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GENETIC STABILITY, AMINO ACID AND POLYAMINE PROFILE ANALYSES IN RADIATA PINE SOMATIC EMBRYOS MATURATED AT HIGH TEMPERATURES

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Abstract:	Applying stress factors such as high temperatures during the different stages of somatic embryogenesis is either important interesting to uncover the molecular mechanisms involved in stress response and adaptation, and as a strategy to produce plants adapted to harsh environmental conditions derived from climate changes. In this sense, the present work aims to study the effect of high temperatures applied during maturation of somatic embryogenesis in the ploidy stability, the amino acid and polyamine profiles of the somatic embryos obtained and in the morphological characteristics of the somatic plantlets. The results revealed that the maturation temperature did not affect the morphology of the resulting somatic plantlets, neither the ploidy and genome size of phenotypically normal somatic embryos, whose ploidy and DNA content levels were similar to those found in mature zygotic embryos. Nonetheless, a slight but significant reduction of the genome size of aberrant somatic embryos was observed. Of the 21 amino acids detected significant differences depending on the maturation temperature were found for glycine, arginine, lysine and ornithine. These last three amino acids are precursors of the polyamines detected. Regarding this, putrescine levels were higher in somatic embryos from the highest maturation temperature (5 min pulse at 60 ºC), however the amount of this polyamine in all samples was much lower than spermidine, spermine and cadaverine. In conclusion, the different temperatures applied did not led to substantial changes in the ploidy level, endogenous PAs of the somatic embryos developed, or in the morphology of the somatic plantlets. Significant changes in the endogenous amino acids were observed, which may be linked not only to PAs metabolism but to another metabolic pathways involved in stress response.	
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GENETIC STABILITY, AMINO ACID AND POLYAMINE PROFILE ANALYSES IN RADIATA PINE SOMATIC EMBRYOS MATURATED AT HIGH TEMPERATURES.

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Abstract

Applying stress factors such as high temperatures during the different stages of somatic embryogenesis is either important interesting to uncover the molecular mechanisms involved in stress response and adaptation, and as a strategy to produce plants adapted to harsh environmental conditions derived from climate changes. In this sense, the present work aims to study the effect of high temperatures applied during maturation of somatic embryogenesis in the ploidy stability, the amino acid and polyamine profiles of the somatic embryos obtained and in the morphological characteristics of the somatic plantlets. The results revealed that the maturation temperature did not affect the morphology of the resulting somatic plantlets, neither the ploidy and genome size of phenotypically normal somatic embryos, whose ploidy and DNA content levels were similar to those found in mature zygotic embryos. Nonetheless, a slight but significant reduction of the genome size of aberrant somatic embryos was observed. Of the 21 amino acids detected significant differences depending on the maturation temperature were found for glycine, arginine, lysine and ornithine. These last three amino acids are precursors of the polyamines detected. Regarding this, putrescine levels were higher in somatic embryos from the highest maturation temperature (5 min pulse at 60 ºC), however the amount of this polyamine in all samples was much lower than spermidine, spermine and cadaverine. In conclusion, the different temperatures applied did not led to substantial changes in the

ploidy level, endogenous PAs of the somatic embryos developed, or in the morphology of the somatic plantlets. Significant changes in the endogenous amino acids were observed, which may be linked not only to PAs metabolism but to another metabolic pathways involved in stress response.

Keywords: aberrant somatic embryo; embryogenic cell line; *Pinus radiata*; ploidy; somatic embryogenesis; zygotic embryo.

Key message: Maturation temperature doesn´t affect ploidy levels of radiata pine somatic embryos, however, it affects the endogenous levels of glycine, arginine, lysine and putrescine.

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1. Introduction

Radiata or Monterey pine (*Pinus radiata* D. Don) is native to some locations in North America (the United States, Coast of California, Baja California and Mexico). 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. Currently, it is widely cultivated in New Zealand, Australia, Chile, South Africa and Spain. In Spain, plantations of *P. radiata* can be found in the Basque Country region representing 28% of the total wooded forest area, which are intended for forest timber productivity (about 80-85% of the annual timber logging) (HAZI, 2022).

In the present scenario of climate changes, breeding programs are focused on developing highly efficient propagation methods to obtain plants with better tolerance to drought and high temperatures (Da Ros et al. 2021). Clonal plant propagation allows the capture of elite-genotypes (Hazubska-Przybył et al. 2022), but in *Pinaceae* the superior character is only visible after reproductive stage, when the success of clonal propagation decreases (Imanuddin et al. 2020). Somatic embryogenesis (SE) associated to traditional techniques allows the implementation of multi-varietal forestry (MVF), incorporating tested tree varieties in a commercial forest (Park 2002; Montalbán et al. 2011). Furthermore, an important advantage of SE is that embryogenic tissue can be frozen in liquid nitrogen and stored at -80 ºC (Montalbán and Moncaleán 2017) until the corresponding field trials have been carried out. In this way, interesting genotypes can be selected, the cell lines can be thawed, and a clonal selected plant can be obtained again. In this sense, establishing cryobanks of embryogenic cell lines is advantageous, however these cryopreservation techniques require an analysis after the regeneration of the tissue to ensure genetic stability (Martínez et al. 2022).

