Cytokinins are involved in drought tolerance of *Pinus radiata* plants originating from embryonal masses induced at high temperatures

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Running head: Drought tolerance and cytokinins in somatic plants

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Abstract

Vegetative propagation through somatic embryogenesis is an effective method to produce elite varieties and can be applied as a tool to study the response of plants to different stresses. Several studies show that environmental changes during

embryogenesis could determine future plant development. Moreover, we previously reported that physical and chemical conditions during somatic embryogenesis can determine the protein, hormone and metabolite profiles, as well as the micromorphological and ultrastructural organization of embryonal masses and somatic embryos. In this sense, phytohormones are key players throughout the somatic embryogenesis process as well as during numerous stress-adaptation responses. In this work we first applied different high-temperature regimes (30ºC, 4 weeks; 40ºC, 4 days; 50ºC, 5 minutes) during induction of *Pinus radiata* somatic embryogenesis, together with control temperature (23ºC). Then, the somatic plants regenerated from initiated embryogenic cell lines and cultivated in greenhouse conditions were subjected to drought stress and control treatments to evaluate survival, growth and several physiological traits (relative water content, water potential, photosynthesis, stomatal conductance and transpiration). Based on those preliminary results even more extreme high-temperature regimes were applied during induction (40ºC, 4 hours; 50ºC, 30 minutes; 60ºC, 5 minutes) and the corresponding cytokinin profiles of initiated embryonal masses from different lines were analysed. Results showed that the temperature regime during induction had delayed negative effects on drought resilience of somatic plants as indicated by survival, photosynthetic activity and water use efficiency. However, high temperatures for extended periods of time enhanced subsequent plant growth in well-watered conditions. High-temperature regime treatments induced significant differences in the profile of total cytokinin bases, N^6-isopentenyladenine, *cis*-zeatin riboside and *trans*-zeatin riboside. We concluded that phytohormones could be potential regulators of stress-response processes during initial steps of somatic embryogenesis and that they may have delayed implications in further developmental processes, determining the performance of the generated plants.

1 Introduction

Somatic embryogenesis (SE) is currently considered as one of the most successful methods for large-scale vegetative propagation of plants, and especially in some economically relevant woody conifer species such as *Pinus radiata*. SE combined with cryopreservation of embryonal masses (EMs) gives the opportunity for scaling-up production of genetically improved varieties that have been fully tested at field (Santa-Catarina et al., 2012; Montalbán et al., 2016). The rising demand for forest products worldwide may be partially addressed by the deployment of tested varieties through vegetative propagation in intensively managed plantation forests, a strategy referred to as multi-varietal forestry (Park 2002). In the last years, our team has overcome different bottlenecks in conifer SE by adjusting the composition of culture media and the culture conditions in *Pinus radiata* (Montalbán et al., 2010, 2012, 2015, García-Mendiguren et al., 2016a; Montalbán and Moncaleán, 2017, 2018) and also in *Pinus halepensis* (Pereira et al., 2016, 2017).

In vitro setups are often used as models systems for the complex field environments in which plants are subjected to stress (Claeys et al., 2014). Furthermore, it is well known that plant response is highly dependent on both the type and the intensity of stress (Claeys and Inzé, 2013) which can be controlled better and more easily in *in vitro* assays. Likewise, SE has been widely used not only as a proxy for understanding the physiological, biochemical and molecular events occurring during conifer embryo development (Morel et al., 2014; Reza et al., 2018), but also in response to different abiotic stresses (Muilu-Mäkelä et al., 2015; Eliášová et al., 2017; Castander-Olarieta et al., 2019).

Besides, stress is reported to be beneficial and, in some cases, even crucial for the embryogenic competence of different plant species by the activation of the molecular machinery required for the transition of somatic cells to an embryogenic state (Fehér et al., 2003; Ochatt, 2017). Moreover, stress can also act like a selective pressure during initial steps of SE determining the quantity and quality of the somatic embryos (Fehér, 2015; García-Mendiguren et al., 2016a; Pereira et al., 2016; Arrillaga et al., 2019; Castander-Olarieta et al., 2019).

In this regard, the relatively short period of embryogenesis in the life of a tree seems to be a critical stage to modulate plant behaviour *ex vitro* (García-Mendiguren et al., 2017). There is some strong evidence in the model conifer species *Picea abies* that environmental conditions during embryogenesis can establish an epigenetic memory that modulates different developmental traits (Johnsen et al., 2005; Kvaalen and Johnsen, 2008; Yakovlev et al., 2010, 2011). These epigenetic marks can be inherited as a pre-adaption to environmental conditions by subsequent generations as a form of maternal effect (Zas et al., 2013; Gosal and Wani, 2018). This middle-term memory, together with short term (developmental plasticity) and long-term (local adaptation) epigenetic developmental responses, are responsible for the great phenotypic plasticity and adaptation capacity observed in plants (Le-Gac et al., 2018), which pave the way for the production of plants pre-adapted to different environmental conditions (Pascual et al., 2014).

Among all types of stress conditions, heat and drought represent perhaps some of the most common abiotic stresses in plants and they have overlapping roles (Jia et al., 2017). Furthermore, it has been documented that increased tolerance to different kinds of stresses may rely on similar metabolic adjustments like cold and drought stresses (Shinozaki et al., 2003).

Because of climate change, the intensity and frequency of extreme weather events, such as heat waves and long drought periods, are predicted to increase (Duliè et al., 2013). High temperatures and drought are known to alter the fluidity and permeability of cell membranes (Sangwan et al., 2002), to produce imbalances in osmotic and water relations (De Diego et al., 2013; Feller and Vaseva, 2014), to increase the production of reactive oxygen species (ROS) (Larkindale and Knight, 2002) and trigger senescence, inhibition of photosynthesis and programmed cell death (Vacca et al., 2004). All these alterations could have significant implications in the viability, productivity and regeneration of all kind of forests, from planted to semi-natural forests (Allen et al., 2010). Because of the complexity of stress tolerance traits, conventional breeding techniques combined with new biotechnological tools could offer more effective options for improving the performance, quality and health of commercially valued forest species and varieties.

The physiological mechanisms underlying heat and drought stress tolerance are still poorly understood. Integration of environmental stimuli, signal transduction and stress response are partially mediated by intense cross-talk among plant hormones (Wahid et al., 2007), which are considered the most important endogenous substances for modulating physiological, developmental and molecular responses (Wani et al., 2016). Apart from the well documented function of phytohormones in different *in vitro* assays such as organogenesis (Moncaleán et al., 2003, 2005; Montalbán et al., 2011) and SE (Carneros et al., 2017; Zhou et al., 2017; Moncaleán et al. 2018), they are active part in numerous stress response processes (Bielach et al., 2017; Corcuera et al., 2012; De Diego et al., 2012; 2015).

Particularly, cytokinins (CKs) are considered as master regulators during plant growth and development (Kieber and Schaller, 2018) and despite not being traditionally

120 considered part of the stress response machinery, recent research has demonstrated that
121 CKs directly participate in stress tolerance of plants (Wani et al., 2016). Although
122 external application has been the most frequent method to study plant responses to CKs,
123 stress-mediated alteration of endogenous levels of CKs indicates their involvement in
124 abiotic stress, including drought (Kang et al., 2012). Current evidence supports that CKs
125 could be primary receptors in temperature sensing (Černý et al., 2014) and cytokinin
126 (CK) crosstalk with ethylene and other so-called "stress-hormones" such as abscisic
127 acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene, has been observed
128 (O'Brien and Benková, 2013).

