

Different environmental conditions at initiation of radiata pine somatic embryogenesis determine the protein profile of somatic embryos

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Abstract Somatic embryogenesis is an important biotechnological tool in breeding and conservation programmes for woody species. It exists an increasing interest in the improvement of somatic embryogenesis induction in commercial species such as *Pinus radiata*. One approach to obtain markers of successful somatic embryogenesis can be the study of protein profiling in a descriptive study. In order to accomplish this objective, 2-D and MALDI-TOF/TOF-MS analysis were used in the identification and quantification of proteins from *Pinus radiata* D. Don somatic embryos derived from 4 cell lines that were generated under different environmental conditions at initiation stage. Based on the Swiss-Prot database, among the 139 proteins detected, 25 of them appeared only in those cell lines with low initiation percentage at the initial stage of somatic embryogenesis. Only two proteins were detected differentially in cell lines with high initiation rates. Approximately 60% of the proteins identified were related to carbohydrate metabolic process and defence response. This study gives new insights about the proteomics in forest trees, as well as provides clues on the underlying causes of the mechanisms governing *Pinus radiata* somatic embryogenesis process, and can serve as a tool for facing the improvement of the breeding programmes.

Key words: embryogenic potential, MALDI-TOF/TOF-MS, *Pinus radiata*, tree proteomics, 2-D analysis.

Pinus radiata is one of the most economically important forest tree species in the world and it is one of the most cultivated species in the north of Spain, especially in the Basque Country (De Diego et al. 2012). Biotechnology offers new opportunities for improving selection efficiency (balancing genetic gain and genetic diversity), conservation and deployment of new varieties for reforestation. Propagation via somatic embryogenesis (SE) is an effective method in propagating elite plants when combined with other technologies, such as cryopreserving the embryogenic tissue and selecting elite clones in field tests (Santa-Catarina et al. 2012). This system offers the capability to produce unlimited numbers of somatic plantlets (Park 2002) and manufactured seeds (Gupta and Hartle 2015). SE in *P. radiata* was first described by Smith et al. (1994). Lately, in our laboratory, there have been some improvements in different aspects of the process such as initiation (Montalbán et al. 2012), maturation (Montalbán et al. 2010), cryopreservation (Hargreaves et al. 2002) and

organogenesis/SE combined protocols (Montalbán et al. 2011). In this sense, the establishment of a successful SE protocol requires the development of molecular markers that can be used to select cell lines with highly embryogenic potential (Jo et al. 2014; Schlögl et al. 2012).

A deeper understanding of the biological factors influencing conifer SE culture stability and productivity is being actively pursued (Brownfield et al. 2007; Cairney and Pullman 2007; Joy et al. 1997; Silveira et al. 2004a, b; Stasolla et al. 2002) and, in combination with molecular markers, promises to guide the species and genotype-specific refinement of conifer SE tissue handling techniques to promote embryogenic vigour (Robinson et al. 2009). One approach to generate such markers and to better understand the regulation of in vitro embryogenesis is to use global transcript expression or protein profiling (Lippert et al. 2005; Zhao et al. 2015). Indeed, proteome profiling has been successfully applied to the systematic analysis of protein expression in several broad-leaf (Correia et al. 2016) and conifer

Abbreviations: HI, Embryogenic cell lines from treatments producing high percentages of initiation; LI, embryogenic cell lines from treatments producing low percentages of initiation; ECLs, Embryogenic cell lines; EDM, Embryo development medium; EMs, Embryogenic masses; SE, Somatic embryogenesis.

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species (Teyssier et al. 2011) during the different stages of the development of the somatic embryos. Comparative studies using proteomics to find markers have been carried out by several authors for different aspects of the SE process (Correia et al. 2012; Morel et al. 2014; Teyssier et al. 2011; Vale et al. 2014). Proteomic approaches have a great potential to study non-model species, because protein sequences have the advantage of being more conserved, making the high-throughput identification of non-model gene products quite effective by comparison to orthologous proteins (Liska and Shevchenko 2003).

After embryogenic tissue induction, modifications in the patterns of gene expression must occur to redirect cells to a new developmental process (Morel et al. 2014). This event results in the synthesis of new mRNAs and proteins specific to the different stages through which embryo development proceeds (Chugh and Khurana 2002). In conifers, some efforts have recently been made to describe SE development at the molecular level (Jo et al. 2014) and differences in proteins abundance between cell lines with contrasting embryogenic potential have been found (dos Santos et al. 2016). Proteomic studies combining two-dimensional (2D) gel electrophoresis with a high degree of accuracy in protein identification by mass spectrometry (MS), are available for *Cupressus sempervirens* (Sallandrouze et al. 1999), *Picea glauca* (Lippert et al. 2005), *Larix×eurolepis* (Teyssier et al. 2014) and other conifers as it was reviewed by Abril et al. (2011).