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, which is genotype-, developmental stage-, explant- and transcription factor-dependent (Fehér 2019). In conifers, SE has five complex important stages (initiation, proliferation, maturation, germination and acclimatization *ex vitro*) (Montalbán et al. 2016). Maturation is influenced by many factors, such as the osmotic potential of the medium and the temperature (Teyssier et al. 2011; Moncaleán et al. 2018). In previous works carried out in our laboratory, significant differences were observed in the number of somatic embryos (*ses*) obtained in maturation process under different temperatures (Do Nascimento et al. 2020). Indeed, somatic plants obtained from embryonal masses maturated at 50 ºC showed better adaptation to drought stress based on water potential and transpiration (Do Nascimento et al. 2022). This approach is of great interest and could be used as a tool to produce plants with improved characteristics that could meet the actual demand of the agricultural and forestry sectors, which are drastically influenced by climate change.

Nonetheless, *in vitro* propagation techniques can cause loss of genetic homogeneity of the plants produced, and in some cases, morphologically abnormal somatic embryos or plantlets are obtained; this can occur for several reasons, among them, ploidy mutations (Kunitake et al. 1998; Borchert et al. 2007). Molecular analyses and flow cytometry have been used in many species to evaluate the trueness-to-type of plantlets regenerated via somatic embryogenesis (Konar et al. 2018; Nunes et al. 2018). However, the effect of the high temperatures exposure during SE on the genetic stability of the regenerated plants has not been tested yet.

Amino acids as well poliamines (PAs) play an important role in plant stress tolerance and morphogenesis and this has been discussed *in vitro* as well as *in vivo* plant development (Steffenon et al. 2020; Lando et al 2019). Proline have traditionally been linked with osmotic adjustment under stress conditions (De Diego et al. 2015). In this sense,*P. pinaster* somatic plants produced under different maturation temperatures not only had different proline basal contents, but they also showed significant differences in the levels of this amino acid under heat stress conditions (Sales et al. 2022). Similarly, experiments carried out in *P. radiata* with heat-primed embryonal masses suggested that other amino acids such as isoleucine could also be implicated in stress responses (Castander-Olarieta et al. 2019). On the other hand, PAs, small aliphatic amines, such as putrescine (Put), spermidine (Spd), spermine (Spm) and cadaverine (Cad), have as primary precursors ornithine, arginine or lysine and the different routes are species dependent (Kuznetsov et al. 2007).

PAs can play a regulatory role in the growth and development of plants, and they have been reported as metabolic hallmarks or interacting with other metabolic pathways in response to abiotic stress (Alcázar et al. 2020). In the case of arginine or ornithine derived PAs, they have been reported to be implicated in osmotic adjustment (Ozturk et al. 2021) or detoxification of reactive oxygen species (Seo et al. 2019). In the case of Cad, it also modulates plant development; however, when it comes to stress response, it has been reported either acting as a stress protectant (Tomar and Arora 2021) or exacerbating stress damage (Jancewicz et al. 2016). In both cases, plant development and stress response, a cross-talk between Cad and Put derived PAs has been suggested (Liu et al. 2014). Furthermore, PAs play important functions in the tolerance of plants to high temperature (Goyal and Ashtir, 2010).

In this sense, our hypothesis is that embryogenic cultures exposed to different temperatures during maturation could show differences in somatic embryo and plant development. To verify it, the effect of high temperatures during maturation stage of SE was evaluated on morphological characteristics of developed somatic plantlets. The analyses of the ploidy stability, amino acids and polyamines content are also presented and discussed in terms of how in vitro high temperatures can affect metabolism of *in vitro* somatic plant conversion.

2. Materials and Methods

2.1. Plant material and maturation experiment

The *ses* and somatic plants were obtained according to Do Nascimento et al. (2020). Briefly, immature female cones of *P. radiata* were collected from open-pollinated trees in a orchard established by Neiker-BRTA, Deba (Spain). Megagametophytes were isolated from seeds and used for initiation and proliferation of embryogenc cultures as described by Castander-Olarieta et al. (2022). Maturation experiments were carried out according to the procedure described in Do Nascimento et al. (2020), where embryogenic cultures were exposed to different temperatures (MT) and times (control 23 ºC for 16 weeks, 40 ºC for 90 min, 50 ºC for 30 min and 60 ºC for 5 min). After this, all cultures were kept in darkness at 23 ºC for 16 weeks, when the mature *ses* were germinated and the plantlets were acclimatized according to Montalbán and Moncaleán (2019). For the conversion to plantlets, the *ses* 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).

2.2. Ploidy assessment by flow cytometry

First, the ploidy level of fresh *ses* versus *ses* stored at -80 ºC was evaluated. For this purpose, a pool of fresh normal phenotype mature ses and a pool of fresh aberrant phenotype ses were compared with a pool of normal ses and a pool of aberrant *ses* stored at -80°C for 8 weeks, each pool included *ses* from 3 different embryogenic cell lines (ECLs).

