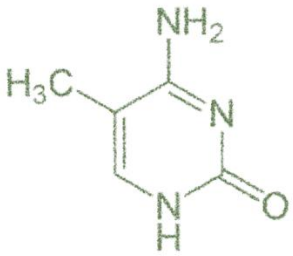

Embriogénesis somática de *Pinus* spp. bajo condiciones de estrés abiótico: modelo para el estudio de los mecanismos que controlan la tolerancia

Antonia Maiara Marques do Nascimento **2022**

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Universidad del País Vasco

Euskal Herriko Unibertsitatea

Embriogénesis somática de *Pinus* spp. bajo condiciones de estrés abiótico: modelo para el estudio de los mecanismos que controlan la tolerancia

Directoras

Paloma Moncaleán

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Antonia Maiara Marques do Nascimento (2022)

A Dios por todas las oportunidades.

A mis padres: Raimunda y Manoel.

A mis hermanos: Manoel y Marciel, y a mi hermana: Aldiana y Niqueias.

A Rubén y a Teodoro.

Dedico y ofrezco

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INDEX

ABBREVIATIONS

ABA	abscisic acid
A_N	instant net photosynthesis
ANOVA	analysis of variance
Arg	L-arginine
Asn	L-asparagine
Cad	cadaverine
CC	carbohydrate concentrations
CS	carbohydrate sources
DW	dry weight
df	degrees of freedom
E	instant leaf transpiration
EC_f	electrolytic conductivity final
EC_i	electrolytic conductivity initial
ECL*s*	established cell lines
EC_t	electrolytic conductivity total
EDM	Embryo Development Medium
E.L.	electrolyte leakage
EMs	embryonal masses
ESI	electrospray ionization source
FW	fresh weight
glm	general linear model
Gln	L-glutamine
g_s	stomatal conductance
GT	greenhouse's temperature
HPLC	high-performance liquid chromatography
I	irrigation condition
LE	length of somatic embryo
MA	mixes of amino acids
MIX I	550 mg L^{-1} of L-glutamine, 525 mg L^{-1} of L-asparagine, 175 mg L^{-1} of L-arginine, 17.5 mg L^{-1} of L-proline, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine
MIX II	1100 mg L^{-1} of L-glutamine, 1050 mg L^{-1} of L-asparagine, 350 mg L^{-1} of L-arginine, 35 mg L^{-1} of L-proline, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine
MIX III	2200 mg L^{-1} of L-glutamine, 525 mg L^{-1} of L-asparagine, 175 mg L^{-1} of L-arginine, 17.5 mg L^{-1} of L-proline, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine
MM	maturation medium
MRM	multiple reaction monitoring
MT	maturation temperatures
NAE	abnormal somatic embryos
NI	no irrigation
NNE	number of normal mature somatic embryos
ns	non significant
PAs	polyamines
PEG	polyethylene glycol
Pro	L-proline

Put	putrescine
R	Pearson's correlation coefficient
RWC	relative water content
SE	somatic embryogenesis
SE	standard error
ses	somatic embryos
Spd	spermidine
Spm	spermine
TET	ten-eleven translocation
TW	turgid weight
UI	under irrigation
WE	width of somatic embryo
Ψ_{leaf}	water potential
175 mal	175 mM of maltose
175 suc	175 mM of sucrose
350 mal	350 mM of maltose
350 suc	350 mM of sucrose
5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine

INTRODUCTION

INTRODUCTION

1. *Pinus radiata* D. Don and *Pinus halepensis* Mill.

The family Pinaceae belongs to the conifers group (order Coniferales - around 615 species), with 11 genera and 232 species that are characterized by flattened and needle-like leaves, being naturally present in the Northern Hemisphere (Farjon, 2010). In general, the Pinaceae family stands out among the conifers for its economic and ecological importance, the genus *Pinus* (around 110 species) occupies the first place within the family (Farjon, 2010, 2018; Kaundun and Lebreton, 2010). In Europe, the forests are composed approximately of 334 million m^3 of pine species influencing hydrological and biogeochemical cycles (Sánchez-González, 2008; Global Forest Resources Assessment 2020, 2020). Moreover, the pine species are base for wood-based industries, provide firewood, resins, comestible seeds, pulp, paper and other non-timber forest products (Sánchez-González, 2008; Farjon, 2010, 2018; Neis et al., 2019; Li et al., 2020).

Radiata or Monterey Pine (*Pinus radiata* D. Don) (Figure 1A), also known as Californian pine, Remarkable pine or Insignis pine, is native to some locations in North America (the United States, Coast of California, Baja California, and Mexico) (Farjon, 2010; Mead, 2013; Dwyer, 2021). Although the native regions of this species are severely fragmented, *P. radiata* is one of the most widely cultivated pine species in the world for its appreciated timber value, and currently, it is widely cultivated in New Zealand (1,494,429 ha) (NEFD, 2020), Australia (775,000 ha) (Downham and Gavran, 2020), Chile (1,285,640 ha) (INFOR, 2020), South Africa (57,000 ha) (Mead, 2013) and Spain (263,271 ha) (MITECO, 2015a). In Spain, huge plantations of *P. radiata* can be found in the Basque Country region (Figure 2A) (109,440 ha), representing 28% of the total wooded forest area, which are intended for forest timber productivity (about 80-85% of the annual timber logging) (HAZI, 2020). In addition to wood production, *P. radiata* is important in several areas. Culturally, its management is a source of employment and wealth; environmentally, improves air quality and prevents erosion; and in the supply of products, it is a source of fiber and pulpwood, in addition to other functions (Mead, 2013).

Figure 1. (A) *Pinus radiata* D. Don tree in seed orchard established by Neiker-BRTA in Deba (Spain); **(B)** apical buds and **(C)** immature green cones of *P. radiata*.

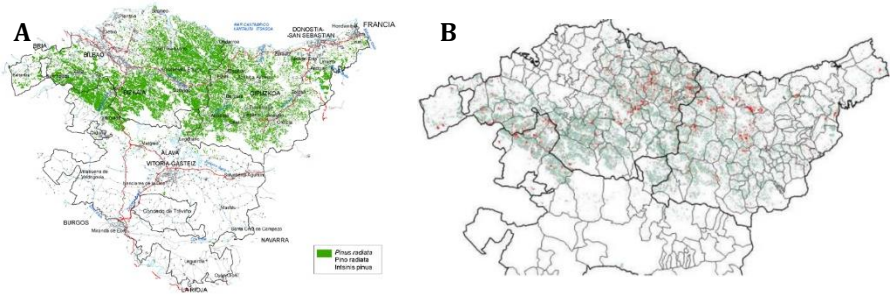

Figure 2. (A) *Pinus radiata* D. Don area in The Basque Country (Spain) (Inventario forestal de la Comunidad Autónoma del País Vasco 2005). **(B)** Evolution of *Pinus radiata* D. Don distribution between 2018 (red) and 2020 (green) (Inventario forestal del País Vasco-2020).

P. radiata tree is a quick-growing with 30 to 50 m tall with gray bark, the dark-green leaves (needles) are in fascicles in groups of three (Figure 1B), 8 to 15 cm long, in dense clusters persisting 3 to 4 years, the cones (Figure 1C) may be solitary or in clusters and have seeds maturing in 2 years, the cones may have 7 to 15 cm long and scales rounded on their exposed portion (Bussmann et al., 2020; Dwyer, 2021). The growth of *P. radiata* is influenced by environmental conditions such as winter rain (> 600 mm per year) and relatively dry summers, soil depth (> 60 cm), well-drained soils, but not soggy soils, fertile and acid soils (Mead, 2013). However, it is known that ecotypes planted in the Basque Country are more sensitive to water stress than others from original provenances (De Diego et al., 2013, 2015). Furthermore, there was a reduction in *P. radiata* plants of 9,438 ha from 2018 to 2020 (HAZI, 2020) (Figure 2B) in the Basque Country due to the premature felling caused by the pathogenic fungi *Dothistroma septosporum* and *Dothistroma pini* (Elvira-Recuenco et al., 2020). Although it is now affected by biotic stress

they usually come combined with abiotic ones as a consequence of climate change (Shahzad et al., 2021).

On the other hand, Aleppo pine (*Pinus halepensis* Mill.) (Figure 3A), also known as Pino de Alepo, Pino Carrasco (Spanish), is native from the Mediterranean area (Farjon, 2010). This species has been used to restore degraded areas and it is widely planted for soil and water conservation purposes (Olarieta et al., 2000) in many countries in Northern Africa, Middle East and Southern Mediterranean Europe (Kadri et al., 2013; Rigane et al., 2019). Huge plantations of *P. halepensis* can be found in Algeria (1,158,533 ha) (Benouadah et al., 2018), Tunisia (361,222 ha) (DGF, 2010; Jaouadi et al., 2019), France (222,000 ha) (Hervé, 2016), Italy (226,101 ha) (Chambel et al., 2013) and Spain (2,080,000 ha) (Alberdi et al., 2016). In Spain (Figure 4), *P. halepensis* is the species of pine that occupies a greater surface at the national level, being widely distributed by the coastal provinces between sea level and 1,000 m altitude such as in the Comunidad Valenciana (467,929 ha), Cataluña (349,327 ha) (16,79%), and Castilla La Mancha (321,934 ha), that represent 54.76% of the total *P. halepensis* area (MITECO, 2015b). In the Basque Country region, *P. halepensis* occupies an area of 546 ha (MITECO, 2015b).

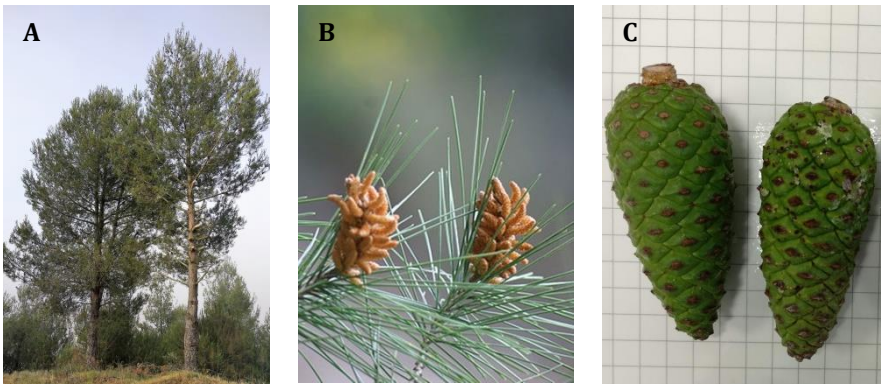

Figure 3. (A) *Pinus halepensis* Mill. trees in seed orchards established in Berantevilla (Spain); **(B)** apical buds (from: euforgen.org) and **(C)** immature green cones of *P. halepensis*.

Figure 4. *Pinus halepensis* Mill. D. area in Spain. (MITECO, 2015. Los pinares de pino carrasco).

Unlike *P. radiata*, *P. halepensis* trees are not used particularly in the forestry industry because the timber density is too high, but it is a species with high forest importance for reforestation programs preventing soil erosion and improving water infiltration (Chambel et al., 2013; Gazol et al., 2017; Quinto et al., 2021). In Spain, due to its important ecological plasticity, *P. halepensis* is also extensively used for afforestation in North-Western areas of the Iberian Peninsula, very often out of its natural habitat range (Abelló, 1988; Voltas et al., 2018). In addition, the seeds of *P. halepensis* are used in human consumption (Jaouadi et al., 2019), the wood is used in the pulp and paper industry (Hassan et al., 2020) as well as for firewood and others uses (Antonović et al., 2018; Llorenç et al., 2020).

The *P. halepensis* tree is on average 20 m tall, with gray-brown bark, the dark-green needle-like leaves (6-12 cm long) formed in pairs persisting for 2 to 3 years, the cones (7-12 cm long) (Figure 3B) are single or in clusters with 2-3 female cones and are mostly serotinous cones with seeds are 5-6 mm long (Lev-Yadun, 1992; Farjon, 2010; Chambel et al., 2013; Balaguer-Romano et al., 2020; Trstenjak et al., 2020). *P. halepensis* grows in semi-arid and sub-humid climates (350–700 mm annual rainfall) with -2 and 10 °C as absolute minimum temperatures and limestone shallow soils (Chambel et al., 2013). Nevertheless, factors such as extreme drought, as well as forest fires, will put some populations of *P. halepensis* at risk of extinction in south-eastern Spain and the interaction between these two factors can alter the post-fire recovery resilience of *P. halepensis* in the future (Chambel et al., 2013; Elvira et al., 2021).

Given the current climate scenario with rising temperatures and drought, for the survival of forest trees with economically important characteristics, breeding programs must select trees that are tolerant to climate changes (Da Ros et al., 2021; Rai et al., 2021). For that, it is necessary to obtain trees with higher stress tolerances. The best way to obtain selected individuals with respect to a character is vegetative propagation (Hazubska-Przybył et al., 2022), but in Pinaceae, when the tree has shown the interest characters, it has changed its phase and therefore its ability to be propagated conventionally has decreased (Imanuddin et al., 2020). For this reason, advanced countries in the forestry sector develop genetic breeding programs in which they combine traditional techniques with new biotechnological tools such as *in vitro* culture through organogenesis and somatic embryogenesis (SE) (Montalbán et al., 2011; Rosvall, 2019; Hazubska-Przybył et al., 2022; Liang et al., 2022).

2. Somatic Embryogenesis

SE is defined as a process in which a bipolar structure develops from a non-zygotic cell without vascular connection to the original tissue that resembles a zygotic embryo but lacks an endosperm and outer covering (Von Arnold et al., 2002). SE is based on cellular totipotency, in which it is possible to produce a whole new plant from a single cell through internal and external stimuli, but it is dependent on the genotypes, developmental stage, explants and transcription factors (Fehér, 2019; Su et al., 2021).

SE was first reported in *Daucus carota* L. cell suspensions by Steward et al. (1958) and Reinert (1958) and, since then, the potential for somatic embryogenesis has been shown for a great variety of plants species. In conifers, the first report of SE was reported for *Picea abies* L. Karst. (Chalupa, 1985; Hakman et al., 1985), and since then numerous protocols have been developed for other conifers species, including *Pinus* (Klimaszewska et al., 2016).

The SE has five complex important stages (initiation, proliferation, maturation, germination, and acclimatization *ex vitro*) (Tao et al., 2021). The initiation and proliferation of embryonal masses (EMs) start from somatic cells, which are stimulated to dedifferentiate and proliferate as embryogenic tissue (von Arnold et al., 2019). *Pinus*

species have cleavage polyembryony in which the most common way to initiate embryogenic tissue is from immature zygotic embryos before cotyledon differentiation, with whole megagametophytes being commonly used as explants (Abrahamsson et al., 2018).

After the initiation and proliferation of EMs, the maturation of somatic embryos (ses) is a complex process that is influenced by many factors, such as the osmotic potential of the medium and temperature (Teyssier et al., 2011; Moncaleán et al., 2018; Valencia-Lozano et al., 2021). The maturation of ses of various *Pinus* spp. is routinely promoted by using media with very high gellan gum concentrations (up to 12 g L^{-1}) (Peng et al., 2021) and carbohydrate (up to 350 mM) (Garin et al., 2000), as well as a high abscisic acid (ABA) content (up to 121 μM) (González-Cabrero et al., 2018). After the maturation stage, the normal mature ses obtained are separated from the residual tissue and transferred to a germination medium that most often lacks of growth regulators (Montalbán et al., 2010; Salaj et al., 2019; Ferreira et al., 2022). After transfer to a germination medium, ses develop similarly to zygotic embryos, and when they have developed roots, they are transferred *ex vitro* under controlled conditions decreasing humidity progressively and thus ensuring successful acclimatization (Montalbán and Moncaleán, 2019). However, one common problem related to SE, especially in woody trees, is the low conversion of ses to plantlets, as a result of incomplete maturation, because only those mature ses with a normal morphology that have accumulated sufficient storage products will be able to convert into whole viable somatic plants (Etienne et al., 2013; Valencia-Lozano et al., 2021).

In our research group, some studies were carried out to optimize, through modification of components in the culture medium (mineral salts, nitrogen source, and plant growth regulators), some phases of the SE process (initiation and proliferation) in *P. radiata* (Montalbán et al., 2010, 2012). Furthermore, we have developed for the first time a SE methodology for *P. halepensis* (Montalbán et al. 2013). In addition, it was also tried to induce stress tolerances by modifying temperature conditions and water availability in the initiation and proliferation stages for *P. radiata* and *P. halepensis* (García-Mendiguren et al., 2016; Pereira et al., 2016, 2017, 2021; Castander-Olarieta et al., 2019). For *P. radiata*

and *P. halepensis*, it was reported that heat stress at the initiation and proliferation of SE, respectively, caused an increase in ses production (Pereira et al., 2017; Castander-Olarieta et al., 2019). Furthermore, the heat stress caused lasting effects at the plant level of *P. radiata* with changes in the cytokinin profile, as well as in the profile of heat shock proteins and proteins involved in translation, methylation, post-transcriptional regulation, and sugar/lipid metabolism (Castander-Olarieta et al., 2020, 2021a, 2021b).

Likewise, during the last years, in our laboratory, we demonstrated in *P. radiata* that the application of different maturation temperatures (18, 23 and 28 °C) led to different hormonal profiles and did not affect the *ex vitro* survival plantlets (Moncaleán et al., 2018). In addition, the application of lower maturation temperatures (18 °C) in *P. pinaster* Aiton resulted in somatic plants with a lower increase in ABA levels, faster and higher proline increase, no reduction in active cytokinin, and better recovery of the instant net photosynthesis (A_N) rate when subjected to subsequent *ex vitro* heat stress (45 °C for 3 h/day for 10 days) (Sales et al., 2022). However, although plantlet regeneration was achieved in *P. radiata* and *P. halepensis*, it is important to achieve a further optimization of these protocols with regard to physical and chemical conditions throughout all the different stages, especially in maturation, since the maturation stage is one of the biggest bottlenecks of SE (Montalbán et al., 2010).

Furthermore, studies to improve germination have been carried out, but the physiological mechanisms that control the increase in germination success have not yet been well elucidated (Montalbán and Moncaleán, 2019). These phenomena are limiting factors for the complete regeneration of plantlets and the implementation of SE for mass propagation which is the reason why further studies, focused on the morphological, physiological as well as epigenetic aspects of SE, are needed to overcome these problems.

3. Abiotic Stress in Plants

Climatic changes affect rainfall variability and cause an increase in temperatures, which can increase water deficit conditions, affecting all organisms, especially plants for being sessile organisms that cannot migrate elsewhere when food and water are limited (Imran et al., 2021). In this context, abiotic stress trigger signal transduction networks that alter

many physiological responses that can compromise the survivance of the plants (Erbilgin et al., 2021; Devireddy et al., 2021; Nantongo et al., 2022). For example, the water stress in *P. sylvestris* L. seedlings caused a decrease in the A_N, transpiration (E), stomatal conductance (g_s) and a strongly impaired leaf water potential (Ψ_{leaf}) (Kreuzwieser et al., 2021). Similar results were reported for *P. tabulaeformis* Carr. seedlings under moderate drought; however, a severe drought caused a death rate of more than 95% of seedlings (Wang et al., 2021). A combination of severe stresses (drought+heat) in *P. elliottii* var. *elliottii* × *P. caribaea* var. *hondurensis* hybrid provoked a stronger reduction on the net CO_2 assimilation rate and transpiration rate, and the plants presented higher levels of lipid peroxidation (Dias et al., 2022). In *P. radiata*, the drought response provoked a decrease in the physiological parameters that were ecotype-dependent (De Diego et al., 2012, 2015) and clon-dependent (Rodríguez-Gamir et al., 2019). Similarly, the drought stress affected the vital metabolic processes in *P. halepensis* plants, such as E, A_N, and respiration at their early development stage, as well as provoked changes in chlorophyll pigment contents and an accumulation of organic solutes (Ghazghazi et al., 2022). Furthermore, a combination of severe stresses (drought+heat) in seedlings of *P. halepensis* caused the death of most of the seedlings due to the cessation of dark respiration (Birami et al., 2021).

Furthermore, abiotic stress in plants influences cellular and molecular changes that affect plant growth and consequently productivity, with differences between resistant and tolerant genotypes (Bita and Gerats, 2013; Thanmalagan et al., 2022). For example, in *P. taeda* L. differences between tolerant vs. sensitive genotypes to water stress at a gene-by-gene level were found with upregulated transcripts under drought conditions (Li et al., 2021). In addition, in *P. pinaster* Ait. tolerant individuals considered pre-adapted to drought expressed stress-related genes (drought-related genes, specifically hormone-regulated genes, genes involved in signaling pathways and stress protection) that were detected only in the latter stages on sensitive individuals subjected to drought (de María et al., 2020).

On the other hand, plants have developed mechanisms to survive environmental stresses that are carried out by altering the expression level of some genes through the introduction of epigenetic modifications, such as histone variants, small RNAs, long

noncoding RNAs and DNA methylation generating/regulating phenotypic plasticity (Liu et al., 2015; Roy, 2016; Jeremias et al., 2018; Thiebaut et al., 2019; Miryeganeh, 2021). In this regard, DNA methylation involves the covalent modification of the residue cytosine of the DNA by the addition of a methyl group in the fifth carbon position of a cytosine ring, resulting in the 5-methylcytosine (5-mC) (Golubov and Kovalchuk, 2017; Koç et al., 2020), driving different responses according to species and plant development stages (Liu and He, 2020). The high global DNA methylation (around 38.8%) levels detected in P. *sylvestris* megagametophytes were associated with the plant adaptation to climate change, especially in the harsh north of Finland (Alakärppä et al., 2018).

Methylation marks can be removed through DNA demethylation that can result in the dilution of 5-mC during DNA replication, or in the oxidation of methylated cytosines that occurs through the ten-eleven translocation (TET) family of enzymes with the first oxidation product being 5-hydroxymethylcytosine (5-hmC) (Ito et al., 2011; Golubov and Kovalchuk, 2017; Wu and Zhang, 2017). Although the biological function of the 5-hmC is still controversial in plants (Golubov and Kovalchuk, 2017), recently in our research group, it was detected for the first time the presence of 5-hmC in the *Pinus* genus that was associated with an establishment of epigenetic memory and its levels have changed with heat stress in *P. radiata* somatic plants (Castander-Olarieta et al., 2020; Pereira et al., 2021).

Increasing resilience to adverse conditions in plants through epigenetic memory is a promising strategy that has been the focus of many studies on forest tree species (Yakovlev et al., 2010, 2011, 2016; Carneros et al., 2017; Yakovlev and Fossdal, 2017; Fox et al., 2018; Klupczyńska and Ratajczak, 2021; Pérez-Oliver et al., 2021). In this context, the zygotic and somatic embryo development and seed maturation can be key periods for the formation of stable epigenetic marks (Johnsen et al., 2005b; Kvaalen and Johnsen, 2008). For the first time, Johnsen et al. (2005a) reported that exposure to different temperatures and photoperiods (short days and high and low temperatures) during the development of seeds of the progeny of *Picea abies* L. Karst., caused the development of a memory mechanism, which, by regulating adaptive plasticity, could counteract the harmful effects of a rapidly changing climate. Thus, changes in environmental conditions,

such as photoperiod and temperature, during embryogenesis result in the formation of epitypes, and epigenetic memory is propagated mitotically, resulting in significant and lasting phenotypic changes in the seasonal time of bud phenology (Carneros et al., 2017; Yakovlev et al., 2020).

The application of heat stress aimed at establishing an epigenetic memory in plants varies with exposure time and temperature (Bita and Gerats, 2013). In *P. radiata* and *P. halepensis* SE, changes in the *in vitro* culture environment as the increase of temperature in the first stages of SE led to changes at a cellular level, different metabolite profiles (cytokinins, sugars) in EMs and ses (Castander-Olarieta et al., 2019, 2021a, 2021b; Pereira et al., 2020); in addition to generating an epigenetic memory with a decrease in DNA methylation/hydroxymethylation and differential expression of stress-related genes in somatic plants obtained (Castander-Olarieta et al., 2020). In this regard, in *P. radiata* and *P. halepensis*, high global DNA methylation (around 40%) was found, and in the highest temperatures applied in megagametophytes in the SE process led to hypomethylation of both embryonal masses and needles of somatic plants (Castander-Olarieta et al., 2020; Pereira et al., 2021). Also, Pérez-Oliver et al. (2021) reported that priming at 50 °C at the SE induction phase is a promising strategy to improve heat resilience in maritime pine and heat exposure of two-year-old maritime pine plants derived from primed embryogenic masses showed better osmotic adjustment and higher increases in chlorophyll, soluble sugars and starch contents. Therefore, in light of recent works, epigenetic changes seem to be one of the main issues around climate change, but there is a need for more intensive research, which is the least known area among plants, especially forest trees (Klupczyńska and Ratajczak, 2021).