Von Aderkas and Bonga (2000) and Neilson et al. (2010) reviewed that stress has the potential to induce or improve SE in cultures of species that have hitherto been recalcitrant. In this sense, Bonga et al. (2010) pointed that lowering or increasing temperatures may improve initiation and proliferation stage as they act as a mild stress that favors cell reprogramming. Additionally, Fehér (2015) reviewed that temperature exerts a selective pressure in the early stages of the process that leads to lower rates of initiation, but higher rates of proliferation and maturation. Therefore, proteomics is a very useful tool for studying stress tolerance, allowing the identification and quantification of stress-tolerance associated proteins (Pinheiro et al. 2014); it opens the possibility to use protein markers in breeding programs for improving selection. In a previous study carried out in *Pinus radiata* (García-Mendigüen et al. 2015) and *Pinus halepensis* (Pereira et al. 2016), we observed that different environmental conditions (temperature and water availability) at initial stages of SE led to different success at initiation stage.

Taking into account all this information, the overall objective of this study was to analyse whether different environmental conditions in SE initiation phase result in differences at proteomic level (of different protein patterns) in the somatic embryos produced.

Materials and methods

Plant material

Embryonal masses (EMs) were generated from one-year-old green female cones of *Pinus radiata* D. Don, collected in June 2012, from four open-pollinated trees in a seed orchard established by Neiker-Tecnalia in Deba-Spain. The seeds were surface-sterilized and megagametophytes containing immature zygotic embryos were excised out following Montalbán et al. (2012).

The megagametophytes were cultured in embryo development medium (EDM) (Walter et al. 1994) at nine different physico-chemical environments (temperature and gellan gum concentrations). Different concentrations of gellan gum were added to increase or reduce water availability of the medium (2, 3 or 4 g/l Gelrite®) and the explants were cultured at three different temperatures: 18, 23 or 28°C (Figure 1). After 4 to 8 weeks, EMs were subcultured every 2 weeks; proliferation medium had the same composition as initiation medium, but at this stage of the process both gellan gum (4.5 g/l) and temperature (23°C) were the same for all cultures. Following four subculture periods, actively growing EMs were recorded as embryogenic cell lines (ECLs) and subjected to maturation following Montalbán et al. (2010). All cultures were kept in darkness at 23°C. In order to analyse the results, initiation percentages were taken from García-Mendigüen et al. (2015).

Water availability in culture media

The water availability in initiation conditions was determined as follows, sterilized filter papers (Whatman no. 2, 7 cm) were weighed and then transferred to different initiation conditions (Figure 1). After 4 weeks, the filter paper discs were re-weighed, and the amount of water absorbed was recorded in milligrams. The data for water availability in the different initiation conditions were subjected to ANOVA to determine differences among maturation treatments. Multiple comparisons were made using Duncan's *post-hoc* test ($\alpha=0.05$).

Protein extraction

For total protein extraction, a modified procedure based on the work described by Zhang et al. (2009) and Correia et al. (2012) was applied. Samples (200 mg) were grounded to a fine powder in liquid nitrogen. While still frozen, the powder was suspended in 4 ml of cold acetone containing 0.2% (w/v) dithiothreitol (DTT) and 10% (w/v) trichloroacetic acid (TCA). The suspension was then transferred to clean centrifuge tubes, incubated overnight at -20°C , and centrifuged for 30 min at $20,000\times g$ (4°C). The supernatant was carefully decanted, and the remaining protein pellet was washed twice in cold acetone (containing 0.2% DTT), incubated for 30 min at -20°C , centrifuged 30 min at $20,000\times g$ (4°C), and vacuum-dried. The resulting pellet was resuspended in 500 μl per tube of an isoelectric focusing (IEF) solubilization buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS (3[(3-cholamidopropyl)-dimethylammonio]-propanesulfonic acid), 1% (w/v) DTT,