Then, normal phenotype and aberrant phenotype *ses* obtained from three ECLs subjected to four MT (23, 40, 50 and 60 °C) were analyzed; based on results from the abovementioned experiment, the *ses* were stored at -80 ºC for eight weeks; also, as control, zygotic embryos from the same mother trees used to generate the ECLs were studied. Nuclear DNA content analysis of *ses* was conducted as described above..

For the preparation of nuclear suspensions, 2-4 embryos per sample (approximately 10 mg) were extracted in 1 ml of Woody Plant Buffer (Loureiro et al. 2007) together with young leafs of *Vicia faba* (2C=26.9 pg). The nuclei were released by making rapid cuts in the tissues with a scalpel blade for approximately 15 seconds. The resulting extract was filtered to remove large debris (CellTrick® 50 µm nylon filter) and 0.5 ml of the filtrate was transferred to an Eppendorf vial. Nuclear suspensions were treated with 50 µl/ml of RNAase (Sigma, St.Louis, USA) and stained with 50 µl/ml of the nuclear counterstain propidium iodine (PI) (Fluka). Once labelled, the suspensions were vortexed for 2 seconds and incubated on ice for 2 min before analysis.

Nuclei suspensions were analyzed in a CytoFLEX flow cytometer (Beckman Coulter) according to Martínez et al. (2022). In brief, samples were injected at 60 µl/min and excited with a blue (488 nm) diode

laser. Forward light scattering signal (FSC) and PI fluorescence emission (588 nm±42nm) were acquired and analyzed using CytExpert 2.1 Software (Beckman Coulter).

Intact nuclei were distinguished from other fluorescent signals (e.g., partial nuclei and other debris) by plotting PE-fluorescence versus FSC and doublets were discarded in FSC-area versus FSC-high graphs. Thus, the nuclei populations were plotted in PE-fluorescence histograms and ploidy level examined from Go/G1 peak patterns. Mean fluorescence emission and coefficient of variation (CV) were calculated for each identified peak. A minimum of 2000 nuclei were analyzed per sample.

The DNA index and the nuclear DNA content were calculated from the relative position of the G0/G1 peaks of embryos and internal reference material, applying the following equations:

DNA Index (DI) = [(F G0/G1 of the sample)]/ [(F G0/G1 reference)]

F: Average population fluorescence

Nuclear DNA content (2C): = Sample DI x reference genome size (*V. faba* 2C = 26.9 pg; 1 pg = 978 Mbp).

2.3. Free amino acids content determination

Amino acid quantification was carried out according to Astarita et al. (2003), with some modifications. The *ses* (200 mg) from five ECLs maturated at the different MT were grinded in 6 mL of ethanol 80% (v/v) and concentrated in a SpeedVac freeze dryer (45°C). After that, ultrapure water was added to resuspend the samples in a total of 2 mL, followed by centrifugation (15000 g, 10 min, 4°C). The supernatant was collected and stored in -20°C freezer for future analysis. Amino acids were derivatized with o-phthaldialdehyde in a borate buffer (400 mM pH 9.5 adjusted with NaOH), filtered with 0.45 µm membrane and submitted to high-performance liquid chromatography (HPLC) quantification. Mobile phases included A) buffered (50mM of sodium acetate and 50mM sodium phosphate) methanol/tetrahydrofuran/water 2/2/100 (v/v/v) pH 8.1 adjusted with acetic acid and B) methanol/water 35/65 (v/v). The gradient started at 20% B until 32 min, changed to 100% B until 71 min, was maintained in 100% B until 93.5 min, when it was returned to 20% B for reconditioning to the next injection until 106 min. The flow rate was constantly 1 $mLmin^{-1}$ and oven temperature were maintained at 40°C. Stationary phase was a Luna 5 µm C18 100 Å column with 250x4.6 mm equipped with a pre-column, both from Phenomenex (Allcrom, Brasil). The analyses were carried out in a Shimadzu Prominence HPLC equipped with a fluorescent detector calibrated to excitation of 250 nm and emission of 480 nm wavelengths. To determine amino acids concentration, peak areas of 20 µL samples were compared to peak areas of correspondent amino acid standards, all in triplicates.

2.4. Free polyamines content determination

The *ses* obtained from five ECLs maturated at the different MT were analyzed for free polyamine content for *P. radiata*. Polyamine (PA) quantification was carried out according to Silveira et al. (2004) with modifications. Samples (200 mg of fresh weight) were briefly grounded in 1.4 mL of 5% perchloric acid (v/v) in Precellys® shaker. After 1 h, the extraction solution was centrifuged for 20 min (15000 *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 (15000 *g*, 4 °C). Both extraction solutions were merged, homogenized and frozen at -20°C for future analyses. Derivatization was performed according to Silveira et al. (2004), where 40 µL

of each sample were 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 organic phase with polyamines was taken to a SpeedVac freeze dryer for 40 min at 40 °C. Finally, pellets were suspended in 175 µL of acetonitrile, filtered with 0.45 µm membrane, and then subjected to 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 until 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 $mLmin^{-1}$ and oven temperature was maintained at 40 °C. Stationary phase was a 5 µm Shim-pack CLC-ODS(M) 100Å column with 250x4.6 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 Pas levels 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.

