

# DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell culture

Carlos Noceda · Terézia Salaj · Marta Pérez ·  
Marcos Viejo · Maria Jesús Cañal · Ján Salaj ·  
Roberto Rodriguez

Received: 12 January 2009 / Revised: 8 June 2009 / Accepted: 7 July 2009 / Published online: 25 July 2009  
© Springer-Verlag 2009

**Abstract** Embryogenic cell lines initiated from immature zygotic embryos of *Pinus nigra* Arn. ssp. *Austriaca* were characterized in terms of macromorphological traits (colour, bipolar structures formation, germination ability) and their embryogenic potential was defined as high, medium or null. Quantification of global genomic DNA methylation revealed the existence of specific DNA methylation levels for the determined embryogenic potentials. The line considered as effectively embryogenic, i.e., with the ability to develop the whole embryogenic program and producing plants, showed the lowest methylation levels. There was also proved the existence of an inverse relationship between total contents of free PAs and embryogenic potential, being the highest contents of free putrescine and spermidine in the non-embryogenic line and the lowest in

the effectively embryogenic one. Relationships among DNA methylation levels, profiles of free individual polyamine contents and embryogenic potentials based on the ability to produce well-formed somatic embryos with effective plant conversion are discussed.

**Keywords** Embryogenic potential · Epigenetic plasticity · Genomic DNA methylation · Polyamines · Somatic embryogenesis

## Abbreviations

dSAM *S*-adenosyl methionine decarboxylase  
PAs Polyamines  
DAP 1,3-diaminopropane  
Put Putrescine  
Spd Spermidine  
Spm Spermine

Communicated by U. Lüttge.

C. Noceda · M. Pérez · M. Viejo · M. J. Cañal (✉) ·  
R. Rodriguez  
Lab. Fisiología Vegetal, Dpto. B.O.S., Facultad de Biología,  
Universidad de Oviedo, C/Catedrático Rodrigo Uría, s/n, 33071,  
Oviedo, Asturias, Spain  
e-mail: mjcanal@uniovi.es

## Present Address:

C. Noceda  
Research Unit on Breeding, Genetic and Physiology of Forest  
Trees, I.N.R.A., 2163 avenue de la Pomme de Pin, CS 40001,  
Ardon, 45075 Orleans Cedex 2, France

T. Salaj · J. Salaj  
Institute of Plant Genetics and Biotechnology,  
Slovak Academy of Sciences, Akademická 2,  
P.O. Box 39 A, 950 07 Nitra, Slovak Republic

M. J. Cañal · R. Rodriguez  
Intituto Universitario de Biotecnología de Asturias  
(IUBA asociado con CSIC), Oviedo, Asturias, Spain

## Introduction

Somatic embryogenesis is an alternative propagation technique to traditional procedures, presenting numerous applications in plant breeding (Von Arnold et al. 2002), but problems like induction window, lost of embryogenic potential, variable ability of plant conversion, lost of competence along subcultures and cryopreservation limit the production that this technique offers. Somatic embryogenesis in *Pinus nigra* Arn. has been successfully achieved and the embryogenic tissues are being maintained in long-term cultures (Salajova and Salaj 1992; Jasik et al. 1995; Salajova et al. 1999; Salajova and Salaj 2005; Salajova et al. 2005).

The embryogenic tissues of this species are characterised by the presence of numerous somatic embryos capable of maturation and subsequent plantlet regeneration (Salajova et al. 1999) although the maturation has been cell line dependent. Structural investigations have revealed different cytological and morphological features of somatic embryos in different cell lines (Salajova and Salaj 2001). The morphology of somatic embryos profoundly affected the maturation capacity (Salajova and Salaj 2005). Analytical methods have shown that the cell lines differ in the extracellular proteins released to the liquid medium in suspension cultures (Petrovska et al. 2004).

Production of embryos under in vitro conditions is a key experimental system to study reactivation and cell division, tissue organization, morphogenic competence or rejuvenation (Dudits et al. 1991) and offers an in vitro experimental system to study embryogenesis (Berros et al. 2005), cell differentiation and totipotency (Gaj 2004; Quiroz-Figueroa et al. 2006). Somatic embryogenesis induction and development have been investigated (Dodeman et al. 1997) in model systems, but remains unclear for most of the agroforestry important species.

Cell differentiation and development are controlled through temporal and spatial activation and silencing of specific genes. In contrast to animal development, plant development is plastic and strongly influenced by biotic and abiotic factors. Therefore, plants require specific interaction between developmental programs and the signalling pathways that are triggered from external stimuli. This interaction is coordinated epigenetically, i.e., at the level of chromatin organization (Causevic et al. 2006). In the last few years, methylation of genomic DNA has become a centre of scientific attraction due to its importance in understanding animal and plant development (Razin and Riggs 1980). Specific changes in total DNA methylation in plants were proved to be related to the developmental phase (juvenile and mature-vegetative versus mature-reproductive) (Fraga et al. 2002b) being also possible to relate the specific methylation status to the further in vitro morphogenic ability (Valledor et al. 2007).