4. Chemical Environment in Tissue Culture

Various types of basal media were employed for the maturation stage of *Pinus* spp. and the most frequently used nutrient media by our research group is the Embryo Development Medium (EDM) (Walter et al., 2005) and DCR medium (Gupta and Durzan, 1985) for *P. radiata* and *P. halepensis* SE, respectively (Montalbán et al., 2010, 2013).

In the culture medium, the carbon source is essential for the growth and development of *in vitro* grown cultures of many plant species because it plays multiple roles, such as C-skeletons source, energy of plant cells (Carlsson et al., 2017; Song et al., 2018; Gulzar et al., 2020), osmotic regulator (Klimaszewska et al., 2016), stress protectors and/or signaling molecules (Lipavska and Konradova, 2004). The common carbon source is provided by the supplementation of sucrose in the culture medium (Carlsson et al., 2017; Kaur et al., 2022) due to it is the predominant saccharide in the phloem sap in most plant species, its cheap and easy availability to obtain (Lipavska and Konradova, 2004; Yaseen et al., 2013). In addition, sucrose has rapid hydrolysis with a high supply of hexoses and storage compounds (Blanc et al., 2002) and has been hypothesized to have stimulatory effects on embryonic development, some of which are not replaceable by another carbon source such as glucose, fructose or their combinations (Iraqi and Tremblay, 2001). On the other hand, when other carbon sources were tested (e.g. lactose, mannitol, sorbitol, glucose, fructose, maltose) they were less efficient in most cases, although in some species the best results were achieved when some of these carbon sources were tested (Lipavska and Konradova, 2004). In this regard, unlike sucrose, maltose presents a slow hydrolysis, to which, the low supply of hexoses was a biochemical signal reported as favorable for the formation of ses of *Hevea brasiliensis* Mull. Arg. (Blanc et al., 2002). The number of ses of *Larix olgensis* Henry increased with the presence of sucrose (75 g L^{-1}) in the maturation medium but decreased significantly when sucrose was replaced with the same concentration of maltose (Song et al., 2018). On the other hand, in *P. uncinata subsp. uliginosa* G.E. Neumann Businský, the maturation medium supplemented with 6% maltose on which presented the highest number of early-precotyledonary/cotyledonary ses, while the lowest number of somatic embryos formed was recorded on media enriched with 6% sucrose (Vlašínová et al., 2017). In addition, Song et al. (2018) reported that a low concentration of sucrose is favorable for synchronization of development of ses, but it is not favorable for generating high-quality ses. In *P. radiata* maturation it was reported that the 175 mM increase in sucrose concentration significantly increased osmolality in the culture medium and those media with higher osmolality produced the highest number of ses (Montalbán et al., 2010).

In addition to the carbon source, the presence of nitrogen in the maturation medium is essential for the growth and development of plants, as they provide building blocks for the synthesis of a multitude of biomolecules, such as proteins, nucleic acids and chlorophyll (Carlsson et al., 2017). In the maturation medium, nitrogen is added in the form of inorganic nitrogen (NH_4^+ and NO_3^-) and organic nitrogen, whose main sources of organic nitrogen in the medium are in the form of casein hydrolysate, amino acid or amino acids mixture (Montalbán et al., 2010; Pullman and Bucalo, 2014; Dahrendorf et al., 2018). The Gln is one of the amino acids that support the growth of cells that have a high energy demand and synthesize large amounts of proteins and nucleic acids, such as embryogenic cells (Elmeer, 2013). The addition of Gln + NO_3^- in the maturation medium of *Picea abies* L. promoted the largest and most developed ses with prominent cotyledons when compared with those maturated in medium without Gln with a high germination rate (Dahrendorf et al., 2018). Although casein hydrolysate can be a source of several elements (calcium, phosphate, various microelements, vitamins, and mainly a mixture of amino acids), they are by nature relatively undefined supplements that can produce toxic substances that inhibit the growth of plant cells (George et al., 2008; Elmeer, 2013). In *P. radiata*, a mixture of various amino acids (L-glutamine - Gln, L-asparagine - Asn, L-arginine - Arg, L-proline - Pro, L-citrulline, L-ornithine, L-lysine and L-alanine) in the maturation medium improved the number and the quality of the ses obtained when compared with those maturated in medium supplemented with casein hydrolysate (Montalbán et al., 2010).

As mentioned above, the incorporation of carbohydrate and nitrogen sources has previously been studied in the SE process of conifers (Montalbán et al., 2010; Carlsson et al., 2017; Dahrendorf et al., 2018). However, there is still a paucity of information about the uptake and assimilation of nitrogen and carbon during the maturation stage in *P. radiata* and *P. halepensis*, as well as how these two factors interfere in the accumulation of accumulated solutes of ses, such as polyamines (PAs), and the subsequent stages of SE.

5. Morphological, Biochemical and Physiological Mechanisms that Govern Somatic Embryogenesis and the Stress Response

As mentioned above, the application of heat stress in the early stages of SE and zygotic embryogenesis can cause the establishment of an epigenetic memory that improves plant survival under stressful conditions (Johnsen et al., 2005a; Pérez-Oliver et al., 2021). Thus, studies that identify early changes in phenotype and physiological and biochemical parameters after the application of thermal and/or water stress help forest breeding programs to develop strategies to increase the tolerance/resistance of plants to stressful environments (San-Eufrasio et al., 2020; Blanco-Martínez et al., 2022; Yang et al., 2022).

Phenotyping of the plantlets obtained, through morphological attributes, such as length, width and number of secondary roots, provide a selection of plantlets with high quality that are able to survive and have a vigorous growth after the acclimatization process (Johnson and Cline, 1991; Grossnickle and MacDonald, 2018). Depending on the physicochemical conditions applied during the induction of SE, changes were observed at the morphological level and in the accumulated metabolites (Castander-Olarieta et al., 2019; Pereira et al., 2020). Furthermore, variations in these parameters studied in ses are associated with later success in plant conversion and acclimatization (Moncaleán et al., 2018). In *P. radiata*, ses that germinated early or with abnormally shaped cotyledons were classified as abnormal, while ses that were yellowish-white, with a distinct hypocotyl region and at least three cotyledons were classified as normal and subsequently germinated and produced viable plants (Garin et al., 2000; Montalbán et al., 2010).

Likewise, the content of PAs is frequently found in ses and in plants that are considered as one of the main signals of stress responses (Eliášová et al., 2018; Shahid et al., 2020; Mikuła et al., 2021). Putrescine (Put), spermine (Spm) and spermidine (Spd) are the three abundant PAs that are involved in the metabolic and biochemical processes of plants such as stress management, RNA transcription, protein synthesis, tolerance to biotic and abiotic stress as well as in the somatic embryo development (Minocha et al., 2004; Silveira et al., 2004; Gemperlová et al., 2009; Kumar et al., 2022; Pramanick et al., 2022). Cadaverine (Cad), another form of polyamine, has rarely been reported in conifers (De

Oliveira et al., 2015) but has been associated with modulating primary plant root growth in *Arabidopsis* (Gibbs et al., 2021) as well as stimulating callus formation and growth, and stress-induced proteins when *Brassica juncea* L. seeds cultivated under stress conditions (Tomar and Arora, 2021). Therefore, in order to establish the implication of PAs in SE in *Pinus* spp., it is necessary to analyze their content in the ses obtained.

Within the most studied physiological parameters, the Ψ_{leaf}, relative water content (*RWC*) and and the *E* have been considered good indicators of the water status in studies focused on the analysis of the effects of water deficit on plants (Jones, 2007). The Ψ_{leaf} is a good candidate to estimate the water status of plants because it is physically defined and allows experiments to be easily repeated (Kramer and Boyer, 1995). In *Pinus* spp., the Ψ_{leaf} can vary from values close to zero when the plants were watered to very negative values (around to -3 MPa) when the plants were under severe water stress (De Diego et al., 2012; Salmon et al., 2020; Wang et al., 2022). On the other hand, the *RWC* is an estimate of cellular hydration that shows the changes in cellular volume that could be affecting interactions between macromolecules and organelles (González and González-Vilar, 2001). Values of *RWC* below 80% imply usually water potential of -1.5 MPa or less, which would produce changes in the metabolism with the ceasing of the A_N (González and González-Vilar, 2001). The high temperatures promote an increase in water loss via *E* (Sadok et al., 2021). In this context, the plants can be isohydric species that maintain a relatively stable Ψ_{leaf} precisely because of their stricter stomatal control or anisohydric species that show a looser regulation of *E* with a decrease in Ψ_{leaf} as the soil water content decreases (McDowell et al., 2008). In conifers, *P. edulis* Engelm. and *Juniperus osteosperma* (Torr.) Little are examples of isohydric and anisohydric species, respectively, in which the first species may be more susceptible to carbon starvation and more vulnerable to sustained drought, while the second species may be more susceptible to hydraulic failure and acute or severe droughts (McDowell et al., 2008; Anderegg and Anderegg, 2013; Meinzer et al., 2014).

Furthermore, a relationship between an increase in the abiotic stress (high temperature and drought) and changes associated with gas exchange parameters has been reported in the literature for many plants species (Lima Neto et al., 2017; Augustine and Reinhardt,

2019; McDowell et al., 2019; Francesca et al., 2022). The increases in temperatures/drought stress cause a decrease in g_s of plants that reduce the rates of CO_2 entry and water vapor exit from leaves, as well as a decrease in A_N, hydration, and biomass accumulation that reduces crop yield (Augustine and Reinhardt, 2019). However, the relationship between the regulation of stomatal opening/closing and the regulation of water potential varies with the water status of the plants as well as the iso/anisohydric status of plant responses to drought (Martínez-Vilalta and Garcia-Forner, 2017).

In addition to water and gas parameters, the electrolyte leakage (*E.L.*) accompanies plant response to stresses as it is an indicator of membrane injury derived from cellular membrane failure (Bajji et al., 2002). During ideal plant growth conditions, antioxidant activity maintains homeostasis, although reactive oxygen species are produced by photosystems I and II (Guadagno et al., 2017). However, under severe or prolonged environmental stresses, such as drought, the antioxidant capacity can be depleted, causing cell necrosis due to cell membrane failure (Guadagno et al., 2017). In this respect, an increase in the relative *E.L.* of needles of *P. sylvestris* seedlings of the drought stress treatment in the mid-growing season indicated severe cell membrane injuries, which was associated with the reduction of chlorophyll fluorescence and increased mortality, as compared to drought stress treatment in the early-growing season and control saplings (Qian et al., 2021).

6. General Hypothesis and Objectives

Based on this information the general hypothesis of the present work was the following:

- The modification of the physical environment during the SE maturation stage can trigger the formation of a stable epigenetic memory, leading to the obtention of somatic plants with altered ability to deal with stress in *Pinus* spp.

To this purpose, the objectives were:

1. To study the effect of high temperatures on the success of the SE process.

2. To analyze the morphological features derived from the heat-stress response in ses and somatic plants.

3. To determine through physiological analyses and their interconnections if the resulting somatic plants present modified stress resilience under conditions of water stress and thermal stress.

4. To investigate if the application of heat has caused alterations in epigenetic marks in somatic plants.

5. To evaluate the influence of the different amino acid mixture and carbohydrate sources in the maturation stage of the SE.

6. To analyze the polyamines profile and the morphological characteristics of germinated somatic embryos of *P. radiata* and *P. halepensis*.

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CHAPTER 1

Pinus spp. somatic embryo conversion under high temperature: effect on morphological and physiological characteristics of plantlets

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1. INTRODUCTION

In the current climate change scenario, research is needed to enable plants to have greater water use efficiency in different environmental conditions (Pareek et al., 2020) and to develop drought tolerance (De Diego et al., 2015) and thermotolerance (Priya et al., 2019). This thermotolerance can be achieved by changing the conditions in the different stages of somatic embryogenesis (SE) without it being necessary to enforce any change in the DNA, but only by allowing the formation of epigenetic memory (Yakovlev et al., 2016; Taïbi et al., 2017).

Pinus halepensis is an important forest tree for reforestation in Mediterranean regions, because it has adapted to drought conditions with high temperatures (Gazol et al., 2017; Manrique-Alba et al., 2020). On the other hand, *Pinus radiata* D. Don is a forest tree of economic importance due to the capacity it has for intensive use of its wood (Fuentes-Sepúlveda et al., 2020). Preliminary studies with *P. radiata* species have reported that the high heat and drought tolerance in environments with climate variation is dependent on the ecotype (De Diego et al., 2012; 2015). The same observation has been made with Basque Country/Spain ecotypes, which are more sensitive to water stress compared to ecotypes from other parts of the world (De Diego et al., 2012; 2015).

SE is a biotechnological tool which consists in the dedifferentiation of somatic cells and their subsequent cell re-differentiation in somatic embryos (ses), through genetic reprogramming (Fehér, 2015). Based on the morphogenetic response, SE is widely used to obtain large amounts of cloned material from elite material in response to an external stress stimulus (Arnholdt-Schmitt et al., 2016; Almeida et al., 2020; Castander-Olarieta et al., 2020; Gao et al., 2020; Maruyama et al., 2020; Pires et al., 2020; Raza et al., 2020).

In recent years, several studies have reported the influence of high temperature in the induction of SE in *Pinus* spp. (García-Mendiguren et al., 2016; Pereira et al., 2018; Arrillaga et al., 2019; Castander-Olarieta et al., 2019). In this regard, the effect of different temperatures applied in the initiation (Kvaalen and Johnsen, 2008; Yakovlev et al., 2016; Pereira et al., 2018; Castander-Olarieta et al., 2019) and maturation (Yakovlev et al., 2016; Moncaleán et al., 2018; Arrillaga et al., 2019) stages of SE process has been reported at 18,

23, or 28 °C. Recently, our research group has reported that somatic plants of *P. radiata* coming from EMs initiated at different temperatures have a different behavior in the greenhouse under water stress conditions (Castander-Olarieta et al., 2020).

Taking into account the abovementioned studies, in this work, we used higher maturation temperatures than those described in previous studies with *P. radiata* and *P. halepensis*. Thus, the objective of this work was to evaluate the influence of high temperatures (23 (control temperature), 40, 50, and 60 °C, after 12 weeks, 90, 30, 5 min, respectively) applied during the maturation stage of the SE in terms of both quantity and morphology of the ses obtained of *P. radiata* and *P. halepensis*. Moreover, we carried out a physiological evaluation of the obtained *P. radiata* plantlets subjected to heat and water stress in the greenhouse in order to test the possible improvement related to heat and water stress tolerance.

2. MATERIALS AND METHODS

2.1. Experiment I

EMs obtained from immature female cones of *P. radiata*, were collected from four mother trees in a seed orchard established by Neiker-BRTA in Deba (Spain) and *P. halepensis* cones were collected from five mother trees in Berantevilla (Spain). The immature seeds were extracted and surface sterilized following Montalbán et al. (2012). Seed coats were removed and intact megagametophytes excised out aseptically were initiated and proliferated following the protocol described by Montalbán et al. for *P. radiata* (Montalbán et al., 2012) and for *P. halepensis* (Montalbán et al., 2013).

For *P. radiata* SE, the basal medium was Embryo Development Medium (EDM) (Duchefa Biochemie, Amsterdam, Netherlands) (Walter et al., 2005). The maturation of EMs was performed following the protocol described by Montalbán et al. (2010). The EMs were briefly suspended in liquid basal medium devoiding plant growth regulators and then filtered on a filter paper in a Büchner funnel. Each filter paper, with 0.08 g of EMs, was placed in the corresponding maturation media. The basal medium was supplemented with 60 g L^{-1} of sucrose, 9 g L^{-1} of Gelrite® (Duchefa Biochemie, Amsterdam, Netherlands), 60 µM of

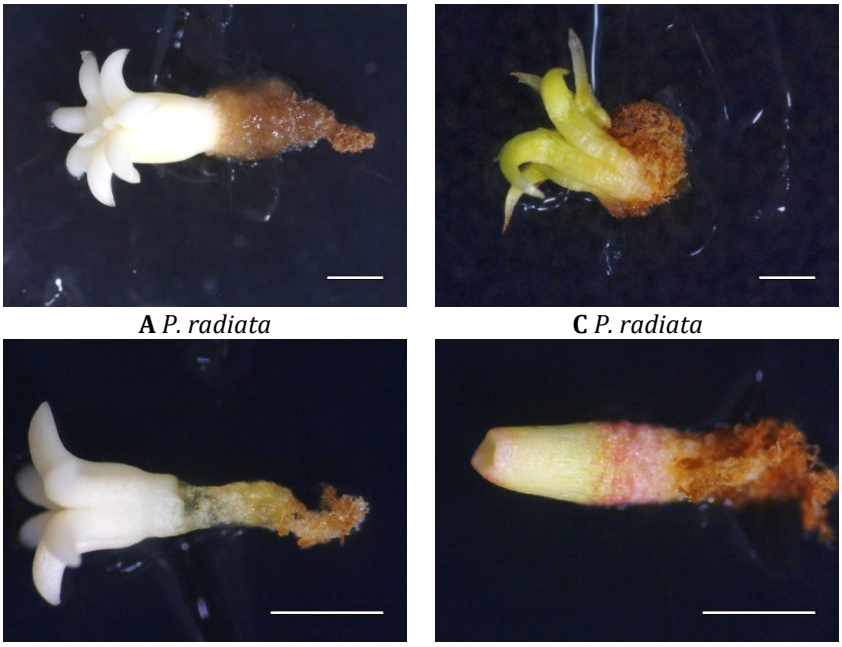
abscisic acid (ABA), and the amino acid mixture of EDM medium used for initiation and proliferation (550 mg L^{-1} of L-glutamine, 525 mg L^{-1} of asparagine, 175 mg L^{-1} of arginine, 19.75 mg L^{-1} of citrulline, 19 mg L^{-1} of ornithine, 13.75 mg L^{-1} of lysine, 10 mg L^{-1} of alanine, and 8.75 mg L^{-1} of proline) (Walter et al., 2005).

For *P. halepensis* SE, the basal medium used was DCR medium (Duchefa Biochemie, Amsterdam, Netherlands) (Gupta and Durzan, 1985) supplemented with 75 μM of ABA, 60 g L^{-1} of sucrose, 9 g L^{-1} of Gelrite® and the amino acid mixture of the EDM medium. The maturation of the 0.08 g of EMs/ plate was carried out as described above.

The cultures were kept at different maturation temperatures (MT) (23 (control temperature), 40, 50, and 60 °C) during different incubation periods (12 weeks, 90, 30, 5 min, respectively). Based on previous studies, we observed that extreme temperatures cannot be applied over extended periods as the ECL*s* are killed. Once the different treatments had finished, all cultures were kept in darkness at 23 °C.

Germination and acclimatization of cotyledonary ses were performed according to Montalbán and Moncaleán (2019).

Therefore, four different temperatures were tested in six established cell lines (ECL*s*) (R2, R9, R16, R49, R130, and R138 for *P. radiata* and H2, H23, H48, H60, H153, and H204 for *P. halepensis*), in a factorial design with eight repetitions (plates) per treatment and ECL. After 16 weeks from the beginning of the experiments, the number of normal mature somatic embryos (NNE) (for *P. radiata* and *P. halepensis*, Figure 1A,B, respectively) and abnormal somatic embryos (NAE) for *P. radiata* and *P. halepensis*, Figure 1C,D, respectively) per 0.08 g of EMs were counted. NAE displayed abnormal morphology, manifested by precocious germination, and in some cases by a lack of cotyledons (Montalbán et al., 2010). Additionally, the length (LE) and width (WE) of 960 NNE was measured. After two months in germination medium, the percentage of the germination for the NNE was calculated.



A *P. radiata* **C** *P. radiata*

B *P. halepensis* **D** *P. halepensis*

Figure 1. Somatic embryos showing distinct morphologies: normal somatic embryo (NNE) for *Pinus radiata* D. Don **(A)**; normal somatic embryo (NNE) for *Pinus halepensis* Mill. **(B)**; abnormal somatic embryo (NAE) for *P. radiata* **(C)**; abnormal somatic embryo (NAE) for *P. halepensis*, bar = 2 mm **(D)**.

2.2. Experiment II

Six-month-old radiata pine plants (Figure 2) were analyzed in October 2019. Twenty plants were used per MT; one fourth of each MT were randomly selected and submitted to the following four treatments: greenhouse temperature (GT) at 23 °C and under an irrigation rate of three times per week (UI); GT at 23 °C and without irrigation (NI); GT at 40 °C and UI; GT at 40 °C and NI. Irrigation conditions (I) were maintained for two weeks, and GT at 40 °C two hours/day for five days.

Figure 2. *Pinus radiata* D.Don plantlets obtained from embryonal masses maturated at high temperatures during the hardening stage in the greenhouse, bar = 8 cm.

Summarizing, in this analysis 16 treatments were considered for each species (four MT × two GT × two I) in a factorial with five repetitions (plants), totaling 80 plants analyzed.

After two weeks from the beginning of the greenhouse experiments, plantlets from the greenhouse with a temperature at 23 °C with or without irrigation or with a greenhouse temperature at 40 °C with or without irrigation, started to present external symptoms of drought stress such as needle epinasty or apical curvature (De Diego et al., 2012). Subsequently, the leaf water status and gas exchange parameters were measured. The water potential (Ψ_{leaf}, MPa) of one needle per plant, was measured at predawn using a Scholander chamber (Skye SKPM 1400) and the pressure-equilibration technique (Scholander et al., 1965).

The response of instant net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), and instant leaf transpiration (E, mmol H_2O m^{-2} s^{-1}) were quantified at midday with a LI-6400XT Portable Photosynthesis System (Li-Cor Biosciences) equipped with the 6400-05 Clear Conifer Chamber (Li-Cor Biosciences).

2.3. Statistical Analysis

For experiment I, ECLs were considered as a block in the model to decrease variability. Deviance analysis was performed with the X^2 test ($p < 0.05$) to assess the effect of temperature on the parameters studied. According to the data distribution, Poisson (NNE and NAE) or Gamma (LE and WE) distribution for *P. radiata*, and Poisson (NNE and NAE) and normal (LE and WE) were used for *P. halepensis*. The data were analyzed using R software®, version 3.6.1. (R Core Team, 2017) using the general linear model (glm) function.

To assess the effect of maturation temperature on the percentage of germinated NNE and the percentage of surviving plants, a logistic regression and the corresponding analysis of deviance was conducted. The cell line was included in the model to cope with variability. When required, a quasibinomial family was considered to deal with overdispersion.

For experiment II, an analysis of variance was conducted to assess the effects of MT, GT, and I on Ψ_{leaf}, A_N, g_s, and E. Full models with the complete interaction MT × GT × I were fitted.

After the analysis of variance was conducted, differences in means between treatment combinations were assessed using the Tukey *post-hoc* test ($\alpha = 0.05$) adjusted for multiple comparisons.

3. RESULTS

3.1. Experiment I

3.1.1. *P. radiata*

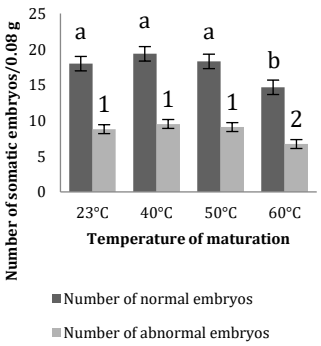
The application of high temperatures caused statistically significant differences for the NNE and NAE (Table 1). However, the application of high temperature was not statistically significant for the LE and WE (Table 1).

Table 1. Analysis of deviance for the effect of different maturation temperatures in the number of normal (NNE) and abnormal somatic embryos (NAE) per 0.08 g of embryonal masses; the length (LE-mm) and width of normal embryos (WE-mm) of *Pinus radiata* D.Don.

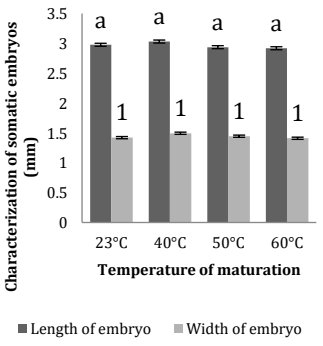
Source	df	NNE		NAE		LE		WE	
		X^2 Test	*p*-Value	X^2 Test	*p*-Value	X^2Test	*p*-Value	X^2 Test	*p*-Value
T	3	34.69	≤0.05 *	27.56	≤0.05 *	0.04	>0.05 ^{ns}	0.09	>0.05 ^{ns}

* Significant differences at $p \leq 0.05$; ^{ns} nonsignificant; df—degrees of freedom.

