroxymethylcytosine (5-hmC, %), and **c** the 5-hmC/5-mC ratio (5-hmC/5-

mC $\times 100$, %). Pearson correlations between stomatal conductance (g_s , $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) and **d** 5-mC, **e** 5-hmC and **f** the 5-hmC/5-mC $\times 100$. Finally, Pearson correlations between instant transpiration (E , $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) and **g** 5-mC, **h** 5-hmC and **i** the 5-hmC/5-mC $\times 100$. *, **: Significant correlations at $p \leq 0.05$ or $p \leq 0.01$, respectively, according to a *t* test

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 1-year and 3-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 6-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 matured 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 matured 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 García-Fórner 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 × 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, 2-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 3 h per 12 days) in the greenhouse (Pérez-Oliver et al. 2021). Furthermore, in our study, the plants coming from EMs matured at 50 °C compared with those plants coming from EMs matured at 23 °C had a higher g_{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 CO₂ 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 matured 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 matured 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 2-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 1-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 $\Psi_{leaf\ final}$ 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 μ M 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 $\Psi_{leaf\,final}$ and E_{final} . Somatic plants kept at heat stress (40 °C, 10 days) in the greenhouse had lower 5-hmC levels than plants kept at 23 °C. 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.

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