Furthermore, it was confirmed that the regain of morphogenic ability by reinvigoration techniques was linked to the recovery of specific DNA methylation status (Fraga et al. 2002a). DNA methylation is essential for both morphogenesis and proliferation during somatic embryogenesis (Lo Schiavo et al. 1989; Santos and Feveireiro 2002; Causevic et al. 2005). The existence in carrot nuclear extracts of protein factors exhibiting specific affinities for conventional and non-conventional methylation acceptor sites were also described (Pitto et al. 2000) showing that their activities are modulated during vegetative cell growth and somatic embryo development. In this context, a question arises whether the maturation capacity of *Pinus nigra*

embryogenic cell lines is dependent on the methylation status of tissues.

Mainly due to their polycationic character, the polyamines (PAs), and specially the long chain ones, have been implicated in a wide range of biological processes including cell division and differentiation, somatic embryogenesis, senescence and environmental stress (Kaur-Sawhney et al. 2003; Fraga et al. 2004; Alcázar et al. 2006; Kusano et al. 2007). There is a possible relationship between PAs and DNA methylation, since their biosynthetic pathways share the enzyme decarboxylated *S*-adenosylmethionine (dSAM) as a common substrate (Fraga et al. 2002a).

In the case of *Pinus radiata*, a decrease in putrescine (Put) with respect to the long chain polyamines spermidine (Spd) and spermine (Spm), Put/(Spd + Spm) ratio during ageing could lead to a concomitant fall of dSAM levels (Fraga et al. 2002c), and consequently to a higher activation of the DNA methyltransferase, which would partially explain the usual gene silencing (Rey et al. 1994) observed during maturation.

The loss of embryogenic competence associated with differential expression of age-dependending genes constitutes a limiting factor for a successful clonal propagation of adults. The knowledge of competence bases in plants, where morphogenesis is stable but not static, is based on information about differentiation plasticity. On this matter, epigenetic mechanisms are fundamental to understand in a short time the plant developmental paradigm from a practical and basic point of view. Taking this into account, the aim of this work is to set if genomic DNA global methylation allows the characterization of different potential embryogenic lines. Furthermore, DNA methylation can be related with PAs contents through dSAM levels. Thus, the quantification of free PAs as a complementary indicator of somatic embryogenesis process is proposed.

## Materials and methods

### Plant material

Embryogenic cell lines were initiated from immature zygotic embryos of *Pinus nigra* Arn. ssp. *Austriaca*. Green cones were collected from open-pollinated trees growing in natural stands (Drazovce, Nitra, Slovak Republic). The collected cones were stored in plastic bags for 5–10 days at 4°C. Afterwards, the immature seeds were excised and surface sterilised with 10% (v/v) hydrogen peroxide for 10 min and rinsed three to four times with sterile distilled water. The megagametophytes containing immature embryos were excised under sterile conditions and eight of them were placed per Petri plate.

### Embryogenic induction and establishment

For embryogenic cultures initiation, medium DCR (Gupta and Durzan 1985), supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/l), *N*<sup>6</sup>-benzyladenine (0.5 mg/l), casein hydrolysate (500 mg/l), glutamine (50 mg/l) and sucrose (20 g/l), was used. Before autoclaving, the pH of the medium was adjusted to 5.7, and 3 g/l Gelrite (Duchefa) were added to solidify. The cultures were kept in darkness at 25°C along all the culture phases.

### Maintenance and multiplication of embryogenic tissues

For long-term culture, the induced cell lines were maintained on DCR medium as previously described for the initiation. Transfers to fresh media were regularly done at 2–3 weeks intervals during 6 months. Afterwards, the embryogenic competence of each single line was tested according to the following traits:

- Colour and consistence of tissues.
- Presence of conform bipolar structures, observed by the light microscopy.
- Cellular organization of embryonal mass and suspensor: cellular configuration was studied with an Axio-plan 2 (Carl Zeiss, Germany) microscope using two different cytological techniques: (a) Martoja and Martoja staining (Martoja and Martoja 1967), according to which selected pieces were fixed with 45% acetic acid during 2 s and then stained with 1% (w/v) orceine in acetic acid for 5 min; (b) small pieces of tissues were stained with 2% (w/v) acetocarmine, and then squashed and covered by cover slip.
- Production, after maturation phase, of well-formed somatic embryos per gram of fresh mass.
- Embryo germination ability (as a percentage of germinating embryos).
- Embryogenic potential, determined on the basis of the ability of different lines to organize, produce embryos and germinate plants.

### Somatic embryo maturation

Maturation of embryos occurred on DCR basal medium supplemented with 95 µM abscisic acid and 9% maltose, finally gelified with Gelrite (0.4%) according to Salajova et al. (1999).

### Somatic embryo germination

Mature cotyledonary somatic embryos were desiccated for 2–3 weeks (Salajova and Salaj 2005) and then germinated

in an hormone-free medium containing 30 g/l maltose (Duchefa) and 1% (w/v) activated charcoal.

### Genomic DNA methylation analysis

During the maintenance of embryogenic tissues, samples for different lines were collected after 8 days of transference to fresh DCR medium, frozen in liquid nitrogen and then stored at –80°C until use. Genomic DNA methylation analyses were done using Fraga et al. (2002d) protocol.

### DNA extraction

Genomic DNA extraction was carried out with a plant genomic DNA extraction kit (DNeasy Plant MiniKit, Qiagen) following the instructions of the supplier with some modifications to obtain a RNA-free DNA (Hasbún et al. 2008).