2.5. Morphological characteristics

At the end of germination experiment the number of needles and secondary roots were counted in eight plantlets from six established cell lines (ECLs) subjected at different MT (three treatments and the control). Length of the plantlets, length of the aerial part, width of needles, stem diameter, and length of primary root were measured in mm using a digital caliber (Fowler High Precision).

2.6. Data collection and statistical analysis

To determine if there were differences in the nuclear content between the samples at -80 ºC versus the fresh samples an analysis of variance (ANOVA) was carried out for normal and aberrant *ses*. ANOVA was also performed to determine if there were differences between normal and aberrant *ses* from different MT.
Data on amino acid and polyamine contents were subjected to ANOVA to elucidate if the MT or the ECLs tested influence these contents. This analysis was also performed to compare the contents of the different PAs, in this case, data were log(x) transformed to meet homocedasticity. Finally, ANOVA was conducted to assess the effect of MT on the morphological characteristics of somatic plantlets. Data on stem diameter were log transformed and data on number of roots were square root transformed to meet homocedasticity. When necessary, Tukey´s post-hoc test was performed to determine differences between groups.

3. Results

3.1. Ploidy assessment by flow cytometry

The degree of ploidy was determined by comparing the position and number of peaks appearing in the PE fluorescence histogram.
Fresh and frozen *ses* showed an identical pattern, with two fluorescence peaks occupying an invariable relative position in the histogram (Fig. 1). The first corresponded to nuclei in Go/G1 phase of the internal

standard (*V. faba*) and the second to G0/G1 nuclei of *P. radiata*. The existence of a single population of *P. radiata* nuclei in a stable position with respect to the standard indicated that all the embryos analyzed were diploid (2C) and guaranteed genetic stability at this level for the entire set of material analysed.

Both the nuclear DNA content (estimated from the DI) and the corresponding CVs were similar in fresh and frozen embryos (Table 1). Statistical analysis confirmed that there were no significant differences between both types of material in normal and aberrant *ses* (Supplementary material, Table S1).

Table 1 DNA index and nuclear genome size in fresh normal and aberrant phenotype somatic embryos and in normal and aberrant somatic embryos stored at -80ºC for eight weeks. Mean ± S.E.

Storage	Morphology	DNA content (pg/2C)	CV (%)	DNA index
Fresh	Normal	49.57 ± 0.71	4.45	1.84 ± 0.03
-80 ºC	Normal	49.64 ± 0.70	4.35	1.85 ± 0.03
Fresh	Aberrant	49.11 ± 0.46	4.09	1.82 ± 0.02
-80 ºC	Aberrant	48.40 ± 0.22	4.17	1.80 ± 0.01

When the ploidy level of embryos subjected to different MT treatments was assessed, no differences related to morphology or MT were observed in the PE-fluorescence cytograms: the peak pattern of samples was identical to that shown in Fig. 1 (see Figure S1) and consistent with a 2C genome (Fig. S1). The same pattern was observed for zygotic embryos (Supplementary Material, Fig. S2).

For normal ses, the DI ranged between 1.84 and 1.87, which indicates that the genome size of the ses from the four MT tested ranged from 49.48 to 50.32 pg/2C (Table 2). These values were similar to those recorded for zygotic embryos, being the average DI and DNA content 1.85 and 49.81, respectively. In the case aberrant ses, the DI oscillated between 1.74 and 1.81 and the relative size of the genome in the range of 46.85-48.77 pg/ 2C (Table 2). Statistically, no significant effect of the MT or the interaction between MT and morphology was observed. Following this analysis significant differences were found for the morphology of the ses, however when the effect of morphology was evaluated within each MT, the statistical analysis revealed a significant reduction in the genome size of aberrant embryos compared to normal ones when MT was 40 ºC and no statistically significant differences for the rest of MT (Supplementary material, Tables S2 and S3).

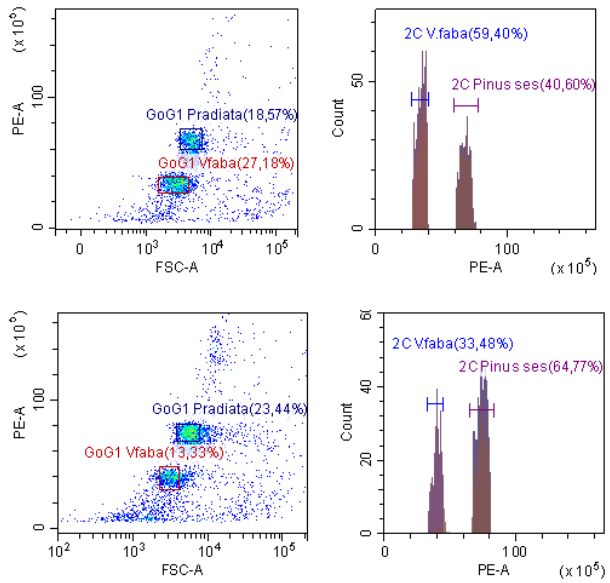

Fig. 1 Flow cytometry analyses to evaluate the genetic stability between morphologically normal fresh and frozen *Pinus radiata* somatic embryos. The 2C nuclei populations at G0G1 corresponding to *P. radiata* and *Vicia faba* (internal standard) are shown in the forward scatter (FSC) vs. PE (fluorescence) cytogram and in the PE histogram. In the upper graphs fresh embryos are shown, the lower graphs show frozen embryos.