129 The aim of this work was to evaluate if application of high-temperature regimes during
130 initiation of radiata pine SE, which are known to reduce water availability (García-
131 Mendiguren et al., 2016a; Moncaleán et al., 2018), could result in the production of
132 somatic plants with different adaptation to drought stress. Isoprenoid CKs profiles were
133 investigated to assess the possible involvement of these phytohormones in the early
134 response of initiated EMs to applied temperature stress determining the ongoing SE
135 steps as well as *ex vitro* plant behaviour.

136 ## 2 Materials and methods

137 ### 2.1 Plant material production

138 #### 2.1.1 Drought experiment

139 The somatic plants employed for this experiment originated from the procedure
140 described in Materials and Methods section (Experiment 1) from Castander-Olarieta et
141 al. (2019). Briefly, one year-old green female cones of *Pinus radiata* D.Don were
142 collected in July 2016 from 4 genetically different open-pollinated trees in a seed

orchard established by Neiker-Tecnalia in Deba (Spain; latitude: 43º16´59"N, longitude: 2º17´59"W, elevation: 50 m). Immature cones and seeds were processed following Montalbán et al. (2012) and the resulting dissected megagametophytes were placed horizontally in Petri dishes (9 mm x 14 mm) containing 19 ml of sterile EDM initiation medium (Walter et al., 2005) supplemented with 3.5 gL^{-1} gellan gum (Gelrite®; Duchefa). Eight megagametophytes were employed per Petri dish and transparent plastic film was used for a proper closure of Petri dishes. At this point, megagametophytes were incubated at different temperature regimes: 23ºC (8 weeks, control), 30ºC (4 weeks), 40ºC (4 days) and 50ºC (5 minutes). The Petri dishes containing the culture medium were pre-warmed before the start of the incubation period. Once finished, all the megagametophytes were kept at 23ºC for 8 weeks in darkness. Further SE steps were carried out at standard conditions following the procedures described by Moncaleán et al. (2018) (Fig. 1). For this experiment only well-formed mature somatic embryos with similar morphologies were employed. After germination, somatic plantlets were transferred to 43 cm^3 (35 mm x 25 mm) individual pots containing blond peat moss (Pindstrup): perlite (7:3, v/v) and acclimatized in a greenhouse under controlled conditions (T = 23 ±2ºC and RH = 70 ± 5%) (Fig. 1). Growing saplings bigger than 5 cm were transplanted to 2.18 L (90 mm x 270 mm) pots containing blond peat moss (Pindtrup): perlite (8:2, v/v) and 3 g L^{-1} Osmocote® Topdress fertilizer (Everris), and watered regularly for one year until they had a minimum leader shoot length of 10 cm to conduct the drought experiment (Fig. 1).

2.1.2 Hormone analyses

The plant material used for the hormone analysis originated from the procedure described in Materials and Methods section (Experiment 2) from Castander-Olarieta et al. (2019). In this experiment green cones collected in July 2017 were employed.

Sampled mother trees and procedures were the same as in 2.1.1 but applying adjusted temperature regimes based on the results obtained from the drought experiment: 23ºC (8 weeks, control), 40ºC (4 hours), 50ºC (30 minutes) and 60ºC (5 minutes). In this case, just before maturation, part of the proliferating EMs (three ECLs per treatment and 40 mg from 4 different EMs of the same ECL) was put aside and frozen in liquid nitrogen. The samples were stored at -80ºC until extraction, purification and quantification of endogenous phytohormones following the methodology described in Moncaleán et al. (2018).

2.2 Drought experiment

2.2.1 Experiment design, plant survival and growth rate

Two-year-old somatic saplings growing in the greenhouse and generated under the conditions previously described in section 2.1.1 were involved in a 3-months drought stress experiment between June to September 2019. Seven plants, each one from a different ECL, were randomly selected per treatment and per duplicate, comprising a total of 42 plants (7 ECLs x 2 biological replicates x 3 treatments). Half of them (each biological replicate) were subjected to a drought stress treatment by the complete suppression of watering, and the remainder were kept watered weekly (control plants). These 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. The ECLs from the control temperature regime treatment (23ºC, 8 weeks) originated from the 4 mother trees employed in section 2.1.1, while the ECLs from the temperature regimes of 30ºC for 4 weeks and 50ºC for 5 minutes originated from 2 and 3 mother trees, respectively. All plants were watered to maximum retention capacity of the substrate before the start of the experiment and drought conditions were maintained for 12 weeks until plantlets from each treatment

started to present external symptoms of drought stress such as needle epinasty or apical curvature (De Diego et al., 2012). After that period, all saplings were rewatered and plant survival was recorded one month later.

Total aerial height (cm) of each plant was measured at the beginning and at the end of the drought treatment (12 weeks) and the growth rate (GR) was calculated as follows:

$$\mathbf{GR\ (\%) = ((H2\text{-}H1) / H1) \times 100}$$

where H represents the height of the plants at the beginning (H1) and at the end (H2) of the experiment.

2.2.2 Plant water potential and relative water content

Plant water potential was determined at the beginning and at the end of the treatment at predawn (from 5:00 to 7:00 a.m.) (Ψ_{pd}, MPa) from needles collected from the apical area using a Scholander chamber (PMS Instrument Company) and the pressure-equilibration technique (Scholander et al., 1965).

Relative water content (RWC (%)) was measured in two needles collected from the apical area of each sapling at the end of the drought period following the method described by De Diego et al., (2012). At harvesting time, needle fresh weight (FW) was recorded and then samples were immersed in de-ionized water and maintained overnight in dark. On the second day, after carefully removing the excess of water from needles surface 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) recorded. RWC was estimated using the following equation:

RWC (%) = (FW – DW) / (TW – DW) × 100

2.2.3 Gas exchange parameters

Stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), instant leaf transpiration (E, mmol H_2O m^{-2} s^{-1}) and instant net photosynthesis (A_N, µmol CO_2 m^{-2} s^{-1}) were measured at the beginning and at the end of the drought period at midday using the LI-6400XT Portable Photosynthesis System (Li-Cor Biosciences) equipped with the 6400-05 Clear Conifer Chamber (Li-Cor Niosciences). Intrinsic water use efficiency (WUE, µmol CO_2 $mmol^{-1}$ H_2O) was determined as the ratio between A_N and E.