There was a tendency to increase the NNE (Figure 3A) and the NAE (Figure 3A) from MT 23 °C to MT 50 °C, and the NNE decreased significantly at 60 °C.



A



B

Figure 3. Somatic embryos obtained per 0.08 g of embryonal masses submitted in different maturation temperatures (23, 40, 50, and 60 °C, after 12 weeks, 90, 30, 5 min, respectively). Number of normal and abnormal somatic embryos **(A)**; the length and width of *Pinus radiata* D. Don normal embryos. Different letters or numbers show significant differences by the Tukey–Kramer test ($p \leq 0.05$) **(B)**.

Although nonsignificant differences were found between MT for LE and WE (Table 1), a similar trend was observed for NNE and NAE. The highest LE and WE were recorded in ses from MT 40 (3.03 mm for LE and 1.49 for WE) and 23 °C; the 50 °C treatment showed intermediate values and treatment at 60 °C presented the lowest (2.92 mm for LE and 1.42 mm for WE). We also observed that the longest embryos were also the widest (Figure 3B).

The application of high temperature was not statistically significant for the germination of ses and the survival percentage of somatic plants in the greenhouse (44%, 51%, 46%, and 51% for 23, 40, 50, and 60 °C, respectively). However, plantlets were obtained from all the tested maturation treatments (63%, 55%, 60%, and 63% from temperatures 23, 40, 50, and 60 °C, respectively) (Figure 4A,B) and a total of 614 plantlets were acclimated in a greenhouse (Figure 4C).

A **B** **C**

Figure 4. Germination and acclimatization of somatic plantlets from *Pinus radiata* D. Don obtained from different embryogenic cell lines and maturation temperatures. Plantlets after two months in germination medium, bar = 1 cm **(A)**; plantlets after three months cultivated in the germination medium, bar = 2 cm **(B)**; plantlets derived from normal cotyledonary somatic embryos growing in the greenhouse, bar = 2 cm **(C)**.

3.1.2. *P. halepensis*

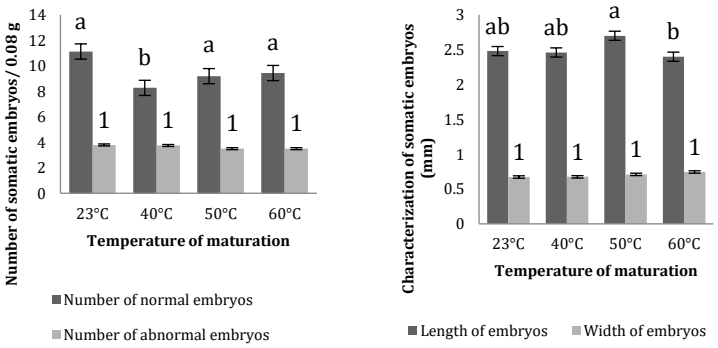
Maturation temperature affected significantly the NNE and LE. On the contrary, MT was not statistically significant for the NAE and WE (Table 2).

Table 2. Analysis of deviance for the effect of different maturation temperatures in the number of normal (NNE) and aberrant somatic embryos (NAE) per 0.08 g of embryonal masses; the length (LE-mm) and width of normal embryos (WE-mm) of *Pinus halepensis* Mill.

Source	df	NNE		NAE		LE		WE	
		X^2 Test	*p*-Value	X^2 Test	*p*-Value	X^2 Test	*p*-Value	X^2 Test	*p*-Value
T	3	21.15	≤0.05 *	0.98	>0.05 ^{ns}	2.44	≤0.05 *	0.17	>0.05 ^{ns}

* Significant differences at $p \leq 0.05$; ^{ns} nonsignificant; df—degrees of freedom.

Unlike in radiata pine, the increase in the MT from 23 to 40 °C did not promote an increase in NNE, but a decrease in their production was observed (Figure 5A). On the other hand, no significant differences were found between the control MT (23 °C) and temperatures of 50 or 60 °C (Figure 5A).



A **B**

Figure 5. Somatic embryos obtained per 0.08 g of embryonal masses submitted in different maturation temperatures (23, 40, 50, and 60 °C, after 12 weeks, 90, 30, 5 min, respectively). Number of normal and abnormal somatic embryos **(A)**; the length and width of normal embryos from *Pinus halepensis* Mill. Different letters or numbers show significant differences according to the Tukey–Kramer test ($p \leq 0.05$) **(B)**.

Although no significant differences were detected between MT for the NAE (Table 2) (Figure 5A), the MT 50 °C promoted a significant increase in LE when compared to 60 °C, whereas MT 23 and 40 °C led to intermediate values (Figure 5B). A similar tendency was observed in WE, but in this case no significant differences were found between MT (Figure 5B).

In total, 262 viable plantlets were obtained in this experiment (Figure 6C). The application of high temperature was not statistically significant for the germination percentage of ses and the survival of somatic plants in the greenhouse (22%, 30%, 24%, and 34% for 23, 40, 50, and 60 °C, respectively). Furthermore, in all ECLs and MTs, a high acclimatization

rate was obtained (91.82%, 92.61%, 98.08%, and 88.91% from temperatures of 23, 40, 50, and 60 °C, respectively).

Figure 6. Germination and acclimatization of somatic plantlets from *Pinus halepensis* Mill. obtained from different embryogenic cell lines and maturation temperatures. Somatic plantlets after two months in germination medium, bar = 1 cm **(A)**; plantlets cultivated after three months in the germination medium, bar = 2 cm **(B)**; plantlets derived from normal cotyledonary somatic embryos growing in the greenhouse, bar = 5 cm **(C)**.

3.2. Experiment II

3.2.1. Water Potential and Gas Exchange Parameters

Plants coming from all treatments applied during the maturation stage survived after the drought and thermic stress in the greenhouse (Figure 7). Significant differences were observed for the effect of MT on the initial and final evaluation of water potential in plants subjected to different stress conditions (Table 3); but in the first evaluation, no significant differences were observed for gas exchange parameters (Table 3).

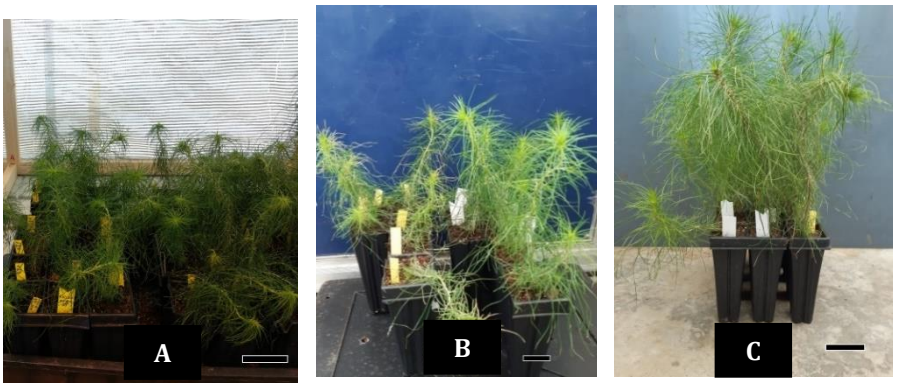


Figure 7. *Pinus radiata* D. Don somatic plants*:* showing needle epinasty and apical curvature at greenhouse temperature of 40 °C under irrigation or nonirrigated (left and right, respectively), bar = 6 cm **(A)**; plants not irrigated submitted to 40 and 23 °C in greenhouse (left and right, respectively),

bar = 3 cm **(B)**; plants not irrigated submitted to 23 and 40 °C in greenhouse (left and right, respectively), after rewatering, bar = 6 cm **(C)**.

Table 3. Analysis of variance (ANOVA) for the effect of different maturation temperatures (MT) and greenhouse temperatures (GT) on the initial and final water potential (Ψ_{leaf} initial and Ψ_{leaf} final, respectively) in plants of *Pinus radiata* D. Don under irrigation and/or no irrigation conditions (I).

ANOVA		
Effect	**df**	**Ψ_{leaf} initial**
MT	3	≤0.05 *
Effect	**df**	**Ψ_{leaf} initial**
MT	3	≤0.05 *
GT	1	≤0.05 *
I	1	>0.05 ^{ns}
MT × GT	3	>0.05 ^{ns}
MT × I	3	>0.05 ^{ns}
GT × I	1	>0.05 ^{ns}
MT × GT × I	3	>0.05 ^{ns}

* Significant differences at $p \leq 0.05$; ^{ns} nonsignificant at $p \leq 0.05$; df—degrees of freedom.

Statistically significant differences were also found for GT for the final assessment of water potential (Table 3). At the end of the experiment, water potential in plants at GT of 23 °C was significantly lower (−0.3 MPa) than Ψ_{leaf} final in plants at GT of 40 °C (−0.26 MPa) by Student's *t*-test ($p < 0.05$). This implies that plants originating from EMs subjected to different MTs had a tolerance to heat stress in the greenhouse, as plants exposed to GT of 40 °C were more hydrated compared to plants exposed to GT of 23 °C. The irrigation regime did not show significant differences for this parameter.

A decrease in water potential was observed in plants from ECLs matured in MTs of 23 °C (Figure 8A). Similarly, the same pattern of decline was observed when assessing water potential after water and heat stress conditions, which in turn was less than the initial water potential (Figure 8A). For this characteristic, the data fit the model with a determination coefficient above 98% ($R^2 = 0.98$).

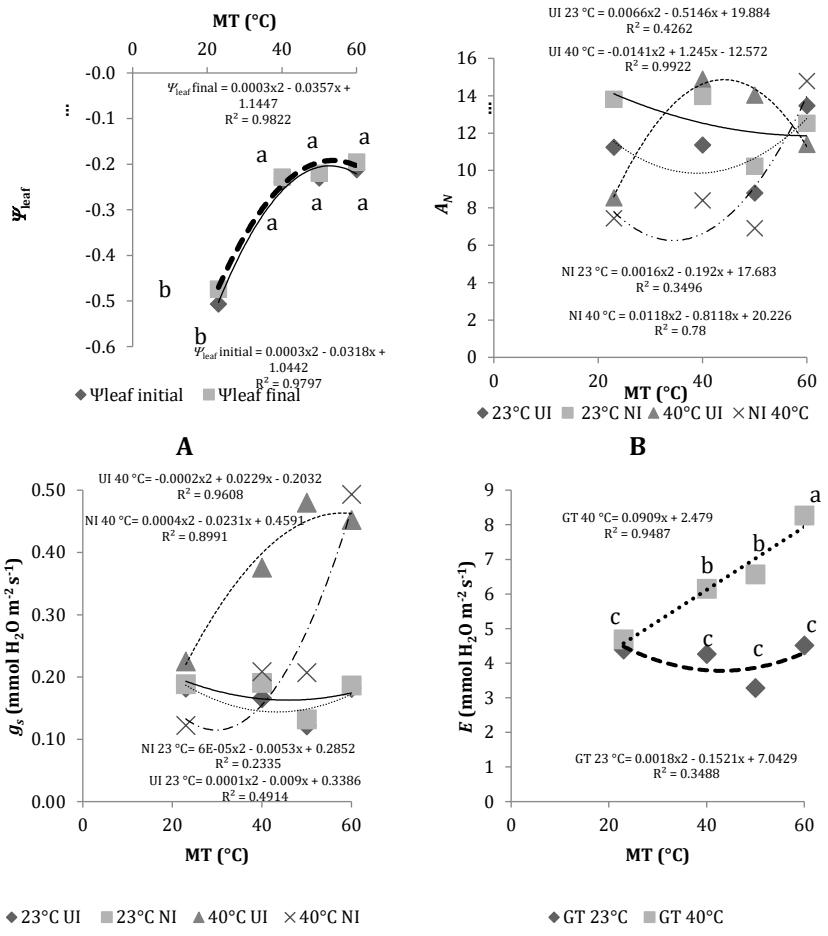


Figure 8. Water and gas exchange parameters in plants with greenhouse temperature (23 or 40 °C) under (UI) irrigation or no irrigation (NI) conditions (I) obtained from EMs of *Pinus radiata* D. Don submitted to different maturation temperatures (MT). Leaf water potentials (Ψ_{leaf}) initial and final (applying of these treatments) **(A)**, instantaneous net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}) **(B)**, stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) **(C)**, and instant transpiration (E, mmol H_2O m^{-2} s^{-1}). Different letters show significant differences by the Tukey–Kramer test ($p \leq 0.05$) **(D)**.

In the first evaluation, there were no significant statistical differences for A_N, g_s, and E (Table 4). By contrast, in the second evaluation, the A_N presented significant statistical differences for the effects MT, MT × GT, GT × I, and in the triple interaction MT × GT × I (Table 5). Stomatal conductance was statistically significant for all the factors and instant transpiration was statistically significant for all the factors assessed, except MT × I and the triple interaction (Table 5).

Table 4. ANOVA for the effect of different maturation temperatures (MT) in the instantaneous net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), and instant transpiration (E, mmol H_2O m^{-2} s^{-1}) in plants of *Pinus radiata* D. Don.

ANOVA Gas Exchange Initial				
Effect	**df**	A_N	g_s	E
MT	3	>0.01 ^{ns}	>0.01 ^{ns}	>0.01 ^{ns}

^{ns} nonsignificant at $p \leq 0.05$; df—degrees of freedom.

Table 5. ANOVA for the effect of different maturation temperatures (MT), greenhouse temperature (GT), and irrigation or no irrigation conditions (I) in the instantaneous net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O $m^{-2}s^{-1}$), and instant transpiration (E, mmol H_2O $m^{-2}s^{-1}$) in plants of *Pinus radiata* D. Don.

ANOVA Gas Exchange Final				
Effect	**df**	A_N	g_s	E
MT	3	≤0.01 **	≤0.001 ***	≤0.001 ***
GT	1	>0.01 ^{ns}	≤0.001 ***	≤0.001 ***
I	1	>0.01 ^{ns}	≤0.01 **	≤0.01 **
MT × GT	3	≤0.05 *	≤0.001 ***	≤0.001 ***
MT × I	3	>0.01 ^{ns}	≤0.01 **	>0.01 ^{ns}
GT × I	1	≤0.01 **	≤0.001 ***	≤0.001 ***
MT × GT × I	3	≤0.01 **	≤0.01 **	>0.01 ^{ns}

*; **; *** Significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively; ^{ns} nonsignificant at $p \leq 0.01$; df—degrees of freedom.

As we can see in Table 6, the relative effect of heat and water stress did not induce statistically significant differences in instantaneous liquid photosynthesis between any treatments, except those plants originating from ECLs submitted to maturation temperatures of 23 and 50 °C (Figure 8B).

Table 6. Effect of different maturation temperatures (MT-°C), greenhouse temperature (GT-°C), and irrigation (1) or no irrigation (2) conditions (I) on the instantaneous net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O $m^{-2}s^{-1}$) in plants of *Pinus radiata* D.Don.

Effect (MT-GT-I)	A_N	g_s
40-40-1	14.89 ^{a}	0.38 ^{b,c}
60-40-2	14.79 ^{a}	0.49 ^{a}
50-40-1	14.04 ^{a}	0.48 ^{a,b}
40-23-2	13.97 ^{a}	0.19 ^{c,d}
23-23-2	13.82 ^{a}	0.19 ^{c,d}
60-23-1	13.46 ^{a}	0.18 ^{c,d}
60-23-2	12.52 ^{a}	0.19 ^{c,d}
60-40-1	11.40 ^{a}	0.45 ^{a,b}
40-23-1	11.35 ^{a}	0.17 ^{c,d}
23-23-1	11.24 ^{a}	0.18 ^{c,d}
50-23-2	10.24 ^{a}	0.13 ^{d}
50-23-1	8.78 ^{a}	0.12 ^{d}
23-40-1	8.54 ^{a}	0.23 ^{c,d}
40-40-2	8.39 ^{a}	0.21 ^{c,d}
23-40-2	7.44 ^{b}	0.12 ^{d}
50-40-2	6.90 ^{b}	0.21 ^{c,d}

Different letters within a column show significant differences in the means observed by Tukey–Kramer's *post hoc* test ($p \leq 0.05$).

However, plants have greater variability with statistically significant differences in stomatal conductance (Table 6). In addition, the plants that maintained higher stomatal conductance compared to those under stress conditions or otherwise, were plants

originating from ECLs exposed to high maturation temperatures (0.49 mmol H_2O m^{-2} s^{-1} by 60 °C) (Figure 8C) (Table 6).

At the end of the experiment, under high temperature conditions in a greenhouse, we observed a significantly increase in the instant transpiration in plants originating from ECLs subjected to MT at 60 °C, followed by 50 and 40 °C. We also observed that this increase was greater under conditions of heat stress than in nonstressed plants (Figure 8D). This parameter also showed statistically significant differences in plants growing in the greenhouse at 40 °C under irrigation, with an increase in transpiration in this cultivation condition (Table 7).

Table 7. Effect of different greenhouse temperatures (GT, °C) and irrigation (UI) or no irrigation (NI) conditions (I) in the instant transpiration (E, mmol H_2O $m^{-2}s^{-1}$) in plants of *Pinus radiata* D. Don.

Effect (GT (°C)-I)	*E*
40-UI	7.19 [a]
40-NI	5.61 [b]
23-NI	4.26 [b,c]
23-UI	3.96 [c]

Different letters show significant differences in the means observed by Tukey–Kramer's *post hoc* test ($p \leq 0.05$).

4. DISCUSSION

The formation of somatic embryos is affected by several factors, such the genetic background and the cultivation conditions (Breton et al., 2005; Lelu-Walter et al., 2006). In this study, the same ECLs (five for each species), were subjected to different maturation treatments, in order to try to minimize the genetic impact on the results obtained. In our experiments we observed that, in both species, embryos were formed with normal and abnormal morphologies. Merino et al. (2018) in Scots pine attributed the formation of ses with normal and abnormal morphology to the difference of transcripts in embryogenic cells. However, the increase in temperature did not significantly promote the formation of abnormal embryos.

In this work, we observed that the NNE was not affected by high temperatures applied at the beginning of the maturation stage, except at 60 °C and 40 °C in which we observed a reduction in NNE for *P. radiata* and *P. halepensis*, respectively. Kvaalen and Johnsen (2008) and García-Mendiguren et al. (2016), when working with *Picea abies* and *P. radiata*, respectively, also reported an increase in the number of embryos obtained when

high temperatures were applied in the initiation stage. In contrast, Arrillaga et al. (2019), in proliferation and maturation stages *Pinus pinaster* SE showed that control temperature (23 °C) provoked the best results in terms of the number of embryos developed.

Castander-Olarieta et al. (2019) in *P. radiata* showed that somatic embryos originating from EMs initiated at 50 °C for 30 min had a higher LE. This is in agreement with our results for Aleppo pine where the longest embryos were found at this temperature.

The germination mechanism of cotyledonary somatic embryos is complex and an alternative in this case are studies with post-maturation treatments (Maruyama and Hosoi, 2012) because in *P. halepensis* (22%, 30%, 24%, and 34% for 23, 40, 50, and 60 °C, respectively) we observed a low percentage rate of germination compared to *P. radiata* (44%, 51%, 46%, and 51% for 23, 40, 50, and 60 °C, respectively).

Stresses applied to EMs in late SE stages, besides inducing different responses between species of conifers, can also guarantee an improvement in the germination of cotyledonary somatic embryos (Stasolla and Yeung, 2003). The germination rate changes according to species and treatments. This result was also described in other conifer species (Niskanen et al., 2004; Carneros et al., 2009; Arrillaga et al., 2019). In this work, we observed that the high MTs did not affect either the germination of ses or the acclimatization of plants in both species of *Pinus*.

The conditions of cultivation during the maturation stage in the SE are crucial for the regeneration of the somatic embryos in quality plantlets with an *ex vitro* survival capacity (Yang et al., 2020). We obtained the highest acclimatization (≤88.91%) independently of the line or temperature for *P. halepensis* in relation to *P. radiata* (ranging from 55.84% to 63.47% for temperature). The maturation of EMs in ses and their subsequent conversion to plants, in both species, prove the tolerance of these EMs to high temperatures.

The pressures exerted by high temperatures and drought affect numerous defense mechanisms in plants (Gratkowska-Zmuda et al., 2020), such as changes in photosynthetic machinery (Johnová et al., 2016). This can happen at an embryogenic level with the formation of an epigenetic memory (Yakovlev et al., 2016) even at the plant level.

In this work, we observed that the different MTs applied to EMs promoted ses without significant physical anomalies. However, *P. radiata* plants originated from these treatments responded physiologically in different ways (Figure 8) when they were subjected to drought and heat stress in the greenhouse.

Taiz and Zeiger (2010) reported that water stress significantly affects stomatal conductance more than photosynthesis, corroborating our results (Table 7). In addition, we also observed that *E* was affected by heat and water stress (Figure 8D and Table 7).

It is possible that plants originating from ECLs subjected to high temperatures in the maturation stage have developed epigenetic mechanisms that allowed a better response to stress (Correia et al., 2013), since the plants maintained or adapted their water and gas exchange parameters to adverse temperature conditions and water stress to which they were subjected (Figure 8).

In this work, plants from all MT submitted to GT of 23 °C showed a similar behavior for g_s and *E*. However, when subjected to heat stress (40 °C), plants originating from EMs subjected to high MTs (40 and 60 °C) had a significant increase in g_s and *E*, reinforcing once again the possibility of "priming" in ECLs subjected to high temperatures. In natural conditions, similar behavior is observed in the apical meristem of plants, which is the center of the morphogenesis of the aerial part and is in constant cell division. The environmental restrictions, for example, heat and drought, perceived by these meristematic regions, trigger changes in the epigenetic state of the plants, developing a memory which, when under stress conditions again, enables the plants to have better tolerance (Sow et al., 2018).

In conclusion, it is possible to modulate the tolerance to stress by applying high temperatures during the final stages of the embryogenic process. Future experiments will be carried out to analyze methylation, physiological and biochemical aspects in one-year old plants to assess whether the plant characteristics endure over time.

5. CONCLUSIONS

P. radiata and *P. halepensis* EMs produced ses at high maturation temperatures. High maturation temperatures compared to the control temperature (23 °C) did not affect the morphological characteristics of the embryos obtained, except for the NNE in both species, NAE and LE in *P. radiata* and *P. halepensis*, respectively. The plants obtained from these somatic embryos survived drought and heat stresses in the greenhouse. Moreover, plants originated from EMs submitted to a maturation temperature of 40 and 60 °C, presented better adaptation to drought and heat stress based on the water and gas exchange parameters analyzed in this experiment. Studies will be carried out to characterize possible epigenetic marks in the obtained plants caused by heat stress applied during the embryogenic process and drought and heat stress applied in plants in greenhouses.

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CHAPTER 2

High temperature and water deficit cause epigenetic changes in somatic plants of *Pinus radiata* D. Don

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1. INTRODUCTION

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

DNA methylation consists in the addition of a methyl group to the fifth carbon of the cytosine ring and is considered to be one of the most important epigenetic mechanisms (Tang et al. 2020; Korotko et al. 2021). The classical epigenetic signature, 5-methylcytosine (5-mC), plays important regulatory functions such as regulation of gene expression (Akhter et al., 2021) and has recently been reported as an epigenetic marker in response to heat stress, in mammals and plants (Entrambasaguas et al., 2021; Wang et al., 2022). The 5-hydroxymethylcytosine (5-hmC) is another form of cytosine DNA modification and is obtained from the iterative oxidation of 5-mC through the ten-eleven translocation proteins (Wu and Zhang 2017). In mammals and plants, the 5-hmC has been linked to demethylation processes (Richa and Sinha 2014; Shi et al., 2017), but in plants, the biological function of the 5-hmC is still controversial (Kumar et al., 2018). The first discovery of 5-hmC in *Pinus* was reported by our research team in *Pinus radiata* D. Don and more recently noticed in *P. halepensis* Mill., which was associated with an establishment of epigenetic memory (Castander-Olarieta et al., 2020; Pereira et al., 2021).

In previous studies, our research team has reported that high temperatures modulate the different stages of SE in relation to morphology, biochemical and physiological status of embryonal masses (EMs), somatic embryos (ses), and somatic plants of *Pinus* spp. (García-Mendiguren et al., 2016; Castander-Olarieta et al., 2019; Do Nascimento et al., 2020).

Castander-Olarieta et al. (2020) reported that the application of heat stress during SE initiation of *P. radiata* provoked the formation of a stable epigenetic memory with a long-term change in the methylation status of the resulting somatic plants. On the other hand, the application of heat stress during SE maturation of *P. radiata* did not affect the number of ses but provoked a significant increase in the stomatal conductance (g_s) and instant leaf transpiration (E) in the resulting somatic plants (Do Nascimento et al., 2020). However, how these mechanisms are also implicated in the epigenome of *ex vitro* somatic plants obtained from ses maturated at high temperatures remains to be elucidated.