### Genomic DNA hydrolysis

DNA samples were denatured and then 1 µl of 10 mM ZnSO<sub>4</sub> and 2 µl of nuclease P1 (Sigma S.A., 200 units/ml in 30 mM C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>Na) were added, mixture was incubated overnight at 37°C; 1.20 µl of Tris (0.5 M, pH 8.3) and 0.20 µl alkaline phosphatase (357 units/ml and 2.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, Sigma) were then added and the mixture was incubated for additional 2 h at 37°C. Samples were centrifuged at 15,000g for 20 min and stored at 4°C.

### HPCE analysis

An uncoated fused silica capillary (Waters Chromatography, S.A. 600 × 0.075 mm<sup>2</sup> I.D. effective length 570 mm) was used in a capillary electrophoresis system (Capillary Ion Analyzer, Waters Chromatography S.A.) connected to a Millennium data processing station (Waters Chromatography S.A.). The running buffer was 48 mM NaHCO<sub>3</sub>, pH 9.6 containing 60 mM SDS (Fraga et al. 2002b). Running temperature was 20°C and operating voltage 17.5 kV. Absorbance was monitored at 254 nm on-column. Before each run, the capillary was conditioned by washing first with 1 M NaOH for 2 min, and then with 1 mM NaOH for 1 min. Finally, it was filled with running buffer for 3 min. Buffer and washing solutions were filtered through 0.22 µm pore size filters. Hydrolyzed samples were injected hydrostatically for 15 s at a height of 9.8 cm upon the cathode.

All samples were analysed twice and six replicates per line were used. Quantification of the relative methylation of each DNA sample was performed as the percentage of 5-methyldeoxycytidine (5-mdC) of respect with the total

deoxycytidines (dC), methylated and unmethylated: 5-mdC peak area  $\times$  100/(dC peak area + 5-mdC peak area).

#### Polyamine analysis

Samples from the embryogenic tissues were frozen in liquid nitrogen, lyophilised for 48 h and stored at  $-80^{\circ}\text{C}$  until used. Extraction, purification and quantifying of free (not covalently bound to other molecules) PAs, including the long chain PAs degradation product 1,3-diaminopropane (DAP), were carried out according to the methodology described by Fraga et al. (2003).

#### Polyamine extraction

Twenty-five milligrams of lyophilised material were homogenized in perchloric acid 5% (v/v) and centrifuged. The supernatant contained the free fraction of PAs.

#### Dansylation of polyamine

Aliquots of 200  $\mu\text{l}$  of the supernatant were mixed with 200  $\mu\text{l}$  of a saturated sodium carbonated solution and 400  $\mu\text{l}$  of dansyl chloride (5 mg/ml acetone). The mixture was incubated for 12 h in darkness. After that, the excess of dansyl chloride was removed with 100  $\mu\text{l}$  of proline solution (100 mg/ml in Milli-Q<sup>®</sup> water) and the mixture was incubated in darkness during 30 min. Then, dansylated polyamines were extracted with toluene.

#### HPLC analysis

Separation and quantification of dansylated polyamines were carried out by reverse-phase high performance liquid chromatography (HPLC) according to the method described by Marcé et al. (1995) with some modifications. The HPLC system consisted of a pump (Model 600E), a microprocessor controller (Model 600), a fluorescence detector (Model 474) and an auto-sampler (Model 717 Plus) all from Waters Chromatography S.A., connected to a processing data station Millennium 2010 (Waters Chromatography S.A.). A Kromasil 5  $\mu\text{m}$  ( $250 \times 4 \text{ mm}^2$ ) reverse-phase column was used.

Aliquots of 20  $\mu\text{l}$  of samples, previously filtered through a 0.45  $\mu\text{m}$  filter (Millipore S.A.) were injected. The mobile phase was acetonitrile 190 UV/gradient quality (Romil S.A.) and water and the polyamines were separated through the following elution gradient: 68% acetonitrile for 4 min, then 1 min linear gradient to until reach 100% acetonitrile, which was kept constant for 4 min, and finally 1 min linear gradient until reach 68% acetonitrile (initial conditions). The flow rate was constant at 1.5 ml/min. Eluted polyamines were excited at 350 nm and fluorescence emission

was monitored at 500 nm. All samples were analysed by duplicate and three analytical replicates by sample were taken. Results are given as mean  $\pm$  SE. Put, Spd, Spm and 1,7-diamino heptane (HTD) used as standards were purchased from Sigma S.A.

#### Losses in the process

In order to determine the losses along the extraction and dansylation, 0.925 KBq of  $^{14}\text{C}$ -Spm (Amersham España S.A.) were added to initial extracts. Aliquots of 25  $\mu\text{l}$  were taken at each step and sample radioactivity was quantified in a Vallac 1409 scintillation counter. Results were corrected taking into account losses measured for each sample.

#### Statistical Analysis

Statistical processing of genomic methylation and polyamine analyses were performed with SigmaStat 3.1 for Windows (Systat Software Inc., San José, CA). Normality and variance homogeneity were checked by Kolmogorov–Smirnov and Bartlett tests, respectively. Quantitative factors were contrasted by one-way analysis of variance (ANOVA) and post hoc Holm-Sidak test ( $P \leq 0.001$ ).