Table 2 DNA content (pg/2C; M ± SE), coefficient of variation (CV, %) and DNA index (M ± SE) in normal and aberrant somatic embryos of *Pinus radiata* from different maturation temperatures (23, 40, 50 and 60 ºC) and in zygotic embryos from the same mother trees of the ECLs used in this study.

Temperature (ºC)	Morphology	DNA content (pg/2C)	CV (%)	DNA index
23	Normal	49.64 ± 0.70	4.35	1.85 ± 0.03
40	Normal	50.32 ± 0.79	4.56	1.87 ± 0.03
50	Normal	50.05 ± 0.67	4.59	1.86 ± 0.02
60	Normal	49.48 ± 0.50	4.27	1.84 ± 0.02
23	Aberrant	48.40 ± 0.22	4.17	1.80 ± 0.01
40	Aberrant	46.85 ± 0.99	4.65	1.74 ± 0.04
50	Aberrant	48.77 ± 0.92	4.38	1.81 ± 0.03
60	Aberrant	48.42 ± 0.22	4.42	1.80 ± 0.01
Mature Zygotic embryos		49.81 ± 0.52	5.87	1.85 ± 0.02

3.2. Free amino acids

Among all the amino acids analyzed, the following did not present significant differences regardless of the MT: alanine, GABA, glutamine, histidine, methionine, phenylalanine, tyrosine, tryptophane, valine (above

90 µg/g FW), aspartate, citrulline, isoleucine, serine, threonine (between 120 and 400 µg/g FW), asparagine, glutamate and leucine (above 410 µg/g FW), (Supplementary material Table S5).

In the case of arginine, glycine, lysine, and ornithine significant differences were found depending on the MT (Table 3). Diverse patterns could be observed for these amino acid contents. Arginine and ornithine contents were significantly higher in *ses* from MT 50°C than in *ses* from MT 23 and 60 ºC. For glycine, *ses* maturated at 40°C and 60°C showed significantly higher values than those maturated at 23°C. The contents of lysine showed high variability. Due to this, despite high contents of this amino acid were found in *ses* from MT 60 ºC, the Tukey post hoc test did not detect differences between MT (Table 3).

Table 3 Free amino acids (µg/g FW) in *Pinus radiata* D. Don somatic embryos maturated under different maturation temperatures (23 °C, 12 weeks; 40 °C, 4 h; 50 °C, 30 min; 60 °C, 5 min).