2.3 Extraction, purification and quantification of endogenous cytokinins

Samples obtained in section 2.1.2 were analysed for the following 25 CKs types: N^6-Isopentenyladenine (iP), N^6-Isopentenyladenosine (iPR), N^6-Isopentenyladenine7-glucoside (iP7G), N^6-Isopentenyladenine-9-glucoside (iP9G), and N^6-Isopentenyladenosine-5'-monophosphate (iPMP), *cis*-Zeatin (cZ), *cis*-Zeatin riboside (cZR), *cis*-Zeatin *O*-glucoside (cZOG),), *cis*-Zeatin-7-glucoside (tZ7G), *cis*-Zeatin-9-glucoside (cZ9G), *cis*-Zeatin riboside *O*-glucoside (cZROG), *cis*-Zeatin riboside-5'-monophosphate (cZRMP), *trans*-Zeatin (tZ), *trans*-Zeatin riboside (tZR), *trans*-Zeatin *O*-glucoside (tZOG), *trans*-Zeatin-7-glucoside (tZ7G), *trans*-Zeatin-9-glucoside (tZ9G), *trans*-Zeatin riboside *O*-glucoside (tZROG), *trans*-Zeatin riboside-5'-monophosphate (tZRMP), Dihydrozeatin (DHZ), Dihydrozeatin riboside (DHZR), Dihydrozeatin *O*-glucoside (DHZOG), Dihydrozeatin-7-glucoside (DHZ7G), Dihydrozeatin-9-glucoside (DHZ9G), Dihydrozeatin riboside *O*-glucoside (DHZROG), Dihydrozeatin riboside-5'-monophosphate (DHZMP). Isoprenoid CK types and their functional groups are shown in Table 1.

238 Each sample was divided in two technical replicates of 10 mg and were analysed
239 according to the protocol described by Svačinová et al. (2012), using miniaturized
240 purification (pipette tip solid-phase extraction). Samples were extracted in 1 ml of
241 modified Bieleski solvent and homogenized using a MM 301 vibration mill (Retsch
242 GmbH & Co. KG, Haan, Germany) at a frequency of 27 Hz for 5 min at 4ºC after
243 adding 3 mm ceria-stabilized zirconium oxide bead. Samples were extracted with the
244 addition of stable isotope-labelled internal standards (0.2 pmol for base, ribosides and 9-
245 and 7-glucoside CKs; 0.5 pmol for *O*-glucoside and CK nucleotides). The extracts were
246 ultrasonicated for 3 min and incubated at 4 °C with continuous shaking for 30 min at 20
247 rpm. After centrifugation (15 min, 20,000 rpm, 4°C), from the supernatants of each
248 sample, another three technical replicates of 300 µl were transferred onto Stage Tips
249 and purified according to the aforementioned protocol, consisting of C18, SDB-RPS,
250 and Cation-SR sorbents (Empore^{TM}). As a result, our experiment was carried out using
251 3 ECLs per treatment and 6 technical replicates per ECL, comprising a total number of
252 72 samples analysed.

253 Prior to loading the samples, the StageTip sorbents were conditioned with 50 µl acetone
254 (by centrifugation at 2,000 rpm, 10 min, 8°C), 50 µl methanol (2,000 rpm, 10 min, 8°C),
255 50 µl water (2,200 rpm, 15 min, 8°C), equilibrated with 50 µl 50% (v/v) nitric acid
256 (2,500 rpm, 20 min, 8°C), 50 µl water (2,500 rpm, 20 min, 8°C) and 50 µl modified
257 Bieleski solvent (2,500 rpm, 20 min, 8°C). After the application of 300 µl of sample
258 (3,500 rpm, 30 min, 8ºC), the tips were washed using 50 µl of water and methanol
259 (3,500 rpm, 20 min, 8°C). Samples were then eluted with 50 µl of 0.5 M NH_4OH in
260 60% methanol (3,500 rpm, 20 min, 8°C) and eluates were collected into new clean
261 microcentrifuge tubes, evaporated to dryness and dissolved in 30 µl of mobile phase
262 prior to UHPLC-MS/MS analyses.

Mass analysis was carried out following the procedure described by Moncaleán et al. (2018), using an Acquity UPLC® System (Waters, Milford, MA, United States), and a triple-quadrupole mass spectrometer Xevo™ TQ-S MS (Waters MS Technologies, Manchester, United Kingdom). All MS data were processed using the MassLynxTM software with TargetLynx™ program (version 4.2. Waters, Milford, MA, United States), and compounds were quantified by standard isotope dilution analysis (Rittenberg and Foster, 1940).

2.4 Statistical analysis

The results from all the physiological traits analysed during the drought experiment (growth rate, relative water content, water potential, A_N, g_s, E and WUE), a usual analysis of variance was conducted to assess the effect of the treatments on each parameter. A Tukey´s post-hoc test ($\alpha = 0.05$) was used for multiple comparisons. In the case of growth rate (for watered plants) and RWC (for plants subjected to drought stress), the analysis of variance did not fulfil the normality hypothesis, and thus, a Kruskal-Wallis test was performed.

Regarding the data obtained from the hormonal study, several models were considered for each hormone type. For cZR, the same usual analysis of variance described in the drought experiment section was performed, followed by multiple comparisons based on Tukey's post-hoc test ($\alpha = 0.05$).

To assess the effect of temperature regimes during the induction step on the levels of iPR, iPMP and cZ, the embryogenic cell line (ECL) was included in the model as a random effect. The inclusion of the ECL improved the fit and helped to analyse the effect of treatments more accurately by accounting for heteroscedasticity in the data. For total CK ribosides, the same model was performed, but including additional

variance parameters for each level of temperature to correct for heteroscedasticity. In the case of total CK bases, total CK nucleotides and DZ, different variance parameters for each cell line were used to correct for heteroscedasticity.

In relation to tZR and DZR, the same procedure as described above for CK ribosides was followed, that is, a linear mixed effects model including the ECL as a random effect with different variance parameters for each temperature level. Multiple comparisons were also based on a Tukey post-hoc test ($\alpha = 0.05$) and predictable linear functions of the coefficients with *p*-values conveniently adjusted (Benjamini and Hochberg, 1995). The analysis of total iP and iP bases was equally conducted but considering different variance parameters for each cell line (ECL) in normal and logarithmic scale respectively.

3 Results

3.1 Drought stress experiment

The application of 40ºC for 4 days resulted in the complete failure of embryogenic tissue initiation. As a result, no plants could be obtained from that treatment and thus, the drought experiment was carried out only with the other three treatments (23°C, 8 weeks, control; 30°C, 4 weeks; 50°C, 5 min).

All the ECLs used in this experiment presented germination rates around 70-80% and the acclimatization success was >90% for all the plants originating from different induction treatments.

In general terms, comparing regularly watered plants with plants subjected to the drought period, clear differences were observed for all the parameters tested. Survival was 100% in watered plants, while plants subjected to drought stress presented survival

rates of 66.7%. Growth rates of stressed plants decreased almost 3 times in relation to control plants, changing from an average growth rate of 77.9% ± 8 to 28.2% ±2.9, respectively (Table 2). Average Ψ_{pd} of plants at the beginning of the experiment (watered to maximum retention capacity of the substrate) was -0.63 ± 0.02 MPa, while the same parameter at the end of the drought period was -1.4 ± 0.08 MPa (Table 2), ranging from -2 MPa for the most stressed plants to -0.9 MPa for the less stressed. Likewise, RWC of plants in drought conditions was about 13% lower than control plants, and all gas exchange parameters analysed (A_N, g_s and E) decreased drastically after 12 weeks (Table 2).

Considering watered plants, no significant differences were detected (ANOVA, not shown) for most physiological traits between control (23°C, 8 weeks) and tested induction treatments (temperature regimes of 30°C, 4 weeks and 50°C, 5 min). Only growth rate was significantly higher for plants originating from the induction treatment at 30°C compared to the 23°C and 50°C treatments (Fig. 2).