## Results and discussion

#### Characterization of cell lines

The embryogenic lines obtained showed different degrees of embryogenic competence.

Based on the previously defined traits and after 6 months of culture, it was possible to discriminate among lines according to their morphology and embryogenic potential (Table 1). Only the line E196, renamed as LE2, showed considerable somatic embryo development. Consequently, this line was considered as effectively embryogenic with high embryogenic potential, developing the whole embryogenic program and finally plants.

Line E177, renamed as LE1, developed a limited number of precotyledonary embryos (10 not conformed embryos produced/grams of fresh weight, g.fw) without germination ability. Therefore, because only first events of embryogenic program were developed, this line was considered as low embryogenic potential. Line E181, renamed as LNE, was considered as null embryogenic potential line with absence (0 mature somatic embryos produced/g.fw) of any embryogenic development.

Line LE2 was morphologically characterized by white and mucilaginous tissue (Fig. 1a) and its cultures were plenty of bipolar structures (80 mature somatic embryos produced/g.fw), i.e., somatic embryos showing an



**Table 1** Classification of embryogenic lines based on the described characteristics

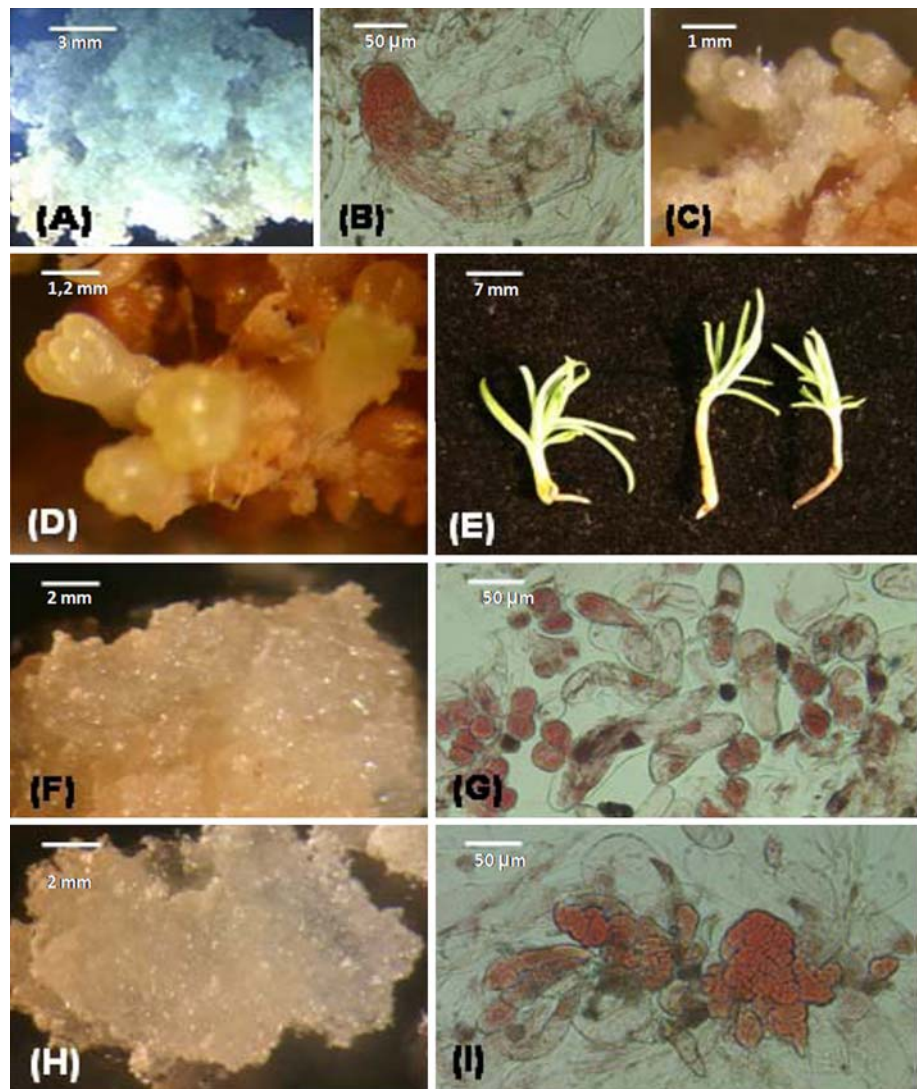
Lines	Callus, colour and consistence	Presence of bipolar structures	Organisation of embryonal part cells	Organisation of suspensor cells	Number of mature somatic embryos produced/g.fw	Germinated somatic embryos	Embryogenic potential
E196 <sub>LE2</sub>	White colour and mucilaginous consistence	+++	Tightly packed meristematic cells	Organized into bundles	80	50%	High LE2
E177 <sub>LE1</sub>	White colour and mucilaginous consistence	++	Aggregates of loosely connected meristematic cells	Without organization into bundles	10 Not conformed embryos	Abnormal	Middle LE1
E181 <sub>LNE</sub>	Yellowish colour and very soft consistence	No	Meristematic cells without connexion	Without organization into bundles	0	0	Null LNE

g.fw: grams of fresh weight

+ Shows approximately 20 bipolar structures/g.fw

**Fig. 1** Macro- and Micromorphological aspects and cell organisation of embryogenic lines derived from immature zygotic embryos of *Pinus nigra* Arn.