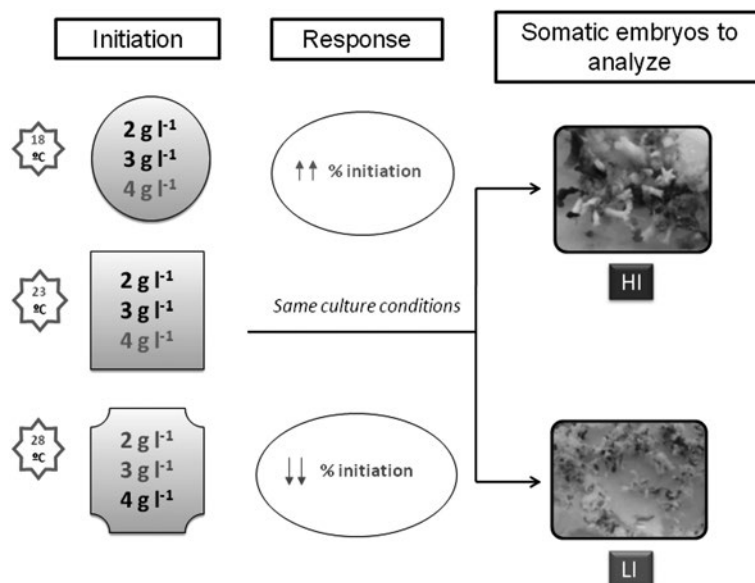


Figure 1. Graphic representation of the production of somatic embryos initiated on EDM supplemented with 2, 3 or 4 g/l Gelrite® and stored at three different temperatures (18, 23 or 28°C). Explants initiated at 18 and 23°C and 4 g/l gellan gum led to high initiation percentages (HI) and explants initiated at 28°C and 2 and 3 g/l gellan gum led to low initiation percentages (LI). Proliferation and maturation stages were carried out at standard culture conditions.

and 1% (v/v) IPG buffer]. The suspension was sonicated and incubated at room temperature for 2 h in a rotary shaker and then centrifuged at $20,000\times g$ for 1 h to remove the insoluble material. The total protein concentration was assessed using the 2-D Quant Kit (GE Healthcare; Amersham Biosciences) according to the manufacturer's guidelines and using BSA as a standard. Samples were aliquoted and stored at -20°C until further processing.

2-D electrophoresis and image analysis

Before sample loading, 13 cm-long immobilized pH gradient (IPG) strips (pH 3–10 NL; GE Healthcare; Amersham Biosciences) were hydrated overnight on an IEF solubilisation buffer. The extracted proteins ($250\ \mu\text{g}$) of somatic embryos were individually loaded onto the strips with a cup-loading system (Bio-Rad). Isoelectric focusing was performed using the Protean IEF cell (Bio-Rad) programmed as follows: 500 V for 1 h, 8,000 V for 2 h (applying an increasing gradient), and 8,000 V for 4 h. The strips were then equilibrated twice successively in an equilibration solution [50 mM Tris-HCl, 6 M urea, 10% (v/v) glycerol, 2% (w/v) SDS, and trace amounts of bromophenol blue], in which the first equilibration contained 2% (w/v) DTT and the second 2.5% (w/v) iodoacetamide. The second-dimension SDS-PAGE was performed in 10% acrylamide gels employing the Bio-Rad System. Proteins were stained using colloidal Coomassie blue G-250 dye (Candiano et al. 2004), and the stained gel images were acquired with a Spot Cutter (Bio-Rad) in the white-trans mode. Gel images were imported into Proteomweaver™ 2-D Analysis Software 4.0 (Bio-Rad), and the spots were detected and matched through the entire matchset. After matching, gel images were normalized using the “Local Regression Model” algorithm,

available in Proteomweaver 4.0. In order to find significant differences between the groups of samples under study, protein spots intensities were subjected to a one-sample *t*-test ($p < 0.05$). When a spot was present only in HI or LI somatic samples, it was excised manually from gels and identification was carried out with the specific sample.

Protein identification by MALDI-TOF mass spectrometry

Tryptic digestion

The gel pieces were washed three times with 25 mM ammonium bicarbonate/50% ACN, one time with ACN and dried in a SpeedVac (Thermo Savant). Twenty-five microliters of $10\ \mu\text{g}/\text{ml}$ sequence grade modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate was added to the dried gel pieces and the samples were incubated overnight at 37°C . Extraction of tryptic peptides was performed by addition of 10% of formic acid (FA)/50% ACN three times being lyophilised in a SpeedVac (Thermo Savant).

Mass spectrometry

Tryptic peptides were resuspended in $10\ \mu\text{l}$ of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α -cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/0.1% formic acid. Aliquots of samples ($0.5\ \mu\text{l}$) were spotted onto the MALDI sample target plate. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 4,500 Da with ca. 1,500 laser shots. For each sample spot, a dependent data

acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition.

Database search

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses an internal MASCOT software (v2.1.0 Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data. Swiss-Prot nonredundant protein sequence database (October 2014) was used for all searches under taxonomy green plants and *Pinus*. Database searches parameters as follows: carbamidomethylation and propionamide of cysteine as a variable modification as well as oxidation of methionine, and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 25 ppm and fragment ion mass tolerance was 0.3 Da. Positive identifications were accepted up to 95% of confidence level.