With respect to drought conditions, significant differences were observed for three of the 8 physiological traits evaluated (survival, A_N and WUE) (Table 3). It is worth mentioning that plants originating from standard induction conditions (23°C, 8 weeks) showed few visual symptoms of drought stress, with slight needle epinasty in some cell lines, but not apparent apical curvature. Plants originating from the 30°C treatment for 4 weeks presented the most heterogeneous aspect. Some plants presented similar symptoms to control plants, while others showed clear apical curvature and needle epinasty. Plants originating from the temperature regime of 50°C for 5 min were the ones presenting the greatest drought stress symptoms (Fig. 3). Accordingly, clear differences in plants survival were detected. 100% of the plants coming from control conditions (23°C, 8 weeks) recovered after rewatering, while plants from induction

335 treatments at 30ºC and 50ºC showed lower survival rates of 57.1% and 42.9%,
336 respectively.

337 A_N and WUE presented significant differences among treatments at the end of the
338 drought period. Plants coming from ECLs initiated at 50ºC showed significant lower A_N
339 values than those from control conditions (23°C), even reaching values near 0 for most
340 of the individuals. Plants originating from the 30°C induction treatment showed
341 intermediate values and high variability, denoting that some plants were close to the
342 values obtained at 50ºC while others were more like those at 23ºC (Fig. 4a). Regarding
343 WUE, similar results were obtained, being the 50ºC induction treatment the one with
344 the lowest values and 23ºC the one with the highest ones. In this case the difference
345 observed between the 50°C and control treatments was also statistically significant (Fig.
346 4b).

347 Despite not statistically significant, it is noticeable that the differences obtained in the
348 case of g_s, *E* and growth rate showed *p*-values of 0.0568, 0.0537 and 0.0645
349 respectively (Table 3), so we could assume that they are on the verge of statistical
350 significance. All of them followed the same tendency previously described for A_N and
351 WUE. Plants originating from the 50°C treatment showed the lowest values and control
352 plants (23°C treatment) the highest ones. Considering the 30ºC treatment, plants
353 presented intermediate values for g_s and E, while the results in the case of growth were
354 very similar to those of control plants (Table 3).

355 **3.2 Hormone analyses**

356 The levels of CK N-glucosides (7G/9G) and most of CK *O*-glucosides (OG), which are
357 known to be irreversible and storage metabolites of the active forms, were under limit of
358 detection in all samples analysed. In the case of tZ, tZRMP, DHZMP and cZRMP, more

than the 40% of the samples analysed were under limit of detection as well, thus no statistical analysis could be performed. Because of the lack of some CK groups, the analysis of total CK types could not be performed either.

Regarding the amount of each CK type, it should be noted that the most abundant cytokinins in EMs were iP-type CKs, and specially the precursor forms (iPMP), along with cZ-type CKs in the base form (cZ). The levels of tZ and DHZ-type CKs were much lower than the two groups previously mentioned, and in the case of these two CK types the most abundant functional groups were the ribosides (tZR) and bases (DHZ), respectively (Table 4).

Analysing the results by functional groups and considering the effect of the treatments, significant differences were found only for CK bases (cZ, tZ, DHZ, iP). No significant differences could be observed in the case of CK ribosides and CK nucleotides. The concentration of total CK bases was significantly lower in EMs produced under 40ºC treatment with respect to EMs originating from control treatment (23ºC) or treatment at 60ºC for 5 minutes (Fig. 5a, Table 4). The treatment at 50°C resulted in intermediate values (Fig. 5a), however not significant compared to other treatments. Among the CK bases, significant differences were especially observed for iP with low concentrations observed in samples originating from the 50ºC and 40ºC treatments and higher levels observed for the control (23ºC) and 60ºC treatment (Fig. 5b; Table 4). No significant differences were detected for other CK bases (cZ, tZ, DHZ) among temperature treatments but similar trends were observed. Both DHZ and cZ- type CKs were found at lower concentrations in EM samples produced under the 40°C induction treatment (Table 4). In the case of DHZ, the differences were on the verge of statistical significance ($p = 0.079$).

Some significant differences were also found for CK ribosides (a transport form of CKs). Although no significant differences in total CK ribosides could be detected among treatments (Table 4), a heterogeneous behaviour was observed for the different riboside-types. Opposite patterns were observed for ribosides cZR and tZR. cZR followed similar trend as base forms (see Fig. 5; Table 4), i.e. significant lower levels following the 40°C and 50°C treatments compared to control treatment (23°C). The concentration of this hormone was found at intermediate values for EMs produced under treatment 60ºC (Fig. 6a, Table 4). In contrast, tZR showed significantly higher concentration after treatment at 60°C compared to control (23°C) and 40°C treatments. The 50ºC treatment resulted in intermediate values (Fig. 6b; Table 4).

Finally, it should be mentioned that no differences were observed for iPMP, the most abundant hormone type in EMs. iPMP is the precursor of the active form iP which was shown to be affected by the temperature regime during induction (Fig. 5b; Table 4).

4 Discussion

This study provided experimental evidence confirming that the application of high temperatures during initiation of SE can result in altered behaviour of plants *ex vitro*, both at standard (growth rate) and drought conditions (survival, A_N, WUE). Moreover, different hormonal profiles at initial steps of the embryogenic process were observed. Unfortunately, these different hormonal profiles are unknown for the tested induction treatments in the case of the drought experiment except the control (23°C, 8 w).

As sessile organisms, plants are continuously and widely exposed to external stimuli such as diverse extreme weather conditions or pathogens. Stress is even exacerbated in trees because of their long lifespan. As a result, plants developed different survival strategies to deal with stress by modifying some of their morphological and

407 physiological traits (Xia et al., 2015). Among these fine-tuned strategies, perhaps one of
408 the most sophisticated and promising ones is the so-called plant priming (Conrath et al.,
409 2015).

410 At control conditions, plants coming from EMs initiated at 30ºC for 4 weeks presented
411 significantly higher growth rates compared to control treatment (23°C, 8 weeks),
412 suggesting that long induction treatments at increased but quite moderate temperatures
413 could establish some kind of "memory" in initiated embryogenic cells. This "memory"
414 may persist throughout the SE process, triggering subsequent growth in the resulting
415 plants, as already observed by Kvaalen and Johnsen (2008) in *Picea abies*. These
416 authors reported increased leader shoot lengths during the second growth season when
417 high temperatures were applied during SE. Several studies in conifers (spruces)
418 (Johnsen et al., 2005; Webber et al., 2005) pointed out that different temperature
419 regimes during both zygotic and somatic embryogenesis can affect the vegetative
420 development of seedlings for years. In Norway spruce it has been shown that the
421 temperature regime during embryogenesis is involved in the timing of bud set and thus
422 cold acclimation. Therefore, the embryogenesis period could be crucial for plant
423 priming.

424 The air temperature has been shown to positively influence growth and A_N in temperate
425 or boreal trees (Way and Oren, 2010). As evidenced in Norway spruce, it could be
426 possible that high temperatures during embryogenesis could act as a priming agent that
427 prepares plants for a future scenario of higher temperatures, in which an improvement
428 of the photosynthetic capacity is required by the increase in electron transport capacity
429 and/or greater heat stability of Rubisco activase (Sage and Kubien, 2007).