**a** Embryogenic tissue containing bipolar structures from cell line LE2. **b** Well-formed somatic embryo from cell line LE2. **c** Precotyledonary somatic embryo from cell line LE2. **d** Cotyledonary somatic embryos from cell line LE2. **e** Plantlets regenerated from somatic embryos from LE2 cell line. **f** Non-embryogenic tissue from cell line LNE. **g** Meristematic cells intermingled with long vacuolised cells from cell line LNE. **h** Embryogenic tissue with low regeneration potential from cell line LE1. **i** Somatic embryos from tissue with low regeneration potential. The embryonal mass is composed of loosely aggregated mass of meristematic cells. The suspensor cells are attached to the embryonal mass without organisation into bundles



embryonal mass (Fig. 1b) formed by tightly packed meristematic cells and a suspensor formed by long, vacuolised cells organized into bundles. Somatic embryos generation and development followed the conifer morphogenetic pattern (Van Zyl et al. 2002), i.e., starting from precotyledonary stages (Fig. 1c), which are followed by mature cotyledonary somatic embryo differentiation (Fig. 1d) and, finally, germination and plant conversion (Fig. 1e) with a germination percentage over 30%.

Nevertheless, the null embryogenic potential line (LNE) is constituted by yellowish calluses with very soft consistence (Fig. 1f) and composed of unorganised cells without potential to develop cotyledonary somatic embryos and, according to results of Jasik et al. (1995), with no presence of neither bipolar structures or suspensor organisation; occasionally meristematic cells appeared (Fig. 1g).

The remaining line (LE1) showed intermediate characteristics, i.e., embryogenic masses with mucilaginous consistence and white colour (Fig. 1h) with the presence of less bipolar structures than LE2. Somatic embryos of this line showed a partial differentiation of embryonal masses (Fig. 1i) and suspensors. In spite of the existence of differentiation process in their tissues, this line do not express effective plant conversion ability.

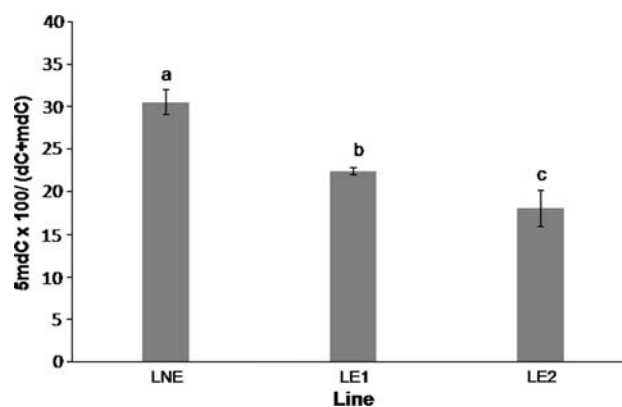
Characterization of studied embryogenic lines has allowed the definition of, at least, two stages in embryo development: cell proliferation (guarantees tissue permanence and the generation of cell populations with embryogenic competence), and differentiation and development of embryonic structures (embryonal masses and suspensors) which reveal the polyembryo phenomenon typical of Gymnosperms.

*Pinus nigra* early somatic embryos were defined as bipolar structures with an embryonal mass and a secondary suspensor (Jasik et al. 1995). Generally, somatic embryos deriving from these structures have germination ability and give plant regeneration. Only the line with higher embryogenic potential (LE2) develops effective somatic embryogenesis including plant conversion.

#### Methylation levels of cell lines

The quantification of global DNA methylation by the use of different methods during somatic embryogenesis was done by several groups (Santos and Fevereço 2002; Chakrabarty et al. 2003; Baurens et al. 2004; Leljak-Levanic et al. 2004; Tchordadjieva and Pantchev 2004; Yamamoto et al. 2005), being results in conifers scarce (Van Zyl et al. 2002; Klimaszewska et al. 2009).

The quantification of global genomic DNA methylation in *Pinus nigra*, shows an inverse relationship between DNA methylation levels and embryogenic potential (Fig. 2). Thus, higher methylation levels (30% of 5mdC)



**Fig. 2** Percentage of methylated cytosines in genomic DNA in the indicated cell lines. Different letters in columns indicate significant differences among means ( $P < 0.001$ , ANOVA and post hoc Holm-Sidak tests). LNE: Cell line without embryogenic potential. LE1: Medium embryogenic potential line. LE2: High embryogenic potential line

corresponded to the line without embryogenic capacity (LNE) and gradually decreased with the increase of the embryogenic potential (LE1 and LE2, with 22% and 18% of 5mdC, respectively).

Only LE2 line showed well-conformed bipolar structures which developed complete plants. Cell line LE1 developed pre-cotyledonary and cotyledonary somatic embryos. We can state that in *Pinus nigra* tissues, low levels of methylation are coincident with total somatic embryo development and conversion. Low rate of developed bipolar structures and nule plant conversion ability seems to be, in this species, determined by higher DNA methylation status.