Results

Characterization of ECLs

According to the initiation percentages, explants cultured at 18 and 23°C (Figure 2A) led to high initiation percentages (17–13%, respectively; data from García-Mendiguren et al. 2015), in contrast to those cultured at 28°C (Figure 2B), which showed lower rates of initiation success (4%). When gellan gum was set at 4 g/l the highest initiation percentage was obtained (16%), compared to explants initiated at either 2 or 3 g/l which showed initiation values of 9% (García-Mendiguren et al. 2015). For this study, somatic embryos from ECLs initiated at 18 or 23°C and 4 g/l gellan gum were classified as “high initiation” (HI) and those from ECLs initiated at 28°C and 2 or 3 g/l gellan gum were named as “low initiation” (LI). Somatic embryos (Figure 2C) from ECLs initiated at 23°C and 2 g/l gellan gum were considered as control.

Somatic embryos of two ECLs coming from HI, two ECLs from LI, and one ECL from Control (three replicates each) were collected and fast-frozen in liquid nitrogen before being kept at -80°C for further analysis (Figure 1).

Water availability

Due to the interaction observed between gellan gum concentration and temperature at initiation stage of the process, water content in all the treatments assayed is shown (Figure 3). EMs cultured at 28°C with 2 g/l de gellan gum had available a higher amount of water than the rest of treatments chosen for protein quantification (18°C-4 g/l, 23°C-4 g/l, 28°C-3 g/l).

Comparison of 2D Gels and protein identification

The 2-D protein extraction protocol resulted in

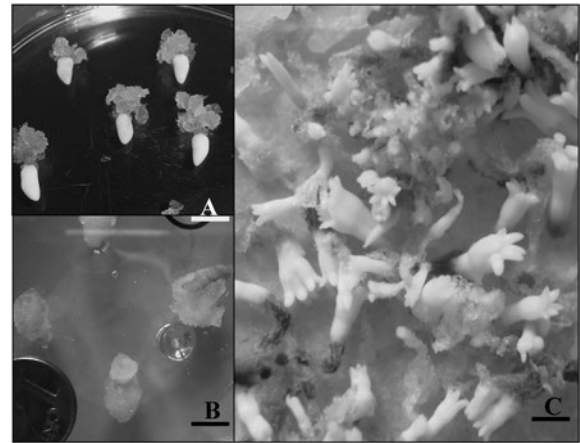


Figure 2. *Pinus radiata* somatic embryogenesis: (A) embryogenic tissue in megagametophytes cultured at 23°C on EDM after 6 weeks, bar represents 10 mm, (B) embryogenic tissue in megagametophytes cultured at 28°C on EDM after 6 weeks, bar length=8 mm, (C) mature somatic embryos produced in HI and LI, bar length=5 mm.

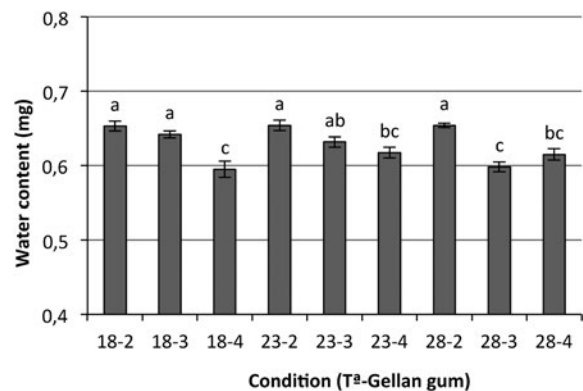


Figure 3. Water content in Petri dishes containing different gellan gum concentrations (2, 3 and 4 g/l). Different letters represents significance at $\alpha=0.05$.

an average of 0.39 ± 0.07 mg of protein in HI and 0.36 ± 0.11 mg of protein in LI per 200 mg of fresh somatic embryos, and 0.43 ± 0.04 mg of protein from 200 mg of fresh somatic embryos in Control. Coomassie-stained 2-DE gels revealed that most of the spots were distributed in the range between pH 3.0 and pH 10.0. The 2-DE images of LI and HI showed different spot distribution (Figures 4, 5).

139 spots were detected. Most of proteins (75) were common to the three treatments (HI, LI and Control). 25 proteins detected were unique to the LI, while only 2 spots were unique to the HI, and no spots were unique to the Control (Figure 6). These 27 spots that were present only in LI or HI further selected and picked from the gels and submitted to MALDI-TOF/TOF-MS analysis. Following MALDI-TOF/TOF-MS analysis and database search, 11 differentially abundant proteins were precisely identified from the 27 selected spots. All of these identified proteins came from LI samples. Table