Regarding the results obtained from the drought experiment, it is noticeable that Ψ_{pd} exhibited significant reductions at the end of the treatment, validating our experimental design. In the same way, all the other physiological parameters analysed decreased in line with $\Psi_{pd,}$ as previously postulated in conifers by other authors (Brodribb and Cochard, 2009). With such a water stress, the decrease by almost 3 times in growth rate (77.9 ± 13.9 for watered plants; 28.2 ± 2.9 for stressed plants) is similar to that observed in other conifer species (Turtola et al., 2003). Likewise, the strong decrease in g_s and the other gas-exchange parameters (A_N and E) over a narrow range of Ψ_{pd} has already been reported in *Pinus radiata* and interpreted as an evidence of strongly isohydric stomatal response to water deficit (De Diego et al., 2012, 2015; Brodribb and McAdam, 2013). In agreement with those studies, the plants that seemed more sensible to drought stress reached Ψ_{pd} values of -2 MPa, which is considered the turgor lost point for this species (De Diego et al., 2012), whereas the most tolerant specimens kept around values of -1 MPa and did not usually present any apparent loss of turgor.

In spite of experimental conditions perfectly adapted to efficient drought test, no statistically significant differences were found for water-related parameters among treatments (Ψ_{pd} and RWC). Significant differences were only observed for 2 non-independent physiological traits, instant net photosynthesis (A_N) and intrinsic water use efficiency (WUE = A_N/E). Accordingly, both traits followed the same pattern: plants originating from the highest temperature induction treatment (50°C) with short exposure periods (5 min) exhibited a significant decrease. Other parameters such as E, g_s or growth rate followed the same trend (lowest values for the 50°C treatment) but were at the verge of statistical significance. Even though the differences detected for plants originating from the longer 30ºC treatment (4 weeks) were not significant, a slight decrease in all parameters mentioned before was observed.

These results suggest that for similar water status, plants originating from high-temperature induction treatment (50°C, 5 min) tended to close stomata earlier, which allows a better maintenance of water status and avoid hydraulic failure (De Diego et al., 2012). Nevertheless, for extended drought periods carbon starvation can occur (Mcdowell et al., 2014). According to the carbon starvation hypothesis, when plants close stomata to prevent desiccation, photosynthetic carbon uptake diminishes to values near zero (Mcdowell et al., 2014). Continued demand for carbohydrates to maintain metabolism can deplete carbohydrate reserves, which are completely necessary to maintain both the molecular machinery required to face drought, such as the production of resin (Lewinsohn et al., 1993), but also some morphological adjustments such as fine root production (Villar-Salvador et al., 2004; Aaltonen et al., 2017). The mechanisms by which plants originating from control temperature treatment (23°C, 8 weeks) and to a lesser extent moderate temperature treatment during induction (30°C, 4 weeks), can maintain favourable water status without early stomata closure could be multiple, starting from structural differences such as fine root and stomata density (Ewers et al., 2000; Mitton et al., 1998), or molecular changes such as the accumulation of compatible sugars, amino acids and polyamines (De Diego et al., 2013).

So, surprisingly the application of high temperatures during SE induction (either moderate for quite long period or high for a brief period), besides which are known to reduce water availability (García-Mendiguren et al., 2016a; Moncaleán et al., 2018), gave rise to plants with reduced capacity to face drought. There are several reports suggesting that increased tolerance to different kinds of stresses rely on similar metabolic adjustments, e.g. drought and freezing tolerance. In pine for example, protection against drought was accompanied by induction of superoxide dismutase (SOD) activity (Alonso et al., 2001), along with an increase in the expression of

chaperones and late embryogenesis abundant proteins (Wang et al., 2003). These responses were also observed in other plants exposed to low temperatures (Rubio et al., 2002). Likewise, in Norway spruce, freezing tolerance is strictly correlated with drought tolerance, and this correlation is partially based on high SOD activities (Blödner et al., 2005). Other authors found out in *Quercus ilex* a significant increase in heat tolerance by drought exposure supporting that the mechanisms developed during acclimation to both stresses are strongly related, as both involve synthesis of heat shock proteins (Gimeno et al., 2009).

However, it has been reported that population differences in drought resistance are more related to the climate of origin (pedoclimatic adaptation and genetic background), while heat stress responses are governed by phenotypic plasticity and acclimation to environmental growing conditions (Marias et al., 2017). All this considered, deeper understanding of the molecular mechanisms involved in the altered behaviours observed among plants originating from different temperature regimes should be required.

To this purpose, a deep analysis of the CK profile of EMs produced under high temperature regimes was performed. Many studies have been published on conifer SE development during the last decades, but relatively little work has been carried out about the involvement of endogenous phytohormones during the process, and most of such studies are addressing different issues, i.e. the comparison of embryogenic and non-embryogenic calluses or genotypes/explants with different embryogenic ability (Bravo et al., 2017; Arrillaga et al., 2019). Even less information is available about the effect of stress on the hormonal profile over the course of SE, as most of the studies are carried out at plant level.

In this study most of the well documented isoprenoid CK forms and conjugates were observed, including bioactive bases, transport (CK riboside forms) and storage (CK N- and *O*- glucoside forms) forms, as well as CK nucleotides as biosynthetic precursors. However, no CK N-glucosides (7-G/9-G), the irreversible and inactive end products of cytokinin metabolism, could be detected, suggesting that the CK N-glucosyltransferase pathway, which takes part in numerous developmental processes such as growth of conifer buds (Zhang et al., 2003) or their organogenesis *in vitro* (Montalbán et al., 2013), is insignificant in SE, as already observed in other conifer studies such as Norway spruce (Vondrakova et al., 2018) or Douglas-fir (Gautier et al., 2019).

All the rest of CK forms observed (bases, transport and precursors) were at very low concentrations, which is in accordance with other studies executed in *Acca sellowiana* (Pescador et al., 2012) and *Picea abies* (Vondrakova et al., 2018) in which a drastic drop in the CK profile was observed during EM proliferation stage. In our experiments, the iP and cZ-types represented the major endogenous CKs in EMs. The highest amounts of cZ-types were in the active forms (cZ), while the most abundant form in the case of iP was the biosynthetic precursor iPMP. In spite of being considered CK derivatives with low biological activity in *Arabidopsis* (Nishiyama et al., 2011), cZ has been demonstrated to be the dominant form of CK in somatic embryos of radiata pine (Moncaleán et al., 2018) and cotyledons of stone pine (Moncaleán et al., 2005). cZ is an abundant and active CK in most monocotyledonous species (Nishiyama et al., 2011) and plays an important role during the development of seeds of eudicots (Tomaž and Marina, 2010). Regarding iP forms, studies carried out during early SE in the tree fern *Cyathea delgadii* showed greater concentrations of Z-type CKs compared to iPs (Grzyb et al., 2017). Nevertheless, results obtained from hormonal analysis performed during SE in cotton highlight the importance of iP during redifferentiation and embryogenesis

induction (Zeng et al., 2007). Besides, in peach seeds, nucleotides and ribosides of the iP-type are predominant and are involved in embryo formation (Arnau et al., 1999).