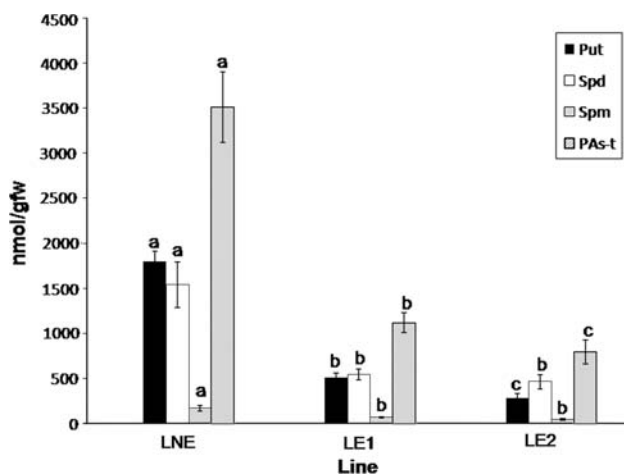
Lower DNA methylation levels could be explained on the basis of (a) DNA methylation involves generally gene silencing and (b) embryogenic program is a multiprocess where gene regulation must be properly regulated. Regarding this subject, cDNA array analysis of 373 genes during *Picea abies* somatic embryogenesis showed the existence of a phase-dependent gene regulation (Van Zyl et al. 2002). At least 35 genes changed their expression during somatic embryogenic development, from which 22 were directly associated with embryo pattern formation. Consequently, we could suppose that the line with effective somatic embryogenesis (LE2) would express whole embryogenic program development genes in addition to maintenance genes. Contrarily, line LNE would express supporting genes exclusively and it would have somatic embryogenesis program genes silenced, which agree with results obtained in leaf-derived non-embryogenic calli of *Eleutherococcus senticosus* Maxim (Chakrabarty et al. 2003).

Although recently, in an attempt to study the biological characterization of young and aged embryogenic cultures

of *Pinus pinaster* Ait. Klimaszewska and collaborators (2009) showed, in the culture conditions tested, both in the presence or absence of demethylation drug 5-aza C, inconsistent changes on DNA methylation along culture; the need of specific hypomethylated status for somatic embryogenesis development was published dealing with *Dactylis glomerata* embryogenic and non-embryogenic calli (Tchorbadjieva and Pantchev 2004) by the use of isoschizomers DNA restriction. Most authors (Finnegan et al. 1996; Van Zyl et al. 2002; Chakrabarty et al. 2003; Tchorbadjieva and Pantchev 2004) underline that DNA methylation percentage is related with the correct differentiation and developmental processes. Results obtained in *Pinus nigra* indicate that epigenetic regulation system could permit bipolar structures formation when 5-mdC level is not higher than 18%.

#### Levels of free polyamines in cell lines

There was an inverse relationship between total contents of free PAs (Fig. 3) and embryogenic potential. Consequently, free contents of each individual PA were the highest in non-embryogenic line (LNE), which was characterized as callogenic and without embryogenic ability. Contrarily, LE2 line contained the lowest levels of total free PAs, due to the lowest Put contents of all the analysed lines. Furthermore, in the lines with embryogenic ability (LE1 and LE2), free Spd contents are the lowest without significant differences between them. Free Spm was the less abundant free polyamine and DAP contents are approximately zero in all the lines.



**Fig. 3** Contents of individual and global free polyamines and DAP analysis. Different letters indicate significant differences among means into the same colour columns ( $P < 0.001$ , ANOVA and post hoc Holm-Sidak tests). LNE: cell line without embryogenic potential; LE1: medium embryogenic potential line; LE2: high embryogenic potential line, Put: putrescine; Spd: spermidine; Spm: spermine; PAs-t: titer of free fraction polyamines

These results agree with the decrease in Put contents observed in calli derived from leaves of germinated seeds of the angiosperm eggplant after embryogenic potential acquisition (Yadav and Rajam 1998).

Due to PAs polycationic nature, they have got polianions binding ability (Kaur-Sawhney et al. 2003), especially in the case of the tetraamine (Spm). There has been demonstrated that most of Spm could be join to chromatin (Hirasawa and Suzuki 1985). This fact could explain, at least partially, the low levels of free Spm, especially in embryogenic lines. The contents of Spm and Spd were similar in embryogenic lines (LE1, LE2), being Spd the majoritary polyamine in the most embryogenic line, which possessed also the lowest Put levels. Non-embryogenic line showed a different situation because Put and Spd had similar contents being the highest of all the lines. These facts could be related with PAs functions attributed by diverse authors in researches made in angiosperms (Berros et al. 1997), and gymnosperms (Minocha et al. 1999). If it is admitted that Put favours cell division (Schons and Brasil 1995), its high levels in LNE fit in the callogenic character without embryogenic feature of this line.

According to the results, Put and Spd contents (Spm had low and constant levels) can be used as criteria for the physiological characterization of somatic embryogenesis in *Pinus nigra* Arn., where low levels of Put and Spd were concomitant with the development of the embryogenic program reaching the highest embryogenic potential when Spd levels were higher than Put.