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

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

2. MATERIALS AND METHODS

2.1. Plant Material

Immature female cones of *P. radiata* were collected in a seed orchard established by Neiker-BRTA in Deba (Spain), and EMs were initiated and proliferated following the

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

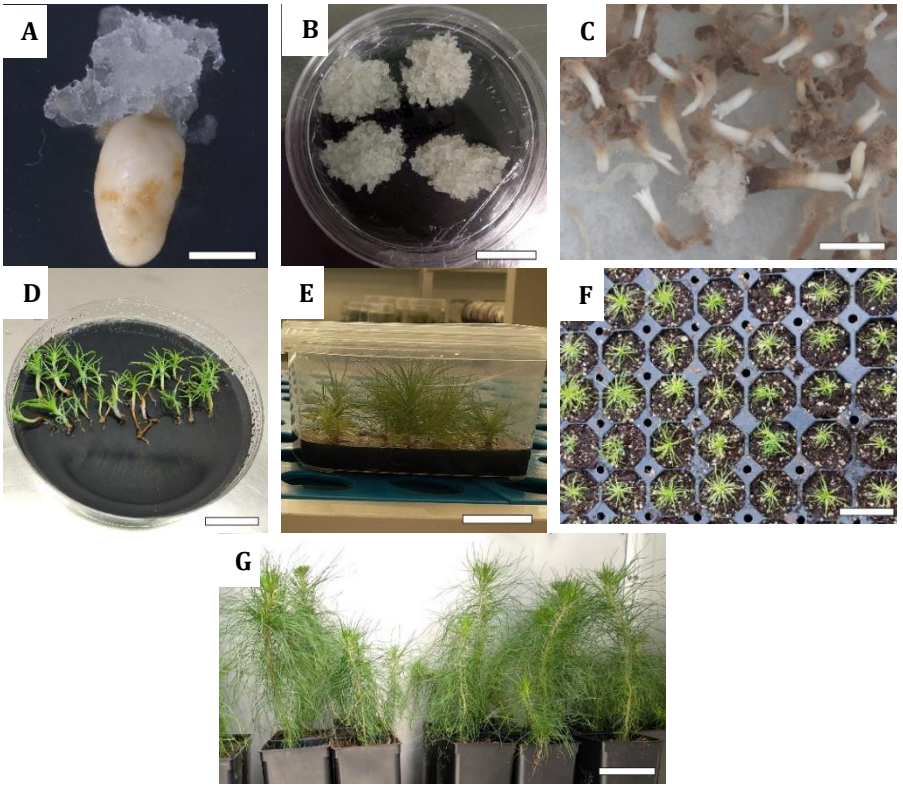

Figure 1. Somatic embryogenesis in *Pinus radiata* D. Don: Extrusion of embryonal masses (EMs) (bar = 5 mm; eight weeks) **(A)**, proliferation of EMs (bar = 2 cm; 12 weeks) **(B)**, cotyledonary somatic embryos at the end of maturation stage after heat stress application (bar = 5 mm; 12 weeks) **(C)**, germination of somatic embryos in Petri dishes (bar = 2 cm; eight weeks) **(D)**, germinated somatic embryos in an Ecobox® (bar = 4 cm; four weeks) **(E)** and; acclimatization of plantlets in the greenhouse (bar = 5 cm; eight weeks) **(F)**. *P. radiata* plants obtained from embryonal masses maturated at high temperatures (23 and 50 °C) before the drought experiment in the greenhouse (bar = 9 cm) **(G)**.

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

2.2. Growth Conditions

Fifteen months old somatic plants (Figure 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 (Figure 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.

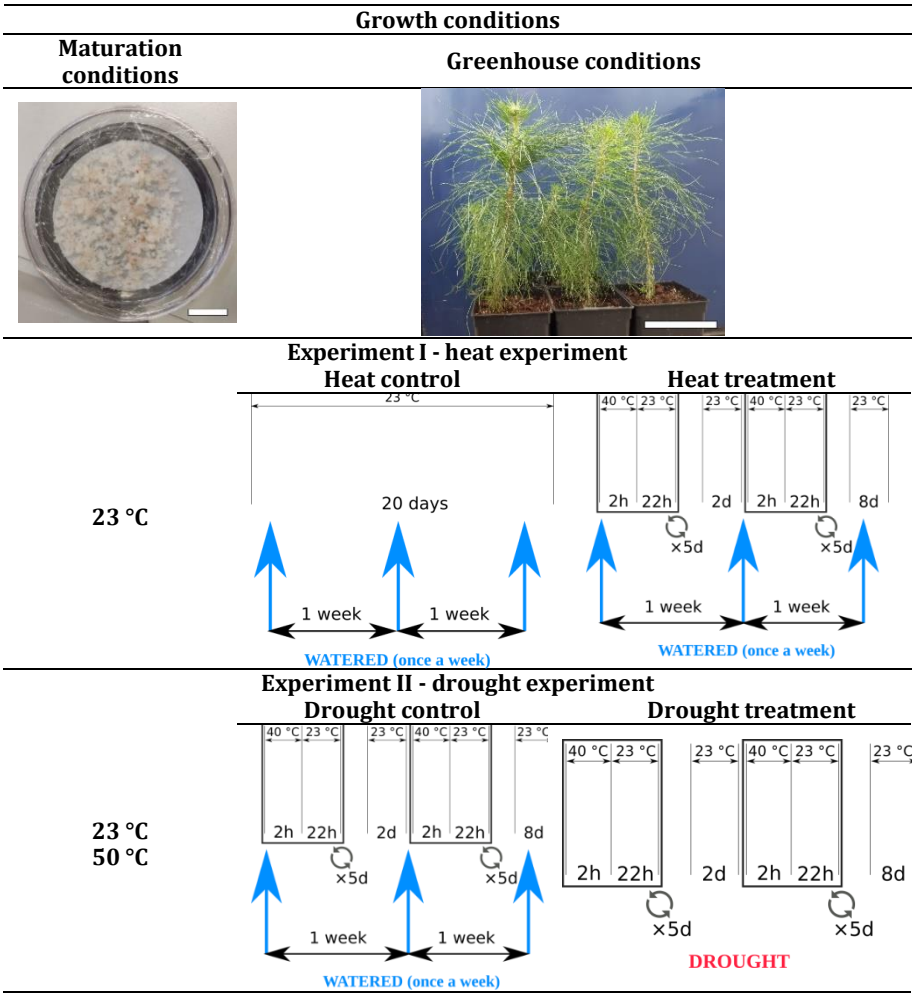

Figure 2. Experimental design for different *in vitro* (Maturation conditions) and *ex vitro* (heat experiment and drought experiment) conditions in the maturation of embryonal masses of *Pinus radiata* D. Don (bar = 2 cm) and the growth of somatic plants obtained (bar = 9 cm), respectively. d days;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

2.2.1. Experiment I - Heat Experiment

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

2.2.2. Experiment II - Drought Experiment

The drought experiment comprised a total of 20 plants. Five plants obtained from maturation at 50 °C and five plants from the same genotypes maturated at control conditions were subjected to a drought stress treatment by the complete suppression of watering, and the remaining plants (five plants obtained from maturation at 50 °C and five plants from the same genotypes maturated at control conditions) were watered weekly (Figure 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 (Figure 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).

2.3. Water Parameters and Gas Exchange Parameters

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).

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 24h (EC_f). Thereafter, samples were autoclaved for 10 min at 121 °C and cooled at room temperature to measure the total electrical conductivity (EC_t). The *E.L.* was calculated according to the following equation:

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

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

2.4. 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 high-performance liquid chromatography (HPLC) system coupled to a 6410 Triple Quad mass spectrometer from Agilent Technologies (Santa Clara, CA, USA). The chromatographic separation was performed on a Zorbax SB-C18 column

(2.1 × 100 mm, 3.5 µm, Agilent Technologies). The mobile phase was 11% methanol and 0.1% formic acid in water and 5 µL of samples were injected in the column at a flow rate of 0.1 mL min^{-1}. The electrospray ionization source (ESI) was operated in the positive ion multiple reaction monitoring mode (MRM) set to an ion spray voltage of 3500 V, 40 psi for nebulizer and source temperature at 350 °C. The intensity of specific MH+→fragment ion transitions was recorded (5-mC m/z 242→126, 5-hmC m/z 258→142 and C m/z 228→112). Identification of cytosine, 5-mC and 5-hmC were assessed by injection of commercial standards (5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set, Zymo Research, Irvine, CA, USA) under the same LC-ESI-MS/ MS-MRM conditions. The measured percentage of 5-mC and 5-hmC in each experimental sample was calculated from the MRM peak area divided by the combined peak areas for 5 plus 5-hmC plus cytosine (total cytosine pool). In the case of 5-hmC, its percentage in respect to the total modified cytosine pool was also calculated.

2.5. 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).

3. RESULTS

3.1. Physiological Parameters

3.1.1. Experiment I - Heat Experiment

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

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

Experiment I - heat experiment				
	Effect	**df**	**F-value**	***p*-value**
Ψ_{leaf}	TC	1	33.09	≤0.001 ***
RWC (%)	TC	1	0.47	>0.05 ^{ns}
E.L. (%)	TC	1	2.65	>0.05 ^{ns}
g_s	TC	1	2.57	>0.05 ^{ns}
E	TC	1	96.64	≤0.001 ***
Experiment II - drought experiment				
Before drought experiment				
	Effect	**df**	**F-value**	***p*-value**
$\Psi_{leafinitial}$	MT	1	8.03	≤0.05 *
$RWC_{initial}$ *(%)*	MT	1	0.19	>0.05 ^{ns}
$E.L._{initial}$ *(%)*	MT	1	4.98	≤0.05 *
$g_{sinitial}$	MT	1	1.22	>0.05 ^{ns}
$E_{initial}$	MT	1	1.86	>0.05 ^{ns}
After drought experiment				
$\Psi_{leaffinal}$	MT	1	13.86	≤0.01 **
	I	1	20.25	≤0.001 ***
	MT x I	1	0.03	>0.05 ^{ns}
RWC_{final} *(%)*	MT	1	1.26	>0.05 ^{ns}
	I	1	0.74	>0.05 ^{ns}
	MT x I	1	0.30	>0.05 ^{ns}
$E.L._{final}$ *(%)*	MT	1	2.85	>0.05 ^{ns}
	I	1	4.93	>0.05 ^{ns}
	MT x I	1	4.25	>0.05 ^{ns}
g_{sfinal}	MT	1	0.04	>0.05 ^{ns}
	I	1	242.63	≤0.001 ***
	MT x I	1	8.21	≤0.05 *
E_{final}	MT	1	1.81	>0.05 ^{ns}
	I	1	245.67	≤0.001 ***
	MT x I	1	11.41	≤0.01 **

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

3.1.2. Experiment II - Drought Experiment

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

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

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

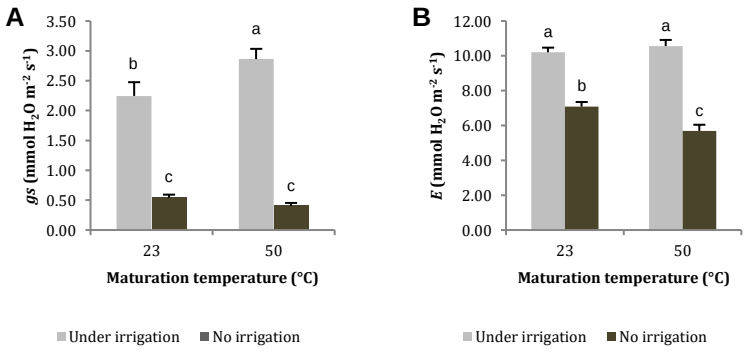

Figure 3. Physiological parameters in plants under irrigation or no irrigation conditions obtained from embryonal masses of *Pinus radiata* D. Don submitted to different maturation temperatures (23

°C or 50 °C). Stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) **(A)**; and instant transpiration (E, mmol H_2O m^{-2} s^{-1}) **(B)**. Bars indicate standard errors. Different letters show significant differences according to the Tukey's *post hoc* test ($p \leq 0.05$).

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

3.2. Global DNA Methylation/hydroxymethylation Analysis

3.2.1. Experiment I – Heat Experiment

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

Table 2. Analysis of variance (ANOVA) to assess the effect of different temperature conditions (TC, 23 °C or 40 °C) in the greenhouse on the methylation and hydroxymethylation rates of needles of plants obtained from embryonal masses of *Pinus radiata* D. Don maturated at 23 °C in the Experiment I – heat experiment. And ANOVA for the effect of different maturation temperatures (MT, 23 °C or 50 °C) on the methylation and hydroxymethylation rates of needles of *P. radiata* plants grown under irrigation or no irrigation conditions (I).

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

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

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

methylation of *P. radiata* plants was lower with heat stress in the growth conditions assayed.

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

Experiment I – heat experiment			
Treatment (°C)	**5-mC %**	**5-hmC %**	**5-hmC/5-mC × 100**
23	38.40 ± 0.23 [a]	0.0129 ± 0.0006 [a]	0.0335 ± 0.0014 [a]
40	37.98 ± 0.75 [a]	0.0089 ± 0.0015 [b]	0.0233 ± 0.0035 [b]
Experiment II - drought experiment			
Treatment	**5-mC %**	**5-hmC %**	**5-hmC/5-mC × 100**
23 °C -UI	37.98 ± 0.75	0.0089 ± 0.0015	0.0233 ± 0.0035
50 °C -UI	38.77 ± 0.39	0.0114 ± 0.0014	0.0292 ± 0.0034
23 °C -NI	37.83 ± 0.34	0.0094 ± 0.0018	0.0247 ± 0.0047
50 °C -NI	38.60 ± 0.31	0.0115 ± 0.0007	0.0298 ± 0.0020

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

3.2.2. Experiment II - Drought Experiment

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

3.3. Correlation Analysis Between Global 5-mc%, 5-hmc%, and Physiological Parameters

3.3.1. 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 (Figure 4A). However, when the plants obtained from EMs maturated at 23 °C grew at 40 °C in the greenhouse significantly negative correlations between the 5-hmC and Ψ_{leaf} and 5-hmC/5-mC ratio and Ψ_{leaf} were observed (Figure 4B).

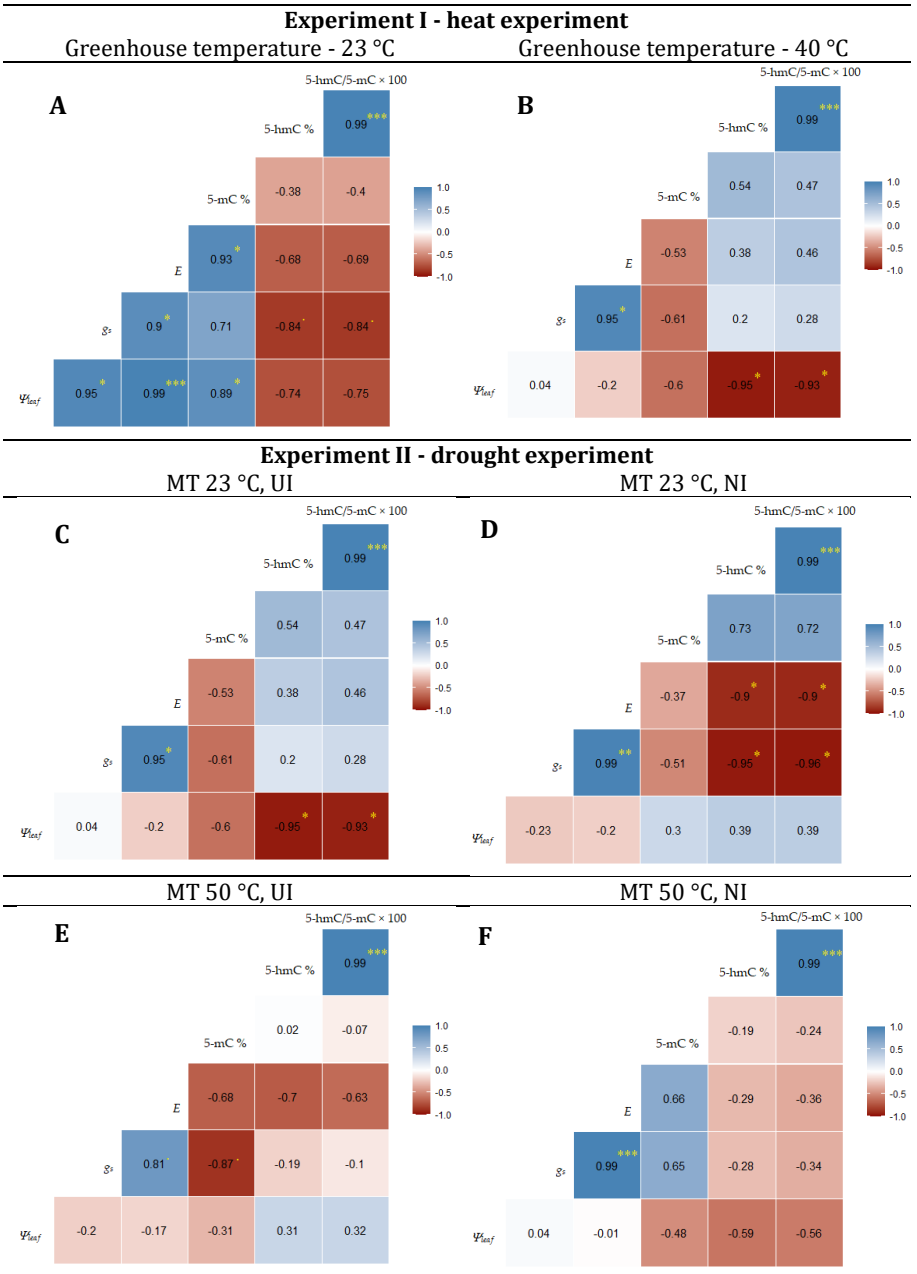

Figure 4. Heat map of Pearson's correlation coefficients pair between global 5-methylcytosine (5-mC, %), 5-hydroxymethylcytosine (5-hmC, %), the 5-hmC/5-mC ratio (5-hmC/5-mC × 100, %), water potential (Ψ_{leaf}, MPa), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) and instant transpiration (E, mmol H_2O m^{-2} s^{-1}) in *Pinus radiata* D. Don plants obtained from embryonal masses maturated at 23 °C and kept at 23 °C **(A)** and 40 °C **(B)**; 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 H_2O m^{-2} s^{-1}) E (mmol H_2O 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. MT 23 °C, UI **(C)**; MT 23 °C, NI **(D)**; MT 50 °C,

UI **(E)**; and MT 50 °C, NI **(F)**. *; **; *** Significant differences at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively, according to the *t* test.

3.3.2. Experiment II - Drought Experiment

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

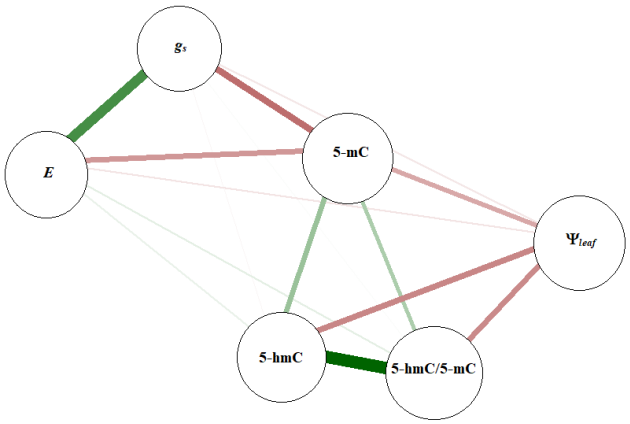

Figure 5. Pearson's correlation network of global 5-methylcytosine (5-mC, %), 5-hydroxymethylcytosine (5-hmC, %), the 5-hmC/5-mC ratio (5-hmC/5-mC, %) and physiological parameters in plants of *Pinus radiata* D. Don; red lines represent negative correlation with $R < -0.57$ and green lines represent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

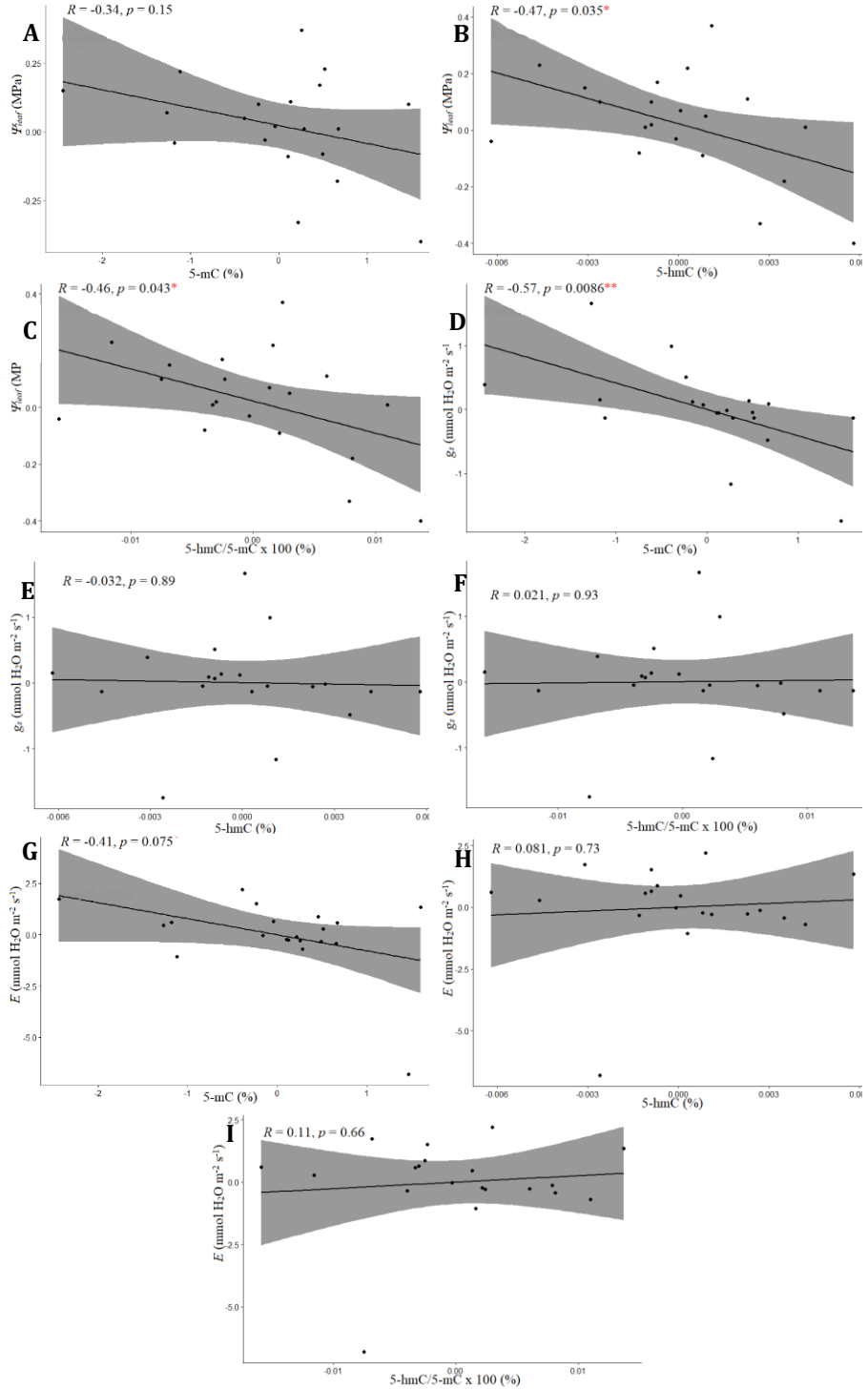

Figure 6. Pearson correlation pair between relationships between water potential (Ψ_{leaf}, MPa) and 5-methylcytosine (5-mC, %) **(A)**, 5-hydroxymethylcytosine (5-hmC, %) **(B)**, and the 5-hmC/5-mC

ratio (5-hmC/5-mC x 100, %) **(C)**. Person correlations between stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) and 5-mC **(D)**, 5-hmC **(E)** and the 5-hmC/5-mC x 100 **(F)**. Finally, Pearson correlations between instant transpiration (E, mmol H_2O m^{-2} s^{-1}) and 5-mC **(G)**, 5-hmC **(H)** and the 5-hmC/5-mC x 100 **(I)**. *; ** Significant correlations at $p \leq 0.05$ or $p \leq 0.01$, respectively, according to a t test.