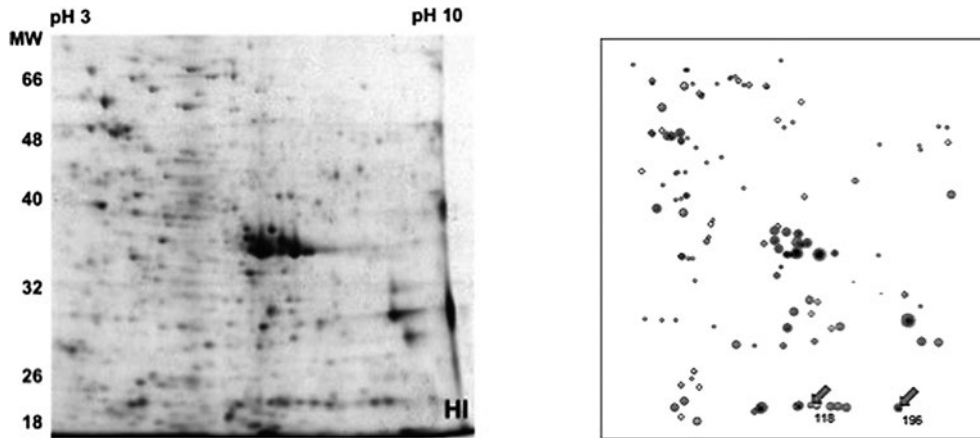


Figure 4. Two-dimensional gels of HI proteins extracted using the TCA/acetone protocol; 250 μg of proteins are separated in the first dimension on an immobilized nonlinear 3–10 pH gradient and in the second dimension on a 10% acrylamide-SDS gel. The gel shown was stained with colloidal Coomassie brilliant blue. The molecular weight of protein standards is indicated on the left of the panel. The spots were analyzed by Proteomweaver™ 2-D Analysis Software 4.0, and a master 2-DE proteome map from the comparative analysis of HI gel was obtained for this group. Proteins represented with arrows and spot number were up-regulated in this group of samples. These proteins were selected for further processing by MALDI-TOF/TOF-MS analysis.

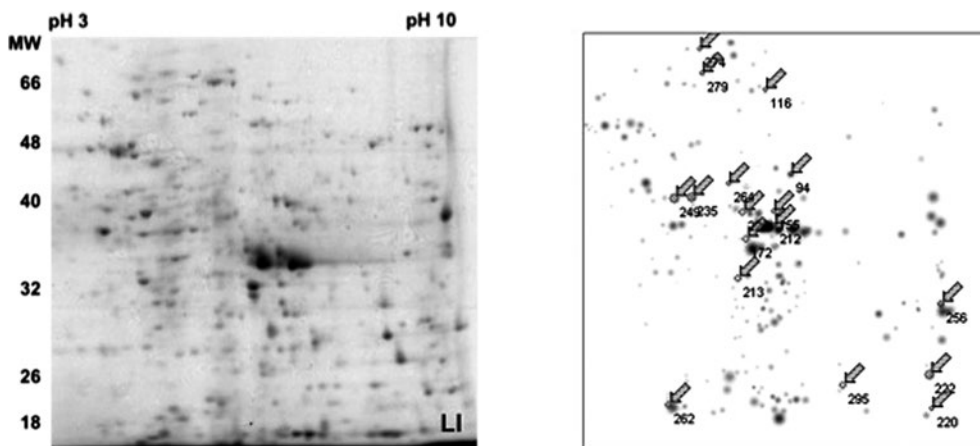


Figure 5. Two-dimensional gels of LI proteins extracted using the TCA/acetone protocol; 250 μg of proteins are separated in the first dimension on an immobilized nonlinear 3–10 pH gradient and in the second dimension on a 10% acrylamide-SDS gel. The gel shown was stained with colloidal Coomassie brilliant blue. The molecular weight of protein standards is indicated on the left of the panel. The spots were analyzed by Proteomweaver™ 2-D Analysis Software 4.0, and a master 2-DE proteome map from the comparative analysis of LI gel was obtained for this group. Proteins represented with arrows and spot number were up-regulated in this group of samples. These proteins were selected for further processing by MALDI-TOF/TOF-MS analysis.

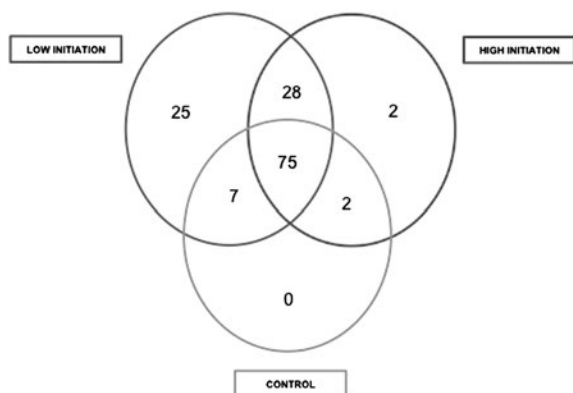


Figure 6. Venn diagram showing distributions of the total number of spots (139) between the embryogenic cell lines from treatments producing high percentages of initiation (HI), embryogenic cell lines from treatments producing low percentages of initiation (LI) and Control cell lines.