Concerning the effect of temperature in the hormonal profile of EMs, several differences were observed among treatments for total CK bases and some CK forms such as iP, cZR and tZR. In the case of total CK bases, iP and cZR, a decreasing tendency was detected when applying higher temperature regimes than control (23ºC, 8 weeks), especially for those applied for the longest periods of time (40ºC-4 hours and 50ºC-30 minutes). On the other hand, tZR levels showed the opposite behaviour, presenting the highest values at the highest temperature regime (60ºC, 5 min), following an increasing tendency as temperature rose. These results support the idea that despite not being the best-studied hormone group for plant stress-responses, CKs actively participate in the transduction of signals (Argueso et al., 2009). These signals are triggered by various environmental stimuli, among which temperature plays a crucial role (Pavlů et al., 2018) potentially via fluxes of Ca^{2+} ions (Černý et al., 2014).

The differences observed in the hormonal profile could explain the varying behaviour observed throughout the whole SE under the same conditions reported in Castander-Olarieta et al. (2019), but also in previous studies when high temperatures and/or different agar concentrations were used. As observed in Moncaleán et al. (2018) and Fraga et al. (2016), high concentrations of cZR and active CKs in general are correlated with high production of mature embryos, which is in disagreement with the results obtained in the linked study Castander-Olarieta et al. (2019). In this case, the application of high temperatures led to a slight but not significant increase in the production of somatic embryos, the same treatments that provoked a decrease in the amount of most CKs in the present work. However, the longest exposure treatments (50ºC, 30 min) gave rise to a great amount of barrel-shaped cotyledonary somatic

553 embryos, which usually present low germination and rooting rates, suggesting that CKs
554 not only regulate the production of somatic embryos but also influence their
555 morphology. On the other hand, Moncaleán et al. (2018) showed that the highest
556 success in the SE process coincided with low levels of iP types, as observed in our
557 study.

558 At plant level, a decline in endogenous CK levels in reaction to stress has long been
559 observed (Shashidhar et al., 1996), which is in accordance with the results obtained for
560 EMs in this study, and also in previous ones (Moncaleán et al., 2018). CKs and ABA
561 appear to have antagonistic roles, among others, in controlling stomatal function
562 (Moncaleán et al., 2007; Kohli et al., 2013). Many studies have detected that heat stress
563 induces a rapid but transient increase in active CK contents, followed by its significant
564 depletion, suggesting that CKs could serve as first signal for thermomorphogenesis
565 (Skalák et al., 2016). Afterwards, hormones classically related to stress such as ABA
566 and SA seem to be the main agents involved in early impacts of stress in radiata pine
567 (Escandón et al., 2016). As a result, CKs seem to exert a negative role on stress
568 responses and a reduction in CK levels in short-term stress exposures has been
569 demonstrated to improve drought and salt tolerance (Nishiyama et al., 2011; Bielach et
570 al., 2017). Those plants usually present higher water contents, but not only because of a
571 strict stomatal regulation, but also because of a better root system, since CKs are known
572 to negatively regulate root growth and lateral root formation (Ramireddy et al., 2018).

573 However, for acclimation and recovery of plants, CKs seem to take active part by
574 promoting a reduction in the oxidative damage and activating the stomata opening
575 required to recover physiological functions and growth (Escandón et al., 2016). De
576 Diego et al. (2015) also postulated that zeatin ribosides could act as protective
577 molecules against drought. In fact, CKs trigger the accumulation of heat-shock proteins

(Černý et al., 2011), and specifically, tZR has been proved to diminish membrane electrolyte leakage and activate the antioxidant system (Liu et al., 2002). In line with this information, the decrease in initiation and proliferation rates observed under high induction temperatures and long exposures in previous and linked studies (Castander-Olarieta et al., 2019) could be influenced to a large extent by the general decrease detected in the CK content of EMs, since micromophological and ultrastructural analyses revealed the presence of oxidative damage in embryogenic cells. In the same way, an increase in the levels of tZR when applying 60ºC for 5 minutes could be the reason for the preservation of big, well organized embryogenic structures observed in Castander-Olarieta et al. (2019).

In parallel, García-Mendiguren et al. (2016b) showed that the stressful conditions during the initiation phase of radiata pine SE continued to influence the maturation phase by the presence of chaperones, heat-shock proteins and osmotically induced proteins in mature somatic embryos. These results correlate with the increased levels of tZ riboside observed in EMs initiated under high temperatures, reinforcing its role as activator of the antioxidant system.

This study provides deeper information about the effect of temperature on SE at a physiological level, about a possible role of CKs in this process, and laid the foundation for a better understanding about the relationship between environmental conditions during SE and plant behaviour months later. However, further research is required at plant level to elucidate which are the molecular/physiological mechanisms involved in drought tolerance.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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7 Author contributions

PM and IM conceived and planned the experiments. ACO prepared all the plant material and carried out the drought experiment. CP, AP, IPe, IPa, ON and MS executed the hormonal analysis. TG and MDU carried out the statistical analyses. ACO wrote the manuscript and all authors provided critical feedback and helped shape the research, analyses and manuscript.

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Figures

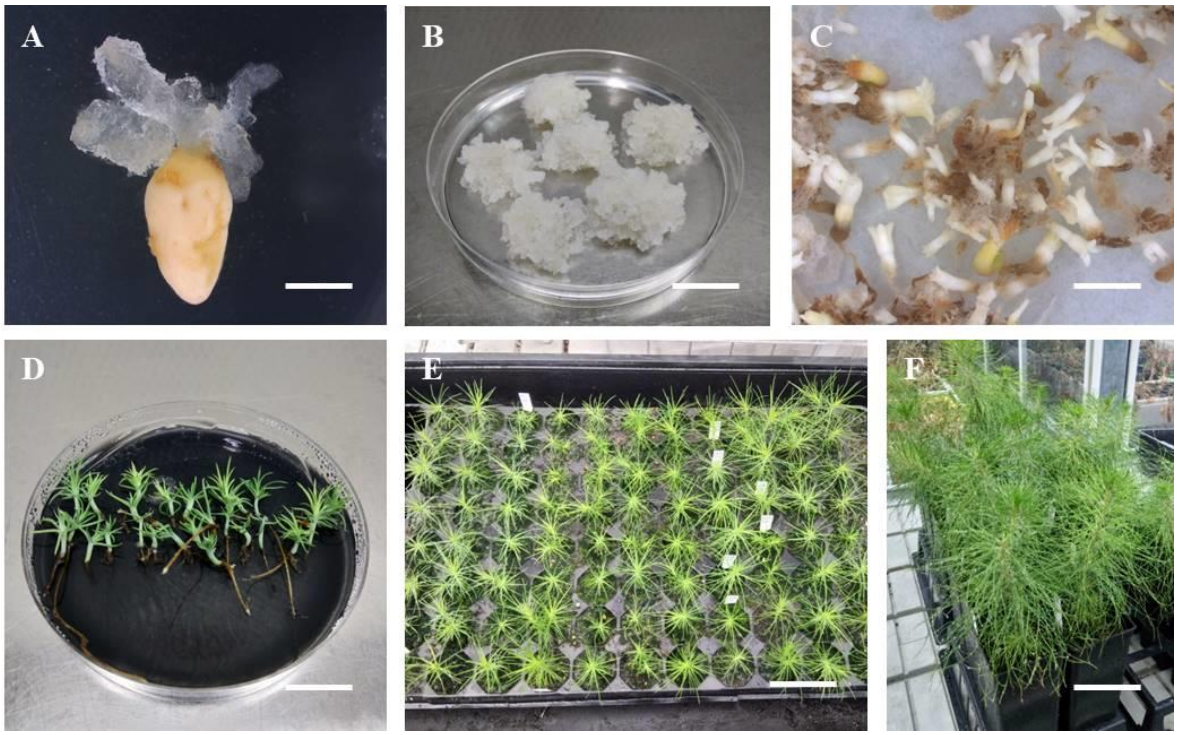
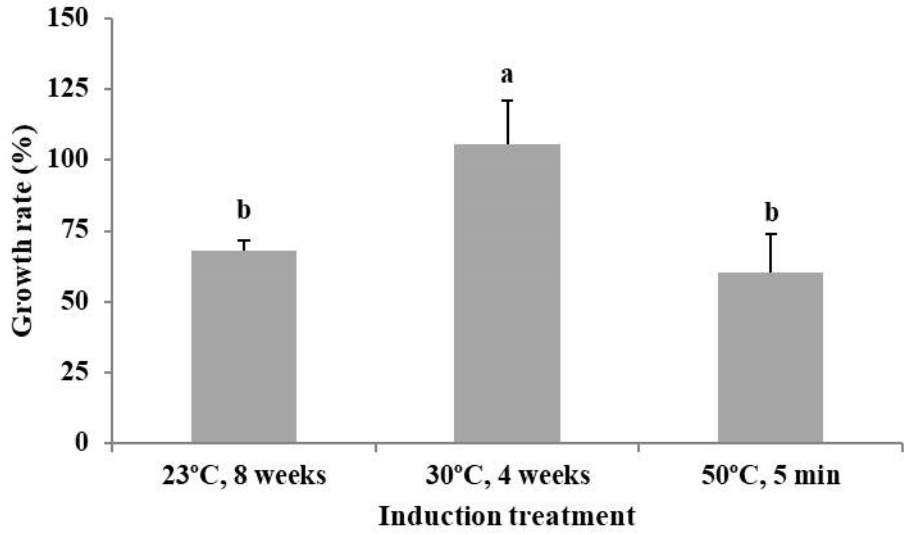