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 (Figure 4C,D,E), except in the somatic plants obtained from EMs maturated at 50 °C under drought conditions (Figure 4F). Furthermore, those plants obtained from maturation temperature at 23 °C and maintained in irrigation conditions had a significantly negative correlation between the $\Psi_{leaf\text{final}}$ and 5-hmC contents, as well as, a significantly negative correlation with the 5-hmC/5-mC ratio was observed (Figure 4C). However, when the plants obtained from this temperature of maturation (23 °C) were maintained under drought stress, a significantly negative correlation was observed between the $g_{s\text{final}}$ with 5-hmC contents and $g_{s\text{final}}$ with the 5-hmC/5-mC ratio (Figure 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 (Figure 4D). On the other hand, the plants obtained from EMs maturated at 50 °C and maintained in irrigation conditions had a marginally negative correlation between $g_{s\text{final}}$ and 5-mC contents ($p = 0.057$) (Figure 4E). However, the plants obtained from EMs maturated at 50 °C and maintained in drought stress did not show a significant correlation between the physiological parameters ($\Psi_{leaf\text{final}}$, $g_{s\text{final}}$ and E_{final}) and the global 5-mC, 5-hmC and the 5-hmC/5-mC ratio (Figure 4F).

4. DISCUSSION

High temperature is one of the main forms of abiotic stress in nature (Bita and Gerats 2013). Although is often aggravated by additional abiotic stresses such as drought, it is important to study the independent action and biological consequences of high temperature to alleviate the effects of combined abiotic stress in plants (Bita and Gerats 2013; Lamaoui et al., 2018; Imran et al., 2021). In this work, when one-year and three-month-old *P. radiata* somatic plants were grown at 40 °C in the greenhouse, their Ψ_{leaf} was significantly lower than the Ψ_{leaf} of the plants growing at 23 °C. In contrast, we reported in a previous study (Do Nascimento et al., 2020) that the Ψ_{leaf} of six-month-old *P. radiata*

somatic plants growing at 23 °C was significantly lower (-0.3 MPa) than in those growing at 40 °C (-0.26 MPa) in the greenhouse, indicating that the Ψ_{leaf} is not constant under the same growing conditions, but it varies with the age of the *P. radiata* somatic plants as reported in other conifers (Rosner et al., 2019).

Drought triggers an instructional mechanism that guides the epigenetic machinery, causing plants to respond more quickly and effectively to a subsequent drought event (Colaneri and Jones 2013; Akhter et al., 2021). The degree of resistance to drought and, consequently, changes in physiological parameters depend on several factors such as phenological stages and exposure time (Ozturk et al., 2021). Our results in the drought experiment showed that *P. radiata* somatic plants derived from EMs maturated at high maturation temperature (50 °C, 30 min) showed higher $\Psi_{leaf\text{final}}$ and a significant lower E_{final} when exposed to drought conditions, indicating that these somatic plants showed a much higher degree of regulation of water loss under drought conditions than those obtained from EMs maturated at 23 °C. In line with these findings, similar reductions in E and Ψ_{leaf} of somatic plants obtained from EMs of *P. radiata* initiated at high temperatures (40 °C, 4 h; 50 °C, 30 min; 60 °C, 5 min) and maintained under water stress were observed by Castander-Olarieta et al., (2021). In this way, in a short time, the regulation of water loss in isohydric plants, such as *P. radiata*, allows plants to limit the transpiration losses and, thus, keep Ψ_{leaf} within tolerable limits (Martínez-Vilalta and Garcia-Forner 2017), avoiding complete stomatal closure without severe restrictions on carbon assimilation and tissue preservation against dehydration (Meinzer et al., 2014). Furthermore, a reduction in E in *Fragaria x ananassa* Duch. plants under saline stress was related to a state of pre-adaptation to saline and/or drought stress, preserving tissues from dehydration and a more effective adjustment to the hyperosmotic environment (Orsini et al., 2012).

The abovementioned results confirmed the hypothesis that stresses applied at different stages of SE can induce memory in the ses that is maintained at later stages of plant development (Do Nascimento et al., 2020; Castander-Olarieta et al., 2021; Pérez-Oliver et al., 2021). Similarly, two-year-old *P. pinaster* Ait. plants derived from EMs induced at high initiation temperature (50 °C, 3 h) showed an improvement in defense mechanisms

against drought stress with a better osmotic adjustment and higher chlorophyll, soluble sugars and starch contents when exposed to high temperatures (45 °C, for three hours per 12 days) in the greenhouse (Pérez-Oliver et al., 2021). Furthermore, in our study, the plants coming from EMs maturated at 50 °C compared with those plants coming from EMs maturated at 23 °C had a higher $g_{s\text{final}}$ under irrigation conditions. This high g_s has been reported to be beneficial for pine growth (Urban et al., 2017). In *P. tabulaeformis* Carr. inoculated with ectomycorrhizal fungi, although the increase in g_s could increase the loss of water, it also permitted that the seedlings, that grew under drought could be carried out more photosynthesis by means of increasing CO_2 absorption (Augé et al., 2015; Gehring et al., 2017; Wang et al., 2021a).

In conifers, low *E.L.* values associated with high *RWC* values were related to a greater capacity of plants to survive in drought conditions (Mantova et al., 2021). In this work, under irrigation, the plants obtained from maturation at 50 °C showed a significantly higher $E.L._{\text{initial}}$ (%) (10.80 ± 2.02) than those maturated at 23 °C (5.77 ± 1.01), but these differences were not observed when plants were submitted to drought. These values of *E.L.* were considered low when compared with other works with *P. radiata* and other conifers subjected to biotic and abiotic stress (De Diego et al., 2012). F or example, in *P. radiata* plants subjected to biotic stress conditions (infection by *Trichoderma viride* and *Fusarium circinatum*), there was an increase in *E.L.* (approximately 26%) characterizing damage to cell membranes in the stressed plants when compared to control plants (Amaral et al., 2019). Also, an increase of *E.L.* (> 40%) in *Pseudotsuga menziesii* Mirb. Franco plants submitted to drought was reported (Mantova et al., 2021). In addition, the *RWC* contents were similar in all treatments showing higher than 86%. Likewise, in *P. pinaster* somatic plants obtained from ses embryos maturated at different temperatures (18, 23, or 28 °C) and kept under heat stress (45 °C for 3 h/day for 10 days) the *RWC* was not significantly affected regardless of its origin (Sales et al., 2022). The maintenance of *RWC* values in *Pinus* needles is necessary for normal physiological processes, but contrary to our results, in two-year-old *P. sylvestris* needles obtained from mature trees, the *RWC* values decreased by about 27% with high-temperature stress (55 °C, 10 min) (Gette et al., 2020).

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

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

We observed that changes in the DNA methylation/hydroxymethylation contents were significantly correlated with changes in the physiological parameters. This fact supports the idea that physiological responses during abiotic stress can be influenced by methylation/hydroxymethylation changes, as previously suggested Castander-Olarieta et al. (2020). In this work, within each treatment, the $\Psi_{leaf\text{final}}$ significantly correlated with methylated and hydroxymethylated DNA, being water and thermal dependent; since it is an essential component of any biological system (Singh et al., 2020). Furthermore, the presence or absence of irrigation exerted changes in the state of correlation between

these variables in agreement with Colaneri and Jones (2013). However, they reported that drought stress, caused for the addition of polyethylene glycol in the culture medium, imposed on *Arabidopsis* seedlings triggered changes in DNA methylation with hypermethylation of protein-coding genes related to stress responses. Methylation has also been associated with aquaporins, which are proteins that regulate the movement of water across cell membranes, but the methylation does not interfere with the intrinsic permeability of aquaporin to water in *Arabidopsis* (Santoni et al., 2006).

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

5. CONCLUSIONS

Plants obtained from EMs submitted to a maturation temperature of 50 °C (30 min) presented better adaptation to drought stress based on the $\Psi_{leaf\text{final}}$ and E_{final}. Somatic plants kept at heat stress (40 °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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CHAPTER 3

The chemical environment at maturation stage in *Pinus* spp. somatic embryogenesis: implications in the polyamine profile of somatic embryos and morphological characteristics of the developed plantlets

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1. INTRODUCTION

Somatic embryogenesis has been suggested as the most promising method for vegetative propagation for a large number of conifers (Gupta et al., 1993), and it can help to capture the greatest benefits from traditional breeding programs by multiplying trees with desirable characteristics for plantation forestry (Pullman and Bucalo, 2014).

Pinus radiata D. Don is one of the most planted pine species in the world, and it is widely used to produce quality wood (Escandon et al., 2017; Fuentes-Sepulveda et al., 2020). Alternatively, *Pinus halepensis* Mill. is a species with high forest importance for reforestation because it can survive under low precipitation levels, and it has higher tolerance to thermal and drought stress (Gazol et al., 2017; Manrique-Alba et al., 2020; Quinto et al., 2021).

Previous studies carried out in our laboratory have demonstrated that *P. radiata* and *P. halepensis* showed different responses to abiotic stress such as drought and high temperature (do Nascimento et al., 2020). Also, in previous experiments, we have focused on the optimization of somatic embryogenesis (SE) processes. In this sense, the optimization of initiation and proliferation stages of SE in *P. radiata* (Montalbán et al., 2010, 2012) and *P. halepensis* (Montalbán et al., 2013) has been our objective in recent years. Recently, some studies focused on promoting and improving the embryogenic process in terms of quality and quantity of somatic embryos (ses) obtained by reduction of water availability in *P. radiata* (Garcia-Mendiguren et al., 2016; Castander-Olarieta et al., 2020) and *P. halepensis* (Pereira et al., 2016, 2017; do Nascimento et al., 2020) have been carried out. However, the rate of germination (%) and the subsequent conversion into plantlets is sometimes low (do Nascimento et al., 2020), and additional research is needed to increase somatic plantlet regeneration (Montalbán and Moncaleán, 2019).

The source of organic nitrogen is an important component of the culture media for the success of the SE process, and in this sense, amino acids are commonly used to improve the maturation of ses, modifying its type and concentration (Gerdakaneh et al., 2011; Satish et al., 2016; Dahrendorf et al., 2018). As Maruyama et al. (2021) reported, the presence of organic nitrogen improved the production of ses. Casein and glutamine are

usually used in the SE of conifers (Afele and Saxena, 1995; Pullman and Bucalo, 2014; Dahrendorf et al., 2018), but, in some species, such as *Pinus patula* Schiede ex Schltdl. and Cham. or *P. radiata*, it has been seen that mixtures of amino acids can be more beneficial for the process (Malabadi and Van Staden, 2005; Montalbán et al., 2010).

The beneficial effect of chemical environment modification in a certain stage of the process can be witnessed not only in that stage but also in the following ones (Dahrendorf et al., 2018; Castander-Olarieta et al., 2019; do Nascimento et al., 2020). In this case, the presence of a carbon source in the culture medium is necessary due to the heterotrophy conditions in which the cells grow (Yaseen et al., 2013). In addition, the carbohydrates used in the SE increase the osmolality of the culture medium (Smeekens et al., 2010; Kube et al., 2014). The most common source of carbohydrates in the culture medium is sucrose, but other carbohydrates such as maltose have been used in other *Pinus* species such as *Pinus nigra* Arn. (Salaj et al., 2019). Therefore, due to the different responses observed in the SE of various species, it is necessary to carry out a study about the effect of different carbohydrates and concentrations (Yang et al., 2020).

Polyamines (PAs) are considered to be a class of compounds related to cellular proliferation (Bais and Ravishankar, 2002; Jiménez, 2005) and play an important role in the regulation of SE (Silveira et al., 2004; Jin et al., 2020; Sundararajan et al., 2021). Moreover, PAs could be associated with the stress caused in plant cells, protecting them and upregulating stress-related genes (Alcazar et al., 2020; Kielkowska and Adamus, 2021). PAs levels vary according to each stage of SE, for example, during the intense cell division observed in the proliferation phase; higher diamine putrescine (Put) contents were reported (Minocha et al., 2004), whereas, in the maturation stage, where intense cell differentiation occurs, higher levels of triamine spermidine (Spd) and tetraamine spermine (Spm) were observed (Minocha et al., 2004; Gemperlova et al., 2009). In conifers, the accumulation of high levels of PAs was correlated also with the conversion of ses in plants (Gemperlova et al., 2009). Moreover, PAs have been considered as markers of SE competence in several species, such as *Oryza sativa* L. (Shoeb et al., 2001), *Medicago sativa* L. (Huang et al., 2001), and *Panax ginseng* C.A. Meyer (Monteiro et al., 2002).

In plants, the three most common PAs are Put, Spd, Spm (Bouchereau et al., 1999; Minocha et al., 2014), and cadaverine (Cad), which are rarely reported (Jancewicz et al., 2016). Additionally, as the amino acids are precursors of PAs biosynthesis, the exogenous addition of amino acids in the medium culture can directly influence PA profiles (Bagni and Tassoni, 2001; Pullman and Bucalo, 2014). The biosynthesis of Put, Spd, and Spm is directly related to arginine and ornithine (de Oliveira et al., 2018), while the biosynthesis of Cad is associated with lysine and ornithine (Liu et al., 2014). In *Araucaria angustifolia* Bert. O. Ktze., the ratio of PAs determined the transition from embryonal masses (EMs) to ses (Farias-Soares et al., 2014).

The aim of this work was to evaluate the influence of the different amino acid mixtures and carbohydrate sources, applied during the maturation stage of the SE, on the number and morphology of the ses obtained, their PAs profile, and the morphological characteristics of the *P. radiata* and *P. halepensis* plantlets developed.

2. MATERIALS AND METHODS

2.1. Plant Material

Embryonal masses (EMs) were obtained from immature cones of *P. radiata*, collected from four mother trees in a seed orchard set-up by Neiker-BRTA in Deba (Spain; latitude: 43°16’59” N, longitude: 2°17’59” W, elevation: 50 m) and of *P. halepensis*, collected from five mother trees in Berantevilla (Spain; latitude: 42°40’57.14” N, longitude: 2°51’29.95” W, elevation: 473 m). The immature seeds of both species were extracted and surface sterilized, following Montalbán et al. (2010, 2012). Seed coats were removed, and intact megagametophytes were excised out aseptically and cultured on initiation media for both species; for *P. radiata*, the basal medium was Embryo Development Medium (EDM; Walter et al., 2005), and for *P. halepensis*, the basal medium used was the DCR medium (Gupta and Durzan, 1985). Initiation and proliferation were carried out following the protocols described by Montalbán et al. (2012, 2013) for *P. radiata* and *P. halepensis*, respectively. Five established cell lines (ECLs) were used for each experiment and species.

2.2. Amino Acid Supplementation Experiment

The maturation of *P. halepensis* and *P. radiata* EMs was carried out following the method described in Montalbán et al. (2010, 2013), respectively. Briefly, the EMs were suspended in a liquid basal medium without plant growth regulators and then filtered on a filter paper in a Büchner funnel. Aliquots containing 0.08 g of EMs on the filter papers were placed on each maturation media. The basal media (DCR for *P. halepensis* and EDM for *P. radiata*) were supplemented with 175 mM of sucrose, 9 g L^{-1} of Gelrite®, and 75 µM of abscisic acid (ABA) for *P. halepensis* or 60 µM of ABA for *P. radiata*; these maturation media were supplemented after autoclaving with different amino acid mixtures as described in Table 1: MIX I (Control) (Walter et al., 2005), MIX II – two times the concentration in the MIX I of the L-glutamine (Gln), L-asparagine (Asn), L-arginine (Arg), and L-proline (Pro); and MIX III – 4-fold the concentration in the MIX I of the Gln (Table 1).

Table 1. Different combinations of amino acids used in the maturation media (EDM and DCR) for *Pinus radiata* D. Don and *Pinus halepensis* Mill.

Amino acids	Treatments (mg L^{-1})		
	MIX I (Control)	MIX II	MIX III
L-glutamine	550	1100	2200
L-asparagine	525	1050	525
L-arginine	175	350	175
L-proline	17.5	35	17.5
L-citrulline	19.75	19.75	19.75
L-ornithine	19	19	19
L-lysine	13.75	13.75	13.75
L-alanine	10	10	10

Both, *P. radiata* and *P. halepensis* cultures were kept at 23 °C and in darkness for 16 weeks on maturation media.

The germination of cotyledonary ses and the acclimatization of plantlets were performed according to Montalbán and Moncaleán (2019). Briefly, the ses were germinated for 8 weeks on half macronutrients LP [Quoirin and Lepoivre (1977), modified by Aitken-Christie et al. (1988)], supplemented with 2 g L^{-1} of activated charcoal and 9 g L^{-1} of Difco Agar granulated (Becton and Dickinson). First, petri dishes (90 mm × 15 mm) were used as containers, with the root caps of the ses pointing downward at an angle of approximately 60°. After this, the germinated plantlets were subcultured in the same medium for another month, but, in EcoBox® (Eco2Box/green filter: a polypropylene

vessel with a "breathing" hermetic cover, 125 mm × 65 mm × 80 mm, Duchefa). The cultures were kept at 23 °C under 16-h photoperiod at 120 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TFL 58 W/33; Philips, France) (Montalbán and Moncaleán, 2019).

After the germination, the plantlets were acclimatized in 43-cm^3 pots, containing blond peat moss (Pindstrup, Ryomgård, Denmark): vermiculite (8:2, v/v) in the greenhouse under controlled conditions.

2.3. Carbohydrate Supplementation Experiment

P. radiata and *P. halepensis* EMs obtained from the same material and methods as in Amino acid supplementation experiment were used. The basal media described in Amino acid supplementation experiment were supplemented with the MIX I (Table 1) and two different carbohydrate sources at two concentrations (four treatments) were assayed: 175 mM of sucrose (175 suc - control); 175 mM of maltose (175 mal); 350 mM of sucrose (350 suc) or 350 mM of maltose (350 mal). Cultures were kept at 23 °C and in darkness for 16 weeks. Germination and acclimatization followed the same methodology described in the material and methods of Amino acid supplementation experiment.

2.4. Osmolality of the Maturation Media

The osmolality (mosm kg^{-1}) of all maturation media was measured at the onset of the experiment using a Micro-Osmometer Automatic (Löser Messtechnik, Berlin, Germany) according to the manufacturer's instructions. Aliquots containing 100 μL of each maturation medium without Gelrite® were measured and the measurements were replicated three times (Montalbán et al., 2010).

2.5. Free Polyamine Content Determination

Germinated ses obtained after two weeks in germination media were analyzed for free PA content for *P. radiata* (Amino acid supplementation experiment) and *P. halepensis* (Amino acid supplementation experiment and Carbohydrate supplementation experiment). Germinated ses of *P. radiata* were not analyzed for Carbohydrate supplementation

experiment, because we only obtained viable germinated ses for the control treatment. PA quantification was carried out according to Silveira et al. (2004) with some modifications. Samples (200 mg of fresh weight) were briefly grounded in 1.4 mL of 5% perchloric acid (v/v) in a Precellys® shaker. After 1 h, the extraction solution was centrifuged for 20 min (15.000 *g*, 4 °C) and supernatant was collected. The pellet was once again suspended in 0.2 mL of perchloric acid and centrifuged for 20 min (15.000 *g*, 4 °C). Both extraction solutions were merged, homogenized and frozen at -20 °C for future analysis. Derivatization was performed according to Silveira et al. (2004), where 40 µL of each sample was mixed with 20 µL of diaminoheptane 0.05 mM, 50 µL of saturated sodium carbonate solution and 100 µL of dansyl chloride in acetone 1.8 mM. After 50 min of incubation in the dark at 70 °C, 25 µL of proline was added to the solution followed by another 30 min incubation at room temperature. After that, 200 µL of toluene was added, the solution was vigorously shaken and 175 µL of the superior organic phase with PAs was taken to a SpeedVac freeze dryer for 40 min at 40 °C. Finally, pellets were suspended in 175 µL of acetonitrile and were ready for high-performance liquid chromatography (HPLC) quantification. Mobile phases were composed of A) 10% (v/v) acetonitrile/ultrapure water pH 3.5 adjusted with HCl and B) 100% of acetonitrile. The gradient started at 65% of B and lasted for 11 min; it was raised to 100% B for up to 25 min, maintained in 100% B until 35.5 min and then returned to 65% B until the end at 44 min. The flow rate was constantly 1 mL min^{-1} and oven temperature was maintained at 40 °C. Stationary phase was a 5 µm Shim-pack CLC-ODS (M) 100Å column with 250 x 4.6 mm equipped with a pre-column, both from Shimadzu®. The analyses were carried out in a Shimadzu Prominence HPLC equipped with a fluorescent detector configured to excitation of 340 nm and emission of 510 nm wavelengths. To determine PA concentration, peak areas of 20 µL samples were compared to triplicates of peak areas of correspondent standards of Put, Spd, Cad and Spm, bought from Sigma-Merck®. The 1,7-diaminoheptane (Sigma-Merck®) was used as an internal standard. Total free PAs were obtained by the sum of the individual PAs. Moreover, based on the fact that Put is a precursor to Spd and Spm (Mustafavi et al., 2018), the Put/(Spd+Spm) ratio was calculated. The final concentration of PAs was expressed in mmol μg^{-1}.

2.6. Data Collection and Statistical Analysis

For both species, *P. radiata* and *P. halepensis*, the number of normal (NNE) (white to yellowish, non-germinating, with a distinct hypocotyl region, and at least three cotyledons) and abnormal mature ses (NAE) (germinating precociously, with fewer than three cotyledons or bearing abnormally-shaped cotyledons) (Montalbán et al., 2010) per gram of embryogenic tissue was recorded. Also, the length (mm) (LE) and width (mm) (WE) of 240 (Amino acid supplementation experiment on each species) and 320 (Carbohydrate supplementation experiment on each species) NNE, and the LE/WE ratio of NNE were calculated. Embryos were obtained in three of the five maturated lines in both experiments and species. From the total of ses obtained, one half of them was germinated for the morphological analysis and the other half was germinated for the determination of free PAs content. After 2 months in a germination medium, the germination rate for the NNE was calculated. Before *ex vitro* planting, the length of plantlets (mm), the length of aerial part (mm), the width of needles (mm), the stem diameter (mm), and the root length (mm) were measured with a digital caliber, and the number of secondary roots was counted in 10 plantlets per treatment. The percentage of acclimatization was calculated after 2 months in *ex vitro* conditions.

For Amino acid supplementation experiment, three different amino acid mixtures were tested in five ECLs for each species (R2, R9, R54, R82, and R137 for *P. radiata* and H48, H51, H149, H153, and H204 for *P. halepensis*) in a factorial with four repetitions (plates) per maturation condition and embryogenic cell line. In carbohydrate supplementation experiment, four different carbohydrates sources and concentrations were tested in five ECLs for *P. radiata* (R2, R9, R54, R82, and R137) and for *P. halepensis* (H48, H51, H149, H153, and H204) in a factorial with four repetitions.

For the effect of the ECLs on each of the variables of this study, an analysis of deviance was performed, followed by a Tukey *post hoc* test ($\alpha = 0.05$), adjusted for multiple comparisons. To obtain robust conclusions, ECL factors were introduced into all the models as a block variable to reduce variability and analyze the effect of the culture medium more accurately.

In Amino acid supplementation experiment, the width of needles and stem diameter, for *P. radiata*, and *P. halepensis*, respectively, were square root transformed, and an ANOVA was performed. Multiple comparisons were made using the Tukey *post hoc* tests ($\alpha = 0.05$). The same occurred in the carbohydrate supplementation experiment, for *P. halepensis*, in length of plantlets, width of needles, and the stem diameter.

In the amino acid supplementation experiment, for *P. radiata*, the NNE, length of plantlets, stem diameter, root part length, and number of secondary roots did not fulfill homoscedasticity and normality assumptions for ANOVA, and therefore, a Kruskal–Wallis was performed. The same occurred for the characteristics: *P. halepensis*—NNE, NAE, length of normal embryo, width of normal embryo, width of needles, and number of secondary roots in the amino acid supplementation experiment; *P. radiata*—length of normal embryo and the LE/WE ratio of ses in the carbohydrate supplementation experiment; and *P. halepensis*—NNE, NAE, length of normal embryo, width of normal embryo, the LE/WE ratio of ses, root part length, and number of secondary roots in the carbohydrate supplementation experiment.

For free PA content determination, the data obtained were *log* transformed, and an ANOVA was performed, followed by Tukey *post hoc* tests ($\alpha = 0.05$), adjusted for multiple comparisons.

The data were analyzed using R software®, version 3.6.1. (R Core Team, 2017).

3. RESULTS

3.1. Osmolality of the Maturation Medium

Statistically significant differences in the osmolality were observed between all the culture media with different compositions of amino acids and carbohydrates (Table 2). The osmolalities of the maturation media (EDM and DCR) were significantly higher when they contained 350 mM of sucrose compared with other carbohydrate and amino acid treatments (Table 3). Both of the maturation media supplemented with 175 mM of maltose showed the lowest values of osmolality (Table 3). Statistically significant differences were

observed when comparing the osmolality of MIX I (control) with the media supplemented with MIX II and MIX III (Table 3).