Table 1. Analysis of variance of the water availability (mg) in EDM used at *Pinus radiata* embryonal mass initiation at nine different environmental conditions (2, 3 and 4 g l^{-1} , stored at 18, 23 and 28°C).

Source	df	F test	p value
Temperature (T)	2	1.971	0.153
Gellan gum (G)	2	29.216	<0.001
T×G	4	5.040	0.002

1 provides the putative names of proteins, the plant organism from which the protein has been identified, the Swiss-Prot database accession numbers, the values for theoretical molecular mass and pI and the number of peptide matches ($\geq 95\%$). Proteins were identified on the basis of the databases available in Swiss-Prot for other conifer species, namely, *Picea sitchensis*, *Pinus koraiensis*, *Pinus pinaster* or *Pinus sylvestris*, other plant species such as *Arabidopsis thaliana*, *Oryza sativa*, *Alnus glutinosa*,

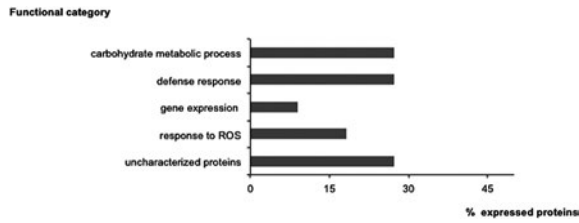


Figure 7. Proportion of proteins expressed in somatic embryos of *Pinus radiata* in LI conditions depending on their functional categories. Percentage has been calculated respect to the total number of identified proteins.

Mesembryanthemum crystallinum or *Zea mays* and other species like *Pseudomonas*. The identification of *Pinus radiata* proteins that could be unique of this species or specifically modified may have failed because of the lack of analogues in the databases. In this way, de novo interpretation of mass spectrometry data, independently of database information, could be helpful in future experiments. It should also be observed that for one spot (enolase) more than one protein was identified. Based on the Swiss-Prot database, identified proteins in LI were organized into different functional categories (Figure 9). A prominent group of proteins involved in defense responses, such as the osmotically inducible protein OsmC, chaperon protein and vicilins, was identified. Proteins associated with other functional groups, such as proteins related to response to ROS and carbohydrate metabolic process, such as enolase or phosphoglycerate kinase or proteins related to gene expression, were also found in lower proportions (Figure 7).

Discussion

The strategy of conducting *Pinus radiata* initiation stage under different environmental conditions was carried out to focus mainly on early molecular mechanisms that could be involved in subsequent physiological modifications promoting somatic embryo development, as reflected in protein profiles. We evaluated and compared the protein patterns in somatic embryos coming from ECLs from treatments producing high percentages of initiation (18–23°C, 4 g/l, HI) and low percentages of initiation (28°C, 2–3 g/l, LI). It should be noted that six of the identifications obtained in the somatic embryos with low initiation percentages were based on homologies with proteins from conifer species, including *Pinus* sp. close relatives, according to SwissProt database (Table 1). The fact that for one spot (enolase) more than one protein was identified may be caused by the degradation products of some proteins (Teyssier et al. 2011) or by the presence of multiple isoforms of a protein (Lippert et al. 2005). The presence of protein isoform with different expression patterns during seed maturation has already been reported in barley (Finnie et al. 2006).

In LI, samples coming from EMs cultured with high and low water availability conditions, proteins involved in the glycolysis/gluconeogenesis pathway, in particular with carbon and phosphate metabolism (enolase, enolase 2, phosphoglycerate kinase) were found, suggesting a more active respiratory pathway as it was previously suggested in *Acca sellowiana* (Fraga et al. 2013). Our results are not in accordance with Teyssier et al. (2011) when they reported a higher level of this type of proteins only in somatic embryos produced under high water availability conditions.

Other studies, carried out in *Populus euramericana* (Bonhomme et al. 2009), described a decreased level of proteins involved in carbon metabolism in plants growing under water deficit conditions. In this sense, our experiments showed that water content does not seem to determine the protein profile of somatic embryos from EMs subjected to different environmental conditions during the initiation stage. Hence, it is likely that the initiation temperature could determine the presence or absence of certain proteins in the somatic embryos obtained.