Amino acids	**Temperature of maturation (°C)**			
(µg/g FW)	**23**	**40**	**50**	**60**
Alanine	14.31 ± 2.99 ^{a}	16.10 ± 2.36 ^{a}	15.60 ± 0.45 ^{a}	13.47 ± 2.25 ^{a}
Arginine	46.32 ± 0.07 ^{bc}	54.17 ± 2.81 ^{ab}	56.34 ± 3.29 ^{a}	24.62 ± 0.39 ^{c}
Asparagine	497.11 ± 11.86 ^{a}	419.37 ± 97.71 ^{a}	675.50 ± 6.24 ^{a}	653.74 ± 97.94 ^{a}
Aspartate	197.00 ± 27.41 ^{a}	242.95 ± 53.78 ^{a}	218.34 ± 17.04 ^{a}	238.98 ± 49.16 ^{a}
Citrulline	206.22 ± 88.70 ^{a}	325.75 ± 108.73 ^{a}	288.14 ± 29.42 ^{a}	332.90 ± 51.51 ^{a}
GABA	38.60 ± 10.83 ^{a}	56.96 ± 18.82^{a}	38.95 ± 3.28 ^{a}	37.80 ± 10.09 ^{a}
Glutamine	83.76 ± 23.50 ^{a}	65.75 ± 10.81 ^{a}	74.46 ± 11.59 ^{a}	71.90 ± 13.33 ^{a}
Glutamate	517.48 ± 87.90 ^{a}	560.45 ± 54.77 ^{a}	494.36 ± 82.85 ^{a}	558.99 ± 95.27 ^{a}
Glycine	66.45 ± 12.03 ^{b}	266.81 ± 35.51 ^{a}	165.94 ± 15.22 ^{ab}	202.20 ± 21.09 ^{a}
Histidine	20.13 ± 2.27 ^{a}	24.83 ± 4.75 ^{a}	29.49 ± 4.86 ^{a}	22.71 ± 4.19 ^{a}
Isoleucine	386.31 ± 42.19 ^{a}	397.85 ± 47.37 ^{a}	381.20 ± 8.90 ^{a}	368.47 ± 60.71 ^{a}
Leucine	560.20 ± 39.19 ^{a}	647.90 ± 70.29 ^{a}	581.21 ± 62.94 ^{a}	677.41 ± 146.36 ^{a}
Lysine	174.20 ± 19.32 ^{a}	135.50 ± 14.30 ^{a}	107.61 ± 21.33 ^{a}	413.53 ± 134.61 ^{a}
Methionine	39.47 ± 6.37 ^{a}	42.28 ± 7.04 ^{a}	39.86 ± 3.72 ^{a}	38.21 ± 7.75 ^{a}
Ornithine	46.16 ± 7.79 ^{b}	86.92 ± 5.36 ^{ab}	113.69 ± 7.38 ^{a}	58.18 ± 19.28 ^{b}
Phenylalanine	23.40 ± 3.92 ^{a}	19.48 ± 2.62 ^{a}	20.30 ± 2.64 ^{a}	25.28 ± 0.80 ^{a}
Serine	317.75 ± 38.90 ^{a}	315.45 ± 78.96 ^{a}	304.67 ± 7.03 ^{a}	315.77 ± 36.17 ^{a}
Tyrosine	9.51 ± 1.68 ^{a}	9.42 ± 2.19 ^{a}	10.82 ± 2.78 ^{a}	7.23 ± 1.09 ^{a}
Threonine	137.23 ± 9.98 ^{a}	129.12 ± 13.91 ^{a}	137.20 ± 13.17 ^{a}	130.90 ± 15.57 ^{a}
Tryptophane	5.92 ± 0.81 ^{a}	7.19 ± 1.46 ^{a}	7.20 ± 1.45 ^{a}	6.88 ± 1.99 ^{a}
Valine	10.23 ± 3.01 ^{a}	12.24 ± 4.05 ^{a}	13.68 ± 2.74 ^{a}	12.24 ± 3.19 ^{a}

Data are presented as mean values ± SE. Significant differences within a line at $p<0.05$ are indicated by different letters.

3.3. Free polyamines

Four PAs types were detected in radiata pine *ses*: Put, Spd, Spm and Cad. A significantly higher content of Spd than of Spm was detected, and a significantly higher content of the latter than of Cad. The Put content was significantly lower than the rest, being two orders of magnitude below the Spd content (Fig. 2, Supplementary material Table S6).

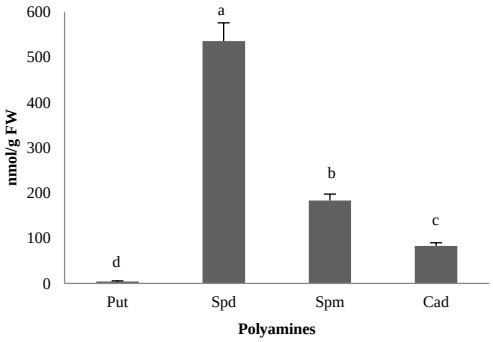

Fig. 2 Putrescine (Put), Spermidine (Spd), Spermine (Spm) and Cadaverine (Cad) contents (mean +S.E.) in *Pinus radiata* D. Don somatic embryos. Significant differences at $p<0.05$ are indicated by different letters.

When the effect of temperature on each polyamine content was analyzed, significant differences were only found for Put (Supplementary material, Table S7). Somatic embryos submitted to 60 ºC had a significantly higher Put content than those maturated at the other temperatures tested (Fig. 3).

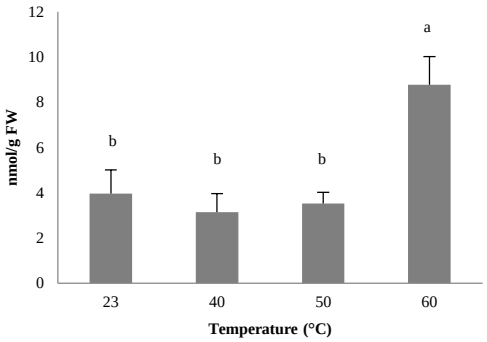

Fig. 3 The effect of maturation temperature (23 °C, 12 weeks; 40 °C, 4 h; 50 °C, 30 min; 60 °C, 5 min) on putrescine content (mean + S.E.) of *Pinus radiata* D. Don somatic embryos. Significant differences at $p<0.05$ are indicated by different letters.

3.4. Morphological characterization of plantlets

The MT did not have a significant effect on any of the morphological parameters measured (Supplementary material, Table S8). Significant differences were only found between the ECLs for the following parameters: number of needles, length of aerial part, stem diameter and number of roots. Three genotypes presented the highest values (R2, R16 and R49) and one genotype the lowest ones (R130) for these parameters; the other two (R9 and R138) showed intermediate to low values depending on the parameter (Table 4). A significant interaction between the ECL and the MT was observed for the number of roots, however the ECLs showed the trend mentioned above (Supplementary material, Fig. S3).