Table 2. Analysis of deviance for the osmolality (mosm kg^{-1}water) of the different maturation media (MM) for *Pinus radiata* D. Don (EDM medium) and *Pinus halepensis* Mill. (DCR medium).

Treatment	df	EDM medium		DCR medium	
		***F*-value**	***P*-value**	***F*-value**	***P*-value**
MM	5	3751	≤0.05 *	3388	≤0.05 *

* Significant differences at $p \leq 0.05$; df Degrees of freedom. The values in the table correspond to the Kruskal–Wallis test.

Table 3. Osmolality (mosm kg^{-1} water) of the maturation media (M ± *SE*) for *Pinus radiata* D. Don (EDM medium) and *Pinus halepensis* Mill. (DCR medium).

Treatment	EDM medium (±*SE*)	DCR medium (±*SE*)
Control (175 mM of sucrose + MIX I)	277 ± 0.00 [d]	260 ± 0.67 [d]
175 mM of maltose	240 ± 0.58 [e]	226 ± 0.88 [e]
350 mM of sucrose	484 ± 3.18 [a]	499 ± 1.00 [a]
350 mM of maltose	428 ± 2.08 [b]	411 ± 2.65 [b]
MIX II	294 ± 1.00 [c]	278 ± 2.96 [c]
MIX III	293 ± 0.88 [c]	272 ± 0.33 [c]

Different letters within a column show significant differences in the means observed by a Kruskal–Wallis test. MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln. *SE* - standard error.

3.2. Amino Acid Supplementation Experiment

Regarding the amino acid mixture used in the maturation media, statistically significant differences were found for all the parameters evaluated in *P. radiata* (NAE, LE, WE, germination of ses, and acclimatization of plantlets) except for the NNE and LE/WE ratio (Table 4).

Table 4. Analysis of deviance for the effect of the different mixes of amino acids (MA) in the maturation stage in: the number of normal and abnormal somatic embryos per 0.08 g of embryonal masses, the morphological characteristics of normal embryos, the germination of somatic embryos, the morphological characteristics and the acclimatization of plantlets of *Pinus radiata* D. Don and *Pinus halepensis* Mill., respectively.

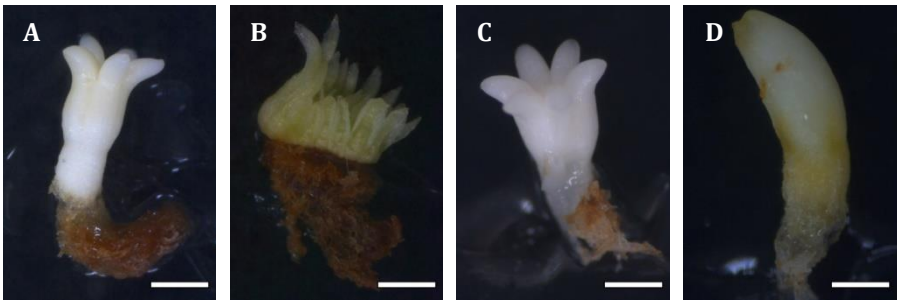
Characteristics	Source	df	*Pinus radiata*		*Pinus halepensis*	
			***F*-value**	***P*-value**	***F*-value**	***P*-value**
Number of normal embryos	MA	2	3.85	>0.05 [ns]	0.37 [1]	>0.05 [ns]
Number of abnormal embryos	MA	2	6.06	≤0.01**	0.98 [1]	>0.05 [ns]
Length of normal embryos	MA	2	6.29	≤0.01**	52.48 [1]	≤0.05 *
Width of normal embryos	MA	2	13.24	≤0.05 *	51.21 [1]	≤0.05 *
Length /Width ratio (mm)	MA	2	18.40	>0.05 [ns]	21.45	>0.05 [ns]
Germination of somatic embryos	MA	2	16.61	≤0.001 ***	22.59	≤0.01**
Length of plantlets (mm)	MA	2	4.47	>0.05 [ns]	0.58	>0.05 [ns]
Length of aerial part (mm)	MA	2	0.59	>0.05 [ns]	0.13	>0.05 [ns]
Width of needles (mm)	MA	2	1.78	>0.05 [ns]	5.48 [1]	>0.05 [ns]
Stem diameter (mm)	MA	2	10.12	≤0.05 *	1.16	>0.05 [ns]
Root part length (mm)	MA	2	2.73	>0.05 [ns]	1.44	>0.05 [ns]
Number of secondary roots	MA	2	7.11	≤0.05 *	0.18 [1]	>0.05 [ns]

Acclimatization of plantlets	MA	2	56.85	≤0.001 ***	19.33	>0.05 ns

*; **; *** Significant differences at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively; ns Non-significant at $p \leq 0.05$; MA mixes of amino acids; df Degrees of freedom. [1] The values in the table correspond to the Kruskal–Wallis test.

In the case of *P. halepensis*, the different mixtures of amino acids produced statistically significant differences for the LE, WE, and germination rate of NNE (Table 4). However, the mixture of different amino acids did not show statistically significant differences for the NNE, NAE, the LE/WE ratio, and acclimatization of plantlets (Table 4).

Figures 1A,B show normal and aberrant morphologies of ses of *P. radiata*, respectively. For *P. radiata*, although non-significant differences were found among the different combinations of amino acids when compared with the production of NNE (Table 4), an increase of the NNE in MIX II and MIX III (240 and 317 ses g^{-1} fresh weight, respectively), compared with the control (MIX I – 157 ses g^{-1} fresh weight), was observed (Figure 2A). The same tendency was found for the NAE, with a statistically significant increase for NAE in media supplemented with MIX II and MIX III when compared to MIX I (Figure 2A). Moreover, we observed that treatments MIX II and MIX III induced an increase in LE and WE when compared to control treatment (MIXI) (Figures 2B,D,E,F). With respect to the LE/WE ratio, non-significant differences were found between the different combinations of amino acids (Figure 2C).



P. radiata *P. halepensis*

Figure 1. Somatic embryos showing different morphologies: *Pinus radiata* D. Don normal somatic **(A)**, bar = 1.44 mm; *P. radiata* abnormal somatic embryo **(B)**, bar = 1.85 mm; *Pinus halepensis* Mill. normal somatic embryos **(C)**, bar = 0.84 mm; *P. halepensis* abnormal somatic embryos **(D)**, bar = 0.60 mm.

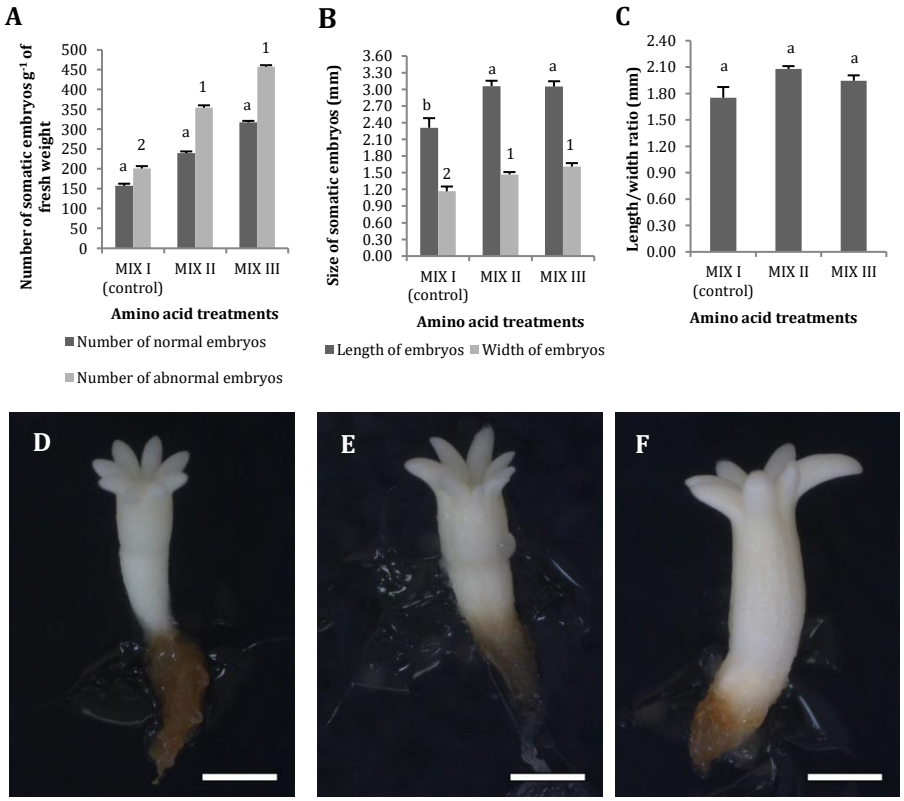


Figure 2. *Pinus radiata* D. Don somatic embryos of maturation media supplemented with different mixes of amino acids. Number of normal and abnormal somatic embryos **(A)**; the length and width of normal embryos **(B)**; and the length/width ratio of normal embryos **(C)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters or numbers. *Pinus radiata* normal somatic obtained from maturation media supplemented with different mixes of amino acids. MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

Figures 1C,D show normal and aberrant morphologies of ses of *P. halepensis*, respectively. For *P. halepensis*, although nonsignificant differences were found among the different mixtures of amino acids for NNE and NAE (Table 4), the highest NNE and NAE were recorded in control conditions (68 ses g^{-1} fresh weight for NNE and 178 ses g^{-1} fresh weight for NAE); MIX II showed intermediate values, and MIX III presented the lowest values (23 ses g^{-1} fresh weight for NNE and 109 ses g^{-1} fresh weight for NAE) (Figure 3A).

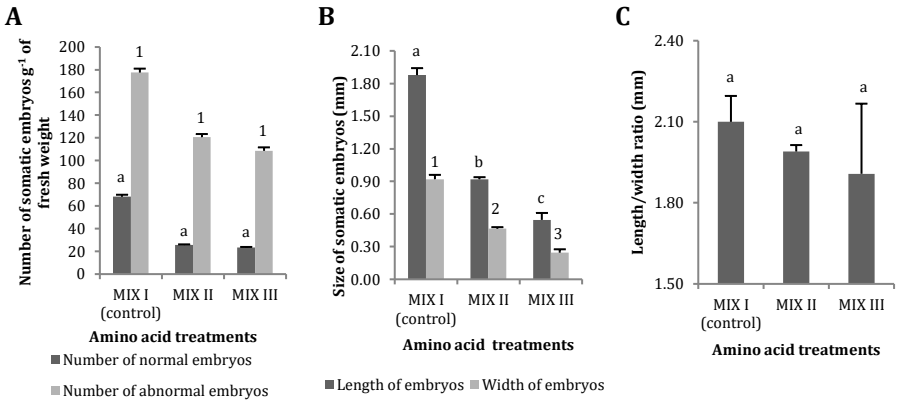


Figure 3. *Pinus halepensis* Mill. somatic embryos obtained per gram of embryonal masses matured in different mixes of amino acids. Number of normal and abnormal somatic embryos **(A)**; the length and width of normal embryos **(B)**; the length/width ratio of normal embryos **(C)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters or numbers. MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

P. halepensis ses developed in control conditions (MIX I) were significantly wider and longer than those obtained in MIX II and MIX III. Significantly lower LE and WE values were obtained when the maturation medium was supplemented with a 4-fold concentration of Gln (MIX III) (Figure 3B), although the LE/WE ratio did not show significant differences (Figure 3C).

As observed in Figure 4A, the best germination percentage of *P. radiata* was obtained in those embryos coming from ECLs maturated on a medium supplemented with double the concentration of Gln, Asn, Arg, and Pro (MIX II), but not in relation to control treatment (MIX I). However, the treatment with 4-fold of Gln (MIX III) promoted a significant decrease of the ses germination with respect to MIX II (Figure 4A). Plantlets of *P. radiata* survived after acclimatization with significantly higher percentage of acclimatization when coming from ses maturated on a culture medium supplemented with MIX II (99%) and MIX III (89.29%) than compared with control (MIX I) (49.21%) (Figures 4B, 5).

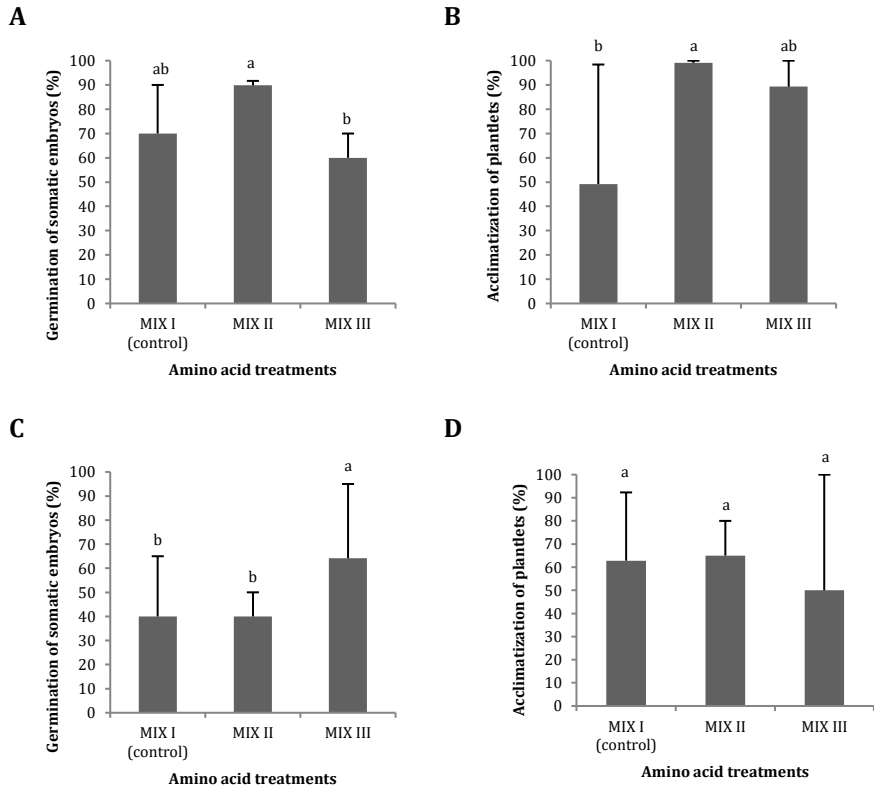


Figure 4. *Pinus radiata* D. Don germination (%) of somatic embryos maturated in a medium supplemented with different mixes of amino acids **(A)**. Acclimatization (%) of plantlets of *P. radiata* obtained from somatic embryos maturated in a medium supplemented with different mixes of amino acids **(B)**. Germination (%) of somatic embryos of *Pinus halepensis* Mill. maturated in a medium supplemented with different mixes of amino acids **(C)**. Acclimatization (%) of plantlets of *P. halepensis* obtained from somatic embryos maturated in a medium supplemented with different mixes of amino acids **(D)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters. MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

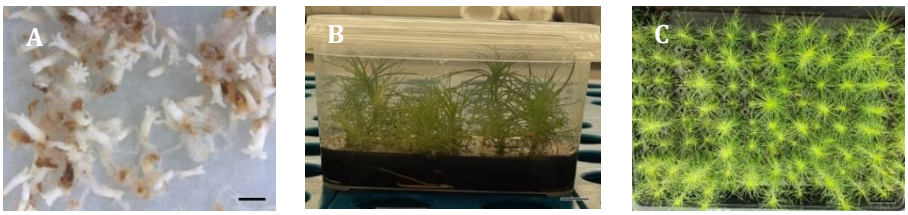


Figure 5. Maturation, germination and acclimatization of somatic embryos from *Pinus radiata* D. Don embryogenic cell lines. Somatic embryos after four months in a maturation medium **(A)**, bar = 3 mm; Somatic plantlets after three months in a germination medium **(B)**, bar = 1.4 cm; plantlets derived

from normal cotyledonary somatic embryos growing in the greenhouse **(C)**, bar = 2.35 cm. The maturation medium was supplemented with MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

P. halepensis germination significantly increased in those embryos coming from ECLs maturated in the presence of 4-fold the concentration of Gln (MIX III) (64%) when compared to results obtained with other amino acid combinations (Figure 4C). Significant differences were not observed for the survival of plantlets in the greenhouse (62.82, 65, and 50% for MIX I, MIX II, and MIX III, respectively) (Figure 4D).

Statistically significant differences for the stem diameter and the number of secondary roots were observed in *P. radiata* (Table 4). The mean length of the plantlets ranged from 38.13 to 47.66 mm, and this value was divided into the aerial part and the root part (Table 5). In this sense, the width of the needle varied between 0.27 and 0.32 mm (Table 5). The MIX II and MIX III promoted a significant increase of the stem diameter when compared to the control (Figure 6A). The same tendency was observed with the number of secondary roots where the MIX II promoted a significant increase in the number of secondary roots (Figures 6B,C).

Table 5. Morphological characteristics of *P. radiata* D. Don somatic plantlets developed from ECLs maturated in a culture medium supplemented with different amino acid compositions (M±*SE*).

Morphological characteristics	MIX I - control	MIX II	MIX III
Length of plantlets (mm)	38.13 ± 6.72	47.17 ± 5.83	47.66 ± 5.86
Length of aerial part (mm)	6.13 ± 0.59	6.91 ± 0.52	6.28 ± 0.48
Width of needles (mm)	0.28 ± 0.32	0.32 ± 0.02	0.27 ± 0.01
Root length (mm)	26.58 ± 4.04	20.68 ± 4.69	24.40 ± 4.66

MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln. *SE* - standard error.

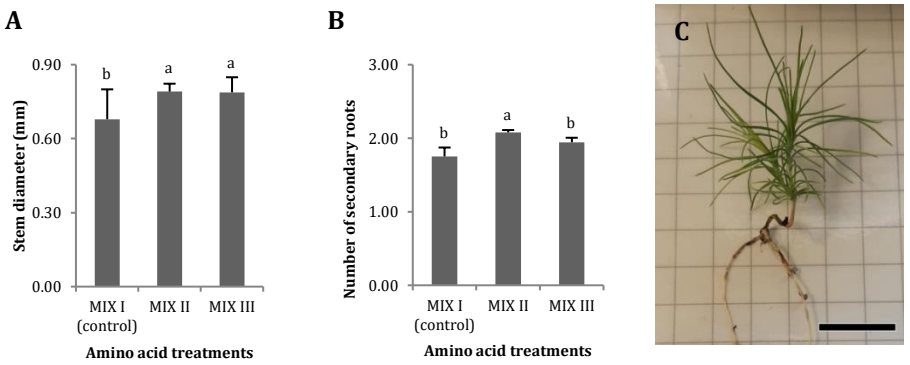


Figure 6. Morphological characteristics of *Pinus radiata* D. Don plantlets, stem diameter (mm) **(A)** and number of secondary roots **(B)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters or numbers. Plantlet obtained from somatic embryos maturated in EDM medium with MIX I **(C)** and MIX II **(D)**, bar = 2 cm. MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

When evaluating the morphological characteristics of the germinated ses developed from ECLs of *P. halepensis* maturated in the presence of different combinations of amino acids, no statistically significant differences were observed (Tables 4, 6).

Table 6. Morphological characteristics of *Pinus halepensis* Mill. somatic plantlets developed from ECLs maturated in a culture medium supplemented with different amino acid compositions (M±*SE*).

Morphological characteristics	MIX I - control	MIX II	MIX III
Length of plantlets (mm)	77.96 ± 11.50	97.63 ± 15.37	92.98 ± 12.76
Length of aerial part (mm)	6.94 ± 1.24	7.58 ± 1.09	6.82 ± 1.05
Width of needles (mm)	0.31 ± 0.03	0.27 ± 0.03	0.23 ± 0.01
Stem diameter (mm)	0.68 ± 0.05	0.79 ± 0.06	0.79 ± 0.07
Root length (mm)	38.09 ± 10.23	64.64 ± 13.27	57.19 ± 10.47
Number of secondary roots	2 ± 0.36	2 ± 0.31	2 ± 0.29

MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln. *SE* - standard error.

3.3. Carbohydrate Supplementation Experiment

The carbohydrate sources significantly affected the NNE, the NAE, and the LE (Table 7). For NAE, WE, and the LE/WE ratio, a significant interaction between the carbohydrate sources and concentrations was observed (Table 7). Finally, the carbohydrate source and the carbohydrate concentration had a significant effect on the germination rates, but there was no interaction between the previously mentioned factors (Table 7).

Table 7. Analysis of deviance for the effect of the different carbohydrate sources (CS) and concentrations (CC) in maturation stage in the number of normal and abnormal somatic embryos per 0.08 g of embryonal masses; the morphological characteristics of normal embryos; the germination of somatic embryos of *Pinus radiata* D. Don and *Pinus halepensis* Mill., respectively; and the effect of the different CS in the germination of somatic embryos, the morphological characteristics and the acclimatization of plantlets *P. halepensis*.

Characteristics	Source	df	*Pinus radiata*		*Pinus halepensis*	
			F-value	*P*-value	*F*-value	*P*-value
Number of normal embryos	CS	1	5.15	≤0.05 *	2.80 [1]	>0.05 [ns]
	CC	1	0.67	>0.05 [ns]	0.90	>0.05 [ns]
	SC x CC	3	1.93	>0.05 [ns]	2.80	>0.05 [ns]
Number of abnormal embryos	CS	1	11.30	≤0.01**	16.89 [1]	≤0.001***
	CC	1	1.45	>0.05 [ns]	0.27	>0.05 [ns]
	SC x CC	3	7.48	≤0.01**	16.90	≤0.001***
Length of normal embryos	CS	1	4.22 [1]	≤0.05 *	23.44 [1]	≤0.05 *
	CC	1	0.78	>0.05 [ns]	31.57	≤0.05 *
	SC x CC	3	4.25	>0.05 [ns]	28.13	≤0.05 *
Width of normal embryos	CS	1	0.44	>0.05 [ns]	32.10 [1]	≤0.001***
	CC	1	0.65	>0.05 [ns]	20.19	≤0.001***
	SC x CC	3	20.17	≤0.01**	35.21	≤0.001***
Length /Width ratio (mm)	CS	1	5.86 [1]	≤0.05 *	12.88 [1]	≤0.05 *
	CC	1	1.08	>0.05 [ns]	3.16	>0.05 [ns]
	SC x CC	3	10.14	≤0.05 *	17.21	≤0.05 *
Germination of somatic embryos	CS	1	36.69	≤0.001***	82.11	≤0.001***
	CC	1	0.51	≤0.001***	-	-
	SC x CC	3	0.51	>0.05 [ns]	-	-
Length of plantlets (mm)	CS	1	-	-	1.21	>0.05 [ns]
Length of aerial part (mm)	CS	1	-	-	0.89	≤0.05 *
Width of needles (mm)	CS	1	-	-	0.47	>0.05 [ns]
Stem diameter (mm)	CS	1	-	-	1.07	>0.05 [ns]
Root part length (mm)	CS	1	-	-	2.56 [1]	>0.05 [ns]
Number of secondary roots	CS	1	-	-	4.76 [1]	≤0.05 *
Acclimatization of plantlets	CS	1	-	-	20.15	≤0.001***

*; **; *** Significant differences at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively; [ns] Non-significant at $p \leq 0.05$; df Degrees of freedom. [1]The values in the table correspond to the Kruskal–Wallis test.

Two types of carbohydrates in both concentrations produced normal and aberrant embryos in *P. radiata*. However, the use of sucrose in the maturation medium, regardless of the concentration, resulted in a significantly higher NNE (Figure 7A). In this regard, we also observed the highest NAE in the medium of maturation with sucrose but only at a concentration of 175 suc (control) in relation to maltose (Figure 7A). In addition, the same tendency was observed for LE and WE (Figure 7B), with the development of longer and wider ses in the maturation medium with sucrose, regardless of the concentration. The ses maturated in media with sucrose presented a significantly higher LE/WE ratio than those obtained from a medium with 350 mal (Figure 7C).