Fig. 1 Extrusion of *Pinus radiata* embryonal masses from megagametophytes (bar = 4.5 mm; 8 weeks) (A), proliferating embryonal masses (bar = 2.4 cm; 12 weeks) (B), cotyledonary somatic embryos (bar = 5.5 mm; 12 weeks) (C), germinated somatic plantlets (bar = 2.3 cm; 5 weeks) (D), acclimatized somatic seedlings (bar = 5.5 cm; 8 weeks) (E) and two-year-old somatic plants ready for the drought experiment (bar = 6 cm) (F).

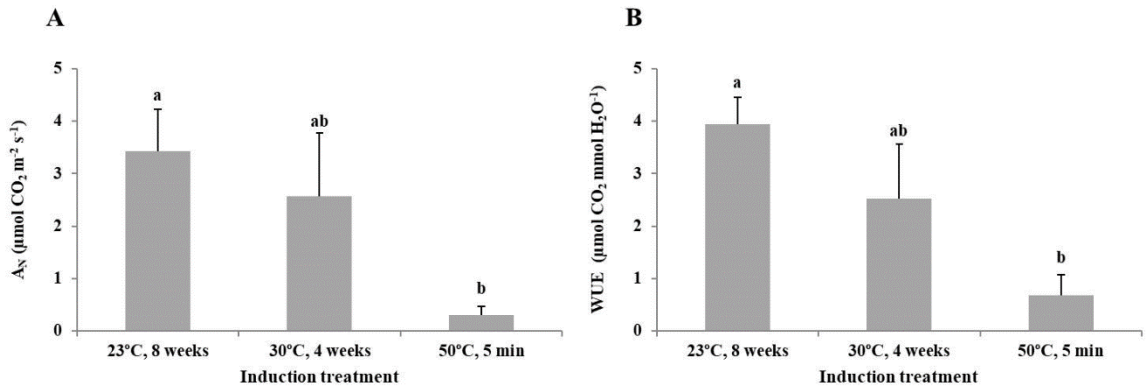


990

991 Fig. 2 Growth rate (%) of two-year-old somatic plants produced under different induction
992 temperature regimes (23ºC, 8 weeks, control; 30ºC, 4 weeks; 50ºC, 5 minutes) and grown
993 in the greenhouse for 12 weeks in watered conditions. Seven plants were used per
994 treatment and data are presented as mean values ± SE. Significant differences at $p < 0.05$
995 are indicated by different letters.

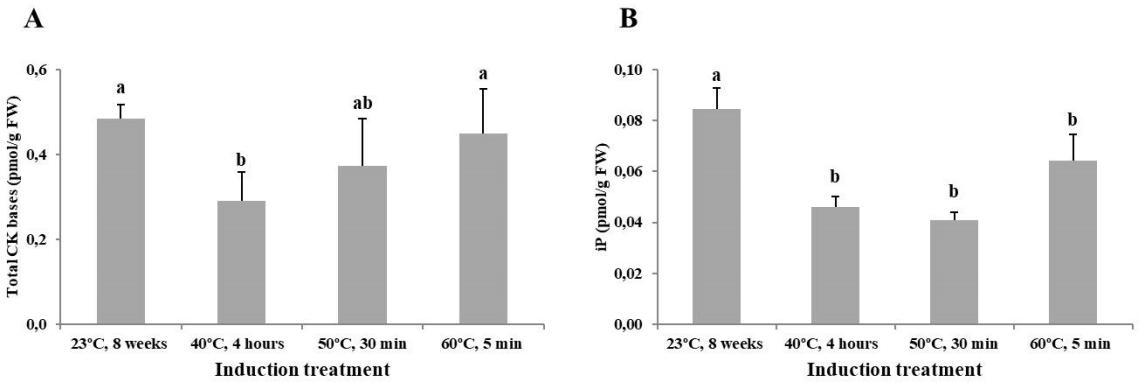
996

997 Fig. 3 Two-year-old *P. radiata* somatic plants originating from the induction treatment of
998 50°C for 5 min grown in either well-watered control condition (bar = 2.72 cm) (A) or
999 drought-stress conditions for 12 weeks (bar = 2.5 cm) (B). See the apparent needle epinasty
1000 and apical curvature in drought condition.



1001

1002 Fig. 4 Instant net photosynthesis (A, A_N) and water use efficiency (B, WUE) of two-year-
1003 old *P. radiata* somatic plants originating from different induction treatments (23ºC, 8
1004 weeks, control; 30ºC, 4 weeks; 50ºC, 5 minutes) and grown in drought-stress conditions for
1005 12 weeks. Seven plants were used per treatment and data are presented as mean values ±
1006 SE. Significant differences at $p < 0.05$ are indicated by different letters.



1007

1008 Fig. 5 Effect of temperature treatment (23ºC, 8 weeks; 40ºC, 4 hours; 50ºC, 30 minutes;
1009 60ºC, 5 minutes) on the levels of total CK bases (A) and N^6-isopentenyladenine (iP) (B) in

P. radiata EMs. Three biological replicates were used per treatment and data are presented as mean values ± SE. Significant differences at $p < 0.05$ are indicated by different letters.