Enolase (s249) has been found only in LI and it is a ubiquitous enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, which is the only dehydration step in the glycolytic pathway, occurring at the end of glycolysis (Vanderstraeten et al. 1991). This enzyme has been found during late SE in *Picea glauca* and *Carica papaya*, suggesting that this protein can be used as a marker of embryo maturation (Lippert et al. 2005; Vale et al. 2014); However, in our study we obtained somatic embryos in HI and LI which may indicate that in this case this protein do not act as a marker of embryogenicity. Phosphoglycerate kinase (s226) is an enzyme that catalyzes the reversible transfer of a phosphate group from ATP to 3-phosphoglycerate (Fraga et al. 2013). In our experiments, these proteins were not found in HI and Control. In previous studies, García-Mendiguren et al. (2015) in *Pinus radiata* and Pereira et al. (2016) in *Pinus halepensis* described 28°C as the worst condition to initiate EMs. The fact that enolase and phosphoglycerate kinase were found only in LI is in accordance with findings reported by Morel et al. (2014) who suggested that glycolysis is enhanced under unfavourable conditions. Moreover, glycolysis and carbohydrate metabolism related proteins, like Enolase, have been observed in *Pinus radiata* biosynthetically active tissues acting as a strong energy sink (Valledor et al. 2010). Furthermore, the proteins involved in the glycolytic pathway appear to be a negative regulator of transcription factors involved in abiotic stress responses (Morel et al. 2014). On the other hand, the presence of proteins involved in glycolysis pathway is also due to the fact that embryogenesis is a developmental process connected with biosynthesis of many compounds

Table 2. Proteins expressed in somatic embryos coming from low initiation (LI) percentage of *Pinus radiata* identified by MALDI-TOF/TOF-MS.

Spot ID ^a	Protein ID	Species	Assession No. ^b	Theoretical mass ^c (Da)/pI	Protein/ion score C.I. ^d %	Peptide matches ^e (≥95%)
s174	Probable mediator of RNA polymerase II transcription subunit 37c	<i>Arabidopsis thaliana</i>	MD37D_ARATH	71342/5.03	99.75	1
	Putative uncharacterized protein	<i>Picea sitchensis</i>	B8LRY5_PICSI	71266/5.07	99.60	19
s274	Chaperone protein ClpB2, chloroplastic	<i>Oryza sativa ssp. japonica</i>	CLPB2_ORYSJ	108917/6.25	94.80	1
	Putative uncharacterized protein	<i>Picea sitchensis</i>	A9NQA7_PICSI	31167 /5.13	96.04	1
s249	Enolase	<i>Mesembryanthemum crystallinum</i>	ENO_MESCR	48318/5.62	98.81	1
	Enolase 2	<i>Zea mays</i>	ENO2_MAIZE	48132/5.7	98.81	1
	Enolase	<i>Alnus glutinosa</i>	ENO_ALNGL	47567/5.41	98.81	1
	Enolase	<i>Oryza sativa ssp. japonica</i>	ENO_ORYSJ	47942/5.41	98.81	1
	Putative uncharacterized protein	<i>Picea sitchensis</i>	B8LL07_PICSI	48107/5.78	99.36	20
	Putative uncharacterized protein	<i>Picea sitchensis</i>	B8LQR0_PICSI	48121/5.78	99.36	20
	Putative uncharacterized protein	<i>Picea sitchensis</i>	COPQ35_PICSI	48130/5.86	99.23	20
s226	Phosphoglycerate kinase	<i>Picea sitchensis</i>	A9NVA3_PICSI	52905/8.52	100	28
	Phosphoglycerate kinase	<i>Picea sitchensis</i>	B8LKQ1_PICSI	49153/8.26	100	25
	Phosphoglycerate kinase	<i>Pinus pinaster</i>	F4MKM1_PINPS	52829/8.84	100	24
s220	Vicilin	<i>Pinus koraiensis</i>	W5IDC2_PINKO	46161/5.74	100	12
s295	Putative uncharacterized protein	<i>Picea sitchensis</i>	A9NPM2_PICSI	12966/7.66	90.11	10
s172	Uncharacterized protein	<i>Pseudomonas sp.</i>	I4N9X1_9PSED	6193/10.12	90.34	7
s239	Putative uncharacterized protein	<i>Picea sitchensis</i>	A9NPC3_PICSI	22635/5.32	96.33	19
s155	OsmC family protein	<i>Pseudomonas sp.</i>	I4N7V9_9PSED	14794/6.73	97.89	15
S179	Caryophyllene/humulene synthase	<i>Pinus sylvestris</i>	B4XAK4_PINSY	66686/5.12	94.93	25
s212	Vicilin	<i>Pinus koraiensis</i>	W5IDC2_PINKO	46161/5.74	100	1

^a Each entry contains a spot number that corresponds to the protein spots marked in Figure 2. ^b Database accession numbers according to SwissProt. ^c Theoretical masses (Da) and pI of differently expressed proteins. ^d Mascot protein or ion (in the cases of one peptide match) scores, r.

and cell structures, and glycolysis supplies energy and synthesizes several metabolic intermediaries (Rode et al. 2011).