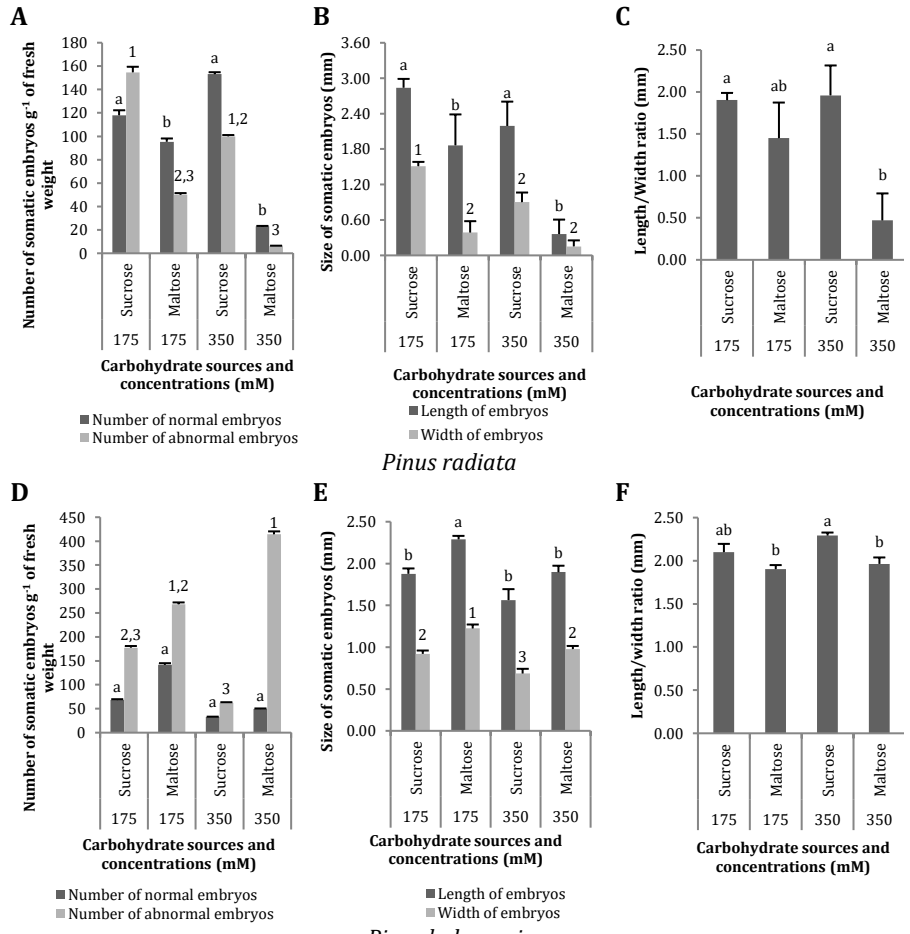


Figure 7. *Pinus radiata* D. Don and *Pinus halepensis* Mill. somatic embryos obtained per gram of embryonal masses matured in different carbohydrate sources and concentrations. Number of normal and abnormal somatic embryos **(A, D)**; the length and width of normal embryos **(B, E)**; and the length/width ratio of normal embryos **(C, F)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters or numbers.

In *P. halepensis*, the NNE was not affected by the different carbohydrate treatments tested (Table 7 and Figure 7D). However, NAE, LE, WE, and the LE/WE ratio were significantly affected by the interaction between the carbohydrate source and the concentration (Table 7). The medium with the highest maltose concentration produced a significantly higher number of NAE than the sucrose media (Figure 7D).

The LE and the WE of *P. halepensis* obtained in a maturation medium supplemented with 175 mal were significantly higher than the LE obtained after maturation with other carbohydrate sources or concentrations (Figure 7E). As noted in Figure 7F, a significantly

higher LE/WE ratio was observed in ses maturated with the highest concentration of 350 suc when compared to results obtained in ses from media supplemented with maltose. Thus, higher concentrations of carbohydrates caused the formation of less developed embryos (Figure 8).

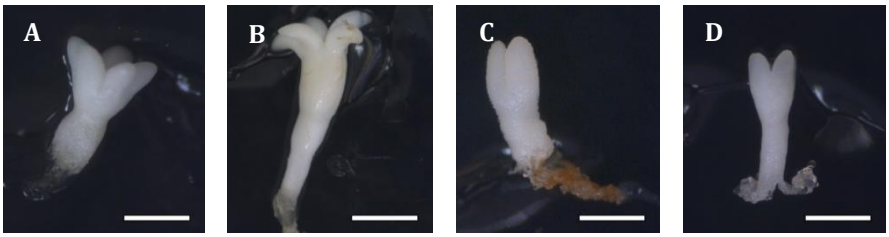


Figure 8. Somatic embryos of *Pinus halepensis* Mill. maturated in DCR medium supplemented with: 175 mM of sucrose **(A)**, bar = 1 mm; 175 mM of maltose **(B)**, bar = 1.57 mm; 350 mM of sucrose **(C)**, bar = 0.57 mm; and 350 mM of maltose **(D)**, bar = 1 mm.

The use of 175 suc promoted a germination percentage of 70%, while the other treatments promoted a percentage lower than 15% for ses of *P. radiata* (Figure 9A). However, the ses maturated at a maturation medium with a high concentration of maltose did not promote the germination of ses (Figure 9A). Viable plantlets were only obtained in those embryos germinated in culture media with 175 suc where the acclimatization percentage was 49.21%.

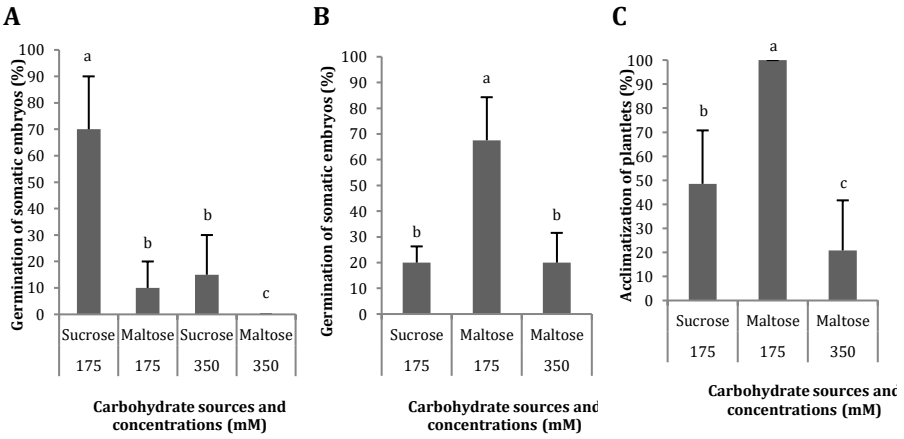


Figure 9. Germination (%) of somatic embryos of *Pinus radiata* D. Don maturated in a medium supplemented with different carbohydrate sources and concentrations **(A)**. Germination (%) of somatic embryos of *Pinus halepensis* Mill. maturated in a medium supplemented with different carbohydrate sources and concentrations **(B)**. Acclimatization (%) of plantlets of *P. halepensis* obtained from somatic embryos maturated in a medium supplemented with different carbohydrate

sources and concentrations **(C)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters.

The ses of *P. halepensis* maturated with 175 mal showed a germination percentage significantly (Table 7) higher than the rest of the treatments assayed (Figure 9B). However, the ses maturated with 350 suc did not germinate. The plantlets of *P. halepensis* from the maturation treatment with 175 mM of maltose survived after acclimatization in a significantly higher percentage (100%) than those that came from a treatment with 175 suc (control) (Table 7 and Figures 9C, 10). In contrast, the worst results were obtained in the treatment with a higher concentration of maltose (Figure 9C).

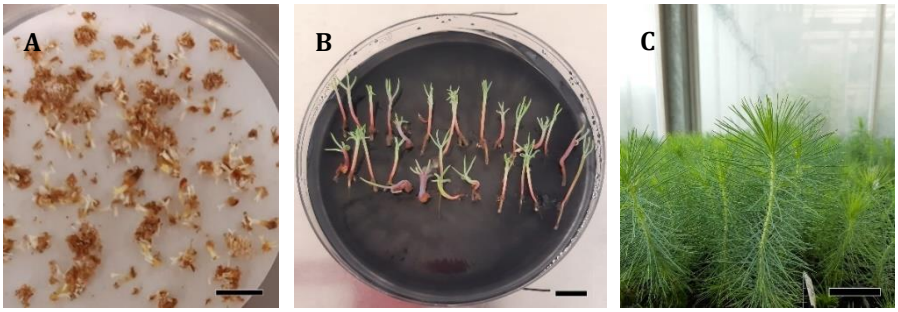


Figure 10. Maturation, germination and acclimatization of somatic embryos from *Pinus halepensis* Mill. embryogenic cell lines. Somatic embryos after four months in a maturation medium **(A)**, bar = 1 cm; Somatic plantlets after three months cultivated in a germination medium **(B)**, bar = 1 cm; plantlets derived from normal cotyledonary somatic embryos growing in the greenhouse **(C)**, bar = 3 cm. The maturation medium was supplemented with 550 mg L^{-1} of L-glutamine, 525 mg L^{-1} of L-asparagine, 175 mg L^{-1} of L-arginine, 17.5 mg L^{-1} of L-proline, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine and 175 mM of maltose.

The length of aerial part and number of secondary roots were significantly affected by the different carbohydrate sources, and no differences were observed for the other morphological characteristics studied in *P. halepensis* (Table 7). It was observed that 175 mal promoted an increase in the aerial part and number of secondary roots in comparison with 175 suc (Figures 11A,B). Although significantly statistical differences were not found for the other morphological characteristics, an increase in the length of plantlets was observed in those obtained from the ses developed in a maturation medium supplemented with 175 mal (Table 8 and Figure 11C). The same results were observed for stem diameter and the root length; but the largest width of the needle was observed in 175 suc (Table 8).

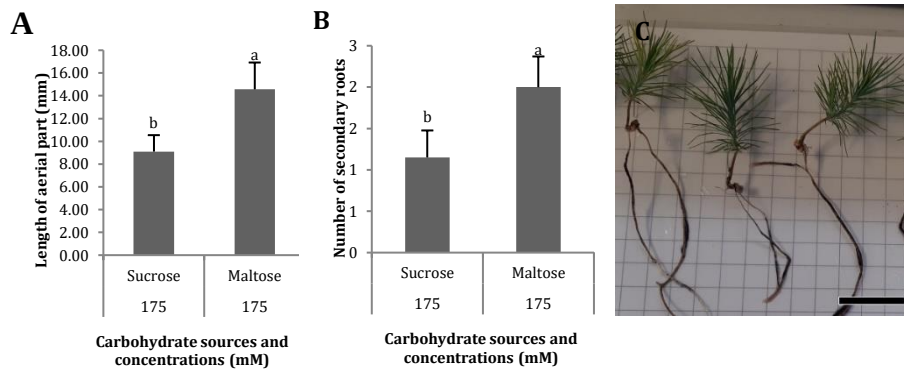


Figure 11. Morphological characteristics of *Pinus halepensis* Mill. plantlets, length of aerial part (mm) **(A)** and number of secondary roots **(B)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters or numbers. Plantlets of *Pinus halepensis* Mill. obtained from somatic embryos maturated in EDM medium supplemented with 175 mM of maltose **(C)**, bar = 3 cm.

Table 8. Morphological characteristics of *Pinus halepensis* Mill. somatic plantlets developed from ECLs maturated in a culture medium supplemented with different carbohydrate sources.

Morphological characteristics	175 mM of sucrose	175 mM of maltose
Length of plantlets (mm)	59.29 ± 10.57	74.19 ± 9.80
Width of needles (mm)	0.27 ± 0.03	0.25 ± 0.02
Stem diameter (mm)	0.66 ± 0.03	0.75 ± 0.06
Root length (mm)	28.24 ± 7.84	44.69 ± 8.25

SE - standard error.

3.4. Free Polyamine Content Determination

The addition of different combinations of amino acids in the maturation medium did not provoke statistically significant differences in the ses germination of *P. radiata*, as well as no statistically significant differences were observed for the interaction between amino acid mixtures and the amounts of PAs (Table 9). However, the only statistically significant differences were found when the content of individual Put, Cad, Spd, and Spm was analyzed (Table 9). Conversely, total free PAs and the ratio Put/(Spd + Spm) did not show significant differences (Table 9 and Figures 12A,B). A higher amount of total free PAs was observed, with means ranging from 300.09 to 368.80 mmol μg^{-1} (Figure 12A). Within the total Free PAs, Cad accounted for half the amount of the total quantity followed by Spd (Figure 12C). However, the ses germinated from *P. radiata* had three times less Spm when compared with the other PAs previously mentioned, while the amount of Put was the lowest free PAs detected (Figure 12C). Plantlets of *P. radiata* contained large amounts of Cad and Spd, intermediate amounts of Spm, and low amounts of Put (Figure 12C).

Table 9. Analysis of variance for the effect of the mixes of amino acids (MA) and the free individual polyamine (PAs) levels; and for the effect of the mixes of MA on the total free PAs and the ratio Put/(Spd+Spm) in germinated somatic embryos of *Pinus radiata* D. Don and *Pinus halepensis* Mill.

PAs content	Source	df	*Pinus radiata*		*Pinus halepensis*	
			F-value	*P*-value	*F*-value	*P*-value
Free PAs (mmol μg^{-1})	MA	2	0.68	>0.05 ^{ns}	1.04	>0.05 ^{ns}
	PAs	3	190.99	≤0.001 ***	54.04	≤0.001 ***
	MA x PAs	6	0.44	>0.05 ^{ns}	3.21	≤0.05 *
Total free PAs (mmol μg^{-1})	MA	2	0.49	>0.05 ^{ns}	0.76	>0.05 ^{ns}
Ratio Put/(Spd+Spm) (mmol μg^{-1})	MA	2	0.28	>0.05 ^{ns}	3.23	>0.05 ^{ns}

*; *** Significant differences at $p \leq 0.05$ or $p \leq 0.001$, respectively; ^{ns} Non-significant at $p \leq 0.05$; df Degrees of freedom; Put, putrescine; Spd, spermidine; Spm, spermine. Mixes of amino acids: MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

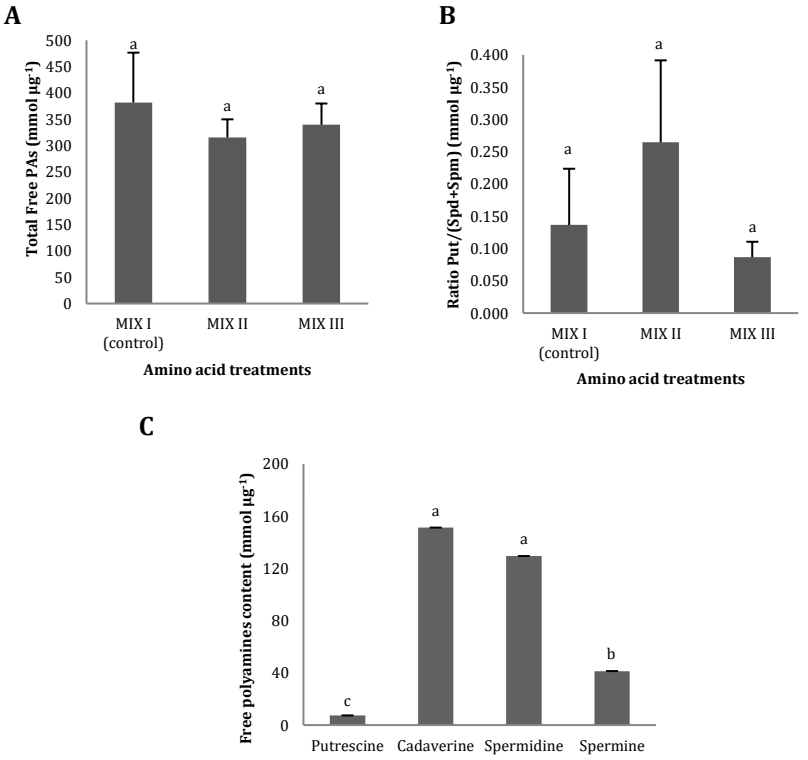


Figure 12. Polyamines (PA) levels (mmol μg^{-1}) in germinated somatic embryos of *Pinus radiata* D. Don obtained from somatic embryos maturated in media supplemented with different mixes of amino acids. Total Free PAs **(A)**; the ratio Put/(Spd+Spm) **(B)**; and the individual free PAs **(C)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters. MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

Statistically significant differences were observed in PAs and the interaction between the amino acid mixtures and free PAs in germinated ses of *P. halepensis* (Table 9). However, no statistically significant differences were observed for the total free PAs and the ratio Put/(Spd + Spm) (Table 9 and Figures 13A,B). The highest concentrations of total free PAs were observed in ses from MIX II treatment (Figure 13A). The amounts of Cad, Spd, and Spm did not change during the germination of ses obtained from maturation media supplemented with different combinations of amino acids (Figure 13C). The level of these three PAs exhibited a slight increase in MIX I compared to the other treatments (Figure 13C). In this case, the amount of Spd increased slightly, followed by Cad (Figure 13C). In contrast, the Put concentration exhibited a considerable significant decrease in MIX I and MIX III (Figure 13C). Exogenous application of amino acids of MIX II in the maturation medium promoted similar amounts of four PAs analyzed in the germinated ses (Figure 13C).

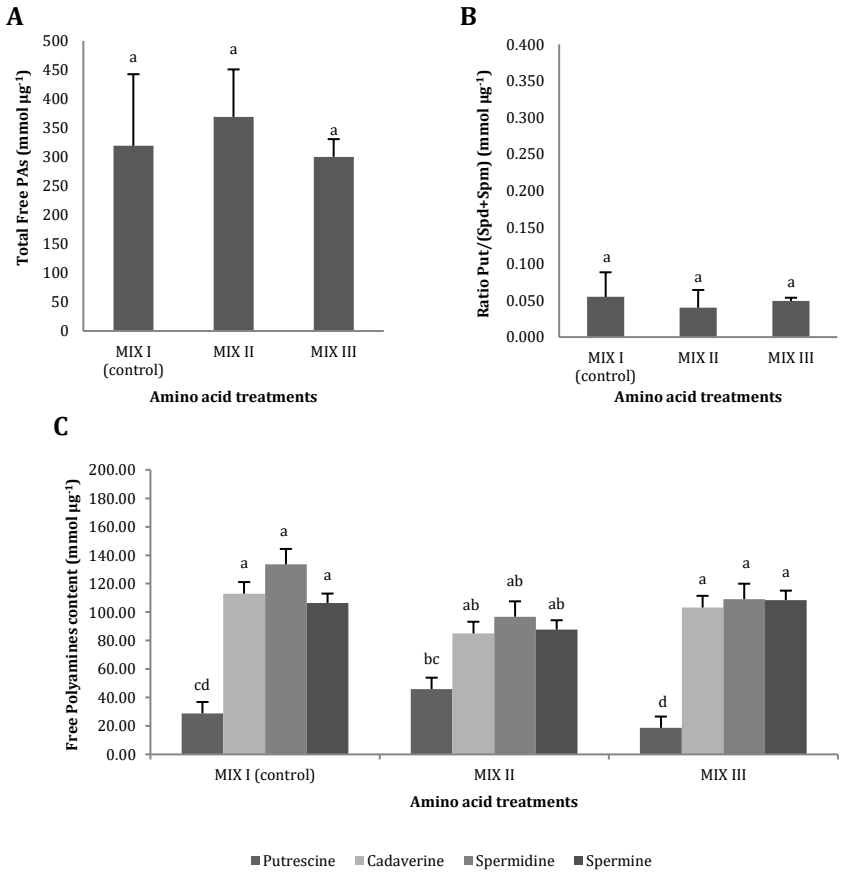


Figure 13. Polyamine (PAs) levels (mmol µg^{-1}) in germinated somatic embryos of *Pinus halepensis* Mill. obtained from somatic embryos maturated in media supplemented with different mixes of amino acids. Total Free PAs **(A)**; the ratio Put/(Spd+Spm) **(B)**; and the individual free PAs **(C)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters. MIX I (Control) – 550 mg L^{-1} of L-glutamine (Gln), 525 mg L^{-1} of L-asparagine (Asn), 175 mg L^{-1} of L-arginine (Arg), 17.5 mg L^{-1} of L-proline (Pro), 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine and 10 mg L^{-1} of L-alanine; MIX II - twice the concentration in the MIX I of the Gln, Asn, Arg and Pro; and MIX III – 4-fold the concentration in the MIX I of the Gln.

For *P. halepensis*, statistically significant differences were observed for the different sources of carbohydrates, PAs, the interaction between carbohydrate sources, and carbohydrate concentrations and the triple interaction between carbohydrate sources, carbohydrate concentrations, and PAs (Table 10).

Table 1. Analysis of variance for the effect of the different carbohydrates sources (CS) and concentration (CC), and the free polyamine levels (PAs) (mmol µg^{-1}); and for the effect of the different carbohydrate sources (CS) and concentration (CC) in the total free PAs (mmol µg^{-1}) and the ratio Put/(Spd+Spm) in germinated somatic embryos of *Pinus halepensis* Mill.

PAs content	Source	df	*F*-value	*P*-value
Free PAs	CS	1	7.58	≤0.001 ***

	CC	1	0.88	>0.05 ^{ns}
	PAs	3	38.17	≤0.001 ***
	SC x CC	1	4.22	≤0.05 *
	CS x PAs	3	2.21	>0.05 ^{ns}
	CC x PA	3	2.40	>0.05 ^{ns}
	CS x CC x PAs	3	10.28	≤0.001 ***
Total free PAs	CS	1	1.88	>0.05 ^{ns}
	CC	1	0.00	>0.05 ^{ns}
	SC x CC	1	0.10	>0.05 ^{ns}
Ratio Put/(Spd+Spm)	CS	1	0.00	>0.05 ^{ns}
	CC	1	1.85	>0.05 ^{ns}
	SC x CC	1	13.87	≤0.01 **

*; *** Significant differences at $p \leq 0.05$ or $p \leq 0.001$, respectively; ^{ns} Non-significant at $p \leq 0.05$; df Degrees of freedom. Put, putrescine; Spd, spermidine; Spm, spermine.

The total free PAs were not affected by the carbohydrate sources, carbohydrate concentrations, or the interaction between these factors (Table 10 and Figure 14A). On the contrary, the ratio Put/(Spd + Spm) was affected by the interaction carbohydrate sources × carbohydrate concentration (Table 10). The *P. halepensis* germinated ses maturated in media with 350 suc or 175 mal showed significantly higher Put/(Spd + Spm) ratios than those obtained in a medium with the lowest maltose concentration (Figure 14B). Additionally, an intermediate value for Put/(Spd + Spm) ratios was observed in the germinated ses maturated in control conditions (175 suc) (Figure 14B). Germinated ses coming from maturation media with different carbohydrate sources and concentrations showed statistical higher values of all PAs, except Put (Figure 14C). In this case, levels of Put presented a marked decrease in germinated ses maturated in maturation media 350 mal when compared with the germinated ses maturated in maturation media 175 mal and 350 suc (Figure 14C). Cad, Spd, and Spm detected in germinated ses maturated in different carbohydrate sources and concentrations presented a similar behavior, but with an increase in the Cad amount in the germinated ses maturated in maturation media with 350 suc (Figure 14C).

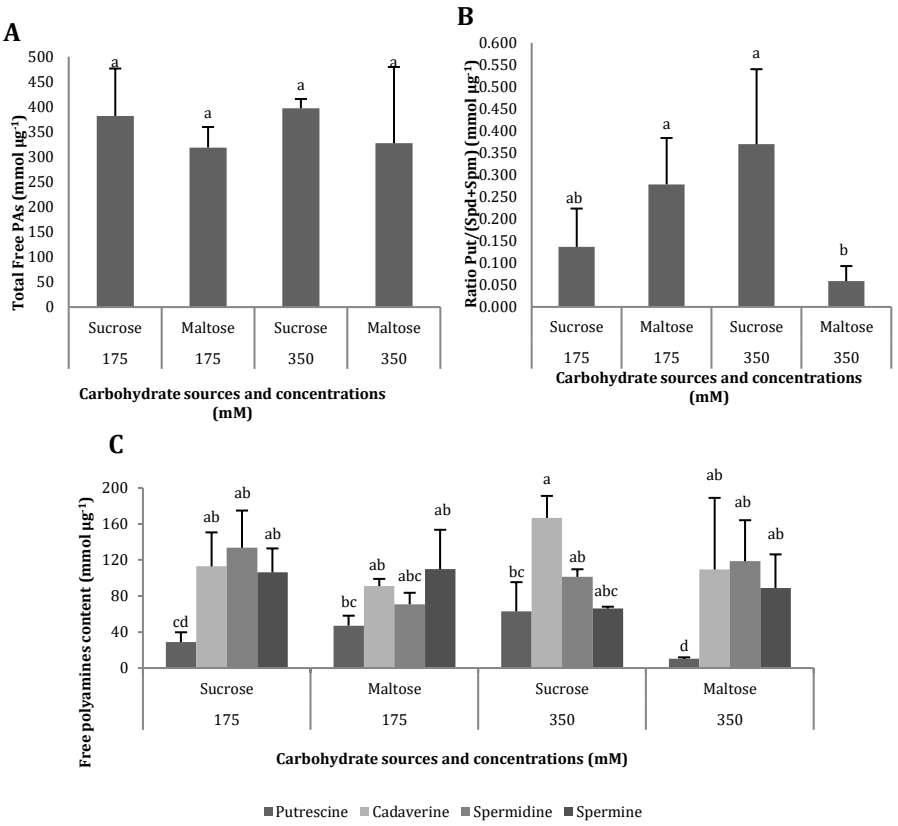


Figure 14. Polyamine (PAs) levels (mmol µg^{-1}) in plantlets of *Pinus halepensis* Mill. obtained from germinated somatic embryos maturated in media supplemented with different carbohydrate sources and concentrations. Total Free PAs **(A)**; the ratio Put/(Spd+Spm) **(B)**; and the Free PAs **(C)**. Bars indicate standard errors. Significant differences at $p \leq 0.05$ are indicated by different letters.