Despite the hormonal and PAs profiles published (Klimaszewska et al. 2009) dealing with young and aged cultures of *Pinus pinaster* in somatic embryogenesis, it is well known that endogenous and exogenous plant growth regulators contents affect correct induction and development of responses mediated by DNA methylation events or other processes as was reported by Lo Schiavo et al. (1989), Minocha et al. (1999) and Leljak-Levanic et al. (2009). With this respect, it is worth to say that dSAM is involved in long chain PAs (Spd and Spm) biosynthesis and DNA methylation competitive inhibition because it is an aminopropyl donator during long chain PAs biosynthesis and, at the same time, it acts as a competitive inhibitor of the DNA methyl transferase. According to such a scheme higher contents of long chain PAs would have some association with higher methylation levels. In fact, the results suggest a relationship among DNA methylation levels, long chain PAs contents and embryogenic potential. The concomitancy between effective embryogenic potential and higher DNA methylation could be explained on the basis of the gene regulation hypothesis through specific methylation of gene promoters (Valledor et al. 2007). According to the results, it could also exist a molecular base to explain indirectly, by means of the connexion



between PAs biosynthesis and DNA methylation, the concomitancy between higher contents of free long chain PAs and embryogenic potential.

**Acknowledgments** The project was supported by Spanish national projects AGL-2004-00810; CIT-010000-2007-5; the Slovak Grant Agency VEGA, Proj. No. 2-5022-25; and COST 843-STSM at Oviedo University to T. Salajova. FICYT foundation supported the fellowship of the young researchers M. Pérez and M. Viejo.

## References

- Alcázar R, Marco F, Cuevas JC, Patron M, Ferrando A, Carrasco P, Tiburcio AF (2006) Involvement of polyamines in plant response to abiotic stress. *Biotechnol Lett* 28:1867–1876
- Baurens FC, Nicolleau J, Legrave T, Verdeil JL, Monteuis O (2004) Genomic DNA methylation of juvenile and mature *Acacia mangium* micropropagated in vitro with reference to leaf morphology as a phase change marker. *Tree Physiol* 24:401–407
- Berros B, Álvarez C, Rodríguez R (1997) Effect of putrescine synthesis inhibitors on somatic embryogenesis in hazelnut. *J Appl Bot* 71:69–138
- Berros B, Hasbún R, Radojevic L, Salajova T, Cañal MJ, Rodríguez R (2005) Protocol for hazelnut somatic embryogenesis. In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants, Springer, Dordrecht, pp 413–426
- Causevic A, Delaunay A, Ounnar S, Righezza M, Delmotte F, Brignolas F, Hagège D, Maury S (2005) DNA methylating and demethylating treatments modify phenotype and cell wall differentiation state in sugarbeet cell lines. *Plant Physiol Biochem* 43:681–691
- Causevic A, Gentil MV, Delaunay A, Abu El-Saud W, García Z, Pannetier C, Brignolas F, Hagège D, Maury S (2006) Relationship between DNA methylation and histone acetylation levels, cell redox and cell differentiation states in sugarbeet lines. *Planta* 224:812–827
- Chakrabarty D, Yu KW, Paek KY (2003) Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (*Eleutherococcus senticosus*). *Plant Sci* 165:61–68
- Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. *J Exp Bot* 48:1493–1509
- Dudits D, Bögre L, Gyögyey J (1991) Molecular and cellular approaches to the analysis of plant embryo development from somatic cells in vitro. *J Cell Sci* 99:475–484
- Finnegan E, Peacock W, Dennis E (1996) Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc Natl Acad Sci USA* 93:8449–8454
- Fraga M, Cañal MJ, Rodríguez R (2002a) In vitro morphogenetic potential of differently aged *Pinus radiata* trees correlates with polyamines and DNA methylation levels. *Plant Cell Tissue Organ Cult* 70:139–145
- Fraga M, Rodríguez R, Cañal MJ (2002b) Genomic DNA methylation-demethylation during aging and revigoration of *Pinus radiata*. *Tree Physiol* 22:813–816
- Fraga M, Rodríguez R, Cañal MJ (2002c) Phase-change related epigenetic and physiological changes in *Pinus radiata* D. Don. *Planta* 215:672–678
- Fraga M, Uriol E, Diego LB, Berdasco M, Esteller M, Cañal MJ, Rodríguez R (2002d) High-performance capillary electrophoretic method for the quantification of 5-methyl 2'-deoxycytidine in genomic DNA: application to plant, animal and human cancer tissues. *Electrophoresis* 23:1677–1681
- Fraga M, Rodríguez R, Cañal MJ (2003) Reinvigoration of *Pinus radiata* is associated with partial recovery of juvenile-like polyamine concentrations. *Tree Physiol* 23:205–209
- Fraga M, Berdasco M, Diego LB, Rodríguez R, Cañal MJ (2004) Changes in polyamine concentration associated with aging in *Pinus radiata* and *Prunus persica*. *Tree Physiol* 24:1221–1226
- Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Growth Regulat* 43:27–47
- Gupta PK, Durzan DJ (1985) Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). *Plant Cell Rep* 4:177–179
- Hasbún R, Valledor L, Rodríguez JL, Santamaría E, Ríos D, Sánchez M, Cañal MJ, Rodríguez R (2008) HPLC quantification of 5-methyl-2'-deoxycytidine in genomic DNA: methodological optimization for chesnut and other woody species. *Plant Physiol Biochem* 46:815–822
- Hirasawa E, Suzuki Y (1985) Occurrence of spermine in chromatin of *Zea mays*. *Plant Growth Regul* 3:239–245
- Jasik J, Salajova T, Salaj J (1995) Developmental anatomy and ultrastructure of early somatic embryos in European black pine (*Pinus nigra* Arn.). *Protoplasma* 185:205–211
- Kaur-Sawhney R, Altabella T, Tiburcio AF, Galston AW (2003) Polyamines in plants: an overview. *J Cell Mol Biol* 2:1–12
- Klimaszewska K, Noceda C, Pelletier G, Label P, Rodríguez R, Lelu-Walter MA (2009) Biological characterization of young and aged embryogenic cultures of *Pinus pinaster* (Ait.). *In Vitro Cell Dev Biol Plant* 45:20–33
- Kusano T, Yamaguchi K, Berberich T, Takhshi Y (2007) Advances in polyamines research in 2007. *J Plant Res* 120:345–350
- Leljok-Levanic D, Bauer N, Mihaljevic S, Jelaska S (2004) Changes in DNA methylation during somatic embryogenesis in *Curcubita pepo* L. *Plant Cell Rep* 23:120–127
- Leljok-Levanic D, Mihaljevic S, Jelaska S (2009) Variations in DNA methylation in *Picea Omorika* (Panc) Purk. embryogenic tissue and the ability for embryo maturation. *Propagat Ornament Plant* 9(1):3–9
- Lo Schiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. *Theor Appl Genet* 77:325–331
- Marcé M, Brown D, Capell T, Figueras X, Tiburcio A (1995) Rapid high-performance liquid chromatographic method for the quantification of polyamines as their dansyl derivatives: application to plant and animal tissues. *J Chromatogr B Biomed Appl* 666:329–335
- Martoja R, Martoja M (1967) Initiation aux techniques de l'histologie animal. Masson et Cie, Paris
- Minocha R, Smith D, Reeves C, Steele K, Minocha S (1999) Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. *Physiol Plant* 105:155–164
- Petrovska B, Moravčíková J, Obert B, Salaj T, Petrova A, Salaj J (2004) Suspension cultures of flax-extracellular proteins and morphogenic response. In: Biotechnology, as theory and practice in horticulture, Book of abstracts and programme, Fifth IVCHB symposium, Debrec, Hungary, p 110
- Pitto L, Cernilogar F, Evangelista M, Lombarda L, Miarelli C, Rocci P (2000) Characterization of carrot nuclear proteins that exhibit specific binding affinity towards conventional and non-conventional DNA methylation. *Plant Mol Biol* 44:659–673
- Quiroz-Figueroa FR, Rojas-Herrera R, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell Tiss Organ Cult* 86:285–301