The length of the plantlets (from 54 to 90 mm), the width of the needles (from 0.23 to 0.51 mm) and the length of the roots (from 26 to 51mm) did not present significant differences between MT treatments or ECLs (Supplementary material, Table S9).

Table 4 Morphological characteristics (mean ± S.E) in different embryogenic cell lines (ECLs). Different letters within a column indicate significant differences by Tukey´s post hoc test.

ECLs	Number of needles	Length of aerial part	Stem diameter	Number of roots
R2	24.41 ± 0.81 a	35.12 ± 1.59 a	1.33 ± 0.05 ab	4.31 ± 0.53 ab
R9	21.53 ± 1.03 abc	30.96 ± 1.27 ab	1.23 ± 0.06 bc	1.69 ± 0.37 c
R16	20.22 ± 1.10 abc	33.85 ± 1.82 a	1.54 ± 0.08 a	4.72 ± 0.73 ab
R49	22.94 ± 0.98 ab	36.46 ± 2.22 a	1.33 ± 0.06 ab	6.72 ± 0.79 a
R130	18.09 ± 1.19 c	26.05 ± 1.81 b	1.07 ± 0.05 c	2.41 ± 0.68 c
R138	19.66 ± 1.01 bc	31.91 ± 2.53 ab	1.20 ± 0.07 bc	3.78 ± 0.74 bc

4. Discussion

In this work the impact of different temperatures treatments (23 °C, 12 weeks; 40 °C, 4 h; 50 °C, 30 min; 60 °C, 5 min) on SE maturation stage has been studied. First, the ploidy level in somatic embryos obtained at these temperatures was assessed; all the normal *ses* analysed corresponded to diploid material. The aberrant *ses* show similar ploidy levels, however those from MT 40 ºC had significantly lower DNA content; these differences being also observed between *P. pinaster* normal and aberrant *ses* (Marum et al. 2009). As reported by these authors, being these differences below 3%, polyploidization does not appear to have occurred during SE process; however, the possibility of aneuploidy could not be totally excluded. In the present study as control, the ploidy levels of zygotic embryos from the same mother trees of the ses was checked. The average values for the DNA content and the DNA index were similar to those found in our *ses* and slightly superior to those reported by O´Brien et al. (1996) and comparable to the results by Wakamiya et al. (1993).

From the 21 amino acids detected, differences in their content depending on the MT were found for only four of them: arginine, glycine, lysine and ornithine. These amino acids were reported to have an important role as nitrogen reserve in *Picea obovata* and *Pinus sylvestris* buds. Castander-Olarieta et al. (2019) found significant differences for another four amino acids (isoleucine, leucine, histidine and tyrosine) and observed changes at the proteome level for enzymes involved in the synthesis of isoleucine (Castander-Olarieta et al. 2022) when similar temperature pulses were applied at SE initiation stage. Our amino acid average values were much higher (twice to fifty times higher, depending on the amino acid) than those reported in the abovementioned study. This may be due to the fact that in both cases, the data were given in fresh weight and ECLs are mostly water (around 10% dry weight, Peng et al. 2020) whereas *ses* have dry weight values around 30% (data not shown). If compared with other studies carried out in our laboratory with radiata pine somatic plantlets after six weeks of germination, the amino acid profiles vary considerably (Castander-Olarieta et al. 2023). In that work it was shown that arginine is one of the most abundant free amino acids, contrasting with the results obtained at embryo level. This is in accordance with Cañas et al. (2006), where they observed that storage proteins found in pine embryos are rich in arginine, which is released and found at high levels as free forms after germination.

It is known that the metabolite profiles vary along maturation progression in somatic end zygotic embryogenesis (Morel et al. 2014), however it is also proven that the environmental conditions such as temperature can have an impact in these profiles (Pereira et al. 2023). In this sense, glycine was present at significantly higher concentrations in ses from MT 40 and 60 ºC when compared with the control in the present study. This result is opposite to those reported by Pereira et al. (2023) for the same amino acid and culture temperatures in *P. halepensis* ECLs.

Lysine was also present in a higher amount in *ses* from MT 60 ºC. In *P. obovata* buds its contents were related to a cryoprotectant role for cell membranes (Alaudinova and Mironov 2018), in addition, some catabolites of this amino acid are implicated in osmoprotection in bacteria and plants (Tomar et al. 2013). Apart from this, lysine decarboxylation results in Cad (Jancewicz et al. 2016). Cadaverine´s catabolism leads to the synthesis of some alkaloids involved in plant protection against biotic stresses (Jancewicz et al. 2016). Regarding abiotic stress, Kuznetsov et al. (2002) suggested that together with ethylene, it could be involved in the long distant translocation of stress signal in plants. However, despite the higher accumulation of lysine in *ses* from the highest MT, the Cad content in *ses* did not vary with temperature. This diamine has also been reported to be related to seed´s germination. The levels found in mature *ses* were lower than those described by Do Nascimento et al. (2021) in somatic plantlets (after two weeks of germination) from the same species; this would be in accordance with Shalaby (2000), who reported an increase in Cad content during germination in legume seeds.