4. DISCUSSION

Different amino acid or carbohydrate combinations significantly affected the osmolality of the maturation culture medium; it is common in the conifers SE process to restrict the water availability of the medium (increasing gellan gum concentration), modifying the osmolality in order to cause a shift in the developmental program of EMs and promote the formation of ses (Kvaalen and Johnsen, 2008; Garcia-Mendiguren et al., 2016). In our experiments, the best environment to achieve proper conversion of ses in plantlets was observed in maturation media with osmolalities between 277 (175 mM of sucrose) and 294 mosm kg^{-1} water (MIX II) for *P. radiata* and between 226 (175 mM of maltose) and 272 mosm kg^{-1} water (MIX III) for *P. halepensis*. However, and in agreement with Montalbán et al. (2010), who obtained good results at osmolalities around 270 mosm kg^{-}

^{1} water, the medium osmolality alone did not explain the results obtained because, in other media with similar osmolalities, such good results were not obtained in both species. In our work, the highest osmolalities (above 400 mosm kg^{-1} water) did not increase the maturation success; in contrast, in *Pseudotsuga menziesii* (Mirb.) Franco raising medium osmolality to 450 mosm kg^{-1} water provoked an improvement in the vigor and morphology of ses developed (Gupta and Pullman, 1991). In this sense, the improvement in the number of ses in *P. radiata* and in *Cryptomeria japonica* D. Don was obtained in lower water availability in maturation media caused by 10 g L^{-1} of gellan gum and 17.5% of polyethylene glycol (PEG), respectively (Moncaleán et al., 2018; Maruyama et al., 2021). However, in *Pinus taeda* L., the maturation medium with 13% of PEG 8,000 and the osmolality ranging from 227 to 233 mmol kg^{-1} allowed an increase in the number of ses (Pullman et al., 2003).

When the size of the *P. radiata* somatic embryos was analyzed, we found that the maturation with MIX I (550 mg L^{-1} of Gln, 525 mg L^{-1} of Asn, 175 mg L^{-1} of L-Arg, 17.5 mg L^{-1} of Pro, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine, and 10 mg L^{-1} of L-alanine) promoted the formation of smaller ses, showing lower acclimatization success months later (Figure 4B). In contrast, germinated ses maturated on MIX II (1,100 mg L^{-1} of Gln, 1,050 mg L^{-1} of Asn, 350 mg L^{-1} of L-Arg, 35 mg L^{-1} of Pro, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine, and 10 mg L^{-1} of L-alanine) promoted larger embryos, with a larger stem diameter and an increase in the number of roots in the germinated ses, improving the acclimatization to *ex vitro* conditions of this species (Figure 4B). Similar to our results with *P. radiata*, Afele and Saxena (1995) reported an increase in the size of the mature somatic embryos and plantlet development of *Picea pungens* Engelm. in the culture medium supplemented with Asn. The combination of several amino acids (Gln, Asn, Arg, L-citrulline, L-ornithine, L-lysine, L-alanine, and Pro) was also beneficial in *C. japonica* SE, and the increase of concentration of several amino acids (2 g L^{-1} of Gln, 1 g L^{-1} of Asn, and 0.5 g L^{-1} of Arg) was essential to improve an efficient maturation of ses (>1,000 ses g^{-1} fresh weight) (Maruyama et al., 2021). In addition, the inclusion of a mixture of amino acid solution with 17 amino acids (Gln, alanine, Pro, lysine, glycine, Arg, leucine, phenylalanine, serine,

isoleucine, valine, histidine, threonine, tyrosine, Asn, tryptophan, and cysteine) in the maturation medium of *P. patula* tended to increase the production of ses and the later conversion of ses in plantlets (Malabadi and Van Staden, 2005). *P. halepensis* ses developed in culture media with MIX III (2,200 mg L^{-1} of Gln, 525 mg L^{-1} of Asn, 175 mg L^{-1} of L-Arg, 17.5 mg L^{-1} of Pro, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine, and 10 mg L^{-1} of L-alanine) were smaller and showed better germination rates (Figure 4C). These results are in agreement with those found in *Pinus strobus* L. where an increase in the concentration of Gln in the maturation medium for an embryogenic line resulted in an increase in the number of ses (Garin et al., 2000). Our findings show the important role of Gln in the maturation of *P. halepensis*, enhancing the germination of ses obtained from the maturation medium with a 4-fold increase in this amino acid concentration. This result coincides with some authors who report the use of Gln as one of the main sources of organic nitrogen for SE in *Plinia peruviana* Poir. Govaerts, *Pinus koraiensis* Siebold, and Zucc. and *Viola canescens* Wall. Ex. Roxb. (Silveira et al., 2020; Gao et al., 2021; Khajuria et al., 2021). In agreement with the improvements observed in the germination of *P. halepensis* ses, it was reported that the Gln also influenced the growth of proembryogenic masses in *Picea abies* L. Karst. (Carlsson et al., 2017) and *P. peruviana* (Silveira et al., 2020), increasing production of ses and consequent conversion to viable plants in date palm (ElDawayati et al., 2018), as well as induction of secondary ses in *Quercus suber* L. (Rahmouni et al., 2020).

In relation to the carbohydrate source in a maturation medium, our results showed a different response in *P. radiata* and *P. halepensis*. In this sense, production, germination, and acclimatization of *P. radiata* ses were better in the presence of sucrose than in maltose in agreement with results obtained in *Pinus pinaster* Ait. (Ramarosandratana et al., 2001; Klimaszewska et al., 2007). Although, in our results, we did not see an improvement in the NNE at high carbohydrate concentrations in *P. radiata*, in *P. strobus*, the increase in its concentration (350 mM) produced a high amount of ses (Garin et al., 2000). Unlike *P. radiata*, the presence of 175 mM of maltose in *P. halepensis* maturation media promoted an increase in the size of ses, with a significant improvement in their germination rates. In this sense, plantlets obtained from *P. halepensis* ses developed in maturation media

with 175 mM of maltose had an increase in the aerial part, and number of secondary roots and 100% of acclimatization was observed. Our results for *P. halepensis* are in agreement with those found in other *Pinus* species such as *P. patula* (Malabadi and Van Staden, 2005), *Pinus thunbergii* Parl. (Sun et al., 2021), and *Pinus elliottii* Engelm. (Yang et al., 2020).

Scott et al. (1995), studying the metabolism of maltose and sucrose in the embryogenesis of microspores isolated from *Hordeum vulgare* L., reported a higher assimilation of carbon in the culture medium, containing sucrose than in those containing maltose, probably because sucrose is well assimilated in both gymnosperms and angiosperms (Ameri et al., 2020; De Sousa et al., 2020; Peng et al., 2020; Shirin et al., 2020). However, different authors have shown controversial results; in this sense, Scott et al. (1995) associated the induction of embryogenesis with lower rates of substrate accumulation obtained from culture media containing maltose, and Carrion Pereira et al. (2019) described maltose as more efficient than sucrose to regenerate *Urochloa brizantha* cv. "Marandu" plants. Even when there are no reports describing morphological characteristics of ses with the concentration of maltose besides ours for *P. halepensis*, an increase in the production of the number of ses in *Pinus massoniana* Lamb. and *P. elliottii* has been reported (Yang et al., 2020; Xia et al., 2021). Similar to our results for *P. halepensis*, Salaj et al. (2019) reported that the presence of maltose in the maturation medium was essential for the formation ses with a consequent conversion of *P. nigra* plantlets.

In addition to morphological characteristics, the changes in the maturation medium also affected the PAs contents in germinated ses. PAs are extremely important for plants and are related to abiotic and biotic stress tolerance (Zou et al., 2021). Also, the PAs vary between species and the developmental stages of SE (Minocha et al., 1999; Silveira et al., 2004; Kuznetsov and Shevyakova, 2007; de Oliveira et al., 2020; Botini et al., 2021). In our work, the amount of PAs did not change with different treatments assayed in *P. radiata*. On the contrary, we observed that the amount of Put was more affected by the different treatments during the maturation stage than other PAs in *P. halepensis*. Moreover, this is the first report about the presence of endogenous Cad in *Pinus* spp. In this sense, concentrations of Cad were observed in the germinated ses obtained in both species and regardless of the treatments assayed. Cad is a lysine decomposition product, rare in

plants, and associated with the accumulation of other PAs (Liu et al., 2014; Jancewicz et al., 2016). In our studies, the presence of Cad could not be associated with worse acclimatization, although, in *Pinus sylvestris* L., the root formation decreased in the presence of exogenous Cad (Niemi et al., 2002). In *Arabidopsis thaliana* L. Heynh., the presence of exogenous Cad was associated with the alterations in the morphological characteristics that modulate plant development, as well as environmental stress responses (Liu et al., 2014). In addition, in the same species, Cad inhibited primary root growth, and it was related to skewing, waving, and lateral root formation (Strohm et al., 2015). Moreover, it is possible to study the relationship of the Cad content with future stress experiments in Pinus, knowing that the Cad can improve the tolerance of abiotic stress in several species with an overexpression of the genes responsible for stress tolerance (Rajpal and Tomar, 2020).

Furthermore, differences between PAs contents were reported as important in SE. The reduction in Put content and the increase in other PAs (Spd, Spm, and Cad) during SE enabled the correct development of ses; for this reason, those differences have been used as important biochemical parameters for the selection of cell lines with embryogenic potential in *A. angustifolia* and *P. radiata* (Minocha et al., 1999; Jo et al., 2014; de Oliveira et al., 2015). In this work, regardless of the treatment of amino acids mixture, we observed an increase of Cad in relation to Put and Spm, but not in relation to Spd in the germinated *P. radiata* ses. Also, levels of Put were the lowest of all PAs detected in germinated ses; a similar profile was reported by Mikula et al. (2021) in *Cyathea delgadii* Sternb. SE process in which profiles of Cad and Spd increased in relation to other PAs. In agreement with these results and in opposition to ours, Minocha et al. (1999) reported that the most abundant PAs during the development of zygotic and ses in *P. radiata* was Spd in relation to Put and Spm. In contrast to our results, in the maturation of *Picea glauca* (Moench) Voss, the amount of Put was higher than Spd (Kong et al., 1998).

In *P. halepensis*, in MIX I, there were higher amounts of Cad, Spd, and Spm than in the remaining combinations, although there were no significant differences. In this sense, *Vicia faba* L. was reported that foliar application with different concentrations of amino acids mixture [Arg (5.2–6.2%), Pro (2.23–3.5%), Tiroanine (3.05–3.56%), Aspartic acid

(3.2–3.45%), Serine (3.76–4.49%), Glutamic acid (7.24–9.12%), Lysine (1.87– 2.45%), Alanine (2.16–2.20%), Cysteine (1.87–2.45%), Valine (2.8–3.1%), Methionine (0.23–0.3%), Isoleucine (1.26–1.7%), Leucine (1.98–2.8%), Tyrosine (0.48–1.02%), Phenylalanine (1.03–1.78%), lysine (1.39–2.3%), and Histidine (0.42–0.9%)] improved the PAs contents in the plants grown under seawater salinity stress (Sadak and Abdelhamid, 2015). Similar levels of Put and the other PAs were observed in germinated ses of P. *halepensis* from a maturation medium with an increase of 350 mg L^{-1} of L-Arg content (MIX II). However, with 2,200 mg L^{-1} of Gln (MIX III) in the maturation medium, a decrease in Put was observed in the germinated ses for *P. halepensis*. Our results are in agreement with those reported by Nieves et al. (2008), which showed that the addition of Arg in the culture medium of *Saccharum* spp. is related to the changes in the amount of PAs, especially Put. The observed levels of Put in our results can be explained due to PAs derived from amino acids through decarboxylation, and the Put can be synthesized from the arginine (Bagni and Tassoni, 2001; de Oliveira et al., 2015). Furthermore, the Put is a direct substrate for Spd and Spm (Mustafavi et al., 2018). For example, in embryogenic masses of *P. sylvestris*, the highest detected PA was Put compared to Spd and Spm (Salo et al., 2016). High amounts of Put appeared both in embryogenic lines that produced ses and in those that did not produce ses, while Spd was directly associated with the embryogenic lines and was able to produce ses in *P. sylvestris* (Salo et al., 2016). In *P. nigra*, the increase in the total amounts of free PAs was related with a lower embryogenic potential (Noceda et al., 2009). Meanwhile, the Spm was crucial to regulate the stress responses in *A. thaliana*, *Rosa damascena* Mill., and *Citrus reticulata* L., among others (Yamaguchi et al., 2007; Shi et al., 2010; Hassan et al., 2018; Hasan et al., 2021).

4. CONCLUSION

An improvement in the germination of ses, conversion of ses in plantlets, and the acclimatization of plantlets were obtained in *P. radiata* and *P. halepensis* with alterations in the amino acid mixture or carbohydrate sources/concentrations in the maturation medium. Furthermore, changes in the maturation medium were not affected by the amount of PA levels in the ses of *P. radiata* but changed in *P. halepensis*. Cadaverine was detected in germinated ses of *P. radiata* and *P. halepensis*.

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GENERAL DISCUSSION

GENERAL DISCUSSION

This work provided knowledge about how changes in the physical and chemical environment during the maturation of ses of *Pinus* spp. can cause morphological, physiological and biochemical changes in the ses obtained, determining not only the success of the different stages of the process of the SE but also the performance of regenerated plants exposed to different stress conditions.

It was observed that the increase in temperatures during the maturation phase in *Pinus* spp. did not cause a reduction in the number of ses obtained. According to our results, Castander-Olarieta et al. (2019) and Pereira et al. (2020b) also reported that pulse-type treatments at higher temperatures (60 °C, 5 min) but early stages of SE, in the long-term, increased ses production in *P. radiata* and *P. halepensis*, respectively. Contrary to our work, Moncaleán et al. (2018) reported that 23 °C during the maturation stage in *P. radiata* caused a significantly higher number of ses when compared to a higher temperature (28 °C). Therefore, the application of high temperatures during the maturation process improves not only the quality but the quantity of ses obtained.

The application of high temperatures during the maturation phase also provoked delayed responses in the obtained *P. radiata* somatic plants, in accordance to the results reported in other conifer species (Sales et al., 2022). Although statistically significant differences were found in the membrane injury (*E.L.*) (Bajji et al., 2002) between the somatic plants obtained from EMs maturated at 23 and 50 °C, the *E.L.* values were low and the damage of cell membranes did not compromise the plants' response to drought.

Furthermore, the most tolerant six-month-old *P. radiata* somatic plants were those that came from higher maturation temperatures (40, 50 and 60 °C) at which they maintained their Ψ_{leaf}, and increased the g_s and *E* under water stress and heat stress. However, when fifteen-month-old *P. radiata* somatic plants obtained from EMs maturated at 50 °C were kept under water stress, they maintained Ψ_{leaf} while there was a decrease in *E*. These two adjustments in physiological parameters are characteristic of species that avoid stress, also called isohydric (McDowell et al., 2008). On the contrary, *P. radiata* somatic plants that were maturated at control temperature of maturation (23 °C) had a similar

adjustment of *E* but with a decrease in Ψ_{leaf} characterizing a lower plant tolerance to water stress (De Diego et al., 2012).

Analysis of DNA methylation studies of *P. radiata* somatic plants were carried out in order to validate the epigenetic memory hypothesis given its influence on stress response and adaptation (Sow et al., 2021; Sales et al., 2022). It was observed that the 5-mC content did not vary according to the applied stresses, either in the maturation phase or in the somatic plants. The 5-mC was correlated with numerous genes responsible for triggering tolerance responses to abiotic stresses and physiological responses (Zhao et al., 2022). In this work, for *P. radiata*, it was observed that control plants (coming from the maturation temperature of 23 °C and growing at 23 °C in the greenhouse) had a positive correlation between 5-mC and Ψ_{leaf} and *E* showing that the somatic plants with the highest methylation levels showed the highest expression of Ψ_{leaf} and *E*.

On the other hand, the content of 5-hmC, recently found in the genus *Pinus* (Castander-Olarieta et al., 2020; Pereira et al., 2021), varied with increasing temperature in the greenhouse in somatic plants from embryogenic masses matured at 23 °C. In this sense, Castander-Olarieta et al. (2021b) recently reported that the contents of 5-hmC are temperature dependent. Additionally, our results showed that the 5-hmC can actively participate in the stress response correlated with physiological parameters. Under stress conditions it was also observed that 5-hmC content had a negative correlation with Ψ_{leaf}. Furthermore, the increase in 5-hmC levels triggers a timely reduction of stomatal opening, helping to maintain tissue water level under drought, as 5-hmC had a negative correlation with g_S and *E*. All these data provide insights into the physiological and methylation responses affected by abiotic stress that help plants tolerate water and heat stress.

In addition to changes in temperature influencing the production of ses during the maturation phase, supplementation with different sources and concentrations of carbohydrate or amino acids in the maturation medium also caused changes in morphological and biochemical characteristics of the resulting ses. In this sense, Gao et al. (2022) reported that the changes of supplementation of carbohydrates (presence or absence of maltose) in the maturation medium also provoked an increase in the

methylation levels that led to an increase in the ses production of *Pseudotsuga gaussenii* Flous.

In *P. radiata* maturation, the medium with MIX II (1,100 mg L^{-1} of Gln, 1,050 mg L^{-1} of Asn, 350 mg L^{-1} of L-Arg, 35 mg L^{-1} of Pro, 19.75 mg L^{-1} of L-citrulline, 19 mg L^{-1} of L-ornithine, 13.75 mg L^{-1} of L-lysine, and 10 mg L^{-1} of L-alanine) led to an increase in the production of ses, in the germination of ses, in the diameter of the stem and in the number of secondary roots that led to a 100% acclimatization rate of the resulting plantlets. Likewise, the different amino acid mixture was effective in both ses induction and plantlet regeneration in many studies with angiosperms and gymnosperms (Stuart and Strickland, 1984; Ashok Kumar and Murthy, 2004; Malabadi and Van Staden, 2005; Montalbán et al., 2010; Nookaraju and Agrawal, 2013; Shashi and Bhat, 2021). On the other hand, in *P. halepensis* maturation, it was observed that the medium supplemented with MIX I led to an increase in the length and width of the ses but the medium supplemented with 4-fold of Gln concentration (MIX III) promoted a 60% acclimatization rate of the plantlets obtained. Similarly, many studies reported that the Gln was essential for the production of ses and conversion of plantlets in conifers (Carlsson et al., 2017; Rahmouni et al., 2020), as the Gln can participate in the synthesis of other amino acids, serves as a nitrogen transporter metabolite (Corredoira et al., 2019). Thus, it was observed that *P. radiata* and *P. halepensis* metabolized amino acids in different ways during the maturation stage and this has effect in the subsequent stages, since the balanced increase of several amino acids (Gln, Asn, L-Arg and Pro) increased the production of ses and conversion of plantlets in *P. radiata*, while the increase in Gln (MIX III) in the maturation medium increased the acclimatization of the *P. halepensis* plantlets.

Additionally, in relation to the carbohydrate sources, the increase in the osmolarity of the medium of *P. radiata* with sucrose caused an increase in the production, width, length and germination of ses. In addition, an increase in the germination and acclimatization rate of the resulting plantlets was observed for ses maturated in a medium with 175 mM of sucrose. Likewise, the high osmolality of the medium induced by sucrose (50 g L^{-1}) improved the maturation and the number of ses in other conifers species (Jiang et al., 2021). Moncaleán et al. (2018) also reported that lower water availability in the

maturation medium, in their case provoked by the supplementation of 10 g L^{-1} of gellan gum, caused a significant increase in the number of ses obtained for *P. radiata*. However, in *P. halepensis* maturation, it was observed that a lower osmolarity in culture media supplemented with 175 mM of maltose led to an increase in the length and width of the ses. In addition, the ses maturated in medium supplemented with 175 mM of maltose had an increase in the germination rate and 100% acclimatization rate of the plantlets obtained was observed. Similarly, the beneficial effect of maltose on the maturation of other conifer species has been previously reported (Li et al., 1998; Klimaszewska and Cyr, 2002; Santos et al., 2002; Ma et al., 2012). For example, in *P. taeda*, when sucrose was replaced by maltose (inferior to PEG as an osmotic agent) in the maturation medium, an increase in the frequency of maturation was reported, as well as an increase in the quality and quantity of the ses obtained (Li et al., 1998). Thus, it can be deduced that the addition of 175 mM of sucrose or 175 mM of maltose in the maturation medium of *P. radiata* or *P. halepensis*, respectively, represents a promising strategy to increase the quality and quantity of the ses obtained, and consequently, the success in acclimatizing of the plantlets obtained.

The PAs are significantly affected by the surrounding environment (Regla-Márquez et al., 2016). In addition, the accumulation of PAs in the SE has been related to different response mechanisms such as embryogenic competence (Giacomolli Polesi et al., 2022), endogenous hormones regulation (Lai et al., 2022), ses formation (Sundararajan et al., 2021), as well as plantlet regeneration (Aydin et al., 2016). In this study, it was observed that the level of PAs in germinated ses changed according to the amino acid mixture or carbohydrate sources and concentrations used in the maturation medium. In this sense, we observed for the first time in *Pinus* the presence of Cad in concentrations similar to Spd and Spm as a general trend. Although it is considered rare in plants (Jancewicz et al., 2016), in this work, Cad appears to be a common polyamine in *Pinus* spp. since it is present in both species, *P. radiata* and *P. halepensis*. Similarly, Cad was a common PAs in ses of *Capsicum chinense* Jacq. (Regla-Márquez et al., 2016) and *A. angustifolia* (De Oliveira et al., 2015). Cad is a diamine derived from lysine produced in the chloroplast stroma (Herminghaus et al., 1991), which under stress conditions plays the role of a stress signal

in the interaction between plant organs, but different from our results, its accumulation compensates for the decrease in the content of PAs in the Put family (Spd and Spm) in *Mesembryanthemum crystallinum* L. (Kuznetsov et al. 2002). On the other hand, Put was the polyamine with the lowest value observed among the PAs. In this regard, it has been described that the decrease in the Put accumulation in plants was compensated by Cad accumulation (Icekson et al., 1986; Shevyakova et al., 2001). However, for *P. halepensis*, the levels of the four PAs were similar for the germinated ses obtained in the medium with 175 mM of maltose, indicating that in this species a similar proportion of these PAs was essential for the improvement in the germination and acclimatization of the resulting plantlets.

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CONCLUSIONS

CONCLUSIONS

1. The application of high maturation temperature improved the quality and quantity of ses embryos of *Pinus* spp.

2. Plants obtained from EMs submitted to a maturation temperature of 40 and 60 °C, presented better adaptation to drought and heat stress based on the water and gas exchange parameters analyzed.

3. Plants obtained from EMs submitted to a maturation temperature of 50 °C (30 min) presented better adaptation to drought stress based on the $\Psi_{leaf\text{final}}$ and E_{final}.

4. Somatic plants kept at heat stress (40 °C, 10 days) in the greenhouse had lower 5-hmC levels than plants kept at 23 °C.

5. 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.

6. An improvement in the germination of ses, conversion of ses in plantlets, and the acclimatization of plantlets were obtained in *P. radiata* and *P. halepensis* with alterations in the amino acid mixture or carbohydrate sources/concentrations in the maturation medium (MIX II or 175 mM of sucrose for *P. radiata*, while MIX I or 175 mM of maltose for *P. halepensis*).

7. 175 mM of maltose promoted 100% acclimatization of the *P. halepensis* plantlets.

8. Furthermore, changes in the maturation medium did not affect the amount of PAs levels in the ses of *P. radiata* but changed in *P. halepensis*.

9. It is demonstrated for the first time the presence of Cad in germinated ses of *P. radiata* and *P. halepensis*.

Embriogénesis somática de *Pinus* spp. bajo condiciones de estrés abiótico: modelo para el estudio de los mecanismos que controlan la tolerancia

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