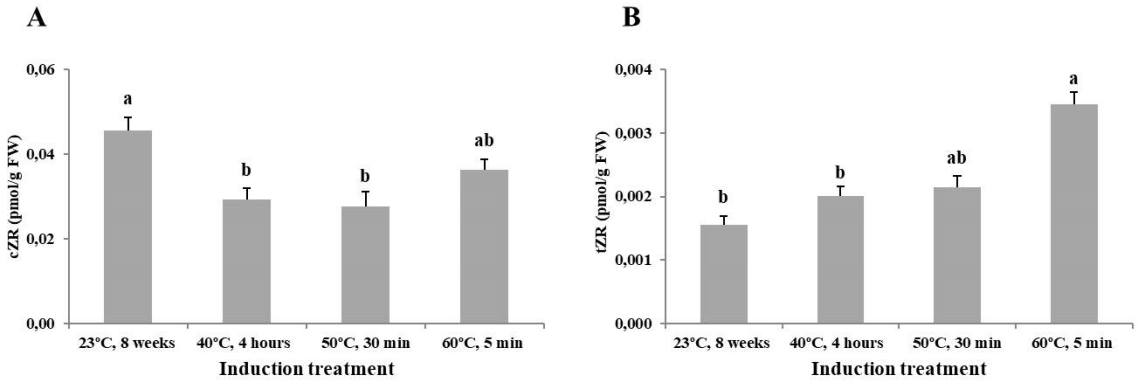


Fig. 6 Effect of temperature treatment (23ºC, 8 weeks; 40ºC, 4 hours; 50ºC, 30 minutes; 60ºC, 5 minutes) on the levels of cZR (A) and tZR (B) in *P. radiata* EMs. Three biological replicates were used per treatment and data are presented as mean values ± SE. Significant differences at $p < 0.05$ are indicated by different letters.

Tables

Table 1 Classification of isoprenoid CK types by functional groups analysed by mass spectrometry.

	Functional group				
Isoprenoid CK type	**Bases**	**Transport**	**Precursors**	**Storage**	**Irreversible metabolites**
N^6-Isopentenyladenine	iP	iPR	iPMP		iP7G, iP9G
cis-Zeatin	cZ	cZR	cZRMP	cZOG, cZROG	tZ7G, cZ9G
trans-Zeatin	tZ	tZR	tZRMP	tZOG, tZROG	tZ7G, tZ9G
Dihydrozeatin	DHZ	DHZR	DHZMP	DHZOG, DHZROG	DHZ7G, DHZ9G

Table 2 Survival, growth rate and physiological parameters of two-year-old *P. radiata* somatic plants grown in well-watered (W) or drought stress (D) conditions for 12 weeks. 21 plants were used for each condition (seven plants from each treatment, each one from a different ECL). Data are presented as mean values ± SE.

	Survival	**Growth rate**	**Ψ_{pd}**	**RWC**	**A_N**	**g_s**	**E**	**WUE**
	(%)	(%)	(MPa)	(%)	(μmol CO_2 m^{-2} s^{-1})	(mmol H_2O m^{-2} s^{-1})	(mmol H_2O m^{-2} s^{-1})	(μmol CO_2 $mmol^{-1}$ H_2O)
W	100	77.9 ± 13.9	-0.63 ± 0.02	76.9 ± 1.3	17.4 ± 0.9	0.52 ± 0.06	5.45 ± 0.29	3.22 ± 0.15
D	66.7	28.2 ± 2.9	-1.4 ± 0.08	63.7 ± 4.1	1.8 ± 0.9	0.02 ± 0.005	0.58 ± 0.11	2.38 ± 0.49

Ψ_{pd}, water potential; RWC, relative water content; A_N, instant net photosynthesis; g_s, stomatal conductance; E, instant leaf transpiration; WUE, water use efficiency

Table 3 ANOVA of different physiological parameters obtained after the drought period (12 weeks) according to the 3 temperature induction treatments from which plants were obtained. Seven two-year-old plants from different ECLs were tested per treatment.

Source	**df**	**F value**	***p* value**
Growth rate			
Temperature (T)	2	3.21	0.064
Ψ_{pd}			
Temperature (T)	2	1.39	0.274
A_N			
Temperature (T)	2	3.63	0.047
g_s			
Temperature (T)	2	3.38	0.057
E			
Temperature (T)	2	3.45	0.054
WUE			
Temperature (T)	2	5.45	0.014
RWC*			
Temperature (T)	2	2.23*	0.327*

Ψ_{pd}, water potential; RWC, relative water content; A_N, instant net photosynthesis; g_s, stomatal conductance; E, instant leaf transpiration; WUE, water use efficiency
*The values for RWC in the table correspond to the Kruskal-Wallis test. The normality assumption for this variable could not be assumed

Table 4 Detectable endogenous cytokinins (pmol g^{-1} FW) in *P. radiata* EMs initiated under different induction treatments (23ºC, 8 weeks; 40ºC, 4 hours; 50ºC, 30 minutes; 60ºC, 5 minutes). Three ECLs per treatment and 6 technical replicates per ECL were used. Data are presented as mean values ± SE. Significant differences within a row at $p < 0.05$ are indicated by different letters.

Cytokinins (pmol/g FW)	Induction treatment (ºC)			
	23	**40**	**50**	**60**
iP	0.084 ± 0.008^{a}	0.046 ± 0.004^{b}	0.041 ± 0.003^{b}	0.064 ± 0.01^{b}
iPR	0.012 ± 0.005^{a}	0.009 ± 0.002^{a}	0.009 ± 0.002^{a}	0.01 ± 0.003^{a}
iPMP	0.387 ± 0.260^{a}	0.314 ± 0.136^{a}	0.303 ± 0.1^{a}	0.249 ± 0.062^{a}
cZ	0.299 ± 0.106^{a}	0.223 ± 0.112^{a}	0.23 ± 0.06^{a}	0.237 ± 0.047^{a}
cZR	0.045 ± 0.003^{a}	0.029 ± 0.003^{b}	0.028 ± 0.003^{b}	0.036 ± 0.002^{ab}
tZR	$0.0016 \pm 1 \times 10^{-4b}$	$0.002 \pm 2 \times 10^{-4b}$	$0.0022 \pm 2 \times 10^{-4ab}$	$0.0034 \pm 2 \times 10^{-4a}$
DHZ	0.059 ± 0.008^{a}	0.037 ± 0.029^{a}	0.073 ± 0.046^{a}	0.097 ± 0.01^{a}
DHZR	0.007 ± 0.001^{a}	0.009 ± 0.003^{a}	0.008 ± 0.002^{a}	0.015 ± 0.01^{a}
Total CK bases*	0.484 ± 0.034^{a}	0.291 ± 0.069^{b}	0.367 ± 0.112^{ab}	0.449 ± 0.106^{a}
Total CK ribosides**	0.058 ± 0.011^{a}	0.048 ± 0.014^{a}	0.046 ± 0.012^{a}	0.059 ± 0.011^{a}
Total CK nucleotides***	0.241 ± 0.065^{a}	0.584 ± 0.302^{a}	0.367 ± 0.162^{a}	0.506 ± 0.314^{a}
Total iP****	0.479 ± 0.252^{a}	0.368 ± 0.142^{a}	0.353 ± 0.096^{a}	0.323 ± 0.066^{a}

*Total CK bases: iP, cZ, tZ, DHZ; **Total CK ribosides: iPR, cZR, tZR, DHZR; ***Total CK nucleotides: iPMP, cZMP, tZMP, DHZMP; ****Total iP: iP, iPR, iPMP