In the case of arginine and ornithine, the highest values were obtained at MT 50 ºC. These two amino acids are the precursors of Put, which is produced directly from ornithine by ornithine decarboxylase or indirectly from arginine by arginine decarboxylase via agmatine (Kumar et al. 1997). Arginine and ornithine together with Put and Spd have been reported to be stress-related metabolites in many plant species, with several roles, in the case of these amino acids, in cellular cultures submitted to stress they can serve as substrate for Put biosynthesis; but also, as suggested by Liebsch et al. (2022) may represent a pool of reduced carbon that can feed the tri-carboxylic acid cycle. Matsunaga et al. (2021) also reported in *Triticum aestivum* that depending on the growth stage of the plant the amino acids accumulated during heat stress were different, these authors also observed an accumulation of arginine and linked it to recovery from stress due to their involvement in the urea cycle (and detoxification of NH_4^+), as other authors have postulated for ornithine (Blume et al. 2019).

Polyamines, specifically Put, Spd, Spm and Cad have demonstrated a fundamental role during seed formation and development of somatic embryos (De Oliveira et al. 2015) and contribute to the accumulation of reserve substances, particularly proteins and triglycerides, which are then used during embryonic germination (Baron and Stasolla 2008). In *P. radiata*, Cad has been detected for the first time in our laboratory by Do Nascimento et al. (2021). Changes in the profile of PAs have been detected along the process of SE in *Picea abies* (Serapiglia et al. 2008), *P. rubens* (Minocha et al. 1993), *Pinus taeda* (Silveira et al. 2004), and *Araucaria angustifolia* (Steiner et al., 2007). Even, it has been demonstrated the improvement of SE with the exogenous applications of PAs (Dutra et al. 2013). Do Nascimento et al. (2020) found that the highest temperatures applied during maturation in this experiment (60°C, 5 min) led to a significantly lower number of *ses*. Interestingly, the same *ses* showed the highest Put levels, which follows the same pattern described by Jo et al. (2014), where they correlated high Put levels in embryonal masses

of *Araucaria angustifolia* with low embryogenic capacity. In pines, however, Peng et al. (2022) showed that cell lines with no embryogenic capacity, or lost embryogenic capacity due to aging, presented lower Put contents than highly productive cell lines. As a result, it would be interesting to confirm whether the increased profile of Put in *ses* maturated at higher temperatures is a result of higher levels of this polyamine in embryonal masses, and if this is the cause of lower embryo production rates.

Besides, Put, Spd and Spm are the three most common PAs implicated in the response to abiotic stress (Yang et al., 2007). These authors observed high levels of Put at an early stage of drought stress, followed later by an accumulation of Spd and Spm in drought-resistant cultivars of rice. Rajpal and Tomar (2020) pointed out that in many cases, just one of the three polyamines showed an apparent enhancement. In our study, Put showed significant higher values in ses from MT 60 ºC. It must be noted in one hand that the *ses* were analysed 16 weeks later from the stress application and on the other hand, that the Put content in samples from all MT was very small when compared with Spd or Spm levels. In *Picea rubens* and *P. abies ses* a higher proportion of these two PAs was also observed (Minocha et al. 2004, Fischerova et al. 2022). In radiata pine (Minocha et al 1999) observed that the content of Spd and Put tend to equal at late stages of maturation but in none of these species the Put content was as low as that observed in our samples. On the contrary, in previous studies carried out in *P. radiata* SE at control temperature (23 ºC), Cad and Spm, showed significant differences with the levels of Spm and Put (Do Nascimento et al. 2021).

Morphological features were not affected by temperature treatments, only genotype influenced some features; contrary, in previous experiments Castander-Olarieta et al. (2019) showed that different initiation temperatures can modify both the size and the shape of the somatic embryos obtained later during maturation in radiata pine. Similarly, Do Nascimento et al. (2021) observed that the supplementation of maturation medium with different amino acids or carbohydrates led to significant differences in some parameters of the somatic plantlets as the stem diameter or the number of secondary roots.

In conclusion, the different temperatures applied did not led to substantial changes in the ploidy level, endogenous PAs of the *ses*, or in the morphology of the somatic plantlets. Significant changes in the endogenous amino acids were observed, which may be linked not only to PAs metabolism but to another metabolic pathways involved in stress response. Further research is needed to confirm this latter hypothesis together with physiological analyses in the resulting plants.

Authors Contributions: Conceptualization, IAM, PM; Ploidy analyses: ACO, AMMN, SSA, AH; amino acid and polyamine analyses; LGP, NS, MPG; statistical analyses: IAM, AMM; writing and original draft preparation: IAM, ACO, PM; visualization and resources: ACO, AMM, SSA; funding acquisition: PM. All authors revised and agreed the final version of the manuscript.

Declarations

The authors have no competing interests to declare that are relevant to the content of this article.

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Key message: Maturation temperature doesn´t affect ploidy levels of radiata pine somatic embryos, however, it affects the endogenous levels of glycine, arginine, lysine and putrescine.

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