Chaperone proteins (s274) were also present in LI samples. Chaperones are known as heat shock proteins (HSPs), whose biological role is to assist folding of unfolding or misfolded proteins under stress conditions (Efeoğlu 2009). These proteins are also known for their roles in the maturation, as they act as oxidative stress regulators and help in the maintenance of the protein structure (Marsoni et al. 2008). Moreover, the presence of HSPs indicates that some kind of abiotic stress is occurring in somatic embryos coming from EMs generated at 28°C (Lee and Schöffl 1996; Teysier et al. 2011). In a similar way, the osmotically inducible protein OsmC (s155) was also identified in LI, and it is involved in stress responses, particularly in defence against oxidative stress caused by exposure to organic hydroperoxides (Lesniak et al. 2003). ROS are important signalling molecules reported to act in plant responses to biotic and abiotic stresses (Delledone et al. 2001) and in the development of SE of conifers (Zhang et al. 2010). The generation of hydrogen peroxide and its regulation by the activation of antioxidant enzymes have been recorded at the beginning of SE formation (Lilik et al. 2005; Ma et al. 2012a, b). It was also shown that the redox state of the culture medium had an important effect on Norway spruce embryo yield and quality

(Belmonte et al. 2005). Based in these observations we can discuss that the presence of OsmC protein in somatic embryos initiated at high temperature could be helpful for cell development of a redox homeostasis system and maintenance of cell differentiation.

Probable mediator of RNA polymerase II transcription subunit 37c (s174) is a component of a coactivator involved in the regulation of transcription of RNA polymerase II-dependent genes, and it acts as a bridge to convey information from gene-specific regulatory proteins to the basal RNA polymerase II transcription machinery (www.uniprot.org/uniprot/Q9LHA8).

Caryophyllene/humulene synthase protein (s179), found in somatic embryos initiated at 28°C and 2–3 g/l gellan gum, is an enzyme that regulates the formation of cyclic terpenes through the cyclization of linear terpenes, which is related to the secondary metabolism. Accumulation of primary and secondary metabolites has been reported as one of the main mechanism of plants to tolerate osmotic stress in Scots pine (Muilu-Mäkelä et al. 2015), and this secondary metabolism is reportedly more active in plants under stresses that lead to increased production of free radicals (Edreva et al. 2008). According with our results, Teysier et al. (2011) also found more abundant proteins involved in secondary metabolism in hybrid larch somatic embryos obtained at low gellan gum concentrations.

Proteins involved in functions of storage of nutritious

substrates such as vicilin (s212, s220) were also found in LI samples. Apart from the aforementioned storage function of vicilins, they are also considered potential biomarkers for early selection of ECLs with high embryogenic potential with positive responsiveness to maturation conditions (dos Santos et al. 2016). This fact is in agreement with our findings because EMs generated at 28°C were those with the highest amount of embryos developed at the end of maturation stage (García-Mendiguren et al. 2015). Additionally, these proteins have been also proposed to have a defensive role as part of legume lectins (Ribeiro et al. 2014). Studies carried out by Fraga and co-workers (2013) in *Acca sellowiana* found out that a vicilin-like storage protein caused reduced vigor in the development of derived plants in off-types embryos. As we have previously mentioned, we did not observe abnormalities on the morphology of the somatic embryos obtained, but the accumulation of these proteins in LI embryos can be related to the high temperatures and high water availability in which the lines were originated.

Some studies have been carried out concerning the comparison of somatic embryos from different culture conditions at maturation stage (Morel et al. 2014; Teyssier et al. 2011) but as far as we know, this is the first report of a 2-DE proteomic analysis of conifer somatic embryos initiated at different environmental conditions. Protein patterns found in somatic embryos cultured under unfavourable environmental conditions suggest that those conditions of the initiation stage continue to influence the maturation phase by the presence of proteins involved in stress and metabolic responses. The high temperature and water availability that induced low initiation rates (García-Mendiguren et al. 2015) promoted the abundance of glycolytic enzymes and stress related proteins, such as chaperones and osmotically induced proteins. Nevertheless, the lack of protein identification for HI conditions, in addition to a lack of molecular information related to conifer SE at the protein level prevented us to make a deeper analysis of the effect of environmental variations in *Pinus radiata* SE process.

Despite the fact that this is a descriptive study on the proteins present in somatic embryos from EMs generated unfavourable conditions, this information could be useful to enhance the information available of proteins involved in SE process. In addition, if future studies confirm the hypothesis that unfavourable conditions during initiation produce plantlets better adapted to ex vitro conditions, the proteins described in this work could be used as biomarkers. Further studies will be required to clarify the long-term effect of the environmental variations at initial stages of the process.

Conclusions

To our knowledge, this is the first report in which a 2-DE proteomic analysis has been carried out in conifer somatic embryos generated under different environmental conditions. The presence of proteins involved in stress and metabolic responses indicate that changing environmental conditions seem to influence all the somatic embryogenesis process. While limited in scope, this study contributes to enhance the existing information of proteins involved in SE process.

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