- Razin A, Riggs A (1980) DNA methylation and gene function. *Science* 210:604–610
- Rey M, Díaz-Sala C, Rodríguez R (1994) Polyamines as markers for juvenility in filbert. *Acta Hort* 351:233–237
- Salajova T, Salaj J (1992) Somatic embryogenesis in European black pine (*Pinus nigra* Arn.). *Biol Plant* 36:213–218
- Salajova T, Salaj J (2001) Somatic embryogenesis and plantlet regeneration from cotyledon explants isolated from embling and seedlings of hybrid firs. *J Plant Physiol* 158:747–755
- Salajova T, Salaj J (2005) Somatic embryogenesis in *Pinus nigra*: embryogenic tissue initiation, maturation and regeneration ability of established cell lines. *Biol Plant* 49:333–339
- Salajova T, Salaj J, Kormutak A (1999) Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. *Plant Sci* 145:33–40
- Salajova T, Rodríguez R, Cañal MJ, Diego LB, Berdasco M, Radojevic L, Salaj J (2005) Protocol of somatic embryogenesis of *Pinus nigra* Arn. In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants. Springer, Dordrecht, pp 81–93
- Santos D, Fevereço P (2002) Loss of DNA methylation affects somatic embryogenesis in *Medicago truncatula*. *Plant Cell Tissue Organ Cult* 70:155–161
- Schons J, Brasil O (1995) Poliaminas na embriogênese somática em cenoura (*Daucus carota* L.). *Sci Agric* 52:534–536
- Tchorbadjieva M, Pantchev I (2004) DNA methylation and somatic embryogenesis of orchardgrass (*Dactylis glomerata* L.). *Bulg J Plant Physiol* 30:3–13
- Valledor L, Hasbún R, Meijón M, Rodríguez JL, Santamaría E, Viejo M, Berdasco M, Feito I, Fraga M, Cañal MJ, Rodríguez R (2007) Involvement of DNA methylation in tree development and micropropagation. *Plant Cell Tissue Organ Cult* 91:75–86
- Van Zyl L, Bozhkov P, Clapham D, Sederoff R, Von Arnold S (2002) Up, down and up again is a signature global gene expression pattern at the beginning of gymnosperm embryogenesis. *Gene Expr Patterns* 3:83–91
- Von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell Tissue Organ Cult* 69:233–249
- Yadav J, Rajam M (1998) Temporal regulation of somatic embryogenesis by adjusting cellular polyamine content in eggplant. *Plant Physiol* 116:617–625
- Yamamoto N, Kobayashi H, Togashi T, Mori Y, Kikuchi K, Kuriyama K, Tokuji Y (2005) Formation of embryogenic cell clumps from carrot epidermal cells is suppressed by 5-azacytidine, a DNA methylation inhibitor. *J Plant Physiol* 162:47–54